Untersuchungen zur Aufklärung physiologischer und pathophysiologischer Wirkungen nutritiver Aktivatoren von Peroxisomenproliferator-aktivierten Rezeptoren

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1 Einleitung und Fragestellung

Nahrungsfette enthalten als Hauptkomponente Fettsäuren. Diese dienen den Körperzellen als Energiesubstrate sowie als Vorstufe für die Synthese komplexer Lipide (z.B. Phospholipide, Glykolipide) und biologisch hochaktiver Mediatoren (z.B. Eicosanoide). Darüber hinaus üben Fettsäuren vielfältige weitere Funktionen aus, indem sie oder ihre Metabolite als transkriptionelle Regulatoren der Genexpression wirken und damit maßgeblich physiologische Prozesse wie Stoffwechsel, Zelldifferenzierung und Wachstum beeinflussen [1-3]. Als bedeutende Mediatoren der Wirkung von Fettsäuren auf die Gentranskription wurden die Peroxisomenproliferator-aktivierten Rezeptoren (PPARs) beschrieben [4, 5]. Diese erstmalig von Issemann & Green [6] identifizierten kernständigen Rezeptoren, die in den Subtypen α, β/δ und γ vorkommen, sind Transkriptionsfaktoren, die durch Bindung eines Liganden aktiviert werden. Gottlicher et al. [4] und Auwerx [5] wiesen erstmalig nach, dass nicht nur synthetische Substanzen wie Fibrate und Thiazolidinedione, sondern auch bestimmte Fettsäuren und Fettsäurederivate wie Eicosanoide als Liganden und Aktivatoren der PPARs wirken. Struktur und funktionelle Bedeutung der PPARs wurden in den letzten Jahren weitestgehend aufgeklärt und in einer großen Zahl an Übersichtsartikeln zusammengestellt [7-13]. Wesentliche transkriptionelle Wirkmechanismen der PPARs sind die Transaktivierung und die Transrepression. Die Transaktivierung, die über die Bindung funktionell aktiver PPAR/RXR-Heterodimere an definierte DNA-Konsensussequenzen (PPAR *response element*, PPRE) im Promotorbereich von Genen zustande kommt, spielt vor allem bei der Regulation von Genen des Lipidstoffwechsels und der Glukosehomöostase eine Rolle [9, 11, 14]. Darüber hinaus üben PPARs auch inhibitorische Wirkungen auf die Gentranskription aus, indem sie die DNA-Bindung bestimmter Transkriptionsfaktoren (z.B. NF-κB, AP-1, STAT1, STAT3) durch Protein-Protein-Wechselwirkungen oder durch Bindung für die Gentranskription essentieller Cofaktoren verhindern. Über diesen als Transrepression bezeichneten Wirkmechanismus werden entzündungshemmende Wirkungen der PPARs vermittelt [15-18]. Aufgrund ihrer zentralen Funktionen in der Kontrolle des Lipid- und Lipoproteinstoffwechsels, des Energiehaushalts, der Glukosehomöostase und inflammatorischer Prozesse werden kardiovaskuläre Erkrankungen und metabolische Störungen (Typ 2-Diabetes mellitus, metabolisches Syndrom) durch PPARs günstig beeinflusst [19-22]. Dies wird mittlerweile im Falle der hypolipidämisch wirksamen Fibrate und der antidiabetisch wirkenden Thiazolidindione für die Pharmakotherapie von Fettstoffwechselstörungen bzw. Diabetes mellitus genutzt.

Als potente PPAR-Agonisten wurden Konjugierte Linolsäuren (engl. *conjugated linoleic acids*, CLA) und chemisch modifizierte Fettsäuren wie oxidierte Fettsäuren und zyklische

Fettsäuremonomere identifiziert [23-30]. CLA sind positionelle und geometrische Isomere der Linolsäure mit konjugierten Doppelbindungen. Diese werden überwiegend über den Verzehr von Wiederkäuerprodukten (Fleisch, Milch) aufgenommen [31-35], da sie im Pansen des Wiederkäuers durch mikrobielle Biohydrogenierung aus Linolsäure und im Gewebe durch Δ9-Desaturierung aus der im Pansen gebildeten trans-Vaccensäure entstehen [36-38]. Daneben spielen für die menschliche Ernährung auch synthetische, als Nahrungsergänzungsmittel eingesetzte CLA-Präparate, eine Rolle. Solche Produkte bestehen zu 60-90% aus CLA, die als freie Fettsäuren oder zu Triglyzeriden verestert angeboten werden [39]. Im Gegensatz zu Wiederkäuerprodukten, deren CLA-Anteil überwiegend aus c9t11-CLA (90%) und einem Minoranteil weiterer Isomere besteht [40, 41], sind in den meisten CLA-Präparaten mit jeweils etwa 45% des CLA-Anteils gleich hohe Anteile an c9t11-CLA und t10c12-CLA und zahlreiche weitere Isomere in geringen Konzentrationen enthalten.

Oxidierte Fettsäuren und zyklische Fettsäuremonomere werden während der oxidativen Modifizierung von Speisefetten, z.B. während der küchentechnischen Zubereitung oder infolge unsachgemäßer Lagerung [42-45], aber auch im Rahmen der industriellen Raffination von Speisefetten gebildet [45-50]. Von besonderer Relevanz für die Aufnahme oxidierter Fettsäuren und zyklischer Fettsäuremonomere in der Humanernährung sind frittierte Lebensmittel [43], welche teilweise beträchtliche Mengen des thermisch behandelten Frittierfettes enthalten (bis zu ein Drittel der Trockenmasse [43]).

CLA haben großes ernährungsmedizinisches Interesse erlangt, da sie eine Vielzahl an günstigen biologischen Eigenschaften im tierischen Organismus ausüben [51-55]. Gleichwohl bestehen aber auch Hinweise für die Existenz ungünstiger Wirkungen von CLA. So wurde eine Beeinträchtigung der Insulinsensivität, Förderung der Hyperinsulinämie, Induzierung von oxidativem Stress sowie eine Absenkung des HDL-Cholesterins beschrieben [39, 55-61]. Obwohl für einige der beobachteten Effekte von CLA bereits Mechanismen auf molekularer Ebene aufgeklärt werden konnten, sind die Wirkmechanismen anderer Effekte noch größtenteils unverstanden. Die molekulare Grundlage für die in tierexperimentellen Studien beobachtete antiatherogene Wirkung der CLA ist beispielsweise bislang nur unzureichend bekannt. Da zahlreiche Hinweise existieren, dass pharmakologische PPARα- und PPARγ-Agonisten eine gefäßprotektive Wirkung aufweisen [21, 62, 63], lässt sich vermuten, dass CLA als natürlich in der Nahrung vorkommende PPAR-Liganden ihre antiatherogenen Wirkungen zumindest partiell über die Aktivierung von PPARs entfalten. Darüber hinaus ist bekannt, dass nicht nur CLA-Isomere selbst, sondern auch deren Metabolite eine hohe biologische Aktivität besitzen [64, 65]. Somit müssen diese ebenfalls als potenzielle Mediatoren der antiatherogenen

Effekte von CLA in Betracht gezogen werden.

Ebenso wie CLA zeigen oxidierte Fette vielfältige biologische Wirkungen im tierischen Organismus. So konnten Hinweise für die Induzierung von oxidativem Stress, eine Beeinträchtigung der Glukosetoleranz und eine proatherogene Wirkung festgestellt werden [66-71]. Auch eine Beeinflussung der Schilddrüsenfunktion durch oxidierte Fette wurde nachgewiesen [72-74]. Allerdings bewirken oxidierte Fette, ähnlich wie Fibrate, eine Absenkung der Plasmatriglyzeride und weisen somit auch günstige Wirkungen auf [75-79]. Letztere Wirkung wird auf die Aktivierung des hepatischen PPARα und konsekutiv gesteigerte peroxisomale und mitochondriale β-Oxidation von Fettsäuren in der Leber zurückgeführt [79], wodurch die Synthese und Sekretion triglyzeridreicher Lipoproteine absinkt. Der PPARα-aktivierende Effekt des oxidierten Fettes aber auch von CLA könnte möglicherweise bei einer alkoholischen Steatose (Fettleber) von therapeutischem Nutzen sein, da diese auf eine Inhibierung der transkriptionellen Aktivität des PPARα zurückzuführen ist. Hierauf deuten zumindest Untersuchungen hin, dass unter Gabe pharmakologischer PPARα-Agonisten eine durch chronische Ethanolfütterung verursachte Fettleber verhindert werden kann [80, 81]. Ob oxidierte Fette, ähnlich wie oxidierte Fettsäuren, den PPARγ aktivieren und darüber inflammatorische Prozesse modulieren, ist ferner bislang unbekannt.

Als Folge einer Supplementierung mit CLA oder oxidiertem Fett konnte ferner eine Reduktion des Milchfettgehalts bei laktierenden Tieren bzw. stillenden Frauen beobachtet werden [82-88]. Dieser Effekt, dessen Ursachen nur unzureichend verstanden sind, muss kritisch betrachtet werden, da die Milch für den säugenden Nachkommen die einzige Nahrungs- und Energiequelle darstellt und damit entscheidend das postnatale Wachstum beeinflusst. Mitursächlich für diesen Effekt oxidierter Fette bzw. von CLA könnte die Aktivierung des PPARα in der Leber sein, da über die resultierende Induzierung PPARα-responsiver Gene Einfluss auf Fettsäuretransport, -aufnahme und -abbau in der Leber ausgeübt wird [89, 90]. Physiologischerweise ist während der Laktation ein erhöhter Fluss an nichtveresterten Fettsäuren vom Fettgewebe zur Milchdrüse, mit dem Ziel Substrat für die Milchfettsynthese bereitzustellen, zu beobachten [91]. Dieser Substratstrom könnte als Folge einer hepatischen PPARα-Aktivierung durch CLA bzw. oxidierte Fette hin zur Leber verschoben sein. Allerdings sind entsprechende Untersuchungen bislang nicht aus der Literatur bekannt.

Insgesamt deuten diese Beobachtungen daraufhin, dass CLA und oxidierte Fette eine Quelle biologisch hochaktiver Substanzen für den Organismus darstellen und vielfältige, sowohl günstige als auch ungünstige, Wirkungen im Organismus ausüben. Allerdings erfordert eine

umfassende und kritische Abschätzung des Präventions- und Risikopotenzials dieser Nahrungsfette, dass weitere Wirkungen von CLA und oxidierten Fetten identifiziert sowie deren Wirkmechanismen untersucht werden. Die Identifizierung weiterer potenzieller Risiken ist insbesondere vor dem Hintergrund bedeutsam, dass mittlerweile CLA als Schlankheitsmittel bzw. Muskelaufbausubstanz durch zahlreiche Internetanbieter, Drogerien und Supermärkte vertrieben werden. Im Rahmen der Risikoabschätzung sollte auch die Wirkung dieser Fette auf den trächtigen Organismus und die Möglichkeit einer transplazentaren Wirkung auf den Fetus untersucht werden. Zahlreiche Studien belegen, dass ungünstige maternale Ernährungsinterventionen während der Trächtigkeit über eine langfristige Programmierung des embryonalen bzw. fetalen Stoffwechsels auch die Disposition für bestimmte Stoffwechselerkrankungen im späteren Leben erhöhen können [92-97].

Die vorliegende Arbeit beschäftigt sich schwerpunktmäßig mit der Aufklärung physiologischer und pathophysiologischer Wirkungen von CLA und oxidierten Fetten in zellulären Systemen und im Tiermodell. Aufgrund der Aktivierbarkeit von PPARs durch diese Nahrungsfette/-fettsäuren und der zentralen Rolle von PPARs in der Vermittlung von physiologischen und pathophysiologischen Wirkungen stehen Prozesse, die durch diese Transkriptionsfaktoren beeinflusst werden, im Vordergrund.

Insgesamt liegen dieser Arbeit 15 Originalarbeiten zugrunde. Das Ziel dieser Originalarbeiten war die Beantwortung der folgenden Fragen:

- Modulieren CLA PPARα- bzw. PPARγ-regulierte Prozesse in Zellen, die an atherosklerotischen Gefäßveränderungen beteiligt sind?
- Sind CLA-Metabolite in CLA-behandelten Zellen der Gefäßwand nachweisbar?
- Sind CLA und oxidiertes Fett in der Lage, über die Aktivierung des hepatischen PPARα der Entstehung einer alkoholischen Steatose entgegenzuwirken?
- Modulieren oxidierte Fette inflammatorische Prozesse im Darmepithel über die Aktivierung des PPARγ?
- Bewirken maternal verabreichte oxidierte Fette eine transplazentare Aktivierung des PPARα im Fetus?
- Welche Wirkmechanismen liegen dem milchfettsenkenden Effekt von CLA bzw. oxidiertem Fett im laktierenden Organismus zugrunde?

2 Eigene Originalarbeiten (A1-A15)

A1 **Ringseis R**, Muller A, Herter C, Gahler S, Steinhart H, Eder K. CLA isomers inhibit TNFα-induced eicosanoid release from human vascular smooth muscle cells via a PPARγ ligand-like action. *Biochimica et Biophysica Acta – General subjects* 2006;1760:290-300.

A2 **Ringseis R**, Gahler S, Herter C, Eder K. Conjugated linoleic acids exert similar actions on prostanoid release from aortic and coronary artery smooth muscle cells. *International Journal for Vitamin Nutrition Research* 2006;76:281-289.

A3 **Ringseis R**, Gahler S, Eder K. Conjugated linoleic acid isomers inhibit platelet-derived growth factor-induced NF-κB transactivation and collagen formation in human vascular smooth muscle cells. *European Journal of Nutrition* 2008 (im Druck).

A4 Schleser S, **Ringseis R**, Eder K. Conjugated linoleic acids have no effect on TNF alpha-induced adhesion molecule expression, U937 monocyte adhesion, and chemokine release in human aortic endothelial cells. *Atherosclerosis* 2006;186:337-344.

A5 **Ringseis R**, Wen G, Saal D, Eder K. Cis-9, trans-11- and trans-10, cis-12-conjugated linoleic acid isomers reduce cholesterol accumulation in acetylated LDL-induced mouse RAW264.7 macrophage foam cells. *British Journal of Nutrition* (eingereicht).

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A7 **Ringseis R**, König B, Leuner B, Schubert S, Nass N, Stangl G, Eder K. LDL receptor gene transcription is selectively induced by t10c12-CLA but not by c9t11-CLA in the human hepatoma cell line HepG2. *Biochimica et Biophysica Acta – Molecular and Cell Biology of Lipids* 2006;1761:1235-1243.

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A11 **Ringseis R**, Muschick A, Eder K. Dietary oxidized fat prevents ethanol-induced triacylglycerol accumulation and increases expression of PPARα target genes in rat liver. *Journal of Nutrition* 2007;137:77-83.

A12 **Ringseis R**, Piwek N, Eder K. Oxidized fat induces oxidative stress but has no effect on NF-κB-mediated proinflammatory gene transcription in porcine intestinal epithelial cells. *Inflammation Research* 2007;56:118-125.

A13 **Ringseis R**, Gutgesell A, Dathe C, Brandsch C, Eder K. Feeding oxidized fat during pregnancy up-regulates expression of PPARα-responsive genes in the liver of rat fetuses. *Lipids in Health and Disease* 2007;6:6.

A14 **Ringseis R**, Saal D, Muller A, Steinhart H, Eder K. Dietary conjugated linoleic acids lower the triacylglycerol concentration in the milk of lactating rats and impair the growth and increase the mortality of their suckling pups. *Journal of Nutrition* 2004;134:3327-3334.

A15 **Ringseis R**, Dathe C, Muschick A, Brandsch C, Eder K. Oxidized fat reduces milk triacylglycerol concentrations by inhibiting gene expression of lipoprotein lipase and fatty acid transporters in the mammary gland of rats. *Journal of Nutrition* 2007;137:2056-2061.

A1 **Ringseis R**, Muller A, Herter C, Gahler S, Steinhart H, Eder K:

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CLA isomers inhibit TNFα-induced eicosanoid release from human vascular smooth muscle cells via a PPARγ ligand-like action

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Abstract

Conjugated linoleic acids (CLAs) were reported to have anti-atherogenic properties in animal feeding experiments. In an attempt to elucidate the molecular mechanisms of these anti-atherogenic effects, the modulatory potential of CLA on cytokine-induced eicosanoid production from smooth muscle cells (SMCs), which contributes to the chronic inflammatory response associated with atherosclerosis, has been investigated in the present study. *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA were shown to reduce proportions of the eicosanoid precursor arachidonic acid in SMC total lipids and to inhibit cytokine-induced NF-κB DNA-binding activity, mRNA levels of inducible enzymes involved in eicosanoid formation ($cPLA_2$, COX-2, mPGES), and the production of the prostaglandins PGE_2 and PGI_2 by TNFα-stimulated SMCs in a dose-dependent manner. The effect of 50 μmol/L of either CLA isomer was as effective as 10 μmol/L of the PPARγ agonist troglitazone in terms of inhibiting the TNFα-stimulated eicosanoid production by SMCs. PPARγ DNA-binding activity was increased by both CLA isomers compared to control cells. Moreover, it was shown that the PPARγ antagonist T0070907 partially abrogated the inhibitory action of CLA isomers on cytokine-induced eicosanoid production and NF-κB DNA-binding activity by vascular SMCs suggesting that PPARγ signalling is at least partially involved in the action of CLA in human vascular SMCs. With respect to the effects of CLA on experimental atherosclerosis, our findings suggest that the anti-inflammatory effect of CLA is at least partially responsible for the anti-atherogenic effects of CLA observed in vivo.

Keywords: Conjugated linoleic acid; Atherosclerosis; Human coronary artery smooth muscle cell; Eicosanoid; TNFα; Troglitazone

1. Introduction

Conjugated linoleic acids (CLAs), which are a naturally occurring group of positional and geometric isomers of linoleic acid, were reported to have anti-atherogenic properties in animal feeding experiments using mice [1,2], rabbits [3], and hamsters [4]. However, using the C57BL/6 mouse model of atherosclerosis feeding CLA even increased the development of atherosclerotic fatty streaks [5]. Regarding that the effects of CLA on experimental atherosclerosis are very controversial and that the molecular mechanisms of the observed anti-atherogenic effects are largely unknown suggests that further research is required to elucidate the potential effects of CLA on atherosclerosis.

Vascular smooth muscle cells (SMC) play an important role in the pathogenesis of atherosclerosis and are the predominant cell type in atherosclerotic plaques. Changes in SMC phenotype, migration, and proliferation are considered critical steps in atherosclerosis [6], nevertheless, the impact of CLA on either of these processes in SMCs has not yet been studied. Changes in SMC phenotype are observed in response to vascular injury, a process called SMC activation, and are characterized by a change from a contractile, nongrowing to a secretory, proliferating phenotype [7,8]. SMC activation is mediated by a variety of stimuli such as tumor necrosis factor (TNF)-α, which are secreted during pathological conditions, e.g., the inflammatory process associated with the development of atherosclerosis, and leads to cyclooxygenase (COX)-2 induction. There is evidence that COX-2 expression contributes

Abbreviations: CLA, conjugated linoleic acid; HCASMC, human coronary artery smooth muscle cells; TNFα, tumor necrosis factor α

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to vascular SMC proliferation and migration and is responsible for the majority of excessive inflammatory prostanoid production [9]. The excessive production of vasoactive inflammatory mediators by activated vascular SMCs is considered to mediate vascular dysfunction and contribute to the chronic inflammatory response associated with atherosclerosis [10]. Therefore, since CLAs were shown to inhibit stimulus-induced COX-2 gene expression and/or eicosanoid release in endothelial cells [11] and monocytes/macrophages [12,13], it might be of great importance with respect to the role of CLA in atherogenesis to study the effect of CLA on stimulus-induced COX-2 gene expression and eicosanoid release from vascular SMCs.

The aim of this study, therefore, was to investigate the effects of two different isomers of CLA, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, which together contribute to more than 90% of total CLA in foods [14], on the production of PGI_2 and PGE_2, which are the major prostanoids produced by SMCs [15,16], in human coronary artery SMCs (HCASMC). In order of elucidating the molecular mechanisms regulating eicosanoid synthesis, we further investigated the effects of these CLA isomers on gene expression of cytosolic phospholipase A_2 ($cPLA_2$), COX-1, COX-2, prostaglandin (PG) I synthase (PGIS), and membrane-bound PGE synthase (mPGES). PGIS and mPGES are enzymes located downstream of COX-2 in the metabolism of the eicosanoid precursor arachidonic acid. The effect of CLA treatment on expression of these enzymes has not yet been investigated. Both prostaglandin synthases compete for the same substrate PGH_2, the arachidonic acid derived product of COX, and catalyze its transformation into PGI_2 and PGE_2, respectively. The inhibitory effect of CLA on stimulus-induced COX-2 induction in macrophages was demonstrated to be partially mediated by a PPARγ-dependent inhibition of NF-κB activation [12,13], which is due to the potential of CLAs to bind and activate PPARs. Thus, in an attempt to clarify the potential involvement of PPARγ in vascular SMCs, we investigated the effect of CLA on cytokine-induced eicosanoid production in the presence of the specific PPARγ antagonist T0070907.

2. Materials and methods

2.1. Chemicals

Cis-9, *trans*-11 CLA (≥96% pure) and *trans*-10, *cis*-12 CLA (≥98% pure) were obtained from Cayman Chemicals (Ann Arbor, MI). Preparation of the stock solutions of fatty acids and of the test media was performed as described previously [11]. MTT (3-[4,5-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue), TNFα (tumor necrosis factor α), and DMSO (dimethylsulfoxide) were purchased from Sigma-Aldrich (Taufkirchen, Germany). The PPARγ agonist troglitazone was purchased from Calbiochem (Bad Soden, Germany). The PPARγ antagonist T0070907 was purchased from IBL-Hamburg (Hamburg, Germany).

2.2. Cell culture

Human coronary artery smooth muscle cells (HCASMC) from a 40-year-old male Caucasian donor were obtained from PromoCell and cultured in SMC Growth Medium 2 containing 5% fetal calf serum, 0.5 μg/L epidermal growth factor, 2.0 μg/L basic fibroblast growth factor, 5 mg/L Insulin, 50 mg/L gentamicinsulfate and 50 μg/L amphotericin B (all from PromoCell, Heidelberg, Germany). Cells were passaged after reaching confluence by using trypsin/EDTA. After trypsinization, trypsin neutralizing solution was added to prevent enzymatic damage to the cells. Only cells from passages 3 to 6 were used for this study.

For all experiments, HCASMCs were seeded at a density of 6.000 cells/cm^2 and incubated for 3 d until reaching 70–80% confluence. Subsequently, cells were incubated with 5, 50 or 100 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA or 10 μmol/L of troglitazone for various incubation times (3 to 24 h) in the presence or absence of TNFα (10 ng/mL). For experiments using the PPARγ inhibitor T0070907, the incubation media was additionally supplemented with 1 μmol/L of T0070907. Cells treated with TNFα (10 ng/mL) in the absence of fatty acids were used as controls ("stimulated control"). Cells treated without TNFα and without fatty acids were used as "unstimulated controls". When using troglitazone and/or T0070907, the concentration of the solvent DMSO in the incubation media was 0.1%. In those experiments, the same amount of DMSO was added to control cells (vehicle control) and the cells treated with CLA isomers.

2.3. Cell viability

Cell viability after treatment as aforementioned was examined by the MTT assay [17].

2.4. Fatty acid analysis

For determination of the fatty acid composition of cellular total lipids SMCs were treated with or without fatty acids for 24 h without addition of TNFα. Afterwards SMCs were washed with phosphate-buffered saline, harvested using a cell scraper, and pelleted by centrifugation (170×*g* for 5 min). The cell pellet was stored at −80 °C until lipid extraction. For lipid extraction cell pellets were suspended in 1 mL bidistilled water and lipids were extracted with 3 mL chloroform/methanol (2/1, v/v+0.001% BHT) and transmethylated with 5% potassium methylate solution in methanol. GC-FID analysis for analysis of fatty acids methyl esters (FAME) was performed using an Agilent 6890 GC equipped with a split/splitless injector at 230 °C, a flame ionization detector at 260 °C, an autosampler (Agilent Technologies, Waldbronn, Germany) and a CP SIL 88 column (100 m, 0.25 mm, 0.2 μm film thickness, Varian, Darmstadt, Germany). Hydrogen was used as carrier gas at a constant flow rate of 1 mL/min. For FAME analysis with GC-MS analysis a Trace GC hyphenated with a PolarisQ ion-trap mass-spectrometer (Thermo Electron GmbH, Dreieich, Germany) was used. Column type and temperature programming were the same as for GC-FID analysis. Electron impact ionization was used at 70 eV in positive mode with an ion volume temperature of 200 °C. Helium was used as carrier gas at a constant flow rate of 1 mL/min. PolarisQ was operated in full scan mode from 50 to 650 amu. Conjugated fatty acid isomers were separated using Ag^+-HPLC-DAD as described recently in detail [18].

2.5. Measurement of eicosanoid release

For determination of the release of PGI_2 and PGE_2, HCASMCs were incubated in 24-well plates in phenol red-free SMC Growth Medium 2 for 24 h as indicated above. After the 24 h incubation period, cell medium was collected and assayed for the eicosanoids 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2, and PGE_2 using EIA-kits (No. 515211, 514010, Cayman Chemicals, Ann Arbor, MI). Eicosanoid concentrations were corrected for protein concentrations in the cell lysate as measured by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

2.6. Analysis of mRNA expression

For the evaluation of time-course of mRNA expression of the genes investigated cells were cultured in 6-well plates in the presence or absence of TNFα (10 ng/mL) for 3, 8 and 24 h. According to the results of time-course experiments analysis of CLA-mediated effects on TNFα-induced mRNA expression was performed after 8 h of incubation in the presence or absence of CLA isomers and/or TNFα. Afterwards, total RNA of the cells was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from

the optical density at 260 and 280 nm (SpectraFluor Plus, Tecan, Crailsheim, Germany). cDNA synthesis and semi-quantitative RT-PCR for the estimation of mRNA expression of PPARγ, $cPLA_2$, COX-1, COX-2, mPGES, PGIS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization were performed using a PCR thermocycler (Biometra, Göttingen, Germany) as described previously [19]. The number of PCR cycles was determined in preliminary experiments ensuring that relative quantification of mRNA expression was performed within the linear range of amplification of each PCR product. PCR primers were obtained from Roth (Karlsruhe, Germany). The primer sequences used for RT-PCR were as follows (forward, reverse): PPARγ (5′-GCAGGAGCAGAGCAAAGAGGTG-3′, 5′-AAATATTGCCAAGTCGCTGTCATC-3′), $cPLA_2$ (5′-GAGCTGATGTTTGCAGATTGGGTTG-3′, 5′-GTCACTCAAAGGAGACAGTGGATAAGA-3′), COX-1 (5′-CAGCTCCTGGCCCGCCGCTT-3′, 5′-GTGCATCAACACAGGCGCCTC-3′), COX-2 (5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′, 5′-AGATCATCTCTGCCTGAGTATCTTT-3′), mPGES (5′-CTCTGCAGCACGCTGCTGG-3′, 5′-GTAGGTCACGGAGCGGATGG-3′), PGIS (5′-AGGAGAAGCACGGTGACATC-3′, 5′-GCAGCGCCTCAATTCCGTAA-3′), GAPDH (5′-GACCACAGTCCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTAG-3′).

2.7. Measurement of NF-κB- and PPARγ-DNA binding activities

For the measurement of NF-κB transactivity cells were seeded in 25 cm^2 tissue culture flasks and incubated for 8 h as indicated above. For the measurement of PPARγ transactivity cells were treated for 24 h in the presence or absence of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA or troglitazone. Afterwards nuclear extracts were prepared with a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol and protein concentrations were determined as aforementioned. NF-κB- and PPARγ-DNA binding activities in the nuclear extracts were determined by the Transcription Factor assays TransAM™ NF-κB Family Kit and TransAM™ PPARγ Kit (Active Motif), respectively. For the measurement of NF-κB activity, binding activities of the NF-κB subunit p65 was determined.

2.8. Statistical analysis

Student's *t*-test was used to compare means of treatments with those of control. Differences of $P<0.05$ were considered significant.

3. Results

3.1. Effects of treatment on viability of HCASMCs

Viability of HCASMCs was not affected by treatment with 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA or 10 μmol/L of troglitazone and co-treatment with 10 ng/mL of TNFα for 24 h relative to control treatment or vehicle control treatment (0.1% DMSO) (data not shown). In addition, cell viability was not affected by simultaneous treatment with 1 μmol/L of the PPARγ antagonist T00709070 for 24 h relative to control treatment. However, incubating cells with 5 μmol/L of the PPARγ antagonist significantly reduced cell viability to about 80% of controls (data not shown).

3.2. Fatty acid composition of HCASMC total lipids

Incubating HCASMCs with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA resulted in a significant incorporation of the respective CLA isomer into total cell lipids in a concentration-dependent manner when compared to control incubations ($P<0.05$; Table 1). In addition, treatment of cells with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA resulted in a strong increase in the proportions of conjugated C16:2 and conjugated C20:2 fatty acids in a concentration-dependent manner relative to control treatment ($P<0.05$). The proportions of the eicosanoid precursor arachidonic acid (C20:4 n-6) and other long-chain polyunsaturated fatty acids, C20:3 n-6, C22:4 n-6, and C22:5 n-3, were reduced by treatment of cells with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA relative to control treatment ($P<0.05$).

Table 1
Fatty acid composition of total lipids of HCASMCs cultured in the presence or absence (control) of 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA

Treatment	control	*cis*-9, *trans*-11 CLA		*trans*-10, *cis*-12 CLA	
		5 μmol/L	50 μmol/L	5 μmol/L	50 μmol/L
C14:0	2.9±0.3	3.1±0.3	2.7±0.4	3.2±0.3	3.0±0.3
C16:0	22.6±1.2	22.9±2.0	19.8±2.1	23.9±2.0	21.2±1.7
C16:2 c7t9	<0.1	0.4±0.2 *	1.4±0.4*	<0.1	<0.1
C16:2 t8c10	<0.1	<0.1	<0.1	0.2±0.2*	1.8±0.7*
C18:0	33.5±4.0	33.4±5.7	28.1±5.9	35.1±6.4	32.3±5.5
C18:1	12.7±3.9	12.5±3.9	9.9±2.7	11.8±4.0	8.9±3.1
C18:2 n-6	2.0±0.4	1.9±0.1	1.7±0.4	2.2±0.5	1.5±0.3*
C18:2 c9t11	<0.1	1.6±0.4*	11.6±2.4*	<0.1	0.2±0.1
C18:2 t10c12	<0.1	<0.1	<0.1	0.9±0.2*	10.2±2.4*
C20:0	0.4±0.1	0.4±0.1	0.3±0.1	0.4±0.1	0.4±0.1
C20:2 c11t13	<0.1	0.1±0.1*	1.00±0.3*	<0.1	<0.1
C20:2 t12c14	<0.1	<0.1	<0.1	0.3±0.3*	0.71±0.2*
C20:3 n-6	1.0±0.1	0.8±0.1	0.7±0.1*	0.7±0.1	0.6±0.1*
C20:4 n-6	5.0±1.2	3.7±0.6*	3.5±0.6*	3.3±0.5*	3.0±0.5*
C20:5 n-3	1.0±0.2	1.0±0.2	0.8±0.2	0.8±0.2	0.8±0.2
C22:4 n-6	0.5±0.2	0.3±0.1	0.3±0.1	0.2±0.1*	0.2±0.2*
C22:5 n-3	2.1±0.3	1.7±0.1*	1.7±0.2*	1.6±0.1*	1.5±0.1*
C22:5 n-6	0.3±0.1	0.3±0.2	0.3±0.2	0.3±0.1	0.3±0.2
C22:6 n-3	2.6±0.4	2.5±0.4	2.3±0.4	2.2±0.7	2.1±0.4

HCASMCs were cultured without fatty acids (control) or with 5 and 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA for 24 h. Fatty acids of SMC total lipids are presented as g per 100 g of total fatty acids. Results represent mean±S.D. of five independent experiments.
* Significantly different from control, $P<0.05$.

3.3. Effect of CLA isomers on TNFα-induced eicosanoid release from HCASMCs

Treatment of HCASMCs with 10 ng/mL of TNFα increased the release of 6-keto $PGF_{1\alpha}$ (Fig. 1A) and PGE_2 (Fig. 1B) into the incubation medium relative to unstimulated control treatment ($P<0.05$). Treatment of cells with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA dose-dependently inhibited the TNFα-stimulated increase in the release of 6-keto $PGF_{1\alpha}$ and PGE_2 into the incubation media.

3.4. Effect of simultaneous treatment of CLA isomers or troglitazone with the PPARγ inhibitor T0070907 on TNFα-induced eicosanoid release from HCASMCs

Treatment of cells with 10 μmol/L of the PPARγ agonist troglitazone also inhibited the TNFα-stimulated increase in the release of 6-keto $PGF_{1\alpha}$ (Fig. 2A) and PGE_2 (Fig. 2B); 10 μmol/

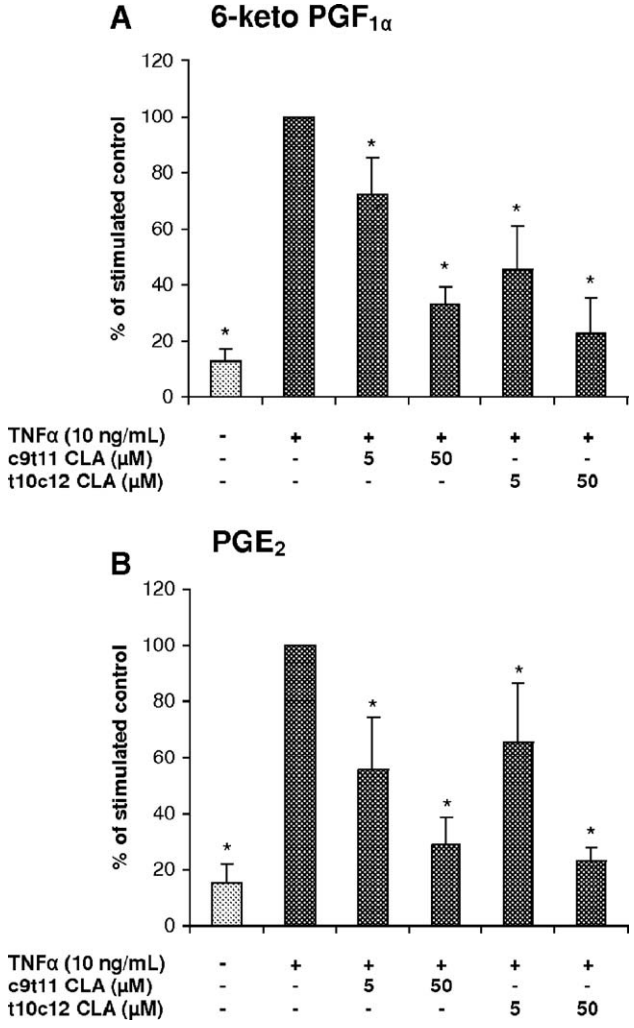

Fig. 1. Effect of 5 or 50 μmol/L of c*is*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA on TNFα-induced release of 6-keto $PGF_{1\alpha}$ (A) and PGE_2 (B) from human coronary artery smooth muscle cells. Cells were incubated simultaneously with fatty acids and TNFα (10 ng/mL) for 24 h. Cells treated with TNFα (10 ng/mL) in the absence of fatty acids were used as controls (stimulated control). Cells treated without TNFα and without fatty acids were used as unstimulated controls. Eicosanoid concentrations were measured by commercial EIA-kits and corrected for protein concentration of cell lysats. Data represent mean ± S.D. of three independent experiments and are expressed as percentage of eicosanoid release of stimulated controls (=100 ± 0%). *Significantly different from stimulated controls, $P<0.05$.

L of troglitazone was as effective as 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA in terms of inhibiting the TNFα-stimulated eicosanoid release. Simultaneous treatment with 1 μmol/L of the PPARγ inhibitor T0070907 partially abrogated the inhibitory effect of CLA or troglitazone on the TNFα-induced release of 6-keto $PGF_{1\alpha}$ and PGE_2 (Fig. 2).

3.5. Time-dependent effect of TNFα on mRNA expression of PPARγ, $cPLA_2$, COX-1, COX-2, mPGES, and PGIS

The time-course of mRNA expression of PPARγ, $cPLA_2$, COX-1, COX-2, mPGES, and PGIS was determined by incubating HCASMCs during 3, 8, and 24 h in the presence or absence of TNFα (10 ng/mL).

As illustrated in Fig. 3A mRNA expression of PPARγ, PGIS, and COX-1 was neither influenced by TNFα-treatment nor the incubation time. In contrast, mRNA expression levels of COX-2, mPGES, and $cPLA_2$ were increased by TNFα after 3 and 8 h of incubation compared to incubation without TNFα. The maximum mRNA level of mPGES was observed in cells after 3 h of incubation with TNFα, whereas maximum levels of COX-2 mRNA and $cPLA_2$ mRNA were observed after 8 h of incubation with TNFα. 24 h after incubation with TNFα mRNA levels of COX-2, $cPLA_2$, mPGES were still increased compared to SMCs treated without TNFα for 3 h; however, after 24 h of incubation the mRNA levels of COX-2, $cPLA_2$, and mPGES in untreated cells (without TNFα) were also increased to similar levels as in TNFα-treated cells.

3.6. Effect of CLA isomers on TNFα-induced mRNA expression of PPARγ, $cPLA_2$, COX-1, COX-2, mPGES, and PGIS

Incubating SMCs with TNFα for 8 h resulted in a marked increase in the mRNA levels of $cPLA_2$, COX-2, and mPGES when compared to incubations without TNFα (Fig. 3B). Treatment of SMCs with 50 μmol/L of *cis*-9, *trans*-11 CLA for 8 h inhibited the TNFα-induced mRNA expression of $cPLA_2$, COX-2, and mPGES. Treatment of SMCs with 50 μmol/L of *trans*-10, *cis*-12 CLA for 8 h inhibited only the TNFα-induced mRNA expression of $cPLA_2$ and mPGES. The mRNA levels of PPARγ, COX-1, and PGIS were not affected by TNFα or treatment with either CLA isomer.

3.7. Effect of CLA isomers, troglitazone and simultaneous treatment with the PPARγ inhibitor T0070907 on TNFα-induced transactivation of NF-κB in HCASMCs

Treatment of HCASMCs with 10 ng/mL of TNFα stimulated NF-κB DNA-binding activity (Fig. 4) compared to unstimulated control treatment ($P<0.05$). Treatment of cells with 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA or 10 μmol/L of the PPARγ agonist troglitazone significantly reduced TNFα-stimulated NF-κB DNA-binding activity relative to stimulated control treatment ($P<0.05$). Simultaneous treatment of HCASMCs with the PPARγ inhibitor T0070907 completely abolished the inhibitory effect of CLA isomers or troglitazone on TNFα-stimulated NF-κB DNA-binding activity.

3.8. Effect of CLA isomers and troglitazone on PPARγ transactivation in HCASMCs

Incubating HCASMCs with 100 μmol/L of *cis*-9, *trans*-11 CLA or 50 or 100 μmol/L of *trans*-10, *cis*-12 CLA as well as 10 μmol/L of troglitazone increased PPARγ DNA-binding activity relative to control incubations (Fig. 5; $P<0.05$).

4. Discussion

CLAs have aroused scientific interest due to their anti-atherogenic properties as observed in animal feeding experiments [1–4]. However, the molecular mechanisms of these

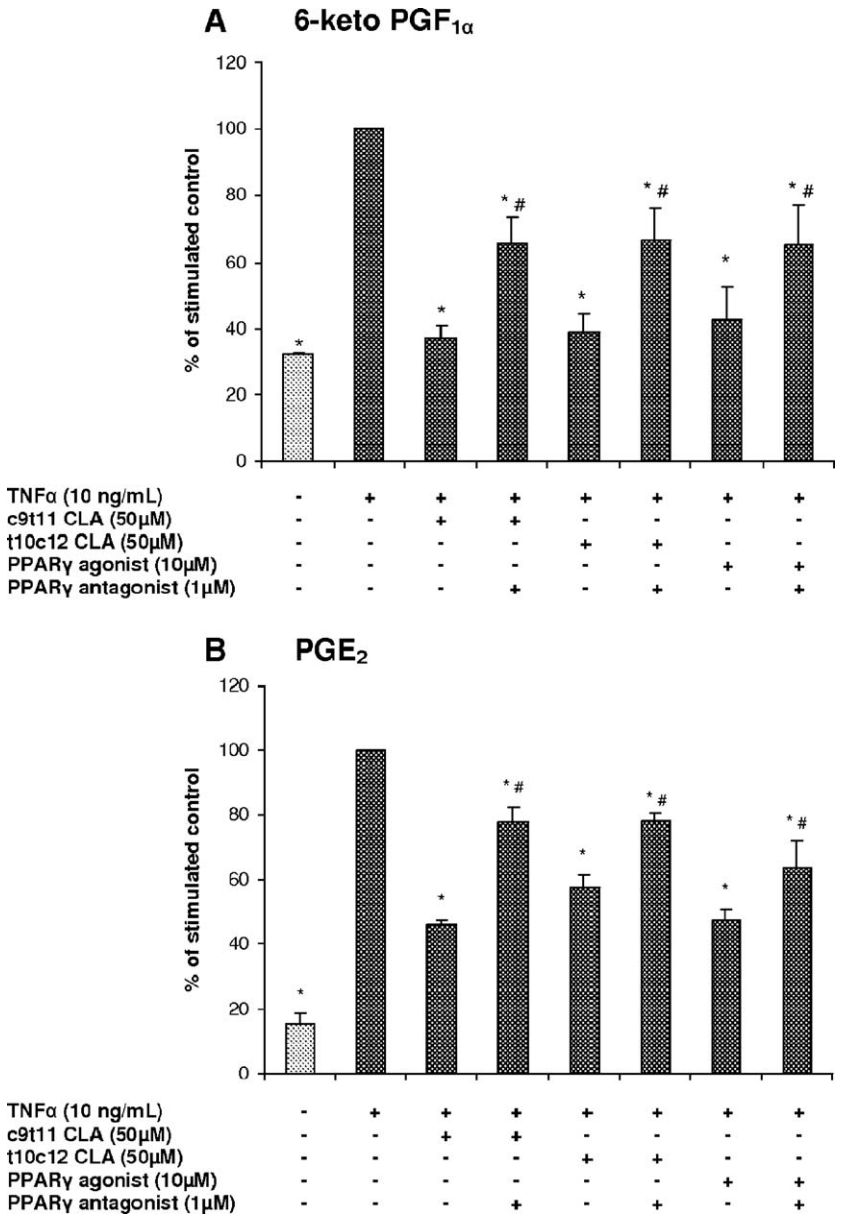

Fig. 2. Effect of 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA or 10 μmol/L of troglitazone (PPARγ agonist) and effect of simultaneous treatment of 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA or 10 μmol/L of troglitazone with the specific PPARγ antagonist T0070907 (1 μmol/L) on TNFα-induced release of 6-keto $PGF_{1\alpha}$ (A) and PGE_2 (B) from human coronary artery smooth muscle cells. Cells were incubated simultaneously with fatty acids or troglitazone and TNFα (10 ng/mL) and/or T0070907 for 24 h. Cells treated with TNFα (10 ng/mL) in the absence of fatty acids were used as controls (stimulated control). Cells treated without TNFα and without fatty acids were used as unstimulated controls. Eicosanoid concentrations were measured by commercial EIA-kits and corrected for protein concentration of cell lysats. Data represent mean ± S.D. of three independent experiments and are expressed as percentage of eicosanoid release of stimulated controls (=100 ± 0%). *Significantly different from stimulated controls, $P<0.05$. #Significantly different from cells treated without T0070907, $P<0.05$.

anti-atherogenic effects are largely unknown. In addition, increased development of experimental atherosclerosis in response to dietary CLA has also been reported in the C57BC/6 mouse model of atherosclerosis [5]. With respect to these controversial observations on experimental atherosclerosis and in view of elucidating potential anti-atherogenic mechanisms of CLA, the present study was performed. Regarding that vascular SMCs play an important role in the pathogenesis of atherosclerosis, the modulatory potential of CLA on eicosanoid production from SMCs has been investigated in the present study.

The main finding of the present study is that CLA isomers inhibit cytokine-induced eicosanoid release from human vascular SMCs in a dose-dependent manner, which is consistent with findings from similar studies using macrophages or endothelial cells [11–13]. To our knowledge, this is the first report demonstrating that CLAs affect SMC function.

The reduction in eicosanoid production by CLA in the present study might be due, at least in part, to the observed reduction of arachidonic acid, which serves as the main substrate for the biosynthesis of prostaglandins, in SMC total lipids following treatment with CLA. It has been reported that

CLA competes with other fatty acids such as linoleic acid for the incorporation into membrane phospholipids but also that CLA interferes with the production of arachidonic acid from linoleic acid [20] resulting in a reduced arachidonate pool and subsequently reduced eicosanoid production.

However, two observations suggest that the effects of CLA on cytokine-induced eicosanoid production are also mediated, at least in part, through other modulatory pathways, e.g., PPARγ-dependent mechanisms. First, both CLA isomers, at physiologically relevant concentrations, as well as the PPARγ agonist troglitazone inhibited the TNFα-stimulated eicosanoid production by HCASMCs. Second, simultaneous treatment of CLA isomers or troglitazone with the specific PPARγ inhibitor T0070907 partially abrogated this inhibitory effect. Indeed, performing PPARγ transactivation assays revealed that both CLA isomers as well as troglitazone were capable of increasing PPARγ DNA-binding activity in HCASMCs in the present study. This is consistent with results from recent studies demonstrating PPARγ transactivation by *cis*-9, *trans*-11 CLA and/or *trans*-10, *cis*-12 CLA [13,21–23]. However, we did not observe an increase of PPARγ mRNA expression by CLA which is in contrast to recent studies showing increased PPARγ gene expression following treatment with CLA isomers [21,22] or troglitazone [24]. However, with respect to the effect of CLA on PPARγ gene expression very conflicting data are reported in the literature, e.g., no alterations [2], increases [21,22] or even decreases [25,26] in PPARγ mRNA abundance were observed following treatment with CLA. These frequently observed differences might be explained by the fact that PPARγ expression also shows a complex regulation by other factors such as growth factors or cytokines, e.g., in human aortic SMCs TGF-β was demonstrated to exert a biphasic effect on PPARγ mRNA expression (early stimulation and late repression) [27] suggesting that differences in the specific experimental conditions (quiescent oder growing conditions, absence or presence of cytokines oder mitogens) and treatment regimes (concentration and time of exposure) might play a causal role for these opposing effects. In addition, differences might be also attributed to cell type-specific actions of CLA and species differences, which have been frequently reported in the literature in connection with CLA. Nevertheless, irrespective of the lack of effect of CLA treatment on PPARγ gene expression the results from the present study but also from others [21–23] clearly demonstrate that CLA isomers transactivate PPARγ. Future studies are required to resolve the contradictory observations regarding the effects of CLA on PPARγ mRNA expression and activity in the present study.

As mentioned above, PPARγ gene expression and activity was also reported to be regulated by cytokines, e.g., TNFα was shown to inhibit PPARγ gene expression and activity due to either PPARγ phosphorylation or inhibition of PPARγ-PPRE (DNA) binding [28,29]. This counteracting effect of TNFα on PPARγ function was shown to cause dedifferentiation and delipidation of 3T3-L1 adipocytes [30] and activation of hepatic stellate cells (HSC) leading to increases in fibrogenic markers [29], respectively. In contrast, we did not observe a decrease in PPARγ gene expression in SMCs following treatment with TNFα suggesting that the counteracting effect of TNFα on PPARγ function is probably also cell type-specific. However, treatment of activated HSCs with synthetic PPARγ ligands was shown to restore PPARγ expression and diminish markers associated with fibrosis [31]. In addition, overexpression of PPARγ was demonstrated to revert phenotypic changes induced by HSC activation [32]. Similar, it has been reported that the PPARγ ligands thiazolidinediones (e.g., troglitazone) attenuate the inhibitory effect of TNFα on differentiation of adipocytes and that overexpression of PPARγ in 3T3-L1 adipocytes abrogates the antiadipogenic effect of TNFα [30]. Therefore, we suggest that CLAs acting as natural PPARγ ligands activated PPARγ in SMCs in the present study, thereby, compensating for a potential TNFα-induced suppression of PPARγ expression. However, future studies should clarify whether CLA is indeed capable of compensating the counteracting effect of TNFα on PPARγ function.

From our aforementioned observations, we suggest that PPARγ signalling is at least partially involved in the modulation of prostaglandin production by CLA in human vascular SMCs. The observation that the inhibitory effect of CLA on cytokine-induced eicosanoid release was not completely abolished by treatment with the specific PPARγ antagonist T0070907 is probably explained by the fact that CLA caused a marked reduction in the concentration of arachidonic acid. The fact that inhibition by troglitazone, which does not effect arachidonate levels in membrane phospholipids, was also not completely blocked by treatment with T0070907, might be attributed due to an insufficient dose of the PPARγ inhibitor to cause complete abrogation. However, although the effect of T0070907 on abrogation of PPARγ mediated effects was shown to increase dose-dependently, a complete abrogation was also not observed in other studies [33]. In the present study, increasing the concentration of T0070907 to 5 μmol/L was accompanied by a reduction of cell viability (data not shown) confirming the findings from a previous study that this PPARγ inhibitor efficiently causes cell death [34]. This suggests that higher doses of T0070907 would have rather caused cell death than completely abolished the inhibitory effect of CLA or troglitazone on cytokine-induced eicosanoid production in vascular SMCs.

Recent studies could demonstrate that the inhibitory effect of CLA on stimulus-induced eicosanoid release from macrophages is mediated by a PPARγ-dependent inhibition of NF-κB activation [12,13,22]. The results from the present study could also demonstrate that treatment of SMCs with either CLA isomer inhibited TNFα-induced NF-κB activation. In addition, we could demonstrate that this inhibition of NF-κB activation could be abolished by simultaneous treatment with the specific PPARγ inhibitor T0070907. This suggests that CLA probably exerts its anti-inflammatory action in SMCs in a similar manner as observed in macrophages. The observed inhibition of NF-κB activation is probably also responsible for the inhibitory effect of CLA isomers on TNFα-induced mRNA expression of COX-2, mPGES, and $cPLA_2$ in the present study. Activation of NF-κB is normally observed upon stimulation with cytokines,

bacterial lipopolysaccharide or mitogenic stimuli and leads to the upregulation of pro-inflammatory genes such as adhesion molecules, chemokines, COX-2, iNOS, $cPLA_2$ [35]. The increased expression of the enzymes COX-2, mPGES and $cPLA_2$, which are co-localized to the endoplasmic reticulum and nuclear envelope [36,37], by TNFα results in the excessive formation of prostanoids [38] as also shown in the present study. Evaluation of CLA-mediated effects on TNFα-induced mRNA expression was performed at 8 h of TNFα-stimulation, representing the time point with the strongest stimulatory effect of TNFα on COX-2, mPGES and $cPLA_2$ mRNA expression as shown by time course experiments. The time course observed for COX-2, mPGES and $cPLA_2$ mRNA expression is characteristic for immediate early response genes such as COX-2 and frequently observed in response to proinflammatory stimuli such as TNFα or IL-1 [39,40]. Surprisingly, although the mRNA levels of those genes were still induced after 24 h of treatment with TNFα which is consistent with observations from others [41,42], no differences in the mRNA accumulation of COX-2, mPGES and $cPLA_2$ were observed between TNFα-treated and untreated SMCs after 24 h as shown by time course experiments. However, time-dependent induction of COX-2 expression in untreated cells has also been observed from others [43] and is probably due to the specific experimental conditions employed. In the present study the growth conditions (subconfluent cells, use of SMC growth medium) employed obviously provided stimulatory conditions on COX-2, mPGES and $cPLA_2$ mRNA expression after 24 h even in the absence of TNFα. It has been reported that growth factors such as EGF and bFGF, which were contained in SMC growth medium herein, or PDGF but also serum increase COX-2 mRNA accumulation in vascular SMCs [8,44], therefore, possibly explaining the increased COX-2, mPGES and $cPLA_2$ mRNA accumulation in untreated SMCs after 24 h.

To our knowledge, the finding that not only stimulus-induced COX-2 expression but also stimulus-induced mPGES expression was reduced by treatment with CLA is novel and is probably explained by the fact that COX-2 and mPGES are

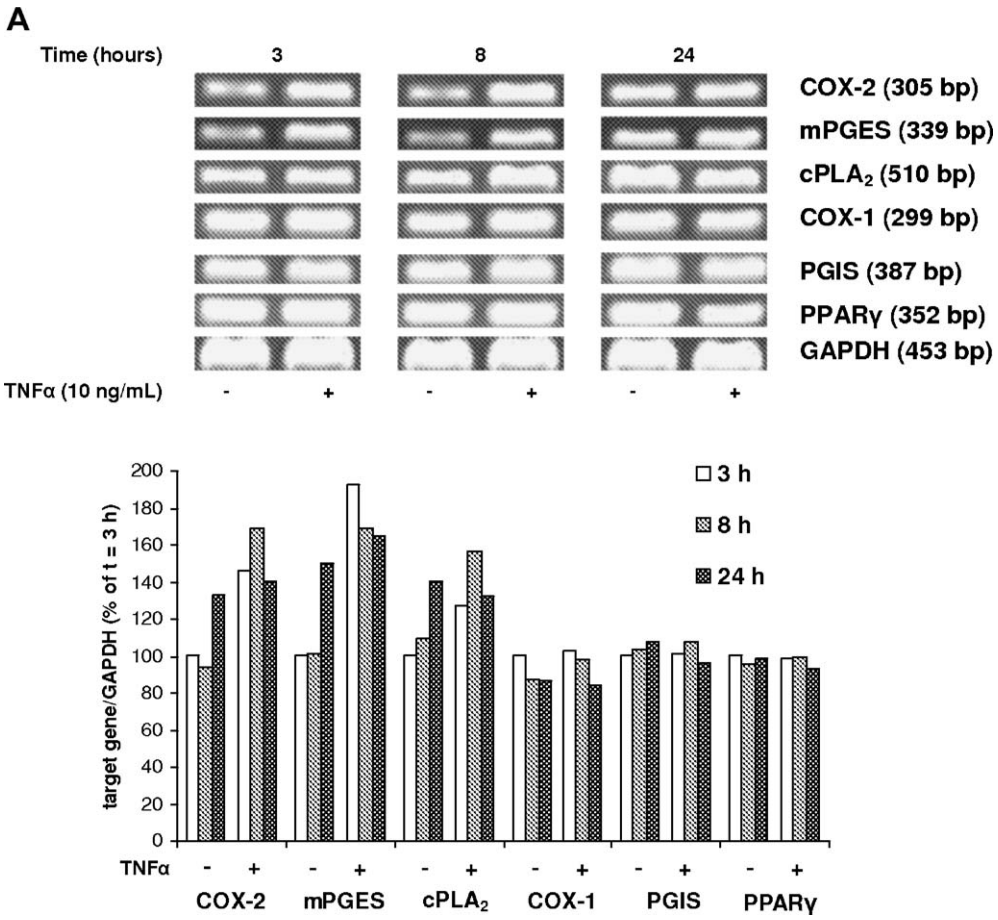

Fig. 3. (A) Time-dependent effect of TNFα on mRNA expression of PPARγ, $cPLA_2$, COX-1, COX-2, mPGES and PGIS in human coronary artery smooth muscle cells. Representative images of PCR product electrophoresis are shown for one independent experiment. Data represent one experiment and are representative for two independent experiments and are expressed as percentage of mRNA expression of cells treated without TNFα for 3 h. (B) Effect of 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA on TNFα-induced mRNA expression of peroxisome proliferator-activated receptor γ (PPARγ), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), cytosolic phospholipase A_2 ($cPLA_2$), prostaglandin I synthase (PGIS), prostaglandin E synthase (PGES) in human coronary artery smooth muscle cells. Cells were incubated simultaneously with fatty acids and TNFα (10 ng/mL) for 8 h. Total RNA was extracted, 1.2 μg total RNA reverse transcribed, and cDNA was subjected to RT-PCR using gene-specific primers as described in Materials and methods. Representative images of PCR product electrophoresis are shown for one independent experiment. Data represent mean±S.D. of at least three independent experiments and are expressed as percentage of mRNA expression of stimulated controls (=100±0%). *Significantly different from stimulated controls, $P<0.05$.

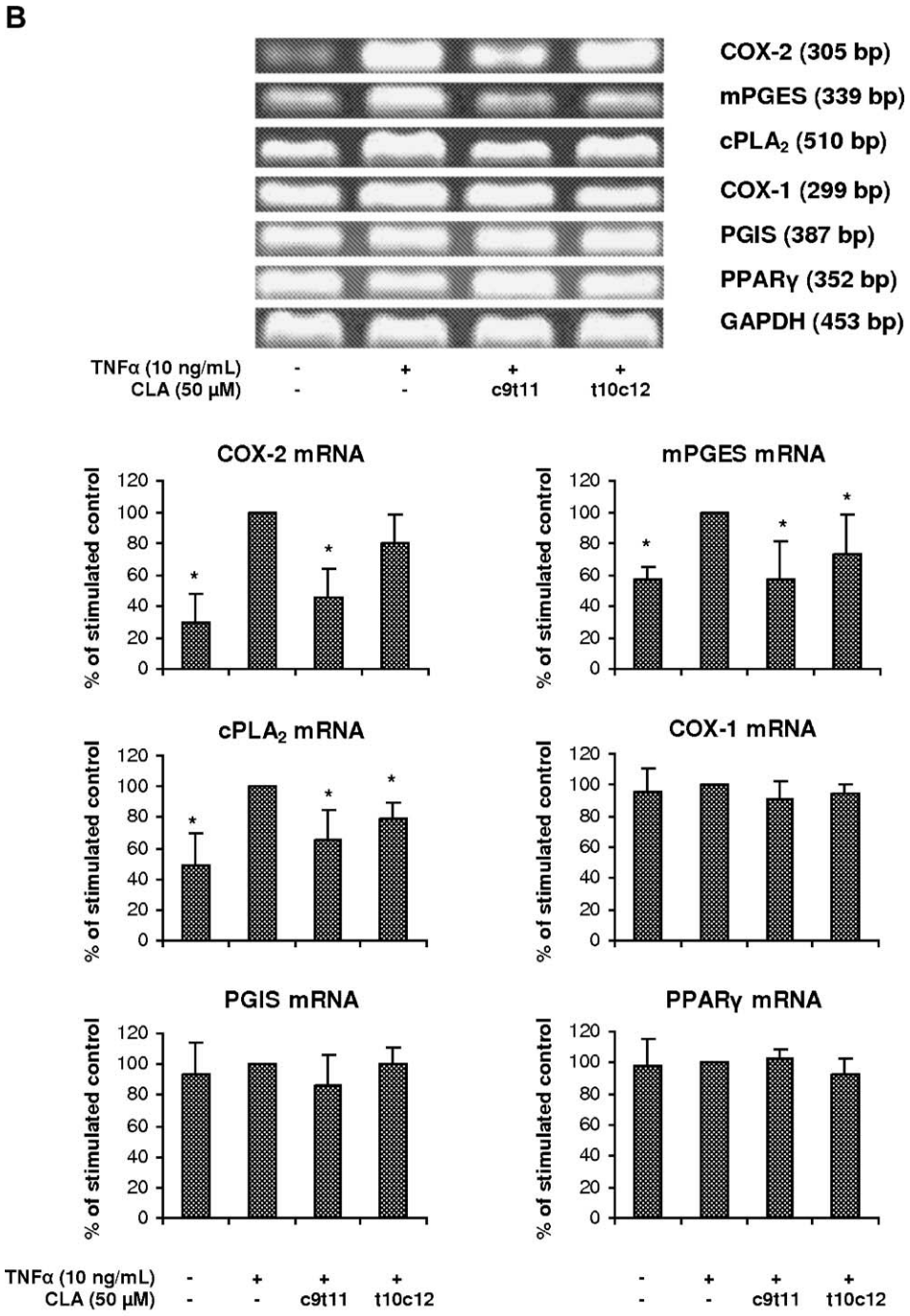

Fig. 3 (*continued*).

functionally-coupled in the cell. Both genes are up-regulated in response to various pro-inflammatory stimuli and were probably down-regulated as a consequence of the anti-inflammatory action of CLA. The observation that PGIS was not influenced by treatment with TNFα or CLA concurs with the fact that PGIS is expressed in a constitutive manner [39]. The markedly increased concentrations of PGI_2 by TNFα in the present study are probably the result of enhanced COX-2 expression concerning that the activity of COX-2 is rate-limiting for the formation of PGI_2 in SMCs and not that of PGIS [13].

COX-2 expression was shown to contribute to vascular SMC proliferation and migration, that are hallmarks in atherosclerosis, and to be responsible for the majority of excessive inflammatory prostanoid production [9], which is involved in the chronic inflammatory response associated with atherosclerosis [10]. Moreover, COX-2 expression was demonstrated to occur early in the development of atherosclerosis in apoE-deficient mice [45]. Therefore, since CLAs were shown to inhibit cytokine-induced COX-2 gene expression and excessive prostanoid production in vascular SMCs in the present study,

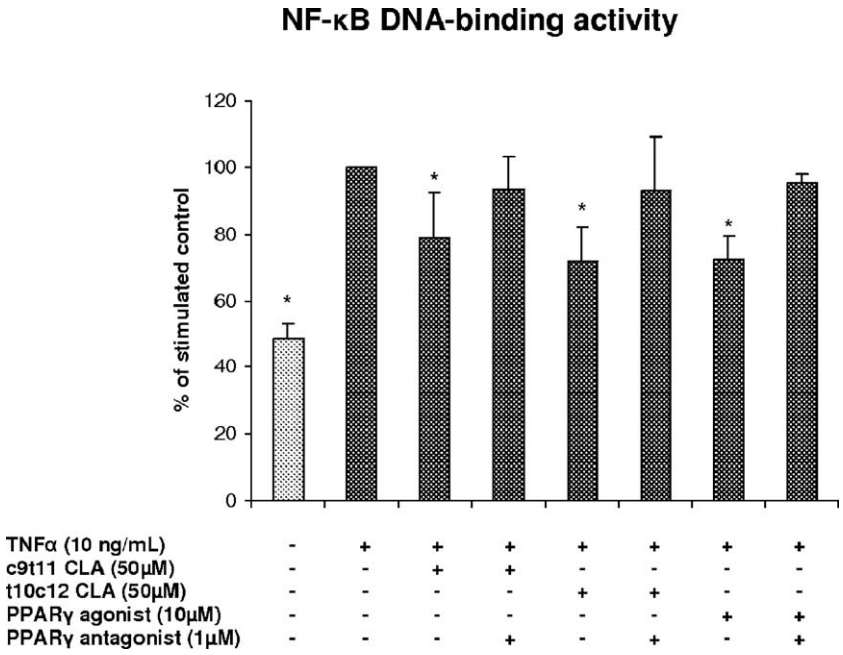

Fig. 4. Effect of 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA or 10 μmol/L of troglitazone (PPARγ agonist) and of simultaneous treatment with the specific PPARγ antagonist T0070907 (1 μmol/L) on TNFα-induced NF-κB DNA-binding activity in human coronary artery smooth muscle cells. Cells were incubated simultaneously with fatty acids or troglitazone and TNFα (10 ng/mL) and/or T0070907 for 8 h. Cells treated with TNFα (10 ng/mL) in the absence of fatty acids were used as controls (stimulated control). Cells treated without TNFα and without fatty acids were used as unstimulated controls. NF-κB DNA-binding activity was determined using an ELISA-based transactivation assay measuring binding of p65 to its NF-κB consensus-binding site. Data represent mean ± S.D. of three independent experiments and are expressed as percentage of NF-κB transactivity of stimulated controls (=100 ± 0%). *Significantly different from stimulated controls, $P < 0.05$.

our findings suggest that the anti-inflammatory action of CLA is at least partially responsible for the anti-atherogenic effects of CLA observed in vivo.

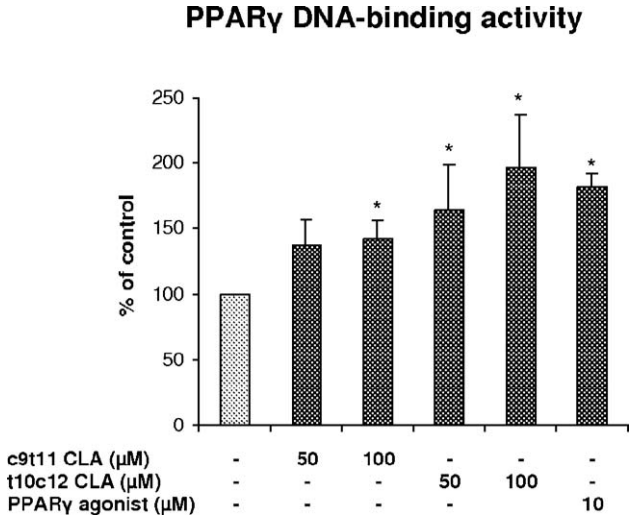

Fig. 5. Effect of *cis*-9, *trans*-11 CLA (50 and 100 μmol/L), *trans*-10, *cis*-12 CLA (50 and 100 μmol/L) and of the PPARγ agonist troglitazone (10 μmol/L) on PPARγ transactivation in human coronary artery smooth muscle cells. Cells were incubated simultaneously with fatty acids or troglitazone for 24 h. Cells treated in the absence of fatty acids or troglitazone were used as controls. PPARγ transactivation was determined using an ELISA-based transactivation assay measuring binding of PPARγ to its specific consensus-binding site. Data represent mean ± S.D. of two independent experiments and are expressed as percentage of PPARγ transactivity of controls (=100 ± 0%). *Significantly different from controls, $P < 0.05$.

Gene expression analysis further revealed that the effect of *cis*-9, *trans*-11 CLA on cytokine-induced mRNA expression of COX-2, mPGES and $cPLA_2$ was slightly stronger than that of the *trans*-10, *cis*-12 CLA isomer although the inhibitory effects on cytokine-induced eicosanoid release were similar for both CLA isomers. This suggests that posttranscriptional effects of CLA may be also involved in the modulation of cytokine-induced prostaglandin formation. Recent studies indicated that not only CLA itself but also conjugated metabolites of CLA exert specific biological activities, e.g., enzyme inhibition, in cell culture systems [46]. Metabolites of CLA, formed via elongation and/or desaturation and β-oxidation, were found in tissues of CLA-fed animals and cultured leukemia cells treated with CLA [46–48]. The present study demonstrates that conjugated metabolites of CLA (conjugated 16:2, conjugated 20:2) are also formed in cells of the vasculature and dose-dependently increase by treatment with CLA. This possibly indicates that the metabolites of CLA are able to cause inhibition of enzymes involved in eicosanoid biosynthesis. However, future studies using highly purified conjugated metabolites of CLA are necessary to study the role of conjugated metabolites in mediating biological effects of CLA in cell culture systems.

In conclusion, this study shows that the CLA isomers *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA inhibit TNFα-induced NF-κB DNA-binding activity and dose-dependently inhibit the production of the prostaglandins PGE_2 and PGI_2 in vascular SMCs. The effect of 50 μmol/L of either CLA isomer was as effective as 10 μmol/L of the PPARγ agonist troglitazone in terms of inhibiting the TNFα-stimulated eicosanoid production

by SMCs. Those concentrations of CLA are considered to be of physiological relevance regarding that serum levels in non-vegetarians were reported to be in the range of 20–70 μmol/L [49]. Moreover, it was shown that the PPARγ antagonist T0070907 abrogated the inhibitory action of CLA isomers on cytokine-induced eicosanoid production and on TNFα-induced NF-κB transactivation in vascular SMCs suggesting that PPARγ signalling is at least partially involved in the action of CLA in human vascular SMCs. With respect to the effects of CLA on experimental atherosclerosis, our findings suggest that the anti-inflammatory action of CLA is at least partially responsible for the anti-atherogenic effects of CLA observed in vivo.

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A2 **Ringseis R**, Gahler S, Herter C, Eder K:

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Conjugated Linoleic Acids Exert Similar Actions on Prostanoid Release from Aortic and Coronary Artery Smooth Muscle Cells

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Abstract: Conjugated linoleic acids (CLAs) are biologically active lipid compounds exerting anti-atherogenic actions *in vivo* without exact knowledge about the underlying mechanisms. Recently, CLAs were shown to lower the release of vasoactive prostanoids from vascular smooth muscle cells (SMCs) which play a central role in atherosclerosis. Since SMCs from different vascular locations were shown to exert differential actions in response to a common stimulus, the present study aimed to explore potential differential effects of CLA isomers on the release of the prostanoids PGE_2 and PGI_2 from coronary artery and aortic SMCs. For this purpose, human aortic and coronary artery SMCs were incubated with 5 and 50 µmol/L of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA for 24 hours and analyzed for fatty acid composition and the release of prostaglandins E_2 and I_2 (PGE_2 and PGI_2). Incubations were performed in the absence (basal conditions) and in the presence of 10 ng/mL of the cytokine tumor necrosis factor-α (TNFα) (cytokine-stimulated conditions). Fatty acid analysis revealed a similar degree of incorporation of CLA isomers and dose-dependent reduction of arachidonic acid in total cell lipids of both types of vascular SMCs following treatment with CLA. The release of PGE_2 and PGI_2 was dose-dependently inhibited by either CLA isomer from both types of vascular SMCs. The inhibitory potential of CLA isomers on the release of prostanoids was slightly different between basal and cytokine-stimulated conditions. In conclusion, the present findings suggest that the action of CLA isomers on the release of vasoactive prostanoids from vascular SMCs is largely independent of the vascular location; e.g., coronary arteries or systemic vasculature (aorta), but partially depends on the pathophysiological status of SMCs. The observed anti-inflammatory effect of CLAs may contribute to the anti-atherogenic actions of CLA.

Key words: Conjugated linoleic acids; coronary artery smooth muscle cells, aortic smooth muscle cells, prostanoids, eicosanoids, PGI_2, PGE_2, TNFα

Introduction

Conjugated linoleic acids (CLAs) are naturally occurring positional and geometric isomers of linoleic acid that have been demonstrated to exert diverse beneficial health effects [1]. Amongst these, the atheroprotective actions of CLA are of particular interest with respect to the high prevalence of atherosclerosis and subsequent cardiovascular events in developed countries. Although there is some evidence from the literature that the anti-

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inflammatory actions of CLA are at least partially involved in their atheroprotective properties [2 – 4], the mechanisms of action are far from being fully understood.

In an attempt to elucidate these mechanisms of action, we have previously demonstrated that CLAs are capable of reducing cytokine-induced release of vasoactive prostanoids from human coronary artery smooth muscle cells (SMCs) via reduction of the prostanoid precursor arachidonic acid and PPARγ-mediated inhibition of NF-κB activation [5]. This is of great significance considering that SMCs play an important role in the pathogenesis of atherosclerosis [6]. During the inflammatory process associated with atherogenesis, vascular SMCs become activated by cytokines and mitogens [6, 7]. This phenotypic switch/modulation results in the excessive production of pro-inflammatory mediators that promote vascular dysfunction and finally, the development of atherosclerotic plaques [8], wherein SMCs are the predominant cell type [6]. Given the phenotypic heterogeneity of vascular SMCs [9] and cell type-specific actions of CLAs frequently observed in the literature [10], it remains to be demonstrated whether the inhibitory action of CLA on prostanoid release from human coronary artery SMCs [5] might be generalized to vascular SMCs from other blood vessels. Raising this point is important because the differential effects of a common stimulus on the growth or function of vascular SMCs cultured from different vascular beds, as well as from different sections of a common blood vessel, are well established [11, 12]. The main reason contributing to these different effects is the phenotype of the cells and their embryonic origin [9]; e.g., SMCs from coronary arteries originate from mesothelial progenitors in the proepicardial organ and these vessels develop in a completely independent manner from vessels of the systemic vasculature (e.g., aorta) [9, 13].

Therefore, the aim of the present study was to investigate the effect of CLA isomers on the production of prostaglandins E_2 and I_2 (PGE_2 and PGI_2) from SMCs of two different locations, coronary arteries and the aorta. PGI_2 and PGE_2 are the two major prostanoids formed by vascular SMCs [14], and basal levels contribute to vascular homeostasis under normal conditions. However, during atherosclerosis development, the excessive formation of PGI_2 and PGE_2 mediates vascular dysfunction and contributes to chronic inflammation in the arterial wall [6]. As isomers, *cis*-9, *trans*-11 CLA, which is the predominant isomer in natural foods [15], and *trans*-10, *cis*-12 CLA, which is one of the main isomers in chemically produced CLA mixtures, were used. Considering that the SMC phenotype also depends on the (patho)physiological status of the blood vessel, we investigated the actions of CLA under basal conditions, reflecting the SMC phenotype in healthy blood vessels, as well as under cytokine-stimulated conditions, reflecting the activated phenotype in atherosclerotic blood vessels. Cytokine stimulation was performed by incubating SMCs with tumor necrosis factor-α (TNFα). TNFα is largely secreted during the inflammatory process associated with the development of atherosclerosis and strongly promotes the excessive formation of prostanoids from activated vascular SMCs *in vivo* and *in vitro*.

Materials and methods

Materials

Cis-9, *trans*-11 CLA (≥ 96% pure) and *trans*-10, *cis*-12 CLA (≥ 98% pure) were obtained from Cayman Chemical (Ann Arbor, MI). Preparation of the stock solutions of fatty acids and of the test media was performed as described previously [4]. MTT (3-[4, 5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue) and TNFα were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Cell culture

Human aortic smooth muscle cells and human coronary artery smooth muscle cells were obtained from PromoCell (Heidelberg, Germany). Both SMC types were cultured in SMC Growth Medium 2 containing 5% fetal calf serum, 0.5 µg/L epidermal growth factor, 2.0 µg/L basic fibroblast growth factor, 5 mg/L Insulin, 50 mg/L gentamycin sulfate, and 50 µg/L amphotericin B (all from PromoCell). Cells were passaged after reaching confluence by using trypsin/EDTA. After trypsinization, trypsin-neutralizing solution was added to prevent enzymatic damage to the cells. Only cells from passages 3 – 6 were used for this study.

For all experiments, SMCs were seeded at a density of 6000 cells/cm^2 and incubated for 3 days until reaching 70 – 80% confluence. Subsequently, cells were incubated with 5 or 50 µmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA for 24 hours in the absence (basal conditions) or presence of 10 ng/mL TNFα (stimulated conditions). Cells treated without fatty acids were used as controls.

Cell count, cell viability, and protein determination

The cell count was determined with a Neubauer chamber after cells were harvested by trypsinization and pelleted by centrifugation (170 × g for 5 minutes). Cell viability after treatment as aforementioned was examined by the MTT assay [16]. In brief, after the 24-hour treatment as indicated above, cell medium was removed and the cells were incubated with medium containing 0.5 mg/mL MTT for 2.5 hours at 37 °C and 5% CO_2 atmosphere. Subse-

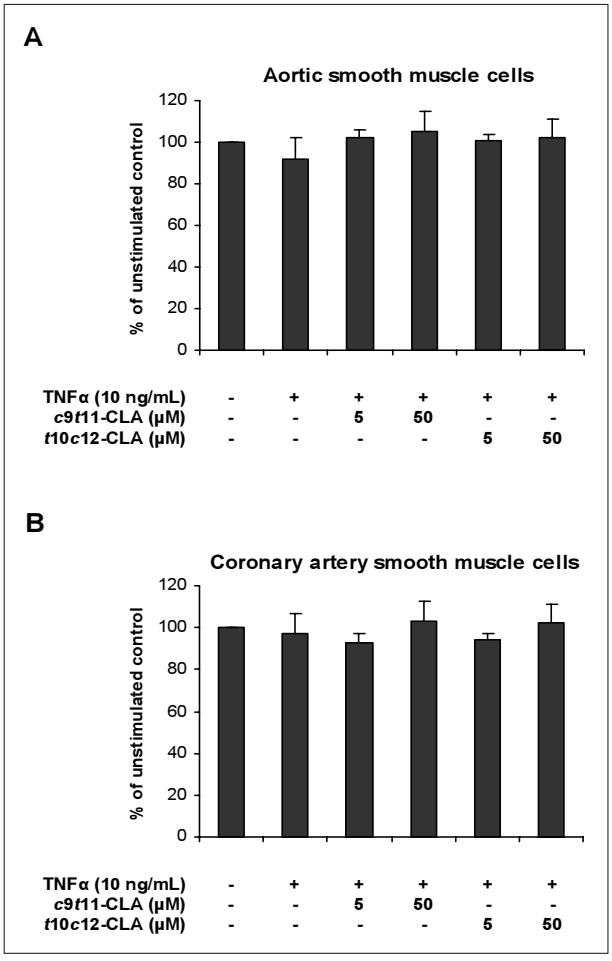

Figure 1: Effect of 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA on cell viability of TNFα-treated human aortic smooth muscle cells (A) and TNFα-treated human coronary artery smooth muscle cells (B) compared to cells treated with TNFα alone or without TNFα (unstimulated control). Data represent mean ± SD of two independent experiments with two repetitions in each experiment and are expressed as percentage of cell viability of unstimulated controls (= 100 ± 0 %).

quently medium was aspirated and 2-propanol (Roth, Karlsruhe, Germany) was added to dissolve the formazan. The supernatants were transferred to a 96-well microtiter plate and the absorbance was read at 595 nm in a microtiter plate spectrophotometer (Tecan, Crailsheim, Germany). Protein concentration of the cell lysates was measured by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) as standard.

Fatty acid analysis

For determination of the fatty acid composition of cellular total lipids, SMCs were treated with or without fatty acids for 24 hours as indicated above. Afterwards SMCs were washed with phosphate-buffered saline (PBS) and total lipids were extracted with hexane/isopropanol (3:2 v/v). The lipid extracts were dried under nitrogen, transmethylated with trimethylsulfonium hydroxide and fatty acid methyl esters (FAMEs), and were separated by gas chromatography (GC) as described previously in detail [17, 18].

Measurement of eicosanoid release

For determination of the release of 6-keto $PGF_{1\alpha}$ and PGE_2, SMCs were incubated in 24-well plates in phenol red-free SMC Growth Medium 2 in the presence or absence of CLA isomers and/or TNFα for 24 hours as indicated above. After the 24-hour incubation period, cell medium was collected and assayed for the eicosanoids 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2, and PGE_2 using EIA-kits (No. 515 211, 514 010, Cayman Chemical, Ann Arbor, MI). Eicosanoid concentrations were corrected for protein concentrations in the cell lysate as measured by the BCA protein assay kit.

Statistical analysis

Data were subjected to one-way ANOVA using Minitab Statistical Software (Minitab, State College, PA). In the case of eicosanoid concentrations, where the differences between variances and means were large, data were transformed to logarithms prior to ANOVA. For statistically significant F values, individual means of treatment groups were compared by Fisher's multiple range test. Means were considered significantly different for $p < 0.05$.

Results

Treatment effects on viability of aortic and coronary artery SMCs

Cell viability of aortic and coronary artery SMCs was not affected by treatment with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA and simultaneous treatment with 10 ng/mL of TNF for 24 hours relative to cells treated with TNFα alone (stimulated controls) or untreated cells (unstimulated controls) (Figure 1).

Effect of CLA isomers on fatty acid composition of aortic and coronary artery SMC total lipids

Analysis of fatty acid composition revealed that proportions of major fatty acids were similar between aortic and coronary artery SMCs with the exception that proportions of C18 : 2 and C20 : 4 were slightly lower in coronary artery SMCs (Table I). In general, effects of CLA on the fatty acid composition of total lipids were similar in both

Table I: Proportions of total saturated, total monounsaturated, and total polyunsaturated fatty acids and major fatty acids of total lipids of human aortic and coronary artery smooth muscle cells cultured in the presence or absence (control) of 5 or 50 µmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA for 24 hours.

Treatment	control	*cis*-9, *trans*-11 CLA		*trans-10*, *cis*-12 CLA	
		5 µmol/L	50 µmol/L	5 µmol/L	50 µmol/L
			g/100 g total FAME		
Aortic smooth muscle cells					
total SFA	39.8 ± 0.9^{a}	35.6 ± 0.9^{b}	29.6 ± 3.3^{c}	36.9 ± 0.6^{b}	33.6 ± 0.7^{c}
total MUFA	34.6 ± 0.2^{a}	34.7 ± 0.4^{a}	24.4 ± 0.9^{b}	35.4 ± 0.8^{a}	26.8 ± 0.6^{b}
total PUFA	25.5 ± 0.7^{c}	29.7 ± 0.5^{b}	46.0 ± 3.8^{a}	27.7 ± 1.4^{b}	39.6 ± 0.2^{a}
C14 : 0	2.0 ± 0.1	1.5 ± 0.1	1.8 ± 0.7	1.4 ± 0.1	2.4 ± 0.5
C16 : 0	20.5 ± 0.2^{a}	19.6 ± 0.3^{a}	15.9 ± 1.9^{b}	20.3 ± 0.7^{a}	17.4 ± 0.1^{b}
C16 : 1 n-7+n-5	6.5 ± 0.1^{a}	4.7 ± 0.3^{b}	4.7 ± 1.5^{b}	4.0 ± 0.4^{b}	4.8 ± 0.2^{b}
C18 : 0	16.9 ± 0.4^{a}	14.1 ± 0.5^{b}	11.5 ± 1.2^{c}	14.5 ± 0.1^{b}	13.2 ± 0.7^{c}
C18 : 1 n-9+n-7	27.7 ± 0.3^{a}	29.4 ± 2.2^{a}	18.6 ± 2.6^{b}	31.0 ± 3.2^{a}	18.6 ± 0.3^{b}
C18 : 2 n-6	4.5 ± 0.1^{a}	3.8 ± 0.9^{a}	3.5 ± 0.5^{ab}	5.4 ± 1.2^{a}	2.5 ± 0.1^{b}
*c*9t11 – CLA	< 0.1^{c}	5.7 ± 1.7^{b}	29.1 ± 2.6^{a}	< 0.1^{c}	< 0.1^{c}
*t*10c12 – CLA	< 0.1^{c}	< 0.1^{c}	< 0.1^{c}	5.0 ± 0.5^{b}	23.8 ± 1.0^{a}
C20 : 4 n-6	8.4 ± 0.2^{a}	5.2 ± 0.6^{b}	3.6 ± 0.2^{c}	4.2 ± 1.1^{b}	3.9 ± 0.1^{c}
C20 : 5 n-3	1.3 ± 0.2	1.5 ± 0.2	0.7 ± 0.1	1.1 ± 0.1	0.8 ± 0.1
C22 : 4 n-6	1.1 ± 0.1	0.7 ± 0.1	0.5 ± 0.2	0.6 ± 0.2	0.4 ± 0.1
C22 : 5 n-3	3.6 ± 0.1	3.7 ± 1.4	2.3 ± 1.1	3.9 ± 0.1	1.6 ± 1.1
C22 : 6 n-3	3.8 ± 0.5	5.5 ± 1.1	2.9 ± 1.5	4.1 ± 0.5	2.1 ± 0.3
Coronary artery smooth muscle cells					
total SFA	39.4 ± 1.4^{a}	37.6 ± 0.9^{b}	30.4 ± 2.3^{c}	38.6 ± 2.2^{ab}	27.3 ± 3.5^{c}
total MUFA	36.9 ± 1.8^{a}	34.3 ± 0.8^{a}	26.7 ± 2.1^{b}	33.5 ± 1.3^{a}	23.7 ± 2.3^{b}
total PUFA	23.8 ± 1.1^{c}	28.1 ± 1.5^{b}	42.9 ± 4.4^{a}	27.9 ± 3.4^{b}	48.9 ± 5.8^{a}
C14 : 0	1.9 ± 0.1	1.8 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.1 ± 0.1
C16 : 0	22.5 ± 1.0^{a}	21.1 ± 0.3^{a}	17.1 ± 1.3^{b}	21.5 ± 1.1^{a}	14.9 ± 2.2^{b}
C16 : 1 n-7+n-5	7.9 ± 1.2^{a}	7.6 ± 0.8^{a}	5.9 ± 0.7^{b}	7.3 ± 0.7^{a}	5.4 ± 0.4^{b}
C18 : 0	14.7 ± 0.6^{a}	14.5 ± 0.6^{a}	11.5 ± 1.0^{b}	15.1 ± 1.0^{a}	10.7 ± 1.3^{b}
C18 : 1 n-9+n-7	27.1 ± 1.2^{a}	25.3 ± 0.9^{a}	19.2 ± 0.9^{b}	24.5 ± 0.8^{a}	16.5 ± 1.7^{b}
C18 : 2 n-6	2.6 ± 0.5^{a}	3.7 ± 0.2^{a}	2.8 ± 0.1^{a}	2.6 ± 0.1^{a}	1.6 ± 0.1^{b}
*c*9t11 – CLA	< 0.1^{c}	3.8 ± 0.5^{b}	23.1 ± 4.1^{a}	< 0.1^{c}	< 0.1^{c}
*t*10c12 – CLA	< 0.1^{c}	< 0.1^{c}	< 0.1^{c}	4.6 ± 0.7^{b}	30.5 ± 5.9^{a}
C20 : 4 n-6	6.8 ± 0.3^{a}	5.2 ± 0.3^{b}	4.0 ± 0.1^{c}	5.8 ± 0.4^{ab}	4.6 ± 0.7^{bc}
C20 : 5 n-3	0.6 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
C22 : 4 n-6	2.0 ± 0.1	2.4 ± 0.9	2.1 ± 0.6	2.1 ± 0.3	1.8 ± 0.3
C22 : 5 n-3	3.0 ± 0.7	2.7 ± 0.7	2.4 ± 0.4	3.2 ± 0.5	2.7 ± 0.2
C22 : 6 n-3	3.3 ± 0.9	3.9 ± 1.0	3.6 ± 1.1	3.8 ± 1.5	2.7 ± 0.8

Results represent mean ± SD of three independent experiments with two repetitions in each experiment. Means within a row not sharing the same superscript letters differ significantly by Fisher´s multiple range test ($p < 0.05$).

types of SMCs. Incubating both SMC types with 5 or 50 µmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA resulted in a dose-dependent incorporation of the respective CLA isomers into total cell lipids when compared to control cells ($p < 0.05$). The proportions of CLA isomers in total cell lipids after 24 hours of incubation were similar between both SMC types. However, at the high concentration of CLA, the proportion of *cis*-9, *trans*-11 CLA was higher than the proportion of *trans*-10, *cis*-12 CLA in total lipids of aortic SMCs, whereas the opposite was found in coronary artery SMCs.

Treating both SMC types with increasing concentrations of CLA isomers decreased the proportions of total saturated fatty acids (SFAs) and total monounsaturated fatty acids (MUFAs) and increased the proportions of total polyunsaturated fatty acids (PUFAs) compared to controls ($p < 0.05$). However, the proportions of total MUFAs were significantly reduced only at the high concentration of CLA in SMCs of both vessels relative to controls. Treating both SMC types with *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA decreased the proportions of C14 : 0, C16 : 0, C16 : 1, C18 : 0, and C18 : 1 in total cell

Table II: Concentrations of PGE_2 and 6-keto $PGF_{1\alpha}$ in the medium of unstimulated and TNFα-stimulated human aortic and coronary artery smooth muscle cells incubated in the absence (control) or presence of 5 or 50 µmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA for 24 hours.

Treatment	TNFα 10 ng/mL	control	*cis*-9, *trans*-11 CLA 5 µmol/L	*cis*-9, *trans*-11 CLA 50 µmol/L	*trans*-10, *cis*-12 CLA 5 µmol/L	*trans*-10, *cis*-12 CLA 50 µmol/L
			nmol/g cell protein			
Aortic smooth muscle cells						
PGE^2						
	–	13.7 ± 4.1^{c}	9.7 ± 2.4^{d}	5.1 ± 0.7^{e}	7.8 ± 1.4^{d}	4.7 ± 1.7^{e}
	+	69.1 ± 6.6^{a}	66.8 ± 6.6^{a}	44.5 ± 6.8^{b}	67.6 ± 9.9^{a}	45.4 ± 5.4^{b}
6-keto $PGF_{1\alpha}$						
	–	1.4 ± 0.3^{d}	1.6 ± 0.5^{d}	1.1 ± 0.5^{d}	1.1 ± 0.3^{d}	0.6 ± 0.1^{e}
	+	15.7 ± 3.7^{a}	12.1 ± 2.5^{ab}	4.1 ± 1.5^{c}	9.1 ± 3.3^{b}	4.1 ± 1.8^{c}
Coronary artery smooth muscle cells						
PGE_2						
	–	158 ± 62^{d}	115 ± 29^{d}	63 ± 14^{e}	123 ± 12^{d}	66 ± 7^{e}
	+	987 ± 53^{a}	551 ± 105^{b}	254 ± 65^{c}	727 ± 184^{b}	209 ± 36^{cd}
6-keto $PGF_{1\alpha}$						
	–	73 ± 19^{d}	72 ± 17^{d}	46 ± 6^{e}	70 ± 11^{d}	25 ± 9^{f}
	+	847 ± 116^{a}	332 ± 97^{b}	195 ± 43^{c}	453 ± 153^{b}	146 ± 25^{e}

Results represent mean ± SD of three independent experiments with two repetitions in each experiment. Means within one eicosanoid of stimulated (+) and unstimulated (–) cells not sharing the same superscript letters differ significantly by Fisher´s multiple range test ($p < 0.05$).

lipids relative to those of controls ($p < 0.05$). The proportions of the eicosanoid precursor arachidonic acid (C20:4 n-6) were markedly reduced by treatment with either CLA isomer at 5 and 50 µmol/L in aortic and coronary artery SMC total lipids ($p < 0.05$). However, the mean reduction in the concentration of arachidonic acid by the high CLA concentration was more pronounced in aortic SMCs (56%) than in coronary artery SMCs (37%). Both CLA isomers were similarly potent in terms of reducing the proportion of arachidonic acid in total cell lipids in SMCs of both vessels. The proportions of the n-3 polyunsaturated fatty acids C20:5, C22:5, and C22:6 in SMC total lipids of both cell types did not differ from their respective controls.

Effect of CLA isomers on eicosanoid release from aortic and coronary artery SMCs under basal and cytokine-stimulated conditions

The effect of treatment of both SMC types with CLA isomers under basal and cytokine-stimulated conditions is shown in Table II. In general, under cytokine-stimulated conditions the release of 6-keto $PGF_{1\alpha}$ and PGE_2 from both types of SMCs was markedly higher than under basal conditions ($p < 0.05$); aortic and coronary artery SMCs had about 5- and 10-fold higher concentrations of PGE_2 and 6-keto $PGF_{1\alpha}$, respectively, in the incubation medium when incubated in the presence of TNFα (stimulated conditions) than in the absence of TNFα (basal conditions).

Differences in the action of CLAs were observed between SMCs under stimulated and basal conditions. Whereas inhibition of the release of 6-keto $PGF_{1\alpha}$ in stimulated SMCs of both vessels was observed at 5 and 50 µmol/L of each CLA isomer ($p < 0.05$), inhibition of the release of 6-keto $PGF_{1\alpha}$ in unstimulated cells was only observed at 50 µmol/L ($p < 0.05$). Similar results were obtained regarding the release of PGE_2 from coronary artery SMCs ($p < 0.05$). However, the opposite was observed in aortic SMCs; inhibition of the release of PGE_2 was observed at 5 and 50 µmol/L of CLA under basal conditions ($p < 0.05$), whilst under cytokine-induced conditions inhibition of the release of PGE_2 was only observed at 50 µmol/L of each CLA isomer ($p < 0.05$).

Moreover, cell type-specific differences were observed regarding the amount of eicosanoids released from SMCs. When related to the cell protein content, basal as well as cytokine-induced release of PGE_2 and 6-keto $PGF_{1\alpha}$ from coronary artery SMCs was about 13- and 50-fold, respectively, higher than the respective release from aortic SMCs. However, no cell type-specific differences were observed regarding the actions of CLAs. Both, under basal and cytokine-stimulated conditions, treatment of SMCs with either CLA isomer resulted in a dose-dependent reduction of the release of PGE_2 and 6-keto

$PGF_{1\alpha}$ ($p < 0.05$). Both CLA isomers exerted similar effects on the release of prostanoids. *Cis*-9, *trans*-11 CLA and t*rans*-10, *cis*-12 CLA dose-dependently lowered the release of PGE_2 from aortic and coronary SMCs, respectively, to similar concentrations in the incubation medium. In contrast, the release of 6-keto $PGF_{1\alpha}$ was more strongly reduced by treatment with *trans*-10, *cis*-12 CLA than with *cis*-9, *trans*-11 CLA; however, the latter effect was only observed under basal conditions ($p < 0.05$), whereas under cytokine-stimulated conditions 50 µmol/L of either CLA isomer was equally potent in terms of reducing the release of 6-keto $PGF_{1\alpha}$ in both SMC types.

Discussion

Although CLAs were repeatedly demonstrated to exert anti-atherogenic actions in various animal models of experimental atherosclerosis, the underlying mechanisms of action are still unclear. Vascular SMCs play a key role in atherogenesis and are the major cellular component of atherosclerotic plaques [6]. Nevertheless, until now little attention has been directed to the potential of CLAs to modulate SMC function. Only one recently published study of our group demonstrated that CLA isomers inhibit cytokine-induced release of vasoactive prostanoids from coronary artery SMCs [5]. Vascular SMCs from different locations, e.g., different blood vessels or different segments of the same blood vessel, were shown to exert differential actions in response to a common stimulus [11, 12]. Therefore, the present study aimed to explore potential differential effects of CLA isomers, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, on the release of vasoactive prostanoids from coronary artery and aortic SMCs. *Cis*-9, *trans*-11 CLA is the predominant isomer in natural foods [15], whereas t*rans*-10, *cis*-12 CLA is one of the main isomers in chemically produced CLA mixtures. Experiments were performed with two different concentrations of CLA (5 and 50 µmol/L), since plasma concentrations in humans vary greatly depending on nutritional habits and due to different isomeric distribution; e.g., plasma levels in non-vegetarians were reported to be in the range of 20 – 70 µmol/L containing about 80% *cis*-9, *trans*-11 CLA and about 10% *trans*-10, *cis*-12 CLA [19].

The results from the present study clearly demonstrate that different CLA isomers inhibit the release of prostanoids from vascular SMCs of different blood vessels. This suggests that the effects of CLA on SMC function in this regard are largely independent of the vascular location of SMCs, which is in contrast to findings from other studies showing that various stimuli (adenosine, 2-hydroxyestradiol) displayed highly differential, even opposing, effects on growth or functional properties of SMCs from coronary arteries compared to SMCs from the aorta [11, 12]. However, the effects of vasoactive mediators such as adenosine on SMC function largely depend on the distribution pattern of membrane-associated receptor subtypes (e.g., adenosine receptors), which vary considerably between SMCs from the coronary artery and the aorta [11]. In contrast, fatty acid analysis of total SMC lipids revealed a similar fatty acid composition as evidenced by similar proportions of total SFAs, total MUFAs, and total PUFAs for coronary artery and aortic SMCs following treatment with CLA. Therefore, in spite of slight differences regarding the incorporation of the two CLA isomers into total cell lipids as well as the reduction of arachidonic acid by CLA in the present study, similar proportions of total SFAs, total MUFAs, and total PUFAs in both SMC types after incubation with both isomers indicate that *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA display similar effects on fatty acid metabolism of SMCs from different vascular locations.

However, the present study provided some indications that the inhibitory potency of CLA on prostanoid release from SMCs is dependent on the (patho)physiological status of the blood vessel. Whereas inhibition of the release of 6-keto $PGF_{1\alpha}$ occurred in stimulated SMCs of both vessels even at the low concentration of CLA, this concentration had no effect in unstimulated cells. Similar results were obtained regarding the release of PGE_2 from coronary artery SMCs in the present study. Such divergent effects of CLA isomers on prostanoid formation between quiescent and stimulated cells have also been reported from others [20], and may be partially explained by different inhibitory potencies of CLA isomers on gene expression and/or enzyme activity of cyclooxygenase (COX) isoforms. COX catalyzes conversion of arachidonic acid into PGH_2, which is the rate-limiting step in the formation of prostanoids. PGH_2 is then further converted by various isomerases and synthases into the biologically active prostaglandins. Various CLA isomers as well as CLA metabolites were reported to inhibit COX activity or gene expression [2, 4, 21, 22]. From the present observations it appears that COX-2, which is the major source of prostanoid production under cytokine-induced conditions, is more effectively inhibited by CLA isomers in vascular SMCs than COX-1, which is largely responsible for prostanoid formation under basal conditions. This assumption is supported by recent findings that *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA were effective at suppressing COX-2 gene expression in aortic endothelial cells and vascular SMCs, respectively, whereas both isomers failed to inhibit COX-1 mRNA expression in these cells [4, 5]. This suggests that the inhibitory potential of

CLA isomers on the release of prostanoids from SMCs is dependent on the (patho)physiological status of the cells; e.g., basal conditions reflecting the situation in healthy blood vessels or cytokine-stimulated conditions reflecting the situation in atherosclerotic blood vessels. Surprisingly, in aortic SMCs, contradictory observations have been made herein with respect to the release of PGE_2, since inhibition of the release of PGE_2 occurred in unstimulated aortic SMCs even at the low concentration of CLA, whereas this concentration had no effect under stimulated conditions. Although this contradictory finding cannot be explained at the moment, this suggests that the effect of CLA isomers on prostanoid formation is, at least in this specific case, dependent on the vascular location of SMCs. Thus, future studies are required to resolve this contradictory observation.

Since arachidonic acid serves as the main substrate for the biosynthesis of eicosanoids via COX, the marked reduction of arachidonic acid by CLA may largely account for the reduced release of prostanoids from coronary artery and aortic SMCs under basal conditions. The reduced arachidonic acid concentrations are presumably largely due to competition of CLA with other fatty acids for the incorporation into membrane phospholipids but also due to interference with the production of arachidonic acid from linoleic acid [4, 23]. Noteworthy, the decline in arachidonate levels was already observed at the low concentration of CLAs in both types of vascular SMCs, with a more pronounced effect being observed in aortic SMCs, indicating that these fatty acids effectively displace arachidonic acid from membrane phospholipids and/or inhibit Δ5- and Δ6-desaturation of linoleic acid. The reduction of arachidonic acid is probably also primarily responsible for the reduced prostanoid release from SMCs by CLAs under cytokine-stimulated conditions. We have previously shown that PPARγ-mediated inhibition of NF-κB activation is partially involved in the reduction of cytokine-induced release of prostanoids by CLAs [5]. Since inhibition of cytokine-induced release of prostanoids by CLAs was not completely abrogated by simultaneous treatment with a synthetic PPARγ antagonist [5], we suggest that reduction of arachidonic acid by CLAs mainly accounts for the reduced release of prostanoids rather than inhibition of NF-κB.

Although CLAs have been reported to exert several isomer-specific effects [24 – 26], the present study provided only little evidence (e.g., the inhibitory effect on the release of 6-keto $PGF_{1\alpha}$ from unstimulated SMCs was more pronounced with *trans*-10, *cis*-12 CLA) confirming this in vascular SMC. Nevertheless, the inhibitory effect of CLAs on eicosanoid release as observed in the present study is consistent with reports from several cell culture and animal feeding experiments using either mixtures or single isomers of CLA [2 – 4, 20, 27, 28]. Moreover, similar to our observations, studies using single isomers of CLA revealed inhibitory actions on prostanoid production for both isomers (*cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) [3, 4, 20]. This indicates that the reduction of eicosanoid production by CLAs is largely independent of the structure of the individual isomer. In contrast to the inhibitory effect on eicosanoid release, CLA was demonstrated by a Swedish group to increase prostaglandin formation (urinary excretion of 8-iso-$PGF_{2\alpha}$ and 15K-DH-$PGF_{2\alpha}$) as a consequence of free radical-induced and COX-catalyzed arachidonic acid oxidation in several independent human studies [29 – 31]. This increase in lipid peroxidation has been observed after supplementation with CLA mixtures [31], *trans*-10, *cis*-12 CLA [30], and *cis*-9, *trans*-11 CLA [29]. Similar findings have been recently published for the apoE-deficient mouse model of atherosclerosis. However, in that study plasma levels of 8-iso-$PGF_{2\alpha}$ were only increased in response to *trans*-10, *cis*-12 CLA, but not in response to *cis*-9, *trans*-11 CLA [32]. In contrast, feeding a 80 : 20-mixture of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA did not alter urinary eicosanoid excretion in the same mouse model [33]. The reasons for these conflicting data are currently unknown, but the use of different formulations of CLAs, heterogenous blends of CLA isomers, different doses, species differences, and the metabolic status of the experimental model may be causative. These reasons may also explain the apparently conflicting results of CLAs on experimental atherosclerosis [32 – 35]. Divergent results have been reported with respect to the outcome (no effect [36], progression [35], or inhibition [32, 33] of atherosclerosis), the CLA source (anti-atherogenic effects have been observed with *cis*-9, *trans*-11 CLA [32], *trans*-10, *cis*-12 CLA [34], and a CLA mixture [33]) as well as the anti-atherogenic mechanisms ascribed to CLAs. One of these possible anti-atherogenic mechanisms might be the observed inhibitory action of CLAs on prostaglandin formation in SMCs, since increased prostaglandin synthesis is associated with atherosclerosis [37], and inhibition of COX has been shown to improve endothelial dysfunction associated with atherosclerosis [38]. Although this inhibitory mechanism alone is insufficient to explain the complex and multiple effects of CLAs on vascular homeostasis, we suggest that this anti-inflammatory effect of CLAs partially contributes to the anti-atherogenic effects of CLAs observed in rabbits, mice, and hamsters.

In summary, the present study demonstrates that physiologically relevant levels of *cis*-9, *trans*-11 CLA and t*rans*-10, *cis*-12 CLA are capable of reducing the release of prostanoids from vascular SMCs originating from different blood vessels. Moreover, the present study shows that the inhibitory potential of CLA isomers on the re-

lease of prostanoids is slightly different between unstimulated and cytokine-stimulated SMCs. These findings suggest that the action of CLA isomers on the release of vasoactive prostanoids from vascular SMCs is largely independent of the vascular location; e.g., coronary arteries or systemic vasculature (aorta), but partially depends on the pathophysiological status of SMCs.

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Conjugated linoleic acid isomers inhibit platelet-derived growth factor-induced NF-κB transactivation and collagen formation in human vascular smooth muscle cells

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Abstract *Background* Atherosclerosis is characterized by extensive thickening of the arterial intima partially resulting from deposition of collagen by vascular smooth muscle cells (SMCs). Polyunsaturated fatty acids stimulate collagen formation through NF-κB activation. *Aim of the study* The present study aimed to explore the effect of conjugated linoleic acids (CLAs) which are known to inhibit NF-κB activation on collagen formation by SMCs. *Methods* Vascular SMCs were cultured with 50 μmol/l of CLA isomers (c9t11-CLA, t10c12-CLA) or linoleic acid (LA) and analysed for collagen formation and NF-κB p50 transactivation. *Results* Treatment with CLA isomers but not LA significantly reduced PDGF-stimulated [^{3}H] proline incorporation into cell layer protein of SMCs without altering cell proliferation. Simultaneous treatment with the PPARγ inhibitor T0070907 abrogated this effect. Treatment of SMCs with c9t11-CLA and t10c12-CLA significantly reduced PDGF-induced NF-κB p50 activation. *Conclusions* CLA isomers inhibit PDGF-stimulated collagen production by vascular SMCs, which is considered to be a hallmark of atherosclerosis, in a PPARγ-dependent manner. Whether inhibition of the NF-κB-pathway is of significance for the reduction of collagen formation by CLA isomers needs further investigation.

Key words conjugated linoleic acid – atherosclerosis – collagen formation – vascular smooth muscle cells – PPARγ

Introduction

Atherosclerosis is characterized by an extensive thickening of the arterial intima, which is the result of the deposition of collagen and other extracellular matrix proteins, lipids, and minerals ultimately leading to the formation of atherosclerotic plaques. The major component of these atherosclerotic plaques is collagen representing up to 60% of the total protein present [16]. Recent studies demonstrated that polyunsaturated fatty acids stimulate collagen formation in fibroblasts through the activation of the redox-sensitive transcription factor nuclear factor-kappaB (NF-κB) and by altering the expression of NF-κB pathway-specific genes and pro-inflammatory target genes such as chemokines and cytokines [12, 14]. Correspondingly, inhibition of NF-κB by parthenolide completely blocked the polyunsaturated fatty acid-induced collagen formation in fibroblasts [14]. Studies in vascular smooth muscle cells (SMC) also implicated a redox-sensitive pathway as the principle mechanism by which oxidized LDL stimulates collagen formation [15].

Conjugated linoleic acids (CLA), a naturally occurring group of positional and geometric isomers of linoleic acid (LA), were demonstrated to exert

potent anti-atherogenic actions in animal models of experimental atherosclerosis without exact knowledge about the underlying mechanisms [18, 35, 36]. It has been demonstrated that treatment with CLA inhibits stimulus-induced NF-κB activation in various cell types including vascular SMCs via a peroxisome proliferator-activated receptor γ (PPARγ)-dependent mechanism [7, 28, 40], suggesting that the anti-inflammatory action of CLA is at least partially involved in the atheroprotective properties of CLA. However, whether the inhibitory action of CLA on stimulus-induced NF-κB activation is also of significance with respect to collagen formation is currently unknown. To our knowledge studies investigating the effect of CLA on collagen formation are not available from the literature.

Therefore, since collagen deposition is considered to be a hallmark of atherosclerosis and in order of elucidating the anti-atherogenic actions of CLA, the present study aimed to explore the effect of CLA isomers on collagen formation in vascular SMCs. Vascular SMCs are an appropriate cell culture model in this context because this cell type is the major source of extracellular protein production within the vessel wall [2]. Since during atherosclerosis development mitogenic signals but also cytokines such as TNFα promote phenotypic modulation of SMCs which leads to SMC migration into the intima and extensive production of collagen [5], the modulatory potential of CLA on collagen formation was studied in the presence of the mitogen platelet-derived growth factor (PDGF). In order of elucidating the effect of CLA on NF-κB-pathway activation we measured DNA-binding of NF-κB subunit p50 (NF-κB p50), and IκBα phosphorylation. In order to address whether PPARγ-signalling is involved in the potential modulation of collagen formation by CLA, we performed the experiments in the presence and absence of T0070907, a potent and selective antagonist of human PPARγ. Since differential effects of a common stimulus on growth or functional properties of vascular SMCs cultured from different vascular beds as well as from different sections of a common blood vessel are well established [31, 41], we used vascular SMCs from two different blood vessels (aorta and coronary artery). As isomers *cis*-9,*trans*-11-CLA (c9t11-CLA), which contributes to more than 90% of total CLA in natural foods such as milk, dairy products, and meat of ruminants [32], and *trans*-10,*cis*-12-CLA (t10c12-CLA), which is one of the main isomers in chemically produced CLA mixtures, were used. LA was used as reference fatty acid. The concentration of fatty acids (50 μmol/l) chosen for cell experiments was largely based on observations from previous cell culture studies demonstrating significant accumulation of CLA in cell lipids and/or induction of potent biological activities [10, 28, 30, 37, 39].

Methods

Chemicals

CLA isomers, c9t11-CLA (≥96% pure) and t10c12-CLA (≥98% pure), were obtained from Cayman Chemical (Ann Arbor, MI, USA). LA (≥99% pure) was purchased from Sigma-Aldrich (Taufkirchen, Germany). Preparation of the stock solutions of fatty acids and of the test media was performed as described previously [10]. In order to avoid oxidation of fatty acids during preparation of stock solutions conversion of fatty acids into their sodium salts was performed after the vials had been thoroughly flushed with nitrogen and under protection from direct light. In addition, fatty acid sodium salts were kept on ice until their addition to the culture medium. Tritium-labelled proline ([2,3-^{3}H] proline) was purchased from Amersham (Buckinghamshire, UK). MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue), PDGF-AB, BSA (bovine serum albumin), and TNFα, were purchased from Sigma-Aldrich. Trichloracetate (TCA) was purchased from Fluka (Buchs, Switzerland). The PPARγ antagonist T0070907 was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Cell culture

Human aortic smooth muscle cells, from a 35-year-old female donor, and human coronary artery smooth muscle cells, from a 40-year-old male caucasian donor, were obtained from PromoCell (Heidelberg, Germany) and cultured in SMC growth medium 2 containing 5% fetal calf serum, 0.5 μg/l epidermal growth factor, 2.0 μg/l basic fibroblast growth factor, 5 mg/l insulin, 50 mg/l gentamicinsulfate and 50 μg/l amphotericin B (all from PromoCell). Cells were passaged after reaching confluence by using trypsin/EDTA. After trypsinization, TNS was added to prevent enzymatic damage to the cells. Only cells from passages 3–8 were used for experiments.

Cell viability

Cell viability after treatment of HAoSMCs and HCASMCs with 50 μmol/l of either c9t11-CLA, t10c12-CLA or LA was examined by the MTT assay [38].

Cell proliferation assay

Cell proliferation was assessed using the Biotrak cell proliferation ELISA system (Amersham, Buckinghamshire, UK) and performed according to the manufacturers protocol. In brief, cells were seeded in 96-well microtitre plates and, after reaching 70–80%

confluence, incubated for 24 hours with labelling medium [100 μM BrdU (5-bromo-2′-deoxyuridine BrdU)] and 50 μmol/l of fatty acids (c9t11-CLA, t10c12-CLA or LA) in the presence of PDGF (10 ng/ml). Cells incubated with labelling medium and PDGF were used as stimulated controls. Cells incubated with labelling medium alone were used as unstimulated controls. At the end of the labelling period, cells were fixed and DNA denatured using a fixative, which was followed by a 30 minutes blocking step. After removing the blocking buffer cells were subsequently incubated with peroxidase-labelled anti-BrdU for 90 minutes at room temperature. The immune complex was detected by the subsequent reaction with TMB and reading the resultant colour at 450 nm in a microtitre plate spectrophotometer.

Collagen formation assay

To assess the effect of treatment on collagen synthesis, we examined the incorporation of [^{3}H] proline into TCA-precipitable proteins according to the method of Dubey et al. [9]. For each experiment SMCs were cultured to 70–80% confluence in 24-well plates and treated with PDGF and fatty acids (c9t11-CLA, t10c12-CLA or LA) for 24 hours. For experiments using the PPARγ antagonist T0070907, treatment of cells with 1 μmol/l of T0070907 was performed simultaneously. Cells treated with PDGF only (10 ng/ml) were used as controls ("stimulated controls"). Cells treated without PDGF and without fatty acids were used as "unstimulated controls". After incubation medium was changed to fresh medium and cells were labelled with [2,3-^{3}H] proline (10 μCi/well) for 24 hours. Afterwards medium was removed, the cell layer was washed twice with PBS, and the cell layer scraped of in ice-cold 10% TCA. Precipitated protein was collected by centrifugation at 14,000 g for 20 minutes. The cell layer precipitate was solubilized in 500 μL of 0.3 N NaOH/0.1% SDS at 37°C for 1 hour, and transferred to scintillation vials. Scintillation fluid (Rotiszint eco plus, Carl Roth, Karlsruhe, Germany) was added, and radioactivity determined in a liquid scintillation counter (Tri-Carb 2100TR, Packard, Meriden, CT, USA).

NF-κB p50 DNA-binding activity

For the measurement of NF-κB p50 transactivity, cells were seeded in 25 cm^2 tissue culture flasks and incubated with fatty acids (c9t11-CLA, t10c12-CLA) in the presence or absence of TNFα (10 ng/ml) or PDGF (10 ng/ml) for 8 hours. Additionally, simultaneous incubations with 1 μmol/l of the PPARγ inhibitor T0070907 were also performed. Afterwards nuclear extracts were prepared with a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol and protein concentrations were determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) with BSA as standard. NF-κB transactivity in the nuclear extracts was determined by the Transcription Factor assay TransAM^{TM} NF-κB p50 (Active Motif).

Immunoblot analysis of phosphorylated form of IκBα (p-IκBα)

For immunoblotting, coronary artery SMCs were plated in 6-well plates and cultured as described above. Cytoplasmic fractions of cells were obtained using the Nuclear Extract Kit from Active Motif according to the manufacturer's protocol, and protein concentrations determined by the BCA assay. Equal amounts of cytoplasmic protein (45 μg) were electrophoresed by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. Loading of equal amounts of protein in each line was verified by Panceau S staining. The membranes were blocked overnight at 4°C in 5% Blotto (5% skim milk in phosphate-buffered saline containing 0.1% Tween), and then incubated with a mouse monoclonal anti-p-IκBα primary antibody (Active Motif) for 2 hours at room temperature. Membranes were washed with 1% Blotto, and incubated with a HRP conjugated secondary antibody anti-mouse IgG (Amersham Biosciences) for 1 hour at room temperature. Afterwards blots were washed again, and bands corresponding to p-IκBα visualized by chemiluminescence (ECL Plus, Amersham Biosciences). The signal intensities were detected with bio-Imaging system (Biostep) and quantified using TotalLab TL100-Quick Start analysis software (nonlinear dynamics).

Statistical analysis

Data were subjected to ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). For statistically significant F values, individual means of the treatment groups were compared by Tukey's test. Means were considered significantly different for $P < 0.05$. Significant effects are denoted with superscript letters. Bars marked with different superscript letters significantly differ.

Results

Treatment with CLA isomers has no effect on the viability of vascular SMCs

Viability of aortic and coronary SMCs was not affected by treatment with 50 μmol/l of c9t11-CLA, t10c12-CLA or LA for 21 hours followed by a 3 hours

treatment with TNFα (10 ng/ml) in the presence of the same fatty acids relative to unstimulated controls; cell viabilities were between 93 and 106% for aortic SMCs and 92 and 105% for coronary artery SMCs, respectively, relative to unstimulated controls (=100%).

Treatment with CLA isomers has no effect on mitogen-induced proliferation of vascular SMCs

Incubation with the mitogen PDGF significantly stimulated proliferation of aortic and coronary artery SMCs compared to unstimulated controls ($P < 0.05$; Fig. 1). However, treatment of both SMC types with 50 μmol/l of either c9t11-CLA, t10c12-CLA or LA did not modulate PDGF-stimulated cell proliferation.

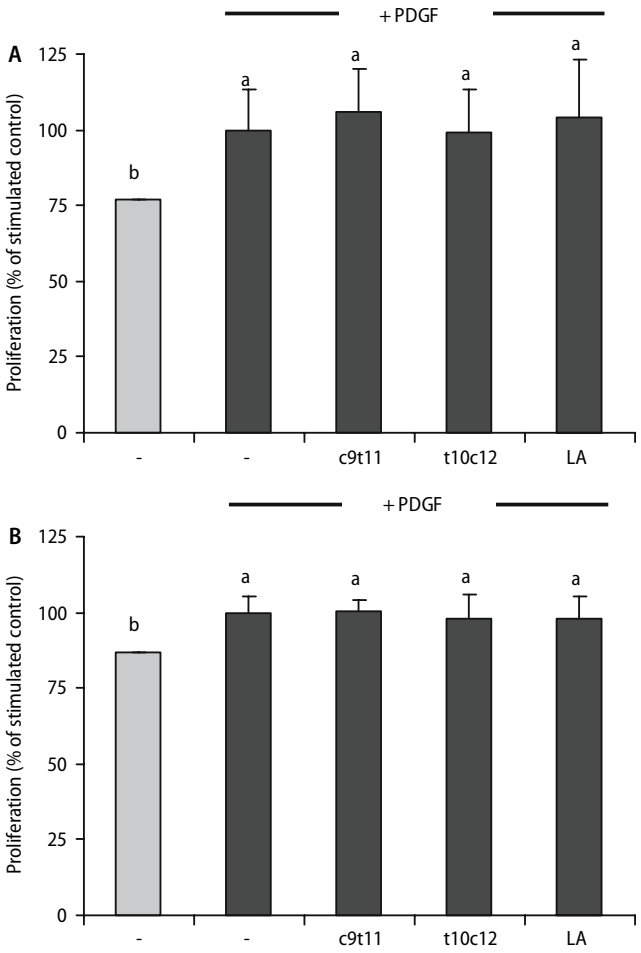

Fig. 1 Effect of treatment for 24 hours with 50 μmol/l of either c9t11-CLA (c9t11), t10c12-CLA (t10c12) or linoleic acid (LA) on PDGF (10 ng/ml)-induced proliferation of aortic SMCs (**A**) and coronary artery SMCs (**B**). Cells treated with PDGF only for 24 hours were used as controls (stimulated controls). Cells treated with medium only for 24 hours were used as unstimulated controls (*grey bars*). Data represent mean ± SD of three independent experiments and are expressed as percentage of cell proliferation of stimulated controls (=100%). Results from statistical analysis (one-way ANOVA followed by Tukey's test) are indicated: Significant effects are denoted with superscript letters. *Bars* marked with different superscript letters significantly differ ($P < 0.05$)

Treatment with CLA isomers reduces mitogen-induced [^{3}H] proline incorporation into cell layer protein of vascular SMCs in a PPARγ-dependent manner

[^{3}H] proline incorporation into the cell layer protein of aortic and coronary artery SMCs was markedly induced by treatment with PDGF when compared to unstimulated controls ($P < 0.05$; Fig. 2A, B). Treat-

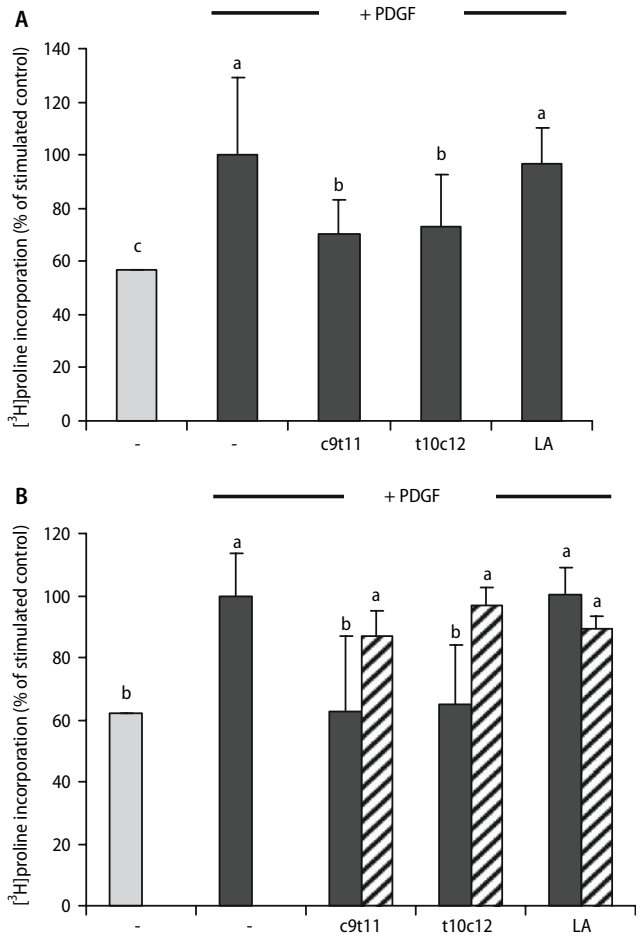

Fig. 2 Effect of treatment for 24 hours with 50 μmol/l of either c9t11-CLA (c9t11), t10c12-CLA (t10c12) or linoleic acid (LA) on PDGF (10 ng/ml)-induced [^{3}H] proline incorporation into the cell layer protein of aortic SMCs (**A**) and coronary artery SMCs (**B**). Effect of simultaneous treatment with 1 μmol/l of the PPARγ antagonist T0070907 is also shown (*shaded bars*). Cells treated with PDGF only for 24 hours were used as controls (stimulated controls). Cells treated with medium only for 24 hours were used as unstimulated controls (*grey bars*). Data represent mean ± SD of three independent experiments and are expressed as percentage of [^{3}H] proline incorporation of stimulated controls (=100%). Results from statistical analysis (one-way ANOVA followed by Tukey's test) are indicated: Significant effects are denoted with superscript letters. *Bars* marked with different superscript letters significantly differ ($P < 0.05$)

ment with 50 μmol/l of either c9t11-CLA or t10c12-CLA significantly reduced PDGF-stimulated [^{3}H] proline incorporation into cell layer protein of aortic and coronary artery SMCs compared to stimulated controls ($P < 0.05$), whereas treatment with 50 μmol/l of LA revealed no effect.

Simultaneous treatment of coronary artery SMCs with 1 μmol/l of the PPARγ inhibitor T0070907 abrogated the inhibitory effect of c9t11-CLA and t10c12-CLA on PDGF-stimulated [^{3}H] proline incorporation into the cell layer protein (Fig. 2B).

Treatment with t10c12-CLA reduces TNFα-induced NF-κB p50 transactivation and treatment with c9t11-CLA and t10c12-CLA reduces PDGF-induced NF-κB p50 transactivation in vascular SMCs

Treatment of coronary artery SMCs with TNFα or PDGF markedly induced DNA-binding of NF-κB p50 compared to unstimulated control treatment ($P < 0.05$; Fig. 3A and B). Incubation of cells with 50 μmol/l of t10c12-CLA significantly reduced TNFα-induced DNA-binding of NF-κB p50 relative to stimulated controls ($P < 0.05$), whereas c9t11-CLA had no effect. Simultaneous treatment of coronary artery SMCs with the PPARγ inhibitor T0070907 completely abolished the inhibitory effect of t10c12-CLA on TNFα-stimulated DNA-binding of NF-κB p50. In addition, treatment of cells with 50 μmol/l of c9t11-CLA and t10c12-CLA but not with LA significantly reduced PDGF-induced DNA-binding of NF-κB p50 relative to stimulated controls ($P < 0.05$). Co-treatment of coronary artery SMCs with the PPARγ inhibitor T0070907 only partially abrogated the inhibitory effect of c9t11-CLA and t10c12-CLA on PDGF-stimulated DNA-binding of NF-κB p50.

Furthermore, immunoblot analysis revealed that treatment of coronary artery SMCs with 50 μmol/l of c9t11-CLA and t10c12-CLA but not with LA inhibited PDGF-induced protein concentration of p-IκBα (Fig. 3C).

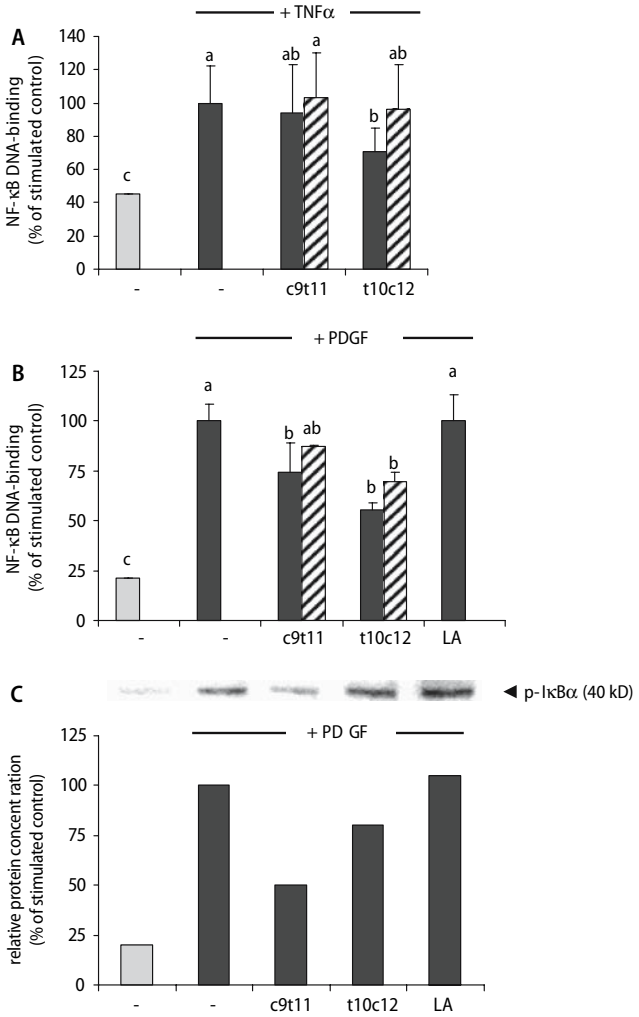

Fig. 3 Inhibitory effect of CLA isomers on stimulus-induced NF-κB activation. **A–B** Effect of treatment with 50 μmol/l of either c9t11-CLA (c9t11), t10c12-CLA (t10c12) or linoleic acid (LA) for 8 hours on TNFα (**A**)- and PDGF (**B**)-induced transactivation of the NF-κB subunit p50 in coronary artery SMCs. Effect of simultaneous treatment with 1 μmol/l of the PPARγ antagonist T0070907 is also shown (*shaded bars*). Cells treated with TNFα (10 ng/ml) or PDGF (10 ng/ml) alone for 8 hours were used as controls (stimulated controls). Cells treated with medium only for 8 hours were used as unstimulated controls (*grey bars*). Data represent mean ± SD of three independent experiments and are expressed as percentage of NF-κB p50 transactivation of stimulated controls (=100%). Results from statistical analysis (one-way ANOVA followed by Tukey's test) are indicated: Significant effects are denoted with superscript letters. *Bars* marked with different superscript letters significantly differ ($P < 0.05$). **C** Effect of treatment with 50 μmol/l of either c9t11-CLA (c9t11), t10c12-CLA (t10c12) or linoleic acid (LA) for 4 hours on PDGF (10 ng/ml)-induced protein concentration of phosphorylated form of IκBα (p-IκBα) in coronary artery SMCs. Representative immunoblot and corresponding densitometric analysis of bands specific to p-IκBα are shown for one independent experiment.

Discussion

CLA are well-known to exert potent anti-atherogenic actions in animal models of atherosclerosis [18, 35, 36]. However, due to the rather descriptive nature of these in vivo-studies the mechanisms of action underlying the anti-atherogenic properties of CLA are only partially understood. Based on cell culture experiments, which are appropriate to provide a more mechanistic inside, some mechanisms of action of CLA such as activation of PPARγ-signalling and inhibition of proinflammatory mediator release have already been proposed [7, 28, 40]. In an attempt to address further potentially novel anti-atherogenic mechanisms of action we focused on collagen formation by vascular SMCs which is considered to be a hallmark in atherosclerosis development since it

significantly contributes to intimal thickening and, finally, atherosclerotic plaque formation [16]. Following a cell-based approach we used vascular SMCs from human aorta and coronary arteries as a suitable model for studying the modulatory potential of CLA on this critical step in atherosclerosis and investigated the effect of two different CLA isomers on collagen production using [^{3}H] proline incorporation. Since the major synthetic destination of proline is collagen, the incorporation of proline into newly synthesized protein provides a reliable index of collagen synthesis [19]. Herein, we could demonstrate for the first time that PDGF-stimulated collagen production by vascular SMCs was markedly inhibited in the presence of the CLA isomers c9t11-CLA and t10c12-CLA. This finding possibly explains, at least in part, the observation from in vivo-studies that CLA reduces atherosclerotic plaque development using animal models of atherosclerosis [18, 35, 36]. In contrast, treatment with LA as a reference fatty acid had no effect on PDGF-stimulated [^{3}H] proline incorporation. This indicates that the inhibitory effect of CLA on collagen production by vascular SMCs is dependent on the specific conjugated dienoic structure of CLA.

Considering that the amounts of total and collagenous protein largely depend on cell growth and cell number, we also investigated the effects of treatment on cell kinetics and cell viability. Effects of treatment with CLA isomers on cell kinetics (proliferation) were determined by estimating cell proliferation from incorporation of 5-bromo-2′-deoxyuridine as a marker of DNA replication. There was no significant difference in cell proliferation between SMCs treated with CLA isomers, LA or PDGF only, confirming that the decreased collagen synthesis by treatment with CLA was not due to changes in cell proliferation or cell number. The finding that the proliferative response of coronary artery SMCs to PDGF stimulation was rather weak is largely explained by the presence of serum containing several factors stimulating cell proliferation during incubation. However, we decided to perform these experiments in the presence of serum because it represents a more physiological situation for the SMCs than the absence of serum. Nevertheless, the proliferative response to PDGF was sufficient to study potential alterations in cell proliferation by CLA. To exclude the possibility that the reduction in [^{3}H] proline incorporation was due to cell death, an MTT assay was performed. CLA isomers as well as LA had no effect on cell viability, thus excluding cytotoxicity as a cause for the reduction in the amounts of collagen by CLA.

Vascular injury as the initial event of atherosclerosis is correlated with nuclear localization of NF-κB. In its latent form, NF-κB exists in the cytosol of unstimulated cells bound to the inhibitory proteins IκBs. Upon activation by cytokines, mitogens or oxidative stress, NF-κB dissociates from the IκBs and translocates to the nucleus where it regulates the expression of genes involved in inflammation [8]. It has been demonstrated that n-6 and n-3 polyunsaturated fatty acids such as arachidonic acid and eicosapentaenoic acid stimulate collagen formation in fibroblasts through the activation of the NF-κB-pathway and NF-κB target gene expression [12, 14]. This is probably explained by the fact that the promoter of the COL1A2 gene which encodes the α2 chain of type I collagen contains at least 2 putative NF-κB binding sites [4]. In addition, the COL1A1 gene which encodes the α1 chain of type I collagen is probably also induced by NF-κB, because both COL1A1 and COL1A2 are highly sensitive to reactive oxygen species (ROS) [26, 27], which are major factors inducing the phosphorylation of IκB and subsequent translocation of NF-κB [1]. Thus, we hypothesized that inhibition of NF-κB transactivation by CLA would inhibit collagen formation in vascular SMCs. Inhibition of stimulus-induced NF-κB transactivation by CLA has been observed in several cell culture models [7, 40]. Similarly, our NF-κB transactivation experiments in the present study revealed that c9t11-CLA and t10c12-CLA inhibit PDGF-induced DNA-binding activity of NF-κB p50 in vascular SMCs. In addition, t10c12-CLA also inhibited TNFα-induced DNA-binding activity of NF-κB p50. We have no explanation that c9t11-CLA was capable of reducing transactivation of NF-κB when stimulated with PDGF but not when stimulated with TNFα. However, since we have recently shown that both CLA isomers decreased transactivation of the p65 subunit of NF-κB [28], which forms transcriptionally active heterodimers with the p50 subunit, we suggest that c9t11-CLA is also a potent inhibitor of NF-κB transactivation in vascular SMCs. Supportive of this assumption is the observation of our recent study, that both CLA isomers strongly reduced TNFα-induced mRNA concentrations of NF-κB target genes cyclooxygenase-2 and cytosolic phospholipase A_2 in vascular SMCs [28]. Although, we did not study the effect of CLA on collagen production during inhibition of the NF-κB-pathway, the present results suggest that NF-κB inhibition is possibly involved in the inhibitory effect of CLA on collagen production. Future experiments using selective inhibitors of the NF-κB-pathway like parthenolide, which blocks polyubiquitination and degradation of the IκB complex, should clarify this question.

To further investigate the molecular mechanism underlying the inhibition of NF-κB transactivation by CLA in vascular SMCs, we determined relative protein concentrations of the phosphorylated form of IκBα, p-

IκBα. IκBα phosphorylation in response to different stimuli (mitogens, cytokines, and ROS) results in the degradation of IκBα and subsequent release of activated NF-κB, which translocates into the nucleus and increases transcription of NF-κB target genes. Hence, inhibition of IκBα phosphorylation results in a reduced DNA-binding of activated NF-κB. Thus, the finding from the present study that both CLA isomers, in particular c9t11-CLA, decreased PDGF-induced protein concentrations of p-IκBα indicates that the inhibitory effect of CLA on NF-κB activation is at least partially due to inhibition of IκBα phosphorylation. Similar findings have also been reported from other groups using different experimental models [13, 22, 24]. According to these reports inhibition of phosphorylation of IκBα by CLA is mediated by blocking IκB kinase (IKK)- and Akt-, a serine/threonine kinase, signalling [13, 22, 24]. Future studies have to show whether these signalling pathways are also blocked by CLA in vascular SMCs.

Since recent studies demonstrated that PPARγ agonists inhibit collagen synthesis from myofibroblasts, activated hepatic stellate cells (HSC), and vascular SMCs [3, 11, 42], we further investigated the effect of CLA isomers on SMC collagen formation in the presence of the synthetic PPARγ antagonist T0070907. The present study clearly demonstrated that the inhibitory effect of CLA isomers on collagen formation could be abrogated by T0070907 indicating that PPARγ-signalling is indeed involved in the inhibitory effect of CLA on SMC collagen formation. Inhibition of collagen synthesis by PPARγ activators from myofibroblasts, HSCs, and vascular SMCs has been shown to involve interruption of TGF-β signalling [3, 11, 42] by directly interfering with the Smad3-signalling pathway [11]. TGF-β is a potent stimulator of collagen production through the induction of connective tissue growth factor (CTGF) gene expression and PDGF production, and both, CTGF and PDGF, are potent stimulators of collagen production. Since it has been further demonstrated that the suppression of TGF-β-signalling and gene expression of CTGF is mediated through reducing oxidative stress [42], and NF-κB has been described as the primary mediator of oxidative stress it appears to be possible that the reduced collagen production by CLA in vascular SMCs is due to the observed PPARγ-dependent inhibition of NF-κB. In line with this assumption is the finding that inhibition of NF-κB activity by the antioxidant (-)-epigallocatechin-3-gallate in activated HSCs was accompanied by the interruption of TGF-β signal transduction and a reduced collagen production [5]. Moreover, the PPARγ activator 15-d-PGJ2, which reduced TGF-β-induced CTGF expression and collagen formation in human aortic SMCs [11], was also reported to inhibit NF-κB [33]. In addition, direct inhibition of NF-κB transactivation by PPARγ activation [22] without involving TGF-β signalling, might also be causative for the reduction of collagen biosynthesis by CLA, because the COL1A2 gene is a NF-κB target gene as mentioned above. Nevertheless, since we did not address the exact molecular link between the CLA-mediated inhibition of NF-κB and SMC collagen synthesis herein and corresponding studies dealing with vascular SMCs are not available from the literature further research is required to support our hypothesis.

Due to the essential role of PPARγ for inhibition of collagen formation [11] it was not surprising that LA, which is a relatively weak activator of PPARγ compared with CLA, did not reduce collagen formation from vascular SMCs. However, another reason partially explaining the lack of effect of LA on collagen formation might be that CLA, in contrast to LA, markedly reduces membrane concentrations of arachidonic acid due to displacement of arachidonic acid from membrane phospholipids and inhibits metabolism of arachidonic acid by COX and lipoxygenase enzymes to biologically active metabolites (prostaglandins, thromboxans, leukotrienes) in vascular SMCs and various other cell types [10, 17, 28, 29]. Because these metabolites are supposed to be mediators of pathological fibrotic conditions increasing the formation of collagen by stimulating profibrotic factors such as TGF-β1 [21], antagonism of specific eicosanoid receptors or selective inhibition of COX-2 resulted in attenuation of fibrosis under different pathological conditions concomitant with a decrease in TGF-β1 [20, 25, 34]. Thus, we propose that inhibition of arachidonic acid metabolism by CLA beside PPARγ activation might also contribute to the reduced collagen formation by CLA and might largely explain the differential action of CLA compared to LA in this cell system.

Similar observations regarding the effect of CLA on collagen formation and NF-κB target gene expression in aortic and coronary artery SMCs suggest that the effects of CLA on collagen production are largely independent of the vascular location of SMCs (aorta vs. coronary artery) which is in contrast to findings from other studies showing that various stimuli such as adenosine or 2-hydroxyestradiol displayed differential, even opposing, effects on growth or functional properties of SMCs from coronary arteries compared to SMCs from the aorta [31, 41]. However, this discrepancy might be explained by the fact that uptake and concentrations of CLA are similar in SMCs from different vascular locations after treatment with CLA as observed in a recent study of our group [29], whereas the effects of adenosine on SMC function largely depend on the distribution pattern of membrane-associated receptor subtypes (e.g. adenosine

receptors) which vary considerably between SMCs from the coronary artery and the aorta [31].

In summary, the present study revealed for the first time that the CLA isomers c9t11-CLA and t10c12-CLA, but not LA, inhibited PDGF-stimulated collagen production by vascular SMCs. Moreover, the study showed that simultaneous treatment with a PPARγ antagonist abrogated the inhibitory effect of CLA isomers on SMC collagen formation, indicating that PPARγ-signalling might be involved in the mediation of this cellular effect of CLA. This might also explain why LA which is only a weak PPARγ activator had no effect on SMC collagen formation. Since activation of the NF-κB-pathway has been implicated in the stimulatory effect of oxidized LDL and n-6 and n-3 polyunsaturated fatty acids on collagen production in vascular SMCs and fibroblasts, respectively, the observation that treatment with CLA causes inhibition of the NF-κB-pathway suggests that this anti-inflammatory effect is possibly involved in the inhibition of collagen production by CLA in vascular SMCs. However, direct proof for the involvement of NF-κB inhibition in the reduced collagen formation has to be given. Deposition of collagen and other extracellular matrix proteins in the vessel wall largely contributes to the formation of atherosclerotic plaques, ultimately leading to acute clinical manifestations of atherosclerosis such as myocardial infarction and stroke. Thus, the reduced collagen production by vascular SMCs treated with CLA might explain, at least in part, the observation from in vivo-studies that CLA reduces atherosclerotic plaque development using animal models of atherosclerosis [18, 35, 36]. In conclusion, the present results provide insight into a new mechanism of action of CLA relating the anti-inflammatory effect of CLA to inhibition of collagen formation from vascular SMCs.

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Conjugated linoleic acids have no effect on TNFα-induced adhesion molecule expression, U937 monocyte adhesion, and chemokine release in human aortic endothelial cells

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Abstract

Leukocyte recruitment and adhesion to the endothelium are critical steps in the early phase of atherosclerosis. Synthetic ligands of peroxisome proliferator-activated receptors (PPARs) were shown to reduce cytokine-stimulated leukocyte–endothelial cell interactions by inhibiting the NF-κB mediated inflammatory response. Conjugated linoleic acids (CLA), which are natural ligands of PPARs, were demonstrated to have anti-inflammatory and anti-atherogenic properties in vivo. With a view to elucidating the anti-atherogenic mechanisms of CLA, the present study aimed to explore the effects of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA on cytokine-induced chemokine release, surface expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and U937 monocyte adhesion in human aortic endothelial cells (HAEC). Treatment of HAECs with 2 ng/mL of TNFα markedly increased expression of adhesion molecules, U937 monocyte adhesion, and release of the monocyte chemoattractant protein (MCP)-1. However, treatment of HAECs with either CLA isomer or linoleic acid did not modulate the cytokine-induced expression of ICAM-1, VCAM-1, and E-selectin, U937 cell adhesion and MCP-1 release. In addition, both CLA isomers and linoleic acid slightly increased PPARγ DNA-binding activity, but did not alter DNA-binding activity of NF-κB. In conclusion, CLA isomers showed no effect on cytokine-induced monocyte–endothelial cell interactions and on the molecular mechanisms regulating these processes in HAEC. This study suggests that anti-atherogenic effects of CLA observed in vivo are probably not mediated by reduced monocyte–endothelial cell interactions.

Keywords: Conjugated linoleic acid; Atherosclerosis; Human aortic endothelial cells; Adhesion molecules; Monocyte adhesion; VCAM-1; ICAM-1; E-selectin; MCP-1; Monocyte chemoattractant protein-1

1. Introduction

Adhesion of circulating monocytes to endothelial cells and transendothelial migration of monocytes are early steps in the atherosclerotic process and are mediated by inducible cell adhesion molecules such as E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, which are expressed on the surface of endothelial cells. Increased expression of adhesion molecules favours monocyte–endothelial cell interactions and is observed in atherosclerotic lesions and plaques [1,2]. It is also known that plasma concentrations of circulating adhesion molecules are elevated in patients with cardiovascular diseases [3]. The recruitment of monocytes into sites of atherosclerosis are mediated by chemokines, which chemoattract circulating blood cells such as monocytes and lymphocytes. Monocyte chemoattractant protein (MCP)-1 is a potent chemoattractant for monocytes and was demonstrated to be expressed by endothelial cells in early atherosclerotic lesions [4].

Abbreviations: CLA, conjugated linoleic acid; HAECs, human aortic endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; INF-γ, interferon-γ; MCP-1, monocyte chemoattractant protein; PPAR, peroxisome proliferator-activated receptors; TNFα, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule

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Pharmacological ligands of peroxisome proliferator-activated receptors (PPARs) were shown to reduce cytokine-stimulated surface expression of adhesion molecules, leukocyte adhesion and chemokine release in endothelial cells, thus making them attractive drugs for the treatment of atherosclerosis [5]. Conjugated linoleic acids (CLAs), which are naturally occurring fatty acids and known to bind and activate PPARα and -γ [6,7], were reported to have anti-atherogenic properties in animal feeding experiments using apo E knockout mice [8] or rabbits [9]. However, the molecular mechanisms of these anti-atherogenic effects are largely unknown. CLAs were also demonstrated to exert anti-inflammatory effects that are mediated, at least in part, by a PPARγ-dependent inhibition of NF-κB activation [6,10,11]. Activation of NF-κB is critical for the induction of adhesion molecules and several other inflammatory markers [12]. Inhibition of the NF-κB mediated inflammatory response by CLA may therefore potentially attenuate pro-atherogenic processes such as adhesion molecule expression and chemokine release in the endothelium. A recent study showed that *cis*-9, *trans*-11 CLA reduces the adhesion of human gastric carcinoma cells to extracellular matrices by reducing the protein expression of VCAM-1 and ICAM-1 [13]. However, the effect of CLA on adhesion molecule expression, leukocyte adhesion and chemokine release in endothelial cells has not yet been investigated, although CLA was shown to modulate endothelial cell function [14,15]. The objective of this study, therefore, was to investigate the effects of two different isomers of CLA, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, on chemokine release, adhesion molecule expression and U937 monocyte adhesion in human aortic endothelial cells (HAEC). Both cell types are widely used for investigating monocyte–endothelial cell interactions [16,17]. HAECs are considered to be a suitable model to study the effects on atherosclerosis regarding that the aorta represents a blood vessel commonly affected by atherosclerosis. The U937 cell line is a model for blood-borne monocytes comprising the great advantage that a virtually unlimited number of relatively uniform cells can be produced from these cell lines [16]. The *cis*-9, *trans*-11 isomer was considered because it is the predominant CLA isomer of milk and dairy products. More than 80% of CLA present in milk, corresponding to 0.1 to 0.7 g per L, exists as *cis*-9, *trans*-11 isomer [18]. *Trans*-10, *cis*-12 CLA exists in milk and most other foods only in traces but is of interest because it also exhibits various biological properties [13,19]. Adhesion molecule expression, monocyte adhesion and chemokine release are strongly induced in response to several inflammatory cytokines such as TNFα, IL-1, and INF-γ [20,21], which are secreted during acute inflammation and the inflammatory process associated with the development of atherosclerosis. We, therefore, evaluated the potential of CLA to modulate the TNFα-stimulated enhancement in adhesion molecule expression, monocyte adhesion and chemokine release. TNFα-stimulated cells treated without fatty acids were used as controls. The effects of CLA were also compared with those of linoleic acid as a reference substance, which was shown to modulate monocyte–endothelial cell interactions [22,23].

2. Materials and methods

2.1. Materials

Cis-9, *trans*-11 CLA (≥96% pure) and *trans*-10, *cis*-12 CLA (≥98% pure) were obtained from Cayman Chemicals (Ann Arbor, MI). Linoleic acid (≥99% pure) was purchased from Sigma–Aldrich (Taufkirchen, Germany). Preparation of the stock solutions of fatty acids and of the test media was performed as described previously [14]. 3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Thiazol blue) and tumour necrosis factor α (TNFα) were purchased from Sigma–Aldrich. The PPARγ agonist troglitazone was purchased from Calbiochem (Bad Soden, Germany).

2.2. Cell culture

Human aortic endothelial cells (HAEC) from a 38-year-old female donor were obtained from PromoCell and cultured in EC Growth Medium MV, composed of EC Basal Medium and supplements (all from PromoCell, Heidelberg, Germany), as described previously [14]. According to PromoCell, these cells were isolated from the ascending aorta or the combination with aortic arch by enzymatic digestion and endothelial cell origin was characterised by Factor VIII-related antigen expression. Only cells from passages 3–6 were used for this study.

U937 monocytic cells (DSMZ, Brauschweig, Germany) were grown in suspension culture in PRMI 1640 medium (containing 0.3 g/L L-glutamine and 2.0 g/L sodium bicarbonate) supplemented with 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany) and 0.5% gentamicin (Gibco/Invitrogen, Karlsruhe, Germany). Cultures were maintained by replacement of fresh medium every 2 days. Cell density was maintained between 1×10^5 and 2×10^6 viable cells/mL.

For all experiments, HAECs were incubated with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid for 20 h followed by a 4 h treatment with TNFα (2 ng/mL) in the presence of the same fatty acids. Cells treated without fatty acids for 20 h and subsequently treated with TNFα for 4 h were used as controls ("stimulated control"). Cells treated without fatty acids for 24 h without addition of TNFα were used as "unstimulated controls". For the measurement of PPARγ DNA-binding activity troglitazone as a positive control (10 μmol/L) was also included in the experiments.

2.3. Cell viability

Cell viability after treatment of HAECs as indicated above was examined by the MTT assay [24].

2.4. Fatty acid analysis

For determination of the fatty acid composition of cellular total lipids HAECs were treated with or without fatty acids for 24 h without addition of TNFα. Afterwards HAECs were washed with PBS and total lipids were extracted with hexane/isopropanol (3:2, v/v). The lipid extracts were dried under nitrogen, transmethylated with trimethylsulfonium hydroxide and FAME were separated by GC as described previously in detail [25].

2.5. Surface expression of adhesion molecules

Determination of cell surface expression of adhesion molecules was performed by enzyme-linked immunosorbent assay (ELISA) as described elsewhere in detail [26]. Briefly, HAEC were cultured to confluence in 96-well plates and incubated as indicated above. Cells were then fixed with 4% formaldehyde and incubated with mouse monoclonal antibodies to ICAM-1 (dilution 1:500), VCAM-1 (1:300) (both from Calbiochem/Merck, Darmstadt, Germany) and E-selectin (1:300; BenderMed Systems, Wien, Austria) for 1 h at room temperature. After incubation of the cell monolayer with a secondary horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Calbiochem/Merck) for 1 h at room temperature, tetramethylbenzidine (Sigma–Aldrich) as substrate was added to each well. Colour development was stopped after 15 min by the addition of phosphoric acid (Stop Reagent, Sigma–Aldrich) and the absorbance of the wells determined on a microplate reader at 450 nm.

2.6. U937 cell adhesion assay

For the quantitative cell adhesion assay [27] U937 monocytes were fluorescently labelled by incubating the cells with 5 μmol/L 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) (Molecular Probes/Invitrogen, Karlsruhe, Germany) in RPMI 1640 medium for 30 min at 37 °C and 5% CO_2 as described elsewhere in detail [16]. After labelling, the U937 monocytes were washed three times with 1% FBS in phosphate-buffered saline (PBS) to remove excess dye. Finally, the cells were resuspended in EC growth medium at a density of 5×10^8 cells/L.

HAECs were cultured to confluence in 24-well plates and incubated as indicated above. HAECs were then incubated with BCECF-labelled U937 monocytes (2.5×10^5 per well) for 30 min at 37 °C. After incubation non-adherent monocytes were removed by washing each well three times with 1% FBS-PBS. The attached monocytes were lysed with 0.5 mL of 50 mmol/L Tris buffer (pH 7.6) containing 0.1% sodium dodecyl sulphate (Sigma–Aldrich). The fluorescence intensity of each well was measured with a fluorescence multiwell plate reader set at excitation and emission wavelengths of 495 and 535 nm, respectively. With each set of experiments a separate plate containing known numbers of U937 cells labelled with BCECF-AM was prepared for determination of a standard curve of fluorescence units per cell.

2.7. Release of the chemokine MCP-1

For determination of the release of MCP-1, HAECs were cultured to confluence in 24-well plates and incubated as indicated above. After incubation supernatants were collected and the concentrations of MCP-1 determined by ELISA (BMS281INST, BenderMed Systems).

2.8. DNA-binding activity of NF-κB and PPARγ

For measurement of transcription factor activities HAECs were cultured to confluence in 25 cm^2 tissue culture flasks and incubated as indicated above. Afterwards nuclear extracts were prepared with a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol. Protein concentrations in the nuclear extracts were determined by the method of Bradford [28] with BSA (Sigma–Aldrich) as standard. NF-κB and PPARγ activities in the nuclear extracts were determined by the Transcription Factor assays TransAM™ NF-κB Family Kit and TransAM™ PPARγ Kit (Active Motif), respectively. These assays facilitated the measurement of binding of NF-κB subunits p65 and p50 and of PPARγ to their respective consensus binding sequences, which are immobilized to a 96-well plate, by primary antibodies specific for p65, p50, and PPARγ, respectively. Subsequent incubation with an HRP-conjugated secondary antibody and a standard developing solution in each well turned the contents blue. After addition of a Stop solution the absorbance of the wells was read at 450 nm in a microplate reader.

2.9. Statistical analysis

Student's *t*-test was used to compare means of treatments with those of control. Differences of $P < 0.05$ were considered significant.

3. Results

3.1. Cell viability

Viability of HAECs was not affected by treatment with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid for 20 h followed by a 4 h treatment with TNFα (2 ng/mL) in the presence of the same fatty acids relative to unstimulated controls; cell viabilities after treatment with TNFα alone (stimulated control) or with TNFα and 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, and linoleic acid were 106 ± 4, 90 ± 4, 92 ± 13, 98 ± 4, 91 ± 8, 102 ± 8, and 97 ± 4% (mean ± S.D. for five independent experiments), respectively, relative to unstimulated

Table 1
Fatty acid composition (g/100 g total fatty acids) of total lipids of HAECs cultured in the presence or absence (control) of 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid

Treatment	Control	*Cis*-9, *trans*-11 CLA		*Trans*-10, *cis*-12 CLA		Linoleic acid	
		5 μM	50 μM	5 μM	50 μM	5 μM	50 μM
C14:0	2.2 ± 0.1	2.1 ± 0.1	1.2 ± 0.1*	2.0 ± 0.1	0.8 ± 0.1*	2.0 ± 0.1	1.1 ± 0.1*
C16:0	19.7 ± 0.4	19.2 ± 0.1	11.5 ± 0.1*	18.5 ± 0.1	8.3 ± 0.2*	19.2 ± 0.4	12.3 ± 0.3*
C16:1	5.2 ± 0.3	4.5 ± 0.1	2.2 ± 0.1*	3.6 ± 0.1*	1.6 ± 0.1*	4.2 ± 0.3*	1.9 ± 0.1*
C18:0	13.2 ± 0.3	12.9 ± 0.2	8.1 ± 0.1*	13.5 ± 0.3	6.7 ± 0.3*	13.2 ± 0.1	8.4 ± 0.2*
C18:1	25.3 ± 0.6	23.0 ± 0.2*	12.3 ± 0.2*	21.2 ± 0.3*	8.8 ± 0.1*	21.1 ± 0.5*	10.0 ± 0.3*
C18:2	1.6 ± 0.2	1.6 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	0.7 ± 0.1	7.9 ± 0.6*	35.9 ± 0.6*
C18:2 *c*9,*t*11	<0.1	5.3 ± 0.4*	35.2 ± 0.8*	<0.1	<0.1	<0.1	<0.1
C18:2 *t*10,*c*12	<0.1	<0.1	<0.1	7.4 ± 1.1*	47.0 ± 0.7*	<0.1	<0.1
C20:0	0.7 ± 0.2	0.7 ± 0.1	0.5 ± 0.1	1.4 ± 0.1*	1.0 ± 0.2	0.7 ± 0.1	0.5 ± 0.1
C20:4 *n*-6	6.4 ± 0.2	5.9 ± 0.1	3.4 ± 0.1*	5.8 ± 0.1	3.4 ± 0.1*	6.4 ± 0.1	4.1 ± 0.1*
C22:4 *n*-6	1.8 ± 0.3	1.8 ± 0.2	1.9 ± 0.1	2.1 ± 0.1	2.2 ± 0.4	2.4 ± 0.1	2.5 ± 0.1
C20:5 *n*-3	1.1 ± 0.1	0.8 ± 0.1	1.5 ± 0.1	1.5 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
C22:5 *n*-3	3.3 ± 0.4	3.2 ± 0.3	2.5 ± 0.3*	3.5 ± 0.4	2.3 ± 0.2*	3.2 ± 0.3	2.3 ± 0.1*
C22:6 *n*-3	7.2 ± 1.2	6.3 ± 1.2	4.5 ± 1.0*	6.4 ± 1.6	5.1 ± 0.8*	6.1 ± 1.6	4.1 ± 1.1*

HAECs were cultured without fatty acids (control) or with 5 and 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid for 24 h. Afterwards total lipids were extracted, transmethylated, and fatty acid methyl esters (FAME) were separated by gas chromatography. Fatty acids of HAEC total lipids are presented as g per 100 g of total fatty acids. Results represent mean ± S.D. of three independent experiments.
* Significantly different from control, $P < 0.05$.

controls (=100%). Treatment of HAECs with 10 μmol/L of troglitazone for 24 h had no effect on cell viability when compared to vehicle control (0.1% DMSO), whereas 20 μmol/L of troglitazone reduced cell viability to 90% of vehicle control (data not shown).

3.2. Fatty acid composition of HAEC total lipids

The proportions of total fatty acids in HAEC total lipids following fatty acid treatments for 24 h are shown in Table 1. Incubating HAECs with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid resulted in a significant incorporation of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, and linoleic acid, respectively, in a concentration-dependent manner when compared to HAECs treated without fatty acids ($P < 0.05$). In addition, HAECs treated with *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA had higher concentrations of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, respectively, compared with HAECs treated with linoleic acid ($P < 0.05$). The proportions of the saturated fatty acids C14:0, C16:0, and C18:0; oleic acid (C18:1); the long-chain polyunsaturated fatty acids C20:4 *n*-6, C22:5 *n*-3, and C22:6 *n*-3 in HAEC lipids dose-dependently decreased by treatment with CLA isomers or linoleic acid relative to control cells ($P < 0.05$).

3.3. Effect on surface expression of adhesion molecules

The effects of TNFα and different fatty acids on surface expression of VCAM-1, ICAM-1, and E-selectin are shown in Fig. 1. Stimulation of HAECs with 2 ng/mL of TNFα caused a strong increase in the expression of all adhesion molecules investigated when compared with unstimulated controls ($P < 0.05$). However, the TNFα-induced expression of VCAM-1, ICAM-1, and E-selectin of HAEC cultured with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid was not different from stimulated controls.

3.4. Effect on U937 monocyte adhesion to HAEC

As shown in Fig. 2, stimulation of HAEC with 2 ng/mL of TNFα for 4 h caused a marked increase in U937 monocyte adhesion to HAEC when compared with unstimulated controls ($P < 0.05$). The TNFα-induced U937 monocyte adhesion, however, did not differ between HAECs cultured with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA or linoleic acid and stimulated controls.

3.5. Effect on MCP-1 release

The effects of TNFα and different fatty acids on MCP-1 release from HAECs are shown in Fig. 3. Treatment of HAECs with 2 ng/mL of TNFα significantly increased the release of the chemokine MCP-1 into the incubation medium relative to unstimulated controls ($P < 0.05$). However, the TNFα-stimulated induction of the MCP-1 release was not altered by treatment of HAECs with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid when compared with stimulated controls.

3.6. Effect on DNA-binding activities of NF-κB subunits p65 and p50

DNA-binding activities of p65 and p50 were strongly induced by treatment of HAECs with 2 ng/mL of TNFα when

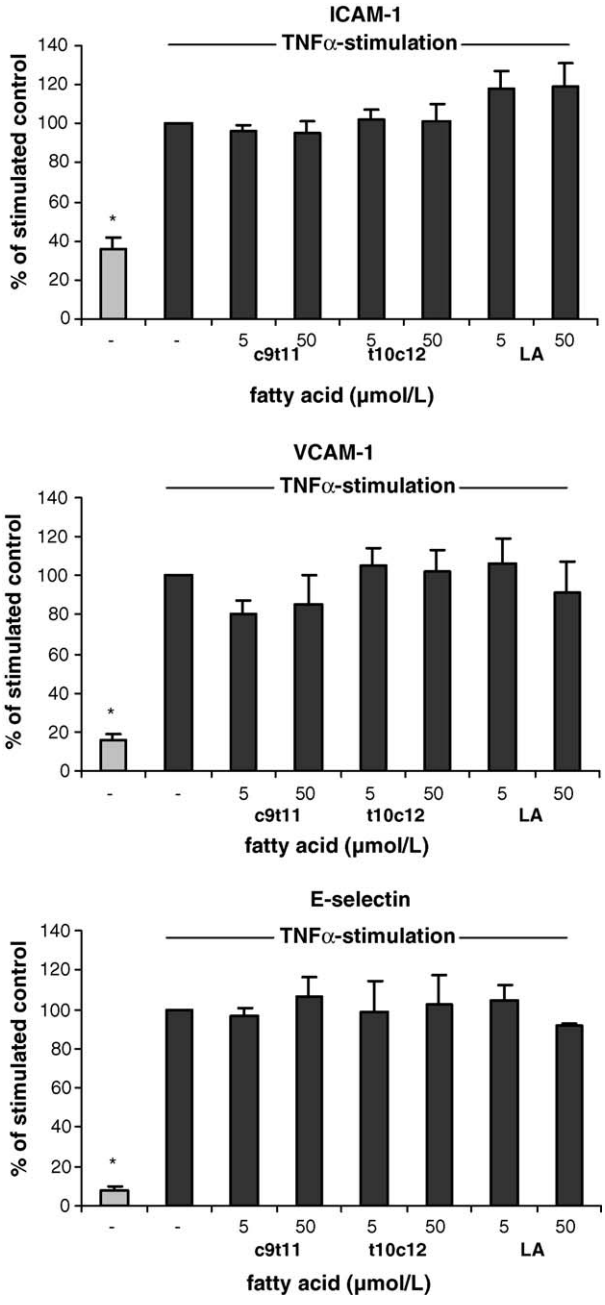

Fig. 1. Treatment of HAECs with *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA has no effect on the TNFα-induced surface expression of adhesion molecules. HAECs were cultured with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid for 20 h followed by a 4 h treatment with TNFα (2 ng/mL) in the presence of the same fatty acids. Cells treated without fatty acids for 20 h and subsequently treated with TNFα (2 ng/mL) for 4 h were used as controls (stimulated controls). Cells treated without fatty acids for 24 h without the addition of TNFα (unstimulated controls) were used to show the cytokine-stimulant effect. HAECs were then incubated with monoclonal antibodies to ICAM-1, VCAM-1, and E-selectin. Secondary HRP-conjugated antibody and substrate were added and absorbance was read at 450 nm. Data represent mean ± S.D. of four independent experiments and are expressed as percentage of adhesion molecule expression of stimulated controls (=100 ± 0%). *Significantly different from stimulated controls, $P < 0.05$.

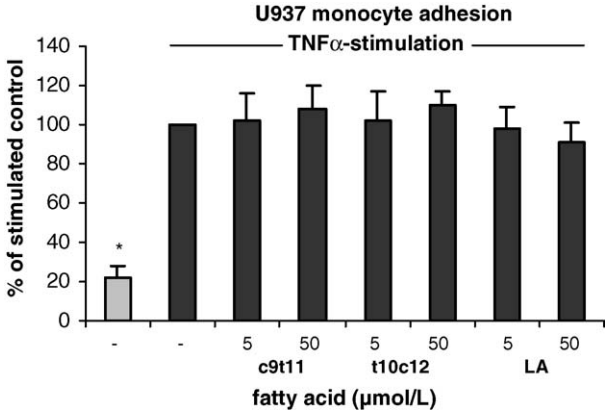

Fig. 2. Treatment of HAECs with *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA has no effect on the TNFα-induced U937 monocyte adhesion to HAECs. HAECs were cultured with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid for 20 h followed by a 4 h treatment with TNFα (2 ng/mL) in the presence of the same fatty acids. Cells treated without fatty acids for 20 h and subsequently treated with TNFα (2 ng/mL) for 4 h were used as controls (stimulated controls). Cells treated without fatty acids for 24 h without the addition of TNFα (unstimulated controls) were used to show the cytokine-stimulant effect. HAECs were then incubated with BCECF-labelled U937 monocytes for 30 min. After removal of non-adherent monocytes attached monocytes were lysed and fluorescence was measured (excitation and emission wavelengths of 495 and 535 nm, respectively). Data represent mean ± S.D. of seven independent experiments and are expressed as percentage of U937 monocyte adhesion of stimulated controls (=100 ± 0%). *Significantly different from stimulated controls, $P < 0.05$.

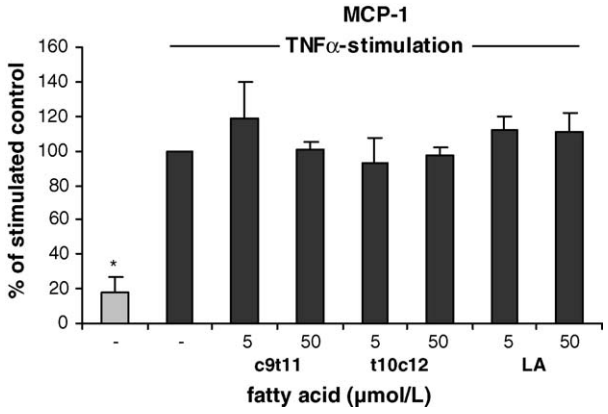

Fig. 3. Treatment of HAECs with *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA has no effect on the TNFα-induced MCP-1 release. HAECs were cultured with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid for 20 h followed by a 4 h treatment with TNFα (2 ng/mL) in the presence of the same fatty acids. Cells treated without fatty acids for 20 h and subsequently treated with TNFα (2 ng/mL) for 4 h were used as controls (stimulated controls). Cells treated without fatty acids for 24 h without the addition of TNFα (unstimulated controls) were used to show the cytokine-stimulant effect. After incubation supernatants were collected and the concentration of MCP-1 was determined by ELISA. Data represent mean ± S.D. of four independent experiments and are expressed as percentage of MCP-1 release of stimulated controls (=100 ± 0%). *Significantly different from stimulated controls, $P < 0.05$.

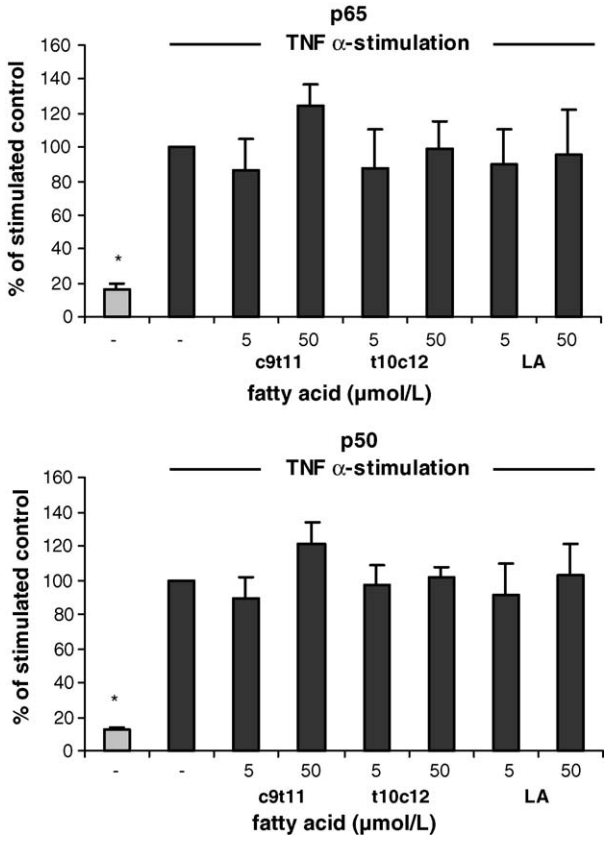

Fig. 4. Treatment of HAECs with *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA has no effect on the TNFα-induced NF-κB DNA-binding activity. HAECs were cultured with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid for 20 h followed by a 4 h treatment with TNFα (2 ng/mL) in the presence of the same fatty acids. Cells treated without fatty acids for 20 h and subsequently treated with TNFα (2 ng/mL) for 4 h were used as controls (stimulated controls). Cells treated without fatty acids for 24 h without the addition of TNFα (unstimulated controls) were used to show the cytokine-stimulant effect. After treatment nuclear extracts were prepared and binding of NF-κB subunits p65 and p50 to NF-κB consensus binding sequence was measured by an ELISA-based assay as described in Section 2. Data represent mean ± S.D. of four independent experiments and are expressed as percentage of NF-κB DNA-binding activity of stimulated controls (=100 ± 0%). *Significantly different from stimulated controls, $P < 0.05$.

compared with unstimulated controls ($P < 0.05$) (Fig. 4). The TNFα-stimulated increase in DNA-binding activities of p65 and p50, however, did not differ between HAECs cultured with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid and stimulated controls.

3.7. Effect on DNA-binding activity of PPARγ

Treatment of HAEC with 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid or 10 μmol/L of troglitazone slightly increased the DNA-binding activity of PPARγ when compared with stimulated and unstimulated controls ($P < 0.05$; Fig. 5).

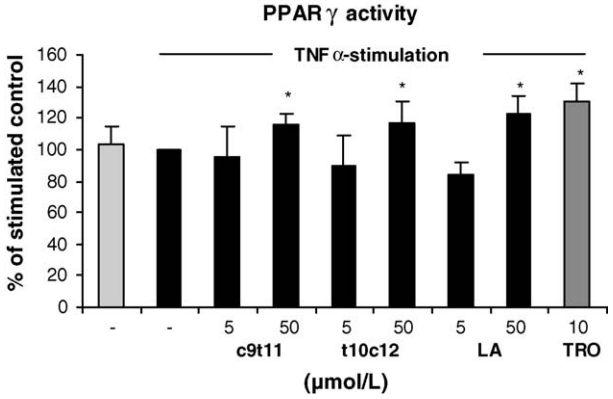

Fig. 5. Treatment of HAECs with *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA has a stimulatory effect on the PPARγ DNA-binding activity. HAECs were cultured with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or linoleic acid or 10 μmol/L of the PPARγ ligand troglitazone (TRO) for 20 h followed by a 4 h treatment with TNFα (2 ng/mL) in the presence of the same fatty acids or PPARγ ligand. Cells treated without fatty acids or troglitazone for 20 h and subsequently treated with TNFα (2 ng/mL) for 4 h were used as controls (stimulated controls). PPARγ DNA-binding activity of cells treated without any exogenous PPARγ ligand for 24 h without the addition of TNFα (unstimulated controls) is also presented. After treatment nuclear extracts were prepared and binding of PPARγ to PPARγ consensus binding sequence was measured by an ELISA-based assay as described in Section 2. Data represent mean ± S.D. of at least four independent experiments and are expressed as percentage of PPARγ DNA-binding activity of stimulated controls (=100 ± 0%). *Significantly different from stimulated controls, $P < 0.05$.

4. Discussion

Adhesion of monocytes to the arterial wall, followed by transmigration into the subendothelial space and lipid accumulation, is a key event in the development of atherosclerosis. The interaction of monocytes with the arterial wall is mediated by chemokines such as MCP-1, which chemoattract circulating monocytes, and adhesion molecules such as VCAM-1, ICAM-1, and E-selectin expressed on the surface of endothelial cells. Adhesion molecule expression, monocyte adhesion and chemokine release are strongly induced in response to several inflammatory cytokines such as TNFα, IL-1, and INF-γ [20,21], which are secreted during acute inflammation and the inflammatory process associated with the development of atherosclerosis. The stimulant effect of cytokine-treatment on adhesion molecule expression, monocyte adhesion and chemokine release in HAECs observed in the present study concurs with observations from other studies reported in the literature [16,17]. CLA was shown to exert anti-inflammatory actions [6,10] and anti-atherogenic effects in animal models of atherosclerosis [8,9]. With a view to elucidating potential anti-atherogenic mechanisms of CLA we investigated the effects of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA on adhesion molecule expression, monocyte adhesion and chemokine release in HAECs. We treated the cells with the same concentrations of CLA isomers, 5 or 50 μmol/L as in a previous study performed by our group

[14]. Incubating HAECs with media containing 5 μmol/L of CLA for 24 h resulted in moderate concentrations of CLA in endothelial cell lipids, up to 7 g/100 g of total fatty acids. Such CLA concentrations are in the range that can be achieved in animal tissues by dietary CLA supplementation [29,30]. Incubating HAECs with media containing 50 μmol/L CLA resulted in very high concentrations of CLA in cell lipids of up to 47 g/100 g of total fatty acids. These concentrations are much higher than those obtained in animal tissues by dietary treatments. However, the unaltered cell viability in the present study confirms the results of our previous study that high concentrations of CLA do not alter the morphological appearance and growth characteristics of HAEC. This opens up the possibility of studying the effects of CLA on monocyte–endothelial cell interactions even at unphysiologically high concentrations.

The present study clearly demonstrated that CLAs do not modulate the cytokine-stimulated expression of adhesion molecules, monocyte adhesion and chemokine release in HAECs in the studied concentration range. The expression of adhesion molecules is mainly regulated at the transcriptional level. Although several transcription factors such as NF-κB, AP-1, STAT, SP-1, GATA, and IRF are involved in the transcriptional control of adhesion molecules, activation of NF-κB is considered to be one of the crucial factors for transcriptional induction of adhesion molecules in endothelial cells [12]. Activity of NF-κB, but also of AP-1 and STAT, is negatively regulated by PPARγ, resulting in the well-established observation that pharmacological PPARγ ligands exert anti-inflammatory effects such as reduced adhesion molecule expression, monocyte adhesion and chemokine release [5]. We, therefore, postulated that CLA, as a natural ligand of PPARγ [6,7], would act in a similar manner and counterregulate NF-κB-dependent transcription of pro-inflammatory genes through PPARγ signalling. However, as we observed only a slight PPARγ activation by CLA in this study, it was not surprising that we observed no inhibition of cytokine-induced NF-κB activation and monocyte adhesion by CLA in the present study. Only a slight activation of PPARγ was also observed with the synthetic PPARγ ligand troglitazone suggesting that the HAEC culture used herein shows a relatively weak response to treatment with PPARγ ligands. However, the degree of PPARγ activation after treatment with PPARγ ligands was approximately in the range of that observed within other macrovascular endothelial cells [31,32]. The finding that a strong PPARγ activation and attenuation of the NF-κB stimulated inflammatory response by treatment with CLA was not observed in the present study is in contrast to previous studies using other cell culture models such as monocytes or macrophages [6,10]. However, this difference is probably explained by cell type-specific actions of CLA, which are well documented in the literature [33,34]. Cell type-specific actions of CLA might also explain why CLA reduced ICAM-1 and VCAM-1 protein expression in a human gastric carcinoma cell line [13] but not in HAECs in the present study. However, the lack of effect of CLA on adhesion molecule expression in the present study is consistent with the observation that CLA supplementation compared with linoleic acid supplementation did not alter the serum concentrations of soluble adhesion molecules in healthy volunteers [35].

As regards the effects of linoleic acid, which is frequently used as a control fatty acid when investigating the biological effects of CLA [15,36], no effects on the parameters investigated and no difference between linoleic acid and CLA were demonstrated in HAECs in the present study. Results concerning the effects of linoleic acid on the parameters addressed in the present study reported in the literature are very controversial [22,23,37,38], but are probably also explained by differences in the origin of the cell culture, species differences and different treatment regimes (concentration and time of exposure). Differences might be also explained by the conditions the assay has been performed. For example, linoleic acid had no effect on leukocyte adhesion under static conditions in the present and in a recent study [37], whereas under fluid flow linoleic acid was demonstrated to increase monocyte deformation and adhesion to HUVECs [38]. The present study did, however, clearly show that linoleic acid has no effect on cytokine-induced adhesion molecule expression, monocyte adhesion and chemokine release in HAECs.

In summary, our data suggest that CLA isomers, even at unphysiologically high concentrations, have no effect on cytokine-induced adhesion molecule expression, monocyte adhesion, and chemokine release or on the molecular mechanisms regulating these processes in HAECs. This suggests that the anti-atherogenic effects of CLA observed in animal feeding experiments [8,9] are not explained by reduced monocyte adhesion to the endothelium, which is a crucial step in the early phase of atherosclerosis. This raises the possibility that CLA may alter the process of atherosclerosis during the later stages of atherogenesis or by mechanisms other than those addressed in this study.

Acknowledgement

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A5 **Ringseis R**, Wen G, Saal D, Eder K:

Cis-9, trans-11- and trans-10, cis-12-conjugated linoleic acid isomers reduce cholesterol accumulation in acetylated LDL-induced mouse RAW264.7 macrophage foam cells.

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Abstract

Synthetic activators of PPARα and PPARγ are capable of reducing macrophage foam cell cholesterol accumulation through the activation of genes involved in cholesterol homeostasis. Since conjugated linoleic acids (CLA) were also demonstrated to activate PPARα and γ *in vivo* and *in vitro*, we tested the hypothesis that CLA are also capable of reducing macrophage foam cell cholesterol accumulation. Thus, mouse RAW264.7 macrophage-derived foam cells were treated with CLA isomers, *c*9*t*11-CLA and *t*10*c*12-CLA, and linoleic acid (LA), as reference fatty acid, and analyzed for the concentrations of free and esterified cholesterol, cholesterol efflux and expression of genes involved in cholesterol homeostasis (CD36, ABCA1, LXRα, NPC-1, and NPC-2). Treatment with *c*9*t*11-CLA and *t*10*c*12-CLA, but not LA, lowered cholesterol accumulation, stimulated acceptor-dependent cholesterol efflux, and increased relative mRNA concentrations of CD36, ABCA1, LXRα, NPC-1, and NPC-2 ($P<0.05$). In conclusion, the present study shows that CLA isomers reduce cholesterol accumulation in RAW264.7 macrophage-derived foam cells presumably by enhancing lipid acceptor-dependent cholesterol efflux.

Introduction

Atherosclerosis, the principle cause of coronary heart disease and stroke, is responsible for more than 40% of all deaths in Europe and the United States[1]. Macrophages are centrally involved in the initiation and progression of atherosclerosis and are the predominant cellular component of atherosclerotic lesions[2]. When concentrations of LDL are elevated, macrophages accumulate lots of LDL-derived cholesterol through scavenger receptor-dependent pathways, and, then transform into cholesterol-enriched foam cells. Development of macrophage-derived foam cells is considered to be a critical step in atherogenesis, since foam cells account for the development of fatty streaks which represent the initial stage of atherosclerosis[3].

Recent *in vitro*-studies indicated that synthetic activators of PPARα and PPARγ induce cholesterol removal from macrophage foam cells through the induction of genes involved in cholesterol homeostasis[4,5]. Although PPARγ ligands increase expression of CD36 scavenger receptors[6,7], which mediate uptake of modified LDL, concomitant induction by PPARα and γ ligands of liver X receptor α (LXR α) and cholesterol exporters of the ATP-binding cassette (ABC) family, ABCA1 and ABCG1, results in a net increase in cholesterol efflux from macrophage foam cells[4,5,8,9]. ABCA1 in particular plays a key role in cellular cholesterol efflux from macrophages to apo-AI, the first step in reverse cholesterol transport. This has been demonstrated in patients with a mutated ABCA1 gene, where cholesterol efflux and reverse cholesterol transport are impaired and development of atherosclerosis is accelerated[10-12]. Recent studies also demonstrated that PPARα activators up-regulate proteins involved in intracellular cholesterol trafficking, namely Niemann Pick type C (NPC) proteins-1 and -2[13], which mediate cholesterol transport from the lysosome to the plasma membrane[14,15]. Increased expression of NPC-1 and NPC-2 by PPARα activators increases cholesterol content of the plasma membrane, and,

the efflux of cholesterol to extracellular acceptors such as apo-AI[13]. These *in vitro*-effects of PPARs also explain, at least partially, that treatment with PPARα and γ ligands exerts anti-atherogenic effects *in vivo*[16,17]. Similarly to synthetic agents, conjugated linoleic acids (CLA), a group of positional and geometric isomers of linoleic acid naturally occurring in food derived from ruminants such as milk and milk products, were also demonstrated to activate PPARα and γ[18-20], and inhibited atherosclerosis in animal feeding experiments[21-23]. This suggests that CLA might stimulate cholesterol removal from macrophage foam cells through activation of PPARα and γ, but a recent study from Weldon et al.[24] revealed no effects of CLA on molecular markers of cholesterol homeostasis in human THP-1 macrophage-derived foam cells. Although the lack of effect might be explained by the relatively poor accumulation of CLA isomers in THP-1 cells[24] when compared to other cell types[25-27], cell type-specific actions of CLA, e.g. on PPARγ expression[28-29], might also account for this.

Thus, in the present study we aimed to explore the effect of CLA isomers on cholesterol homeostasis in RAW264.7 macrophage-derived foam cells. These cells have been reported to show pronounced biological activities, partially mediated by a PPARγ-dependent mechanism, in response to treatment with CLA[19,26]. Moreover, these cells are a widely used model to study macrophage foam cell cholesterol homeostasis[30]. RAW264.7 macrophages were transformed to foam cells by treatment with acetylated LDL (acLDL). AcLDL is a well-recognized ligand for multiple scavenger receptors including CD36 and mediates uptake of modified lipoproteins through scavenger receptors in an unregulated fashion, leading to foam cell formation *in vitro*[31]. As parameters of cholesterol homeostasis, we considered the concentrations of free and esterified cellular cholesterol, and mRNA and/or protein expression levels of PPARα and γ as well as important genes involved in the regulation of cholesterol homeostasis (CD36, ABCA1, LXRα, NPC-1, and NPC-2) in acLDL-induced macrophage foam cells. Cholesterol removal from macrophage foam cells was estimated by measurement of HDL-dependent cholesterol efflux. As isomers *cis*-9, *trans*-11 (*c*9*t*11)-CLA, which contributes to more than 90% of total CLA in natural foods such as milk, dairy products, and meat of ruminants[32], and *trans*-10, *cis*-12 (*t*10*c*12)-CLA, which is a minor isomer in natural foods but one of the main isomers in chemically produced CLA mixtures, were used. Linoleic acid (LA) was used as reference fatty acid.

Materials and methods

Materials and reagents

The CLA isomers *c*9*t*11-CLA (≥96% pure) and *t*10*c*12-CLA (≥98% pure) were purchased from Cayman Chemicals (Ann Arbor, MI). LA (≥99% pure) was obtained from Sigma-Aldrich (Taufkirchen, Germany).

Cell culture and treatments

Mouse RAW264.7 cells, obtained from LGC Promochem (Wesel, Germany), were grown in DMEM medium (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum, 4 mmol/L L-glutamine, 4.5 g/L glucose, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate and 0.5% Gentamicin. For experiments RAW264.7 cells were plated in 24-well plates at a density of 2 x 10^5/well. After reaching 80% confluence, cells were treated with 50 µmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA for 24 h. To induce foam cell transformation, cells were incubated with fatty acids and 50 µg/mL acLDL for an additional 24 h. Cells treated without additional fatty acids were used as controls. Experiments were performed between

passages 5 and 20.

Cell viability

The viability of cells after treatment with fatty acids was examined by the MTT assay[33].

Fatty acid analysis of cell total lipids

After treatment of cells as indicated above, cells were washed with PBS and total lipids were extracted with hexane/isopropanol (3:2 v/v). The lipid extracts were analyzed for fatty acid composition as described previously in detail[20].

Isolation and acetylation of LDL

LDL was isolated from fresh human serum by sequential gradient ultracentrifugation[34]. Afterwards LDL was dialyzed for 24 h at 4°C against a buffer containing 0.15 mol/L NaCl and 0.3 mmol/L EDTA, pH 7.4. Acetylation of LDL was performed according to the method of Fraenkel-Conrad[35]. In brief, 1 mL of 0.15 mol/L NaCl containing 3 mg LDL protein was added to 1 mL of a saturated sodium acetate solution, and stirred in an ice water bath. Acetic anhydride (Sigma) was added slowly in small aliquots (2 µL/2 min) equal to 1.5 times the mass of LDL protein, and the mixture was stirred for an additional 30 min. Subsequently, the acLDL was dialyzed for 24 h at 4°C against a buffer containing 0.15 mol/L NaCl and 0.3 mmol/L EDTA, pH 7.4, filter-sterilized, and stored at 4°C.

Cholesterol analysis

After treatment of cells as indicated above, cells were washed twice with PBS, cellular lipids extracted with a mixture of hexane and isopropanol (3:2, v/v), and lipid extracts dried under a stream of nitrogen. Total cholesterol and free cholesterol concentrations were determined using enzymatic assays (Wako Chemicals GmbH, Germany). The concentration of esterified cholesterol was determined by subtraction of the concentration of free cholesterol from that of total cholesterol. Cholesterol concentrations were related to cellular protein as determined by the BCA protein assay kit (Pierce, Rockford, IL). Cellular protein was obtained by lysis of cells in 0.2 mol/L NaOH after extraction of cellular lipids. Results are expressed as µg lipids/mg cellular lipids.

RNA isolation and real-time RT-PCR

After treatment of cells as indicated above, total RNA of the cells was extracted using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. cDNA synthesis and real-time RT-PCR were performed as described recently in detail[36]. Mouse-specific primer sequences were as follows (forward, reverse): β-Actin (5´-ACG GCC AGG TCA TCA CTA TTG-3´, 5´-CAC AGG ATT CCA TAC CCA AGA AG-3´), CD36 (5´-GAA CCT ATT GAA GGC TTA CAT CC-3´, 5´-CCC AGT CAC TTG TGT TTT GAA C-3´), PPARα (5´-CGG CCC CAT ACA GGA GAG CAG-3´, 5´-GGG TGG CAG GAA GGG AAC AGA C-3´), PPARγ (5´-GGC GAG GGC GAT CTT GAC AG-3´, 5´-GGG CTT CCG CAG GTT TTT GAG-3´), ABCA1 (5´-TGC CAG CAA GAC GAA ACA GAC G-3´, 5´-GAC CAG GGC AAT GCA AAC AAA GAC-3´), LXRα (5´-GTA CAA CCC CGG CAG TGA GAG C-3´, 5´-GCA GGC GAA GGG CAA ACA C-3´), NPC-1 (5´-GGG GCA TCA GTT ACA ATG CT-3´, 5´-AAA CAC CGC ACT TCC CAT AG-3´), NPC-2 (5´-GAA ATC AGA CCC GAA ATG GA-3´, 5´-CAT CCT GTC TGG TGG AAC CT-3´).

Western blot analysis

After treatment of cells as indicated above, cells were lysed with RIPA lysis buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Sigma), and protein concentrations of lysates determined by the BCA assay. Equal amounts of protein

were electrophoresed by 7.5% SDS-PAGE for ABCA1 and 10% SDS-PAGE for ABCA1, LXRα, PPARγ, and β-Actin, and transferred to a nitrocellulose membrane. The membranes were blocked overnight at 4°C in blocking solution (5% skim milk in Tris buffered saline with Tween-20 [TBS-T]: 50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, 0.1% Tween-20), and then incubated with primary antibodies against CD36 (1:500, HyCult Biotechnology), PPARγ (1:1,000, Upstate), ABCA1 (1:1,000, Novus Biologicals), LXRα (1:500, Affinity Bioreagents), and β-Actin (1:1,000, Novus Biologicals) for 1.5 h at room temperature. The membranes were washed with TBS-T, and incubated with a horseradish peroxidase conjugated secondary anti-mouse IgG antibody (1:2,000, Amersham Biosciences) or anti-rabbit IgG antibody (1:10,000, Amersham Biosciences) for 1,5 h at room temperature. Afterwards blots were developed using ECL Plus (Amersham Biosciences). The signal intensities of specific bands were detected with Bio-Imaging system (Biostep) and quantified using TotalLab TL100-Quick Start analysis software (nonlinear dynamics).

Cholesterol efflux measurement

Cholesterol efflux assays were performed as described with minor modifications[30]. 24 h after plating, RAW264.7 macrophages were pre-treated with medium containing 10% low density lipoprotein-deficient serum for 24 h. Subsequently, RAW264.7 cells were incubated with fatty acids in the absence or presence of acLDL (50 µg/mL) and 1 µCi ^{3}H-cholesterol (Amersham-Biosciences) per well in medium containing 0.2% bovine serum albumin for 24 h. After incubation, medium was aspirated, cells were washed two times with PBS, and incubated with fatty acids in medium containing 0.2% bovine serum albumin for a further 8 h. Cells were washed again with PBS, and incubated in the presence or absence of 50 µg/mL HDL as cholesterol acceptor for 4 h. After incubation, medium was collected and cells were lysed using 0.2 M NaOH, and medium and lysates were counted for ^{3}H-activity. Cholesterol efflux was calculated as the percentage of ^{3}H-activity in the medium from the total ^{3}H-activity in medium and lysate as described[37].

Statistical analysis

Student`s t-test was used to compare means of treatments with those of control. Differences of $P<0.05$ were considered significant.

Results

Effects of treatment on viability of RAW264.7 macrophage-derived foam cells

Treatment with 50 µmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA had no affect on the viability of RAW264.7 cells compared to control treatment (data not shown).

Incorporation of c9t11-CLA, t10c12-CLA and LA in RAW264.7 macrophage-derived foam cells

Incubation of RAW264.7 macrophages with 50 µmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA resulted in a marked incorporation of the respective fatty acids into total cell lipids compared to control cells (**Table 1**). However, the increase in the proportion of C18:2*c*9*t*11 after treatment with *c*9*t*11-CLA was less pronounced than the increase in the proportions of C18:2*t*10*c*12 and C18:2 (n-6) after treatment with *t*10*c*12-CLA and LA, respectively.

Table 1. Concentrations of c9t11-CLA, t10c12-CLA or LA in total lipids of RAW264.7 macrophage foam cells cultured in the absence (control) or presence of 50 µmol/L of c9t11-CLA, t10c12-CLA or LA for 24 h[1]

Treatment	control	c9t11-CLA	t10c12-CLA	LA
Fatty acid		g/100 g total FAME		
LA	1.4 ± 0.1	2.2 ± 0.3	2.1 ± 0.1	13.0 ± 0.6
c9t11-CLA	<0.1	9.2 ± 0.5	0.1 ± 0.1	<0.1
t10c12-CLA	<0.1	0.1 ± 0.1	13.3 ± 0.5	<0.1

[1] Values are means ± SD, n = 2.

Effects of treatment on cholesterol concentrations in RAW264.7 macrophage-derived foam cells

Treatment of acLDL-induced RAW264.7 macrophage-derived foam cells with either *c*9*t*11-CLA or *t*10*c*12-CLA lowered concentrations of esterified cholesterol but not of free cholesterol compared to control cells (P<0.05; **Figure 1**). Treatment with LA had no effect on the concentrations of free or esterified cholesterol in acLDL-induced RAW264.7 macrophage-derived foam cells.

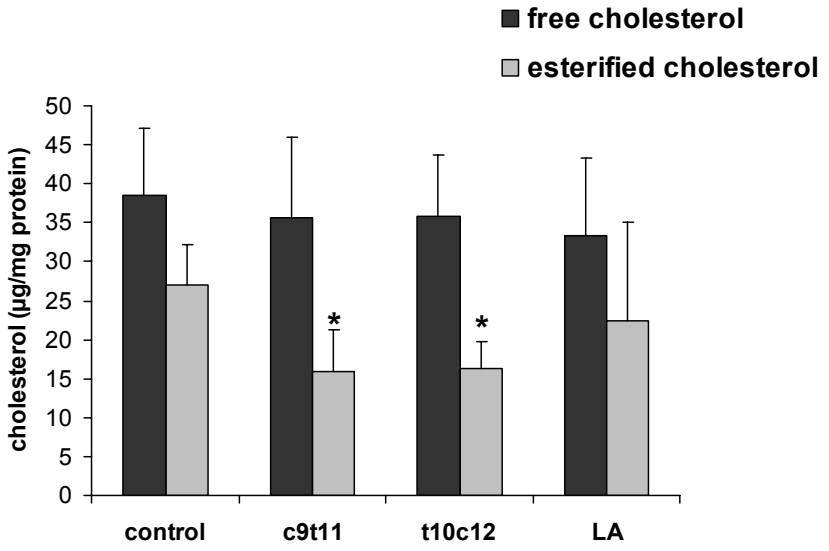

Figure 1.
Effect of treatment with 50 µmol/L of either c9t11-CLA, t10c12-CLA or LA on total, free, and esterified cholesterol concentrations in acLDL-induced RAW 264.7 macrophage-derived foam cells. Cells treated without fatty acids were used as controls. Data represent means ± SD of at least three independent experiments.
*Significantly different from control, P<0.05.

Effects of treatment on relative mRNA concentrations of genes involved in cholesterol homeostasis in RAW264.7 macrophage-derived foam cells

Relative mRNA concentrations of CD36, ABCA1, LXRα, and NPC-2 were significantly increased by both *c*9*t*11-CLA and *t*10*c*12-CLA (P<0.05; **Figure 2**), whereas that of NPC-1 was only increased by *t*10*c*12-CLA compared to control cells (P<0.05). Treatment of RAW264.7 macrophage-derived foam cells with LA did not alter mRNA concentrations of the genes investigated (P<0.05). Relative mRNA concentration of PPARα in RAW264.7 macrophage-derived foam cells was not altered by treatment with either fatty acid. Relative mRNA concentration of PPARγ in RAW264.7 macrophage-derived foam cells was significantly elevated by incubation with *c*9*t*11-CLA (P<0.05), but not by incubation with *t*10*c*12-CLA or LA.

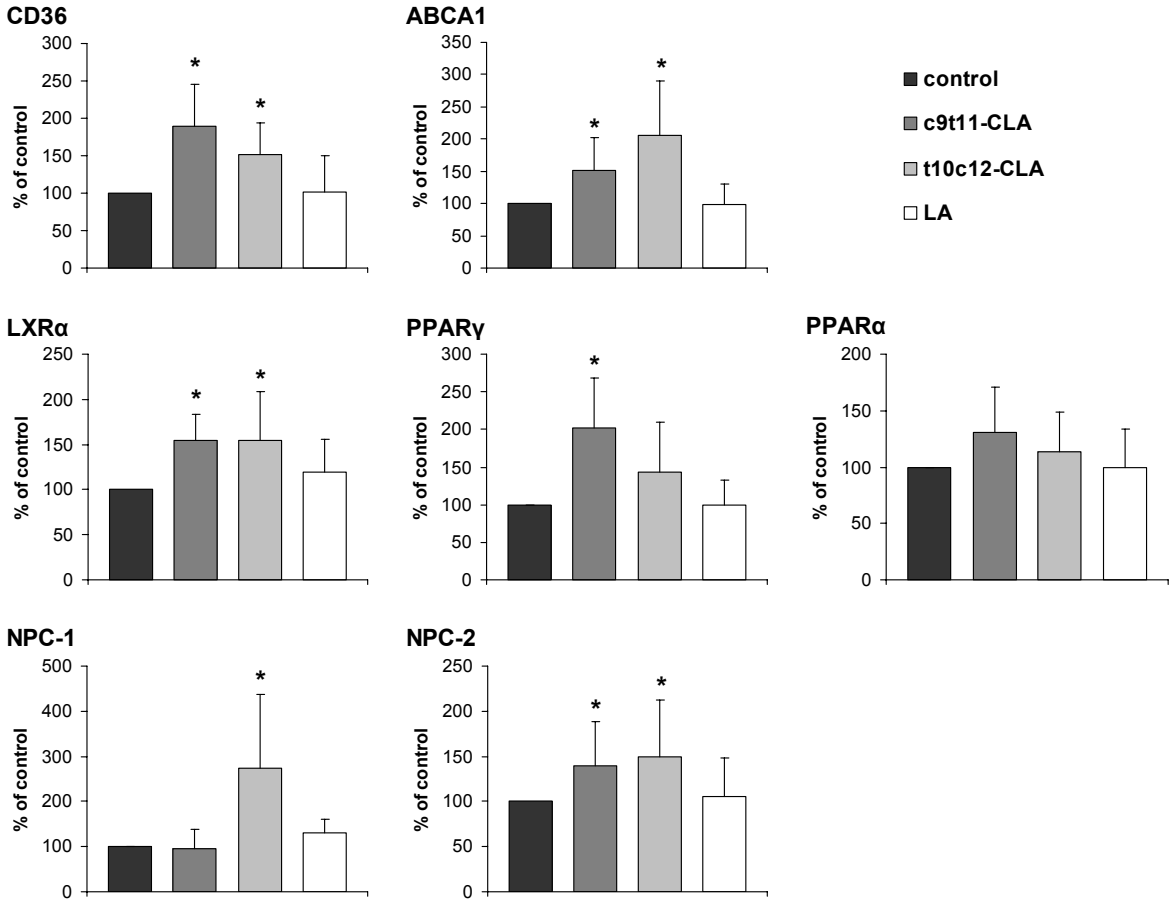

Figure 2.
Effect of treatment with 50 µmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA on relative mRNA concentrations of genes involved in cholesterol homeostasis in acLDL-induced RAW264.7 macrophage-derived foam cells. Cells treated without fatty acids were used as controls. Data represent mean ± SD of at least three independent experiments and are expressed as percentage of mRNA abundance of controls (= 100 ± 0 %). *Significantly different from control, $P<0.05$.

Effects of treatment on relative protein concentrations of ABCA1, LXRα and PPARγ in RAW264.7 macrophage-derived foam cells

Since mRNA data indicated an induction of genes involved in cholesterol efflux by CLA we also determined relative protein concentrations of ABCA1 and its transcriptional activator LXRα in western blot experiments. PPARγ protein was also considered because of the marked effect of *c*9*t*11-CLA on its mRNA concentration. Unfortunately, we had no access to antibodies against NPC-1 and NPC-2 which are not commercially available yet. Our western blot experiments revealed that only the protein concentration of ABCA1 was significantly increased by *t*10*c*12-CLA ($P<0.05$; **Figures 3A and B**), whereas *c*9*t*11-CLA tended to increase the protein concentration of ABCA1. Relative protein concentrations of LXRα and PPARγ were not altered by treatment with either CLA isomers or LA.

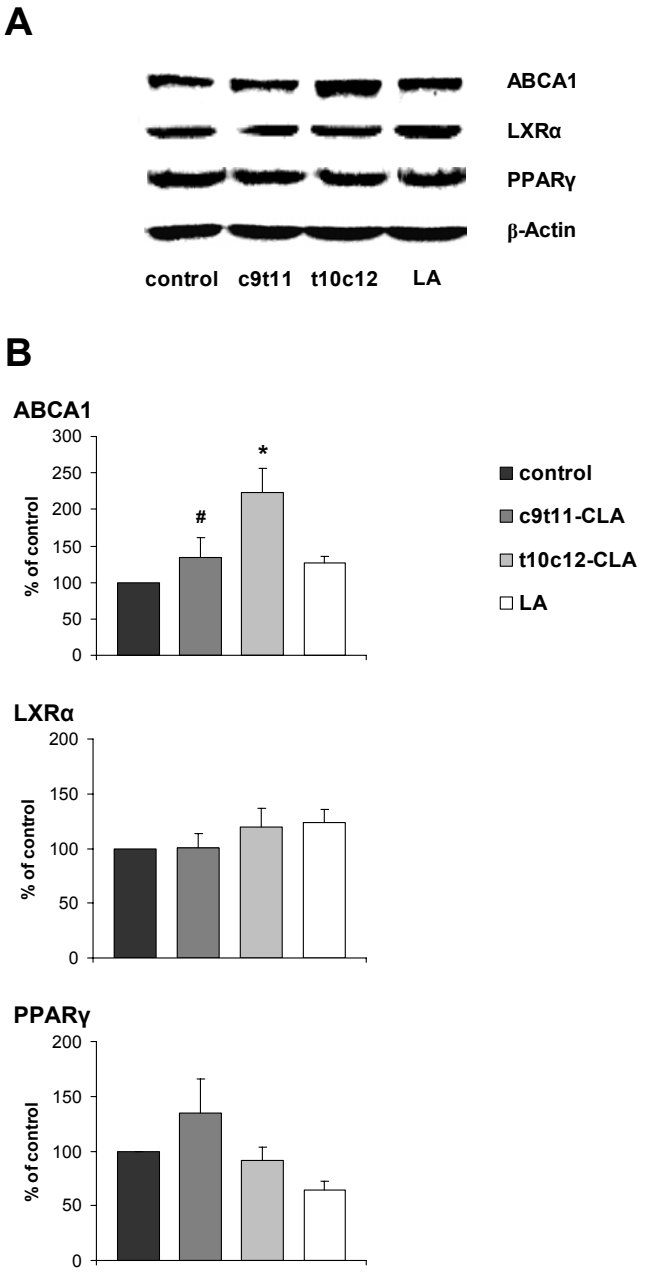

Figure 3.
Effect of treatment with 50 µmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA on relative protein concentrations of ABCA1, LXRα, and PPARγ in acLDL-induced RAW264.7 macrophage-derived foam cells. Cells treated without fatty acids were used as controls.
A, Representative immunoblots specific to ABCA1, LXRα, PPARγ, and β-Actin which was used for normalization are shown.
B, Graphs represent data from densitometric analysis and represent mean ± SD of at least three independent experiments. Data are expressed as percentage of protein concentration of controls (= 100 ± 0 %).
*Significantly different from control, $P<0.05$, #Data tends to differ from control, $P<0.15$.

Effects of treatment on cholesterol efflux from RAW264.7 macrophage-derived foam cells

In the presence of HDL, cholesterol efflux from acLDL-induced RAW264.7 macrophage-derived foam cells was significantly increased by *c*9*t*11-CLA and *t*10*c*12-CLA compared to control treatment ($P<0.05$; **Figure 4**), but not by LA. In the absence of HDL cholesterol efflux was not different between control cells and cells treated with fatty acids.

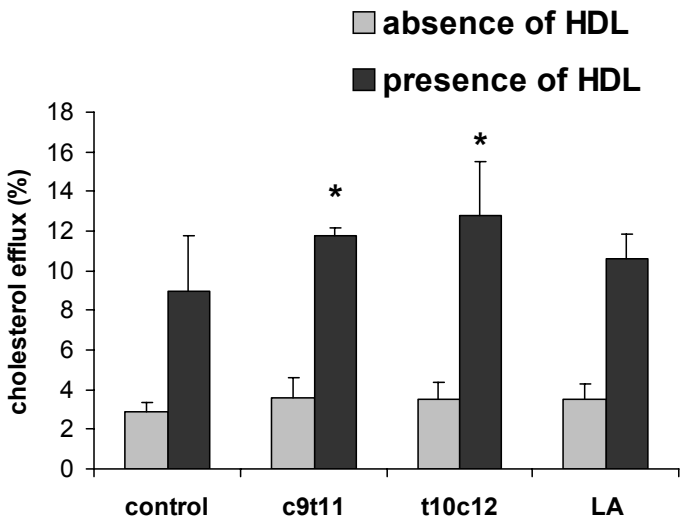

Figure 4.
Effect of treatment with 50 µmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA on cholesterol efflux in acLDL-induced RAW264.7 macrophage-derived foam cells in the presence and absence of HDL. Cells treated without fatty acids were used as controls. Efflux is expressed as counts media/(counts media + counts lysate) x 100. Data represent mean ± SD of at least three independent experiments.
*Significantly different from control, $P < 0.05$.

Discussion

Pharmacological PPARα and γ ligands have been demonstrated to induce cholesterol removal from macrophage foam cells through alterations in the expression of genes involved in cholesterol homeostasis[4,5,8,9,13]. Since CLA are also well-known activators of PPARα and PPARγ[18-20], the present study aimed to explore the effect of CLA isomers on cholesterol homeostasis in RAW264.7 macrophage-derived foam cells. The present study clearly demonstrated that *c*9*t*11-CLA and *t*10*c*12-CLA lowered concentrations of esterified cholesterol, the storage form of cholesterol in macrophage-foam cells, and stimulated HDL-dependent cholesterol efflux in mouse RAW264.7 macrophage-derived foam cells. The reduction of cholesterol accumulation by CLA isomers in RAW264.7 macrophage-derived foam cells was evidenced by a decline in the concentrations of esterified and total cholesterol. Thus, the finding that lowering of macrophage cholesterol accumulation and induction of HDL-dependent cholesterol removal could be observed with CLA isomers, which has also been reported in response to pharmacological PPAR ligands[4,5,8,9,13], strengthens our assumption that CLA isomers are capable of affecting macrophage foam cell cholesterol homeostasis through their potential to activate PPARs. In connection with recent findings that treatment with CLA isomers failed to reduce cholesterol accumulation in human THP-1 macrophage-derived foam cells[24], the results from the present study clearly indicate that the effect of CLA on macrophage cholesterol accumulation is cell type-specific. The reason for these cell type-specific effects of CLA cannot be definitely ruled out, but differences in the cellular uptake and incorporation of CLA isomers between RAW264.7 and THP-1 cells might be causative. Whereas in THP-1 macrophages only a poor incorporation of CLA isomers in total cell lipids after treatment with CLA isomers has been reported[24], treatment of RAW264.7 macrophage foam cells with CLA isomers in the present study resulted in a marked incorporation of CLA isomers. However, since up-regulation of CD36 by CLA isomers has been observed in that recent report[24] other reasons such as differences in the expression pattern of PPAR-subtypes between these two cell lines might also apply[18,38]. Nevertheless, to resolve these contradictory results between RAW264.7 and THP-1 cells with certainty further research is required.

On the molecular level, reduction of cholesterol accumulation in cultured macrophages by PPARα and PPARγ activators has been explained by increased

expression of LXRα, which in turn activates expression of LXRα target genes such as macrophage cholesterol exporters, namely ABCA1 and ABCG1, and, thereby, stimulates cholesterol efflux to extracellular acceptors for reverse cholesterol transport[4,5,8,9]. A recent study further demonstrated that PPARα activation stimulates postlysosomal mobilization of cholesterol by induction of gene expression of NPC-1 and NPC-2[13]. Both proteins control intracellular trafficking of cholesterol from the late endosomal compartment and lysosome, respectively, to the plasma membrane[14,15]. It has been suggested[13] that this results in an enrichment of cholesterol in the plasma membrane and an enhanced availability of cholesterol at the cell membrane, which also contributes to increased macrophage cholesterol efflux and reverse cholesterol transport by PPARα activators. Hence, the increased mRNA expression of ABCA1, LXRα, NPC-1, and NPC-2 in response to CLA isomers as observed in the present study is associated with the reduced cholesterol accumulation in RAW264.7 macrophage-derived foam cells. The induction of ABCA1 by CLA isomers could also be observed at the protein level although this effect was only significant for *t*10*c*12-CLA whereas *c*9*t*11-CLA only showed a tendency towards an increased ABCA1 protein concentration. However, this observation is in the line with the more pronounced effect of *t*10*c*12-CLA on the elevation of ABCA1 mRNA concentration compared with *c*9*t*11-CLA. Surprisingly, we could not detect an increased protein concentration of LXRα in response to *t*10*c*12-CLA or *c*9*t*11-CLA although both CLA isomers elevated the mRNA concentration of this transcription factor. Although we do not know the exact reason for this finding it has to be considered that the elevation of LXRα mRNA concentration by CLA isomers was only about 50%, a degree that can hardly be demonstrated in western blot experiments. Nevertheless, collectively the present data suggest that reverse cholesterol transport is presumably stimulated by natural PPAR ligands deriving from food like CLA. The observation from a recent study that plasma HDL cholesterol concentrations and ABCA1 gene expression were increased in the aorta of hamsters fed *c*9*t*11-CLA is indeed indicative of the potential of CLA to increase the capacity for reverse cholesterol transport[39].

The present study further shows that treatment with LA, a frequently used reference fatty acid when studying the effects of CLA[24,40], has no effect on the esterified cholesterol fraction and cholesterol removal and only slight effects on mRNA expression of genes involved in cholesterol homeostasis in RAW264.7 macrophage-derived foam cells. This indicates that the inhibitory effect of CLA isomers on macrophage foam cell cholesterol accumulation is dependent on the specific conjugated dienoic structure of CLA. In part the lack of effect of LA might be explained by the fact that LA is a weaker PPARα and PPARγ activator than *c*9*t*11-CLA or *t*10*c*12-CLA[18]. Consistent with this finding is the observation that LA, in contrast to *c*9*t*11-CLA or *t*10*c*12-CLA, did not induce the PPARγ target gene CD36 in RAW264.7 and THP-1 macrophage-derived foam cells[24]. Similar observations have been reported from Yu et al.[19] using RAW264.7 macrophages.

In conclusion, the present study shows that *c*9*t*11-CLA and *t*10*c*12-CLA reduce cholesterol accumulation in RAW264.7 macrophage-derived foam cells probably by stimulating HDL-dependent cholesterol efflux. The CLA-induced increase in cholesterol efflux from macrophage foam cells is presumably explained by the up-regulation of LXRα and the LXRα target gene ABCA1, which operates on cholesterol export to extracellular acceptors such as apo-AI/HDL. Enhanced mRNA expression of NPC-1 and NPC-2 in response to treatment with CLA isomers probably also contributes to the observed lowering of cholesterol accumulation in RAW264.7 cells, since both proteins mediate intracellular cholesterol transport to the plasma

membrane and increase the availability of cholesterol for efflux through extracellular acceptors. Since treatment with CLA isomers also caused up-regulation of CD36, which mediates cholesterol uptake from modified LDL particles[41], we suggest that the concomitant up-regulation of ABCA1, which mediates cholesterol removal from cells, was probably more pronounced leading to a net efflux of cholesterol. Because extensive accumulation of cholesterol by macrophage foam cells in the arterial wall leads to atherosclerosis, the present findings in connection with other beneficial effects of CLA in macrophages[19,26,42] might in part explain the anti-atherogenic actions of CLA as observed in animal models of experimental atherosclerosis[21-23].

Acknowledgment
RR participated in the design of the study, supervised lipid and gene expression analyses and efflux experiments, participated in the interpretation of the results and prepared the manuscript; GW performed western blot experiments; DS determined lipid and mRNA concentrations and performed cholesterol efflux experiments; KE conceived of the study and its design, coordinated work, participated in the interpretation of the results, and helped to draft the manuscript. There was no specific funding for this study and there is not any potential conflict of interest.

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Troglitazone but not conjugated linoleic acid reduces gene expression and activity of matrix-metalloproteinases-2 and -9 in PMA-differentiated THP-1 macrophages

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Abstract

Gene expression and activity of matrix-metalloproteinases (MMP)-2 and -9 in macrophages are reduced through peroxisome proliferator-activated receptor γ (PPARγ)-dependent inhibition of NF-κB. Since conjugated linoleic acids (CLAs) are PPARγ ligands and known to inhibit NF-κB via PPARγ, we studied whether CLA isomers are capable of reducing gene expression and gelatinolytic activity of MMP-2 and -9 in PMA-differentiated THP-1 macrophages, which has not yet been investigated. Incubation of PMA-differentiated THP-1 cells with either *c*9*t*11-CLA, *t*10*c*12-CLA or linoleic acid (LA), as a reference fatty acid, resulted in a significant incorporation of the respective fatty acids into total cell lipids relative to control cells ($P<.05$). Treatment of PMA-differentiated THP-1 cells with 10 and 20 μmol/L troglitazone but not with 10 or 100 μmol/L *c*9*t*11-CLA, *t*10*c*12-CLA or LA reduced relative mRNA concentrations and activity of MMP-2 and MMP-9 compared to control cells ($P<.05$). DNA-binding activity of NF-κB and PPARγ and mRNA expression of the NF-κB target gene $cPLA_2$ were not influenced by treatment with CLA. In contrast, treatment of PMA-differentiated THP-1 cells with troglitazone significantly increased transactivation of PPARγ and decreased DNA-binding activity of NF-κB and relative mRNA concentration of $cPLA_2$ relative to control cells ($P<.05$). In conclusion, the present study revealed that CLA isomers, in contrast to troglitazone, did not reduce gene expression and activity of MMP-2 and -9 in PMA-differentiated THP-1 macrophages, which is probably explained by the observation that CLA isomers neither activated PPARγ nor reduced DNA-binding activity of NF-κB. This suggests that CLA isomers are ineffective in MMP-associated extracellular matrix degradation which is thought to contribute to the progression and rupture of advanced atherosclerotic plaques.

Keywords: Conjugated linoleic acid; Peroxisome proliferator-activated receptor γ; Matrix-metalloproteinase; NF-κB; Macrophages; Atherosclerosis

1. Introduction

Most of the acute clinical manifestations of atherosclerosis such as myocardial infarction and stroke are due to the rupture of advanced atherosclerotic plaques. Activated macrophages within the inflamed atherosclerotic plaques play a key role in inducing plaque rupture by secreting matrix-metalloproteinases (MMPs) that degrade the extracellular matrix and therefore contribute to destabilization of atherosclerotic plaques [1–3]. MMP-9 (gelatinase B) and MMP-2 (gelatinase A) are predominant MMPs secreted by activated inflammatory macrophages [4,5], and elevated blood levels of these MMPs in patients with coronary artery disease clearly indicate their important role in the atherosclerotic process [6,7]. Therefore, factors that regulate the activation of MMPs are potential therapeutic targets for stabilizing rupture-prone atherosclerotic plaques.

Recent studies demonstrated that peroxisome proliferator-activated receptor γ (PPARγ) plays a critical role in the regulation of MMP activity in human monocytes and macrophages as well as vascular smooth muscle cells. Activation of PPARγ in response to different PPARγ ligands resulted in a decreased phorbol 12-myristate 13-acetate (PMA)-induced MMP-9 expression and gelatinolytic activity in macrophages [8] and a decreased MMP-9 secretion in

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vascular cells [9–11]. PPARγ-mediated inhibition of proinflammatory transcription factors such as NF-κB, which plays an essential role in the regulation of MMP-9 and MMP-2 and other inflammatory responses [12,13], largely constitutes the mechanistic basis for this effect [11,14]. In addition, attenuation of inflammatory processes by pharmacological PPARγ ligands also contributes to the cardiovascular benefits of this class of drugs.

We and others have shown that conjugated linoleic acids (CLA), a group of conjugated isomers of linoleic acid (LA) naturally occurring in food such as milk and meat of ruminants, are capable of inhibiting NF-κB DNA-binding activity and proinflammatory gene transcription in macrophages and smooth muscle cells through activation of PPARγ [15,16]. In addition, CLAs have been demonstrated to exert potent anti-atherogenic actions in animal models of experimental atherosclerosis [17,18]. Although the underlying mechanisms of action are only poorly understood, PPARγ-dependent repression of pro-inflammatory gene expression has been proposed [18]. However, whether CLAs are capable of affecting gene expression and gelatinolytic activity of MMPs has not yet been investigated.

Therefore, the present study aimed to explore the effect of the CLA isomers on gene expression and gelatinolytic activity of MMP-2 and MMP-9 in PMA-treated THP-1 monocytic cells. THP-1 monocytic cells can be induced to differentiate into macrophages following treatment with PMA. During PMA-induced monocyte–macrophage differentiation, which is accompanied by up-regulation of MMP-9 and MMP-2 [5,19,20], cells become flat and adhere to the surface of cell culture plates and exhibit striking morphological similarities to macrophages [21]. Thus, PMA-differentiated THP-1 cells have been widely used as a representative macrophage cell line and used as an investigative model of atherosclerosis in vitro [22]. Since the activity of MMPs is at least in part controlled by a family of endogenous inhibitors called tissue inhibitors of metalloproteinases (TIMPs), we also investigated the effect of CLA on TIMP-1 and TIMP-2, which are also synthesized by macrophages and are effective in binding to the catalytic site and inhibiting the active forms of MMPs [23]. To further elucidate the potential mechanisms of action of CLA on MMP and TIMP expression we also determined the DNA-binding activity of NF-κB, which has been demonstrated to be an important transcriptional regulator of MMP and TIMP genes [12,13,24]. In addition, we determined the mRNA expression of the NF-κB target gene cytosolic phospholipase A_2 ($cPLA_2$) which was recently shown to be down-regulated by CLA isomers in vascular smooth muscle cells [16]. Due to the transrepression activity of PPARγ activation on NF-κB we also analyzed the PPARγ DNA-binding activity and the mRNA expression of the PPARγ target gene CD36 in response to treatment with CLA isomers. As isomers, *c*9*t*11-CLA, which contributes to more than 90% of total CLA in natural foods [25], and *t*10*c*12-CLA, which is one of the main isomers in chemically produced CLA mixtures, were used. As reference substances, LA, which is frequently used when investigating the biological effects of CLA [26,27], and troglitazone, a pharmacological PPARγ ligand, were used.

2. Methods and materials

2.1. Materials and reagents

*c*9*t*11-CLA (≥96% pure) and *t*10*c*12-CLA (≥98% pure) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). LA (≥99% pure), PMA and troglitazone were obtained from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Cell culture and treatments

The human monocytic THP-1 cells were obtained from the American Type Culture Collection (ATCC)/LGC Promochem (Wesel, Germany). THP-1 monocytes were cultured according to the ATCC protocol for propagation in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 4.5 g/L glucose, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate, 0.05 mmol/L 2-mercaptoethanol and 1% penicillin/streptomycin. For the induction of monocyte–macrophage differentiation, cells were seeded (5×10^5 cells/24 wells) in RPMI1640 medium with 50 ng/ml PMA for 72 h as described previously [26]. After differentiation, nonattached cells were removed by aspiration, and the adherent cells were washed with RPMI1640 medium three times. Afterwards, PMA-differentiated THP-1 cells were treated with 10 and 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 1, 10 and 20 μmol/L troglitazone for 6 and/or 24 h. PMA-differentiated THP-1 cells treated without fatty acids or troglitazone were used as control. Incubation media containing fatty acids were prepared by diluting the fatty acid stock solutions (100 mmol/L fatty acid in ethanol) or the troglitazone stock solution (20 mmol/L in DMSO) to the concentrations indicated. Vehicle controls contained 0.1% ethanol or 0.1% DMSO. No differences were observed between vehicle controls and non-vehicle controls in either experiment. For all experiments only cells between Passages 5 and 20 were used.

2.3. Cell viability

The viability of cells after treatment with fatty acids and troglitazone was examined by the MTT assay [28].

2.4. Fatty acid analysis of macrophage total lipids

After treatment of PMA-differentiated macrophages with fatty acids as indicated above, cells were washed with PBS and total lipids were extracted with hexane/isopropanol (3:2 v/v). The lipid extracts were dried under nitrogen, transmethylated with trimethylsulfonium hydroxide, and fatty acid methyl esters (FAME) were separated by GC as described previously in detail [29].

2.5. Reverse transcription-polymerase chain reaction

After treatment of cells as indicated above, the total RNA of the cells was extracted using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. cDNA synthesis and relative quantification of mRNA expression of MMP-2, MMP-9, TIMP-1, TIMP-2, CD36 and $cPLA_2$ compared to the housekeeping gene glyceraldehyde-3-phosphat dehydrogenase (GAPDH) were determined by real-time detection polymerase chain reaction (PCR) as described previously [30]. Relative quantification was performed using the △△Ct method [31]. Ct values of target genes and the housekeeping gene were obtained using Rotorgene Software 5.0. Relative expression ratios are expressed as fold changes of mRNA abundance compared to control cells. The effect of PMA-induced THP-1 monocyte–macrophage differentiation on gene expression of MMPs and TIMPs was also analyzed by semi-quantitative reverse transcription-PCR (RT-PCR) as described recently in detail [16]. The number of PCR cycles was determined in preliminary experiments ensuring that relative quantification of mRNA expression was performed within the linear range of amplification of each PCR product. Sequences of gene-specific primers, obtained from Operon (Köln, Germany) were as follows (forward, reverse): GAPDH (5′-GAC CAC AGT CCA TGC CAT CAC-3′, 5′-TCC ACC ACC CTG TTG CTG TAG-3′), MMP-2 (5′-GGC CCT GTC ACT CCT GAG AT-3′, 5′-GGC ATC CAG GTT ATC GGG GA-3′), MMP-9 (5′-CAA CAT CAC CTA TTG GAT CC-3′, 5′-TGG GTG TAG AGT CTC TCG CT-3′), TIMP-1 (5′-AAT TCC GAC CTC GTC ATC AG-3′, 5′-TGC AGT TTT CCA GCA ATG AG-3′), TIMP-2 (5′-TGA TCC ACA CAC GTT GGT CT-3′, 5′-TTT GAG TTG CTT GCA GGA TG-3′), cPLA2 (5′-GAG CTG ATG TTT GCA GAT TGG GTT G-3′, 5′-GTC ACT CAA AGG AGA CAG TGG ATA AGA-3′), CD36 (5′-GGT GTG GTG ATG TTT GTT GC-3′, 5′-CAG GGC CTA GGA TTT GTT GA-3′).

Table 1
Fatty acid composition of total lipids of PMA-differentiated THP-1 macrophages cultured in the absence (control) or presence of 100 μmol/L of *c*9*t*11-CLA, *t*10*c*12-CLA or LA for 24 h[a]

Treatment	Control	*c*9*t*11-CLA	*t*10*c*12-CLA	LA
	g/100 g total FAME			
Fatty acid				
C14:0	2.6±0.1	1.9 ±0.1*	2.1±0.1*	1.9±0.1*
C16:0	31.5±0.4	23.2±0.9*	26.4±0.4*	22.7±0.5*
C16:1	4.2±0.1	3.3±0.1*	3.0±0.1*	2.6±0.1*
C18:0	13.9±0.1	10.3±0.6*	11.2±0.3*	9.7±0.1*
C18:1	26.3±0.2	20.3±0.4*	19.2±0.2*	16.2±0.3*
C18:2 (n-6)	4.8±0.5	4.6±0.1	3.9±0.1	31.9±0.1*
C18:2*c*9*t*11	<0.1	23.4±0.9*	0.1±0.1	0.1±0.1
C18:2*t*10*c*12	<0.1	<0.1	19.3±0.8*	<0.1
C20:4 (n-6)	4.2±0.1	4.1±0.3	4.9±0.2	5.0±0.1*
C20:5 (n-3)	2.7±0.1	1.4±0.1*	1.8±0.1*	1.3±0.1*
C22:4 (n-6)	0.7±0.1	0.7±0.1	0.5±0.1	0.6±0.1
C22:6 (n-3)	3.9±0.1	1.7±0.3*	2.7±0.1*	2.4±0.1*

[a] Values are means±S.D. (n=2).
* Significantly different from control, P<.05.

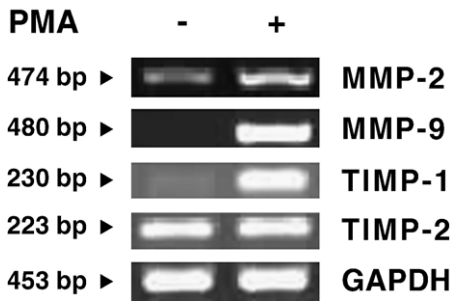

Fig. 1. Relative mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in differentiated and undifferentiated THP-1 cells. Monocyte–macrophage differentiation was induced by treatment of THP-1 cells with 50 ng/ml PMA for 72 h. After incubation, total RNA was extracted, 1.2 μg total RNA reverse transcribed and cDNA was subjected to semiquantitative RT-PCR using gene-specific primers as described in Methods and Materials. Representative images of PCR product gel electrophoresis following RT-PCR analysis are shown for one independent experiment.

2.6. Gelatinolytic zymography

For determination of gelatinolytic activity, cells were treated as indicated above, except that serum-free medium was used. After treatment, culture medium was collected and concentrated (10×) using Vivaspin 500 concentrators (Vivascience, Stonehouse, UK). The gelatinolytic activities of secreted active and pro-form of MMP-2 and -9, respectively, in the concentrated culture medium were analyzed by zymography using 10% polyacrylamide/sodium dodecyl sulphate (SDS) gels containing 0.1% (w/v) gelatin as described by Hawkes et al. [32]. In brief, 30 μl of concentrated culture medium was diluted with 15 μl of 3× nonreducing sample buffer (30% wt/vol glycerol, 187.5 mM Tris-base, 6.9% SDS, 0.15% bromphenol blue, pH 6.8), incubated at 45°C for 15 min, and each lane was loaded with 20 μl of the sample. After electrophoresis, gels were washed two times in 200 ml of 2.5% Triton X-100 at room temperature for 15 min and incubated in development buffer (0.05 M Tris-HCl, 5 mM $CaCl_2$, 0.03% Triton X-100, pH 8.8) for another 15 min at room temperature, followed by an overnight incubation at 37°C in the same buffer to allow digestion of the gelatin substrate. After digestion, gels were rinsed briefly with water, fixed with 200 ml of 40% ethanol and 10% glacial acetic acid for 30 min at room temperature, stained for 2 h with 0.116% Coomassie blue (Sigma) in 25% ethanol and 8% acetic acid, and destained in a solution of 25% ethanol and 8% acetic acid. Gelatinolytic activity was detected as clear bands against the blue-stained background. Gelatinolytic bands were size calibrated with a prestained protein ladder (PAGE Ruler, Fermentas, St. Leon-Rot,

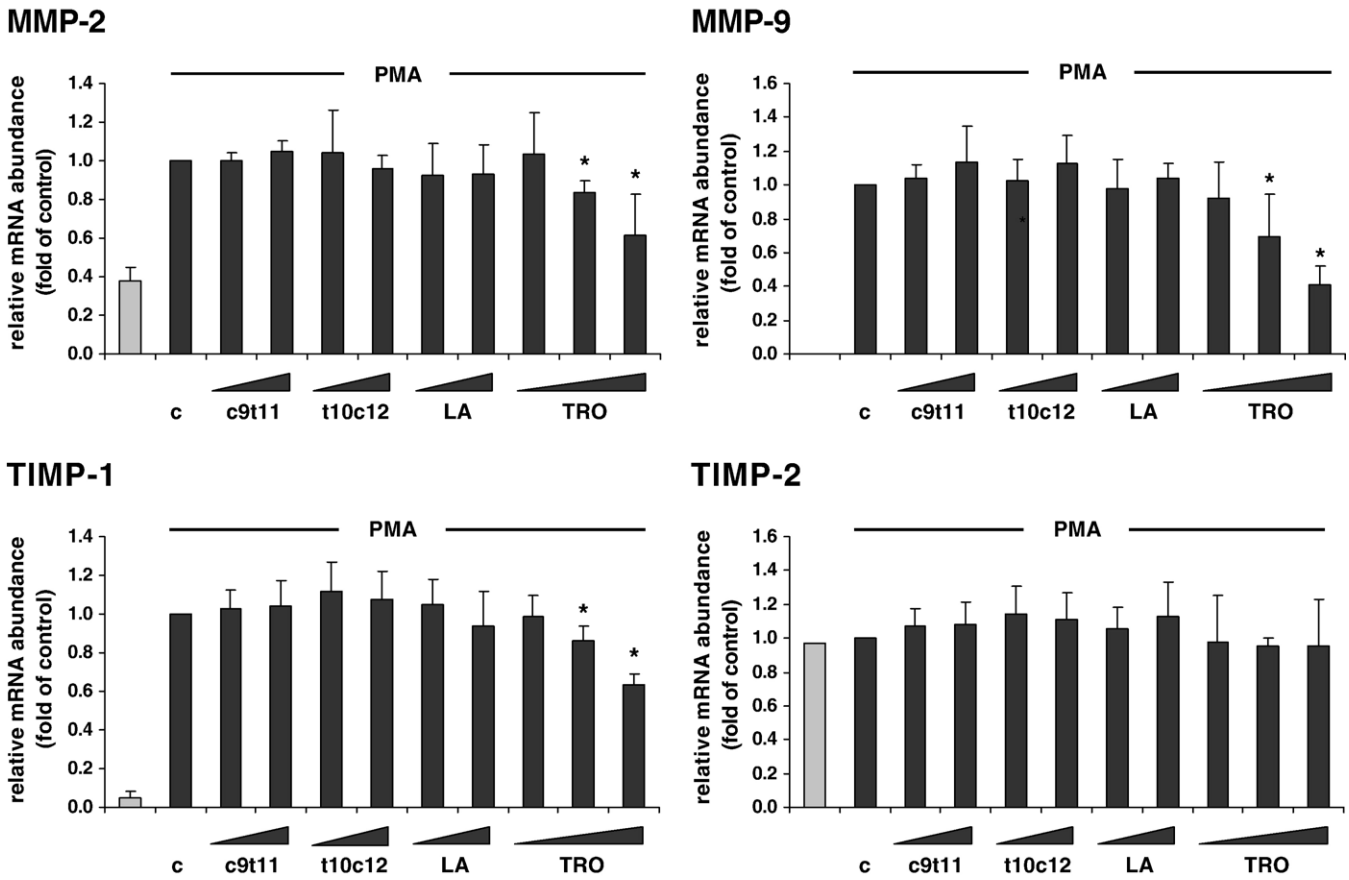

Fig. 2. Effect of 24-h treatment of PMA-differentiated THP-1 macrophages with 10 and 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 1, 10 and 20 μmol/L troglitazone (TRO) relative to vehicle control (c) on mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. Grey bars represent the value of undifferentiated THP-1 monocytes. After incubation, total RNA was extracted, 1.2 μg total RNA reverse transcribed and cDNA was subjected to real-time RT-PCR using gene-specific primers as described in Methods and Materials. Data represent the mean±S.D. of at least three independent experiments measured in quadruplicate and are expressed as fold of mRNA abundance of vehicle control (=1.0±0).

Germany). Gels were photographed and the intensity of the bands determined by densitometric analysis using Gel-Pro Analyzer software (Intas, Upland, CA, USA).

2.7. DNA-binding activity of NF-κB and PPARγ

For the measurement of PPARγ and NF-κB DNA-binding activities, nuclear extracts were prepared from PMA-differentiated THP-1 macrophages treated as indicated above with a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol. Protein concentrations in the nuclear extracts were determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) with BSA as standard. PPARγ and NF-κB transactivities in the nuclear extracts were determined by the transcription factor assays TransAM PPARγ Kit and TransAM NF-κB p50 Kit (both from Active Motif), respectively, according to the manufacturer's protocol. For the measurement of PPARγ and NF-κB transactivities 20 μg of nuclear protein was used in each assay.

2.8. Statistical analysis

For statistical analysis, data were subjected to nonparametric analysis by Friedman test using the Minitab Statistical Software (Minitab, State College, PA, USA). A nonparametric test was used because data of most parameters did not show a normal distribution as evidenced by Shapiro–Wilk test. Differences between means of treatment and control of $P<.05$ were considered significant.

3. Results

3.1. Effects of treatment with PMA on morphology and phenotype of THP-1 cells

THP-1 monocytes treated without PMA for 72 h were round in shape and did not adhere to the plastic surface of cell culture plates. In contrast, THP-1 cells treated with PMA for 72 h became flat and amoeboid in shape, and adhered to the surface of cell culture plates, which is characteristic of the differentiation of monocytes to macrophages.

3.2. Effects of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on viability of PMA-differentiated THP-1 cells

Treatment with 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L troglitazone for 24 h had no effect on the viability of PMA-differentiated THP-1 cells compared to control treatment. The average cell viability after treatment with 100 μmol/L of *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L troglitazone was in the range of 94% and 98% (mean for three independent experiments) of vehicle control cells (=100%).

3.3. Effects of fatty acid treatment on fatty acid composition of PMA-differentiated THP-1 cell total lipids

Incubation of PMA-differentiated THP-1 cells with either *c*9*t*11-CLA, *t*10*c*12-CLA or LA resulted in a significant incorporation of the respective fatty acids into total cell lipids relative to control cells ($P<.05$; Table 1). The incorporation of *c*9*t*11-CLA, *t*10*c*12-CLA or LA was accompanied by a concomitant decrease in the proportions of the saturated fatty acids C14:0, C16:0 and C18:0, and the monounsaturated fatty acids C16:1 and C18:1 relative to control cells ($P<.05$). Treatment with CLA isomers further decreased the proportions of C20:5 (n-3) and C22:6 (n-3) relative to control cells ($P<.05$). Treatment with LA increased the proportions of C20:4 (n-6) compared to control cells ($P<.05$).

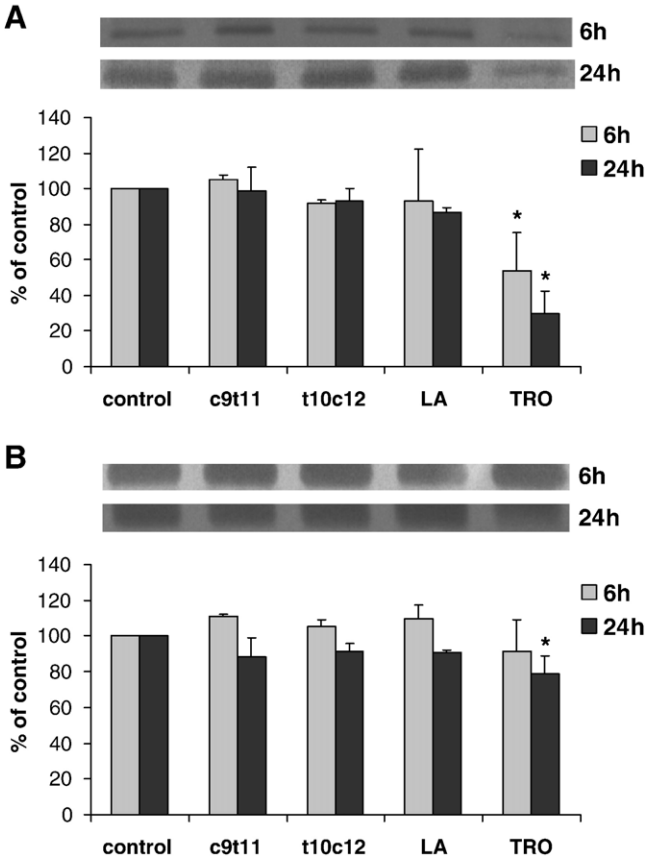

Fig. 3. Effect of treatment (6 h, 24 h) with 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L TRO on secretion of MMP-2 and MMP-9 in PMA-differentiated THP-1 macrophages. After incubation, culture medium was collected, concentrated and assayed for secretion and activation of MMP-2 and MMP-9 by gelatin zymography as described in Methods and Materials. Representative images of gelatin zymography and densitometric analysis of MMP activity are shown for active 66-kDa MMP-2 (A) and active 82-kDa MMP-9 (B). Data from densitometric analysis represent the mean±S.D. of three independent experiments and are expressed as percentage of MMP activity of control (=100±0%).

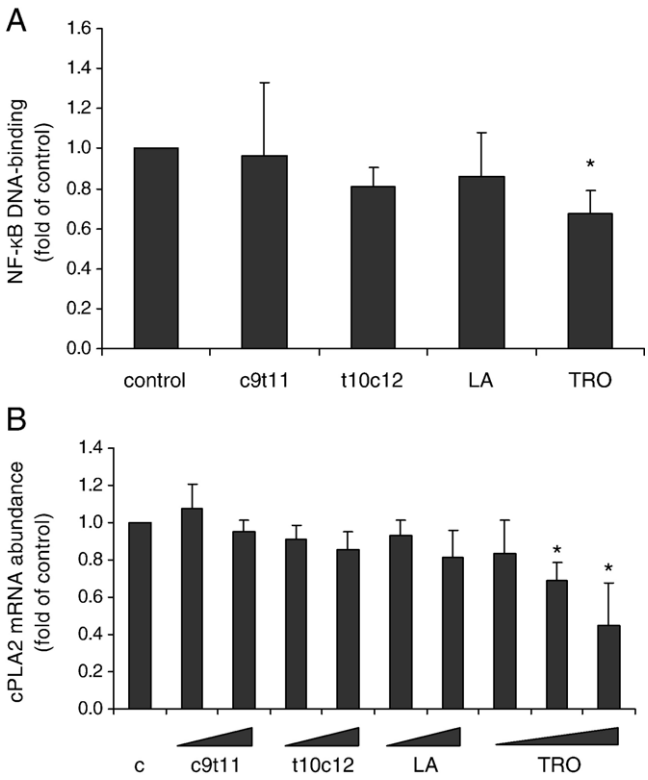

Fig. 4. Effect of 24-h treatment with 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L TRO on DNA-binding activity of NF-κB (A) and effect of 24-h treatment with 10 and 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 1, 10 and 20 μmol/L TRO relative to vehicle control (c) on mRNA expression of the NF-κB target gene $cPLA_2$ (B) in PMA-differentiated THP-1 macrophages. After treatment, nuclear extracts and total RNA were prepared and binding of NF-κB subunit p50 to the NF-κB consensus binding sequence and relative mRNA concentration of $cPLA_2$ were determined by an ELISA-based assay and real-time detection PCR, respectively, as described in Methods and Materials. Data represent the mean± S.D. of three independent experiments and are expressed as fold of vehicle control (=1.0±0).

3.4. Effect of treatment with PMA on mRNA concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 in THP-1 cells

As demonstrated in Fig. 1, relative mRNA concentrations of MMP-2, MMP-9 and TIMP-1 were markedly increased in THP-1 cells treated with PMA for 72 h compared to cells treated without PMA. The most pronounced increase in the relative mRNA concentration was observed for MMP-9 and

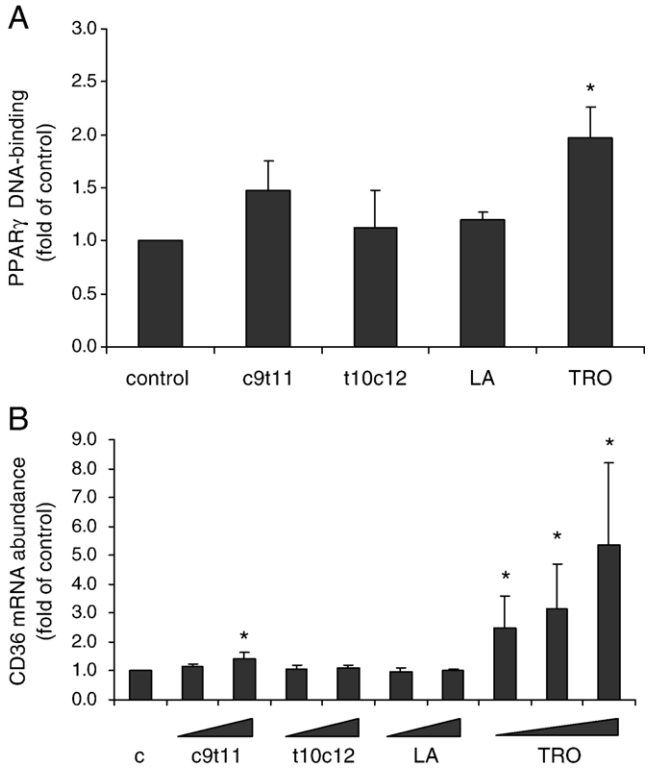

Fig. 5. Effect of 24-h treatment with 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 20 μmol/L TRO on DNA-binding activity of PPARγ (A) and effect of 24-h treatment with 10 and 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA or 1, 10 and 20 μmol/L TRO relative to vehicle control (c) on mRNA expression of the PPARγ target gene CD36 (B) in PMA-differentiated THP-1 macrophages. After treatment, nuclear extracts and total RNA were prepared and binding of PPARγ to the PPAR response element and relative mRNA concentration of CD36 were determined by an ELISA-based assay and real-time detection PCR, respectively, as described in Methods and Materials. Data represent the mean±S.D. of three independent experiments and are expressed as fold of PPARγ DNA-binding activity of vehicle control (=1.0±0).

TIMP-1, which were not or only barely detectable in THP-1 cells treated without PMA. The relative mRNA concentration of TIMP-2 was not different between THP-1 cells treated with or without PMA.

3.5. Effect of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in PMA-differentiated THP-1 cells

As shown in Fig. 2, treatment of PMA-differentiated THP-1 cells with either 10 or 100 μmol/L *c*9*t*11-CLA, *t*10*c*12-CLA or LA for 24 h did not alter relative mRNA concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 compared to control cells. Treatment of PMA-differentiated THP-1 cells with troglitazone for 24 h dose-dependently reduced relative mRNA concentrations of MMP-2, MMP-9 and TIMP-1 compared to control cells ($P<.05$), whereas the relative mRNA concentration of TIMP-2 was not influenced by troglitazone compared to control treatment.

3.6. Effect of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on the activity of MMP-2 and MMP-9 in PMA-differentiated THP-1 cells

Treatment with 100 μmol/L *c*9*t*11-CLA, *t*10*c*12-CLA or LA for 6 or 24 h did not influence the gelatinolytic activities of secreted active MMP-2 (66 kDa) and active MMP-9 (82 kDa) in PMA-differentiated THP-1 cells (Fig. 3). However, treatment with 20 μmol/L troglitazone for 24 h reduced the gelatinolytic activities of secreted active MMP-2 and active MMP-9 in PMA-differentiated THP-1 macrophages ($P<.05$). The decreased activity of active MMP-2 in response to troglitazone was even observed after 6 h of treatment.

3.7. Effect of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on NF-κB DNA-binding activity and cPLA$_2$ mRNA expression in PMA-differentiated THP-1 cells

The DNA-binding activity of NF-κB was not influenced by treatment with 100 μmol/L *c*9*t*11-CLA, *t*10*c*12-CLA or LA but was significantly reduced by treatment with 20 μmol/L troglitazone in PMA-differentiated THP-1 cells compared to control cells ($P<.05$; Fig. 4A). The relative mRNA concentration of the NF-κB target gene cPLA$_2$ was dose-dependently reduced by treatment of PMA-differentiated THP-1 cells with 10 and 20 μmol/L troglitazone ($P<.05$), but not by CLA isomers or LA relative to control cells (Fig. 4B).

3.8. Effect of treatment with c9t11-CLA, t10c12-CLA, LA or troglitazone on PPARγ DNA-binding activity and CD36 mRNA expression in PMA-differentiated THP-1 cells

The DNA-binding activity of PPARγ was not influenced by treatment with 100 μmol/L *t*10*c*12-CLA or LA in PMA-differentiated THP-1 cells relative to control cells (Fig. 5A). Treatment with 100 μmol/L *c*9*t*11-CLA tended to increase PPARγ DNA-binding activity in PMA-differentiated THP-1 cells ($P<.15$), whereas treatment with 20 μmol/L troglitazone significantly increased PPARγ DNA-binding activity in PMA-differentiated THP-1 cells compared to control cells ($P<.05$). The relative mRNA concentration of the PPARγ target gene CD36 was increased by treatment of PMA-differentiated THP-1 cells with 100 μmol/L *c*9*t*11-CLA and 1, 10 and 20 μmol/L troglitazone ($P<0.05$), but not by *t*10*c*12-CLA or LA relative to control cells (Fig. 5B).

4. Discussion

Several cell culture studies revealed that MMP expression and activity in macrophages and other cells of the vascular wall are reduced through PPARγ-dependent inhibition of NF-κB [11,14,33], which is a major regulator of MMP gene expression and activity [12,13,24,34]. Since CLAs are PPARγ ligands and known to inhibit NF-κB-mediated expression of pro-inflammatory genes via PPARγ in various

cell lines [15,16], we studied whether CLA isomers are capable of affecting gene expression and gelatinolytic activity of MMPs in macrophages which have not yet been investigated. As a model system we used the well-established PMA-differentiated THP-1 macrophage cell model, which is widely used to investigate the effects of compounds on macrophage MMP gene expression and activity [20,35,36]. In addition, several studies dealing with CLA have used the THP-1 macrophage cell model [18,26,37].

The present study clearly demonstrated that CLA isomers did not reduce PMA-induced gene expression and gelatinolytic activity of MMP-2 and MMP-9 in the THP-1 macrophage cell model. In addition, gene expression of TIMP-1 and TIMP-2, which are critical for the regulation of MMP activity in macrophages [23,38], was not altered either by treatment with CLA isomers. In contrast, the synthetic PPARγ-ligand troglitazone significantly reduced gene expression and activity of both MMPs which is consistent with findings from recent studies [11,14]. In addition, troglitazone significantly increased PPARγ transactivation and reduced DNA binding of NF-κB which probably constitutes the mechanistic basis for the reduced gene expression and activity of MMPs in response to troglitazone as evidenced in recent studies [11,14]. Consistent with the reduced NF-κB DNA-binding activity in response to treatment of THP-1 macrophages with troglitazone is the observed dose-dependent decrease in the mRNA expression of $cPLA_2$ which is an inflammatory gene known to be regulated by NF-κB. Since neither of the CLA isomers had any effect on PPARγ transactivation, NF-κB DNA-binding activity and mRNA expression of $cPLA_2$, we suggest that this failure is responsible for the unaltered gene expression and activity of MMP-2 and MMP-9 in PMA-differentiated THP-1 macrophages. These findings indicate that pharmacological and natural PPARγ ligands obviously differ with respect to modulation of gene expression and activity of MMPs in THP-1 macrophages. However, these findings do not exclude the possibility that CLA isomers might exert inhibitory effects on other inflammatory genes such as cyclooxygenase (COX)-2 in THP-1 cells. Although it has been demonstrated that CLA reduces COX-2 gene expression and prostaglandin E_2 release via inhibition of NF-κB in vascular smooth muscle cells and RAW264.7 macrophages [16,39,40], it has also been shown that CLA reduces COX-2 levels and prostaglandin biosynthesis in MCF-7 breast cancer cells independently of NF-κB by antagonization of the AP-1 pathway [41]. In addition, findings from Li et al. [39,40] using RAW264.7 macrophages also suggested that *t*10*c*12-CLA potentially inhibits alternative signaling pathways in addition to the NF-κB pathway, e.g., signaling by the mitogen-activated protein kinase family such as extracellular signal-related kinase or c-jun NH_2-terminal kinase, which also contributes to the anti-inflammatory effects of CLA.

With respect to the reason underlying the lack of effect of CLA isomers on the parameters addressed in the present study, we are confident of sufficient treatment (incubation time, CLA concentration) and incorporation of the fatty acids into the cells as demonstrated by markedly increased concentrations of CLA isomers in THP-1 cell total lipids following treatment with CLA. CLA concentrations in THP-1 cell total lipids were similar to those found in other cell types treated with CLA isomers [16,42] and in the range of those causing significant PPARγ transactivation in various cell types [16,27]. We also suggest that failure of treatment with CLA isomers is not due to a low expression of PPARγ, because the PPARγ subtype is abundantly expressed in THP-1 cells [18] and markedly up-regulated during PMA-induced monocyte–macrophage differentiation [43]. One reason possibly explaining the failure of treatment with CLA might be the fact that CLA isomers are comparatively weak PPARγ ligands due to a low binding affinity and, therefore, cause only a weak PPARγ transactivation. In contrast, troglitazone is a synthetic compound known to have a high affinity for PPARγ as evidenced by ligand-binding assays [44]. The latter was also evidenced in the present study by the use of a PPARγ transactivation assay which revealed a marked response by troglitazone but only a weak, but not significant, response by *c*9*t*11-CLA and no response at all by *t*10*c*12-CLA. The relative mRNA level of PPARγ, however, was not affected by treatment with either troglitazone or fatty acids (data not shown) which is consistent with previous reports [18,26]. In contrast, the relative mRNA level of the PPARγ target gene CD36 was not only markedly increased by troglitazone but also by *c*9*t*11-CLA by about 1.5-fold, whereas *t*10*c*12-CLA and LA had no effect. This finding concurs with our observation that *c*9*t*11-CLA but not *t*10*c*12-CLA slightly increased PPARγ DNA-binding activity indicating that at least *c*9*t*11-CLA causes PPARγ transactivation. The observed increase in CD36 mRNA concentration by *c*9*t*11-CLA is consistent with findings from a recent study also using THP-1 macrophages [26]. However, in the study of Weldon et al. [26] increased mRNA concentrations of CD36 were also observed in response to *t*10*c*12-CLA. We cannot explain this discrepancy between our study and the study of Weldon et al. [26] at the moment, but differences in the cell culture conditions applied might be causative. One important difference between our study and the study of Weldon et al. [26] is the use of serum-free medium during ligand treatment of THP-1 cells in the latter study. This might have reduced the competition and interference of the fatty acids added to the medium, e.g., CLA or LA, with those normally found in serum and, therefore, might have increased the ligand availability for PPARγ. Nevertheless, a study using RAW264.7 macrophages also revealed that the relative mRNA concentration of CD36 was only elevated in response to *c*9*t*11-CLA but not to *t*10*c*12-CLA [15], which is therefore largely confirmatory of what we have found herein using THP-1 macrophages. LA, which was used as a reference fatty acid, did not increase PPARγ transactivation and CD36 mRNA level either in THP-1 cells which is probably also

explained by its comparatively low binding affinity for PPARγ [45]. The lower binding affinity of CLA isomers for PPARγ compared to troglitazone might be of decisive importance in the THP-1 macrophage cell model used herein, because other PPAR subtypes such as PPARα and PPARβ/δ are also highly expressed in THP-1 cells [43] and CLA binds to and activates all PPAR subtypes with similar efficiency [46,47]. Therefore, it might be speculated that the low PPARγ activation by CLA in THP-1 cells is due to a competition of the various PPAR subtypes for binding of CLA isomers to their ligand-binding domains. Thus, the effect of CLA on PPARγ would be expected to be higher in a cell type expressing predominantly the PPARγ subtype. Indeed, a recent study [15] demonstrated that several CLA isomers, amongst others *c*9*t*11-CLA and *t*10*c*12-CLA, caused a pronounced activation of PPARγ in RAW264.7 macrophage cells which predominantly express PPARγ, whereas neither PPARα nor PPARβ/δ was detectable. In addition, the aforementioned study [15] revealed that CLA-induced PPARγ transactivation was accompanied by a concomitant decrease in the production of pro-inflammatory products such as nitric oxide, interleukin-6 and tumor necrosis factor-α which is indicative of the potential of CLA isomers to mediate negative regulation of pro-inflammatory transcription factors such as NF-κB or AP-1 in macrophages via PPARγ. Although we cannot definitely rule out the reason for the lack of effect of CLA on MMP expression and activity in THP-1 cells, we are convinced that the macrophage cell model used herein is an appropriate cell model in view of elucidating the anti-atherogenic actions of CLA as observed in vivo [17,18]. THP-1 macrophages, in contrast to RAW264.7 macrophages, may better reflect the in vivo situation because primary monocyte-derived macrophages also express detectable levels of PPARα and PPARβ/γ besides PPARγ [11].

Pathological studies have shown that rupture-prone regions of atherosclerotic plaques are frequently infiltrated with a large number of monocyte-derived macrophages [48]. These plaque-associated macrophages produce large quantities of MMPs, particularly MMP-9 and MMP-2 [5,49], which participate in extracellular matrix degradation and destabilization of plaques, thereby promoting acute cardiovascular events such as myocardial infarction and stroke which are typical late-stage events of atherosclerosis. Because CLA failed to influence macrophage MMP secretion and activity in the present study we suggest that CLA may exert its anti-atherogenic actions by other mechanisms than those addressed in the present study or during earlier stages of atherosclerosis. For instance, several independent studies revealed that CLA exerts lipid-lowering actions (e.g., total and LDL cholesterol) in different animal models and humans subjects [50–53], suggesting that typical atherogenic risk factors are influenced by CLA in a beneficial manner. In addition, CLA has also been shown to have a beneficial effect on insulin sensitivity [54] and glucose homeostasis, e.g., it normalizes an impaired glucose tolerance [46], thereby modulating major metabolic disorders predisposing to atherosclerosis.

In conclusion, the present study revealed that CLA isomers, in contrast to troglitazone, did not reduce gene expression and activity of MMP-2 and -9 in PMA-differentiated THP-1 macrophages. The lack of effect of CLA isomers is probably explained by the observation that CLA isomers neither activated PPARγ nor reduced DNA-binding activity of NF-κB, which is an important regulator of MMP gene expression [12,13,24,34]. In contrast, the inhibitory effect of the pharmacological PPARγ ligand troglitazone on gene expression and gelatinolytic activity of MMP-2 and -9 was accompanied by an increased PPARγ transactivation and a reduced DNA-binding activity of NF-κB. This suggests that CLA isomers are ineffective in MMP-associated extracellular matrix degradation which is thought to contribute to the progression and rupture of advanced atherosclerotic plaques in the late stage of atherosclerosis.

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LDL receptor gene transcription is selectively induced by *t*10*c*12-CLA but not by *c*9*t*11-CLA in the human hepatoma cell line HepG2

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Abstract

Conjugated linoleic acids (CLA) have attracted scientific interest due to their potential beneficial effects on atherosclerosis. Recently, a mixture of CLA isomers was demonstrated to upregulate LDL receptor expression in the human hepatoma cell line HepG2. However, the underlying mechanisms remain to be resolved. Thus, the aim of this study was to elucidate how CLA mediates upregulation of LDL receptor in HepG2 cells and whether this upregulation is isomer-specific. The results revealed that LDL receptor promoter activity and mRNA expression were strongly induced upon treatment with *t*10*c*12-CLA ($P<0.05$), whereas *c*9*t*11-CLA and linoleic acid (LA) had no effect. In addition, only treatment with *t*10*c*12-CLA markedly induced mRNA expression of SREBP-2 and HMG-CoA reductase and slightly induced that of SREBP-1 ($P<0.05$). Using SREBP-2 knockdown cells, we could demonstrate that the effect of *t*10*c*12-CLA on LDL receptor gene transcription was significantly reduced when compared to control cells ($P<0.05$). When using SREBP-1 knockdown cells the effect of *t*10*c*12-CLA on LDL receptor mRNA only slightly decreased compared to control cells. In addition, using different deletion constructs of the LDL receptor gene promoter we showed that the induction of the LDL receptor by *t*10*c*12-CLA is independent of the AP-1 motif in the LDL receptor promoter. In conclusion, the present study revealed that transcriptional activation of the LDL receptor gene by *t*10*c*12-CLA is dependent on the upregulation of SREBP-2 and is probably due to the activation of the SRE-1 in the LDL receptor gene promoter in HepG2 cells. Thus, the decreased plasma cholesterol levels in response to CLA as observed in a limited number of animal and human studies might be explained by an enhanced uptake of VLDL and LDL cholesterol via hepatic LDL receptors. However, it provides no explanation for the outcome of most human studies reporting unaltered or even increased plasma and LDL cholesterol concentrations in response to supplementation with CLA.

Keywords: Conjugated linoleic acid; LDL receptor; Atherosclerosis; SREBP-2; SREBP-1

1. Introduction

Increased plasma cholesterol concentrations are a widely recognized risk factor for atherosclerosis. One of the major determinants of plasma cholesterol concentrations is the activity of the LDL receptor, which mediates cellular uptake and degradation of plasma LDL cholesterol. Although the LDL receptor is expressed in almost all tissues, hepatic LDL receptors are of great importance for LDL clearance from plasma, since about two thirds of LDL receptors in the organism are present in the liver [1]. Recently, conjugated linoleic acids (CLA), which are a naturally occurring group of positional and geometric isomers of linoleic acid (LA), were demonstrated to increase both LDL receptor mRNA and protein abundance in HepG2 cells [2]. This observation might explain why CLA supplementation decreased plasma LDL or VLDL cholesterol levels in healthy human subjects [3,4] and reduced plasma total and LDL cholesterol levels in hamsters [5,6], although several other human studies revealed either no

Abbreviations: CLA, conjugated linoleic acid; LA, linoleic acid; SREBP, sterol regulatory element binding protein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase

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effect [7–10] or even an increase [11] in plasma and LDL cholesterol concentrations in response to supplementation with CLA. Nevertheless, the underlying mechanisms for the CLA-induced upregulation of LDL receptor in HepG2 cells remain largely unresolved.

Although little is known about the regulation of LDL receptor gene transcription by fatty acids, it is well documented that transcription of the LDL receptor gene is regulated by multiple mechanisms. It has been well established that the intracellular cholesterol concentration regulates LDL receptor expression through a negative feedback mechanism [12,13]. In sterol-depleted cells, the sterol regulatory element binding proteins (SREBP)-1 and -2 are activated and bind to the cis-acting sterol regulatory element-1 (SRE-1) in the LDL receptor gene promoter and initiate LDL receptor gene transcription, whereas activation of SREBPs and LDL receptor transcription is inhibited when cholesterol is accumulated in the cell. Yu-Poth et al. [2] concluded from their study that upregulation of LDL receptor by CLA is independent of SREBP-1. However, whether SREBP-2 is involved in the upregulation of LDL receptor has not been addressed in that study, although it is well known that LDL receptor gene transcription is induced by SREBP-2 [14]. Moreover, SREBP-independent pathways that are also known to mediate LDL receptor gene transcription in hepatocytes [15–18] were also not addressed in the aforementioned study [2].

The aim of the present study was to investigate the mechanisms underlying the CLA-induced LDL receptor gene transcription. Following the previous study from Yu-Poth et al. [2] we used HepG2 cells, a hepatoblastoma-derived cell line that mimics normal hepatocytes. Moreover, dietary fatty acids were demonstrated to regulate LDL receptor in this cell line [2,19]. To address the question whether SREBP-1 and/or -2 are involved in the CLA-induced LDL receptor gene transcription we generated SREBP-1 and -2 knockdown cells by RNA interference. Since the AP-1 motif in the LDL receptor promoter has also been implicated as an important target site for the induction of LDL receptor gene transcription [18], we performed reporter gene assays using different deletion mutations of the LDL receptor gene promoter. Moreover, in contrast to Yu-Poth et al. [2] which used a mixture of various CLA isomers, we investigated the effects of single CLA isomers, since divergent effects have been observed with different CLA isomers with respect to blood lipids or inflammation [11,20]. As isomers we used *c*9*t*11-CLA, which is the predominant isomer in natural foods [21], and *t*10*c*12-CLA, which is one of the main isomers in chemically produced CLA mixtures.

2. Materials and methods

2.1. Chemicals

The CLA isomers *t*10*c*12-CLA and *c*9*t*11-CLA were purchased from Cayman Chemical (Ann Arbor, MI, USA). Linoleic acid (LA) was obtained from Sigma-Aldrich (Taufkirchen, Germany). Acetic acid [1,2-^{14}C] (100 mCi/mmol) was purchased from Movarek Biochemicals (Brea, CA). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany). RPMI 1640 medium (containing 0.3 g/L L-glutamine and 2.0 g/L sodium bicarbonate), trypsin-ethylene diamine tetra acetic acid (0.5 g/L trypsin 0.2 g/L EDTA) in Hank's balanced salt solution (HBSS) and gentamicin were obtained from Gibco/Invitrogen (Karlsruhe, Germany).

2.2. Cell culture

HepG2 cells (DSMZ, Braunschweig, Germany) were grown in RPMI 1640 medium supplemented with 10% FBS and 0.5% gentamicin (growth medium) [22].

2.3. Fatty acid solutions

Stock solutions of fatty acids (*c*9*t*11-CLA, *t*10*c*12-CLA, LA) were prepared in ethanol at a concentration of 100 mmol/L. For the preparation of the test media, aliquots of these stock solutions were used. The solvent was evaporated under nitrogen, and the fatty acids were converted into their sodium salts by adding equimolar amounts of sodium hydroxide solution. The solution containing the fatty acid salts was added to the pre-warmed growth medium to achieve the final concentrations of fatty acids (10 and 100 μmol/L).

2.4. Cell count and viability

The cell count was determined with a Neubauer chamber after cells were harvested by trypsinization. Cell viability after treatment of cells without (control) or with 10 and 100 μmol/L of fatty acids (*c*9*t*11-CLA, *t*10*c*12-CLA, LA) was examined by the MTT assay [23].

2.5. RNA isolation and real-time RT-PCR

For the determination of mRNA expression levels of LDL receptor, HMG-CoA reductase, SREBP-1, and SREBP-2 HepG2 cells were plated in 24-well plates at a density of 1.8×10^5 cells/well. 48 h after plating, cells were incubated with medium alone (control) or medium supplemented with 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA for 6 and 24 h, respectively. Following incubations, total RNA of the cells was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. 1.2 μg of total RNA was subjected to first strand cDNA synthesis at 42 °C for 60 min using M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany) and 18mer oligo dT-primer (Operon, Köln, Germany). Relative quantification of target gene mRNA compared to the housekeeping gene GAPDH mRNA was determined by real-time detection RT-PCR using the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). Real-time PCR amplification of cDNA was carried out for 30–40 cycles using Taq DNA Polymerase from Promega (Mannheim, Germany) and gene-specific primers (Operon, Köln, Germany). Each PCR cycle comprised denaturation for 20 s at 95 °C, annealing for 30 s at primer-specific temperature (60–65 °C) and elongation for 55 s at 72 °C. Fluorescence intensity in all real-time PCR probes was measured at the end of the elongation step. Subsequently, melting curve analysis was performed for identification of PCR products. Amplification of only one PCR product of the expected length was confirmed using 1% agarose gel electrophoresis. Relative expression ratios were calculated by the ΔΔCt-method according to [24]. The primer sequences used for PCR were as follows (forward, reverse): GAPDH (5′-GAC CAC AGT CCA TGC CAT CAC-3′, 5′-TCC ACC ACC CTG TTG CTG TAG-3′), SREBP-1 (5′-GTG GCG GCT GCA TTG AGA GTG AAG-3′, 5′-AGG TAC CCG AGG GCA TCC GAG AAT-3′), SREBP-2 (5′-CGC CAC CTG CCC CTC TCC TTC C-3′, 5′-TGC CCT GCC ACC TAT CCT CTC ACG-3′), HMG-CoA reductase (5′-TAC CAT GTC AGG GGT ACG TC-3′, 5′-CAA GCC TAG AGA CAT AAT CAT C-3′), LDL receptor (5′-CCC CGC AGA TCA ACC CCC ACT C-3′, 5′-AGA CCC CCA GGC AAA GGA AGA CGA-3′).

2.6. Transient transfection and dual-luciferase reporter assay

For transient transfections, HepG2 cells were plated in 96-well plates at a density of 8×10^4 cells/well. 24 h after plating, cells were transiently transfected

with either promoter reporter gene construct phLDL2, phLDL4 (both kindly provided from Prof. Dr. Krone, Cologne, Germany) or pAP-1-Luc (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) and control vector pRL-SV40 (Promega, Mannheim, Germany) using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) for 5 h according to the manufacturer's protocol. Subsequently, cells were treated as described in Section 2.5 for 24 h. Afterwards the cells were washed with phosphate-buffered saline and lysed with passive lysis buffer (Promega). Reporter *Firefly*-luciferase and *Renilla*-luciferase activities were determined with the Dual-Luciferase Reporter Assay System from Promega according to the manufacturer's instructions using a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany). For control of background luminescence *Firefly*- and *Renilla*-luciferase activities were also determined in the lysates of nontransfected control cells and subtracted from total luminescence of transfected cells. Data were normalized for transfection efficiency by dividing *Firefly*-luciferase activity of either phLDL2, phLDL4 or pAP-1-Luc by that of *Renilla*-luciferase activity of the cotransfected pRL-SV40 plasmid. Results represent relative light units (RLU) and are expressed as fold induction compared to control treatment.

2.7. RNA interference

Knockdown variants of HepG2 cells expressing low levels of SREBP-1 and SREBP-2, respectively, were produced by small interfering (si) RNA-mediated gene knockdown. For this purpose, HepG2 cells were plated in a 24-well format at a density of 1×10^5 cells/well. 24 h after plating, cells were transfected using the Lipofectamine 2000 Reagent (Invitrogen) and gene-specific Stealth™ RNAi molecules ("knockdown siRNA"; Invitrogen) targeting human SREBP-1 and SREBP-2, respectively, according to the manufacturer's protocol for 24 h. After transfection, medium was replaced with fresh medium and cells were incubated for another 24 h. Afterwards cells were treated without (control) or with 100 μmol/L of *t*10*c*12-CLA for 24 h and analyzed for SREBP-1, SREBP-2, HMG-CoA reductase, and LDL receptor mRNA levels by means of real-time RT-PCR. RNAi molecules were designed by the BLOCK-iT™ RNAi designer (Invitrogen). Sequences were as follows (sense, antisense): SREBP-1 (5′-GAU CUA UGU GGC GGC UGC AUU GAG A-3′, 5′-UCU CAA UGC AGC CGC CAC AUA GAU C-3′) and SREBP-2 (5′-AGG CAG GCU UUG AAG ACG AAG CUA A-3′, 5′-UUA GCU UCG UCU UCA AAG CCU GCC U-3′). To monitor for unspecific knockdown effects control cells were transfected with a control Stealth™ RNAi molecule ("Random siRNA") which matched the knockdown siRNA with respect to length and GC content and was not homologous to any mammalian gene sequence. Sequences of control RNAi molecules were as follows (sense, antisense): SREBP-1 (5′-GAU UGU AGC GGG UCG UUA CGU CAG A-3′, 5′-UCU GAC GUA ACG ACC CGC UAC AAU C-3′) and SREBP-2 (5′-AGG UCG GAG UUC AGA GAA GCA CUA A-3′, 5′-UUA GUG CUU CUC UGA ACU CCG ACC U-3′).

2.8. Measurement of [^{14}C]acetate incorporation into cellular lipids

For experiments designed to study incorporation of [^{14}C]acetate into cellular cholesterol HepG2 cells were seeded in 24-well plates (1.8×10^5 cells/well) and treated 48 h after plating with CLA isomers for 24 h as described in Section 2.5. After treatment, the medium was removed and the cells were incubated for another 2 h in the same medium in the presence of [1,2-^{14}C]acetate (100 mCi/mmol, 0.71 μCi per well). Cells were washed three times with ice-cold PBS and radiolabeled lipids were extracted using hexane/isopropanol (3:2, v/v). Free cholesterol, cholesterol esters and triacylglycerols were separated by TLC on silica TLC aluminium sheets (Merck, Darmstadt, Germany) using a solvent system containing hexan/diethyl ether/acetic acid (80:20:3, v/v/v). Spots were obtained by phosphorimaging using an Imaging plate (Raytest, Straubenhardt, Germany) for 24 h. After scanning the images with a Bio-Imaging-Analyzer (BAS-1500, Raytest) the spots were quantified using Bio-Imaging-Analyzer software. Quantification results of treated samples were related to those of untreated controls, which were adjusted to 100%.

2.9. Statistical analysis

Treatment effects were analyzed by one-way ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). For statistically significant *F*-values, means were compared by Fisher's multiple range test. Means were considered significantly different for $P<0.05$.

3. Results

3.1. Cell viability

As in our previous study [22], treatment of HepG2 cells with 10 or 100 μmol/L of either *c*9*t*11-CLA, *t*10*c*12-CLA or LA did not alter cell viability as determined by the MTT-assay when compared to cells treated with medium alone (=control; data not shown).

3.2. Effect of CLA isomers on relative mRNA concentrations of LDL receptor, HMG-CoA reductase, SREBP-2, and SREBP-1

The relative mRNA concentrations of LDL receptor, HMG-CoA reductase, SREBP-2, and SREBP-1 were significantly induced about 2.7-, 1.7-, 2.0-, and 1.5-fold, respectively, following treatment of HepG2 cells with 100 μmol/L *t*10c12-CLA for 24 h when compared to control cells (Fig. 1A–D). No effect was observed after 6 h of treatment of cells with 100 μmol/L of *t*10*c*12-CLA. Treatment with 100 μmol/L of LA or *c*9*t*11-CLA had no effect on the relative mRNA concentrations of LDL receptor, HMG-CoA reductase, SREBP-2, and SREBP-1 at both time points (6 and 24 h) when compared to control cells.

3.3. Effect of CLA isomers on LDL receptor promoter activity

To investigate whether the AP-1 binding site at −125 in the LDL receptor promoter is involved in the action of CLA isomers on LDL receptor gene transcription we used two different LDL receptor promoter constructs. Using LDL receptor promoter construct phLDL2, which contains the AP-1 binding site at −125, LDL receptor promoter activity was dose-dependently increased by treatment with *t*10*c*12-CLA (2-fold and 5-fold at 10 μmol/L and 100 μmol/L, respectively) relative to control cells (Fig. 2A). Treatment with LA or *c*9*t*11-CLA had no effect on the activity of the LDL receptor promoter construct phLDL2 when compared to control cells. When using the promoter construct phLDL4, which lacked the AP-1 binding site at −125, LDL receptor promoter activity was also dose-dependently increased by treatment with *t*10*c*12-CLA (1.4-fold and 3.5-fold at 10 μmol/L and 100 μmol/L, respectively) relative to control cells, whereas treatment with LA or *c*9*t*11-CLA had no effect (Fig. 2B).

3.4. Effect of CLA isomers on the AP-1 signal transduction pathway

Transfection of HepG2 cells with pAP-1-Luc vector enabled us to measure the binding of transcription factors to the AP-1 binding site, providing a direct measure for the activation of this pathway. As demonstrated in Fig. 3, no alteration of the AP-1 signal transduction pathway was observed after treatment with 10 or 100 μmol/L of either *c*9*t*11-CLA or *t*10*c*12-CLA when compared to control treatment.

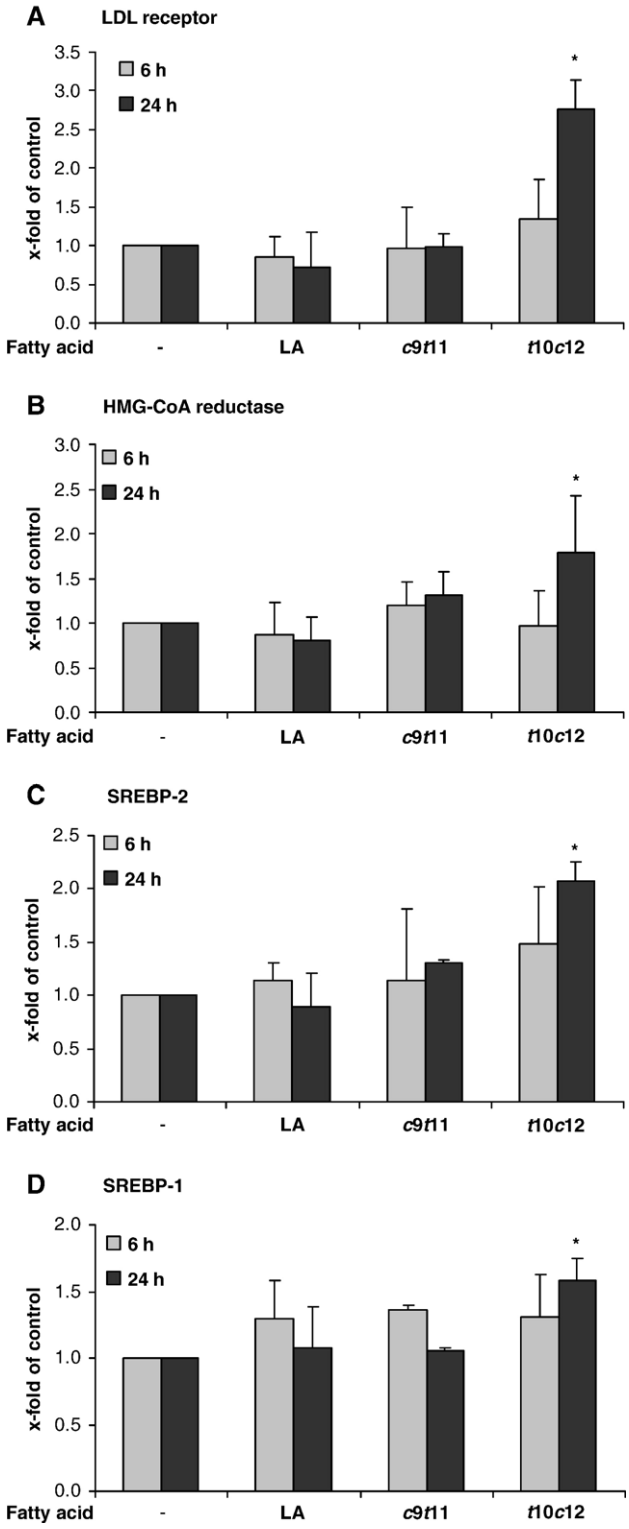

Fig. 1. Effect of 100 μmol/L of either linoleic acid (LA), *c*9*t*11-CLA or *t*10*c*12-CLA on relative mRNA concentrations of LDL receptor (A), HMG-CoA reductase (B), SREBP-2 (C), and SREBP-1 (D) in HepG2 cells. Cells were incubated without (control) or with fatty acids for 6 and 24 h, respectively, and subsequently analyzed for relative mRNA concentrations by real-time RT-PCR. Bars represent means ± SD of three to four independent experiments and are expressed as fold-changes compared to control (= 1.0 ± 0.0). *Significantly different from control, $P < 0.05$.

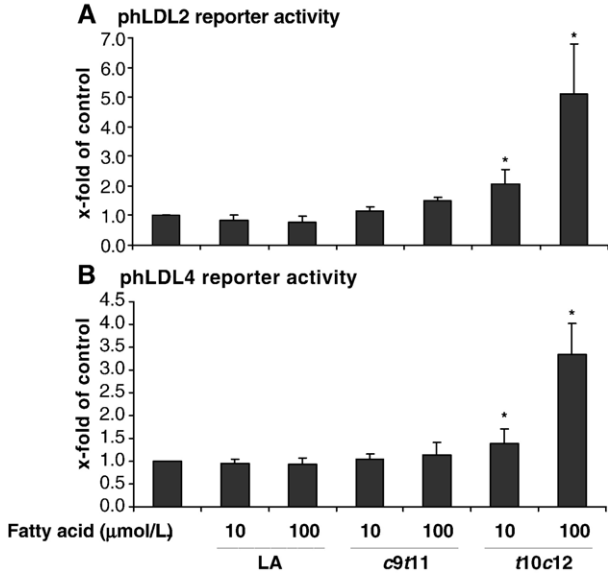

Fig. 2. Effect of 10 and 100 μmol/L of either linoleic acid (LA), *c*9*t*11-CLA or *t*10*c*12-CLA on the activity of the LDL receptor gene promoter in HepG2 cells. Cells were transiently transfected with LDL receptor promoter constructs phLDL2, containing the AP-1 motif at −125, or phLDL4, lacking the AP-1 motif at −125. Subsequently, cells were treated with fatty acids for 24 h and reporter activities of phLDL2 (A) and phLDL4 (B), respectively, were measured using commercial Dual Luciferase Assay. Bars represent means ± SD of three independent experiments and are expressed as fold-changes compared to control (= 1.0 ± 0.0). *Significantly different from control, $P < 0.05$.

3.5. Effect of t10c12-CLA on the relative mRNA concentrations of LDL receptor, HMG-CoA reductase, SREBP-2, and SREBP-1 in SREBP-1 knockdown cells

Transfection of HepG2 cells with knockdown siRNA targeting SREBP-1 caused a reduction of SREBP-1 mRNA concentration of about 60% compared to cells transfected with

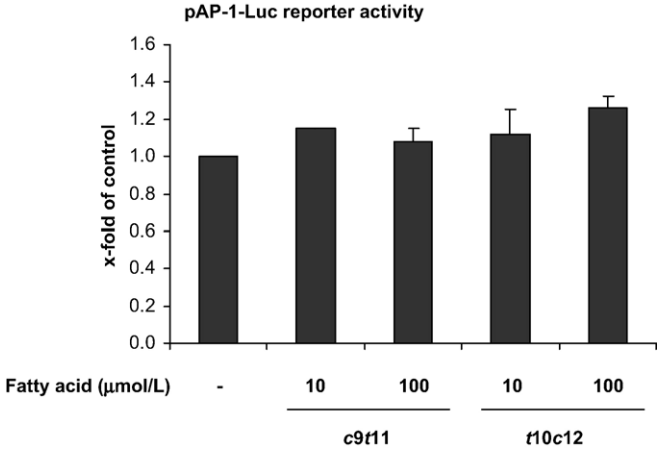

Fig. 3. Effect of 10 and 100 μmol/L of either *c*9*t*11-CLA or *t*10*c*12-CLA on AP-1 transactivation activity in HepG2 cells. Cells were transiently transfected with the AP-1-sensitive reporter vector pAP-1-Luc, and subsequently treated with fatty acids for 24 h. Afterwards, reporter activity was measured using commercial Dual Luciferase Assay. Bars represent means ± SD of three independent experiments and are expressed as fold-changes compared to control (= 1.0 ± 0.0). *Significantly different from control, $P < 0.05$.

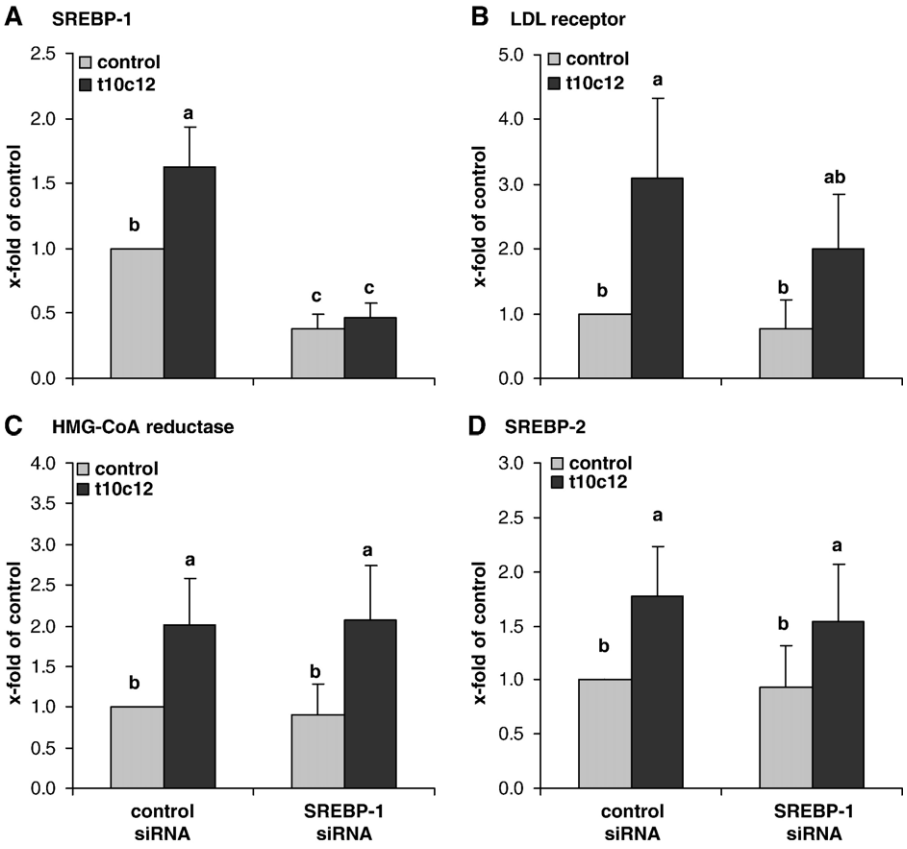

Fig. 4. Effect of 100 μmol/L of *t*10*c*12-CLA on relative mRNA concentrations of SREBP-1 (A), LDL receptor (B), HMG-CoA reductase (C), and SREBP-2 (D) in SREBP-1 knockdown HepG2 cells. SREBP-1 knockdown cells were generated by siRNA-mediated gene silencing. After introduction of knockdown, cells were treated without (control) or with *t*10*c*12-CLA for 24 h and analyzed for relative mRNA concentrations by means of real-time RT-PCR. Bars represent means ± SD of three independent experiments and are expressed as fold-changes compared to control (= 1.0 ± 0.0). [a,b,c]Bars marked with different superscript letters differ significantly, $P < 0.05$.

control siRNA (Fig. 4A). The stimulatory effect of *t*10*c*12-CLA on SREBP-1 mRNA concentration as observed in control cells was completely abrogated in SREBP-1 knockdown cells. The stimulatory effect of *t*10*c*12-CLA on LDL receptor mRNA concentration as observed in control cells was slightly, but not significantly ($P > 0.05$), reduced in SREBP-1 knockdown cells (Fig. 4B). However, LDL receptor mRNA concentration was still induced about 2-fold by treatment with *t*10*c*12-CLA compared to treatment with medium alone in SREBP-1 knockdown cells. The effect of *t*10*c*12-CLA on HMG-CoA reductase and SREBP-2 mRNA concentrations was similar in control cells and SREBP-1 knockdown cells (Fig. 4C and D).

3.6. Effect of t10c12-CLA on the relative mRNA concentrations of LDL receptor, HMG-CoA reductase, SREBP-2, and SREBP-1 in SREBP-2 knockdown cells

Transfection of HepG2 cells with knockdown siRNA targeting SREBP-2 caused a reduction of SREBP-2 mRNA concentration of about 80% compared to cells transfected with control siRNA (Fig. 5A). The stimulatory effect of *t*10*c*12-CLA on SREBP-2 mRNA concentration as observed in control cells was completely abrogated in SREBP-2 knockdown cells. The stimulatory effect of *t*10*c*12-CLA on LDL receptor mRNA concentration as observed in control cells was significantly reduced in SREBP-2 knockdown cells (Fig. 5B). However, LDL receptor mRNA concentration was still induced about 1.7-fold by treatment with *t*10*c*12-CLA compared to treatment with medium alone in SREBP-2 knockdown cells. The stimulatory effect of *t*10*c*12-CLA on HMG-CoA reductase mRNA concentration was completely abrogated in SREBP-2 knockdown cells (Fig. 5C). The effect of *t*10*c*12-CLA on SREBP-1 mRNA concentration was similar in control cells and SREBP-1 knockdown cells (Fig. 5D).

3.7. [^{14}C]acetate incorporation into cellular free cholesterol

[^{14}C]acetate incorporation into cellular free cholesterol of HepG2 cells was significantly induced by treatment with 100 μmol/L of *t*10*c*12-CLA when compared to control cells (Fig. 6). 10 μmol/L of *t*10*c*12-CLA as well as 10 and 100 μmol/L of *c*9*t*11-CLA had no effect on [^{14}C]acetate incorporation into cellular free cholesterol relative to control cells.

4. Discussion

CLA have attracted scientific interest in recent years, since they have been demonstrated to exert diverse beneficial health

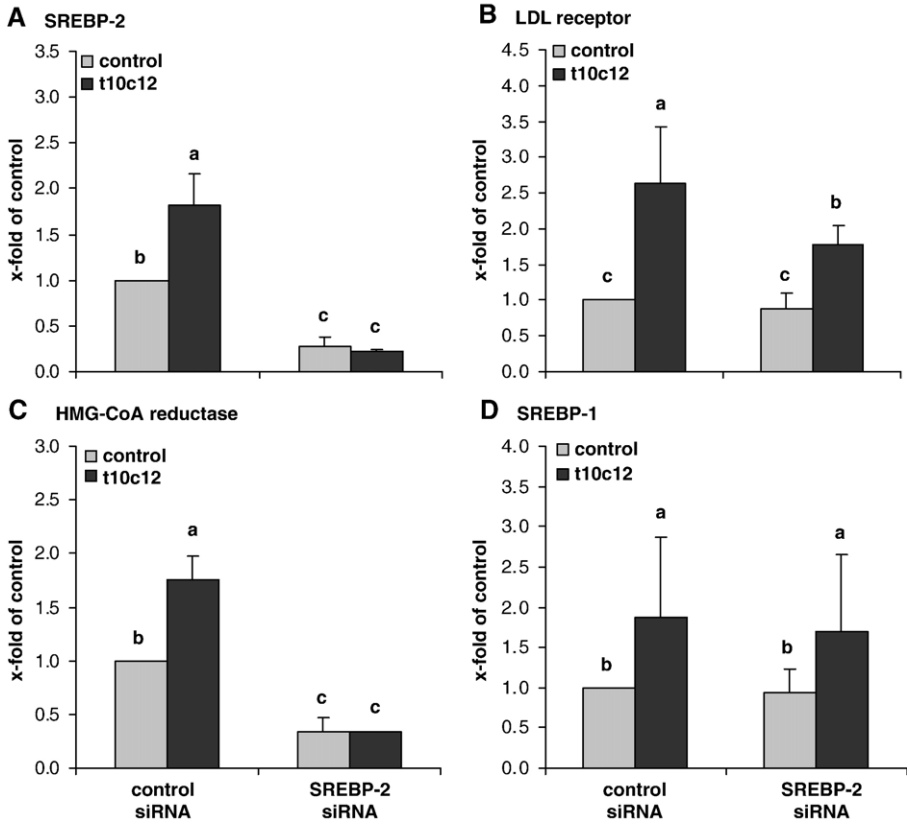

Fig. 5. Effect of 100 μmol/L of *t*10*c*12-CLA on relative mRNA concentrations of SREBP-2 (A), LDL receptor (B), HMG-CoA reductase (C), and SREBP-1 (D) in SREBP-2 knockdown HepG2 cells. SREBP-2 knockdown cells were generated by siRNA-mediated gene silencing. After introduction of knockdown, cells were treated without (control) or with *t*10*c*12-CLA for 24 h and analyzed for relative mRNA concentrations by means of real-time RT-PCR. Bars represent means ± SD of three independent experiments and are expressed as fold-changes compared to control (= 1.0 ± 0.0). [a,b,c]Bars marked with different superscript letters differ significantly, $P < 0.05$.

effects such as inhibition of cancer and atherosclerosis [25–27]. However, the underlying mechanisms for the anti-atherogenic effects of CLA, in particular, are only poorly understood. Recently, a mixture of different CLA isomers was demonstrated to upregulate LDL receptor mRNA and protein expression in the human hepatoma cell line HepG2 [2]. The liver regulates plasma LDL cholesterol levels through the surface expression of LDL receptors which mediate plasma LDL and VLDL clearance [1]. Thus, the upregulation of LDL receptor and consecutively increased LDL clearance from the circulation might contribute to these anti-atherogenic actions of CLA in animal feeding experiments. The aim of this study was to elucidate how CLA mediates upregulation of LDL receptor in HepG2 cells and whether this upregulation is caused by a specific CLA isomer.

The results of the present study clearly demonstrated that CLA induces LDL receptor gene expression in HepG2 cells, which is consistent with the observation from Yu-Poth et al. [2]. Moreover, the present study revealed for the first time that the induction of LDL receptor gene transcription by CLA occurs in an isomer-specific manner: only the *t*10*c*12-CLA was capable of inducing LDL receptor promoter activity and mRNA levels, whereas *c*9*t*11-CLA as well as LA had no effect. The marked increase in the activity of the LDL receptor gene promoter in response to *t*10*c*12-CLA probably suggests that the increased

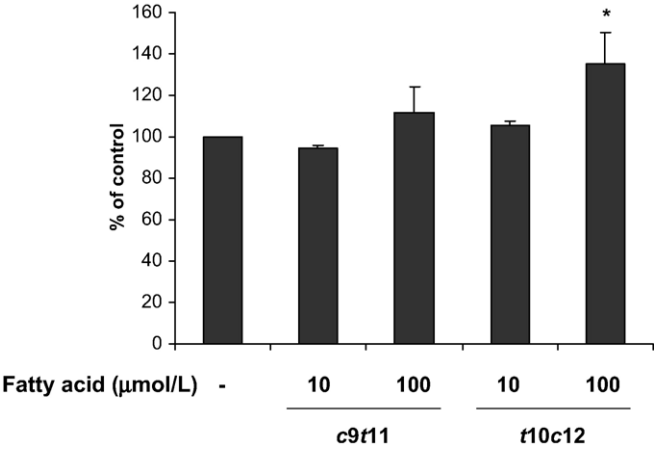

Fig. 6. Effect of 10 and 100 μmol/L of either *c*9*t*11-CLA or *t*10*c*12-CLA on ^{14}C-acetate incorporation into cellular free cholesterol in HepG2 cells. Cells were incubated with fatty acids for 24 h followed by a 2 h incubation with (1,2-^{14}C) acetate. Radiolabeled lipids were extracted using hexane/isopropanol, and free cholesterol was separated by TLC. Spots were obtained by phosphorimaging and quantified by densitometry. Bars represent means ± SD of four independent experiments performed in duplicate and are expressed as percent of control (= 100 ± 0%). *Significantly different from control, $P < 0.05$.

mRNA level of the LDL receptor is primarily due to an increased mRNA synthesis rather than an enhanced mRNA stability of the LDL receptor gene transcript.

The increase in LDL receptor mRNA in response to *t*10*c*12-CLA was accompanied by a substantial increase in SREBP-2 mRNA abundance and only a slight increase in the mRNA abundance of SREBP-1. Since SREBPs are well known to activate LDL receptor transcription by binding to the SRE-1 in the LDL receptor gene promoter [12,13], the increase in SREBP mRNA abundance may account for the observed increase in LDL receptor gene transcription by *t*10*c*12-CLA. Although we did not analyze the mature forms of the SREBPs, which are the transcriptionally active elements binding to the SRE-1, we could demonstrate by use of SREBP-2 knockdown cells, generated by siRNA-directed gene silencing, that the effect of *t*10*c*12-CLA on LDL receptor gene transcription was significantly reduced when compared to control cells expressing normal levels of SREBP-2. When using SREBP-1 knockdown cells the effect of *t*10*c*12-CLA on LDL receptor mRNA only slightly decreased compared to cells treated with control siRNAs. This suggests that the *t*10*c*12-CLA-induced upregulation of LDL receptor mRNA is at least partially mediated by an increased SREBP-2 transcription rather than an increased SREBP-1 transcription. This suggestion also confirms the assumption of Yu-Poth et al. [2] that the upregulation of LDL receptor by CLA occurs independently of SREBP-1. Therefore, we hypothesize that the increase in SREBP-2 mRNA abundance in response to *t*10*c*12-CLA resulted in an increased maturation of the inactive SREBP-2 precursor to the active form translocating to the nucleus and, thereby, activating transcription of the LDL receptor gene. This assumption is strengthened by the observation that HMG-CoA reductase mRNA and presumably also the activity of this enzyme (as evidenced by an increased ^{14}C-acetate incorporation into cellular cholesterol) were also significantly induced by *t*10*c*12-CLA. HMG-CoA reductase is a well known target gene of SREBP-2 [28], and coordinated upregulation of the genes coding for LDL receptor and HMG-CoA reductase is a typical effect in response to activation of SREBP-2 in cultured human cells [29]. This was also confirmed by the results from our SREBP knockdown experiments; *t*10*c*12-CLA exerted a stimulatory effect on HMG-CoA reductase only in control cells expressing normal levels of SREBP-2 or in SREBP-1 knockdown cells, but not in SREBP-2 knockdown cells. Interestingly, the knockdown of SREBP-2 mRNA resulted in a marked reduction in the HMG-CoA reductase mRNA level, whereas the LDL receptor mRNA level remained largely unaffected. We assume that this is in main parts due to the specific cell culture conditions used herein. It has been suggested that AP-1 signal transduction markedly contributes to basal expression of the LDL receptor [18], whereas HMG-CoA reductase gene transcription is largely mediated via SREBP-2 [28]. Given that the culture medium used herein contained 10% fetal bovine serum and expression of c-fos, a member of the AP-1 family of transcription factors, has been demonstrated to be upregulated by serum through the serum-response element [30], we suggest that this is probably responsible for the relatively high basal LDL receptor gene transcription and the lack of effect of SREBP-2 knockdown on LDL receptor mRNA levels in the present study.

From our aforementioned observations, we suggest that an increased transcriptional activation of the SRE-1 in the LDL receptor gene promoter by SREBP-2 is involved in the upregulation of LDL receptor gene transcription by *t*10*c*12-CLA. Whether this is, indeed, the consequence of an increased maturation of the inactive precursor of SREBP-2 into the transcriptional active form cannot be concluded with certainty from the present results and, therefore, should be investigated in future experiments. Alternatively, the mature form of SREBP-2 might have accumulated as a result of a reduced proteolytic degradation by the ubiquitin–proteasome. Namely, it has been suggested that inhibition of proteasome-mediated degradation of SREBP-2 leads to accumulation of nuclear SREBP-2 and therefore increased LDL receptor levels in HepG2 cells [31,32]. Accordingly, future studies should address the molecular events which could account for increased levels of mature SREBP-2 by *t*10*c*12-CLA.

In order to address SREBP-independent mechanisms, which may be relevant for the upregulation of LDL receptor gene transcription by *t*10*c*12-CLA, we also considered possible activation of the AP-1 pathway in response to treatment with CLA. AP-1 is normally induced upon oxidative stress and AP-1 is also capable of mediating SREBP-independent LDL receptor gene transcription via AP-1 binding sites in the LDL receptor gene promoter region [18]. CLA-induced oxidative stress has been observed in Jurkat T cells [33]. In addition, a prooxidant activity of dietary supplementation with *t*10*c*12-CLA has been observed in human studies [34,35]. Thus, we hypothesized that induction of AP-1 may also account for the *t*10*c*12-CLA-induced upregulation of LDL receptors in HepG2 cells. However, using two different LDL receptor promoter constructs (one containing and the other lacking the AP-1 binding site at −125 in the LDL receptor gene promoter) we could demonstrate that the AP-1 binding site is probably not involved in the induction of the LDL receptor promoter activity by *t*10*c*12-CLA. In addition, using a pAP-1-Luc reporter vector designed for monitoring induction of the AP-1 pathway, we could demonstrate that AP-1 signalling was not induced by *t*10*c*12-CLA. Therefore, we exclude the possibility that oxidative stress is involved in the upregulation of LDL receptor gene transcription by *t*10*c*12-CLA.

With respect to the reported effects of human CLA studies on blood lipids the findings from the present cell culture study appear to be in strong contrast. From the present results *t*10*c*12-CLA would be expected to be beneficial and to lower plasma cholesterol concentrations *in vivo* due to enhanced clearance of LDL particles by hepatic LDL receptors. However, most of the human studies reported no effect of supplementation with CLA mixtures on plasma and LDL cholesterol concentrations [7–10], and a recent study even reported an increase in LDL cholesterol concentrations and LDL to HDL cholesterol ratio in response to supplementation with *t*10*c*12-CLA whereas *c*9*t*11-CLA decreased this ratio [11]. Based on this study [11] and on a recently reported proinflammatory effect of *t*10*c*12-CLA [34], a detrimental influence has been attributed to the *t*10*c*12-CLA

isomer by different authors, which is exactly the opposite of what would have been suggested from the present cell culture study. The fact that the results herein do not support the observations from most human studies with respect to blood lipid profiles cannot be explained at the moment. Nevertheless, similar contradictory observations between cell culture and human studies are also well known with respect to other effects of CLA, e.g., whereas inhibitory effects on inflammatory prostanoid release have been observed in several cell culture experiments in response to CLA mixtures and in response to *c*9*t*11-CLA and/or *t*10*c*12-CLA [36–39], a Swedish group reported increases in prostanoid formation in several independent human studies after supplementation with a CLA mixture [40] and with *t*10*c*12-CLA but not with *c*9*t*11-CLA [34]. Nevertheless, a more recent study of that group also found a slight increase in inflammatory markers after supplementation with *c*9*t*11-CLA [41]. This indicates that not only contradictory results between cell culture and human studies are frequently reported in the literature but also that human, animal, and cell culture studies dealing with CLA are often inconsistent with respect to the reported effect and to the active isomer. In parts this inconsistency is attributable to the use of different formulations of CLA, heterogenous blends of CLA isomers, different doses, species differences, and the metabolic status of the experimental model. These reasons may also partially explain the apparently conflicting results of CLA on experimental atherosclerosis [25,42–44]. Namely, the reported studies revealed no effect [45], a stimulatory effect [44] or an inhibitory effect [25,42] on experimental atherosclerosis. Moreover, antiatherogenic effects have been observed with *c*9*t*11-CLA [42], *t*10*c*12-CLA [43] as well as a CLA mixture [25] suggesting that the outcome of those studies cannot be solely ascribed to the use of a specific CLA preparation or a single CLA isomer.

Apart from these reasons partially explaining these contradictory observations from cell culture and human studies it should be also mentioned that the concentrations of CLA isomers used in the present study are rather high when compared to the *in vivo*-situation. As demonstrated in a previous study of our group [22] incubating HepG2 cells with 100 μmol/L of CLA resulted in relatively high concentrations of CLA in cell lipids when compared to those reported from tissues of CLA fed animals [46,47]. In humans, blood concentrations of CLA were reported to be in the range of 20–70 μmol/L [48], consisting mainly of *c*9*t*11-CLA and only to about 10% of *t*10*c*12-CLA. This suggests that especially the concentration of *t*10*c*12-CLA herein is above the range that is normally expected in humans. Nevertheless, the concentrations of CLA used herein did not impair cell viability and are in the range of similar cell culture studies dealing with the CLA's mechanisms of action, and the present study was also conducted to deal with mechanistic questions. Moreover, the concentrations used herein are far below of those concentrations that were used in the study of Yu-Poth et al. [22], which was the starting point of the present study. Taken together, the concentrations of CLA used in the present study should be regarded as rather high, and this should be kept in mind when trying to transfer the data of this cell culture study to the human situation. Furthermore, it is important to note that the activity of hepatic LDL receptors alone is insufficient to explain the complex and multiple effects determining plasma cholesterol concentrations.

In conclusion, the results of the present study demonstrate that exposure to *t*10*c*12-CLA but not to *c*9*t*11-CLA increased LDL receptor mRNA levels and promoter activity and HMG-CoA reductase mRNA levels in HepG2 cells. Moreover, we could demonstrate that the effect of *t*10*c*12-CLA on LDL receptor gene transcription is at least partially dependent on SREBP-2. Since SREBP-2 is responsible for the coordinated upregulation of genes involved in cholesterol biosynthesis and homeostasis, we suggest that *t*10*c*12-CLA probably increases biosynthesis and receptor-mediated uptake of cholesterol. Thus, the decreased plasma VLDL and LDL cholesterol levels in response to CLA as observed in a limited number of animal and human studies [3–6] might be explained by an enhanced uptake of VLDL and LDL via hepatic LDL receptors resulting in an increased clearance of cholesterol from the circulation. However, it provides no explanation for the outcome of most human studies reporting unaltered or even increased plasma and LDL cholesterol concentrations in response to supplementation with CLA. Thus, future studies should address further aspects of lipid and lipoprotein metabolism which may explain the effects of CLA on human blood lipids. In addition, further studies should investigate the exact molecular mechanisms that account for the induction of SREBP-2-dependent gene expression by *t*10*c*12-CLA in HepG2 cells.

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Formation of conjugated linoleic acid metabolites in human vascular endothelial cells

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Abstract

Conjugated linoleic acids (CLAs) are bioactive lipid compounds showing anti-atherogenic actions in cell culture experiments and animal models of atherosclerosis without exact knowledge about the underlying mechanisms. CLAs were recently reported to be further metabolized to bioactive conjugated metabolites indicating that these metabolites are possibly involved in mediating the anti-atherogenic actions of CLA. Regarding the lack of information with respect to the formation of CLA metabolites in the vascular endothelium, which is strongly involved in the process of atherosclerosis, the present study aimed to explore the potential formation of CLA metabolites in vascular endothelial cells. The results from the present study show for the first time that the CLA isomers *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA are metabolized within endothelial cells to β-oxidation products such as CD16:2*c*7*t*9 and CD16:2*t*8*c*10 and elongation products such as CD20:2*c*11*t*13, CD20:2*t*12*c*14 as well as CD22:2*c*13*t*15 and CD22:2*t*14*c*16. Different CD16:2/CLA ratios observed between cells treated with different CLA isomers indicate that the metabolism of CLAs depends on the configuration of the conjugated double bonds. In conclusion, regarding the biological activity reported for CD20:2*t*12*c*14 and other metabolites of CLA, the present results indicate that metabolites of CLA are possibly also involved in mediating the anti-atherogenic actions of CLA.

Keywords: Conjugated linoleic acid; Atherosclerosis; Human aortic endothelial cell; Conjugated dienoic structure; *cis*-9, *trans*-11 CLA; *trans*-10, *cis*-12 CLA

1. Introduction

Conjugated linoleic acids (CLA) are a group of positional and geometric isomers of linoleic acid characterized by the presence of conjugated double bonds. CLAs are naturally found in significant amounts in milk, dairy products and meat of ruminants [1]. The predominant isomer of CLA in natural foods is *cis*-9, *trans*-11 CLA, which contributes to more than 90% of total CLA in food [1], whereas *trans*-10, *cis*-12 CLA is a minor CLA isomer in natural foods. Despite this disparate distribution in natural foods, both CLA isomers were demonstrated to be biologically highly active lipid compounds and have attracted scientific interest due to beneficial health effects such as regression of experimental atherosclerosis [2–6].

Recent studies indicated that CLA isomers are further metabolized within the cell to fatty acids of different chain length possessing the characteristic conjugated dienoic structure of CLAs [7–12]. These CLA metabolites are supposed to originate from elongation, desaturation and peroxisomal or mitochondrial β-oxidation of CLAs [8]. The detection of CLA metabolites in tissues from CLA-fed animals and cell cultures treated with CLA has gained in importance since it has recently been shown that these metabolites of CLA exert potent biological activities [7]. This indicates that the anti-atherogenic effects observed with CLA are presumably not only mediated by CLA itself but also by its conjugated metabolites. Until now little information is available from the literature regarding the formation of CLA metabolites in cells of the vascular wall, which play a key role in the process of atherosclerosis. Only one

Abbreviations: CLA, conjugated linoleic acid; HAEC, human aortic endothelial cells; CD, conjugated diene structure

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study of our group previously demonstrated that treatment of human vascular smooth muscle cells with different CLA isomers results in the isomer-specific formation of conjugated dienoic fatty acids [12].

With respect to the effects of CLA on atherosclerosis, possible metabolism of CLA within the endothelium should be taken into special consideration as well. The endothelium is strongly involved in the initial step of atherosclerosis, which is characterized by endothelial damage and dysfunction leading to a disturbed vascular homeostasis, leukocyte recruitment, smooth muscle cell proliferation, and, finally, development of atherosclerotic plaques [13]. We and others have shown that CLAs are able to modulate endothelium-mediated vascular homeostasis, e.g., reducing release of pro-inflammatory mediators such as prostaglandin E_2 and of the vasoconstrictive agent thromboxane A_2 within vascular endothelial cells and inhibiting platelet aggregation [14–17]. This suggests that the anti-inflammatory and anti-thrombogenic actions of CLA are at least partially responsible for the anti-atherogenic effects of CLA observed in vivo.

Therefore, regarding that CLA metabolites indeed come into question as the active substances mediating the anti-atherogenic effects of CLA observed in vivo, the present study was carried out to gain information about the potential metabolism of CLA in endothelial cells which comprise the cellular component of the endothelium. For this purpose total cell lipids of human aortic endothelial cells (HAECs) treated with two different CLA isomers, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, were analyzed for the formation of CLA metabolites using GC-FID, GC-MS and Ag^+-HPLC.

2. Materials and methods

2.1. Chemicals

Cis-9, *trans*-11 CLA (≥96% pure) and *trans*-10, *cis*-12 CLA (≥98% pure) were obtained from Cayman Chemicals (Ann Arbor, MI). Preparation of stock solutions of fatty acids and of the test media was performed as described previously [17]. MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue) was purchased from Sigma-Aldrich. n-hexane and methanol (both from Merck, Darmstadt, Germany) were of analytical grade and distilled before use. Chloroform for HPLC analysis was purchased from CS Chromatographie (Langerwehe, Germany). Potassium methylate (30–35% in methanol), sulphuric acid (96%) and propionitrile for synthesis were purchased from Merck. Internal standard cis-10-heptadecenoic acid methyl ester was obtained from Sigma (Steinheim, Germany).

2.2. Cell culture

HAECs from a 38-year-old female donor were obtained from PromoCell and cultured in EC Growth Medium MV, composed of EC Basal Medium and supplements (all from PromoCell, Heidelberg, Germany), as described previously [17]. After reaching 70–80% confluence, cells were treated with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA for 24 h. Cells treated without additional fatty acids were used as controls.

2.3. Cell count and cell viability

The cell count was determined with a Neubauer chamber after cells were harvested by trypsinization and pelleted by centrifugation (170×*g* for 5 min). Cell viability after treatment of HAECs as indicated above was examined by the MTT assay [18].

2.4. Analysis of fatty acid composition of total cell lipids

After treatment of HAECs for 24 h as indicated above cells were washed with PBS, harvested using a cell scraper, and pelleted by centrifugation (170×*g* for 5 min). The cell pellet was stored at −80 °C until extraction of total cell lipids.

2.4.1. Extraction of total cell lipids

Cell pellets were suspended in 1 mL bidistilled water and total cell lipids were extracted with 3 mL chloroform/methanol (2/1, v/v+0.001% BHT) after adding 100 μL of the internal standard (100 μg heptadecenoic acid methyl ester in n-hexane, C17:1*c*10). The lower organic phase was transferred into a pyrex-glass-tube and extraction was repeated twice. The combined organic phases were evaporated to dryness under a stream of nitrogen at 40 °C. Lipids were methylated in a two-step procedure. The lipid extract was transmethylated with 5% potassium methylate solution in methanol for 30 min at 60 °C. After cooling to room temperature 3 mL of 1 N methanolic sulphuric acid (2.8 mL 96% sulphuric acid in 100 mL methanol) were added. After vortexing the pyrex-tubes were heated for 15 min at 60 °C for methylation of free fatty acids. After cooling, 3 mL of saturated sodium chloride solution in water and 2 mL n-hexane were added. The fatty acid methyl esters were extracted into the n-hexane phase by vortexing. The upper n-hexane phase was transferred after centrifuging into a 4-mL glass vial and extraction of methyl esters was repeated once. The combined n-hexane phases were evaporated to dryness under a stream of nitrogen and solved in 500 μL n-hexane.

2.4.2. Analysis of fatty acid composition by GC-FID analysis

Fatty acids methyl ester analysis was performed using an Agilent 6890 GC equipped with a split/splittless injector at 230 °C, a flame ionization detector at 260 °C, an autosampler (Agilent Technologies, Waldbronn, Germany) and a CP SIL 88 column (100 m, 0.25 mm, 0.2 μm film thickness, Varian, Darmstadt, Germany). Hydrogen was used as carrier gas at a constant flow rate of 1 mL/min. The temperature of the GC oven was set to 70 °C for 3 min, increased at 8 °C/min to 180 °C, held for 2 min, increased at 4 °C/min to 210 °C, held for 4 min, increased at 2 °C/min to a final temperature of 240 °C and held for 25 min. HP Chemstation software (Rev. A.08.03) was used for data analysis. The sample was injected using a split ratio of 1:10.

2.4.3. Analysis of fatty acid composition by GC-MS analysis

Fatty acid methyl ester analysis was performed using a Trace GC hyphenated with a PolarisQ ion-trap mass-spectrometer (Thermo Electron GmbH, Dreieich, Germany). Column type and temperature programming were the same as for GC-FID analysis. Electron impact ionization was used at 70 eV in positive mode with an ion volume temperature of 200 °C. Helium was used as carrier gas at a constant flow rate of 1 mL/min. PolarisQ was operated in full scan mode from 50 to 650 amu. 1 μL of sample was injected in splitless mode.

2.4.4. Analysis of conjugated fatty acid isomers by Ag^+-HPLC

Conjugated fatty acid isomers were separated using Ag^+-HPLC-DAD. The system consisted of an isocratic Merck-Hitachi L-6000 A HPLC pump equipped with a Thermo Separation Products autosampler and a Biotek Kontron diode-array detector operated at wavelength between 200 and 400 nm. Two Chromspher 5 lipids columns (250 mm×4.6 mm, 5 μm) were used in series with a 50 mm×4.6 mm pre-column of the same column material (Varian, Darmstadt, Germany). 0.2% propionitrile in n-hexane was used as eluent for separation of conjugated fatty acids at a flow rate of 1 mL/min (approximately 80 bar). Columns were equilibrated with fresh eluent 1 h prior to analysis. KromaSystem 2000 software (Version 1.83, Goebel, Ludwigshafen, Germany) was used for data analysis.

2.5. Statistic analysis

Student's *t*-test was used to compare means of treatments with those of control. Differences of $P<0.05$ were considered significant.

3. Results

3.1. Cell viability

Viability of HAECs was not affected by treatment with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA for 24 h as also demonstrated in previous studies of our group using the same cell culture model [17,19]. In the present study cell viabilities after treatment with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA were between 95 and 102% (mean for three independent experiments), respectively, of control cells (=100%).

3.2. Proportions of CLA and conjugated metabolites of CLA in total fatty acids of HAEC total lipids

Treatment of HAECs with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA resulted in a significant incorporation of the respective CLA isomer into total endothelial cell lipids in a concentration-dependent manner relative to control cells ($P<0.05$; Table 1). To a low extent (<1 g/100 g of total fatty acids) *trans*-9, *trans*-11 CLA and *trans*-10, *trans*-12 CLA were also found in total lipids of endothelial cells following treatment with *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA indicative of a slight conversion of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, respectively, into the *trans*/*trans*-isomers. Fig. 1 shows a typical Ag^+-HPLC-DAD chromatogram of the CLA region of HAECs treated with 50 μmol/L of each CLA isomer at a detection wavelength of 234 nm.

At this detection wavelength of 234 nm, which is nearly specific for the CD structure, it was possible to detect various conjugated metabolites of CLA such as the β-oxidation product CD16:2 and the elongation products CD20:2 and CD22:2 in endothelial cell total lipids following treatment with CLA isomers (Fig. 1). By comparison of GC-MS chromatograms of different treatments, it was possible to identify the CLA metabolites by their mass spectra. Fig. 2 shows the partial GC-MS chromatogram (TIC) of the CD16:2 region of HAECs treated with 50 μmol/L of *cis*-9, *trans*-11 CLA (Fig. 2A) and *trans*-10, *cis*-12 CLA (Fig. 2B). Analysis of methyl esters was favored because synthesis of the 4,4-dimethyloxazoline derivatives for structure elucidation is accompanied with compound loss and artefact formation. As demonstrated in Fig. 3 mass spectra of methyl ester isomers show similar fragmentation patterns but the diene fragments (*m*/*z* 67, 81) and the molecular ion [M^+] (e.g., *m*/*z* 266, 322, 350) for identification of chain

Table 1
Proportions of CLA and conjugated metabolites of CLA in total lipids of human aortic endothelial cells cultured in the presence or absence (control) of 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA

Treatment	Control	*cis*-9, *trans*-11 CLA		*trans*-10, *cis*-12 CLA	
		5 μmol/L	50 μmol/L	5 μmol/L	50 μmol/L
CD18:2*c*9*t*11	<0.1	2.5±0.6*	18.1±2.3*	0.3±0.1*	0.4±0.1*
CD18:2*t*10*c*12	<0.1	<0.1	<0.1	2.2±0.2*	19.1±2.4*
CD18:2*t*9*t*11	<0.1	0.5±0.1*	0.8±0.1*	<0.1	0.1±0.1*
CD18:2*t*10*t*12	<0.1	<0.1	<0.1	0.3±0.1*	0.8±0.1*
CD16:2*c*7*t*9	<0.1	0.6±0.1*	1.3±0.1*	<0.1	0.3±0.1
CD16:2*t*8*c*10	<0.1	<0.1	<0.1	0.4±0.1*	1.8±0.2*
CD16:2*t*7*t*9	<0.1	<0.1	0.4±0.1*	<0.1	<0.1
CD16:2*t*8*t*10	<0.1	<0.1	<0.1	<0.1	0.2±0.1*
CD20:2*c*11*t*13	<0.1	0.1±0.1*	1.0±0.1*	<0.1	<0.1
CD20:2*t*12*c*14	<0.1	<0.1	<0.1	0.2±0.1*	1.0±0.1*
CD20:2*t*11*t*13	<0.1	0.1±0.1*	1.0±0.2*	<0.1	0.1±0.1*
CD20:2*t*12*t*14	<0.1	<0.1	<0.1	0.2±0.1*	0.4±0.1*
CD22:2*c*13*t*15	<0.1	0.1±0.1*	0.1±0.1*	<0.1	<0.1
CD22:2*t*14*c*16	<0.1	<0.1	<0.1	<0.1	<0.1
CD22:2*t*13*t*15	<0.1	<0.1	<0.1	<0.1	<0.1

Human aortic endothelial cells were cultured without fatty acids (control) or with 5 and 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA for 24 h. Fatty acids are presented as g per 100 g of total fatty acids. Results represent mean±S.D. of three independent experiments.

* Significantly different from control, $P<0.05$.

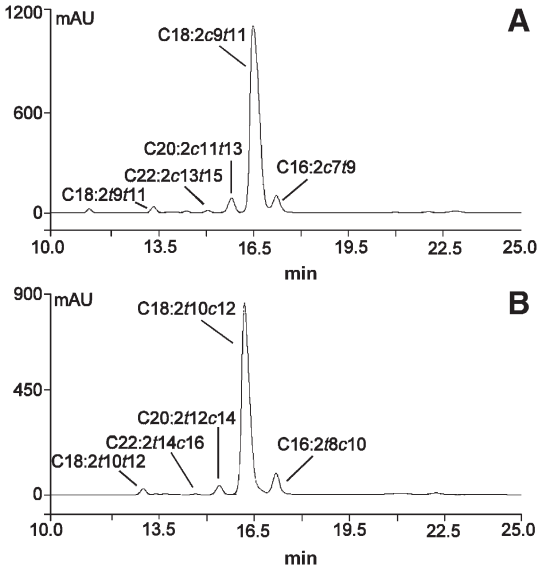

Fig. 1. Partial Ag^+-HPLC-DAD chromatogram at a detection wavelength of 234 nm of conjugated fatty acids of HAEC treated with 50 μmol/L *cis*-9, *trans*-11 CLA (A) or 50 μmol/L *trans*-10, *cis*-12 CLA (B).

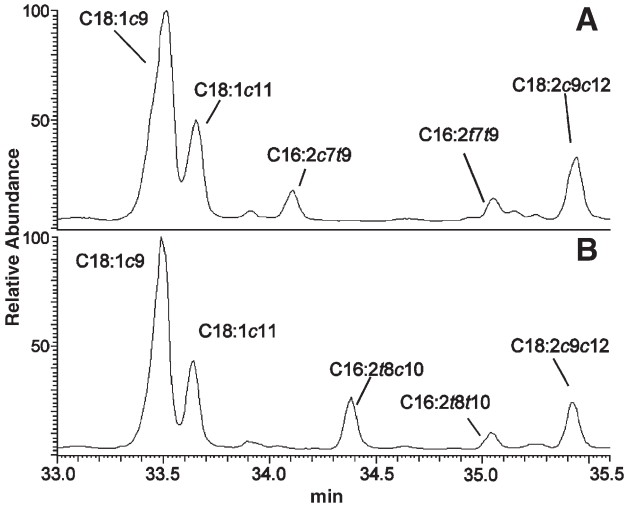

Fig. 2. Partial GC-MS chromatogram (TIC) of CD16:2 region of HAEC treated with 50 μmol/L *cis*-9, *trans*-11 CLA (A) or 50 μmol/L *trans*-10, *cis*-12 CLA (B).

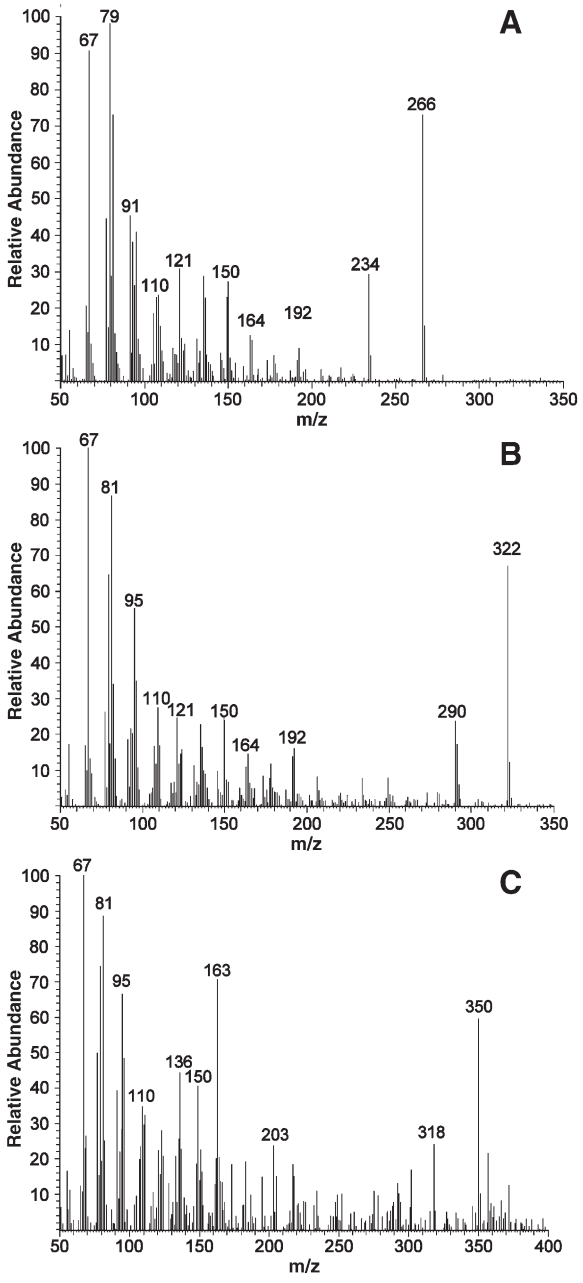

Fig. 3. GC-MS spectra of CLA β-oxidation and elongation products of HAEC treated with 50 μmol/L *cis*-9, *trans*-11 CLA. CD16:2*c*7*t*9 (A), CD20:2*c*11*t*13 (B) and CD22:2*c*13*t*15 (C).

length are of high abundance, therefore, enabling us to reliably identify CD16:2*c*7*t*9 (Fig. 3A), CD20:2*c*11*t*13 (Fig. 3B) and CD22:2*c*13*t*15 (Fig. 3C). As illustrated in Fig. 4, GC-MS analysis was required for correct identification of small amounts of CLA metabolites in endothelial cell total lipids as observed in the present study due to co-eluting major fatty acids; e.g., the CD20:2 metabolites of CLA, CD20:2*c*11*t*13 and CD20:2*t*12*c*14, elute in the region of arachidonic acid.

Treatment with *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA revealed a dose-dependent elevation of the respective CD16:2 β-oxidation products, CD16:2*c*7*t*9 and CD16:2*t*8*c*10, in endothelial cell total lipids when compared to control cells

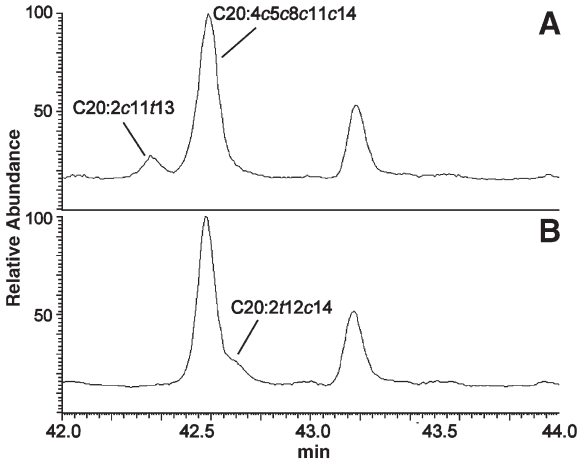

Fig. 4. Partial GC-MS chromatogram (TIC) of CD20:2 region of fatty acids of HAEC treated with 50 μmol/L *cis*-9, *trans*-11 CLA (A) or 50 μmol/L *trans*-10, *cis*-12 CLA (B).

($P<0.05$; Table 1). To a low extent, the *trans*/*trans*-isomers of CD16:2, CD16:2*t*7*t*9 and CD16:2*t*8*t*10, respectively, were also detectable in total lipids of endothelial cells treated with 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA, whereas it was below the limit of detection in endothelial cells treated with 5 μmol/L of either CLA isomer.

Similar to the formation of CD16:2, the elongation products of the respective CLA isomers, CD20:2*c*11*t*13 and CD20:2*t*12*c*14, dose-dependently increased in endothelial cells treated with *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, respectively, relative to control cells ($P<0.05$).

As also observed with the *trans*/*trans*-CD16:2 products of CLA, the *trans*/*trans*-isomers of CD20:2, CD20:2*t*11*t*13 and CD20:2*t*12*t*14, respectively, were also detectable in endothelial cell total lipids following treatment with *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, respectively. C22:2 elongation products of the respective CLA isomers such as CD22:2*c*13*t*15 and CD22:2*t*14*c*16 were also detectable in

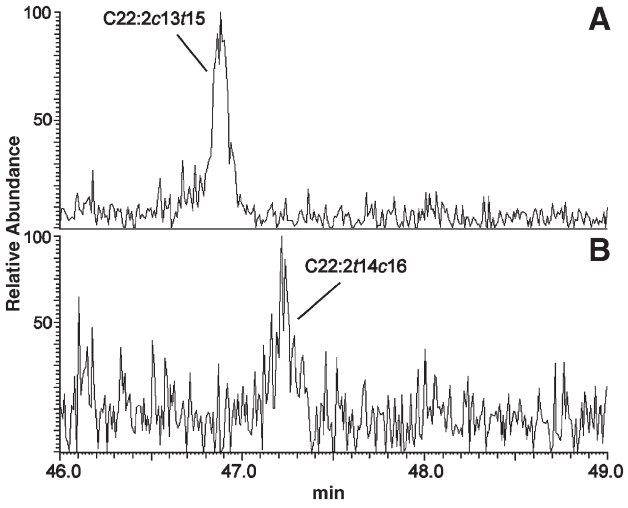

Fig. 5. Partial GC-MS chromatogram (extracted ion *m*/*z* 350) of CD22:2 region of fatty acids of HAEC treated with 50 μmol/L *cis*-9, *trans*-11 CLA (A) or 50 μmol/L *trans*-10, *cis*-12 CLA (B).

trace amounts in total HAEC lipids following treatment with CLA isomers, but were below the limit of quantitation (<0.05% GC-FID). However, by extraction of the specific *m*/*z* 350 trace it was possible to reliably detect these metabolites in endothelial cell total lipids as shown by the GC-MS chromatogram of the CD22:2 region (Fig. 5). According to this, significant amounts of CD22:2*c*13*t*15 were formed by treatment with *cis*-9, *trans*-11 CLA compared to control cells (Fig. 5A), whereas CD22:2*t*14*c*16 following treatment with *trans*-10, *cis*-12 CLA (Fig. 5B) was just detectable. The *trans*/*trans*-isomer of CD22:2, CD22:2*t*13*t*15, was not detectable at all in either of the CLA-treated cells.

3.3. Ratios of C16:2/C18:2 and C20:2/C18:2 in total fatty acids of total cell lipids for the treatment of HAECs with cis-9, trans-11 CLA and trans-10, cis-12 CLA

The respective C16:2/C18:2 and C20:2/C18:2 ratios in endothelial cell total lipids are shown in Table 2. The CD16:2*t*8*c*10/CLA*t*10*c*12 ratio for the *trans*-10, *cis*-12 CLA-treated cells was significantly higher than the CD16:2*c*7*t*9/CLA*c*9*t*11 ratio for the *cis*-9, *trans*-11 CLA-treated cells, whereas the CD20:2*t*12*c*14/CLA*t*10*c*12 ratio for the *trans*-10, *cis*-12 CLA-treated cells did not differ from the CD20:2*c*11*t*13/CLA*c*9*t*11 ratio for the *cis*-9, *trans*-11 CLA-treated cells.

4. Discussion

We have previously reported that CLA metabolites are formed in human vascular smooth muscle cells treated with CLA isomers [12]. With respect to the anti-atherogenic effects of CLA isomers observed in vivo [2–6] and in vascular cells [14–17] and to the biological activities that have recently been observed for conjugated metabolites of CLA [11] our previous findings suggested that the actions of CLA might, at least partially, be caused by its conjugated metabolites. The present study showed for the first time that the CLA isomers, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA, are also metabolized within human vascular endothelial cells resulting in the isomer-specific formation of various metabolites of CLA with 16, 20 and 22 C-atoms, identifiable at the characteristic dienoic structure of CLA, which is retained during metabolism.

For the reported modulation of endothelial cell function by CLA isomers [14–17], the detection of conjugated long-chain fatty acids in endothelial cell lipids such as CD20:2*c*11*t*13, CD20:2*t*12*c*14, CD20:2*t*11*t*13 as well as CD22:2*c*13*t*15 and CD22:2*t*14*c*16, that are derived from enzymatic elongation of CLA [11,12], might be of special biological significance. Both CD20:2*t*12*c*14 alone or a mix of CD20:2*c*11*t*13/*t*12*c*14 as well as *trans*-10, *cis*-12 CLA have been proven to markedly inhibit heparin-releasable lipoprotein lipase (LPL) activity and lipid accumulation in 3T3-L1 adipocytes compared to cells treated without fatty acids or with the non-conjugated C20:2*c*11*c*14 [7,21]. LPL is a vascular endothelium-bound enzyme that is rate determining for the clearance of triacylglycerol-rich lipoproteins and partially regulates free-fatty acid supply to the tissues [20]. Since structure–activity relationship studies revealed that the *trans*-10, *cis*-12 conjugated double bond is essentially required for LPL inhibition [7,21], it might be possible, albeit highly speculative, that CD22:2*t*14*c*16, as detected in endothelial cell lipids in the present study, also interferes with LPL activity and thereby modulates endothelial cell function. However, the amounts of CD22:2 metabolites were generally very low in endothelial cell lipids when compared to the CD20:2 metabolites. Solely by extraction of the specific *m*/*z* 350 trace in the GC-MS analysis, we were capable of definitely identifying CD22:2*c*13*t*15 and CD22:2*t*14*c*16 in endothelial cell total lipids. Therefore, the physiological relevance of the CD22:2 metabolite levels detected with respect to the modulation of endothelial cell function has to be proven. Nevertheless, future studies should address the question of biological properties of these CLA-derived CD22:2 metabolites as well as their physiological relevance.

To analyze the influence of different CLA isomers on fatty acid elongation processes the ratios of CD20:2/CLA were calculated. Similar to vascular smooth muscle cells [12], no differences were observed regarding this ratio in endothelial cell total lipids between *cis*-9, *trans*-11 CLA- and *trans*-10, *cis*-12 CLA-treated cells, suggesting that enzymatic capacities for the conversion of CLAs to CD20:2 metabolites are independent of the configuration of the conjugated double bonds (*cis*/*trans* or *trans*/*cis*). Other typical conjugated elongation/desaturation products of CLA such as CD20:3 or CD20:4, which are produced from CLA by Δ6-desaturation, elongation and further Δ5-desaturation, were not detectable at all in vascular endothelial cells in the present study. This is in contrast to the well-detectable levels of these desaturation/elongation products in hepatic tissues from CLA-fed animals [8,10], but might be explained by the fact that vascular endothelial cells have a comparatively low capacity for fatty acid elongation and/or desaturation relative to hepatocytes.

Concerning the well-detectable amounts of CD16:2 isomers in endothelial cell lipids in the present study, these are supposed to originate, at least in part, from chain-shortening of CLA within the peroxisomes [8]. Chain-shortening within peroxisomes serves to provide shortened fatty acids for further β-oxidation within the mitochondria. The amounts of CD16:2*c*7*t*9 and CD16:2*t*8*c*10 found in total lipids of endothelial cells treated with 50 μmol/L of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA contributed to about 1.3 to 1.8 g/100 g of total fatty acids. These levels of conjugated CD16:2 metabolites are

Table 2
Ratios of C16:2/C18:2 and C20:2/C18:2 in total lipids of human aortic endothelial cells treated with 50 μmol/L of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA

Treatment	*cis*-9, *trans*-11 CLA	*trans*-10, *cis*-12 CLA
CD16:2*c*7*t*9/CLA*c*9*t*11	0.07±0.01[b]	–
CD16:2*t*8*c*10/CLA*t*10*c*12	–	0.09±0.01[a]
CD20:2*c*11*t*13/CLA*c*9*t*11	0.05±0.01	–
CD20:2*t*12*c*14/CLA*t*10*c*12	–	0.05±0.01

Ratios are presented as g per g of total fatty acids. Results represent mean±S.D. of three independent experiments. Means with different superscript letters are significantly different, $P<0.05$.

notably since non-conjugated C16:2, which derives from β-oxidation of linoleic acid, was not detectable at all in control cells. In addition, we have previously observed that the non-conjugated C16:2 β-oxidation product of linoleic acid was also not detectable in vascular smooth muscle cells treated with linoleic acid [12], suggesting that conjugated CD16:2 isomers such as CD16:2*c*7*t*9 and CD16:2*t*8*c*10 are apparently accumulating in endothelial cells treated with CLA isomers. This assumption is strengthened by the observation from others that conjugated β-oxidation products of CLA were also accumulating in microsomal fractions of hepatic tissues or purified hepatic mitochondria incubated with CLA [11,12]. In vitro studies measuring mitochondrial respiration following incubation with different fatty acids revealed that the accumulation of CD16:2 in the mitochondrial matrix is due to a slower oxidation rate of CLA compared to linoleic acid [22,23]. The latter is probably explained by the fact that the oxidation of unsaturated fatty acids requires so-called auxiliary enzymes that work with different efficiencies depending on whether the *cis* or *trans* double bond is even- or odd-positioned [24]. Consequently, differences are also expected regarding the oxidation rate between fatty acids with different conjugated double bonds such as *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA. This, in fact, has been demonstrated in vascular smooth muscle cells in a previous study [12]. In this regard, we have previously postulated that an increased CD16:2*t*8*c*10/CLA*t*10*c*12 ratio as observed for *trans*-10, *cis*-12 CLA-treated cells compared to the CD16:2*c*7*t*9/CLA*c*9*t*11 ratio for *cis*-9, *trans*-11 CLA-treated cells is an indicator for an enhanced β-oxidation capacity for *trans*-10, *cis*-12 CLA compared to *cis*-9, *trans*-11 CLA [12]. Indeed, using purified rat liver mitochondria, it could be demonstrated that the very poor availability of *trans*-3-enoyl-CoA, which is formed during β-oxidation of *cis*-9, *trans*-11 CLA, for the auxiliary enzyme Δ3-*cis*-Δ2-*trans*-enoyl-CoA isomerase is responsible for a comparatively slow oxidation of *cis*-9, *trans*-11 CLA, while the *trans*-10 double bond of *trans*-10, *cis*-12 CLA requires no auxiliary enzyme [22]. In the present study, this instance is obviously the same in endothelial cells treated with different CLA isomers. With respect to the significance of these findings, it has to be pointed out that not only the β-oxidation of CLA itself is slowed following treatment with CLA but also the oxidation rates of other fatty acids are altered [22]. Albeit speculative, this may lead to an altered energy metabolism and in turn to a modulation of endothelial cell function.

As in our previous study dealing with vascular smooth muscle cells [12], the present study further revealed that low amounts of *trans*-9, *trans*-11 CLA and *trans*-10, *trans*-12 CLA as well as *trans*/*trans*-CD16:2 and -CD20:2 metabolites of CLA were found in total lipids of endothelial cells treated with *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA. Therefore, as previously suggested from our group, this apparent conversion of *cis*/*trans*- or *trans*/*cis*-CLA isomers or metabolites into the *trans*/*trans*-CLA isomers/metabolites in endothelial cells is probably due to the greater thermodynamic stability of the conjugated *trans*/*trans*-double bond compared to the conjugated *cis*/*trans*- or *trans*/*cis*-double bond, making the conversion an energetically favored reaction.

Taken together, the results from the present study showed for the first time that the CLA isomers *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA are also metabolized within endothelial cells to C16:2, C20:2 and C22:2 fatty acids, while retaining the conjugated dienoic structure of CLAs. In connection with our previous findings in smooth muscle cells this suggests that CLA isomers are presumably metabolized by the same metabolic/enzymatic pathways such as peroxisomal or mitochochondrial β-oxidation and fatty acid elongation as postulated for smooth muscle cells. This further suggests that metabolism of CLA within cells from the vascular wall is similar. Regarding the strong biological activities of metabolites of CLA, e.g., CD20:2 [11], that were well detectable in endothelial cells in the present study, and the anti-inflammatory and anti-thrombogenic actions of CLA observed in endothelial cells [14–17], this possibly indicates that conjugated metabolites of CLA are also involved in mediating the potent actions of CLA in vascular cells.

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Detection of conjugated dienoic fatty acids in human vascular smooth muscle cells treated with conjugated linoleic acid

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Abstract

Conjugated linoleic acids (CLA) have attracted scientific interest due to their potential beneficial effects on atherosclerosis. Recent studies demonstrated that conjugated metabolites of CLA are found in tissues of CLA-fed animals and cultured cells treated with CLA. This observation has gained in importance since it has recently been shown that these metabolites of CLA exert specific biological activities. Therefore, the present study aimed to explore the potential formation of metabolites of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA and *trans*-9, *trans*-11 CLA in cells of the vascular wall, which has not yet been shown. Examination of fatty acid composition of total cell lipids using Ag^{+}-HPLC, GC-FID and GC-MS analysis revealed a significant isomer-specific formation of conjugated metabolites of CLA such as CD16:2, CD20:2 and CD22:2 in human coronary artery smooth muscle cells treated with various CLA isomers. Different CD16:2/CLA ratios between various CLA isomers as observed in the present study indicate that fatty acid metabolism is differently affected by the configuration of the double bonds. In conclusion, the observation from the present study suggests that the effects of CLA in vascular cells might not only be mediated by CLA itself but also by its conjugated metabolites. Future studies using highly purified conjugated metabolites of CLA are necessary to study their role in mediating biological effects of CLA in cell culture systems.

Keywords: Conjugated linoleic acid; Atherosclerosis; Human coronary artery smooth muscle cell; Conjugated diene structure

1. Introduction

Conjugated linoleic acids (CLA), a naturally occurring group of positional and geometric isomers of linoleic acid, have attracted scientific interest due to their potential beneficial effects on atherosclerosis, carcinogenesis or obesity [1].

With respect to atherosclerosis CLA revealed a regression of pre-established atherosclerosis in mice and rabbits and a modification of lipoprotein metabolism in hamsters [2–4]. Although the molecular mechanisms are largely unknown, it has been proposed that the anti-atherogenic actions comprise reduction of membrane-bound arachidonic acid and peroxisome proliferator-activated receptor (PPAR)-γ-dependent inhibition of NF-κB activation and subsequently reduced prostanoid release [5–7]. Supportive of this hypothesis is the observation that CLA inhibits cytokine-induced cyclooxygenase (COX)-2 gene expression and excessive prostanoid production in vascular smooth muscle cells (SMC) by a PPARγ ligand-like mechanism [8]. COX-2 expression was shown to contribute to vascular SMC proliferation and migration, that are hallmarks in atherosclerosis, and to be responsible for the majority of inflammatory prostanoid production [9], which is also involved in the chronic inflammatory response associated with atherosclerosis [10]. This suggests that the anti-inflammatory action of CLA is at least partially responsible for the anti-atherogenic effects of CLA observed in vivo.

Recent studies could demonstrate that metabolites of CLA, e.g., fatty acids with a conjugated diene structure (CD) such as CD16:2, CD18:3, CD20:2, CD20:3 and CD20:4, are found in tissues of CLA-fed animals and cultured cells treated with CLA [11–15]. These CD fatty acids are supposed to originate from elongation, desaturation and peroxisomal or mitochondrial β-

Abbreviations: CLA, conjugated linoleic acid; HCASMC, human coronary artery smooth muscle cells; CD, conjugated diene structure

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oxidation of CLA [12,14]. Moreover, it has been demonstrated that CD20:2 and CD20:3 exert potent biological activities in cell culture systems [11], indicating that the various effects observed with CLA are presumably not only mediated by CLA itself but also by its conjugated metabolites. Until now, studies investigating the formation of CLA metabolites in cells of the vascular wall, e.g., smooth muscle cells, which play a central role in the process of atherosclerosis, after treatment with CLA are not available from the literature.

Therefore, in the present study, we investigated the formation of metabolites of CLA in human coronary artery SMCs treated with different CLA isomers. Human coronary artery SMCs are considered to be appropriate cell culture models in the context of atherosclerosis regarding that coronary arteries represent blood vessels commonly affected by atherosclerosis. As CLA isomers *cis*-9, *trans*-11 CLA which contributes to more than 90% of total CLA in food [16] as well as *trans*-10, *cis*-12 CLA and *trans*-9, *trans*-11 CLA, which are minor CLA in natural foods, were used. Linoleic acid was also considered because it is frequently used as a reference fatty acid when investigating the biological effects of CLA [17,18].

2. Materials and methods

2.1. Materials

Cis-9, *trans*-11 CLA (≥96% pure), *trans*-10, *cis*-12 CLA (≥98% pure) and *trans*-9, *trans*-11 CLA (≥98% pure) were obtained from Cayman Chemicals (Ann Arbor, MI). Linoleic acid was purchased from Sigma (Taufkirchen, Germany). Preparation of stock solutions of fatty acids and of the test media was performed as described previously [5]. MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; Thiazol blue) was purchased from Sigma-Aldrich. n-hexane and methanol (both from Merck, Darmstadt, Germany) were of analytical grade and distilled before use. Chloroform for HPLC analysis was purchased from CS Chromatographie (Langerwehe, Germany). Potassium methylate (30–35% in methanol), sulphuric acid (96%) and propionitrile for synthesis were purchased from Merck. Internal standard cis-10-heptadecenoic acid methyl ester was obtained from Sigma (Steinheim, Germany).

2.2. Cell culture

Human coronary artery smooth muscle cells (HCASMC) from a 40-year-old male Caucasian donor were obtained from PromoCell (PromoCell, Heidelberg, Germany) and cultured in SMC Growth Medium 2 containing 5% fetal calf serum, 0.5 μg/L epidermal growth factor, 2.0 μg/L basic fibroblast growth factor, 5 mg/L insulin, 50 mg/L gentamicinsulfate and 50 μg/L amphotericin B (all from PromoCell). Cells were passaged after reaching confluence by using trypsin/EDTA. After trypsinization, TNS was added to prevent enzymatic damage to the cells. Only cells from passages 3–7 were used for this study.

After reaching 70–80% confluence cells were treated with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA or linoleic acid for 24 h. Cells treated without additional fatty acids were used as controls.

2.3. Cell count and cell viability

The cell count was determined with a Neubauer chamber after cells were harvested by trypsinization and pelleted by centrifugation (170×*g* for 5 min). Cell viability after treatment of HCASMCs as indicated above was examined by the MTT assay [19].

2.4. Analysis of fatty acid composition of total cell lipids

After treatment of cells for 24 h as indicated above cells were washed with PBS, harvested using a cell scraper, and pelleted by centrifugation (170×*g* for 5 min). The cell pellet was stored at −80 °C until extraction of total cell lipids.

2.4.1. Extraction of total cell lipids

Cell pellets were suspended in 1 mL bidistilled water and total cell lipids were extracted with 3 mL chloroform/methanol (2/1, v/v+0.001% BHT) after adding 100 μL of the internal standard (100 μg heptadecenoic acid methyl ester in n-hexane, C 17:1 cis 10). The lower organic phase was transferred into a Pyrex-glass-tube and extraction was repeated twice. The combined organic phases were evaporated to dryness under a stream of nitrogen at 40 °C. Lipids were methylated in a two step procedure. The lipid extract was transmethylated with 5% potassium methylate solution in methanol for 30 min at 60 °C. After cooling to room temperature 3 mL of 1 N methanolic sulphuric acid (2.8 mL 96% sulphuric acid in 100 mL methanol) were added. After vortexing the Pyrex-tubes were heated for 15 min at 60 °C for methylation of free fatty acids. After cooling 3 mL of saturated sodium chloride solution in water and 2 mL n-hexane were added. The fatty acid methyl esters were extracted into the n-hexane phase by vortexing. The upper n-hexane phase was transferred after centrifuging into a 4-mL glass vial and extraction of methyl esters was repeated once. The combined n-hexane phases were evaporated to dryness under a stream of nitrogen and solved in 500 μL n-hexane.

2.4.2. Analysis of fatty acid composition by GC-FID analysis

Fatty acids methyl ester analysis was performed using an Agilent 6890 GC equipped with a split/splittless injector at 230 °C, a flame ionization detector at 260 °C, an autosampler (Agilent Technologies, Waldbronn, Germany) and a CP SIL 88 column (100 m, 0.25 mm, 0.2 μm film thickness, Varian, Darmstadt, Germany). Hydrogen was used as carrier gas at a constant flow rate of 1 mL/min. The temperature of the GC oven was set to 70 °C for 3 min, increased at 8 °C/min to 180 °C, held for 2 min, increased at 4 °C/min to 210 °C, held for 4 min, increased at 2 °C/min to a final temperature of 240 °C and held for 25 min. HP Chemstation software (Rev. A.08.03) was used for data analysis. The sample was injected using a split ratio of 1:10.

Table 1
Proportions of linoleic acid and conjugated linoleic acid (CLA) in total lipids of human coronary artery smooth muscle cells cultured in the presence or absence (control) of 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA or linoleic acid

Treatment	Control	c9t11-CLA		t10c12-CLA		t9t11-CLA		Linoleic acid	
		5 μmol/L	50 μmol/L	5 μmol/L	50 μmol/L	5 μmol/L	50 μmol/L	5 μmol/L	50 μmol/L
C18:2*c*9*c*12	2.2±0.3	2.1±0.2	1.8±0.4	2.3±0.5	1.6±0.4	2.3±0.8	1.8±0.2	5.4±1.0*	18.2±5.1*
CLA*c*9*t*11	<0.1	1.8±0.5*	13.3±4.3*	0.2±0.1*	0.3±0.1*	0.2±0.2*	0.2±0.1*	<0.1	<0.1
CLA*t*10*c*12	<0.1	<0.1	<0.1	1.0±0.3*	10.3±3.2*	<0.1	<0.1	<0.1	<0.1
CLA*t*9*t*11	<0.1	0.3±0.2*	0.7±0.3*	0.2±0.1*	0.5±0.1*	1.1±0.4*	7.2±2.2*	<0.1	<0.1

Human coronary artery smooth muscle cells were cultured without fatty acids (control) or with 5 and 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA or linoleic acid for 24 h. Fatty acids are presented as g per 100 g of total fatty acids. Results represent mean±S.D. of five independent experiments.

* Significantly different from control, $P<0.05$.

Table 2
Proportions of conjugated metabolites of CLA in total lipids of human coronary artery smooth muscle cells cultured in the presence or absence (control) of 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA or linoleic acid

Treatment	Control	c9t11-CLA		t10c12-CLA		t9t11-CLA		Linoleic acid	
		5 μmol/L	50 μmol/L	5 μmol/L	50 μmol/L	5 μmol/L	50 μmol/L	5 μmol/L	50 μmol/L
C16:2*c*7*c*10	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
CD16:2*c*7*t*9	<0.1	0.4±0.3*	1.5±0.7*	<0.1	0.1±0.1	<0.1	<0.1	<0.1	<0.1
CD16:2*t*8*c*10	<0.1	<0.1	<0.1	0.4±0.1*	1.9±0.7*	<0.1	<0.1	<0.1	<0.1
CD16:2*t*7*t*9	<0.1	0.2±0.1*	0.5±0.1*	<0.1	0.2±0.1*	0.4±0.2*	1.8±0.5*	<0.1	<0.1
C20:2*c*11*c*14	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.4±0.2*	2.2±0.7*
CD20:2*c*11*t*13	<0.1	0.2±0.1*	1.1±0.4*	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
CD20:2*t*12*c*14	<0.1	<0.1	<0.1	0.5±0.4*	1.1±0.2*	<0.1	<0.1	<0.1	<0.1
CD20:2*t*11*t*13	<0.1	0.1±0.2*	0.5±0.5*	0.1±0.1*	0.3±0.3*	0.3±0.1*	0.6±0.3*	<0.1	<0.1
C22:2*c*13*c*16	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
CD22:2*c*13*t*15	<0.1	0.1±0.1*	0.1±0.1*	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
CD22:2*t*14*c*16	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
CD22:2*t*13*t*15	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Human coronary artery smooth muscle cells were cultured without fatty acids (control) or with 5 and 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA or linoleic acid for 24 h. Conjugated dienoic fatty acids are presented as g per 100 g of total fatty acids. Results represent mean±S.D. of five independent experiments.

* Significantly different from control, $P<0.05$.

2.4.3. Analysis of fatty acid composition by GC-MS analysis

Fatty acid methyl ester analysis was performed using a Trace GC hyphenated with a PolarisQ ion-trap mass-spectrometer (Thermo Electron GmbH, Dreieich, Germany). Column type and temperature programming were the same as for GC-FID analysis. Electron impact ionization was used at 70 eV in positive mode with an ion volume temperature of 200 °C. Helium was used as carrier gas at a constant flow rate of 1 mL/min. PolarisQ was operated in full scan mode from 50 to 650 amu. 1 μL of sample was injected in splitless mode.

2.4.4. Analysis of conjugated fatty acid isomers by Ag^+-HPLC

Conjugated fatty acid isomers were separated using Ag^+-HPLC-DAD. The system consisted of an isocratic Merck-Hitachi L-6000 A HPLC pump equipped with a Waters 717 autosampler and a Waters 996 diode-array detector operated at wavelength between 210.4 and 395.4 nm. Three Chromspher 5 lipids columns (250 mm × 4.6 mm, 5 μm) were used in series with a 50 mm × 4.6 mm pre-column of the same column material (Varian, Darmstadt, Germany). 0.2% propionitrile in n-hexane was used as eluent for separation of conjugated fatty acids at a flow rate of 1 mL/min (approximately 80 bar). Millennium[32] software (Version 3.20, Waters, Eschborn, Germany) was used for data analysis.

2.5. Statistical analysis

Student's *t* test was used to compare means of treatments with those of control. Differences of $P<0.05$ were considered significant.

3. Results

3.1. Cell viability

Viability of HCASMCs was not affected by treatment with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA or linoleic acid for 24 h. Cell viabilities after treatment with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA and linoleic acid were between 95 and 106% (mean for three independent experiments), respectively, of control cells (=100%).

3.2. Proportions of CLA and linoleic acid in total fatty acids of SMC total lipids

Analysis of fatty acid composition of total cell lipids following incubation of HCASMCs with 5 or 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA or linoleic acid revealed a significant incorporation of the respective CLA isomer or linoleic

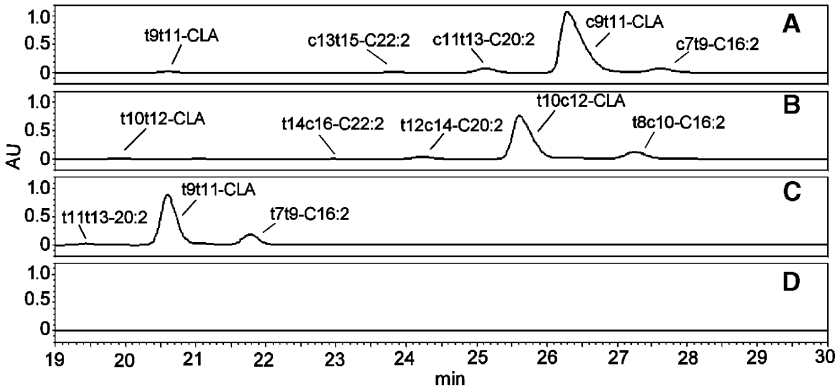

Fig. 1. Partial Ag^+-HPLC-DAD chromatogram at a detection wavelength of 234 nm of conjugated fatty acids of HCASMC treated with 50 μmol/L *cis*-9, *trans*-11 CLA (A), 50 μmol/L *trans*-10, *cis*-12 CLA (B), 50 μmol/L *trans*-9, *trans*-11 CLA (C) and 50 μmol/L linoleic acid (D).

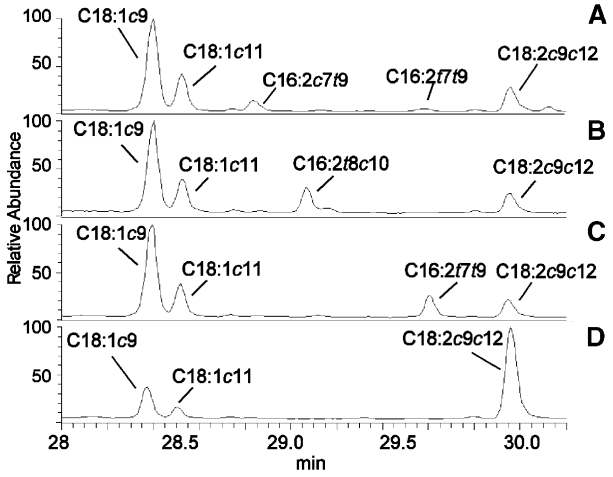

Fig. 2. Partial GC-MS chromatogram (TIC) of CD16:2 region of HCASMC treated with 50 μmol/L *cis*-9, *trans*-11 CLA (A), 50 μmol/L *trans*-10, *cis*-12 CLA (B), 50 μmol/L *trans*-9, *trans*-11 CLA (C) and 50 μmol/L linoleic acid (D).

acid into total cell lipids in a concentration-dependent manner when compared to HCASMCs treated without fatty acids ($P<0.05$; Table 1).

3.3. Proportions of conjugated metabolites of CLA in total fatty acids of SMC total lipids

Analysis of fatty acid composition of total cell lipids following treatment with *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA or *trans*-9, *trans*-11 CLA showed a dose-dependent elevation of the respective CD16:2 β-oxidation products, CD16:2*c*7*t*9, CD16:2*t*8*c*10 and CD16:2*t*7*t*9, and of the respective CD20:2 elongation products, CD20:2*c*11*t*13, CD20:2*t*12*c*14 and CD20:2*t*11*t*13, of CLA in total cell lipids relative to control cells ($P<0.05$; Table 2). A CLA-derived conjugated C20:3 metabolite was only detectable in cells treated with 50 μmol/L *cis*-9, *trans*-11 CLA but was below the limit of quantitation. Fig. 1 shows a typical Ag^{+}-HPLC chromatogram of the CLA region of HCASMC treated with 50 μmol/L of each CLA isomer at a detection wavelength of 234 nm, which is nearly specific for the CD structure. By comparison of GC-MS chromatograms of different treatments it was possible to identify the metabolites by their mass spectra. Fig. 2 shows the partial GC-MS chromatogram (TIC) of the CD16:2 region of HCASMCs treated with 50 μmol/L of each CLA isomer. Analysis of methyl esters was favored because synthesis of the 4,4-dimethyloxazoline derivatives for structure elucidation is associated with compound loss and artefact formation. Unfortunately, mass spectra of methyl ester isomers as illustrated in Fig. 3 show similar fragmentation patterns but the diene fragments (*m*/*z* 67, 81) and the molecular ion [M^{+}] (e.g., *m*/*z* 266, 322, 350) for identification of chain length are of high abundance. Identification of small amounts of CLA metabolites is aggrevated by co-eluting major fatty acids. CD20:2 metabolites elute in the region of arachidonic acid (Fig. 4), whereas CD16:2 metabolites elute in the C18:1 region. Therefore, GC-MS analysis is essential for correct identification.

The respective C22:2 elongation products C22:2*c*13*t*15, C22:2*t*14*c*16 and C22:2*t*13*t*15 in total cell lipids were also detectable in trace amounts but below the limit of quantitation (<0.05% GC-FID). By extraction of the specific *m*/*z* 350 trace, it was possible to detect these metabolites in HCASMC total lipids. Fig. 5 shows partial GC-MS chromatogram of the CD22:2 region. Significant amounts of C22:2*c*13*t*15 were formed by treatment with *cis*-9, *trans*-11 CLA (Fig. 5A), whereas the C22:2*t*14*c*16 and C22:2*t*13*t*15 by treatment with *trans*-10, *cis*-12 CLA (Fig. 5B) and *trans*-9, *trans*-11 CLA (Fig. 5C), respectively, were just detectable.

Treatment with linoleic acid resulted in a significant elevation of the respective C20:2*c*11*c*14 elongation product

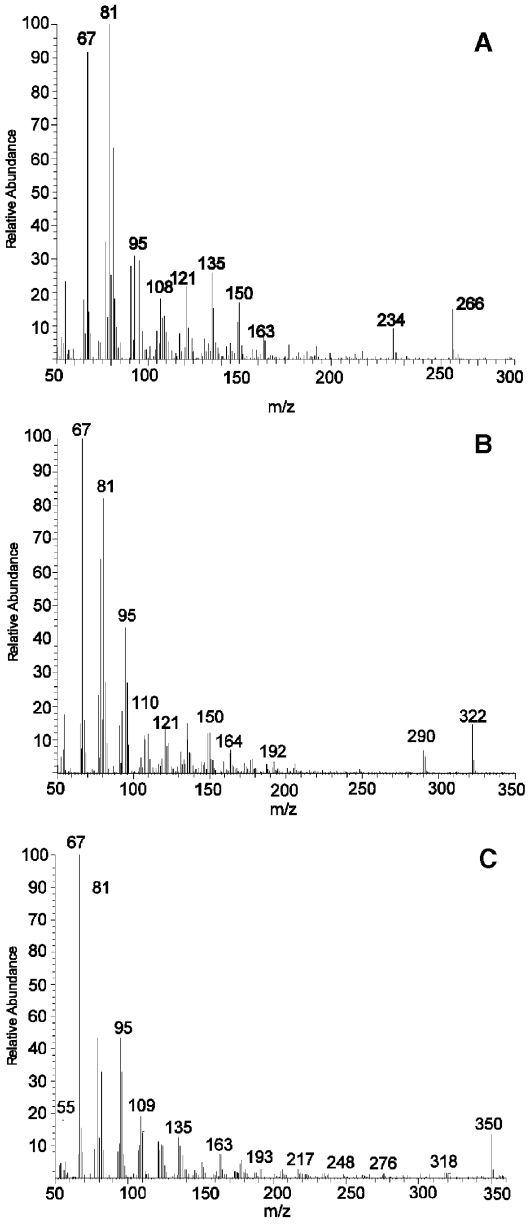

Fig. 3. GC-MS spectra of CLA β-oxidation and elongation products of HCASMC treated with 50 μmol/L *cis*-9, *trans*-11 CLA. C16:2*t*7*c*9 (A), C20:2*c*11*t*13 (B) and C22:2*c*13*t*15 (C).

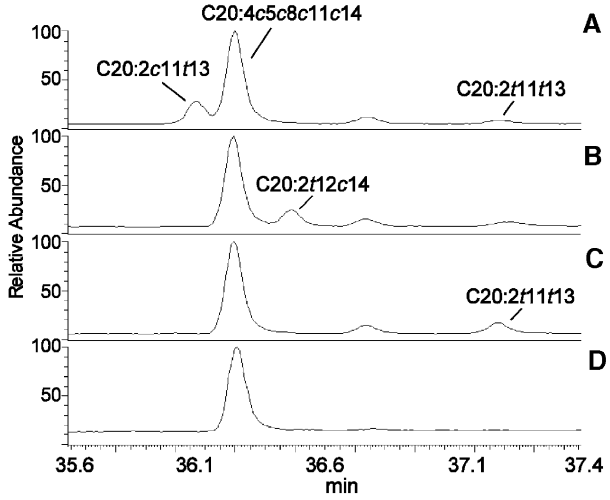

Fig. 4. Partial GC-MS chromatogram (TIC) of CD20:2 region of fatty acids of HCASMC treated with 50 μmol/L *cis*-9, *trans*-11 CLA (A), 50 μmol/L *trans*-10, *cis*-12 CLA (B), 50 μmol/L *trans*-9, *trans*-11 CLA (C) and 50 μmol/L linoleic acid (D).

in total cell lipids, whereas the C16:2*c*7*c*10 β-oxidation product and the C22:2*c*13*c*16 elongation product of linoleic acid was below the limit of detection.

3.4. Ratios of C16:2/C18:2 and C20:2/C18:2 in total fatty acids of total cell lipids for the treatment of SMCs with CLA isomers or linoleic acid

The respective C16:2/C18:2 and C20:2/C18:2 ratios in HCASMC total lipids are shown in Table 3. The respective CD16:2/CLA ratios increased in the following order: CD16:2*c*7*t*9/CLA*c*9*t*11 < CD16:2*t*8*c*10/CLA*t*10*c*12 < CD16:2*t*7*t*9/CLA*t*9*t*11. No C16:2/linoleic acid ratio could be calculated for the linoleic acid-treated cells due to the absence of detectable C16:2 levels.

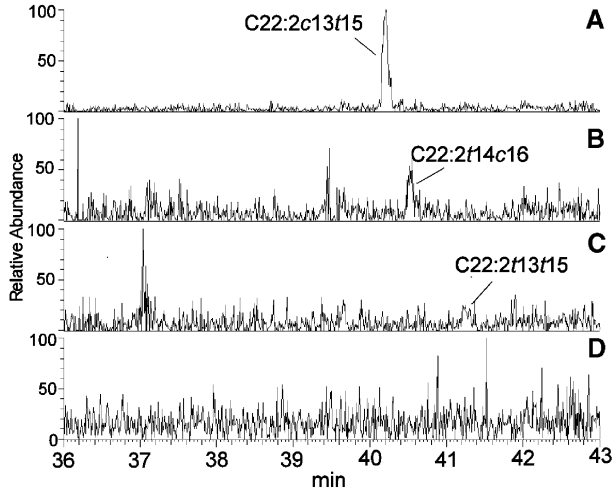

Fig. 5. Partial GC-MS chromatogram (extracted ion *m*/*z* 350) of CD22:2 region of fatty acids of HCASMC treated with treated with 50 μmol/L *cis*-9, *trans*-11 CLA (A), 50 μmol/L *trans*-10, *cis*-12 CLA (B), 50 μmol/L *trans*-9, *trans*-11 CLA (C) and 50 μmol/L linoleic acid (D).

Table 3
Ratios of C16:2/C18:2 and C20:2/C18:2 in total lipids of human coronary artery smooth muscle cells treated with 50 μmol/L of *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-9, *trans*-11 CLA or linoleic acid

Treatment	Linoleic acid	c9t11-CLA	t10c12-CLA	t9t11-CLA
C16:2*c*7*c*10/C18:2*c*9*c*12	n.d.	–*	–*	–*
CD16:2*c*7*t*9/CLA*c*9*t*11	–*	0.12 ± 0.02[c]	–*	–*
CD16:2*t*8*c*10/CLA*t*10*c*12	–*	–*	0.21 ± 0.02[b]	–*
CD16:2*t*7*t*9/CLA*t*9*t*11	–*	–*	–*	0.29 ± 0.04[a]
C20:2*c*11*c*14/C18:2*c*9*c*12	0.12 ± 0.03	–*	–*	–*
CD20:2*c*11*t*13/CLA*c*9*t*11	–*	0.09 ± 0.01	–*	–*
CD20:2*t*12*c*14/CLA*t*10*c*12	–*	–*	0.11 ± 0.02	–*
CD20:2*t*11*t*13/CLA*t*9*t*11	–*	–*	–*	0.11 ± 0.02

Ratios are presented as g per g of total fatty acids. Results represent mean ± S.D. of five independent experiments. Means with different superscript letters are significantly different, $P < 0.05$. n.d.—Not detectable.
* Not determined.

The respective CD20:2/CLA ratios in total cell lipids did not differ between each other as well as compared to the C20:2/linoleic acid ratio.

4. Discussion

It has been demonstrated that various conjugated metabolites of CLA are found in tissues of CLA-fed animals and cultured cells treated with CLA [11–15]. This observation has gained in importance since it has recently been shown that these metabolites of CLA exert specific biological activities [11]. The present study aimed to explore the potential formation of metabolites of CLA in cells of the vascular wall, which has not yet been shown. Thus, this is the first study demonstrating the existence of CLA metabolites in vascular SMCs treated with different CLA isomers.

Examination of fatty acid composition of total cell lipids using Ag^{+}-HPLC, GC-FID and GC-MS analysis in the present study enabled us to reliably detect various isomer-specific conjugated metabolites of CLA such as CD16:2, CD20:2 and CD22:2 in total lipids of human vascular SMCs treated with *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA and *trans*-9, *trans*-11 CLA. To our knowledge, the finding that CD22:2 metabolites of CLA are found in total lipids of cells treated with CLA is novel, whereas the detection of CD16:2 and CD20:2 metabolites of CLA has been reported previously for leukemia cells treated with CLA and various tissues of CLA-fed animals [11,12]. CD16:2 isomers are proposed to originate, at least in part, from chain-shortening of CLA within the peroxisomes [12]. CD20:2 isomers are probably derived from enzymatic elongation of CLA as also suggested from others [11]. As C22:2*c*13*c*16 is the elongation product of C20:2*c*11*c*14 we propose that conjugated C22:2 was generated from conjugated C20:2 via enzymatic elongation. Although the amounts of CD22:2 metabolites were very low the existence of significant amounts of C22:2*c*13*t*15 in total lipids of SMCs treated with *cis*-9, *trans*-11 CLA could be reliably demonstrated by GC-MS analysis. Markedly lower levels of C22:2*t*14*c*16 and C22:2*t*13*t*15 in total lipids of SMCs treated

with *trans*-10, *cis*-12 CLA and *trans*-9, *trans*-11 CLA, respectively, probably indicate that the elongation of C20:2 metabolites is differently affected by the configuration of the double bonds (*cis*/*trans*, *trans*/*cis* or *trans*/*trans*). The fact, that the unconjugated C22:2*c*13*c*16 in total lipids of cells treated with linoleic acid was apparently not detectable at all suggests that the rate of elongation also depends on the existence of isolated or conjugated double-bonds.

As aforementioned, CD16:2 isomers are proposed to originate from chain-shortening of CLA within the peroxisomes. The finding that the conjugated C16:2 metabolites of CLA were detectable in total lipids of cells treated with CLA isomers whereas the respective unconjugated C16:2 product of linoleic acid was not suggests that CD16:2 is apparently accumulating in vascular cells treated with CLA. Since chain-shortening in the peroxisomes is normally followed by further β-oxidation of the shortened fatty acid within the mitochondria, the observed accumulation of CD16:2 fatty acids indicates that mitochondrial β-oxidation of CLA is probably inhibited by CLA or its metabolites. This suggestion is confirmed by the observation that conjugated β-oxidation products were also accumulating in microsomal fractions of hepatic tissues incubated with CLA [11]. The obviously reduced β-oxidation capacity for CLA compared to linoleic acid might be a further indication of differences in the metabolism between unsaturated fatty acids with isolated (e.g., linoleic acid) and conjugated (e.g., CLA) double bonds as recently suggested [11].

Different CD16:2/CLA ratios in total cell lipids between various CLA isomers as observed in the present study are further indicators that fatty acid metabolism is differently affected by the configuration of the double bonds. However, an increased CD16:2/CLA ratio as observed for *trans*-9, *trans*-11 CLA compared to *trans*-10, *cis*-12 CLA or *cis*-9, *trans*-11 CLA might reflect an enhanced capacity for chain-shortening of *trans*-9, *trans*-11 CLA and their metabolites within the peroxisome and, additionally, might indicate a reduced mitochondrial β-oxidation of the shortened CLA isomer. Thus, future studies are required to clarify the contribution of peroxisomal compared to mitochondrial β-oxidation for the formation of CD16:2 fatty acids from CLA.

No differences were observed in the ratios of CD20:2/CLA in total cell lipids between the different CLA isomers as well as between the CD20:2/CLA ratio and the C20:2*c*11*c*14/linoleic acid ratio. This suggests that the capacity of human vascular cells to elongate various CLA isomers or linoleic acid to CD20:2 and C20:2n-6, respectively, is similar. However, conjugated elongation/desaturation products of CD20:2 such as CD20:3 or CD20:4 could not be clearly identified in total SMC lipids. Only traces of a conjugated C20:3 isomer were detectable in total lipids of SMCs treated with 50 μmol/L *cis*-9, *trans*-11 CLA without further elucidation of its structure. This is in contrast to the unconjugated C20:3n-6 and C20:4n-6 elongation/desaturation products which were present in significant amounts. Since the conjugated C20:3 and C20:4 metabolites were detectable in significant amounts in CLA-fed animal tissues and leukemia cells treated with CLA [11,13,14], this probably indicates a low capacity of vascular cells for the generation of these products from CLA.

The present study further revealed that low amounts of *trans*-9, *trans*-11 CLA were found in total lipids of cells treated with *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA suggesting a slight conversion of *cis*/*trans*- or *trans*/*cis*-CLA isomers into the *trans*/*trans*-CLA isomer. In contrast, conversion of *trans*-9, *trans*-11 CLA or *cis*-9, *trans*-11 CLA into *cis*/*trans*- and/or *trans*/*cis*-CLA isomers, respectively, appeared to be marginal. To our knowledge, there are no reports available concerning the enzymatic conversion of CLA-isomers into different CLA-isomers in mammalian tissues or cell culture models. However, it is well known that the *trans*/*trans*-double bond is thermodynamical more stable than the *cis*/*trans*- or the *trans*/*cis*-double bond suggesting that low amounts of *trans*-9, *trans*-11 CLA are probably the result of a slight conversion of *cis*/*trans*- or *trans*/*cis*-CLA isomers due to thermodynamical stabilization of conjugated double bonds. This assumption could also explain the finding that the *trans*/*trans*-metabolites of CLA, CD16:2*t*7*t*9 and CD20:2*t*11*t*13, were found in cells treated with *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA, whereas no *cis*/*trans*- or *trans*/*cis*-CLA metabolites were detectable in cells treated with *trans*-9, *trans*-11 CLA. However, the question whether this thermodynamic conversion occurs already within the cell or later during sample preparation or analysis, although reasonable precautions for sample preparation, storage or handling have been taken, cannot be resolved from the present study.

In conclusion, the present study shows for the first time that conjugated metabolites of CLA, e.g., CD16:2, CD20:2 and CD22:2, are also formed in cells of the vascular wall. As also suggested from others [12,13] CD16:2 presumably originates from peroxisomal β-oxidation of CLA whereas CD20:2 and CD22:2 are probably the result of enzymatic elongation of CLA. Regarding that biological activities have recently been observed for conjugated metabolites of CLA [11] this suggests that the effects of CLA in vascular cells might not only be mediated by CLA itself but also by its metabolites. Moreover, the results suggest that fatty acid metabolism is differently affected by the *cis*/*trans* configuration of conjugated double bonds in human vascular cells. Thus, future studies using highly purified conjugated metabolites of CLA are necessary to study their role in mediating biological effects of CLA in cell culture systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbalip.2005.09.011.

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Short communication

Identification of conjugated linoleic acid elongation and β-oxidation products by coupled silver-ion HPLC APPI-MS

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Abstract

Atmospheric pressure photoionisation (APPI) was used in combination with silver-ion (Ag^+)-HPLC for detection of (conjugated) fatty acid methyl esters (FAME) by tandem-mass spectrometry. APPI-MS of methyl esters of conjugated linoleic acid showed an increase in signal-to-noise ratio by a factor of 40 compared to atmospheric pressure chemical ionization in the positive mode. It was possible to identify double bond position, configuration and chain length of FAME based on chromatographic separation and mass detection. The developed LC–MS method is useful for the analysis of CLA elongation and β-oxidation products, especially with *trans*,*trans*-configuration, which are difficult to analyze by conventional GC–MS techniques.

Keywords: Conjugated linoleic acids; Silver-ion HPLC; Mass spectrometry; Bioactive lipids; Fatty acid metabolism; Atmospheric pressure photoionization

1. Introduction

Conjugated linoleic acids (CLA) are a naturally occurring minor group of positional and geometric isomers of linoleic acid (c9c12-C18:2), which have been shown to have potentially positive nutritional effects such as fat mass reduction in humans [1], anti-atherosclerotic effects in mice [2] and anti-cancerogenic effects in mice [3]. These conjugated fatty acids are formed during the biohydrogenation of linoleic and linolenic acid by microorganism *Butyrivibrio fibrisolvens* in ruminants [4]. Main CLA isomer is the c9t11-C18:2 (rumenic acid) [5] which amounts for nearly 90% of total CLA content in beef and milk and some 19 CLA isomers have been identified in milk fat with double bond position between Δ12Δ14 and Δ7Δ9 in *cis*- and *trans*-configuration [6,7]. The biological mechanisms of action are still subject to intensive research and there is evidence for significant differences in biologic activity of single isomers [8]. Highly sensitive methods for the analysis of fatty acids and their metabolites (such as prostaglandins and isoprostanes) from food and biological matrices have been established in the past such as negative chemical ionization (NCI) GC–MS of their pentafluorobenzyl (PFB) derivatives [9] or isobutane positive chemical ionization (PCI) GC–MS/MS of fatty acid methyl esters (FAME) [10]. Characterization of double bond position and configuration of FAME was established by acetonitrile PCI GC–MS/MS [11]. However, CLA isomers cannot be completely resolved by GC [12] and mass spectral data for structural determination of the fatty acids are often compromised due to co-eluting compounds. Alternatively, silver ion HPLC (Ag^+-HPLC) has become the most favourable method for CLA analysis as it is capable of resolving most isomers [13,14]. Advantage of Ag^+-HPLC is that compounds are separated into a *trans*,*trans*-, a *cis*,*trans*/*trans*,*cis*- and a *cis*,*cis*-group depending on the configuration of double bonds and on the double bond position within each group. The drawback, the carbon chain length has no influence on the separation within such a

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group. This results in co-elution of elongation (C20:2) and β-oxidation (C16:2) metabolites of CLA with other conjugated fatty acids (e.g. c11t13-C18:2 co-elutes with c11t13-C20:2) as shown recently in human coronary artery smooth muscle cells (HCASMC) after incubating with different CLA isomers [15]. Differences in metabolisation of *trans* fatty acids depending on the configuration and position of double bond may explain positive or negative nutritional effects [15–17]. Therefore, Ag^+-HPLC is a very powerful technique to study the fatty acid elongation and desaturation metabolism of conjugated fatty acids but so far additional GC–MS measurements for confirmation of the absence of co-eluting compounds were necessary. Aim of this study was to hyphenate Ag^+-HPLC for separation of CLA and their elongation and β-oxidation metabolites with selective mass-spectrometric detection using lipids from HCASMC as model system. Atmospheric pressure photoionization (APPI) was tested for efficient ionisation of the non-polar methyl ester derivatives and compared with atmospheric pressure chemical ionization (APCI).

2. Experimental

2.1. Samples

c7t9-C16:2 and c11t13-C20:2 β-oxidation and elongation products of CLA have been identified in a previous study by Ag^+-HPLC-DAD, GC–FID and GC–MS as main metabolites in human coronary artery smooth muscle cells (HCASMC) incubated with 50 μM c9t11-CLA [15]. Unfortunately, these metabolites are not commercially available as pure standard compounds at the moment. Therefore, lipid extracts from HCASMC were used as model lipids for HPLC-MS method development. The metabolites were absent in control cells. Lipids were extracted from the cells three times with chloroform/methanol (2/1, v/v) after addition of 100 μL internal standard solution in *n*-hexane (100 μg heptadecenoic acid methyl ester, c10-C17:1). Lipids were *trans*-methylated with 5% potassium methylate solution in methanol (30 min at 60 °C) and subsequent acidic esterification of free fatty acids by 0.5 M sulphuric acid in methanol (15 min at 60 °C) [18]. After addition of saturated sodium chloride solution FAMEs were extracted from the aqueous phase with hexane. Internal standard c10-C17:1 and CLA methyl ester mix (98%) were purchased from Sigma, Seelze, Germany. All solvents, sulphuric acid (96%) and sodium chloride were of analytical grade and purchased from Merck, Darmstadt, Germany. Potassium methylate was obtained as 30–35% solution in methanol from the same supplier.

2.2. Liquid chromatography

HPLC system consisted of an Agilent 1100 LC binary pump, an Agilent 1100 column oven (20 °C), a CTC PAL autosampler and an Agilent 1100 diode-array detector (234 nm). Separation was performed using three Chromspher 5 Lipids columns in series (250 mm × 4.6 mm, 5 μm) with a pre-column (50 mm × 4.6 mm, 5 μm) of the same column material (Varian, Darmstadt, Germany). 0.2% propionitrile in *n*-hexane (both Merck, Darmstadt, Germany) was used as eluent [19] at a flow rate of 0.6 mL min^{-1}. Analysis was performed after cleaning the columns with 8% propionitrile in *n*-hexane for 1 h and equilibrating the columns with the eluent for 1 h.

2.3. Mass-spectrometry

The LC-system was equipped with an API 4000 QTrap mass spectrometer detector (Applied Biosystems, Darmstadt, Germany/MDX Sciex, Toronto, Canada). APPI and APCI interfaces were tested. Experiments were performed either by direct flow injection or by Ag^+-chromatography with an injection volume of 10 μL. For APPI experiments toluene was used as dopant for the ionization. The toluene was co-injected into the APPI ion source via the auxiliary gas (gas2) at a flow rate of 30 μL min^{-1}. The source temperature was 300 °C for APPI and APCI experiments. Needle current for APCI was set to 3 μA. Detector/interface-parameters: full scan parameters: enhanced MS (EMS, Linear Ion Trap (LIT) capability of the tandem MS was used), polarity: positive, scan rate: 4000 amu s^{-1}, LIT fill time: dynamic, scan range: 200–400 amu, curtain gas 10 (arbitrary units), ion transfer voltage IS: 730 V, nebulizer gas (gas 1): 45 psi, auxiliary gas (gas 2): 30 psi, interface heater: on, system pressure 4.8 10e−5 Torr, declustering potential: 50 V. Multiple reaction monitoring (MRM) parameters: polarity: positive, curtain gas: 10 (arbitrary units), ion transfer voltage IS: 730 V, nebulizer gas (gas 1): 45 psi, auxiliary gas (gas 2): 30 psi, interface heaeter: on, collision gas (nitrogen): medium (system pressure 3.8 10e−5 Torr). Precursor/product ions and MRM parameters, which were optimized automatically by injection of CLA methyl ester standard mix using Analyst 1.4.1 software, are listed in Table 1.

Table 1
Multiple reaction monitoring parameters for different FAME

Fatty acid	Precursor ion $[M]^+$ (amu)	Product ion $[M - CH_3OH]^+$ (amu)	Dwell time (ms)	DP	CE	CXP
C18:2	294.26	262.1	70	51	13	16
C16:2	266.30	234.3	70	50	15	13
C18:3	292.30	260.3	70	50	15	13
C20:2	322.33	290.2	70	46	13	16
C20:3	320.30	288.3	70	50	15	13
C20:4	318.30	286.3	70	50	15	13

DP, declustering potential; CE, collision energy; CXP, collision cell exit potential.

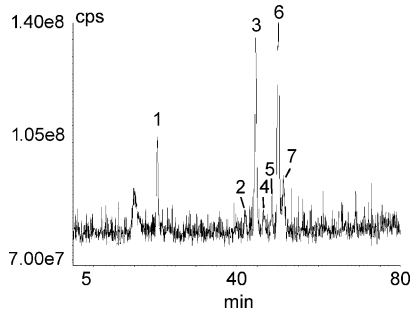

Fig. 1. Total ion chromatogram of methylated lipid fatty acids of HCASMC incubated with 50 μM c9t11-CLA for 24 h. Peak assignment: (1) saturated fatty acid methyl esters (C16:0, C18:0), (2) c11t13-C20:2, (3) c9t11-CLA, (4) c7t9-C16:2, (5) c11-C18:1, (6) c10-C17:1 (internal standard) and (7) c9-C18:1.

3. Results

3.1. Comparison of sensitivity

The signal-to-noise ratio for APPI analysis of a standard CLA methyl ester solution with toluene as dopant was enhanced by a factor of approximately 40 compared to the same concentration analyzed with APCI. Therefore, APPI interface was considered as the most sensitive ionization technique and used for further experiments. Test calibration for CLA methyl ester was linear between 1 μg L^{-1} and 1 mg L^{-1} for direct flow injection analysis in MRM mode (transition *m/z* 294/262) ($r = 0.9993$) reaching detection limits of 1 pg on column.

3.2. Fragmentation of FAME

Photoionization with post-column addition of toluene as dopant showed similar fragmentation pattern for FAME compared to GC–MS with chemical ionization. Ionization of CLA methyl esters lead to the formation of high abundant $[M]^+$ and $[M+H]^+$ ions (*m/z* 294/295, 100%) and main fragments of *m/z* 262 (35%), 110 (10%). A full scan (200–400 *m/z*) total ion chromatogram of methylated lipid fatty acids of human coronary artery smooth muscle cells (HCASMC) incubated with 50 μM c9t11-CLA for 24 h is presented in Fig. 1 and three exemplary mass spectra in Fig. 2, respectively. CLA show typical $[M]^+$ and $[M+H]^+$ ions of *m/z* 294/295 and $[M-32]^+$ fragments of *m/z* 262 (methanol loss) as can be seen in Fig. 2A. Other FAMEs like heptadecenoic acid methyl ester (c10-C17:1, *m/z* 283/250, Fig. 2B) or oleic acid methyl ester (c9-C18:1 *m/z* 296/264, not shown) also form $[M+H]^+$, $[M]^+$ and $[M-32]^+$ ions. Monoenic FAMEs were also separated depending on the position and configuration of double bond (Fig. 1, peaks 5–7). Major saturated fatty acid methyl esters were not separated by the Ag^+-columns and eluted as one peak (Fig. 1, peak 1). Stearic acid methyl ester (C18:0) and palmitic acid methyl ester (C16:0) form only the molecular ions $[M+H]^+$ (Fig. 2C, *m/z* 299 and 271, respectively).

3.3. Analysis of CLA elongation and β-oxidation metabolites in HCASMC

Fig. 3 shows four different MRM traces of HCASMC lipid fatty acids mentioned above. Using these ion traces we screened for fatty acid elongation, β-oxidation and desaturation products (transitions: *m/z* 294/262 C18:2, 266/234 C16:2, 322/290 C20:2, and 292/260 C18:3). Only the incorporated c9t11-CLA (Fig. 3A), its β-oxidation (c7t9-C16:2, Fig. 3B) and its elongation product (c11t13-C20:2, Fig. 3C) were detectable but no desaturation to conjugated linolenic acid (Fig. 3D). The FAs t9t11-CLA (Fig. 3A) and t7t9-C16:2 (Fig. 3B), respectively, were also detectable but not t11t13-C20:2 (Fig. 3C).

4. Discussion

Analysis of *cis*-/*trans*-isomers of fatty acids is of increasing importance as recent studies showed positive and negative nutritional effects depending on the configuration as well as on the position of double bonds [8] and the differences in metabolisation of *trans*,*trans*-CLA isomers [15–17]. *Trans* fatty acids and conjugated fatty acids occur naturally in food of animal origin such as beef, milk, cheese but are also formed during processing and storage of fats [20]. These fatty acids show very similar chromatographic behavior in GC and HPLC [12–14] and are difficult to analyze. This is also the case in respect to other minor lipid oxidation products such as hydroxy- and keto-fatty acids. Ag^+-HPLC with two or three columns in series permits an improved resolution of CLA (especially for the *trans*-/*trans*-isomers) in comparison to GC on polar columns. Fig. 4 shows a CLA methyl ester mixture in a partial Ag^+-HPLC chromatogram and in a partial GC–FID chromatogram from a 100 m CP Sil 88 column.

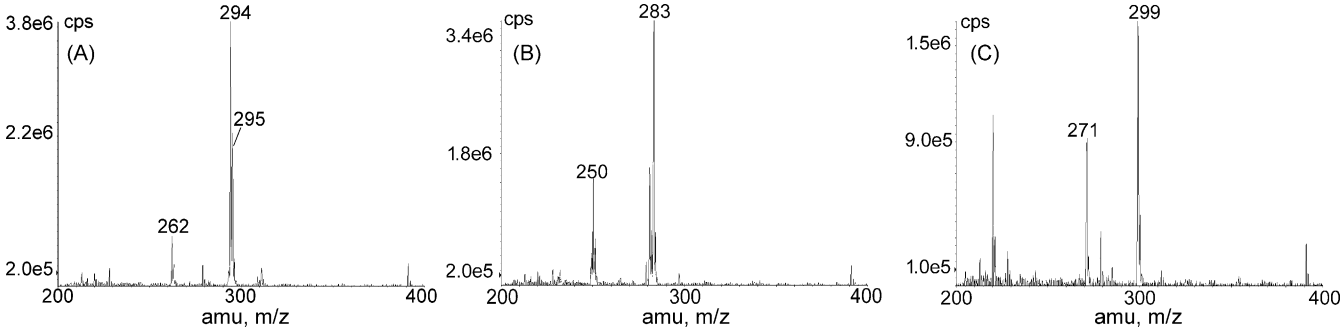

Fig. 2. Full scan mass spectra of FAMEs: (A) c9t11-CLA (peak 3 in Fig. 1); (B) c10-C17:1 (internal standard; peak 6 in Fig. 1); (C) C16:0 and C18:0 (peak 1 in Fig. 1).

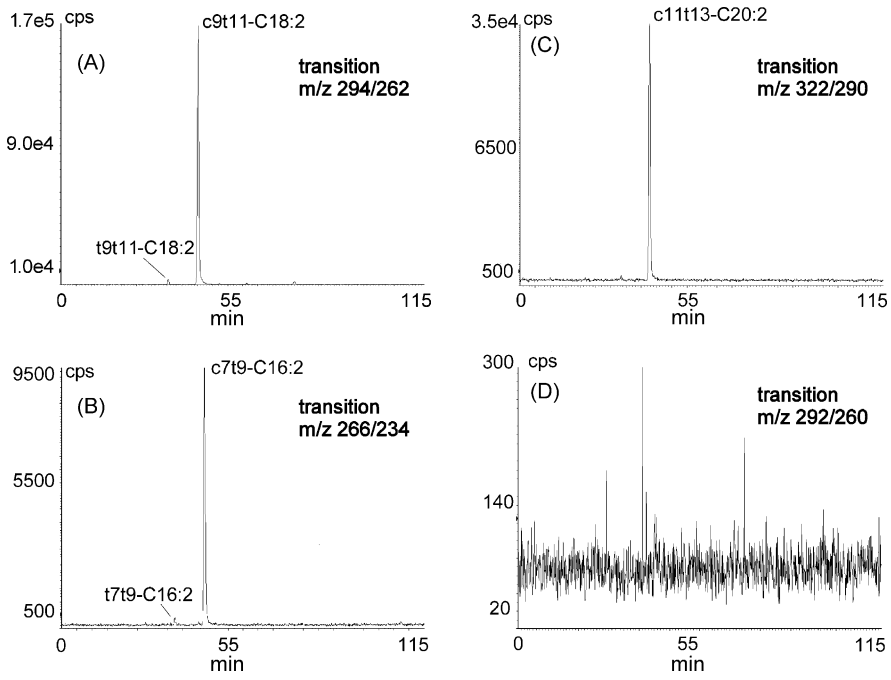

Fig. 3. Extracted ion chromatogram traces of FAMEs in HCASMC incubated with 50 μM c9t11-CLA for 24 h, analyzed by multiple-reaction-monitoring (MRM). (A) C18:2 trace (*m/z* 294/262), (B) C16:2 trace (*m/z* 266/234), (C) C20:2 trace (*m/z* 322/290) and (D) C18:3 trace (*m/z* 292/260).

Ag^+-HPLC offers an improved resolution, especially for the six *trans*,*trans*-CLA-isomers (peaks 1–6) which eluted as two peaks in the GC analysis. In complex (physiological) samples for metabolic studies of *trans*,*trans*-CLA metabolism GC–MS techniques are not sufficient, not because of a lack in sensitivity but of their inability to separate all *trans*-/*trans*-isomers necessary for reliable qualification and quantification. Ag^+-HPLC is superior for this specific analytical problem as Ag^+ ion can form charge-transfer-complexes of different strength and the double bond configuration and position can be exactly determined by their retention times in comparison to known standards [14]. Unfortunately, HPLC of lipids normally requires apolar solvent mixtures which are unfavorable for commonly used atmospheric pressure electrospray ionization (AP-ESI) interfaces in LC–MS. As an alternative, APCI and APPI interfaces are available for ionization of unpolar analytes. APPI was firstly introduced by Robb et al. for LC–MS [21] as an adaptation of the already known photoionization detector (PID) for GC and was successfully used for analysis of e.g. respiratory quinones [22] or phytosterols [23]. After evaporation of the HPLC eluent, compounds are ionized by irradiation of a strong UV-lamp with energy of approximately 10 eV which is not sufficient to ionize the solvents resulting in low background [21]. As APCI has been successfully used in combination with Ag^+-HPLC for triglyceride analysis [24] both interfaces were tested to elucidate differences in the sensitivity of analysis of a CLA mixture. Flow injection analysis of a CLA mixture showed that CLA are very susceptible for APPI analysis resulting in an increase in signal-to-noise ratio by a factor of 40 in comparison to APCI. This sensitivity is sufficient for common analysis of CLA isomers, their metabolites and isomerization products even at detection limits. Stability of retention times is a problem in Ag^+-HPLC for correct peak assignment. We showed recently that propionitrile as modifier greatly enhances the stability of retention times in comparison to the commonly used acetonitrile for Ag^+-HPLC [19]. The stability of the eluent system is sufficient for identification of compounds on the basis of retention times in comparison to an analysis of a standard mixture. APPI was regarded as optimal choice for CLA analysis as the eluent of 0.2% propionitrile in *n*-hexane did not influence sensitivity (data not shown). Background from HPLC grade solvents was detectable in full scan mass spectra (Fig. 1, *m/z* 380, *m/z* 200–230) but did not interfere in MRM mode. Ionization mechanisms and fragmentation patterns for analysis of FAME

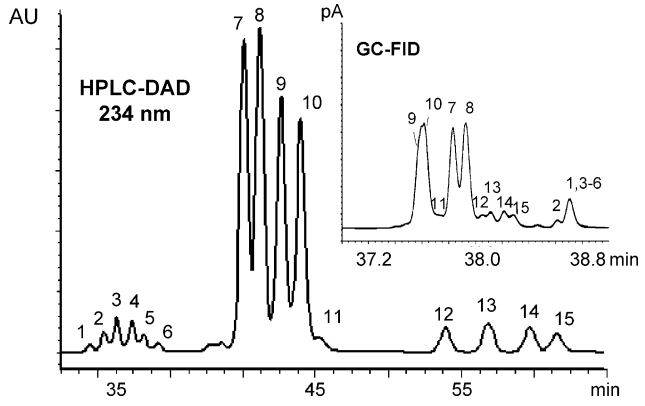

Fig. 4. Partial Ag^+-HPLC chromatogram (three ChromSpher five lipids columns, 1 mL min^{-1} 0.2% propionitrile in hexane as eluent, UV-detection at 234 nm) of a CLA methylester mixture. The insert shows the partial GC-chromatogram of the respective sample separated on a 100 m CPSil 88 GC column. Peak assignment: (1) t12t14-CLA, (2) t11t13-CLA, (3) t10t12-CLA, (4) t9t11-CLA, (5) t8t10-CLA, (6) t7t9-CLA, (7) c11t13-CLA, (8) t10c12-CLA, (9) c9t11-CLA, (10) t8c10-CLA, (11) t7c9-CLA, (12) c11c13-CLA, (13) c10c12-CLA, (14) c9c11-CLA and (15) c8c10-CLA.

are noteworthy. Saturated FAME preferably form the $[M+H]^+$ ions and did not show any further fragmentation. For these stable molecules, ionization occurs by means of a proton transfer via toluene/solvent as dopant. Monounsaturated FAME such as c10-C17:1 preferably form the $[M+H]^+$ ion, fragmenting by a loss of the proton and methanol to the $[M-32]^+$ ion. CLA were shown to form the $[M]^+$ as most abundant ion, followed by methanol loss (*m/z* 262). These differences can be explained by increasing ionization sensitivity of fatty acids with increasing number of double bonds. Mild ionization conditions causing high abundant molecular ion and small fragmentation are volitional for CLA analysis as number, position and configuration of double bonds can be deduced from chromatography [14,15,17]. Therefore, the different techniques must be discussed not only for their sensitivities but also for the structural information obtained. Negative CI-GC–MS of PFB derivatives can be used preferably for sensitive detection of fatty acid and especially their eicosanoid metabolites [9] reaching detection limits of 10 fg but hardly gives any structural information for double bond configuration of closely related compounds as the different CLA isomers. Positive APPI-MS reaches detection limits for CLA in the lower pg range which is an order of magnitude lower than PCI-GC–MS of PFB derivatives [9] or isobutane PCI-GC–MS of FAME [10,25]. Hyphenation of Ag^+-HPLC with APPI-MS detection is convenient way for identification of CLA metabolites. For complete structure elucidation of CLA other fatty acids derivatives have to be synthesized (e.g. 4,4-dimethyloxazoline derivatives) and configuration of the double bonds have to be analysed by (GC)-Fourier Transformation Infrared Spectroscopy (FTIR) [12,25]. These methods require high sample amounts and may be subject to artifact formation as fatty acids have to be derivatized at high temperatures (180 °C for 4–16 h) [26]. Identification of double bond configuration by acetonitrile CI-GC–MS/MS in positive mode is based on the formation of an $[M+54]^+$ ion via a six-membered ring intermediate after reaction with acetonitrile in the gas phase. Collisional dissociation fragments (α and ϖ ions) are characteristic for the double bond position. The α/ϖ ratio can be used for deducing double bond configuration [11]. The six *trans*,*trans*-CLA-isomers ($\Delta7\Delta9$-$\Delta13\Delta15$) elute as two peaks on a CPSil 88 GC column [12,27] the quality of the spectra of these isomers by GC–MS may be comprised at low concentrations. Ag^+-HPLC clearly separates the six isomers. Co-elution does occur but only for compounds with different chain lengths and identical double bond configuration. The resulting mass difference of at least *m/z* 28 is easily detectable by MS compared to a mass difference of 2 *m/z* for co-eluting positional CLA isomers in GC–MS. Ag^+-HPLC-APPI-MS/MS is a suitable tool for detailed analysis of double bond position and configuration for conjugated FAMEs with two double bonds and might become a complementary technique to NCI-GC–MS for specific analytical problems. It was possible to show that no isomerization of c9t11-CLA and its elongation and β-oxidation product to other *cis*-/*trans*-isomers occurred but small isomerization to the C18:2 and C16:2 *trans*,*trans*-isomers, respectively. No *trans*,*trans* isomerization occurred for the c11t13-C20:2 metabolite suggesting that isomerization is not caused by sample preparation. The described HPLC-MS technique is advantageous for analysis of labile compounds because mild chromatographic and ionisation conditions can be applied minimising artifact formation. The FAs c7t9-C16:2 and c11t13-C20:2 were clearly confirmed as main rumenic acid metabolites in HCASMC, which otherwise can hardly be distinguished from the respective C18:2 isomers by diode array detection [6]. Without the further need of RP-HPLC pre-fractionation of interfering compounds [28], the method will help analyzing CLA, *trans*-fatty acids, their metabolites and oxidation products to survey their effects in human nutrition. As requested in the Dietary Guidelines for Americans (2005) "research is needed to determine whether differences exist in the health effects of industrial versus animal sources of *trans* fat" [29], which differ substantially in the isomer distribution of conjugated fatty acid and C18:1 isomers.

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A11 **Ringseis R**, Muschick A, Eder K:

Dietary oxidized fat prevents ethanol-induced triacylglycerol accumulation and increases expression of PPARα target genes in rat liver.

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Dietary Oxidized Fat Prevents Ethanol-Induced Triacylglycerol Accumulation and Increases Expression of PPARα Target Genes in Rat Liver

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Abstract

Alcoholic fatty liver results from an impaired fatty acid catabolism due to blockade of PPARα and increased lipogenesis due to activation of sterol regulatory element-binding protein (SREBP)-1c. Because both oxidized fats (OF) and conjugated linoleic acids (CLA) have been demonstrated in rats to activate hepatic PPARα, we tested the hypothesis that these fats are able to prevent ethanol-induced triacylglycerol accumulation in the liver by upregulation of PPARα-responsive genes. Forty-eight male rats were assigned to 6 groups and fed isocaloric liquid diets containing either sunflower oil (SFO) as a control fat, OF prepared by heating of SFO, or CLA, in the presence and absence of ethanol, for 4 wk. Administration of ethanol lowered mRNA concentrations of PPARα and the PPARα-responsive genes medium chain acyl-CoA dehydrogenase, long chain acyl-CoA dehydrogenase, acyl-CoA oxidase, carnitine palmitoyl-CoA transferase I, and cytochrome P450 4A1 and increased triacylglycerol concentrations in the liver ($P < 0.05$). OF increased hepatic mRNA concentrations of PPARα-responsive genes and lowered hepatic triacylglycerol concentrations compared with SFO ($P < 0.05$) whereas CLA did not. Rats fed OF with ethanol had similar mRNA concentrations of PPARα-responsive genes and similar triacylglycerol concentrations in the liver as rats fed SFO or CLA without ethanol. In contrast, hepatic mRNA concentrations of SREBP-1c and fatty acid synthase were not altered by OF or CLA compared with SFO. This study shows that OF prevents an alcohol-induced triacylglycerol accumulation in rats possibly by upregulation of hepatic PPARα-responsive genes involved in oxidation of fatty acids, whereas CLA does not exert such an effect. J. Nutr. 137: 77–83, 2007.

Introduction

PPARα is a ligand-activated transcription factor that is essentially required for the regulation of hepatic fatty acid metabolism. The important role of PPARα for normal liver function is underscored by the observation that PPARα knockout animals develop fatty liver when fasted (1).

Fatty liver in humans most commonly develops in response to chronic alcohol abuse and is probably the result of both impaired fatty acid catabolism and increased lipogenesis in the liver (2). The latter is probably due to an increased mRNA expression of lipogenic genes such as fatty acid synthase (FAS)[1] by activation of sterol regulatory element-binding protein (SREBP)-1 in response to ethanol feeding (3). The impaired fatty acid catabolism is presumably caused by the blockade of PPARα function by ethanol, because ethanol feeding decreased DNA binding of the transcriptionally active PPARα/RXR heterodimer in the promoter region of PPARα-regulated genes and consequently reduced or failed to induce hepatic mRNA levels of several PPARα-regulated genes involved in fatty acid catabolism [long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), acyl-CoA oxidase (ACO), and liver carnitine palmitoyl-CoA transferase (L-CPT) I] (4). The central role of the disturbed PPARα function in the pathogenesis of alcoholic fatty liver is evident by the observation that administration of pharmacological PPARα agonists to ethanol-fed animals prevented fatty liver by reversing PPARα dysfunction, inducing mRNA levels of several PPARα target genes, and stimulating the rate of fatty acid β-oxidation in the liver (5,6).

In addition to pharmacological treatment of alcoholic liver disease, nutritional modulation of alcoholic fatty liver might also be an attractive approach. Recently, we and others demonstrated in rats that dietary oxidized fats activate hepatic PPARα and PPARα-regulated genes (7,8) and reduce hepatic triacylglycerol concentrations (8,9). Moreover, we demonstrated that dietary oxidized fats reduce mRNA concentrations and activities of lipogenic enzymes in the liver (9). This suggests that actions of dietary oxidized fat might be useful in the prevention of alcoholic fatty liver. However, to our knowledge, no studies are available from the literature in this regard.

[1] Abbreviations used: ACO, acyl-CoA oxidase; CLA, conjugated linoleic acid; FAS, fatty acid synthase; LCAD, long chain acyl-CoA dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; OF, oxidized fat; PPARα, peroxisome proliferator-activated receptor α; SFO, sunflower oil; SREBP-1c, sterol regulatory element-binding protein-1c.

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Therefore, in this study we aimed to explore the effect of dietary oxidized fat (OF), prepared from sunflower oil by heating, on the development of alcoholic fatty liver by use of the well-established Lieber-DeCarli liquid diet for alcohol administration in rats (10). Although differences exist between alcoholic liver disease in rodents and humans, which must be considered when extrapolating the results to the human situation, rats are commonly used as a model for studying the effects of alcohol on hepatic lipid metabolism (11). We used unheated sunflower oil (SFO) as a control fat. Because conjugated linoleic acids (CLA) affect PPARα-dependent gene transcription and are beneficial with respect to prevention from several diseases, we used a CLA supplement containing 60% total fatty acids as CLA as another treatment fat. To estimate the extent of PPARα activation we determined the abundance of mRNA of typical PPARα responsive genes in the liver that are involved in fatty acid catabolism such as MCAD, LCAD, CYP4A1, ACO, and L-CPT I. We also measured mRNA abundance of the muscle form of CPT I (M-CPT I) in the liver, because M-CPT I is also significantly induced in the liver upon treatment with PPARα activators (12). Because the production of ketone bodies is also stimulated by PPARα activation (13), we determined the plasma concentration of β-hydroxybutyrate as a further indicator of PPARα activation.

Materials and Methods

Animals and diets. Forty-eight male Sprague-Dawley rats, with an initial body weight of 251 ± 15 g, were obtained from Charles River. The animals were housed individually in Macrolon cages in a room with controlled temperature (23 ± 1°C), humidity (50–60%), and lighting (0600 to 1800 h). Nutritionally adequate liquid diets were formulated according to the Lieber-DeCarli low fat diet (10). Rats were assigned to 6 dietary groups (n = 8/group) and were fed liquid diets according to a 2-factorial design with 3 different dietary fats (SFO vs. CLA vs. OF), with or without ethanol (− ethanol vs. + ethanol) (**Table 1**). The ethanol-containing diets provided 4.184 MJ of energy per L and consisted of 18% of total energy as protein, 12% as fat, 35% as carbohydrate, and 35% as ethanol. All control rats were fed the same diet as the ethanol-fed rats except that ethanol was replaced isocalorically with maltose dextrin. Both ethanol-fed and control rats ingested identical amounts of nutrients except carbohydrates.

The CLA oil (BASF) contained 60 g CLA isomers (29.3 g *c*9*t*11-CLA, 28.9 g *t*10*c*12-CLA, 1.4 g *c*10*c*12-CLA, 0.3 g *t*9*t*11-CLA) per 100 g CLA oil as analyzed by Ag^+-HPLC-DAD (14). The OF was prepared by heating SFO at 60°C for 25 d, and the extent of lipid peroxidation in the OF was estimated by assaying the peroxide value (POV), concentration of thiobarbituric acid substances (TBARS), concentration of conjugated dienes, concentration of total carbonyls, acid value, and percentage of polar compounds as described recently (15). The concentrations of lipid peroxidation products were (OF vs. fresh SFO): peroxide value (379 vs. 3 mEq O_2/kg), TBARS (13.1 vs. 1.1 mmol/kg), conjugated dienes (274 vs. <1 mmol/kg), total carbonyles (96.9 vs. 2.9 mmol/kg), acid value (5.8 vs. 0.4 g KOH/kg), and polar compounds (27.8 vs. 5.1).

Ethanol-fed rats were acclimated to their diets over a 5-d period by feeding graded ethanol at 12% of total energy on d 1 and 2, 24% on d 3 and 4, and 36% of energy on d 5 and thereafter. Diets were administered daily at 0800 h in graduated rat feeding tubes (Dyets) for 4 wk. The rats from all experimental groups received identical amounts of the isocaloric liquid diets. During the 24 h before sample collection, the liquid diets were given in 2 portions (70% at 0800, and the final 30% at 0600) to minimize variations in feeding patterns between the ethanol-fed rats and the control rats. All experimental procedures described followed established guidelines for the care and handling of laboratory animals (16) and were approved by the council of Saxony-Anhalt.

Sample collection. Four hours after the final portion had been administered, the rats were anesthetized with diethyl ether and killed by decapitation. The liver was excised immediately, and frozen with liquid nitrogen. Blood was collected from the opened neck into heparinized polyethylene tubes (Sarstedt) by the use of heparinized plastic funnels. Plasma was separated from blood by centrifugation (1100 × g; 10 min) at 4°C. For separation of VLDL the plasma density was adjusted to δ = 1006 g/L by adding 0.5 mL of a solution containing 0.195 mol/L sodium chloride and 2.44 mol/L sodium bromide to 1.0 mL of plasma, and centrifuged (19,000 × g; 20 h) at 4°C using a Discovery 90-Ultracentrifuge (Sorvall). Liver, plasma, and VLDL were stored at −80°C pending analysis.

Lipid analysis. Liver lipids were extracted with a mixture of hexane and isopropanol (3:2, v:v) (17). Total cholesterol and triacylglycerol concentrations of liver, plasma, and VLDL were determined using enzymatic

TABLE 1 Composition of isocaloric liquid diets[1]

Component	SFO − ethanol	SFO + ethanol	CLA − ethanol	CLA + ethanol	OF − ethanol	OF + ethanol
			g/L			
Casein	41.4	41.4	41.4	41.4	41.4	41.4
L-Cystine	0.5	0.5	0.5	0.5	0.5	0.5
DL-Methionine	0.3	0.3	0.3	0.3	0.3	0.3
Sunflower oil	13.6	13.6	9.8	9.8	0	0
CLA oil	0	0	3.8	3.8	0	0
Oxidized fat	0	0	0	0	13.6	13.6
Dextrine maltose	173.2	81.1	173.2	81.1	173.2	81.1
Ethanol	0	50	0	50	0	50
Fiber	10	10	10	10	10	10
Mineral mix[2]	8.75	8.75	8.75	8.75	8.75	8.75
Vitamin mix[3]	2.5	2.5	2.5	2.5	2.5	2.5
Choline bitartrate	0.53	0.53	0.53	0.53	0.53	0.53
Xanthan gum	3	3	3	3	3	3

[1] 1 L of diet contained 4.184 MJ.
[2] Mineral mix (mg/L): calcium phosphate dibasic, 4,375; potassium citrate monohydrate, 1,925; sodium chloride, 648; potassium sulphate, 455; magnesium oxide, 210; ferrous sulfate heptahydrate, 43; manganous sulfate hydrate, 40; zinc carbonate, 14; chromium potassium sulfate, 4.82; cupric carbonate, 2.63; sodium fluoride, 0.52; potassium iodate, 0.09; and sodium selenite, 0.09.
[3] Vitamin mix (per L): all-*trans*-retinol, 1.8 mg; cholecalciferol, 10 μg; all-*rac*-α tocopheryl acetate, 30 mg; menadione sodium bisulfate, 200 μg; thiamine-HCl, 1.5 mg; riboflavin, 1.5 mg; pyridoxine-HCl, 1.75 mg; nicotinic acid, 7.5 mg; calcium pantothenate, 4 mg; folic acid, 0.5 mg; biotin, 50 μg; cyanocobalamine, 25 μg; p-amino benzoic acid, 12.5 mg; and inositol, 25 mg.

reagent kits obtained from Merck Eurolab (Refs. 157609990314 and 113009990314). For the measurement of liver total cholesterol and liver triacylglycerols, lipids of the extract were dissolved in Triton X-100 before enzymatic measurement as described by De Hoff et al. (18).

Plasma concentrations of β-hydroxybutyrate. Plasma concentration of β-hydroxybutyrate was measured using a kit from R-Biopharm. (Ref. 10907979035).

RNA isolation and real-time RT-PCR. To determine mRNA expression levels of MCAD, LCAD, PPARα, CYP4A1, ACO, L-CPT I, M-CPT I, SREBP-1c, FAS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization, total RNA was isolated from liver using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. Total RNA (1.2 μg) was subjected to cDNA synthesis using M-MuLV Reverse Transcriptase (MBI Fermentas). To determine mRNA expression levels, real-time detection RT-PCR using the Rotorgene 2000 system (Corbett Research) was applied. cDNA templates (2 μL) were amplified in 100 μL Rotorgene PCR tubes in a final volume of 20 μL containing 500 μmol/L dNTP (Roth), 3.5 mmol/L $MgCl_2$, 1.25 U GoTaq Flexi DNA Polymerase, 4 μL 5× buffer (all from Promega), 0.5 μL 10× Sybr Green I (Sigma-Aldrich), and 26.7 pmol of each primer pair. The PCR protocol comprised an initial denaturation at 95°C for 3 min and 20–35 cycles of amplification comprising denaturation at 95°C for 25 s, annealing at 60°C for 30 s and elongation at 72°C for 55 s. Subsequent melting-curve analysis was performed from 50°C to 99°C with a heating rate of 0.1°C/s and continuous fluorescence measurement. Identification of product length of the amplified product was confirmed using 2% agarose gel electrophoresis. Relative quantification was performed using the ΔΔCt method (19). Ct-values of target genes and the reference gene were obtained using Rotorgene software, version 5.0. GAPDH served as an appropriate reference gene in this experiment because the Ct-values of GAPDH did not differ between treatment groups. Relative expression ratios are expressed as fold changes of mRNA abundance in the treatment group compared with the control group (SFO without ethanol). Sequences of gene-specific primers obtained from Operon were as follows (forward, reverse): GAPDH (5′-GCA TGG CCT TCC GTG TTC C-3′, 5′-GGG TGG TCC AGG GTT TCT TAC TC-3′), SREBP-1c (5′-GGA GCC ATG GAT TGC ACA TT-3′, 5′-AGG AAG GCT TCC AGA GAG GA-3′), FAS (5′-AGG TGC TAG AGG CCC TGC TA-3′, 5′-GTG CAC AGA CAC CTT CCC AT-3′), MCAD (5′-CAA GAG AGC CTG GGA ACT TG-3′, 5′-CCC CAA AGA ATT TGC TTC AA-3′), LCAD (5′-AAG GAT TTA AGG GCA AGA AGC-3′, 5′-GGA AGC GGA GGC GGA GTC-3′), PPARα (5′-CCC TCT CTC CAG CTT CCA GCC C-3′, 5′-CCA CAA GCG TCT TCT CAG CCA TG-3′), CYP4A1 (5′-CAG AAT GGA GAA TGG GGA CAG C-3′, 5′-TGA GAA GGG CAG GAA TGA GTG G-3′), ACO (5′-CTT TCT TGC TTG CCT TCC TTC TCC-3′, 5′-GCC GTT TCA CCG CCT CGT A-3′), L-CPT I (5′-GGA GAC AGA CAC CAT CCA ACA TA-3′, 5′-AGG TGA TGG ACT TGT CAA ACC-3′), and M-CPT I (5′-GCA AAC TGG ACC GAG AAG AG-3′, 5′-CCT TGA AGA AGC GAC CTT TG-3′).

Statistical analysis. Data were subjected to ANOVA using the Minitab Statistical Software. Classification factors were dietary fat, ethanol, and the interaction of both factors (fat × ethanol). In cases of large differences between variances and means, data were transformed to logarithms prior to ANOVA. For *F*-values, individual means of the treatment groups were compared by Fisher's multiple range test. Means were considered significantly different at $P < 0.05$. Values in the text are means ± SD.

Results

Food intake and final body weights. The food intake was the same for each rat due to the controlled feeding regimen used. The amount of liquid diet consumed daily by each rat was 61.4 mL, equivalent to 15.6 g of dry matter. Final body weights were not influenced by dietary treatments ($P = 0.79$; overall, 270 ± 13 g for all treatment groups, $n = 48$).

Liver weights. Liver weights were influenced by the type of fat. Rats fed the OF diets had higher liver weights than rats fed the SFO or the CLA diets ($P < 0.05$; **Fig. 1***A*). Liver weights of rats fed the CLA diets did not differ from those fed the SFO diets. Ethanol did not influence liver weights of the rats, and there was no fat × ethanol interaction.

Triacylglycerol concentrations in liver, plasma, and VLDL and plasma concentrations of β-hydroxybutyrate. Triacylglycerol concentrations in the liver were influenced by the type of fat and by ethanol ($P < 0.05$, Fig. 1*B*). Rats fed the ethanol diets had higher concentrations of triacylglycerols than those fed the control diets ($P < 0.05$). Rats fed the OF diets had lower concentrations of triacylglycerols in the liver than those fed SFO or CLA diets ($P < 0.05$). Hepatic triacylglycerol concentrations of rats fed OF with ethanol were similar to those of rats fed SFO without ethanol. Hepatic triacylglycerol concentrations did not differ between rats fed the SFO and the CLA diets. Triacylglycerol concentrations in plasma and VLDL were influenced by the type of fat, and there was a fat × ethanol interaction ($P < 0.05$, Fig. 1*C*, *D*). Rats fed the OF diets had lower concentrations of triaclyglycerols in plasma and VLDL than those fed the SFO or CLA diets ($P < 0.05$). Rats fed diets with CLA and ethanol had higher triaclyglycerol contrations in plasma and VLDL than those fed CLA without ethanol ($P < 0.05$). In contrast, rats fed diets with SFO and ethanol and those fed the diets with OF and ethanol did not differ in their plasma and VLDL triacylglycerol concentrations from rats fed diets with the respective fats without ethanol. Plasma concentration of β-hydroxybutyrate was influenced by the type of fat and by ethanol (Fig. 1*E*). Rats fed the ethanol diets had a higher concentration of β-hydroxybutyrate

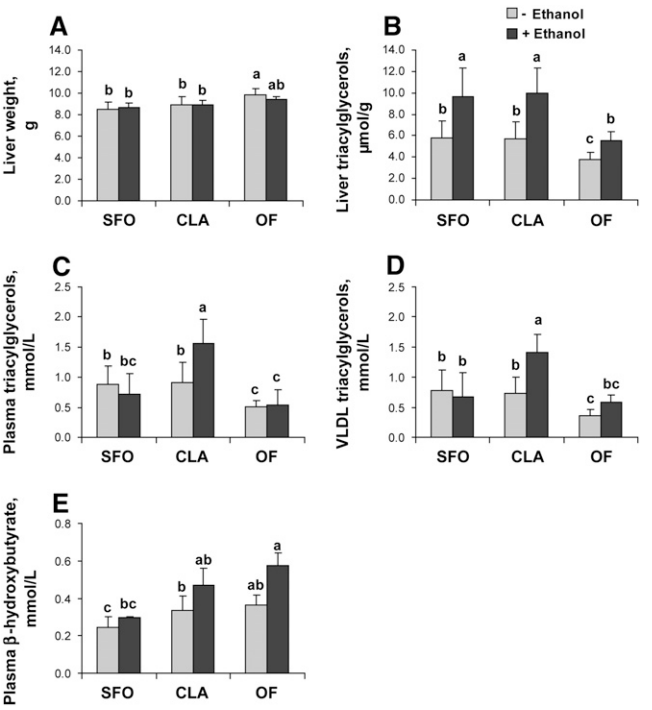

Figure 1 Liver weights (*A*), liver triacylglycerols (*B*), plasma triacylglycerols (*C*), VLDL triacylglycerols (*D*), and plasma β-hydroxybutyrate (*E*) in rats fed liquid diets containing SFO, CLA, or OF with or without ethanol. Bars represent means ± SD, $n = 8$/group. Means without a common letter differ, $P < 0.05$. Significant effects ($P < 0.05$) from 2-way ANOVA: liver weight: fat; liver triacylglycerols: fat, ethanol; plasma triacylglycerols: fat, fat x ethanol; VLDL triacylglycerols: fat, fat × ethanol; plasma β-hydroxybutyrate: fat, ethanol.

than those fed the control diets ($P < 0.05$). Rats fed the OF diet with ethanol had a higher concentration of β-hydroxybutyrate than rats fed SFO with ethanol. Rats fed the CLA diets did not differ in the plasma concentration of β-hydroxybutyrate from rats fed the SFO diets.

Relative mRNA concentrations of PPARα and PPARα-responsive genes in the liver. The relative mRNA concentration of PPARα was influenced by ethanol but not by the type of dietary fat (**Fig. 2**). Relative mRNA concentrations of the PPARα-responsive genes MCAD, LCAD, CYP4A1, ACO, L-CPT I, and M-CPT I were influenced by the type of fat and by ethanol (Fig. 2). Rats fed the ethanol diets had lower relative mRNA concentrations of PPARα and of all PPARα-responsive genes than those fed the control diets ($P < 0.05$). Rats fed the OF diets had higher relative mRNA concentrations of all PPARα-responsive genes than rats fed the SFO or CLA diets ($P < 0.05$). Rats fed the OF diet with ethanol had similar relative mRNA concentrations of all PPARα-responsive genes as rats fed SFO without ethanol. Rats fed the CLA diets did not differ in the relative mRNA concentrations of all PPARα-responsive genes from rats fed the SFO diets.

Relative mRNA concentrations of SREBP-1c and FAS in the liver. Relative mRNA concentrations of SREBP-1c and FAS were influenced by ethanol, and there was a fat × ethanol interaction (**Fig. 3**). In rats fed CLA or OF diets, ethanol increased relative mRNA concentrations of SREBP-1c and FAS ($P < 0.05$). In contrast, in rats fed the SFO diets, ethanol did not alter relative mRNA concentrations of SREBP-1c and FAS.

Discussion

In this study we investigated whether dietary OF and CLA could be useful in the prevention of alcoholic fatty liver. Alcoholic fatty liver is characterized by increased concentrations of triacylglycerols that are the result of an impaired fatty acid catabolism due to a blockage of PPARα and an increased lipogenesis in the liver due to activation of the SREBP-1 pathway (2).

The results of the present study clearly demonstrate that triacylglycerol accumulation in response to ethanol feeding could be markedly reduced in rats by simultaneous administration of dietary OF when compared with CLA or SFO. Moreover, the observation that dietary OF resulted in similar hepatic triacylglycerol levels during ethanol feeding as observed in rats fed SFO or CLA in the absence of ethanol suggests that dietary OF is indeed capable of preventing alcoholic fatty liver disease.

To elucidate the molecular mechanisms underlying these beneficial effects of OF, we investigated mRNA concentrations of fatty acid metabolism–related genes that are altered by ethanol administration. Based on these findings, we suggest that the potential mechanisms of action involved in the prevention of alcohol-induced fatty liver by dietary OF probably involve the restoration of disturbed PPARα function, whereas ethanol-induced SREBP-1c-dependent activation of lipogenesis was not affected by dietary OF. Although we did not measure the protein abundance of the transcriptionally active nuclear form of SREBP-1c, the observation that the SREBP-1c target gene FAS was also not altered by dietary OF supports our assumption that lipogenesis was not affected by dietary OF. However, because ethanol feeding in the SFO and OF group caused an increase in triacylglycerol concentrations only in the liver, and not in plasma and VLDL, it might be also possible that ethanol decreased the VLDL secretion rate (which was not addressed in the present study) from the liver of the SFO and OF group. Thus, future studies should also investigate whether the effect of OF on ethanol-induced fatty liver might be also related to alterations in the secretion rate of apoB-containing VLDL particles.

PPARα activation and function has been shown to be inhibited by ethanol feeding (4,20) as also evidenced in the present study by decreased mRNA levels of PPARα and PPARα-responsive genes such as MCAD, LCAD, ACO, CYP4A1, L-CPT I, and M-CPT I. However, although mRNA expression levels of PPARα-responsive genes were reduced in all ethanol-treated groups when compared with their respective control

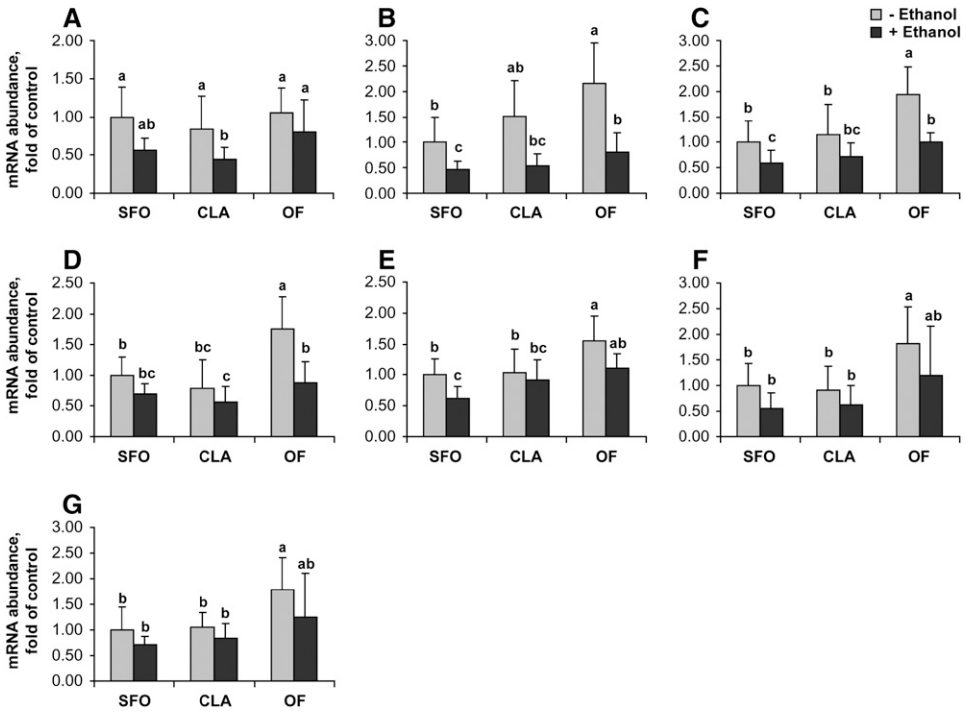

Figure 2 Relative mRNA concentrations of PPARα (*A*) and PPARα-responsive genes MCAD (*B*), LCAD (*C*), ACO (*D*), CYP4A1 (*E*), L-CPT I (*F*), and M-CPT I (*G*) in the liver of rats fed liquid diets containing SFO, CLA, or OF with or without ethanol. SFO without ethanol is considered as control (=1.00). Bars represent means ± SD, $n = 8$/group. Means without a common letter differ, $P < 0.05$. Significant effects ($P < 0.05$) from 2-way ANOVA: PPARα: ethanol; MCAD: fat, ethanol; LCAD: fat, ethanol; ACO: fat, ethanol; CYP4A1: fat, ethanol; L-CPT I: fat, ethanol; M-CPT I: fat, ethanol.

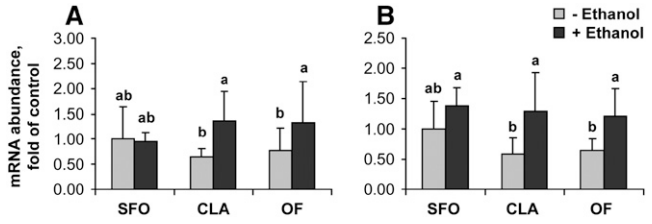

Figure 3 Relative mRNA concentrations of SREBP-1c (*A*) and FAS (*B*) in the liver of rats fed liquid diets containing SFO, CLA, or OF with or without ethanol. SFO without ethanol is considered as control (=1.00). Bars represent means ± SD, $n = 8$/group. Means without a common letter differ, $P < 0.05$. Significant effects ($P < 0.05$) from 2-way ANOVA: SREBP-1c: ethanol, fat × ethanol; FAS: ethanol, fat × ethanol.

groups, administration of OF during ethanol feeding resulted in activation of PPARα-responsive genes such as MCAD, LCAD, and CYP4A1 compared with SFO or CLA. In addition, mRNA abundance of ACO and L-CPT I was increased by administration of OF during ethanol feeding. Interestingly, the mRNA concentration of M-CPT-I, which is normally virtually absent from the liver (21), also tended to be increased ($P = 0.08$) in the liver by treatment with OF during ethanol feeding. Because M-CPT I is also induced in the liver upon treatment with PPARα activators (12), this finding also supports our assumption that OF caused a PPARα response even in the presence of ethanol. Insofar as these enzymes are involved in fatty acid catabolism, we suggest that the increased mRNA expression of these enzymes increased the capacity of the liver to oxidize fatty acids and thus counteracted the elevated levels of triacylglycerols and the diminished PPARα function during ethanol feeding. Similar observations have been made using synthetic PPARα agonists such as WY14,643 and fibrates, respectively (4,5,22). Treatment with WY14,643 restored the ability of the PPARα/RXR heterodimer to bind its specific PPAR response element and induce mRNA levels of many PPARα target genes resulting in a higher rate of fatty acid β-oxidation (4). Consequently, excessive accumulation of triacylglycerols in the livers during ethanol feeding was prevented by these agents (4,5,22). In connection with our aforementioned findings, this suggests that dietary OF prevents fatty liver development by similar mechanisms as reported for synthetic PPARα activators.

As a further parameter indicating a PPARα response, we measured the plasma concentration of the ketone body β-hydroxybutyrate, because it has been shown that production of ketone bodies is also induced upon PPARα activation (2,4) and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase, the key enzyme in ketone body formation, is also a PPARα target gene (13). Thus, the observation that treatment with OF and, to a slight extent, with CLA increased plasma concentration of β-hydroxybutyrate, is also indicative of the induction of a PPARα response. The finding that plasma concentration of β-hydroxybutyrate was increased during ethanol feeding as observed herein is consistent with findings from others (4,23) and reflects stimulation of ketogenesis as a consequence of ethanol administration, similar to the pathogenesis of alcoholic ketoacidosis (24).

Treatment with CLA did not influence ethanol-induced triacylglycerol accumulation compared with SFO. In addition, mRNA concentrations of hepatic PPARα-responsive genes were not altered by CLA compared with SFO. This suggests that CLA has no beneficial effect on alcoholic liver disease, although the ability of CLA to bind and activate PPARα has been well established and the induction of a PPARα response in the liver of rats fed CLA has been demonstrated (25–28). Nevertheless, the effects of CLA on hepatic lipid metabolism in rats reported in the literature are very inconsistent. Whereas some authors demonstrated an induction of a PPARα response in the liver of rats after feeding CLA (27,28), others did not (29), which is similar to the present study. Moreover, different observations have been made with respect to the species investigated. In mice, feeding CLA, predominantly the *t*10*c*12-CLA isomer, resulted in a significant liver enlargement and steatosis in several independent studies (30–32), whereas, in studies with rats, these effects have not been documented. Thus, the lack of effect of CLA in this study cannot be explained currently. However, we assume that the failure of effect of treatment with CLA is not explained by an insufficient dose of CLA because the concentration of CLA in the diet was similar (33) or even higher (26) compared with studies demonstrating transcriptional modulation of PPAR-responsive genes by CLA. It has been suggested that weaker PPAR-ligands like polyunsaturated fatty acids (e.g., linoleic acid or CLA) may not bind to this ligand-activated transcription factor with sufficient affinity to prevent the actions of ethanol compared with high affinity ligands like WY14,643 (4). For instance, 30 μmol/L of linoleic acid induced PPAR/RXR complex formation only to 30% of that observed with 5 μmol/L WY14,643 (34). In contrast, oxidized metabolites of linoleic acid such as 9- and 13-hydroxyoctadecadienoic acid (9-HODE and 13-HODE), which are present in oxidized fats due to formation from PUFA during heat treatment, are considered to be very potent activators of PPARα (35). Thus, this could explain the difference between the effects of OF and CLA on ethanol-induced fatty liver in the present study. In addition, 9-HODE and 13-HODE were also identified as potent PPARγ activators (36). This is noteworthy with respect to fatty liver development, because the pharmacological PPARγ-ligand pioglitazone was also recently reported to prevent alcoholic fatty liver by mechanisms involving restoration of the ethanol-induced downregulation of c-Met and upregulation of SREBP-1c (35).

Although dietary treatment with ethanol and OF is not possible in humans due to ethical reasons, we, like many others (3,5,37–39), used rats as an animal model for the current investigation. The Lieber-DeCarli liquid for administering alcohol orally was an excellent way to reproduce early lesions of alcoholic liver disease such as steatosis (11). Moreover, some of the treatment approaches, when applied to nonhuman primates, prevented signs of alcoholic liver disease in a similar manner as shown in rodent models (11). In the discussion of the results with respect to human nutrition, it must be considered, however, that there are differences between rats and humans with respect to activation of PPARα by treatment with PPARα agonists. In rats, treatment with PPARα agonists cause peroxisome proliferation and a strong upregulation of PPARα target genes (40–43). In contrast, humans, which belong to the "nonproliferating" species such as guinea pigs, swine, or monkeys, have a lower expression of PPARα in the liver and show a weaker response of most of the PPARα target genes to treatment with PPARα agonists (44). Therefore, we expect that the upregulation of hepatic PPARα target genes involved in fatty acid oxidation by treatment with OF in humans may be weaker than in rats. Nevertheless, as PPARα agonists retain a triacylglycerol-lowering effect also in nonproliferating species (45), we assume that OF could also counteract the development of an alcoholic fatty liver in humans. To our knowledge, there is only 1 published study available investigating the effect of PPARα activators on alcoholic fatty liver in humans (5). This pilot study revealed that treatment with 200 mg fenofibrate/d for 4 wk significantly

decreased serum triacylglycerol levels in 8 patients with alcoholic fatty liver, although all patients continued to drink alcohol (>80 g/d) during fenofibrate administration. Serum levels of aspartate aminotransferase, alanine aminotransferase, and γ-glutamyl transpeptidase did not differ before and after treatment with fenofibrate, indicating that liver function was not impaired during short-term treatment with fenofibrate. Although the extent of fatty liver after treatment with fenofibrate has not been reported in that study and a controlled study on a larger number of patients will be necessary to obtain definitive results, it suggests that PPARα activators may be effective for the treatment of hypertriglyceridemia in alcoholics and suitable agents to prevent alcoholic fatty liver.

In conclusion, the present study demonstrated that feeding dietary OF during ethanol administration resulted in markedly lower triacylglycerol accumulation in the liver when compared with CLA or SFO. This suggests that dietary OF is capable of preventing alcoholic fatty liver development and, thus, provides a nutritional approach to modulate this most common liver disease. However, because the uptake of OF has unfavorable effects, such as the impairment of the antioxidant defense system, the uptake of OF for the prevention of human diseases such as alcoholic fatty liver must be considered with caution. Nevertheless, as triacylglycerol accumulation makes the liver more prone to injury by various agents such as drugs and toxins (46), which are presumably involved in the pathogenesis of alcoholic hepatitis and fibrosis (47,48), approaches to prevent or even treat fatty liver are of great importance.

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Oxidized fat induces oxidative stress but has no effect on NF-κB-mediated proinflammatory gene transcription in porcine intestinal epithelial cells

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Abstract. *Objective and design:* The effect of oxidized frying oils on PPARγ which is a potent inhibitor of inflammatory responses in the intestine is currently unknown. Thus, the present study aimed to explore the effect of oxidized frying oil on PPARγ DNA-binding and proinflammatory responses in intestinal epithelial cells.
Material and methods: 18 male pigs were fed two different diets containing either fresh fat or oxidized fat (n = 9 each). After 28 d, intestinal epithelial cells were isolated and analyzed for PPARγ DNA-binding, NF-κB DNA-binding and NF-κB target gene expression. In addition, markers of lipid peroxidation and antioxidant status were determined.
Results: Feeding the oxidized fat slightly, but not significantly, activated PPARγ DNA-binding in intestinal epithelial cells when compared to fresh fat. In addition, oxidized fat increased the concentration of TBARS and reduced the concentrations of α-tocopherol and activities of antioxidant enzymes relative to fresh fat ($P < 0.05$). No effect of the oxidized fat was observed on NF-κB DNA-binding and NF-κB target gene expression in intestinal epithelial cells.
Conclusions: The present study suggests that moderate PPARγ activation and induction of oxidative stress by oxidized frying oil have no implication for NF-κB-mediated proinflammatory gene expression in porcine intestinal epithelial cells.

Key words: Inflammation – Enterocyte – PPARγ – NF-κB – Oxidative stress – Pig

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Introduction

In recent years the contribution of dietary oxidized fats to total energy intake has markedly increased in industrialized countries, mainly due to the increasing consumption of fast foods which contain heated and processed dietary fats such as frying oil. During deep-frying several lipid peroxidation products are formed in the frying oil [1], which are absorbed in the fried food and ingested during consumption. Oxidized frying oil as well as its components (e. g. oxidized fatty acids) were reported to activate the ligand-activated transcription factor peroxisome proliferator-activated receptor α (PPARα) and induce the expression of PPARα target genes *in vivo* and *in vitro* [2–5]. Oxidation products of fatty acids were also demonstrated to activate PPARγ *in vitro* [6], and some of the *in vitro*-effects observed with oxidized fatty acids were shown to be mediated by PPARγ [7]. For instance dietary oxidized fatty acids were shown to enhance production of apolipoprotein A-I (apo A-I), the main apo of HDL particles, from cultured intestinal cells via activation of PPARγ [7]. However, whether dietary oxidized fats are also capable of activating PPARγ and PPARγ-dependent gene transcription *in vivo* is currently unknown.

PPARγ was originally shown to play a critical role in adipocyte differentiation and glucose homeostasis [8]. However, PPARγ has also been implicated as an important modulator of inflammatory responses in various tissues, including the intestinal and colonic mucosa where PPARγ is highly expressed by both epithelial and macrophage cells [9, 10]. Modulation of inflammatory responses by PPARγ is mediated by negatively interfering with a variety of signalling pathways such as nuclear factor-kappa B (NF-κB) [11], which leads to the repression of transcription of genes involved in inflammation [12]. This transrepression activity likely constitutes the mechanistic basis for the anti-inflammatory prop-

erties of PPARγ and probably also explains why pharmacological PPARγ-ligands markedly reduce inflammation in animal models of inflammatory colitis [13–17]. Conjugated linoleic acids, a group of naturally occurring polyunsaturated fatty acids, were also recently shown to ameliorate experimental colitis through a PPARγ-dependent mechanism [18], indicating that dietary fatty acids are indeed capable of mediating PPARγ-dependent effects *in vivo*. However, whether dietary oxidized fats are also capable of attenuating inflammatory responses due to activation of PPARγ is uncertain. Oxidized fats were demonstrated to potently induce oxidative stress [19–21] and oxidative stress has been shown to induce proinflammatory gene expression [22, 23]. In fact, the oxidized fatty acid 13-hydroperoxyoctadecadienoic acid has been shown to induce proinflammatory gene expression via activation of NF-κB in vascular smooth muscle cells [24]. Therefore, the net effect of these postulated, obviously opposing effects of oxidized fat (PPARγ activation and induction of oxidative stress) on inflammatory gene transcription is uncertain and appropriate studies dealing with this question are missing.

The aim of the present study was to explore the effect of a dietary oxidized fat, prepared by heating under deep-frying conditions, on PPARγ DNA-binding activity and its transrepression activity on NF-κB-mediated gene transcription in intestinal epithelial cells of pigs. Intestinal epithelial cells were used since these cells express high levels of PPARγ [9] and are presumably exposed to abundant levels of oxidized fatty acids during intestinal passage after ingestion of a dietary oxidized fat. In addition, oxidized fatty acids were demonstrated to be efficiently taken up by the enterocyte [25]. Regarding the connection between oxidative stress and the induction of inflammatory responses, we also determined the treatment effect on the enterocyte antioxidant defense system, which is sensitive to the induction of oxidative stress [26] and largely contributes to the mucosal defense against diet-derived oxidants [27]. To further elucidate potential PPARγ-dependent effects of oxidized fat *in vivo*, we also analyzed the effect of treatment on plasma and HDL cholesterol concentrations as well as on apo A-I formation. We have previously observed that treatment of rats and guinea pigs with oxidized fat decreased cholesterol concentrations of plasma but increased those of HDL [28, 29]. The latter might be explained by the aforementioned increased production of apo A-I [7].

Materials and methods

Animals and diets

18 male, 8 week old pigs (German Landrace × Large White × Pietrain), obtained from a local breeder, were kept individually in single crates in an air-conditioned pigpen maintained at a temperature of 23 ± 2 °C and 50–60 % relative humidity with lighting from 06.00 to 18.00. After one week of adaptation the pigs were weighed, randomly assigned to two groups of 9 pigs each with mean body weights of 12.0 ± 1.1 kg (control group) and 12.2 ± 0.9 kg (treatment group), and then fed the experimental diets.

The diets were nutritionally adequate for growing pigs (National Research Council, 1998) and contained 14.4 MJ/kg metabolizable energy. The composition of the experimental diet was (g/kg diet): wheat (400), soy bean meal (230), wheat bran (150), barley (98.1), fat (90), and mineral premix (30) including L-lysine, L-threonine, and DL-methionine. The experimental diets varied in the type of fat which consisted of fresh fat in the control group and oxidized fat in the treatment group. The oxidized fat was sunflower oil which was heated at 200 °C for 24 h. The fresh fat was a mixture of sunflower oil and palm oil in a ratio of 93: 7. This ratio was chosen to equalize the fatty acid composition of the fresh fat with that of the oxidized fat, since the heating process caused a loss of PUFA. The major fatty acids in the fresh fat and the oxidized fat as analysed by GC-FID [30, 31] were (g/100 g total fatty acids): palmitic acid (16: 0), 9.0 vs. 6.8; stearic acid (18: 0), 4.0 vs. 4.2; oleic acid [18: 1(n-9)], 24.3 vs. 24.1; linoleic acid [18: 2(n-6)], 60.0 vs. 60.6; and α-linolenic acid [18: 3(n-3)], 0.1 vs. 0.1. Other fatty acids, including trans-fatty acids, were present only in traces (<0.5 g/100 g fatty acids).

The extent of lipid peroxidation in the fats after inclusion into the diets was estimated by assaying the peroxide value (POV) [32], concentration of thiobarbituric acid substances (TBARS) [33], concentration of conjugated dienes [34], concentration of total carbonyls [35], and acid value [32]. For this purpose the fats were extracted from the diets with a mixture of hexane and isopropanol according to the method of Hara and Radin [36]. Since heat treatment of sunflower oil causes a marked loss of the native vitamin E, the vitamin E concentrations of the diets were adjusted to 56 mg α-tocopherol equivalents/kg by individual supplementation of the diets with all-rac-α-tocopherol acetate after determination of the basal α-tocopherol concentrations of the diets (the biopotency of all-rac-α-tocopheryl acetate was considered to be 67 % of that of α-tocopherol). To standardize the feed intake, each pig received daily 700 g of diet throughout the whole experiment. Water was provided by nipple drinking systems ad libitum. The experimental diets were fed for 28 days. All experimental procedures described followed guidelines for the care and handling of laboratory animals and were approved by the regional council of Saxony-Anhalt.

Blood collection and isolation of intestinal epithelial cells

On the day of isolation of intestinal epithelial cells, each animal was fed with 300 g of the respective diets 4 h before killing with a captive bolt pistol, and blood was collected into heparinized polyethylene tubes (Sarstedt, Nürnbrecht, Germany). Plasma and lipoproteins were separated from blood as described previously in detail [37]. The abdomen was immediately opened, and a 35 cm intestinal segment was dissected starting at 30 cm distal to the pyloric sphincter, and flushed two times with ice-cold wash buffer [phosphate-buffered saline (PBS) containing 0.2 mM PMSF, and 0.5 mM DTT, pH 7.4]. The isolation of porcine intestinal epithelial cells was performed by the modified distended intestinal sac technique according to Fan et al. [38]. In brief, the intestinal segments were filled with 100 mL of preincubation buffer (PBS containing 27 mM sodium citrate, 0.2 mM PMSF, and 0.5 mM DTT, pH 7.4), sealed with strings, and filled intestinal segments were incubated in a saline bath (154 mM NaCl) for 15 min at 37 °C. Afterwards, the preincubation buffer was discarded, and the intestinal segments were filled with isolation buffer (PBS containing 1.5 mM Na_2EDTA, 0.2 mM PMSF, 0.5 mM DTT, and 2 mM D-glucose, pH 7.4). Two major cell fractions, consisting of the upper, and the crypt cell fraction, were sequentially isolated from intestinal segments through two consecutive incubations with isolation buffer at 37 °C for 40 (upper cell fraction) and 60 min (crypt cell fraction), respectively. Each cell fraction was collected separately, and washed two times with ice-cold PBS. Afterwards, cells were retained by centrifugation (400 g, 4 min, 4 °C), and immediately frozen at –80 °C. For future preparation of nuclear extracts, RNA isolation, and enzyme activity determinations only the crypt cell fractions were used.

Measurement of PPARγ and NF-κB DNA-binding activities

For the measurement of PPARγ and NF-κB DNA-binding activities nuclear extracts were prepared from enterocytes with a Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol. Protein concentrations in the nuclear extracts were determined

by the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL) with BSA as standard. PPARγ and NF-κB transactivities in the nuclear extracts were determined by the Transcription Factor assays TransAM™ PPARγ Kit and TransAM™ NF-κB p50 Kit (both from Active Motif), respectively, according to the manufacturer's protocol. For the measurement of PPARγ and NF-κB transactivities 20 µg of nuclear protein were used in each assay. From controls (THP-1, COS-7) 5 µg of nuclear protein were assayed. These ELISA-based assays facilitated the measurement of binding of PPARγ and NF-κB subunit p50 to their respective consensus binding sequences (PPAR response element: 5´-AACTAGGNCAAAG-GTCA-3´; NF-κB binding sequence: 5´-GGGACTTTCC-3´), which are immobilized to a 96-well plate, by primary antibodies specific for PPARγ and p50, respectively. Subsequent incubation with a HRP-conjugated secondary antibody and a standard developing solution in each well turned the contents blue. After addition of a Stop solution the absorbance of the wells was read at 450 nm in a microplate reader.

Analysis of mRNA expression

Total RNA of enterocytes was isolated by the Tissue Lyser (Qiagen, Hilden, Germany) using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from the optical density at 260 and 280 nm (SpectraFluor Plus, Tecan, Crailsheim, Germany). cDNA synthesis and semi-quantitative RT-PCR for the estimation of mRNA expression of PPARγ, cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, and glyceraldehyde-3-phosphat dehydrogenase (GAPDH) for normalization, were performed using a PCR thermocycler (Biometra, Göttingen, Germany) as described previously [39]. The number of PCR cycles was determined in preliminary experiments ensuring that relative quantification of mRNA expression was performed within the linear range of amplification of each PCR product. According to this, cDNAs were subjected to 30 (PPARγ), 42 (COX-2), 33 (TNFα), and 20 (GAPDH) PCR cycles of amplification, respectively. For evaluation of mRNA expression of interleukin (IL)-6, inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase, apo A-I, and GAPDH for normalization, real-time RT-PCR was performed using the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia) as described recently [40]. Ct-values were in the range of 32–33 (IL-6), 26–27 (iNOS), 17–18 (SOD), 19–20 (GSH-Px), 18–19 (catalase), and 18–19 (GAPDH), respectively. The PCR primers used for real-time and semi-quantitative RT-PCR were obtained from Operon (Köln, Germany) and Roth (Karlsruhe, Germany), respectively. Primer sequences were as follows (forward, reverse): PPARγ (5´-TGT GTC CCC GCC TTA TTA TTC TGA-3´, 5´-GCC CTC GCC TTT GCT TTG GTC-3´), COX-2 (5´-AGA TCA GAA GCG AGG ACC AG-3´, 5´-GTG TGA GGC GGG TAG ATC AT-3´), TNFα (5´-TCC TCA CTC ACA CCA TCA GC-3´, 5´-TAG TCG GGC AGG TTG ATC TC-3´), GAPDH (5´-AGG GGC TCT CCA GAA CAT CAT CC-3´, 5´-TCG CGT GCT CTT GCT GGG GTT GG-3´), IL-6 (5´-ATG GCA GAA AAA GAC GGA TG-3´, 5´-GTG GTG GCT TTG TCT GGA TT-3´), iNOS (5´-ATG GAA CAC CCC AAA TAC GA-3´, 5´-AGG ATG TTG TAG CGC TGG AC-3´), SOD (5´-TCC ATG TCC ATC AGT TTG GA-3´, 5´-CTG CCC AAG TCA TCT GGT TT-3´), GSH-Px (5´-CAA GAA TGG GGA GAT CCT GA-3´, 5´-GAT AAA CTT GGG GTC GGT CA-3´), catalase (5´-CAG CTT TAG TGC TCC CGA AC-3´, 5´-AGA TGA CCC GCA ATG TTC TC-3´), and apo A-I (5´-CGA TCA AAG ACA GTG GCA GA-3´, 5´-GCT GCA CCT TCT TCT TCA CC-3´).

Determination of apo A-I protein expression

For determination of the relative protein concentration of apo A-I in HDLs, equal volumes (3 µl) of HDL fractions were subjected to 12% SDS-PAGE. Gels were stained with Coomassie and the relative intensity of the band corresponding to apo A-I (29 kDa) was determined by densitometric analysis (Gel-Pro Analyzer software; Intas, Upland, CA, USA).

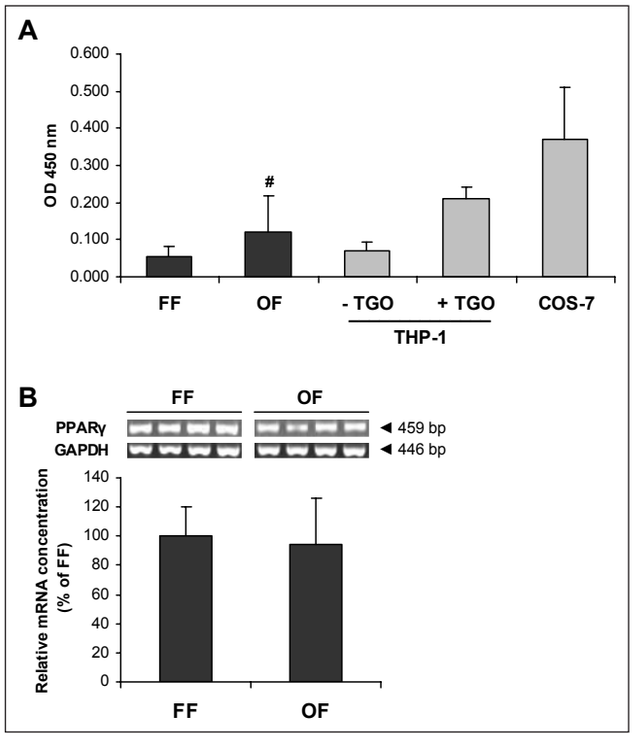

Fig. 1. PPARγ DNA-binding activity (**A**) and relative PPARγ mRNA concentration (**B**) in intestinal epithelial cells isolated from pigs fed oxidized fat (OF) and fresh fat (FF). Bars represent means ±SD of nine pigs per group. In addition, negative and positive controls for the measurement of PPARγ DNA-binding activity are also shown. Control nuclear extracts were prepared from PMA-differentiated THP-1 macrophages treated without (negative control) or with 20 µM of the synthetic PPARγ-agonist troglitazone (TGO; positive control) for 24 h. A further positive control originating from stably PPARγ-transfected COS-7 cells is also shown. Representative images of PPARγ and GAPDH PCR product electrophoresis after semi-quantitative RT-PCR amplification are shown for four animals of each group. Symbols indicate treatment effect according to statistical analysis: $^{\#}$, $P < 0.15$.

Determination of antioxidant status

Concentrations of α-tocopherol in enterocyte lysates were determined by means of high performance liquid chromatography with fluorescence detection according to the method of Balz et al. [41]. In brief, lysed enterocytes were mixed with 1 mL of 10 g/L pyrogallol solution (in ethanol, absolute) and 150 µL of saturated sodium hydroxide solution. This mixture was heated for 30 min at 70 °C, and tocopherols were extracted with n-hexane. Individual tocopherols of the extracts were separated isocratically using a mixture of n-hexane and 1,4 dioxane (94: 6, v/v) as mobile phase at a flow rate of 1 mL/min and a LiChrosorb Si-60 column (5-µm particle size, 250 mm length, 4 mm i. d., Merck) and detected by fluorescence (excitation wavelength: 295 nm, emission wavelength: 330 nm). Concentrations of α-tocopherol were calculated by an external calibration curve and related to the protein concentration of enterocyte lysates as determined by the BCA protein assay kit (Pierce). For determination of antioxidant enzyme activities homogenates were prepared from frozen enterocytes. Frozen enterocytes were thawed in ice-cold PBS and pipetted up and down for several times. Afterwards the lysed enterocytes were centrifuged (600 g, 10 min, 4 °C) and the supernatants, representing homogenates, were used for the measurement of enzyme activities. Enzyme activities were related to the protein concentrations of homogenates as determined by the BCA protein assay kit (Pierce). The activity of GSH-Px in enterocyte homogenates was determined with

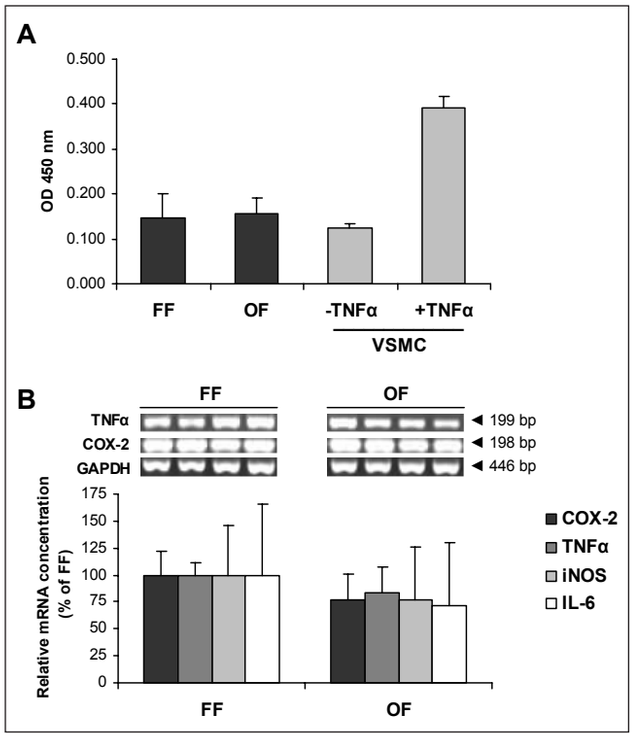

Fig. 2. NF-κB DNA-binding activity (**A**) and relative mRNA concentrations of the NF-κB target genes cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS), and interleukin (IL)-6 (**B**) in intestinal epithelial cells isolated from pigs fed oxidized fat (OF) and fresh fat (FF). Bars represent means ± SD of nine pigs per group.
In addition, negative and positive controls for the measurement of NF-κB DNA-binding activity are also shown. Control nuclear extracts were prepared from vascular smooth muscle cells (VSMC) treated without (negative control) or with 10 ng/mL TNFα (positive control) for 8 h. Representative images of COX-2, TNFα, and GAPDH PCR product electrophoresis after semi-quantitative RT-PCR amplification are shown for four animals of each group. IL-6 and iNOS mRNA concentrations were determined by real-time RT-PCR.

t-butyl hydroperoxide at 25 °C according to the method of Paglia and Valentine [42]. One unit of GSH-Px activity is defined as one μmol reduced β-nicotinamide adenine dinucleotide phosphate oxidized per min. Peroxisomal catalase activity was measured in enterocyte homogenates at 25 °C using hydrogen peroxide as the substrate according to Aebi [43]. One unit of catalase activity is defined as the amount consuming 1 μmol hydrogen peroxide per min. In order to release catalase from peroxisomes homogenates were incubated in an ultrasonic bath for 60 s prior to the measurement of catalase activity. SOD activity in enterocyte homogenates was determined with pyrogallol as the substrate [44]. One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50 %.

Determination of thiobarbituric acid substances (TBARS)

TBARS were measured in enterocyte lysates using a modified version of the TBARS assay [33]. Lysed enterocytes were mixed with TBA reagent (8 g/L TBA with 7 % perchloric acid, 2: 1, v/v) and heated for 60 min at 95 °C. TBARS were extracted with n-butanol, and absorption was measured at 532 nm. Concentrations were calculated via a standard curve with 1,1,3,3,-tetraethoxypropane and related to the protein concentration of enterocyte lysates.

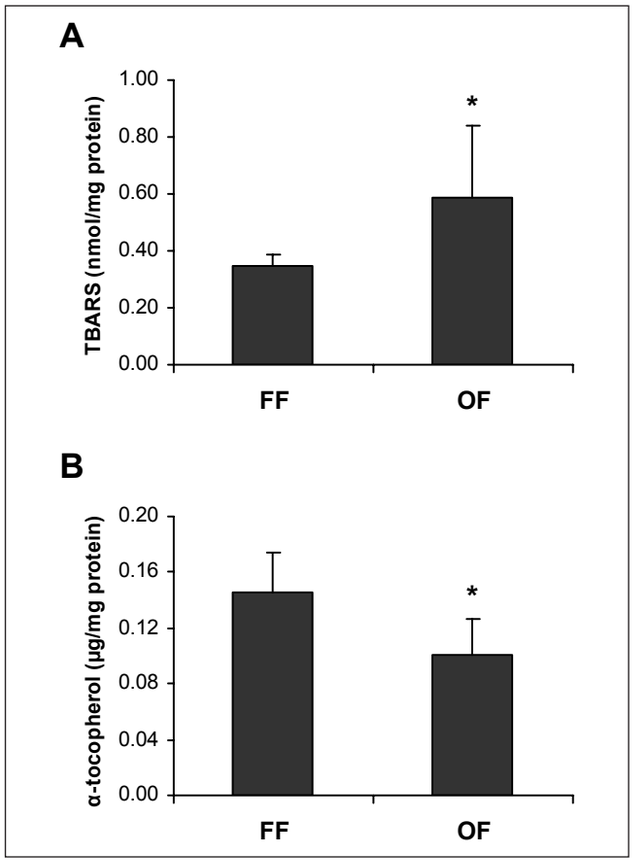

Fig. 3. Concentration of TBARS (A) and α-tocopherol (B) in intestinal epithelial cells isolated from pigs fed oxidized fat (OF) and fresh fat (FF). Bars represent means ±SD of nine pigs per group. Symbols indicate treatment effect according to statistical analysis: *, $P < 0.05$.

Statistics

Student's t-test was used to compare means of treatments with those of control. Differences of $P < 0.05$ were considered significant.

Results

Lipid peroxidation products in experimental fats

The oxidized fat had higher concentrations of peroxides (10.0 vs. 2.5 mEq O_2/kg), TBARS (0.27 vs. 0.01 mmol/kg), conjugated dienes (89.1 vs. 22.7 mmol/kg), and total carbonyles (24.5 vs. 2.5 mmol/kg) and a higher acid value (8.0 vs. 1.6 g KOH/kg) than the fresh fat.

Food intake and final body weights of pigs

The food intake was the same for each pig due to the standardized feeding regimen used. Final body weights did not differ between pigs fed the fresh fat diet (26.0 ± 1.5 g, n = 9) and those fed the oxidized fat diet (25.6 ± 1.4 g, n = 9). Body weight development also did not differ between the treatment groups (data not shown).

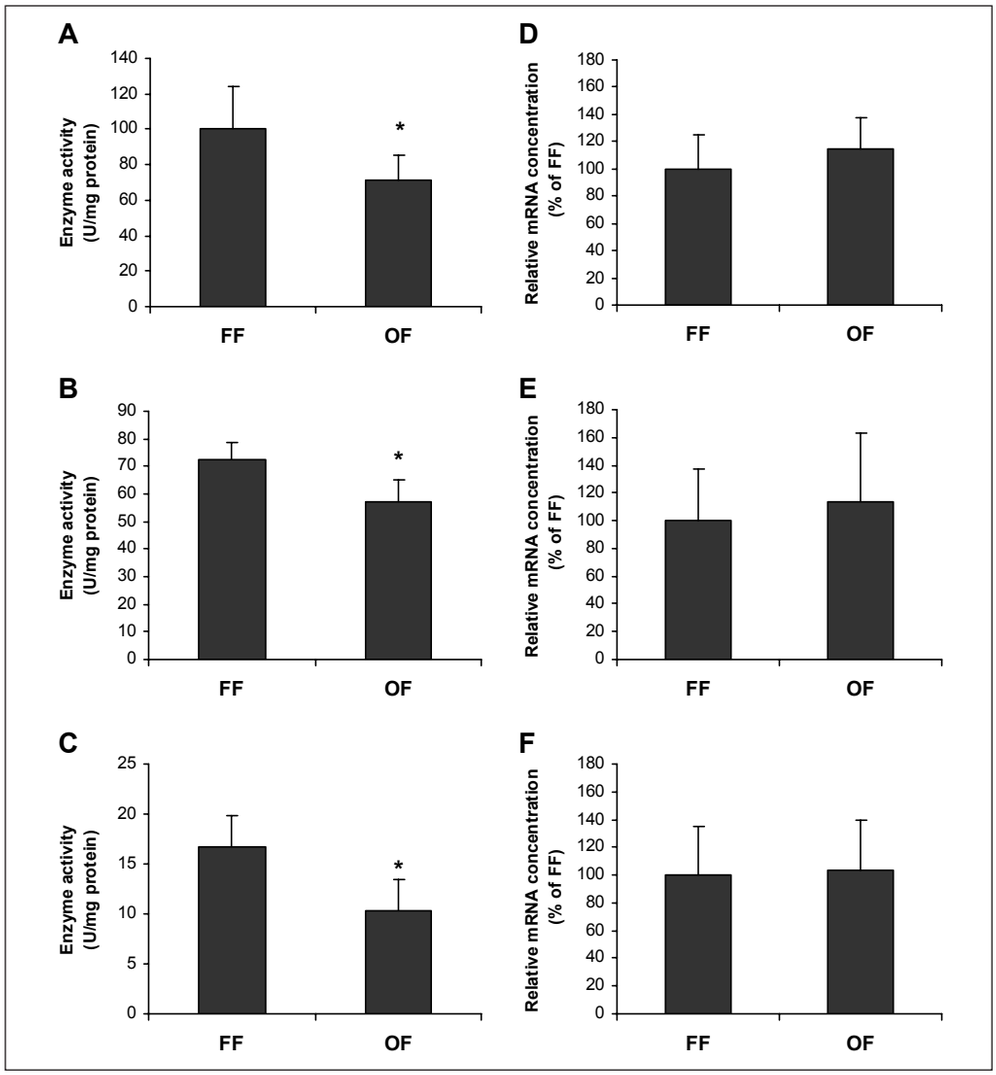

Fig. 4. Activities and relative mRNA concentrations of the enzymatic antioxidants catalase (A, D), glutathione peroxidase (B, E), and superoxide dismutase (C, F) in intestinal epithelial cells isolated from pigs fed oxidized fat (OF) and fresh fat (FF). Bars represent means ±SD of nine pigs per group. Symbols indicate treatment effect according to statistical analysis: *, $P < 0.05$.

Effect of oxidized fat on PPARγ mRNA expression and DNA-binding activity

DNA-binding activity of PPARγ tended to be higher in intestinal epithelial cells from pigs fed the oxidized fat diet than in those of the fresh fat diet ($P < 0.15$; Fig. 1A). Relative mRNA concentrations of PPARγ did not differ between intestinal epithelial cells from pigs fed the oxidized fat diet and the fresh fat diet ($P > 0.05$; Fig. 1B).

Effect of oxidized fat on NF-κB DNA-binding activity and mRNA expression of NF-κB target genes

DNA-binding activity of NF-κB did not differ between intestinal epithelial cells from pigs fed the oxidized fat diet and the fresh fat diet ($P > 0.05$; Fig. 2A). In addition, relative mRNA concentrations of the NF-κB target genes COX-2, iNOS, IL-6, and TNFα were not different between intestinal epithelial cells from pigs fed the oxidized fat diet and the fresh fat diet ($P > 0.05$; Fig. 2B).

Plasma and HDL cholesterol concentrations and apo A-I concentration in HDL

Plasma cholesterol concentration was significantly lower in pigs fed the oxidized fat than in those fed the fresh fat (2.55 ± 0.23 vs. 2.83 ± 0.22 mmol/L, n = 9, $P < 0.05$). HDL cholesterol concentrations did not differ between pigs fed the fresh fat diet and those fed the oxidized fat diet (1.13 ± 0.11 vs. 1.02 ± 0.18 mmol/L, n = 9). Apo A-I concentration in HDL was not different between pigs fed the fresh fat diet and those fed the oxidized fat diet (1.00 ± 0.06 vs. 1.01 ± 0.09, n = 9).

Relative mRNA concentration of apo A-I in intestinal epithelial cells

Relative mRNA concentration of apo A-I in intestinal epithelial cells was not different between pigs fed the fresh fat diet and those fed the oxidized fat diet (1.00 ± 0.59 vs. 0.72 ± 0.38, n = 9).

Effect of oxidized fat on lipid peroxidation and antioxidant status

Concentrations of TBARS were significantly elevated in intestinal epithelial cells from pigs fed the oxidized fat diet when compared to those of the fresh fat diet ($P < 0.05$; Fig. 3A). The concentrations of α-tocopherol were significantly lower in intestinal epithelial cells from pigs fed the oxidized fat diet than in those of the fresh fat diet ($P < 0.05$; Fig. 3B). Activities of the enzymatic antioxidants catalase, GSH-Px, and SOD were significantly reduced in intestinal epithelial cells from pigs fed the oxidized fat diet relative to those of the fresh fat diet ($P < 0.05$; Fig. 4A, 4B, 4C). Relative mRNA concentrations of catalase, GSH-Px, and SOD did not differ between intestinal epithelial cells from pigs fed the oxidized fat diet and the fresh fat diet ($P > 0.05$; Fig. 4D, 4E, 4F).

Discussion

The present study aimed to explore the effect of dietary thermo-oxidized fat on PPARγ DNA-binding activity and its transrepression activity on NF-κB-mediated gene transcription in porcine intestinal epithelial cells. Intestinal epithelial cells were used for this study since these cells (i) express high levels of PPARγ [9], (ii) are presumably exposed to abundant levels of oxidized fatty acids during intestinal passage after ingestion of the thermooxidized fat, and (iii) efficiently take up oxidized fatty acids as readily as unoxidized fatty acids [19], unlike other cells such as endothelial cells, smooth muscle cells, and macrophages [45].

The results of the present study demonstrate that feeding a dietary oxidized fat to pigs caused a moderate, but not significant, increase in PPARγ DNA-binding activity in intestinal epithelial cells which indicates that dietary oxidized fats could indeed activate PPARγ in vivo. In this regard the present study complements the findings from in vitro-studies showing that components of thermo-oxidized fats activate this ligand-activated transcription factor in cultured intestinal cells [6]. Moreover, this finding extends our knowledge on the modulation of signalling pathways by dietary oxidized fat in vivo.

Given that a relatively high amount of nuclear protein, when compared to the assay controls (THP-1, COS-7), was necessary to obtain reliable absorbance values in the PPARγ transactivation assay, the PPARγ DNA-binding activity in the enterocytes was relatively low. However, we excluded the possibility that the weak absorbance values obtained from the enterocyte nuclear extracts are responsible for false-positive results. The absorbance values of the nuclear extracts from the fresh fat group were only slightly lower than those of the THP-1 negative control, suggesting that PPARγ DNA-binding was indeed low in enterocytes of the fresh fat group. In contrast, the mean absorbance value of the oxidized fat group was about 70 % higher than that of the THP-1 negative control. As displayed in Figure 1B, the PPARγ transactivity in THP-1 cells treated with the synthetic PPARγ-agonist troglitazone increased about 200 %, a degree of activation that is consistent with reports from the literature [46]. Therefore, although the standard variation of absorbance values in the oxidized fat group was quite high, we think it is justified to conclude that PPARγ was slightly activated by treatment with oxidized fat.

Since activation of PPARγ has been implicated in the attenuation of inflammatory processes in various tissues, including the intestine [11], by negatively interfering with a variety of signalling pathways such as NF-κB, we focused our studies on the role of dietary oxidized fat on NF-κB-dependent gene transcription. NF-κB plays a key role in inflammatory diseases due to its ability to bind specifically to NF-κB-response elements in the promoters of key inflammatory genes (e. g. COX-2, iNOS, TNFα, and IL-6) and induce their gene transcription [12], which also explains why inhibition of NF-κB often ameliorates the pathology of these diseases [12, 15, 17, 18]. Thus, the finding that DNA-binding activity of NF-κB as well as NF-κB target gene expression in intestinal epithelial cells were not altered by dietary oxidized fat probably indicates that the moderate PPARγ activating effect of dietary oxidized fat has no implication for proinflammatory gene transcription in intestinal epithelial cells. The lack of effect might be explained by an insufficient transrepression of NF-κB by the thermo-oxidized fat in the present study due to the moderate PPARγ activation.

To further elucidate potential PPARγ-dependent effects of the oxidized fat, we also determined enterocyte apo A-I mRNA expression and apo A-I and cholesterol concentrations in the HDL fraction, since a previous study revealed that dietary oxidized fatty acids enhance intestinal cell apo A-I production via PPARγ [7], which might have accounted for an increased HDL synthesis. However, neither increased HDL cholesterol concentrations nor increased apo A-I mRNA or protein concentrations could be observed in the present study. In contrast, recent studies of our group revealed that treatment of rats and guinea pigs with oxidized fat increased HDL cholesterol concentrations [28, 29]. However, whether the lack of effect on HDL cholesterol levels also supports the assumption that the present oxidized fat is only a weak PPARγ activator or whether this is a species-specific effect has to be clarified in future studies. In addition, it has to be considered that plasma HDL concentrations are not only determined by intestinal production of apo A-I, since the liver is also a major site of apo A-I synthesis and nascent HDL particle secretion [47].

The observation that the oxidized fat caused only a slight and non-significant activation of PPARγ could be attributed to the fact that the fat used in this study contained moderate levels of oxidized fatty acids such as 13-hydroxyoctadecadienoic acid and 13-oxooctadecadienoic acid as indicated by the relatively low concentrations of conjugated dienes analysed. This was due to the comparatively mild heat treatment of the fat. However, strong oxidized fats with markedly higher concentrations of conjugated dienes, as usually used in animal experiments [4], do not reflect fats produced during the deep-frying process. Therefore, the oxidized fat in the present study is physiologically more relevant to the situation in humans consuming fried foods. In addition, the moderate PPARγ activation by the oxidized fat could also be explained by the possibility that some of the oxidized fatty acids, which are relatively unstable, have been decomposed or degraded before being absorbed by the enterocyte.

To some extent, the lack of effect on inflammatory gene transcription might also be attributed to the fact that the basal

state of inflammation in the intestinal epithelium of the animals was rather low, which is based on the observation that the number of PCR cycles required to estimate mRNA expression of the inflammatory genes was comparatively high, and the reduction of inflammatory indices in normal healthy animals is expected to be only marginal. The relatively high amount of enterocyte nuclear protein that was necessary to obtain a reliable detection signal in the ELISA-based transactivation assay provides further indication of a relatively low inflammatory state of the intestinal cells. Therefore, future studies should investigate whether thermo-oxidized fat exerts a different effect on inflammatory gene transcription during states of acute intestinal inflammation, e. g. in animal models of experimental colitis.

Since oxidized fats were demonstrated to potently induce oxidative stress [19–21] and in view of the known connection between oxidative stress and proinflammatory gene expression [22, 23], we further investigated whether the administration of oxidized fat could provoke oxidative stress by determining the concentrations of TBARS, a marker of lipid peroxidation, and α-tocopherol, as well as the activities of antioxidant enzymes. Both enzymatic and non-enzymatic antioxidants are sensitive to the induction of oxidative stress and are largely responsible for the defense of the intestinal mucosa against luminal diet-derived oxidants, thereby preventing free radical-mediated damage to cellular components [48]. The present findings (increased lipid peroxidation and depletion of antioxidants such as α-tocopherol) clearly suggest that administration of the dietary oxidized fat induced oxidative stress and therefore impaired the antioxidant defense system of the intestinal epithelial cells. Since oxidative stress in the intestine has also been accompanied by reduced activities of enzymatic antioxidants in enterocytes [49, 50], the reduced activities of catalase, SOD, and GSH-Px as observed in the present study probably provide further evidence for this assumption. However, the slight impairment of the antioxidant defense mechanisms (about 20–25 %) when compared to others [49, 50] suggests that the oxidative stress induced by the oxidized fat was only moderate. This probably indicates that the antioxidant defense system of the intestinal epithelial cells was still sufficient to cope with the oxidative burden of the ingested lipid peroxides and the oxidative stress induced was not strong enough to induce proinflammatory gene transcription. In contrast, strong induction of oxidative stress in intestinal cells leads to a marked activation of proinflammatory transcription factors and destabilization of cell integrity [51]. One explanation for the slight impairment of the antioxidant defense system might be that extracellular detoxifying enzymes in the mucus layer of the intestine are also involved in the protection of enterocytes from direct contact with diet-derived lipid peroxides in the gut lumen [52], and, thus, limit the oxidative burden within the epithelial cell.

With respect to the clinical relevance of the findings from the present study, the oxidized fat used was obviously a mild irritant, only slightly affecting the redox status of the intestinal epithelium. Whether this impairment of the antioxidant defense system might be significant for the integrity of the intestinal epithelium (barrier function) has not been addressed in the present study but should be taken into consideration. It has been suggested that a progressive fall in enzymatic and nonenzymatic antioxidants as observed in the present study precedes the occurrence of damage to intestinal cell constituents [51]. Since the intestinal epithelial cells are primarily responsible for the antioxidant defense of the epithelium against luminal oxidants [27], it is likely that in the presence of stronger irritants, such as toxins or pathogens, the already impaired defense mechanisms of the intestinal epithelial cells are not sufficient to cope with this additional challenge nor to preserve cellular integrity and homeostasis of the intestinal epithelium. Thus, the mild irritation of the gut epithelium by oxidized fats might also be relevant for human health.

Taken together the present study revealed that feeding dietary oxidized fat, prepared under deep-frying conditions, to pigs resulted in a moderate, but not significant, PPARγ activation in intestinal epithelial cells. Moreover, the present study demonstrated that this oxidized fat caused only moderate oxidative stress and slightly impaired the antioxidant defense system of the intestinal epithelium. In conclusion, the results of this study suggest that both effects of the oxidized fat (moderate PPARγ activation and induction of oxidative stress) have no implication for NF-κB-mediated proinflammatory gene expression in porcine intestinal epithelial cells.

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Short paper

Feeding oxidized fat during pregnancy up-regulates expression of PPARα-responsive genes in the liver of rat fetuses

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Abstract

Background: Feeding oxidized fats causes activation of peroxisome proliferator-activated receptor α (PPARα) in the liver of rats. However, whether feeding oxidized fat during pregnancy also results in activation of PPARα in fetal liver is unknown. Thus, this study aimed to explore whether feeding oxidized fat during pregnancy causes a PPARα response in fetal liver. Two experiments with pregnant rats which were administered three different diets (control; oxidized fat; clofibrate as positive control) in a controlled feeding regimen during either late pregnancy (first experiment) or whole pregnancy (second experiment) were performed.

Results: In both experiments pregnant rats treated with oxidized fat or clofibrate had higher relative mRNA concentrations of the PPARα-responsive genes acyl-CoA oxidase (ACO), cytochrome P_{450} 4A1 (CYP4A1), L-type carnitin-palmitoyl transferase I (L-CPT I), medium-chain acyl-CoA dehydrogenase (MCAD), and long-chain acyl-CoA dehydrogenase (LCAD) in the liver than control rats ($P < 0.05$). In addition, in both experiments fetuses of the oxidized fat group and the clofibrate group also had markedly higher relative mRNA concentrations of ACO, CYP4A1, CPT I, MCAD, and LCAD in the liver than those of the control group ($P < 0.05$), whereas the relative mRNA concentrations of PPARα, SREBP-1c, and FAS did not differ between treatment groups. In the second experiment treatment with oxidized fat also reduced triacylglycerol concentrations in the livers of pregnant rats and fetuses ($P < 0.05$).

Conclusion: The present study demonstrates for the first time that components of oxidized fat with PPARα activating potential are able to induce a PPARα response in the liver of fetuses. Moreover, the present study shows that feeding oxidized fat during whole pregnancy, but not during late pregnancy, lowers triacylglycerol concentrations in fetal livers.

Background

In recent years the contribution of dietary oxidized fats to total energy intake has markedly increased in industrialized countries, mainly, due to the increasing consumption of fast food which contain significant amounts of these heated and processed fats. We and others have shown that

oxidized fats are strong activators of peroxisome proliferator-activated receptor α (PPARα) [1-3], a ligand-activated transcription factor that belongs to the family of nuclear receptors. PPARα is centrally involved in the regulation of lipid homeostasis in the liver and is essential for normal liver function [4]. Upon activation of PPARα by a ligand, which includes the fibrate class of hypolipidemic drugs such as clofibrate or fenofibrate, fatty acids, and eicosanoids, the transcription of genes containing a PPAR response element (PPRE) in its promoter region is induced. Typical PPARα-responsive genes in the liver, which are also up-regulated by oxidized fats and its components such as oxidized fatty acids (e.g. hydroperoxy-fatty acids, cyclic fatty acids) [1-3,5,6], include a wide array of genes that are involved in peroxisomal and mitochondrial fatty acid β-oxidation such as L-type carnitin-palmitoyl transferase I (L-CPT I), acyl-CoA oxidase (ACO), cytochrome P_{450} 4A1 (CYP4A1), medium-chain acyl-CoA dehydrogenase (MCAD), and long-chain acyl-CoA dehydrogenase (LCAD) [7,8]. PPARα activation by oxidized fat was also shown to cause liver enlargement due to peroxisome proliferation and reduce triacylglycerol concentrations in liver, plasma, and very low-density lipoproteins in rats [3,9,10]. In part, the triacylglycerol-lowering effect of oxidized fat in the rat is presumably also due to the observed reduction in mRNA concentrations and activities of lipogenic enzymes such as fatty acid synthase (FAS) [9].

During pregnancy treatment of rats with the pharmacological PPARα activator clofibrate has been shown to cause proliferation of peroxisomes [11,12], induction of peroxisomal enzyme activities [13], and induction of CYP4A1 in fetal liver [14] indicating that clofibrate is capable of activating PPARα transplacentally. In addition, pathological changes in newborn rats born to mothers treated with clofibrate during pregnancy [15], and an impaired fetal growth of fenofibrate-treated pregnant rats have been reported [16]. Moreover, in rat and mouse liver epithelial cells treatment with the PPARα agonist WY-14,643 caused up-regulation of proto-oncogenes [17-19], which has been attributed to the hepatocarcinogenic effect of peroxisome proliferators in rodents [18]. However, whether feeding oxidized fat to pregnant rats also results in activation of PPARα and up-regulation of PPARα target genes in fetal livers is unknown from the literature. Moreover, it is unknown whether components of oxidized fats such as oxidized fatty acids are able to substantially pass the placenta and enter the fetus, because it has been shown that the transplacental transport of fatty acids from the maternal diet is highly selective for individual fatty acids, e.g. long-chain polyunsaturated fatty acids such as docosahexaenoic acid and arachidonic acid are preferentially transported through the placenta at the expense of other less important fatty acids [20-22].

Therefore, the present study aimed to explore whether feeding oxidized fat during pregnancy causes a PPARα response in fetal liver as estimated by the up-regulation of typical PPARα-responsive genes such as ACO, L-CPT I, CYP4A1, MCAD, and LCAD and whether the induction of fatty acid catabolism might also affect fetal hepatic triacylglycerol concentrations. We also analyzed the mRNA abundance of the lipogenic transcription factor sterol regulatory-element binding protein (SREBP)-1c and its target gene FAS, because in previous studies administration of oxidized fats has also been demonstrated to reduce mRNA expression of lipogenic enzymes [9]. To address possible adverse effects of treatment with oxidized fat, we also determined the mRNA abundance of the proto-oncogenes c-myc, c-jun, and c-fos.

Since the duration of administration of oxidized fat during pregnancy might also influence the effect on lipid metabolism in the fetal liver, we performed two experiments which varied in the duration of administration of the oxidized fat. In the first experiment we investigated the effect of short-term administration (last 5 d of pregnancy) of oxidized fat on the PPARα response in maternal and fetal liver, whereas in the second experiment the effect of long-term administration (whole pregnancy) was studied.

Results

Fatty acid composition and concentrations of lipid peroxidation products in the experimental fats

The oxidized fat in the short-term experiment had lower proportions of polyunsaturated fatty acids (C18:2) but higher proportions of saturated (C16:0, C18:0) and monounsaturated (C18:1) fatty acids compared to the control fat due to the heat treatment of sunflower oil during preparation of the oxidized fat (Table 1). In the long-term experiment the proportions of C18:2 and C18:1 in the oxidized fat were similar to those in the control fat due to adjustment of fatty acid composition of the control fat, whereas the proportions of saturated fatty acids were lower in the oxidized fat compared to the control fat (Table 2). In both experiments the oxidized fat had higher concentrations of peroxides, TBARS, and conjugated dienes, and a higher acid value than the fat of the control group. The concentrations of lipid peroxidation products (peroxide value and conjugated dienes) in the control fat were higher in the long-term experiment than in the short-term experiment. This is probably due to the fact that fats in the long-term experiment were included into a semi-synthetic diet which contained pro-oxidant minerals such as iron or copper whereas fats in the short-term experiment were administered the rats directly by gastric tube.

Table 1: Characteristics of the experimental fats of the short-term experiment

Treatment group	Control	Clofibrate	Oxidized fat[1]
Fat	SFO[2]		oxidized SFO[2]
Major fatty acids, g/100 g total fatty acids			
C16:0	6.1		9.1
C18:0	3.4		5.3
C18:1 (n-9)	32.6		38.3
C18:2 (n-6)	56.6		44.7
Peroxidation products			
Peroxide value, mEq O_2/kg	3.0		379
Acid value, g KOH/kg	0.4		5.8
Conjugated dienes, mmol/kg	< 0.1		274
TBARS[2], mmol/kg	1.1		13.1

[1]Prepared by heating at a temperature of 60°C for 25 d.
[2]Abbreviations: SFO, sunflower oil; TBARS, thiobarbituric acid-reactive substances.

Final body weights and relative liver weights of pregnant rats

Body weight development was not affected by dietary treatment in both experiments due to the controlled feeding regimen applied. In the short-term experiment, final body weights of pregnant rats at d 21 of pregnancy did not differ between the three treatment groups (control, 300 ± 28 g; oxidized fat, 299 ± 24 g; clofibrate, 307 ± 17 g, mean ± SD). Pregnant rats treated with clofibrate or oxidized fat had higher relative liver weights than control rats (P < 0.05; control, 3.6 ± 0.2 g/100 g body weight; oxidized fat, 4.4 ± 0.3 g/100 g body weight; clofibrate, 4.9 ± 0.2 g/100 g body weight).

In the long-term experiment, final body weights of pregnant rats at d 21 of pregnancy were also not different between treatment groups (control, 342 ± 35 g; oxidized fat, 331 ± 40 g; clofibrate, 328 ± 18 g). Relative liver weights of pregnant rats treated with clofibrate or oxidized fat were also higher than those of control rats (P < 0.05; control, 3.4 ± 0.2 g/100 g body weight; oxidized fat, 4.4 ± 0.5 g/100 g body weight; clofibrate, 4.5 ± 0.3 g/100 g body weight).

Relative mRNA concentrations of PPARα and PPARα-responsive genes in livers of pregnant rats and fetuses

In the short-term experiment, relative mRNA concentrations of PPARα in the liver of pregnant rats and fetuses were not different between treatment groups (pregnant rats: 1.00 ± 0.49, control group; 1.02 ± 0.48, oxidized fat group; 1.26 ± 0.22, clofibrate group; P = 0.439; fetuses: 1.00 ± 0.29, control group; 1.29 ± 0.50, oxidized fat group; 1.15 ± 0.74, clofibrate group; P = 0.653). Pregnant rats treated with oxidized fat had 2.4-, 3.0-, 2.5- and 2.1-fold higher relative mRNA concentrations of ACO, CYP4A1, MCAD, and LCAD, respectively, in the liver than control rats (P < 0.05; Fig. 1). The relative mRNA concentration of L-CPT I did not differ between both groups of rats. Treatment of pregnant rats with clofibrate resulted in 4.8-, 11-, 1.6-, 2.5- and 2.4-fold higher relative mRNA concentrations of ACO, CYP4A1, L-CPT I, MCAD, and LCAD, respectively, in the liver compared to control treatment (P < 0.05). Fetuses of the oxidized fat group had 6.3-

Table 2: Characteristics of the experimental fats of the long-term experiment

Treatment group	Control	Clofibrate	Oxidized fat
Fat	SFO[2] : lard (54:46)		oxidized SFO[2]
Major fatty acids, g/100 g total fatty acids			
C16:0	14.9		10.2
C18:0	9.5		6.2
C18:1 (n-9)	28.9		34.4
C18:2 (n-6)	42.0		44.2
Peroxidation products			
Peroxide value, mEq O_2/kg	6.6		230
Conjugated dienes, mmol/kg	12.3		139
TBARS[2], mmol/kg	1.0		19

[1]Prepared by heating at a temperature of 60°C for 25 d.
[2]Abbreviations: SFO, sunflower oil; TBARS, thiobarbituric acid-reactive substances.

, 9.0-, 6.4-, 1.5- and 2.1-fold higher relative mRNA concentrations of ACO, CYP4A1, L-CPT I, MCAD, and LCAD respectively, in the liver than those of the control group ($P < 0.05$), whereas fetuses of the clofibrate group had 20-, 51-, 12-, 2.8- and 3.0-fold higher relative mRNA concentration of ACO, CYP4A1, L-CPT I, MCAD, and LCAD, respectively in the liver than those of the control group ($P < 0.05$).

In the long-term experiment, relative mRNA concentrations of PPARα in the liver of pregnant rats and fetuses also did not differ between treatment groups (pregnant rats: 1.00 ± 0.49, control group; 1.56 ± 0.99, oxidized fat group; 0.63 ± 0.29, clofibrate group; $P = 0.053$; fetuses: 1.00 ± 0.70, control group; 0.72 ± 0.20, oxidized fat group; 0.75 ± 0.31, clofibrate group; $P = 0.201$). Treatment of pregnant rats with oxidized fat resulted in 4.0-, 4.4-, 2.5-, 2.8- and 2.0-fold higher relative mRNA concentrations of ACO, CYP4A1, L-CPT I, MCAD, and LCAD, respectively, in the liver compared to control treatment ($P < 0.05$; Fig. 1). Pregnant rats treated with clofibrate had 4.8-, 8.3-, 2.0- and 1.9-fold higher relative mRNA concentrations of ACO, CYP4A1, MCAD, and LCAD, respectively, in the liver than control rats ($P < 0.05$), whereas the relative mRNA concentration of L-CPT I did not differ between both treatment groups. Fetuses of the oxidized fat group had 2.2-, 6.0-, 3.2- and 2.0-fold higher relative mRNA concentrations of ACO, CYP4A1, MCAD, and LCAD, respectively, in the liver than those of the control group ($P < 0.05$), whereas the relative mRNA concentration of L-CPT I did not differ between both treatment groups. Fetuses of the clofibrate group had 3.5-, 28-, 18- and 3.9-fold higher relative mRNA concentration of ACO, CYP4A1, L-CPT I, and LCAD, respectively, in the liver than those of the control group ($P < 0.05$), whereas the relative mRNA concentration of MCAD did not differ between both treatment groups.

Relative mRNA concentrations of SREBP-1c and FAS in livers of pregnant rats and fetuses

In the short-term experiment, treatment of pregnant rats with oxidized fat resulted in lower relative mRNA concentrations of FAS in the liver relative to control treatment ($P < 0.05$), whereas the relative mRNA concentration of SREBP-1c did not differ between these two groups (Fig. 2). Relative mRNA concentrations of SREBP-1c and FAS in the liver of pregnant rats treated with clofibrate did not differ from those of control rats. However, pregnant rats treated with clofibrate had higher relative mRNA concentrations of SREBP-1c in the liver than those fed oxidized fat ($P < 0.05$). In fetal livers relative mRNA concentrations of SREBP-1c and FAS did not differ between treatment groups.

In the long-term experiment, relative mRNA concentrations of SREBP-1c and FAS in the liver of pregnant rats and fetuses did not differ between the three treatment groups (Fig. 2).

Concentrations of triacylglycerols in livers of pregnant rats and fetuses

In the short-term experiment, concentrations of triacylglycerols in the liver of pregnant rats did not differ between the three treatment groups (Fig. 3). Fetuses of the clofibrate group had lower concentrations of triacylglycerols in the liver than those of the control and the oxidized fat group ($P < 0.05$); oxidized fat had no effect on the triaclyglycerol concentration in fetal livers compared to control.

In the long-term experiment, treatment of pregnant rats with oxidized fat or clofibrate resulted in lower concentrations of triacylglycerols in the liver compared to control treatment ($P < 0.05$). Fetuses of the oxidized fat group and the clofibrate group also had lower concentrations of triacylglycerols in the liver than those of the control group ($P < 0.05$).

Relative mRNA concentrations of c-myc, c-jun, and c-fos in livers of pregnant rats and fetuses

In the short-term experiment, relative mRNA concentrations of c-myc, c-jun, and c-fos in the liver of pregnant rats and fetuses did not differ between the three treatment groups (Fig. 4).

In the long-term experiment, treatment of pregnant rats with oxidized fat resulted in higher relative mRNA concentrations of c-myc, c-jun, and c-fos relative to control treatment ($P < 0.05$). In fetal livers relative mRNA concentrations of c-myc, c-jun, and c-fos did not differ between treatment groups.

Discussion

The present study demonstrates for the first time that components of oxidized fat with PPARα activating potential are able to induce a PPARα response in the liver of fetuses. Moreover, the present study shows that feeding oxidized fat during whole pregnancy, but not during late pregnancy, lowers triacylglycerol concentrations in fetal livers. Hydroxy- and hydroperoxy-fatty acids such as hydroxyoctadecadienoic acid (HODE) and hydroperoxyoctadecadienoic acid (HPODE) occurring in oxidized fats are very potent PPARα agonists [6,23,24]. These oxidized fatty acids are produced during the early stage of lipid peroxidation, and, due to their low thermodynamic stability, easily decompose at high temperatures. Thus, fats treated at low temperatures have markedly higher concentrations of these primary lipid peroxidation products than fats treated at high temperature [3]. Therefore, in order to provoke a significant PPARα activating effect of the oxidized

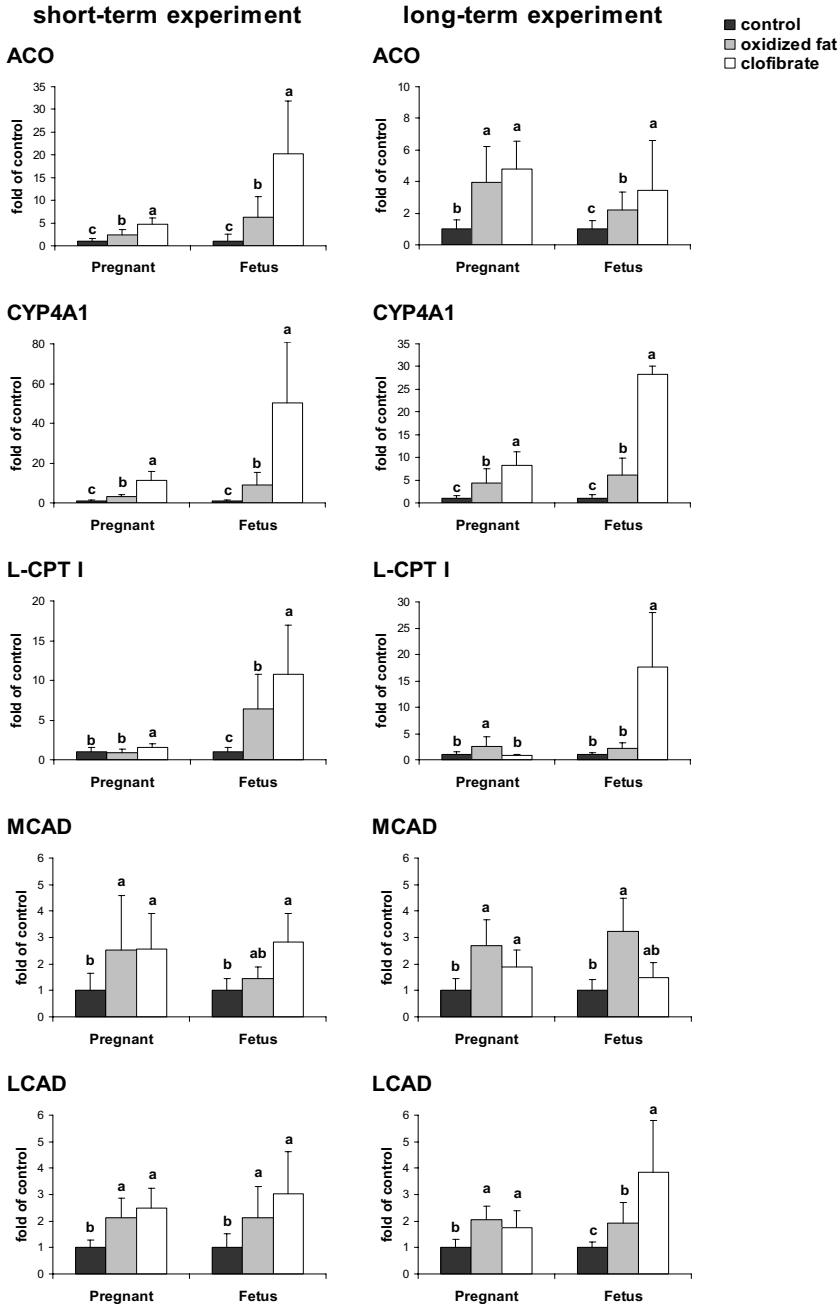

Figure 1

Effects of treatment on relative mRNA concentrations of PPARα-responsive genes in livers of pregnant rats and fetuses. Effect of short-term (d 16 – d 21 of pregnancy, left) and long-term (d 1 – d 21 of pregnancy, right) administration of three different diets (control, oxidized fat, clofibrate) during pregnancy on relative mRNA concentrations of ACO, CYP4A1, CPT I, MCAD, and LCAD in the liver of pregnant rats and fetuses at d 21 of pregnancy. Left, Bars represent mean ± SD (n = 9/group). Right, Bars represent mean ± SD (n = 12/group). Bars marked without a common superscript letter differ ($P < 0.05$). Results from one-way ANOVA (*P*-values): short-term experiment: ACO, $P = 0.0001$ (pregnant), $P = 0.001$ (fetus); CYP4A1, $P = 0.0001$ (pregnant), $P = 0.002$ (fetus); L-CPT I, $P = 0.013$ (pregnant), $P = 0.012$ (fetus); MCAD, $P = 0.042$ (pregnant), $P = 0.001$ (fetus); LCAD, $P = 0.001$ (pregnant), $P = 0.03$ (fetus); long-term experiment: ACO, $P = 0.0001$ (pregnant), $P = 0.015$ (fetus); CYP4A1, $P = 0.0001$ (pregnant), $P = 0.001$ (fetus); L-CPT I, $P = 0.016$ (pregnant), $P = 0.011$ (fetus); MCAD, $P = 0.001$ (pregnant), $P = 0.019$ (fetus); LCAD, $P = 0.001$ (pregnant), $P = 0.001$ (fetus).

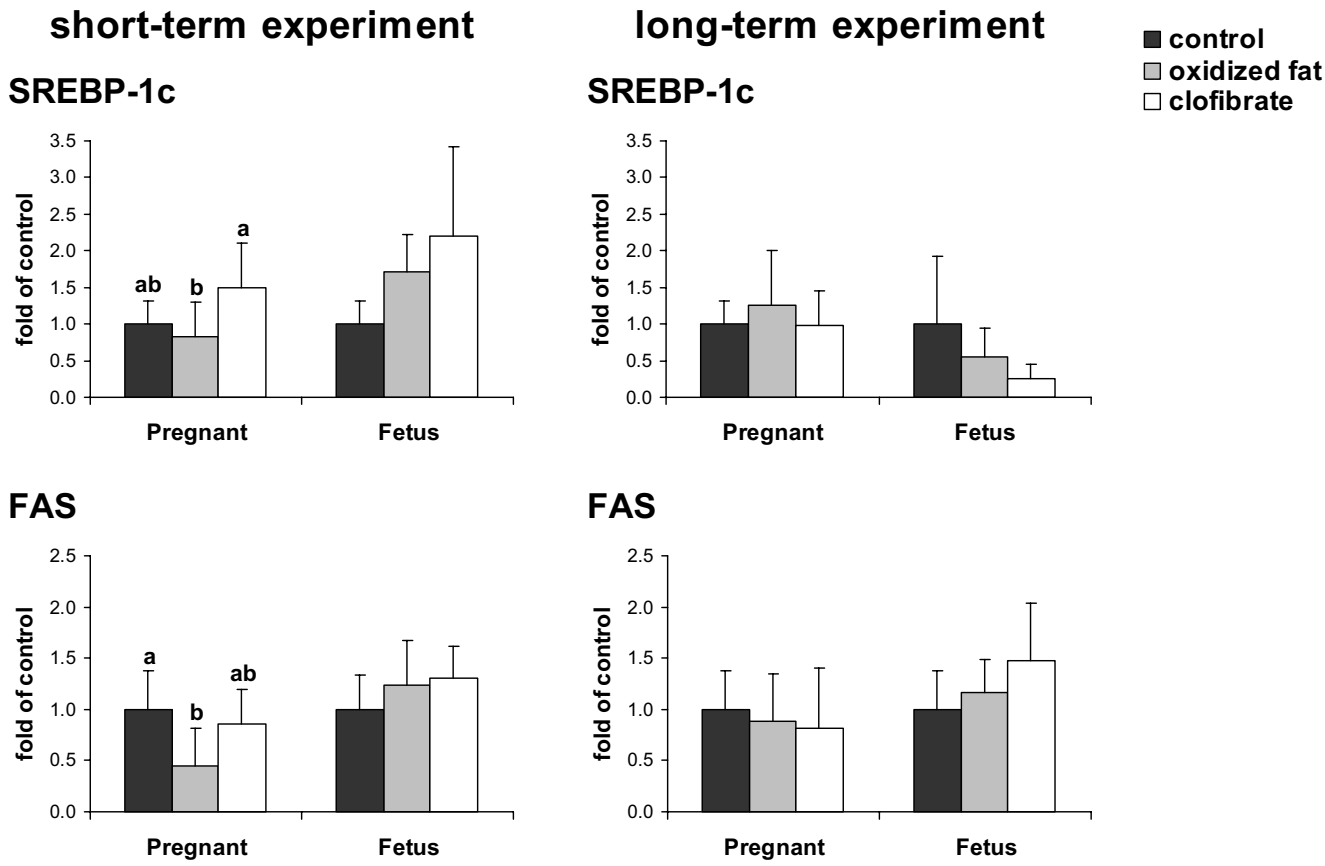

Figure 2

Effects of treatment on relative mRNA concentrations of SREBP-1c and FAS in livers of pregnant rats and fetuses. Effect of short-term (d 16 – d 21 of pregnancy, left) and long-term (d 1 – d 21 of pregnancy, right) administration of three different diets (control, oxidized fat, clofibrate) during pregnancy on relative mRNA concentrations of SREBP-1c and FAS in the liver of pregnant rats and fetuses at d 21 of pregnancy. Left, Bars represent mean ± SD (n = 9/group). Right, Bars represent mean ± SD (n = 12/group). Bars marked without a common superscript letter differ ($P < 0.05$). Results from one-way ANOVA (*P*-values): short-term experiment: SREBP-1c, $P = 0.041$ (pregnant), $P = 0.058$ (fetus); FAS, $P = 0.026$ (pregnant), $P = 0.425$ (fetus); long-term experiment: SREBP-1c, $P = 0.526$ (pregnant), $P = 0.189$ (fetus); FAS, $P = 0.716$ (pregnant), $P = 0.334$ (fetus).

fat, we decided to use a fat treated at a relatively low temperature for a long period. Although we did not determine the concentrations of oxidized fatty acids such as 13-HODE or 13-HPODE in the oxidized fat, the high peroxide value and the high concentration of conjugated dienes indicate that the oxidized fats used in both experiments presumably had high concentrations of hydroxy- and hydroperoxy-fatty acids which may be particularly responsible for the PPARα activating effect of oxidized fats. Nonetheless, other components of heated fats such as cyclic fatty acid monomers which also show a strong PPARα response [5] might be also causative for PPARα activation.

The lower concentrations of primary lipid peroxidation products (peroxides, conjugated dienes) in the oxidized fat used in the long-term experiment compared to that used in the short-term experiment are probably explained by the fact that in the long-term experiment the lipid peroxidation products in the oxidized fat were determined after inclusion into the diet. Primary lipid peroxidation products easily decompose and are partially degraded in the presence of other diet components such as metal ions (e.g. iron, copper) acting as catalysts.

Since unspecific effects might have been caused by a different fatty acid composition of the experimental fats (the heating process caused a loss of polyunsaturated fatty acids), we aimed at equalizing in particular the concentration of the polyunsaturated fatty acid C18:2 (n-6) in the fresh fat and the oxidized fat in the second experiment which lasted during the whole pregnancy. Although the

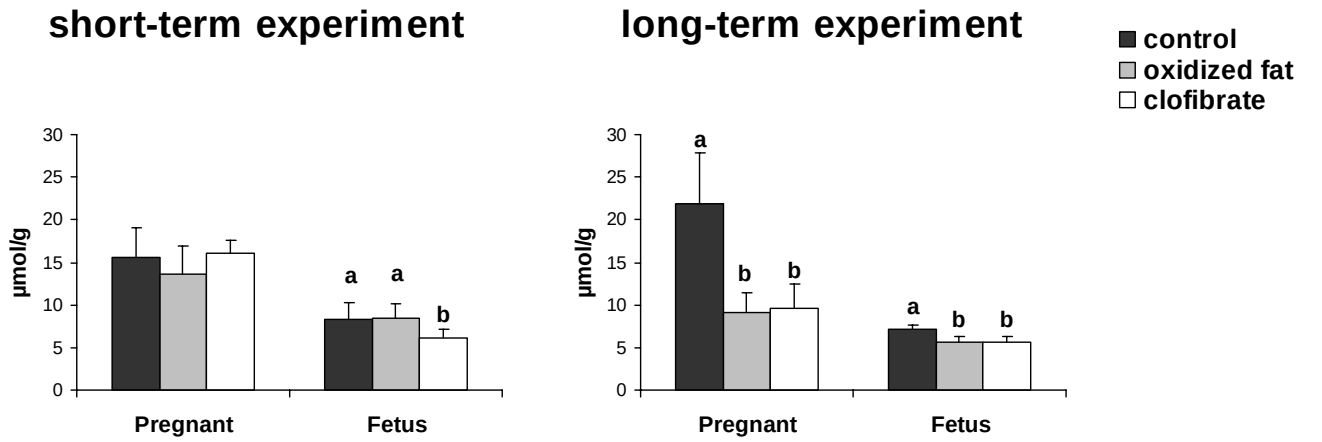

Figure 3

Effects of treatment on triacylglycerol concentrations in livers of pregnant rats and fetuses. Effect of short-term (d 16 – d 21 of pregnancy, left) and long-term (d 1 – d 21 of pregnancy, right) administration of three different diets (control, oxidized fat, clofibrate) during pregnancy on triacylglycerol concentrations in the liver of pregnant rats and fetuses at d 21 of pregnancy. Left, Bars represent mean ± SD (n = 9/group). Right, Bars represent mean ± SD (n = 12/group). Bars marked without a common superscript letter differ ($P < 0.05$). Results from one-way ANOVA (*P*-values): short-term experiment: $P = 0.239$ (pregnant), $P = 0.025$ (fetus); long-term experiment: $P = 0.0001$ (pregnant), $P = 0.045$ (fetus).

concentrations of the long-chain saturated fatty acids C16:0 and C18:0 were consequently decreased in the oxidized fat compared to the fresh fat, we think that these differences are not responsible for the differences in the PPARα response observed between the experimental groups, because saturated long chain-fatty acids were shown to bind and activate PPARα only very weakly compared to polyunsaturated fatty acids such as C18:2 (n-6) [25]. Therefore, we assume that the PPARα response to an oxidized fat might depend on the balance between unoxidized fatty acids with low PPARα transactivation activity and oxidized fatty acids with high PPARα transactivation activity because both types of fatty acids compete for the PPARα-ligand binding site.

A further consequence of the use of lard in the control fat was that the cholesterol content of the control and the clofibrate diet slightly differed from that of the oxidized fat diet. However, based on an average cholesterol concentration of about 80 mg per 100 g lard the control and the clofibrate diet contained less than 0.004% cholesterol. Therefore, we assume that the slight difference in the cholesterol concentration between the experimental diets is negligible, especially since no relation between PPARα activation and dietary cholesterol is known from the literature.

The present study clearly shows that feeding oxidized fat during pregnancy, similar to clofibrate, which was used as a positive control, causes not only a PPARα response in the liver of pregnant animals as shown by liver enlargement and up-regulation of PPARα-responsive genes but also in the liver of the fetuses. Although the induction of the PPARα response by oxidized fat in the fetal liver was not as pronounced as observed with clofibrate, the oxidized fat also caused a strong up-regulation of PPARα-target genes of up to 9-fold in the livers of fetuses which was even more pronounced than the effect of oxidized fat in the livers of pregnant rats. Therefore, these findings suggest that not only pharmacological PPARα activators but also components of oxidized fat are able to sufficiently pass the placenta and activate PPARα in the fetal liver. This finding is novel since the placental transfer of these components of oxidized fat with PPARα activating potential from the maternal diet to the fetus is unknown. Indeed, the transplacental transport of fatty acids is highly selective for individual fatty acids [20-22], but no data are available from the literature with respect to the placental passage of oxidized fatty acids which are presumably decisive for the PPARα activating effect of oxidized fat. Thus, the present results indicate that these critical components of oxidized fats are also sufficiently transported across the placenta. Moreover, the observation from the short-term experiment that up-regulation of mRNA expression of ACO, CYP4A1 and L-CPT I by oxidized fat in the fetal liver was even more pronounced than in the liver of pregnant rats indicates that components of oxidized fat responsible for PPARα activation are presumably transported through the placenta with high preference.

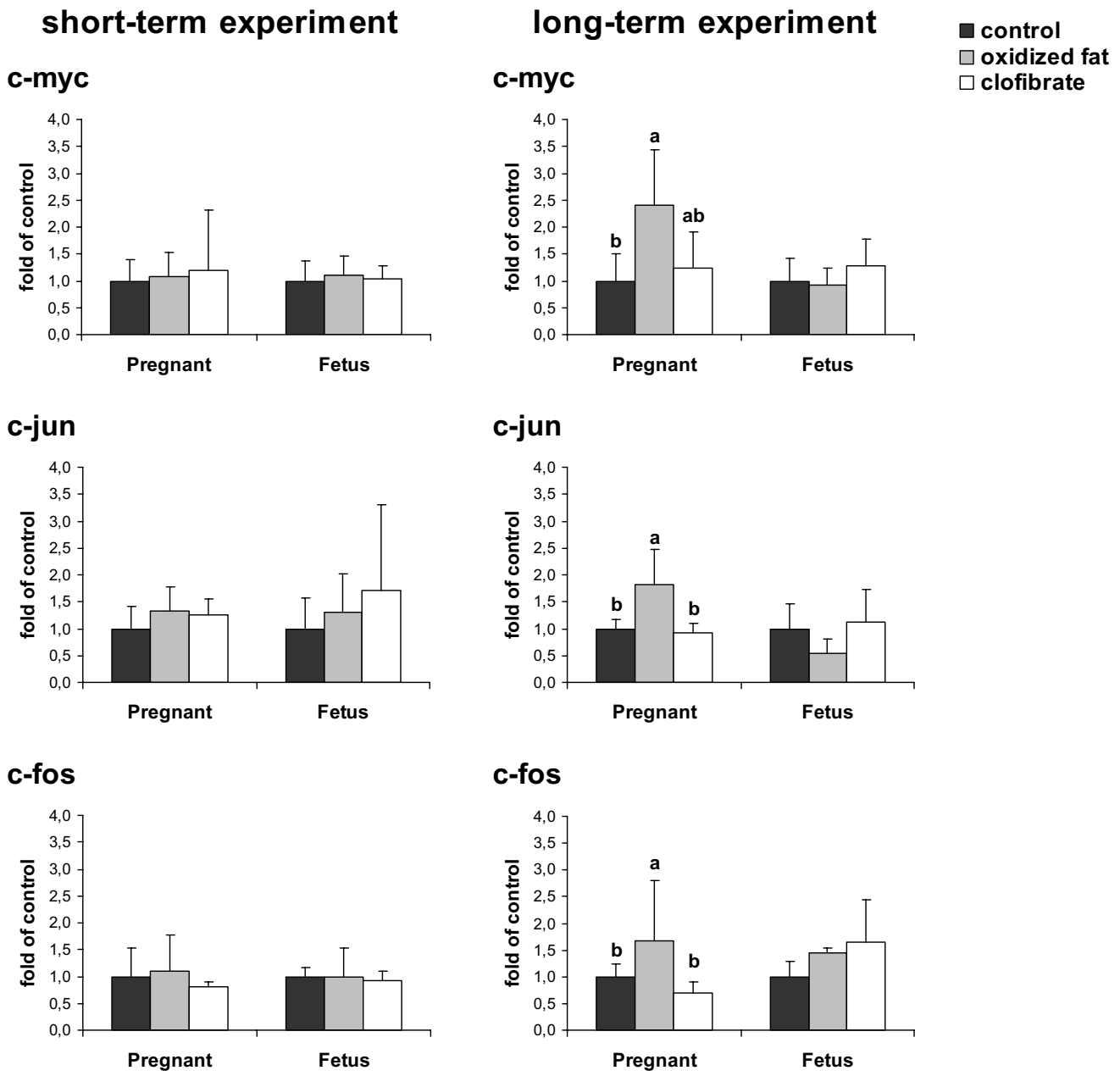

Figure 4

Effects of treatment on relative mRNA concentrations of proto-oncogenes in livers of pregnant rats and fetuses. Effect of short-term (d 16 – d 21 of pregnancy, left) and long-term (d 1 – d 21 of pregnancy, right) administration of three different diets (control, oxidized fat, clofibrate) during pregnancy on relative mRNA concentrations of c-myc, c-jun, and c-fos in the liver of pregnant rats and fetuses at d 21 of pregnancy. Left, Bars represent mean ± SD (n = 9/group). Right, Bars represent mean ± SD (n = 12/group). Bars marked without a common superscript letter differ ($P < 0.05$). Results from one-way ANOVA (*P*-values): short-term experiment: c-myc, $P = 0.880$ (pregnant), $P = 0.807$ (fetus); c-jun, $P = 0.362$ (pregnant), $P = 0.552$ (fetus); c-fos, $P = 0.669$ (pregnant), $P = 0.966$ (fetus); long-term experiment: c-myc, $P = 0.001$ (pregnant), $P = 0.420$ (fetus); c-jun, $P = 0.0001$ (pregnant), $P = 0.086$ (fetus); c-fos, $P = 0.048$ (pregnant), $P = 0.323$ (fetus).

Since activation of hepatic PPARα by clofibrate or oxidized fat has been demonstrated to enhance the fatty acid oxidation capacity in the liver and to lower hepatic triacylglycerol concentrations in non-pregnant rats [1,3,9], we also studied the effect of oxidized fat on the concentrations of triacylglycerols in the fetal liver. Indeed, we could demonstrate that feeding the oxidized fat during pregnancy also reduced the concentrations of triacylglycerols in the fetal liver indicating that the transplacental induction of PPARα responsive genes enhanced the peroxisomal and mitochondrial fatty acid oxidation capacity of the fetal liver. However, this effect has only been observed when the oxidized fat was fed during the whole pregnancy, but not when the oxidized fat was fed for the last 5 d of pregnancy only, although the PPARα responsive genes were markedly up-regulated in fetal livers of pregnant rats treated during either whole or late pregnancy. This suggests that short-term administration of oxidized fat to pregnant rats causes significant alterations on the gene expression level, which have no implications on the phenotypic level, e.g. triaclyglycerol concentrations. In contrast, short-term administration of clofibrate during pregnancy even revealed alterations on the phenotypic level as evidenced by reduced triacylglycerol concentrations in fetal livers. The latter might be attributed to the fact that clofibrate caused a more pronounced activation of PPARα in fetal livers due to a higher affinity for PPARα compared to oxidized fat leading to a marked induction of fatty acid oxidation and a significant lowering of triacylglycerol concentrations in fetal livers even after short-term exposure to clofibrate.

Interestingly, no effect of oxidized fat and even clofibrate on hepatic triacylglycerol concentrations following short-term treatment could be observed in pregnant rats, although clofibrate reduced triacylglycerol concentrations in fetal livers following short-term treatment. The failure of clofibrate or oxidized fat to reduce hepatic triacylglycerol concentrations in pregnant rats in the short-term experiment might be attributed to the fact that treatment was only performed during late pregnancy when significant alterations in lipid metabolism [e.g. hypertriacylglycerolemia as a consequence of enhanced adipose tissue lipolytic activity, enhanced liver production of VLDL particles, and decreased extrahepatic lipoprotein lipase activity [26-29]] occur in the pregnant animal. The enhanced arrival of free fatty acids, which also serve as ligands for PPARα, in the liver during late pregnancy could decrease the availability for the stronger PPARα activators clofibrate or components of oxidized fat with PPARα activating potential to their PPARα-ligand binding site. This in turn could reduce the capability of fibrates or oxidized fat to activate PPARα and consequently, its metabolic effects. This might be important since a substantial body of evidence suggests that not all alterations in gene transcription induced by pharmacological PPARα activators are also induced by fatty acids [30], e.g. some genes that contain a PPRE do not respond to fatty acids but to high-affinity PPARα activators [31-33]. Thus, administration of oxidized fat or clofibrate during late pregnancy might provoke differential effects on lipid metabolism than in virgin or early pregnant rats. The observation that treatment of rats with the triacylglycerol-lowering PPARα agonist fenofibrate during late pregnancy even increased triacylglycerol concentrations in plasma, whereas in virgin rats treatment with fenofibrate caused a reduction in plasma triacylglycerol concentrations [16], is probably supportive of this assumption.

Whether the effect observed with oxidized fat or clofibrate might have been also influenced by the metabolic state, e.g. fasting vs. non-fasting, cannot be answered with certainty. In the present study we decided to perform gene expression analysis of PPARα-responsive genes in the liver in the non-fasting state, because lipolysis of stored triacylglycerols in adipose tissue is strongly activated during fasting, resulting in a marked increase in plasma free fatty acid levels. These free fatty acids act as endogenous PPARα ligands and might have competed with the exogenous ligands (e.g. clofibrate, oxidized fatty acids or cyclic fatty acid monomers) for the PPARα-ligand binding site. Therefore, an altered ratio between endogenous free fatty acids and exogenous oxidized fatty acids as a consequence of the fasting state might have provoked a different PPARα response in the liver of pregnant rats and fetuses than observed in the non-fasting state. However, further studies are required to definitely resolve this question.

Only slight effects have been observed in the present study with respect to the lipogenic transcription factor SREBP-1c and its target gene FAS. Namely, oxidized fat caused a slight reduction in the mRNA abundance of FAS in the liver of pregnant rats, which is consistent with recent findings in non-pregnant rats [9]. However, no effect of oxidized fat has been observed on gene expression of lipogenic enzymes in fetal livers indicating that the reduced hepatic triacylglycerol concentrations in fetuses from pregnant rats treated with oxidized fat are probably largely due to an enhanced fatty acid oxidation capacity due to transplacental activation of PPARα. In part, the failure of oxidized fat on FAS gene expression in fetal livers might be explained by the fact that lipogenesis in fetal livers is generally very low and only increased during late pregnancy [34], and, therefore, probably does not respond to variations in the maternal diet.

Since pathological changes in newborn rats born to mothers treated with clofibrate during pregnancy have been reported [15], treatment with fenofibrate has been shown to impair fetal growth [16], and a link between induced

CYP4A1 expression, peroxisome proliferation, and carcinogenesis in rat livers has been described [35], we also addressed possible adverse effects of treatment with oxidized fat on fetal livers. With respect to the hepatocarcinogenic effect of peroxisome proliferators in rodents it has been suggested that enhanced DNA synthesis as a consequence of up-regulation of proto-oncogenes including c-fos, c-jun, and c-myc might be mechanistically involved [17-19]. In the present study an up-regulation of proto-oncogenes in the livers of pregnant rats has been observed in the long-term experiment, but not in the short-term experiment, indicating that short-term administration of oxidized fat has no impact on mRNA expression of proto-oncogenes. However, in fetal livers mRNA expression of proto-oncogenes was not affected by oxidized fat regardless of the duration of oxidized fat administration suggesting that the oxidized fat is uncritical with respect to hepatocarcinogenesis. Unexpectedly, treatment with clofibrate had no effect on proto-oncogene expression in pregnant rats and fetuses either in the short-term and the long-term experiment, although it has been reported that the high-affinity PPARα-ligand WY-14,643 strongly up-regulated various proto-oncogenes in rat and mouse liver epithelial cells [18,19]. This differential action of WY-14,643 and clofibrate on proto-oncogene mRNA expression cannot be explained at the moment and, therefore, requires further research activities.

Although oxidized fat had no effect on proto-oncogene expression in fetal livers in the present study, oxidized fats might be considered critically in view of inducing oxidative stress in different tissues as shown in recent studies [36,37]. In addition, specific components of oxidized fats such as cyclic fatty acid monomers, which are formed in substantial amounts during domestic frying of frozen foods in sunflower oil [38], are probably toxic, e.g. earlier studies reported that mice receiving cyclic fatty acid monomers as well as rat pups from mothers fed cyclic fatty acid monomers had a higher death rate [39-41].

Conclusion

In conclusion, the present study demonstrates for the first time that components of oxidized fat with PPARα activating potential contained in the maternal diet are able to induce a PPARα response in the liver of fetuses as evidenced by an up-regulation of PPARα target genes. In addition, the present study shows that feeding oxidized fat during whole pregnancy, but not during late pregnancy, lowers triacylglycerol concentrations in fetal livers, probably as a consequence of an enhanced peroxisomal and mitochondrial β-oxidation capacity. Although administration of oxidized fat during pregnancy had not impact on fetal proto-oncogene mRNA expression either after short-term or long-term administration, the observed pronounced transplacental PPARα activation by oxidized fat might be considered critically because of other recently reported adverse effects of treatment with PPARα activators during pregnancy [15,16]. Therefore, further research should be encouraged with respect to possible detrimental effects of oxidized fat on fetal development.

Methods

Animals

Two experiments were carried out with female Sprague-Dawley rats obtained from Charles River (Sulzfeld, Germany). At 11 wk of age, the rats were mated by housing one male rat with two female rats. D 1 of pregnancy was assigned upon observation of sperm in the vaginal smears, at which time rats were randomly assigned to the treatment groups. The short-term experiment was performed from d 16 to d 21 of pregnancy and included 27 pregnant rats with an initial body weight (d 16 of pregnancy) of 297 ± 26 (Mean ± SD) g, which were allotted to three groups of nine rats each. The long-term experiment was performed from d 1 to d 21 of pregnancy and included 36 rats with an initial body weight (d 1 of pregnancy) of 238 ± 27 g, which were allotted to three groups of twelve rats each. Pregnant rats were kept individually in Macrolon cages in a room maintained with controlled temperature (23 ± 1°C), humidity (50–60%), and lighting (0600 to 1800 h). All experimental procedures described followed established guidelines for the care and handling of laboratory animals [42] and were approved by the council of Saxony-Anhalt.

Diets

Short-term experiment

In the short-term experiment, rats received 2 mL of different experimental fats by gavage daily at 0800 h, and, additionally, fed a commercial standard rodent diet (Altromin, Lage, Germany). To standardize food intake, the diets were fed daily in controlled amounts of 16 g per d. The first group (control group) received sunflower oil, the second group (oxidized fat group) oxidized fat (see "preparation of the oxidized fat"), and the third group (clofibrate group) sunflower oil containing 75 mg clofibrate (Fluka, Buchs, Switzerland) equivalent to 250 mg clofibrate per kg body weight. The experimental fats were given for 5 d from d 16 of pregnancy to d 21 of pregnancy. The standard diet was completely consumed by all the rats. Thus, all the rats within this experiment consumed identical amounts of the food.

Long-term experiment

In the long-term experiment, semipurified diets, composed according to the recommendations of ASNS for rats during reproduction [43], were used. The diet consisted of (g/kg diet): casein, 200; cornstarch, 390; saccharose, 198; cellulose, 50; fat, 100; mineral mixture, 40; vitamin mix-

ture, 20; DL-methionine, 2. The type of fat was varied according to a one-factorial design. The first group (control group) received a mixture of sunflower oil and lard (54:46, w/w) was used. This ratio was chosen to equalize the fatty acid composition of the fresh fat with that of the oxidized fat, since the heating process caused a loss of polyunsaturated fatty acids, therefore, excluding that the treatment effects were caused by a different fatty acid composition of the experimental fats. The second group (oxidized fat group) received oxidized fat (see "preparation of the oxidized fat"). The third group (clofibrate group) received the same fat as in the control group, and clofibrate was added to the diet at a concentration of 5 g/kg. The vitamin E concentration of the diets was 50 mg α-tocopherol equivalents per kg diet. To adjust the vitamin E concentration of the diets, the native concentrations of tocopherols of the fats were analyzed. Based on the native concentrations of the fats, diets were supplemented individually with all-rac-α-tocopheryl acetate (the biopotency of all-rac-α-tocopheryl acetate is considered to be 67% of that of α-tocopherol). Diets were prepared by mixing the dry components with the fat and water and subsequent freeze drying. The residual water content of the diet was below 5 g/100 g of diet. In preliminary experiments rats fed diets with clofibrate or oxidized fat ad libitum consumed their diets over a longer period than rats fed control diets, which consequently shortened the fasting period and which itself has a pronounced effect on PPARα-response. Therefore, we decided to administer food daily at 0800 h in controlled amounts to standardize intake and to ensure that rats from all treatment groups had a comparable fasting period. The amount of food administered was 20% less than the amounts of identical diets with fresh fats consumed ad libitum by rats in preliminary studies. The amount of food offered daily was increased continuously during the experiment from 14 g to 17 g. In this feeding system, the food offered was completely consumed by all the rats. Thus, all the rats within this experiment consumed identical amounts of food. The experimental diets were fed from d 1 of pregnancy to d 21 of pregnancy.

In both experiments, water was available *ad libitum* from nipple drinkers during the whole experiment.

Preparation of the oxidized fat

The oxidized fat was prepared by heating sunflower oil at a temperature of 60°C for 25 d. Sunflower oil was filled into a glass beaker and placed into a drying oven set at the intended temperature. Throughout the heating process, air was continuously bubbled through the fat at a flow rate of 650 ml/min. This treatment caused a loss of polyunsaturated fatty acids, and a complete loss of tocopherols and raised the concentrations of lipid peroxidation products in the fats. The extent of lipid peroxidation in the oxidized fat was estimated by assaying the peroxide value (POV) [44], acid value [44], concentration of thiobarbituric acid substances (TBARS) [45], and concentration of conjugated dienes [46]. To assess lipid peroxidation products in the oxidized fat after inclusion into the diet (long-term experiment), the fat was extracted from aliquots of the diets with a mixture of hexane and isopropanol (3:2, v/v) and analysed for peroxide value, concentration of conjugated dienes, and TBARS.

Sample collection

4 h after the final portion had been administered the rats were anesthetized with diethyl ether and killed by decapitation. The liver and fetuses were excised immediately, and frozen with liquid nitrogen. In addition, livers from three randomly taken fetuses per pregnant rat were excised, and frozen with liquid nitrogen. All samples were stored at -80°C pending analysis.

Lipid analysis

Lipids of maternal livers and pools of fetal livers were extracted with a mixture of hexane and isopropanol (3:2, v/v) [47]. Total cholesterol and triacylglycerol concentrations were determined using enzymatic reagent kits obtained from Merck Eurolab (Darmstadt, Germany). Prior to enzymatic measurement, lipids of the extract were dissolved in Triton X-100 as described by De Hoff et al. [48]. Fatty acid composition of experimental fats was determined by GC-FID analysis of fatty acid methyl esters (FAME) as described previously in detail [49].

RNA isolation and real-time RT-PCR

For the determination of mRNA expression levels of PPARα, CYP4A1, ACO, L-CPT I, MCAD, LCAD, SREBP-1c, FAS, c-myc, c-jun, and c-fos total RNA were isolated from maternal liver and fetal liver pools using Trizol™ reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. cDNA synthesis and relative quantification of target gene mRNA compared to the housekeeping gene GAPDH mRNA was determined by real-time detection RT-PCR as described previously [50]. Sequences of gene-specific primers obtained from Operon (Köln, Germany) were as follows (NCBI GenBank; forward, reverse): GAPDH (NM_017008; 5'-GCA TGG CCT TCC GTG TTC C-3', 5'-GGG TGG TCC AGG GTT TCT TAC TC-3'), PPARα (NM_013196; 5'-CCC TCT CTC CAG CTT CCA GCC C-3', 5'-CCA CAA GCG TCT TCT CAG CCA TG-3'), CYP4A1 (M14972; 5'-CAG AAT GGA GAA TGG GGA CAG C-3', 5'-TGA GAA GGG CAG GAA TGA GTG G-3'), ACO (J02752; 5'-CTT TCT TGC TTG CCT TCC TTC TCC-3', 5'-GCC GTT TCA CCG CCT CGT A-3'), L-CPT I (NM_031559; 5'-GGA GAC AGA CAC CAT CCA ACA TA-3', 5'-AGG TGA TGG ACT TGT CAA ACC-3'), MCAD (NM_016986; 5'-CAA

GAG AGC CTG GGA ACT TG-3', 5'-CCC CAA AGA ATT TGC TTC AA-3'), LCAD (NM_012819; 5'-AAG GAT TTA AGG GCA AGA AGC-3', 5'-GGA AGC GGA GGC GGA GTC-3'), SREBP-1c (XM_213329; 5'-GGA GCC ATG GAT TGC ACA TT-3', 5'-AGG AAG GCT TCC AGA GAG GA-3'), FAS (NM_017332; 5'-AGG TGC TAG AGG CCC TGC TA-3', 5'-GTG CAC AGA CAC CTT CCC AT-3'), c-myc (NM_012603; 5'-CTG GAG TGA GAA GGG CTT TG-3', 5'-CAG CAG CTC GAA TTT CTT CC-3'), c-jun (NM_021835; 5'-ACC AAG AAT TCC GTG ACG AC-3', 5'-CAA GGT CAT GCT CTG CTT CA-3'), and c-fos (NM_022197; 5'-CAT CGG CAG AAG GGG CAA AGT AGA G-3', 5'-TGC CGG AAA CAA GAA GTC ATC AAA G-3').

Statistical analysis

Treatment effects were analyzed using one-way ANOVA. For significant *F*-values, means were compared by Fisher's multiple range test. Differences with $P < 0.05$ were considered significant.

List of abbreviations used

ACO, acyl-CoA oxidase; CYP4A1, cytochrome P_{450} 4A1; FAS, fatty acid synthase; LCAD, long-chain acyl-CoA dehydrogenase; L-CPT I, L-type carnitin-palmitoyl transferase I; MCAD, medium-chain acyl-CoA dehydrogenase; PPARα, peroxisome proliferator-activated receptor α; PPRE, PPAR response element; SREBP-1c, sterol regulatory-element binding protein-1c.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

RR participated in the design of the study and in the interpretation of the results and prepared the manuscript.

AG and CD carried out the feeding experiments, quantification of lipid concentrations, and mRNA expression analysis.

CB participated in the design and coordination of the study, and interpretation of the results.

KE conceived of the study and its design, coordinated work, participated in the interpretation of the results, and helped to draft the manuscript.

All authors read and approved the final manuscript.

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A14 **Ringseis R**, Saal D, Muller A, Steinhart H, Eder K:

Dietary conjugated linoleic acids lower the triacylglycerol concentration in the milk of lactating rats and impair the growth and increase the mortality of their suckling pups.

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Dietary Conjugated Linoleic Acids Lower the Triacylglycerol Concentration in the Milk of Lactating Rats and Impair the Growth and Increase the Mortality of their Suckling Pups[1]

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ABSTRACT Recent studies showed that conjugated linoleic acids (CLA) lower triacylglycerol concentrations in the milk of lactating animals. This study was performed to determine the reasons for this phenomenon; we also investigated whether there is a relation between altered lipid metabolism in the liver and the reduction in milk triacylglycerols in rats fed CLA. Two groups of female rats were fed diets containing 0 [sunflower oil (SFO) group] or 14.7 g/kg diet of a CLA mixture (CLA group) at the expense of sunflower oil during growth, pregnancy, and lactation. CLA-fed rats had 49 and 80% lower mRNA concentration and activity of fatty acid synthase, respectively, a 51% lower mRNA concentration of lipoprotein lipase (LPL) in their mammary glands at d 17 of lactation, and a 46% lower milk fat content than SFO rats ($P < 0.05$). Although CLA rats had lower concentrations of triacylglycerols in the liver than SFO rats (20.8 $\pm$ 2.6 vs. 62.6 $\pm$ 27.7 μmol/g, $P < 0.05$), concentrations of triglycerides in plasma, which are the substrates of LPL, did not differ between the groups. Moreover, the number of pups per litter, litter weights, and pup weights at d 17 of lactation were 41, 35, and 22% lower, respectively, in the CLA group than in the SFO group. In conclusion, the present study suggests that dietary CLA reduces triacylglycerol concentrations in the milk via reduced de novo fatty acid synthesis in the mammary gland and an impaired uptake of fatty acids from lipoproteins into the mammary gland. This might be the reason for reduced growth rates and an increased mortality of suckling pups. J. Nutr. 134: 3327–3334, 2004.

KEY WORDS: • *conjugated linoleic acid* • *lactation* • *mammary gland* • *liver* • *rat*

Several studies showed that dietary conjugated linoleic acids (CLAs)[3] exert many biological effects in humans and animals (1–4). In lactating animals and nursing women, dietary CLA caused a reduction in milk fat concentration (2,4,5). Fatty acids used for triacylglycerol synthesis in the mammary gland derive mainly from 2 different sources, either de novo fatty acid synthesis or uptake of fatty acids from circulating lipoproteins, which are released by the action of lipoprotein lipase (LPL). Fatty acids derived from de novo fatty acid synthesis are mainly medium-chain fatty acids with 8–14 carbon atoms, whereas those released from lipoproteins by LPL reflect those of the diet and are typically long-chain fatty acids with 18–22 carbon atoms (6,7). The fatty acid composition of milk lipids can be strongly altered by dietary manipulation of de novo fatty acid synthesis in the mammary gland or the uptake of fatty acids from lipoproteins into the mammary gland. For example, feeding a high-fat diet causes an increase in long-chain fatty acids (>80 g/100 g of total fatty acids) in the milk of rats by stimulating fatty acid uptake from lipoproteins by LPL; feeding a low-fat diet increases the proportions of medium-chain fatty acids (>50 g/100 g of total fatty acids) in the milk by stimulating de novo fatty acid synthesis in the mammary gland (8,9).

Some studies demonstrated that the reduced milk fat concentration of lactating animals fed CLA diets might be due to an inhibitory effect of *trans*-10, *cis*-12 CLA on enzymes involved in de novo fatty acid synthesis (4,5). However, it is not known whether dietary CLA also influences the uptake of fatty acids from lipoproteins. Until now, only one study dealt with the effects of dietary CLA on LPL in mammary glands (4). In that study, *trans*-10, *cis*-12 CLA reduced gene expression of LPL in the mammary gland of dairy cows. This suggests that dietary CLA inhibits the uptake of fatty acids from triglyceride-rich lipoproteins into the mammary gland. Although the effects of dietary CLA on plasma lipids are contradictory (10–13), some studies showed that dietary CLA lowers the concentration of triglyceride-rich lipoproteins in plasma (10,11), which are the substrates of LPL. Therefore, a reduced milk fat concentration could also be the result of reduced concentrations of triacylglycerols in the plasma. Triglyceride-

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[3] Abbreviations used: AcCx, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; BCA, bicinchoninic acid; CL, cardiolipin; CLA, conjugated linoleic acid; EFA, essential fatty acid; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; LPL, lipoprotein lipase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PPARα, peroxisome proliferator-activated receptor α; PS, phosphatidylserine; SM, sphingomyelin; SREBP-1c, sterol regulatory element binding protein-1c.

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rich lipoproteins are formed in the liver and secreted into the blood. There is a close relation between hepatic fatty acid metabolism and the formation of VLDL in the liver. CLA was shown to lower both apolipoprotein B synthesis and triacylglycerol secretion in HepG2 cells (14,15). However, it was also shown that CLA activates hepatic peroxisome proliferator-activated receptor α (PPARα) and enhances β-oxidation of fatty acids in the liver (16–18) and thereby could reduce the concentration of triacylglycerols in plasma. The aim of our study was to study the relation between possible activation of hepatic PPARα and milk fat concentration in lactating rats fed a dietary CLA supplement.

To gain knowledge about milk fat synthesis, we determined the concentrations of triacylglycerols and fatty acids in the milk and gene expression of lipogenic enzymes and LPL in the mammary gland. The concentrations of medium-chain fatty acids (derived from de novo fatty acid synthesis) and long-chain fatty acids (derived from plasma lipoproteins) in particular were determined to yield information about the sources used for milk fat synthesis. To study the hepatic lipid metabolism, we proposed to measure lipid concentrations in the liver (triacylglycerols, cholesterol and phospholipids), hepatic gene expression of PPARα and some of its target genes (acyl-CoA oxidase, catalase), and the expression of genes encoding enzymes involved in de novo fatty acid synthesis (fatty acid synthase, acetyl-CoA carboxylase).

Milk is the only source of nutrients for suckling pups. We assume that a reduced milk fat concentration might affect growth and survival of the offspring during the suckling period. Therefore, we also examined the development and mortality of the suckling pups.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (n = 24, 4 wk old) were obtained from Charles River. They were acclimated to the facility for 2 d and given free access to a stock diet (Altromin). Thereafter, at a mean body weight of 64 ± 4 g, they were randomly assigned to 2 groups (n = 12) and fed the experimental diets. The rats were kept individually in Macrolon cages in a room maintained at a temperature of 23°C and 50–60% relative humidity with lighting from 0600 to 1800 h. At 11 wk of age, the rats were paired with adult male Sprague-Dawley rats (Charles River) for 6 d. At the day of parturition, designated as d 1 of lactation, litters were weighed and then adjusted to 10 pups/dam without differentiation of gender. All experimental procedures described followed guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

Diets and feeding. Semipurified diets, composed according to the recommendations of ASNS (19), were used for rat diets. The basal diet consisted of (g/kg diet): casein, 200; cornstarch, 400; saccharose, 268; cellulose, 30; fat, 40; mineral mixture, 40; vitamin mixture, 20; DL-methionine, 2. Two experimental diets, which differed in fat type, were used. The SFO diet contained 30 g sunflower oil (Palmin) and 10 g soybean oil (C. Thywissen)/kg diet; the CLA diet contained 30 g CLA oil (Strengthfood) and 10 g soybean oil/kg diet. The CLA oil contained 54 g CLA isomers/100 g CLA oil, which is equivalent to 1.47 g CLA/100 g diet. The fatty acid compositions of total lipids of the 2 experimental fats were similar except for the concentrations of 18:2(n-6) and CLA (**Table 1**). Total lipids in the SFO diet contained <0.1 g CLA/100 g total fatty acids, whereas total lipids in the CLA diet contained 40.9 g CLA/100 g total fatty acids. The CLA oil contained a large number of CLA isomers. Among them, *trans*-10, *cis*-12 CLA, *cis*-11, *trans*-13 CLA, *cis*-9, *trans*-11 CLA, and *trans*-8, *cis*-10 CLA were the major CLAs contributing ~65% of total CLA.

The diets were prepared weekly by mixing the dry components with oil and water and subsequent freeze-drying. The water content after freeze-drying was <5 g/100 g diet. The experimental diets were fed for a total of 13 wk. To standardize the feed intake, diets were fed daily in restricted amounts at 0800 h. The amount of food administered in the present study was 15% less than the amounts of diets with identical nutrient composition consumed ad libitum by female rats in preliminary studies. During growth and pregnancy, the rats were fed identical amounts of diets, increasing from 7 g/d to 19 g/d, except for wk 11. In wk 11, when the rats were paired with the male rats, they had free access to the diets. Throughout the period of lactation, the rats had free access to the diets as in an earlier study in our laboratory (20). Water, from nipple drinkers, was available ad libitum.

TABLE 1

Fatty acid composition of total lipids of the experimental diets

Fatty acid	SFO	CLA
	g/100 g fatty acids	
12:0	<0.1	0.1
14:0	0.1	0.2
16:0	7.6	6.1
16:1(n-7)	0.2	0.2
18:0	3.6	2.1
18:1(n-9)	26.6	28.4
18:2(n-6)	57.5	17.1
Total CLA	<0.1	40.9[1]
18:3(n-3)	1.6	1.6
20:5(n-3)	0.6	0.4
22:5(n-3)	0.2	<0.1
22:6(n-3)	<0.1	<0.1

[1] The CLA isomer distribution of the CLA oil was as follows (g/100 g total CLA): *trans*-13, *trans*-15 (0.13); *trans*-12, *trans*-14 (2.22); *trans*-11, *trans*-13 (2.75); *trans*-10, *trans*-12 (5.61); *trans*-9, *trans*-11 (5.41); *trans*-8, *trans*-10 (2.63); *trans*-7, *trans*-9 (3.12); *trans*-6, *trans*-8 (0.18); *cis*-13, *trans*-15 and *trans*-13, *cis*-15 (2.85); *cis*-12, *trans*-13 and *trans*-12, *cis*-13 (0.97); *trans*-11, *cis*-13 (0.62); *cis*-11, *trans*-13 (15.8); *trans*-10, *cis*-12 (18.5); *cis*-9, *trans*-11 (15.6); *trans*-8, *cis*-10 (14.9); *trans*-7, *cis*-9 (0.79); *cis*-11, *cis*-13 (1.99); *cis*-10, *cis*-12 (2.58); *cis*-9, *cis*-11 (2.08); *cis*-8, *cis*-9 (1.30).

Sample collection. On the day of parturition, 3 pups from each dam were randomly separated, anesthetized with diethyl ether, killed by decapitation, and frozen with liquid nitrogen; 2 pups from each dam were used for carcass analysis, and 1 pup from each dam was used for determination of hepatic lipid concentrations. Milk samples were collected on d 10 of lactation at 1000 h as previously reported (21). Milk samples were stored at −20°C pending analysis. On d 17 of lactation, the dams were anesthetized with diethyl ether and killed by decapitation. Blood from the dams was collected into heparinized polyethylene tubes (Sarstedt) and plasma was separated by centrifugation (1100 × g, 10 min) at 4°C and stored at −80°C. The liver and mammary gland were excised immediately, frozen with liquid nitrogen, and stored at −80°C until analysis. For analysis of biochemical variables and growth development, only 10 dams from the SFO group and 8 dams from the CLA group and the corresponding pups were used for the following reasons: first, 1 and 2 rats were not pregnant in the SFO group and the CLA group, respectively; second, 1 dam within each treatment group was excluded from analysis in order to wet-nurse the remaining pups after adjustment to 10 pups per dam; and third, 1 dam within the CLA group was excluded from analysis due to devouring the whole litter directly after parturition. For analysis of CLA isomeric distribution in the milk, only 9 milk samples in the SFO group and 6 milk samples in the CLA group were used due to insufficient sample amounts in 1 and 2 dams, respectively. The remaining milk variables were determined in the milk from 10 and 8 dams in the SFO and CLA groups, respectively.

Lipids in liver, milk, diets and carcass. Total lipids of milk, liver, carcass, and dietary lipids were extracted with a mixture of hexane and isopropanol (3:2, v/v) (22). The fatty acid composition of total lipids in the diet, milk, and the liver of the pups was determined by GC as described recently (23). The amounts of individual CLA in

the CLA-oil and in milk total lipids were determined by GC analysis and silver-ion HPLC according to Sehat et al. (24).

Total cholesterol in liver and plasma and triacylglycerol concentrations in liver, plasma, and milk total lipids were determined using enzymatic reagent kits as described previously (25). Hepatic concentrations of cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM) were quantified by HPLC as previously reported (25).

Carcass composition of newborn pups. Analysis of carcass composition of newborn pups was performed after homogenization and freeze-drying. Head and gastrointestinal tract were removed from newborn pups and not included in the analysis. The hide was not removed from these rats. Carcass total lipids were determined gravimetrically by evaporating the solvent from an aliquot of the hexane-isopropanol extract. Carcass protein was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce; no. 23225).

Milk nutrients. Lactose concentration in the milk was determined by an enzymatic reagent kit from Boehringer (Catalog no. 10986119035). Milk protein was measured as carcass protein. Triacylglycerols in the milk were determined as described above. Milk energy content was calculated based on Atwater factors; lactose and protein each contributed 17 MJ/kg and triacylglycerols contributed 38 MJ/kg.

Enzyme activities in mammary glands and liver. For determination of enzyme activities, aliquots of liver and mammary gland were homogenized in a buffer containing 0.25 mol sucrose/L and 0.1 mol phosphate/L (pH 7.4). Hepatic catalase activity was measured spectrophotometrically in liver homogenate according to Aebi (26). For the measurement of fatty acid synthase (FAS) activity in the mammary gland, homogenates were centrifuged at 105,000 × g for 10 min and the supernatants were used for enzyme assays. FAS activity in the mammary gland was determined according to the method of Nepokroeff et al. (27). Activities were related to the protein concentrations of the samples determined using BCA protein assay kit (Pierce).

Relative mRNA concentrations. Isolation of total RNA from liver and mammary gland and cDNA synthesis were performed as described previously (21). The relative quantities of SREBP-1c, PPARα, FAS, and LPL mRNA compared with glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA were determined by means of semiquantitative RT-PCR as reported previously (28). Relative quantities of AcCx and catalase mRNA were determined by quantitative real-time RT-PCR as described previously (28). Specific primers coding for these genes were obtained from Roth. Results are expressed as the ratio of the target gene mRNA to the reference gene (GAPDH) mRNA. The primer sequences used for RT-PCR were as follows: 5′-GCATGGCCTTCCGTGTTCC-3′ (forward) and 5′-GGGTGGTCCAGGGTTTCTTACTC-3′ (reverse) for rat GAPDH; 5′-TCTTCAACTGGCTGGAGGAAG-3′ (forward) and 5′-TATGCCTTGCTGGGGTTTTCT-3′ (reverse) for rat LPL; 5′-CCTCCCCTGGTGGCTGCTACAA-3′ (forward) and 5′-CCTGGGGTGGGCGGTCTTT-3′ (reverse) for rat FAS; 5′-CCCTCTCTCCAGCTTCCAGCCC-3′ (forward) and 5′-CCACAAGCGTCTTCTCAGCCATG-3′ (reverse) for rat PPARα; 5′-GGAGCCATGGATTGCACATT-3′ (forward) and 5′-AGGAAGGCTTCCAGAGAGGA-3′ (reverse) for rat SREBP-1c; 5′-GATGGGGGCCTGCTCCTGTCCTA-3′ (forward) and 5′-CGCCCCTGGTCGCTTGATGTA-3′ (reverse) for rat AcCx; 5′-CTTTCTTGCTTGCCTTCCTTCTCC-3′ (forward) and 5′-GCCGTTTCACCGCCTCGTA-3′ (reverse) for rat ACO and 5′-TCTTGTTCAGCGACCGAGGGATTC-3′ (forward) and 5′-GGTGGCGGTGAGTGTCTGGGTAAG-3′ (reverse) for rat catalase.

Statistics. Means of the 2 groups were compared by Student's *t* test. Because 2 pups from each dam were used for the determination of the carcass composition, observations within 1 dam were repeated measurements. Therefore, an ANOVA including the factors diet and dam and their interaction was used to evaluate data of carcass composition. Values in the text are means ± SD. Means were considered significantly different at $P < 0.05$.

RESULTS

Food intake and body weight of female rats. The food intake was the same for each rat due to the standardized feeding regimen used, averaging 15.7 g/d during growth and 17.4 g/d during pregnancy. During lactation, the daily food intake also did not differ between groups [33.7 ± 3.7 g/d ($n = 10$) in rats fed the CLA diet and 34.9 ± 2.7 g/d ($n = 8$) in rats fed the SFO diet]. Final body weights did not differ between dams fed the CLA diet (293 ± 42 g, $n = 8$) and those fed the SFO diet (305 ± 24 g, $n = 10$) at d 17 of lactation. The body weight development during growth, pregnancy, and lactation also did not differ between the treatment groups (data not shown).

Liver weights, lipid concentrations, and activities and relative mRNA concentrations of enzymes involved in lipid metabolism. Relative liver weights (expressed per 100 g of body weight) were lower in dams fed the CLA diet than in those fed the SFO diet (**Table 2**). Absolute liver weights tended to be lower in CLA-fed dams than in those fed the SFO diet ($P < 0.15$). Dams fed the CLA diet had higher concentrations of PC, PI, and SM, but lower concentrations of cholesterol and triacylglycerols in the liver than those fed the SFO diet. Hepatic concentrations of PE and PS tended to be higher in CLA-fed dams than in SFO-fed dams ($P < 0.15$),

TABLE 2

Liver weights, lipid concentrations, and activities and relative mRNA concentrations of enzymes involved in lipid metabolism in lactating rats fed diets with either CLA or SFO[1]

	SFO ($n = 10$)	CLA ($n = 8$)
Liver wet weight		
Absolute, *g*	13.5 ± 2.0	11.8 ± 1.8
Relative, *g/100 g body weight*	4.64 ± 0.64	3.87 ± 0.70*
	μmol/g	
Hepatic lipid concentrations		
Total cholesterol	21.1 ± 10.7	8.5 ± 1.2*
Triacylglycerols	62.6 ± 27.7	20.8 ± 2.6*
Cardiolipin	1.24 ± 0.13	1.31 ± 0.18
Phosphatidyl ethanolamine	3.81 ± 0.98	4.60 ± 0.90
Phosphatidyl choline	8.7 ± 1.9	11.8 ± 1.9*
Phosphatidyl inositol	2.25 ± 0.66	3.08 ± 0.42*
Phosphatidyl serine	1.98 ± 1.22	3.35 ± 1.86
Sphingomyelin	0.97 ± 0.25	1.42 ± 0.19*
Total phospolipids[2]	18.9 ± 4.3	25.5 ± 3.6*
	% of GAPDH	
Relative mRNA concentrations		
SREBP-1c	43.0 ± 19.6	58.6 ± 15.6
FAS	64.9 ± 14.2	61.9 ± 9.7
AcCx	0.004 ± 0.002	0.005 ± 0.001
PPARα	36.7 ± 23.6	77.0 ± 16.1*
ACO	1.4 ± 1.2	4.2 ± 2.6*
Catalase	0.07 ± 0.06	0.22 ± 0.13*
	U/mg protein	
Enzyme activity		
Catalase	82 ± 41	174 ± 45*

[1] Results are means ± SD. *Different from rats fed the SFO diet, $P < 0.05$.

[2] Total phospholipids = sum of cardiolipin, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and sphingomyelin.

whereas the concentration of CL did not differ between the 2 groups. The sum of CL, PE, PC, PI, PS, and SM was higher in dams fed the CLA diet than in those fed the SFO diet. The relative mRNA concentrations of PPARα, ACO, and catalase and the activity of catalase in the liver were higher in CLA-fed dams than in SFO-fed dams. mRNA concentrations of SREBP-1c, FAS, and AcCx in the liver did not differ between groups.

Lipid concentrations in plasma. Cholesterol and triacylglycerol concentrations in plasma did not differ between the 2 groups [1.58 ± 0.26 vs. 1.63 ± 0.13 mmol cholesterol/L and 0.60 ± 0.17 vs. 0.56 ± 0.14 mmol triacylglycerols/L in dams fed the CLA diet (n = 8) and those fed the SFO diet (n = 10), respectively].

Activity of FAS and relative mRNA concentrations of FAS and LPL in the mammary gland. The mRNA concentration of LPL in the mammary gland was lower in CLA-fed dams than in SFO-fed dams (**Table 3**). The activity and the mRNA concentration of FAS in the mammary gland were also lower in dams fed the CLA diet than in those fed the SFO diet.

Concentrations of nutrients and energy in the milk. The concentration of triacylglycerols and energy in the milk were lower in rats fed the CLA diet than in those fed the SFO diet. The concentrations of protein and lactose in the milk did not differ between the 2 groups (Table 3).

Fatty acid composition of total lipids in the milk. The proportion of fatty acids with 8–14 carbon atoms in the milk was lower in CLA-fed dams than in SFO-fed dams (**Table 4**). The proportion of C16:0 in the milk did not differ between the groups. The milk of dams fed the CLA diet had higher proportions of 18:0, 18:1 and total CLA but lower proportions of 18:2(n-6) than the milk of dams fed the SFO diet. The major CLA isomers found in the milk of CLA-fed rats were *cis*-11, *trans*-13 CLA, *trans*-10, *cis*-12 CLA, *cis*-9, *trans*-11 CLA and *trans*-8, *cis*-10 CLA (**Fig. 1**). In the rats fed the SFO diet, *trans*-10, *trans*-12 CLA, *trans*-9, *trans*-11 CLA, *trans*-8, *trans*-10 CLA, *trans*-7, *trans*-9 CLA, *cis*-11, *trans*-13 CLA, *trans*-10, *cis*-12 CLA, *cis*-9, *trans*-11 CLA and *trans*-8, *cis*-10 CLA were the only CLA isomers with concentrations > 0.01 g/100 g fatty acids. The *cis*-9, *trans*-11 CLA was found to be the most abundant CLA isomer in the milk of rats fed the SFO diet.

Concentrations of fatty acids in the milk. The milk of the dams fed the CLA diet contained much higher concentrations of total CLA but lower concentrations of 18:2(n-6) than the milk of dams fed the SFO diet [22.6 ± 10.2 vs. 1.0 ± 0.3 mmol CLA/L ($P < 0.05$) and 7.8 ± 3.9 vs. 40.1 ± 7.3 mmol 18:2(n-6)/L ($P < 0.05$) in the dams fed the CLA diet (n = 8)

TABLE 3

Activities and relative mRNA concentrations of enzymes involved in lipid metabolism in the mammary gland and concentrations of nutrients and energy in the milk of lactating rats fed diets with either CLA or SFO[1]

	SFO (n = 10)	CLA (n = 8)
	% of GAPDH	
Relative mRNA concentration		
FAS	88.5 ± 31.2	45.3 ± 5.5*
LPL	51.6 ± 30.7	25.1 ± 10.0*
	U/mg protein	
Enzyme activity		
FAS	2.15 ± 0.42	0.43 ± 0.27*
Milk composition		
Protein, *g/L*	89.2 ± 21.8	91.3 ± 16.6
Triacylglycerol, *g/L*	112 ± 11	60 ± 29*
Lactose, *g/L*	20.8 ± 3.8	21.2 ± 4.1
Energy, *MJ/L*	6.06 ± 0.52	4.16 ± 1.09*

[1] Results are means ± SD. *Different from rats fed the SFO diet, $P < 0.05$.

TABLE 4

Fatty acid composition of total lipids in the milk of lactating rats fed diets with either CLA or SFO[1]

Fatty acid	SFO (n = 10)	CLA (n = 8)
	g/100 g fatty acids	
8:0	3.3 ± 0.8	3.8 ± 0.6
10:0	9.4 ± 1.3	7.6 ± 1.8*
12:0	8.6 ± 1.2	3.9 ± 1.2*
14:0	9.0 ± 2.1	3.4 ± 0.9*
16:0	22.8 ± 2.3	23.6 ± 1.4
18:0	3.4 ± 0.3	5.0 ± 0.5*
18:1(n-9)	21.9 ± 3.5	28.6 ± 3.6*
18:2(n-6)	12.2 ± 1.3	4.4 ± 0.2*
20:2(n-6)	0.7 ± 0.2	0.2 ± 0.1*
20:3(n-6)	0.8 ± 0.1	0.1 ± 0.1*
20:4(n-6)	1.5 ± 0.3	0.6 ± 0.2*
20:5(n-3)	0.1 ± 0.1	0.3 ± 0.1
22:4(n-6)	0.7 ± 0.1	0.2 ± 0.1*
22:6(n-3)	0.1 ± 0.1	0.2 ± 0.1
Total CLA	0.3 ± 0.1	15.9 ± 11.8*
C8–C14	28.5 ± 6.6	17.6 ± 4.3*
C18–C22	41.2 ± 4.6	49.0 ± 8.5

[1] Results are means ± SD. *Different from rats fed the SFO diet, $P < 0.05$.

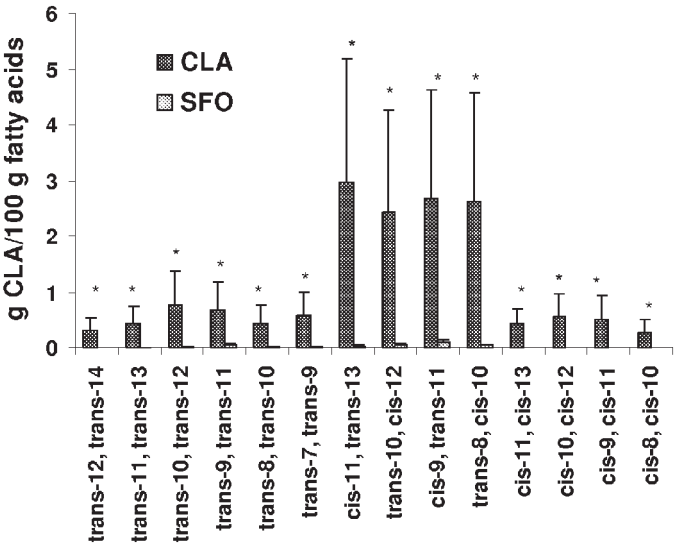

FIGURE 1 Concentrations of CLA isomers in the milk of lactating rats fed diets with either CLA or SFO. Results are means ± SD, n = 9 (SFO) or 6 (CLA). *Different from rats fed the SFO diet, $P < 0.05$. Concentrations of *trans*-12, *trans*-14 CLA, *trans*-11, *trans*-13 CLA, *cis*-11, *cis*-13 CLA, *cis*-10, *cis*-12 CLA, *cis*-9, *cis*-11 CLA, *cis*-8, *cis*-10 CLA in the milk of lactating rats fed the SFO diet were below the detection limit of 0.01 g/100 g fatty acids.

TABLE 5

Development of litters and pups during lactation and carcass composition of newborn pups of rats fed diets with either CLA or SFO

	SFO	CLA
Development of litters and pups during lactation[1]		
d 1 of lactation[2]		
Pups per litter, *n*	13.9 ± 2.6	13.0 ± 3.3
Litter weight, *g*	76.4 ± 11.2	73.3 ± 15.7
Pup weight, *g*	5.6 ± 0.4	5.7 ± 0.4
d 8 of lactation		
Pups per litter, *n*	9.2 ± 2.5	8.9 ± 2.2
Litter weight, *g*	116 ± 39	100 ± 30
Pup weight, *g*	12.3 ± 2.3	11.3 ± 1.7
d 17 of lactation		
Pups per litter, *n*	6.8 ± 1.9	4.0 ± 2.5*
Litter weight, *g*	198 ± 45	129 ± 83*
Pup weight, *g*	30.3 ± 5.8	23.8 ± 6.7*
Carcass composition of newborn pups[3]		
Protein,[4] *g/100 g DM*	56.0 ± 5.0	58.9 ± 4.3
Fat, *g/100 g DM*	14.4 ± 2.5	9.4 ± 1.3*
Protein:fat ratio, *g/g*	4.65 ± 1.28	6.55 ± 0.96*

[1] Results are means ± SD, $n = 10$ (SFO) or 8 (CLA). *Different from rats fed the SFO diet, $P < 0.05$.
[2] Before adjustment to 10 pups per dam.
[3] Results are means ± SD, $n = 20$ (SFO) or 16 (CLA). The effects of dam and dam × diet interaction on data of carcass composition of the newborn pups were not significant ($P > 0.05$).
[4] DM, dry matter.

and those fed the SFO diet ($n = 10$), respectively]. The concentrations of medium-chain fatty acids (C8-C14), fatty acids with 16 carbon atoms (C16), and long-chain fatty acids (C18-C22) were lower in milk of CLA-fed dams than in milk of SFO-fed dams [56 ± 35 vs. 149 ± 17 mmol C8-C14 fatty acids/L ($P < 0.05$), 47.1 ± 22.7 vs. 85.2 ± 10.7 mmol C16 fatty acids/L ($P < 0.05$) and 99 ± 45 vs. 140 ± 23 mmol C18-C22 fatty acids/L ($P < 0.05$) in dams fed the CLA diet ($n = 8$) and those fed the SFO diet ($n = 10$), respectively]. The ratio between medium-chain fatty acids, which derive from de novo fatty acid synthesis in mammary gland, and long-chain fatty acids, which derive from plasma lipids in the milk, was lower in dams fed the CLA diet than in those fed the SFO diet [0.71 ± 0.19 vs. 1.12 ± 0.23 in CLA-fed dams ($n = 8$) and SFO-fed dams ($n = 10$), respectively; $P < 0.05$].

Reproductive performance, development of litters and pups during lactation, and carcass composition and hepatic lipid concentrations of newborn pups. The number of pups per litter, litter weights, and pup weights at d 1 and 8 of lactation did not differ between the groups (**Table 5**). At d 17 of lactation, the number of pups per litter, litter weights, and pup weights were lower in dams fed the CLA diet than in those fed the SFO diet. Newborn pups from rats fed the CLA diet had a higher protein:fat ratio but lower concentrations of total lipids in the carcass than those from rats fed the SFO diet. Carcass protein did not differ between pups from the 2 groups. Hepatic concentrations of triacylglycerols were markedly lower in newborn pups from CLA-fed dams than in pups from SFO-fed dams [7.4 ± 2.1 ($n = 8$) vs. 18.2 ± 8.5 ($n = 10$) μmol/g, $P < 0.05$]. The concentration of cholesterol in the liver of newborn pups tended to be lower in the CLA group than in the SFO group [19.0 ± 8.4 ($n = 8$) vs. 29.2 ± 15.4 ($n = 10$) μmol/g, $P < 0.15$].

Fatty acid composition of total lipids in the livers of pups at d 1 and 17. At d 1 and 17, pups of dams fed the CLA diet had higher proportions of 18:1(n-9), *cis*-9, *trans*-11 CLA, and *trans*-10, *cis*-12 CLA in liver total lipids but lower proportions of 18:2(n-6), 20:4(n-6), 22:4(n-6), and 22:5(n-6) than pups of dams fed the SFO diet (**Table 6**). The proportions of C12:0, C14:0 and C16:0 in total lipids in the liver of the pups at d 1 and 17 did not differ between the groups. The ratio between 20:3(n-9) and 20:4(n-6) in liver total lipids of pups at d 1 did not differ between the groups; at 17 d of age, this ratio was higher in pups of CLA rats than in pups of SFO rats.

TABLE 6

Fatty acid composition of total lipids in the liver of pups of rats fed diets with either CLA or SFO at d 1 and 17 of life[1]

Age of pups	d 1		d 17	
Treatment of dams	SFO	CLA	SFO	CLA
Fatty acid	*g/100 g fatty acids*			
12:0	0.5 ± 0.3	0.2 ± 0.2	2.0 ± 1.2	1.5 ± 0.4
14:0	1.3 ± 0.5	1.1 ± 0.4	4.0 ± 1.4	3.6 ± 0.9
16:0	23.4 ± 1.7	23.4 ± 2.3	24.2 ± 2.3	25.4 ± 2.2
18:0	7.9 ± 2.9	11.6 ± 2.8*	10.0 ± 2.0	10.6 ± 1.4
18:1(n-9)	18.0 ± 3.0	23.6 ± 2.8*	15.4 ± 2.6	25.7 ± 3.2*
18:2(n-6)	9.2 ± 1.4	4.8 ± 0.6*	14.3 ± 0.6	6.9 ± 1.0*
cis-9, *trans*-11 CLA	<0.1	0.6 ± 0.3*	<0.1	1.5 ± 0.3*
trans-10, *cis*-12 CLA	<0.1	0.9 ± 0.5*	<0.1	2.0 ± 0.2*
20:3(n-9)	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
20:4(n-6)	15.4 ± 2.4	10.9 ± 2.9*	14.2 ± 2.5	6.7 ± 2.1*
20:5(n-3)	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.4 ± 0.1*
22:4(n-6)	3.8 ± 1.2	1.0 ± 0.4*	3.3 ± 0.5	0.3 ± 0.2*
22:5(n-6)	4.7 ± 0.9	2.9 ± 0.5*	3.2 ± 0.5	1.3 ± 0.4*
22:6(n-3)	3.3 ± 1.1	5.4 ± 2.7	2.4 ± 0.7	3.0 ± 0.9
20:3(n-9)/20:4(n-6)	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.05 ± 0.01*

[1] Results are means ± SD, $n = 10$ (SFO) or 8 (CLA). *Different from rats fed the SFO diet, $P < 0.05$.

DISCUSSION

In the present study, the effect of a CLA supplement was tested in female rats during growth, pregnancy, and lactation. As in the majority of animal studies dealing with CLA, the CLA supplement used was a mixture of various CLA isomers. The CLA supplement contained 54 g CLA/100 g total fatty acids, which is equivalent to a CLA concentration of 14.7 g/kg diet. *Trans*-10, *cis*-12 CLA, *cis*-11, *trans*-13 CLA, *cis*-9, *trans*-11 CLA, and *trans*-8, *cis*-10 CLA were the major CLAs contributing to the ~65 g/100 g total CLA. The remaining CLA identified consisted of minor isomers in *trans*/*cis*-, *cis*/*trans*-, *trans*/*trans*- and cis/*cis*-configurations. The CLA isomeric distribution of the supplement used in this study differs markedly from the distribution pattern of natural foods. In natural foods such as milk, milk products, or beef, *cis*-9, *trans*-11 CLA is the main CLA isomer. It comprises between 80 and 90 g/100 g total CLA isomers, whereas *trans*-10, *cis*-12 CLA and other CLA isomers exist in very low concentrations. In contrast, most dietary CLA supplements contain a large number of CLA isomers (29). Concentrations of total CLA isomers in the CLA diet, amounting to 14.7 g/kg of diet, were much higher than those of Western human diets. Daily CLA consumption of humans in the United States from natural foods was estimated to be in the range between 150 and 210 mg/d (30), which is equivalent to a CLA concentration in the diet of ~0.3 g/kg dry matter. The present study therefore should be regarded as a model study showing the potential effects of a high supplementation with various CLA isomers rather than the effects of CLA occurring in natural foods.

The *trans*-10, *cis*-12 CLA, rather than the *cis*-9, *trans*-11 CLA is considered responsible for most of the CLA effects on lipid metabolism in animal studies (31–33), but the effects of minor CLA isomers on the metabolism in animals are unknown. Because the CLA supplement used in this study contained a large number of CLA isomers, it is not known which CLA isomers were mainly responsible for the effects of the CLA supplement in this study.

To study the effect of dietary CLA, we added the CLA supplement to the diet at the expense of sunflower oil. Similar approaches were used by several others and are widely accepted for studying the effects of CLA on animal metabolism (34,35). Nevertheless, one should be aware that in such an approach, the concentration of linoleic acid in the CLA diet is lower than in the control diet, and some effects observed after feeding the CLA diet could be due to lower concentrations of linoleic acid rather than to CLA itself. To avoid metabolic confounding due to insufficient dietary linoleic acid concentrations, we supplemented the diets of both treatment groups with 10 g soybean oil/kg diet. The linoleic acid intake of the rats fed the CLA diet, which was 107, 120, and 230 mg/d during growth, pregnancy, and lactation, respectively, was in excess of the requirement of 100 mg linoleic acid/d for pregnant and lactating rats (36). Therefore, we can exclude the possibility that the effects observed in rats fed the CLA diet were due to a deficiency of linoleic acid.

Our study shows that feeding a CLA supplement to rats during pregnancy and lactation strongly reduces milk fat concentration. This finding agrees with other studies in humans and animals (2,4,5). The observation that rats fed the CLA diet had a reduced mRNA level and FAS activity indicates that CLA lowered the rate of de novo fatty acid synthesis in the mammary gland. This indication is confirmed by the observation of markedly reduced concentrations of medium-chain fatty acids with 8–14 carbon atoms in the milk of rats fed CLA. Fatty acids with 8–14 carbon atoms are the main products of de novo fatty acid synthesis in the mammary gland. The observation that dietary CLA reduces gene expression of lipogenic enzymes in the mammary gland of lactating rats agrees with findings of another study (37). That study, moreover, showed that *trans*-10, *cis*-12 CLA is a more potent inhibitor of mammary lipogenesis than *cis*-9, *trans*-11 CLA.

In addition to fatty acids synthesized in the mammary gland, fatty acids released from triglyceride-rich lipoproteins by LPL and taken up into the mammary gland are another important source for milk fat synthesis. Those fatty acids largely reflect those of the diet; they are predominately long-chain fatty acids with 18–22 carbon atoms, either saturated or unsaturated (6,7). The finding that the milk of rats fed the CLA supplement also had reduced concentrations of long-chain fatty acids with 18–22 carbon atoms suggests that the CLA supplement impaired the uptake of those fatty acids into the mammary gland, thereby impairing the synthesis of triglycerides with long-chain fatty acids.

Because the concentration of triglycerides in plasma was not lower in rats fed the CLA supplement than in control rats, we assume that the activity of LPL, the key factor in the uptake of circulating lipids by the lactating mammary gland (38), could have been reduced. For technical reasons, we were not able to determine the activity of that enzyme. However, gene expression analysis revealed that mRNA concentrations of LPL in the mammary gland were significantly lower in rats fed the CLA diet than in control rats. We therefore assume that the activity of this enzyme might have been reduced in rats fed CLA. The finding of our study is consistent with a recent study in cows in which abomasally infused *trans*-10, *cis*-12 CLA caused a significant reduction of LPL mRNA in mammary glands and a reduction in milk fat concentration (4).

Data from this study therefore suggest that the reduced milk fat concentration in the CLA group was the result of the inhibition of both de novo fatty acid synthesis and uptake of fatty acids from lipoproteins into the mammary gland. However, the finding that milk of CLA-fed rats had a lower ratio of medium-chain fatty acids to long-chain fatty acids than milk of SFO-fed rats suggests that inhibition of de novo fatty acid synthesis was more responsible for the impairment than inhibition of the uptake of fatty acids from plasma lipoproteins.

Our study shows that dietary CLA lowers the concentrations of triacylglycerols and cholesterol and increases the concentration of phospholipids in the liver of lactating rats. These effects might be explained by the activation of hepatic PPARα by dietary CLA. It was shown that CLAs are ligands of PPARα (16–18). Activation of PPARα leads to proliferation of peroxisomes and stimulates the expression of genes encoding proteins of mitochondrial and peroxisomal β-oxidation (39). Increased mRNA levels of ACO and catalase, both marker enzymes of peroxisomes, indicate that dietary CLA activated PPARα, caused peroxisome proliferation, and enhanced lipid catabolism in the liver. The activation of PPARα leads to reduced concentrations of hepatic triacylglycerols through an increased oxidation of fatty acids (40). Increased concentrations of phospholipids and reduced concentrations of cholesterol in the liver are other responses to the activation of PPARα, which were also observed in other animal studies examining dietary CLA supplements (10,34,41). The finding that hepatic mRNA concentrations of lipogenic enzymes such as FAS and AcCx were not altered in rats fed CLA suggests that CLA did not influence the rate of de novo fatty acid synthesis in the liver. Typically, enhanced fatty acid oxidation in the liver due to activation of PPARα is associated with reduced plasma triacylglycerol concentrations (40). Therefore, it was surprising that plasma triacylglycerol concentrations did

not differ between rats fed CLA and control rats. However, this observation suggests that the reduced milk fat concentrations of rats fed CLA were not caused by reduced concentrations of plasma triacylglycerols.

The reduction in milk fat concentration resulted in a reduced energy content in the milk. The production of lipids in the milk is of biological importance for the development of the offspring (8,42). Because maternal milk is the only source of nutrients for the suckling pups, it was not surprising that the reductions in fat and energy in the milk were accompanied by a reduced development of the litter and pup weights during lactation. The finding of the present study is in contrast to a previous study in rats that did not report a reduced survival of the offspring during lactation (43). However, differences between the present and the previous study could be due to a different diet composition, e.g., dietary fat contents (60 vs. 40 g fat/kg diet) or isomeric distribution of the CLA supplement. It was demonstrated that a higher fat content in the diet increases lipid accumulation in the pup carcass (8), which increases the energy stores of the suckling pup and probably decreases mortality. The fatty acid analysis of milk triglycerides showed that the concentration of linoleic acid in the milk of CLA dams was much lower than that in the milk of control dams. Therefore, the question arose whether the increased mortality of pups of the CLA dams could be due a deficiency in essential fatty acids (EFA). To test this, we determined the fatty acid composition of liver lipids in pups after birth and at weaning. The amounts of linoleic acid and arachidonic acid in the liver at weaning were lower in pups of CLA dams than in pups of control dams, as expected, but they were clearly higher than those observed in EFA-deficient rats (44). As a consequence of the lower supply of linoleic acid from the milk, the amount of mead acid [20:3(n-9)] and the ratio at weaning between mead acid and arachidonic acid, both sensitive indicators of EFA deficiency, were higher in CLA pups than in SFO pups. Nevertheless, these indicators were markedly lower than those of EFA-deficient rats (44). Therefore, we assume that the increased mortality of pups of CLA dams was not caused by an insufficient intake of EFA in the milk. Rather, the increased mortality of the suckling pups could be the result of a limited supply of the energy that is required for optimal growth of the offspring. Some studies demonstrated that dietary CLA also influences the amount of milk produced. The effects, in this respect, however, are not quite uniform. Some studies reported a reduced milk production by dietary CLA in various animals species (45,46), whereas one reported increased milk production (47). Because the milk of rats fed the CLA diet contained a large number of CLA isomers, we also cannot exclude the possibility that one or more of those isomers had unfavorable effects on the suckling pups.

The observation that small amounts of CLA were also found in the milk of rats fed the SFO diet supports the indication from a previous study that microorganisms in the digestive tract of monogastric animals are able to synthesize CLA (48), which are probably absorbed and incorporated into tissues and milk lipids. CLAs are also synthesized endogenously from vaccenic acid (*trans*-11–18:1) via the Δ9-desaturase reaction (49). However, the absence of vaccenic acid in the SFO diet does not support this notion.

This study shows that the newborn pups of rat dams fed a diet containing CLA had reduced concentrations of triacylglycerols in the liver, reduced amounts of linoleic acid and arachidonic acid in liver lipids, and an increased protein:fat ratio in their carcass compared with pups of control dams. Increases in the protein:fat ratio of the carcass occur in growing or adult animals fed a dietary CLA supplement (10,31,50). Our study shows for the first time that CLA fed to pregnant rats has similar effects on their fetuses.

In conclusion, the present study suggests that administration of a dietary CLA supplement to pregnant and lactating rats strongly reduces milk fat concentration and energy content of the milk, changes body composition of newborn pups, reduces body weight gains of suckling pups, and increases their mortality. Although the results of our study cannot be extrapolated directly to humans, they suggest that commercially available CLA supplements should be considered critically in pregnant and nursing women.

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Oxidized Fat Reduces Milk Triacylglycerol Concentrations by Inhibiting Gene Expression of Lipoprotein Lipase and Fatty Acid Transporters in the Mammary Gland of Rats[1]

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Abstract

Feeding oxidized fats to lactating rats causes a strong reduction of triacylglycerol concentration in the milk. The reason for this, however, has not yet been elucidated. Pregnant Sprague-Dawley rats were assigned to 2 groups of 11 rats each and fed diets containing either fresh fat (FF group) or an oxidized fat (OF group) from d 1 to d 20 of lactation. Concentrations of triacylglycerols and long-chain fatty acids in the milk and weight gain of suckling pups were lower in the OF group than in the FF group ($P < 0.05$). Concentrations of medium-chain fatty acids in the milk and messenger RNA (mRNA) abundance of lipogenic enzymes in the mammary gland did not differ between the 2 groups of rats. However, the OF group had a lower concentration of triacylglycerols and nonesterified fatty acids (NEFA) in plasma and lower mRNA concentrations of lipoprotein lipase and fatty acid transporters in the mammary gland than the FF group ($P < 0.05$). Moreover, the OF group had higher mRNA concentrations of hepatic lipase, fatty acid transporters, and several genes involved in fatty acid oxidation in the liver than the FF group ($P < 0.05$). The present findings suggest that a dietary oxidized fat lowers the concentration of triacylglycerols in the milk by a reduced uptake of fatty acids from triacylglycerol rich-lipoproteins and NEFA into the mammary gland. The study, moreover, indicates that an oxidized fat impairs normal metabolic adaptations during lactation, which promote the utilization of metabolic substrates by the mammary gland for the synthesis of milk. J. Nutr. 137: 2056–2061, 2007.

Introduction

Oxidized lipids as components of heated or fried foods play an important role in human nutrition in industrialized countries. Lipid peroxidation products present in oxidized oils influence animal metabolism in several ways (1–3). Recently, we and others have shown that feeding oxidized oils to rats causes activation of PPARα, which is associated with increased expression of genes involved in oxidation of fatty acids [e.g. acyl-CoA oxidase (ACO),[2] L-type carnitin-palmitoyl transferase I (L-CPT I), medium-chain acyl-CoA dehydrogenase (MCAD), cytochrome P450 4A1 (CYP4A1) (lauric acid omega-hydroxylase)], reduced gene expression of enzymes involved in de novo-fatty acid synthesis [e.g. fatty acid synthase (FAS)], and strongly reduced concentrations of triacylglycerols in liver and plasma (4–7). In lactating rats, feeding oxidized fats caused a strong reduction of milk triacylglycerol concentration, which in turn led to reduced weight gains of suckling pups (8,9). The reason underlying the reduction of milk triacylglycerol concentration in lactating rats by oxidized fat, however, has not yet been clarified.

Milk triacylglycerol synthesis depends on the availability of fatty acids in the mammary gland, which derive from 3 different sources. The first source represents de novo biosynthesis of fatty acids within the mammary gland by the activity of lipogenic enzymes. Medium-chain fatty acids with 8–14 carbon atoms are the main products of this process, which is controlled by the lipogenic transcription factor sterol regulatory element-binding protein (SREBP)-1c (10). Fatty acids released from triacylglycerol-rich lipoproteins by lipoprotein lipase (LPL) and taken up into the mammary gland are another important source for milk triacylglycerol synthesis (11). Those fatty acids reflect those of the diet and are typically long-chain fatty acids with 16–22 carbon atoms, either saturated or unsaturated (12,13). Nonesterified fatty acids (NEFA) in the plasma released from adipose tissue by hormone-sensitive lipase and taken up into the mammary gland by fatty acid transporters are the 3rd source of fatty acids available for milk triacylglycerol synthesis. We have recently shown that fatty acid synthesis in the mammary gland is not influenced

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[2] Abbreviations used: ACO, acyl-CoA oxidase; CYP4A1, cytochrome P450 4A1; FABPpm, plasma membrane fatty acid-binding protein; FAS, fatty acid synthase; FAT/CD36, fatty acid translocase/CD36; FATP, fatty acid transport protein; FF, group fed fresh fat; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HL, hepatic lipase; IDH1, cytosolic $NADP^+$-dependent isocitrate dehydrogenase; L-CPT I, L-type carnitin-palmitoyl transferase I; LPL, lipoprotein lipase; mAspAT, mitochondrial aspartate aminotransferase; MCAD, medium-chain acyl-CoA dehydrogenase; mRNA, messenger RNA; NEFA, nonesterified fatty acids; OF, group fed oxidized fat; POV, peroxide value; RPS9, ribosomal protein S9; SCD, stearoyl-CoA desaturase; SREBP-1c, sterol regulatory element-binding protein-1c.

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by dietary oxidized fats in lactating rats (8). Therefore, we postulate that reduced milk triacylglycerol concentrations in lactating rats fed oxidized fats are due to a decreased uptake of fatty acids from triacylglycerol-rich lipoproteins and/or a reduced uptake of NEFA from plasma. The availability of fatty acids from triacylglycerol-rich lipoproteins for tissues depends on the concentrations of triacylglycerol-rich lipoproteins in plasma and on the activity of LPL (14). To determine whether oxidized fat could reduce the availability of fatty acids from triacylglycerol-rich lipoproteins for the mammary gland, we measured plasma triacylglycerol concentrations and gene expression of LPL in mammary gland. In growing rats, feeding an oxidized fat causes a strong reduction of triacylglycerols in plasma and VLDL by an increased β-oxidation of fatty acids and a reduced de novo-fatty acid synthesis in the liver (6,7). To study whether such an effect also occurs in lactating rats, we determined messenger RNA (mRNA) expression of genes involved in hepatic fatty acid oxidation (ACO, L-CPT I, CYP4A1, and MCAD) and synthesis [SREBP-1c, FAS, stearoyl-CoA desaturase (SCD), cytosolic $NADP^+$-dependent isocitrate dehydrogenase (IDH1)]. To further elucidate the effect of oxidized fat on the uptake of NEFA into the mammary gland, we determined plasma concentrations of NEFA and mRNA expression levels of fatty acid transporters, fatty acid translocase/CD36 (FAT/CD36), fatty acid transport protein (FATP), and mitochondrial aspartate aminotransferase [(mAspAT), also called plasma membrane fatty acid-binding protein (FABPpm)] in the mammary gland.

Materials and Methods

Animals. Twenty-two 13-wk-old female Sprague-Dawley rats with a body weight of 250 ± 17 g (mean ± SD) were obtained from Charles River and randomly assigned to 2 groups of 11 rats each. The rats were kept individually in Macrolon cages in a room maintained with controlled temperature (23 ± 1°C), humidity (50–60%), and lighting from 0600 to 1800 and consumed a commercial standard rodent diet (Altromin) ad libitum. At 14 wk of age, the rats were mated by housing 1 male rat with 1 female rat. At the day of parturition, designated as d 1 of lactation, litters were adjusted to 7 pups/dam and dams were fed the experimental diets until d 20 of lactation. All experimental procedures described followed established guidelines for the care and handling of laboratory animals (15) and were approved by the council of Saxony-Anhalt.

Diets and feeding. Semipurified diets, composed according to the recommendations of American Society for Nutritional Sciences for rats during reproduction (16), were used. The diet consisted of (g/kg diet): casein, 200; cornstarch, 390; saccharose, 198; cellulose, 50; fat, 100; mineral mixture, 40; vitamin mixture, 20; DL-methionine, 2. The type of fat was varied according to a 1-factorial design. The first group [fed fresh fat (FF)] received a mixture of sunflower oil and lard (60:40, wt:wt). We chose this ratio to equalize the concentration of PUFA, mainly C18:2(n-6), of the fresh fat with that of the oxidized fat, because the heating process caused a loss of PUFA. The second group was fed oxidized fat (OF; see "Preparation of the oxidized fat"). The vitamin E concentration of the diets was 50 mg α-tocopherol equivalents per kilogram diet. To adjust the vitamin E concentration of the diets, the native concentrations of tocopherols of the fats were analyzed. Based on the native concentrations of the fats, diets were supplemented individually with all-rac-α-tocopheryl acetate (the biopotency of all-rac-α-tocopheryl acetate is considered to be 67% of that of α-tocopherol). Diets were prepared by mixing the dry components with the fat and water and were subsequently freeze-dried. The residual water content of the diet was <5 g/100 g diet. Food was administered daily at 0800 in restricted amounts to standardize intake.

Experimental diets were fed during the suckling period from d 1 to d 20 of lactation. To standardize food intake, diets were fed in a controlled feeding regimen, whereby each rat received the same amount of diet. The food given was 20% less than the amounts of identical diets with fresh fats consumed ad libitum by rats in preliminary studies. The amount of food offered each day was increased continuously from 20 g at d 1 of lactation to 36 g at the end of the experiment (d 20 of lactation).

Rats consumed water ad libitum from nipple drinkers during the entire experiment.

Preparation of the oxidized fat. The oxidized fat was prepared by heating sunflower oil at 60°C for 25 d. Sunflower oil was poured into a glass beaker and placed into a drying oven set at the intended temperature. Throughout the heating process, air was continuously bubbled through the fat at a flow rate of 650 mL/min. This treatment caused a loss of PUFA and a complete loss of tocopherols and raised the concentrations of lipid peroxidation products in the fats. The extent of lipid peroxidation in the oxidized fat was estimated by assaying the peroxide value (POV) (17), concentration of TBARS (18), and concentration of conjugated dienes (19). To assess lipid peroxidation products in the oxidized fat after inclusion into the diet, the fat was extracted from aliquots of the diets with a mixture of hexane and isopropanol (3:2, v:v) and analyzed for POV, concentration of conjugated dienes, and TBARS.

Sample collection. At d 15 of lactation, milk samples were collected from the dams. After separation from the pups for 1 h, dams were anesthetized i.m. with ketamine (75 mg/kg body weight) and injected i.m. with 1 international units oxytocin to stimulate milk flow. Milking was performed at 1000 h with a milking machine. From each rat, 2–3 mL of milk was obtained from all teats within 10 min through below atmospheric pressure. Samples were stored at −20°C until analysis. At d 20 of lactation, rats were killed by decapitation under light anesthesia with diethyl ether. Blood was collected from the opened neck into heparinized polyethylene tubes (Sarstedt) by the use of heparinized plastic funnels. Plasma was separated from blood by centrifugation (1100 × *g*; 10 min) at 4°C. Liver and mammary gland were excised immediately and shock frozen with liquid nitrogen. All samples were stored at −80°C pending analysis.

Lipid analysis. Triacylglycerol concentrations in liver, milk, and plasma were determined using an enzymatic reagent kit obtained from Merck Eurolab (ref. No. 113009990314). For the measurement of liver and milk triacylglycerols, lipids of the extract, obtained with a mixture of hexane and isopropanol (3:2, v:v) (20), were dissolved in Triton X-100 prior to enzymatic measurement, as described by De Hoff et al. (21). Plasma NEFA concentrations were determined using the enzymatic NEFA C kit from Wako Chemicals (ref. no. 99975406). The fatty acid composition of dietary fats and milk total lipids was determined by GC-flame ionization detector as described recently (22).

RNA isolation and real-time detection PCR. For the determination of mRNA expression levels of PPARα, CYP4A1, ACO, L-CPT I, MCAD, LPL, hepatic lipase (HL), FAT/CD36, FATP, mAspAT, SREBP-1c, FAT, SCD, and IDH1, total RNA was isolated from liver and mammary gland using TrizolP reagent (Invitrogen) according to the manufacturer's protocol. RNA concentration and purity were estimated from the OD at 260 and 280 nm, respectively. cDNA synthesis and relative quantification of target gene mRNA compared with the housekeeping gene mRNA was determined by real-time detection PCR as described previously (23). As housekeeping genes, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the liver and ribosomal protein S9 (RPS9) in the mammary gland. RPS9, in contrast to GAPDH, is a suitable internal reference gene for normalization of gene expression data in mammary tissue (24). Relative quantification was performed using the ΔΔ cycle threshold (Ct)-method (25). Ct values of target genes and the reference gene were obtained using Rotorgene software 5.0. Relative expression ratios are expressed as fold-changes of mRNA abundance in the treatment group (OF) compared with the control group (FF). Sequences of gene-specific primers were as follows (forward, reverse; National Center for Biotechnology Information GenBank): GAPDH (GCA TGG CCT TCC GTG TTC C, GGG TGG TCC AGG GTT TCT TAC TC; NM_017008); PPARα (CCC TCT CTC CAG CTT CCA GCC C, CCA CAA GCG TCT

TCT CAG CCA TG; NM_013196); CYP4A1 (CAG AAT GGA GAA TGG GGA CAG C, TGA GAA GGG CAG GAA TGA GTG G; M14972); ACO (CTT TCT TGC TTG CCT TCC TTC TCC, GCC GTT TCA CCG CCT CGT A; J02752); L-CPT I (GGA GAC AGA CAC CAT CCA ACA TA, AGG TGA TGG ACT TGT CAA ACC; NM_031559); SREBP-1c (GGA GCC ATG GAT TGC ACA TT, AGG AAG GCT TCC AGA GAG GA; XM_213329); FAS (AGG TGC TAG AGG CCC TGC TA, GTG CAC AGA CAC CTT CCC AT; NM_017332); MCAD (CAA GAG AGC CTG GGA ACT TG, CCC CAA AGA ATT TGC TTC AA; NM_016986); LPL (TCC CAC CAC AAC GAA GTA CA, TCA GCC AGG GCA TTA TTT TC; NM_012598); FAT/CD36 (TCG TAT GGT GTG CTG GAC AT, GGC CCA GGA GCT TTA TTT TC; L19658); FATP (GGT AGC AAA TGC ACC CTC AT, CTC CTG CTG TGA TGT GAG GA; U89529); mAspAT (ACC ATC CAC TGC CGT CTT AC, CCC CGA TGC GTA GGT ATT CT; M18467); SCD (CCG TGG CTT TTT CTT CTC TCA, CTT TCC GCC CTT CTC TTT GA; NM_139192); IDH1 (GCT TCA TCT GGG CCT GTA AG, GCT TTG CTC TGT GGG CTA AC; NM_031510); HL (TGC CAA TTT TGT GGA TGC TA, TTA AGC CAT GCT CTG CAA TG; NM_012597); and RPS9 (CAA ATT TAC CCT GGC GAA GA, TCA GGC CCA GAA TGT AAT CC; NM_031108).

Statistical analysis. Values presented in the text are means ± SD. Treatment effects were analyzed using 1-way ANOVA. For significant *F*-values, means were compared by Fisher's multiple range test. Differences with $P < 0.05$ were considered significant.

Results

Characterization of the experimental fats. Palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic acid (18:2) were the major fatty acids in the dietary fat. Other fatty acids were present only in traces. The proportion of 18:2 was similar in both fats. The proportion of 18:1 was slightly higher and the proportions of SFA were slightly lower in the oxidized fat compared with the control fat (**Table 1**). The concentrations of all of the lipid peroxidation products determined were much higher in the oxidized fat than in the fresh fat, both before and after inclusion in the diet. Concentrations of trans-fatty acids, including conjugated linoleic acids, were below the limit of detection of 0.1 g/100 g fatty acids.

Food intake, body weight development of lactating rats, and numbers of pups born. The diet intake was identical for each rat due to the standardized feeding regimen used. The diet intake during lactation averaged 28.9 g/d per rat. The body weight gain of the female rats did not differ between both groups. The maximum body weights observed were 380 ± 27 g (FF group) and 380 ± 28 g (OF group) at the time of parturition. The number of pups born (number per litter) did not differ between the 2 groups (data not shown).

Development of litters during the suckling period. At d 8, 14, and 20 of lactation, litters, standardized to 7 pups/dam, of dams fed diets containing OF were lighter than those fed diets containing FF ($P < 0.05$; **Table 2**). Weight gains of litters from d 1 to d 20 were also lower in dams fed diets containing OF than in dams fed diets containing FF.

Concentrations of triglycerides and fatty acids in the milk. Rats fed diets with OF had lower triacylglycerol concentrations in the milk than rats fed diets with FF ($P < 0.05$; **Table 3**). The concentrations of 16:0, 16:1, 18:0, 18:1, 18:2(n-6), 18:3(n-3), 20:0, 20:2(n-6), 20:3(n-6), 20:4(n-6), 22:4(n-6), 22:5(n-3), 22:6(n-3), and total long-chain fatty acids with 18–22 carbon atoms in the milk were lower in rats fed diets with OF than in those fed diets with FF ($P < 0.05$). In contrast, the concentrations of 8:0, 10:0, 12:0, 14:0, and total medium-chain fatty acids with 8–14 carbon atoms did not differ between the 2 groups.

Liver weights and liver and plasma lipids of lactating rats. Relative liver weights of lactating rats fed diets with OF were higher than those fed diets with FF ($P < 0.05$; **Table 4**). Absolute liver weights did not differ between both groups of rats. Triacylglycerol concentrations in liver and plasma and concentrations of free fatty acids in plasma were markedly lower in rats fed diets containing OF relative to those fed diets containing FF ($P < 0.05$).

Relative mRNA concentrations in the liver and mammary gland of lactating rats. Relative mRNA concentrations of PPARα, ACO, L-CPT I, CYP4A1, and MCAD in the liver of dams fed diets with OF were 1.8-, 5.3-, 2.3-, 5.2-, and 5.6-fold, respectively, of control ($P < 0.05$; **Fig. 1**). In contrast, PPARα mRNA was only barely detectable by real-time RT-PCR in lactating mammary gland. The relative mRNA concentrations of ACO, L-CPT I, CYP4A1, and MCAD in the mammary gland did not differ between both groups (data not shown).

Relative mRNA concentrations of HL, FATP, mAspAT, and FAT/CD36 in the liver of rats fed diets containing OF were 2.2-, 2.0-, 2.1-, and 4.4-fold, respectively, of control ($P < 0.05$; **Fig. 2**). In contrast, rats fed diets containing OF had lower relative mRNA concentrations of LPL, FATP, FABPpm, and FAT/CD36 in the mammary gland than rats fed diets containing FF ($P < 0.05$).

TABLE 1 Characteristics of the experimental fats

	FF	OF
Major fatty acids, *g/100 g total fatty acids*		
16:0	14.1	9.2
18:0	8.5	5.3
18:1	31.4	38.6
18:2 (n-6)	43.9	45.1
Peroxidation products		
Before inclusion in the diet		
POV, *mEq O_2/kg*	9	412
Conjugated dienes, *mmol/kg*	7	197
TBARS, *mmol/kg*	<0.1	7.1
After inclusion in the diet		
POV, *mEq O_2/kg*	26	520
Conjugated dienes, *mmol/kg*	7	139
TBARS, *mmol/kg*	<0.1	6.3

TABLE 2 Weight gain of litters of dams fed FF or OF diets[1]

	FF	OF
Weight of litters	*g*	
d 1	45 ± 3	44 ± 3
d 8	107 ± 12	87 ± 15*
d 14	201 ± 18	149 ± 24*
d 20	282 ± 24	207 ± 30*
Weight gain of litters from d 1 to d 20	238 ± 22	164 ± 29*

[1] Values are means ± SD, $n = 11$. *Different from rats fed FF, $P < 0.05$.

TABLE 3 Concentrations of triacylglycerols and fatty acids in the milk of lactating rats fed FF or OF diets[1]

	FF	OF
	μmol/g milk	
Triacylglycerols	270 ± 48	177 ± 74*
Fatty acids		
8:0	38.3 ± 5.9	38.3 ± 16.7
10:0	76.4 ± 15.7	88.1 ± 36.5
12:0	50.4 ± 13.7	65.3 ± 29.0
14:0	43.1 ± 14.2	56.6 ± 28.4
16:0	171 ± 36	95.8 ± 46.3*
16:1(n-7)	18.0 ± 4.4	6.0 ± 2.3*
18:0	43.8 ± 10.9	21.0 ± 9.2*
18:1(n-9)	187 ± 34	83 ± 34*
18:2(n-6)	144 ± 24	60 ± 28*
18:3(n-3)	2.3 ± 0.6	0.8 ± 0.5*
20:0	0.7 ± 0.2	0.4 ± 0.2*
20:1(n-9)	2.8 ± 1.8	1.5 ± 0.6
20:2(n-6)	5.8 ± 1.5	2.2 ± 1.1*
20:3(n-6)	4.3 ± 1.3	2.1 ± 1.0*
20:4(n-6)	15.0 ± 3.2	5.9 ± 3.1*
22:4(n-6)	3.7 ± 1.1	1.4 ± 0.6*
22:5(n-3)	1.0 ± 0.3	0.5 ± 0.2*
22:6(n-3)	1.1 ± 0.3	0.6 ± 0.3*
Sum of fatty acids		
8–14	208 ± 47	248 ± 108
16	189 ± 40	102 ± 48*
18–22	412 ± 71	179 ± 78*

[1] Values are means ± SD, $n = 11$. *Different from rats fed FF, $P < 0.05$.

The relative mRNA concentrations of SREBP-1c, FAS, SCD, and IDH1 in the liver and mammary gland did not differ between rats fed FF and those fed the OF (data not shown).

Discussion

This study aimed to elucidate the mechanisms underlying the milk fat-reducing effect of oxidized fat in the lactating organism. To better compare the present findings with those of previous studies (8,9), we also used the rat as a model. The rat has been suggested to be a suitable model, because rats, like humans, develop similar changes in lipid metabolism during pregnancy and lactation (26). In accordance with our previous studies (8,9), we used a fat treated at a relatively low temperature for a long period. This reflects a fat that has been stored for a long time under inappropriate conditions (e.g. warm temperatures, light exposure). Those fats contain high concentrations of primary

TABLE 4 Liver weights, triacylglycerol concentrations in liver and plasma, and concentrations of free fatty acids in plasma of lactating rats fed FF or OF diets[1]

	FF	OF
Liver wet weight		
Absolute, *g*	13.2 ± 1.9	14.4 ± 1.7
Relative, *g/100 g body weight*	4.72 ± 0.38	5.14 ± 0.40*
Liver triacylglycerols, *μmol/g*	59.1 ± 28.4	8.8 ± 2.6*
Plasma triacylglycerols, *μmol/L*	0.80 ± 0.22	0.31 ± 0.10*
Plasma free fatty acids, *μmol/L*	0.42 ± 0.09	0.31 ± 0.03*

[1] Values are means ± SD, $n = 11$. *Different from rats fed FF, $P < 0.05$.

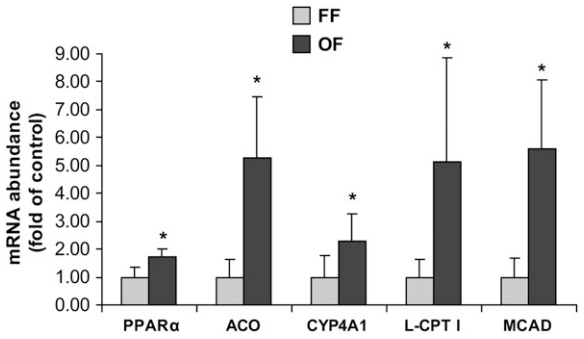

FIGURE 1 Effect of feeding FF or OF diets to lactating rats on mRNA abundance of PPARα and genes involved in fatty acid oxidation, ACO, CYP4A1, L-CPT I, and MCAD in the liver. Bars represent means ± SD ($n = 11$) and are expressed as fold-changes of mRNA abundance in the treatment group (OF) compared with the control group (FF). *Different from rats fed FF, $P < 0.05$.

lipid oxidation products such as hydroxy and hydroperoxy fatty acids that are potent PPARα agonists (27–29). For technical reasons, we did not directly determine the concentrations of these oxidized fatty acids in the dietary fats, but the high POV and the high concentration of conjugated dienes indicate that the OF indeed had high concentrations of these fatty acids. To exclude effects of a different intake of PUFA, we equalized the concentration of linoleic acid of the FF to that of the OF. There were slight differences in the proportions of SFA and monounsaturated fatty acids in both fats, but we assume that these differences contributed less, if at all, to the effects observed in the rats fed the oxidized fat. Because the intake of OF could lead to a

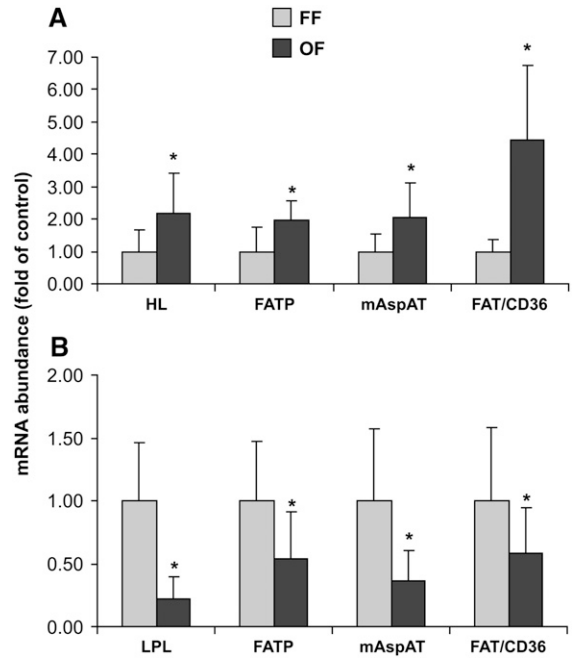

FIGURE 2 Effect of feeding FF or OF diets to lactating rats on mRNA abundance of HL, LPL and fatty acid transporters, FATP, mAspAT, and FAT/CD36 in the liver (*A*) and mammary gland (*B*). Bars represent means ± SD ($n = 11$) and are expressed as fold-changes of mRNA abundance in the treatment group (OF) compared with the control group (FF). *Different from rats fed FF, $P < 0.05$.

diminished food intake (30,31), we used a controlled feeding system in which each rat consumed the same amount of diet to exclude secondary food intake effects. The amount of food administered was 20% less than in preliminary studies but was sufficient to meet the high energy demand of lactating rats even in peak lactation (32).

This study clearly confirmed the findings of our previous study (8) that reduced milk triacylglycerol concentrations by dietary OF are not the result of a reduced de novo-fatty acid synthesis in the mammary gland. This was evidenced by an unaltered mRNA abundance of genes encoding enzymes involved in de novo-fatty acid synthesis such as FAS, SCD, and IDH1 as well as of the lipogenic transcription factor SREBP-1c in the mammary gland of dams fed OF compared with control dams. Furthermore, the concentration of medium-chain fatty acids with 8–14 carbon atoms in the milk, which are mainly synthesized de novo within the mammary epithelial cell (14), did not differ between both groups of dams.

Our study shows that the dietary OF leads to a decreased mRNA abundance of LPL, which mediates utilization of fatty acids from circulating triacylglycerol-rich lipoproteins by the hydrolysis of triacylglycerols, and of fatty acid transporters, which mediate cellular uptake of albumin-bound NEFA, in the mammary gland. In addition, the decreased concentration of plasma triacylglycerols in rats fed OF indicates that the substrate for LPL was also reduced by feeding OF. Fatty acid analysis of milk lipids revealed that the concentrations of long-chain fatty acids with 18–22 carbon atoms, which are hydrolyzed from lipoprotein triacylglycerols by the activity of LPL and largely reflect those of the diet (12,13), were significantly lower in the milk of dams fed OF than in control dams. These findings strongly support our assumption that the reduced milk triacylglycerol concentration in dams fed OF is the consequence of both a diminished uptake of fatty acids from lipoproteins by LPL and a decreased uptake of free fatty acids by fatty acid transporters from plasma into the mammary gland.

Interestingly, in the liver, the mRNA abundance of HL and fatty acid transporters was increased in dams fed OF relative to control dams. This indicates that feeding OF to dams exerted a completely opposing effect on the expression of these genes in the liver compared with the mammary gland. Moreover, relative liver weights and mRNA abundance of PPARα-responsive genes involved in fatty acid catabolism such as ACO, CYP4A1, L-CPT I, and MCAD were increased in the liver of dams fed OF. These findings are in accordance with findings from several recent studies using nonlactating rats and are explained by the strong PPARα-activating effect of OF in the liver (4–6). This effect is also accompanied by induction of peroxisome proliferation contributing to the increase in liver weights in dams fed OF. The strongly reduced triacylglycerol concentrations in the liver of dams fed OF are probably largely explained by the upregulation of hepatic PPARα-responsive genes, which markedly enhances hepatic fatty acid β-oxidation capacity. The latter effect presumably explains that the physiological lactation-induced increase in hepatic triacylglycerol concentrations as observed in lactating rats fed FF was considerably reduced by OF. Because the fatty acid transporters investigated are also PPARα target genes (33,34), we assume that upregulation of those genes in the liver of dams fed OF was also mediated by the PPARα activating effect of OF. Decreased plasma concentrations of NEFA in dams fed OF may be due to an increased uptake of circulating NEFA from the plasma into the liver. Normally, during lactation, the utilization of metabolic substrates such as fatty acids or glucose for the synthesis of milk in the mammary gland is increased and the utilization of substrates for oxidation in other tissues (e.g. liver, skeletal muscle) is reduced (35). The finding that uptake of fatty acids into the liver and their subsequent oxidation increased, whereas uptake of fatty acids into the mammary gland decreased, indicates that the OF impaired normal metabolic adaptations during lactation due to its PPARα-activating effect. Similar observations regarding an impairment of lactation-induced energy-sparing mechanisms (e.g. reduction in heat production by skeletal muscle) by the administration of PPARα activators have been reported from others (36). We cannot exclude the possibility that OF might have provoked similar changes in adaptative thermogenesis, which could be an explanation for the observation that dams fed OF had similar body weights as control dams, although the dams fed OF produced less milk and thus may have required less energy for milk production.

In the mammary gland, the mRNA expression of genes involved in intracellular fatty acid catabolism did not differ between dams of both groups, indicating that reduction in milk triacylglycerols by oxidized fat is not due to an altered rate of fatty acid oxidation in the mammary gland of dams fed OF. However, the PPARα gene shows only a negligible expression in the lactating mammary gland of rodents (37,38), because the physiological expression of PPARα is dramatically decreased in the mammary gland during lactation (38), indicating that oxidation of fatty acids in the lactating mammary gland might be limited. A possible effect of OF on mammary tissue development cannot be ruled out, because we did not perform histological analysis of mammary tissue or determine mammary tissue weights in lactating rats. However, future studies should also address a possible detrimental effect of OF on mammary gland development, because a recent study revealed that pharmacological activation of PPARα during pregnancy impairs mammary gland development and results in a defect of lactation and mortality of the pups (39).

In view of the physiological relevance of milk for the development of the offspring, we also investigated the effect of OF on the development of the suckling pups during lactation. Because maternal milk is the only source of nutrients for the suckling pups, it was not surprising that the reductions in triacylglycerols and, concomitantly, energy in the milk by oxidized fat were accompanied by reduced development of litters during lactation, as also shown previously (8,9). Because the litter weight of lactating rats fed OF was already reduced at d 8 of lactation and thereafter, we assume that the reduction of triacylglycerols in the milk occurred early during lactation, indicating that this effect of OF is mediated even after short-term administration of OF. Therefore, the uptake of oxidized fats by the lactating organism has to be considered critically with regard to the great biological importance of milk fat synthesis for the development of the offspring.

Taken together, this study demonstrates for the first time, to our knowledge, that feeding OF during lactation increases not only the mRNA abundance of genes involved in fatty acid oxidation but also of genes mediating fatty acid uptake in the liver. In contrast, in the mammary gland, the mRNA expression levels of LPL and fatty acid transporters were significantly downregulated by the administration of OF to dams, which was accompanied by significant milk fat reduction. Therefore, these findings suggest that the normal metabolic adaptations during lactation, which promote the utilization of substrates by the mammary gland for the synthesis of milk and reduce the use of substrates for oxidation in other tissues, are disturbed in lactating rats by OF and largely explain the milk fat reducing effect of OF.

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3 Zusammenfassende Diskussion

In den nachfolgenden Abschnitten werden die wichtigsten Ergebnisse der eigenen Arbeiten (**A1-A15**) zusammenfassend dargestellt und deren Relevanz im übergeordneten Kontext diskutiert. Auf eine detaillierte Diskussion der Ergebnisse sei an dieser Stelle verzichtet. Hierfür wird auf die entsprechenden Originalarbeiten verwiesen. Ebenso sind Hintergrund bzw. Zielstellung, Material und Methoden sowie die ausführliche Beschreibung der Ergebnisse den entsprechenden Originalarbeiten zu entnehmen.

3.1 CLA-Isomere als Modulatoren PPARα- und PPARγ-regulierter Prozesse in Zellen der Gefäßwand, Macrophagen und Hepatozyten

Die molekularen Wirkmechanismen, die den antitherogenen Effekten der CLA zugrunde liegen, waren bislang nur unzureichend bekannt. Ihre Aufklärung ist allerdings von großem ernährungsmedizinischem Interesse, da die Atherosklerose und ihre wichtigste Manifestation, die koronare Herzkrankheit (KHK), die häufigste Todesursache in Deutschland darstellen und damit Strategien zur Prävention der Atherosklerose erforderlich sind. Aufgrund der gefäßprotektiven Wirkung pharmakologischer PPARα- und PPARγ-Agonisten lässt sich vermuten, dass CLA als natürlich in der Nahrung vorkommende PPAR-Liganden ihre antiatherogenen Wirkungen zumindest partiell über die Aktivierung von PPARs entfalten. Deshalb wurde der Einfluss von CLA auf solche atherogene Prozesse untersucht, die durch pharmakologische PPARα- bzw. PPARγ-Agonisten moduliert werden. Derartige Prozesse sind die Sekretion inflammatorischer Mediatoren durch Gefäßmuskelzellen [98, 99], die Kollagensynthese und Proliferation von Gefäßmuskelzellen [100-104], die endotheliale Adhäsion zirkulierender Blutzellen und die endotheliale Chemokinsekretion [105-107], die Cholesterinakkumulierung in Macrophagen-Schaumzellen [108-111], die Freisetzung matrixdegradierender Enzyme durch Macrophagen [112-114] und die Cholesterinhomöostase des Hepatozyten [115-120].

3.1.1 Wirkungen in Gefäßmuskelzellen und Endothelzellen

Inflammatorische Prozesse in der Gefäßwand spielen eine Schlüsselrolle in der Pathogenese der Atherosklerose. Hierbei sind neben Zytokinen auch gefäßaktive Stoffe aus der Gruppe der Eicosanoide (Prostaglandine, Leukotriene, Thromboxane) beteiligt, die von immunkompetenten Zellen (Monozyten, Leukozyten), Endothelzellen und glatten Muskelzellen gebildet werden. *In vitro*-Untersuchungen konnten bereits zeigen, dass CLA-Isomere die Freisetzung von Eicosanoiden und anderen gefäßaktiven Substanzen (z.B. Stickstoffmonoxid) aus humanen Endo-

thelzellen modulieren [121-124]. Dies ist im Hinblick auf die pathogenetischen Ereignisse der Atherosklerose bedeutsam, da diese Substanzen in die Blutdruckregulation, die Blutstillung sowie in entzündliche Prozesse involviert sind. Zur Wirkung von CLA in Gefäßmuskelzellen waren bislang keine Studien bekannt, obwohl diese Zellen über die Freisetzung gefäßaktiver Substanzen ebenfalls entscheidenden Einfluss auf die Entstehung der Gefäßdysfunktion und den chronischen Entzündungsprozess in der Gefäßwand ausüben.

Die entsprechend durchgeführten eigenen Untersuchungen ergaben, dass CLA-Isomere zu einer verminderten Prostaglandinfreisetzung aus humanen Gefäßmuskelzellen sowohl unter basalen als auch unter zytokinstimulierten Bedingungen führen [**A1**, **A2**], was weitestgehend mit den Beobachtungen in Endothelzellen und anderen Zellsystemen übereinstimmt [121-126]. Die Verminderung der basalen Prostanoidfreisetzung in Gefäßmuskelzellen, die vor allem für die Aufrechterhaltung der Gefäßhomöostase im intakten Blutgefäß eine wichtige Rolle spielt, ist vermutlich überwiegend auf die Reduzierung des zellulären Arachidonsäurepools sowie verminderter Verfügbarkeit an freier Arachidonsäure zurückzuführen [**A1**, **A2**], da Arachidonsäure als quantitativ bedeutendster Prekursor der Eicosanoidsynthese dient. Untersuchungen an verschiedenen zellulären Systemen ergaben, dass diese Effekte der CLA Folge einer Hemmung der Desaturierung von Linolsäure zu Arachidonsäure, einer verminderten Membraninkorporierung von Arachidonsäure und einer reduzierten $sPLA_2$-katalysierten Arachidonsäurefreisetzung sind [122, 125, 127, 128]. Da CLA-Isomere auch als direkte Inhibitoren von COX-Enzymen beschrieben wurden [65, 129], dürfte ihre Inhibierung zusätzlich die Prostaglandinsynthese in Gefäßmuskelzellen vermindern. Für die Absenkung der zytokininduzierten Eicosanoidfreisetzung durch CLA in Gefäßmuskelzellen dürfte die beobachtete Verminderung der Transkriptkonzentrationen der $cPLA_2$, COX-2 und mPGES verantwortlich sein [**A1**], da diese Gene für Enzyme des Arachidonsäuremetabolismus zu Eicosanoiden kodieren. Obgleich diese Veränderungen auf Transkriptebene nicht auf Proteinebene überprüft wurden, ist aus Studien an RAW264.7-Macrophagen bekannt, dass eine Verminderung der mRNA-Konzentration der COX-2 durch CLA auch mit einer verminderten Proteinexpression der COX-2 korreliert [130]. Zusätzlich vermindert CLA die COX-2-Proteinexpression über eine posttranskriptionelle Regulation des mRNA-Transkripts [130] wie dies in ähnlicher Weise auch für verschiedene pharmakologische Agenzien beschrieben wurde [131, 132]. Bezüglich des zugrunde liegenden Wirkmechanismus konnten die eigenen Untersuchungen zeigen, dass die Verminderung der zytokininduzierten Freisetzung von Prostanoiden aus Gefäßmuskelzellen durch CLA über einen PPARγ-abhängigen Mechanismus erfolgt [**A1**]. Dieser Mechanismus beruht darauf, dass die CLA-vermittelte Aktivierung des PPARγ-Signalweges eine Inhibierung der transkriptionel-

len Aktivität des NF-κB bewirkt [**A1**]. Da der NF-κB als Schlüsseltranskriptionsfaktor in der Regulation inflammatorischer Gene (COX-2, $cPLA_2$, mPGES) fungiert, wird durch dessen Inhibierung konsekutiv die Eicosanoidfreisetzung vermindert. Diese Beobachtung deckt sich mit Befunden in anderen Zellsystemen, in denen CLA ebenfalls eine PPARγ-abhängige Hemmung der NF-κB-Transaktivierung bewirkt [30, 133]. Ebenso stimmt dies mit der Beobachtung überein, dass eine Aktivierung des PPARγ durch pharmakologische PPARγ-Liganden die NF-κB-DNA-Bindungsaktivität und NF-κB-Zielgene (COX-2, iNOS) in Gefäßmuskelzellen inhibiert [134, 135]. Eine Induktion von Enzymen der Eicosanoidsynthese ($cPLA_2$, COX-2, mPGES) wird als Folge einer Gefäßschädigung in aktivierten Gefäßmuskelzellen beobachtet [136, 137]. Die resultierende exzessive Prostanoidfreisetzung beeinträchtigt die Gefäßhomöostase, fördert die Gefäßdysfunktion und bestimmt daher maßgeblich die atherosklerotische Läsionsentwicklung [138-140]. Daher stellt die beobachtete antiinflammatorische Wirkung der CLA in Gefäßmuskelzellen einen potenziell antiatherogenen Effekt dar, der zumindest partiell die inhibitorische Wirkung von CLA auf die atherosklerotische Läsionsentwicklung erklären kann. Die beobachtete Involvierung des PPARγ in der Vermittlung dieses antiinflammatorischen Effektes der CLA unterstreicht den vaskuloprotektiven Einfluss einer PPARγ-Aktivierung. Aufgrund des großen Stellenwerts pharmakologischer COX-2-Inhibitoren in der Behandlung der Atherosklerose und anderer entzündlicher Erkrankungen, deutet die beobachtete COX-2-Hemmung darüber hinaus auf ein großes ernährungspräventives und -therapeutisches Potenzial der CLA für andere entzündliche Erkrankungen hin.

Während die Modulation inflammatorischer Prozesse durch CLA bereits seit längerem Gegenstand intensiver Forschung ist [30, 125, 126, 141, 142], fand die Beeinflussung fibrotischer Prozesse durch CLA bislang kaum Beachtung. Trotzdem sind derartige Prozesse im Rahmen der Atherogenese von enormer Bedeutung, da fibrotische Prozesse zu massiver extrazellulärer Deposition von Proteinen wie Kollagen, Elastin, Glykoproteinen und Proteoglykanen in der Gefäßwand führen und damit entscheidend zur Intimaverdickung und atherosklerotischen Plaqueentwicklung beitragen. Kollagen, insbesondere Typ I-Kollagen, ist dabei das dominierende extrazelluläre Matrixprotein atherosklerotischer Plaques mit einem Proteinanteil von etwa 60% [143]. Da mitogen- bzw. zytokinaktivierte Gefäßmuskelzellen die Hauptproduzenten für Kollagen in der Gefäßwand darstellen [144], wird die Kollagenbildung durch aktivierte Gefäßmuskelzellen als kritische Stufe der Atherogenese angesehen. Die eigenen Untersuchungen an vaskulären Gefäßmuskelzellen konnten erstmalig zeigen, dass CLA-Isomere die mitogenstimulierte Kollagenbiosynthese hemmen [**A3**]. Da pharmakologische PPARγ-Agonisten ebenfalls eine potente Hemmung der Kollagenbiosynthese in Gefäßmuskelzellen

und anderen Zelltypen bewirken [100, 145-147], wurde auch die Involvierung des PPARγ-Signalweges in der Vermittlung dieses CLA-Effektes untersucht. Dabei zeigte sich, dass die CLA-induzierte Hemmung der mitogeninduzierten Kollagenbiosynthese durch Antagonisierung des PPARγ aufgehoben wird [**A3**], was die Bedeutung des PPARγ-Signalweges für diesen zellulären Effekt der CLA deutlich macht. Als Wirkmechanismus für die PPARγ-vermittelte Hemmung der Kollagenbiosynthese konnte die Hemmung der TGF-β-induzierten Expression des *connective tissue growth factors* (CTGF), der als bedeutender Stimulator der zellulären Kollagenbiosynthese wirkt [148-150], identifiziert werden [100]. Da eine Inhibierung des NF-κB mit einer Hemmung des TGF-β-Signalweges und der zellulären Kollagenbiosynthese einhergeht [151, 152], lässt sich vermuten, dass die mit beiden CLA-Isomeren beobachtete Hemmung der TNFα-induzierten NF-κB-DNA-Bindungsaktivität in Gefäßmuskelzellen [**A1**] auch für deren hemmende Wirkung auf die Kollagenbiosynthese verantwortlich ist. Allerdings muss als potenzieller Wirkmechanismus für die Inhibierung der Kollagenbiosynthese durch CLA auch die beobachtete Hemmung des Arachidonsäuremetabolismus durch CLA in Gefäßmuskelzellen in Betracht gezogen werden [**A1**, **A2**]. So konnte gezeigt werden, dass COX- und Lipoxygenaseprodukte der Arachidonsäure wie Prostaglandine, Thromboxane und Leukotriene die Kollagenbildung im Rahmen pathologisch-fibrotischer Prozesse fördern, indem sie die Bildung profibrotischer Faktoren wie TGF-β1 stimulieren [153]. Im Gegensatz dazu führt eine Inhibierung bzw. Antagonisierung spezifischer Eicosanoidrezeptoren zur Abschwächung fibrotischer Prozesse und Verminderung der TGF-β1-Bildung [154-156]. Im Unterschied zur Wirkung hochaffiner PPARγ-Liganden wie Troglitazon oder Rosiglitazon, die neben einer Hemmung der Kollagenbiosynthese auch eine Inhibierung der mitogeninduzierten DNA-Synthese in Gefäßmuskelzellen bewirken [157, 158], war die Wirkung von CLA-Isomeren auf die Kollagenbiosynthese nicht von einer verminderten Zellproliferation begleitet [**A3**]. Dies deutet auf gewisse Unterschiede im Wirkmechanismus von CLA-Isomeren gegenüber hochaffinen PPARγ-Liganden hin, die möglicherweise durch zusätzliche PPARγ-unabhängige Effekte der CLA-Isomere in Gefäßmuskelzellen hervorgerufen werden. Derartige Effekte könnten über eine Beeiflussung der Membranfluidität und membranständiger Rezeptoren vermittelt sein.

Insgesamt zeigen die eigenen Untersuchungen an humanen Gefäßmuskelzellen [**A1**, **A2**, **A3**], dass CLA-Isomere nicht nur inflammatorische Prozesse, sondern auch fibrotische Prozesse über einen PPARγ-abhängigen Mechanismus beeinflussen. Da die Ablagerung von Kollagen und anderer extrazellulärer Matrixproteine in der Gefäßwand maßgeblich zur Bildung atherosklerotischer Plaques und damit zur Entstehung klinischer Manifestationen der Atherosklerose (Myokardinfarkt, ischämischer Apoplex) beiträgt, stellt die CLA-induzierte Hemmung der

Kollagenbildung durch Gefäßmuskelzellen einen günstigen, antiatherogenen Wirkmechanismus dar. Diese gefäßprotektive Wirkung der CLA könnte daher die in tierexperimentellen Studien beobachtete reduzierte Bildung atherosklerotischer Plaques erklären. Im Unterschied zur Wirkung hochaffiner PPARγ-Liganden war die Hemmung der Kollagensynthese durch CLA aber nicht mit einer Hemmung der Gefäßmuskelzellproliferation verbunden, die ebenfalls eine entscheidende Rolle in der Pathogenese atherosklerotischer Plaques und restenosebedingter Gefäßwandläsionen spielt [159-160]. Aufgrund der erwiesenen atheroprotektiven Wirkung einer gehemmten Zellproliferation ist daher anzunehmen, dass die antiatherogene Wirkung pharmakologischer PPARγ-Agonisten derjenigen von nutritiven PPARγ-Agonisten überlegen ist.

Endothelzellen sind insbesondere in der Frühphase der Atherogenese am Entstehungsprozess der Atherosklerose beteiligt. Diese werden als Folge von wiederholt oder kontinuierlich einwirkenden schädigenden Einflüssen (z.B. Bluthochdruck, erhöhtes Serumcholesterin) in ihrer Funktion beeinträchtigt (Endotheldysfunktion). Die Endotheldysfunktion ist durch eine durch Zytokine und Wachstumsfaktoren vermittelte Aktivierung der Endothelzellen gekennzeichnet, die im Wesentlichen die folgenden zwei Prozesse in Gang setzt: Zum einen exprimieren die Endothelzellen verstärkt Adhäsionsmoleküle auf ihrer Zelloberfläche und ermöglichen so den Leukozyten am Endothel zu adhärieren und durch die Endothelbarriere in den Subendothelialraum zu migrieren [161, 162]. Zum anderen bilden sie durch Migration, ausgehend von den Vasa vasorum, neue Gefäße, die in die Intima einwachsen und so für eine Neovaskularisation atherosklerotischer Gefäßabschnitte sorgen. Da die Adhäsionsmoleküle nicht nur von den dem Gefäßlumen zugewandten Endothelzellen exprimiert werden, was der initialen Leukozytenrekrutierung dient, sondern auch von den neovaskulären Endothelzellen, eröffnet sich durch diese Neovaskularisation ein zusätzlicher Weg des Einstroms an Immunzellen in das Entzündungsgebiet und beschleunigt so die Läsionsentwicklung. Die eigenen Untersuchungen zur Wirkung von CLA-Isomeren auf die zytokininduzierte Oberflächenexpression von Adhäsionsmolekülen ergaben keine Hinweise auf eine Modulation der adhäsiven Eigenschaften von humanen Aorta-Endothelzellen durch CLA-Isomere [**A4**]. Dies konnte auch in einem *in vitro*-Monozyten-Endothelzell-Adhäsionstest bestätigt werden [**A4**]. Ferner zeigte sich, dass die TNFα-induzierte endotheliale Freisetzung von Chemokinen wie IL-8 und MCP-1, welche die Rekrutierung zirkulierender Leukozyten zum aktivierten Gefäßendothel vermitteln [163], durch CLA-Isomere nicht verändert wird [**A4**]. In Übereinstimmung mit diesen Befunden konnte auch gezeigt werden, dass sich die DNA-Bindungsaktivität des Transkriptionsfaktors NF-κB, der eine zentrale Rolle in der transkriptionellen Induzierung von Adhäsionsmolekülen und Chemokinen durch Zytokine spielt [164-166], in CLA-behandelten Endothelzellen im Vergleich zu

Kontrollzellen nicht beeinflussen lässt [**A4**]. Trotzdem wurde der PPARγ, der als negativer Regulator des NF-κB-Signalweges die endotheliale Freisetzung von Chemokinen und die Expression von Adhäsionsmolekülen vermindert [106, 165, 167], durch Behandlung der Endothelzellen mit CLA-Isomeren in seiner transkriptionellen Aktivität moderat erhöht [**A4**]. Möglicherweise ist diese nur mäßig ausgeprägte Aktivierung für die fehlende Repression der zytokininduzierten NF-κB-DNA-Bindungsaktivität und Adhäsionsmolekülexpression durch CLA verantwortlich. Diese Annahme wird gestützt durch Befunde an anderen zellulären Systemen, in denen eine deutlich stärkere PPARγ-Transaktivierung durch CLA mit einer potenten Hemmung der stimulusinduzierten NF-κB-Transaktivität verbunden war [30, 133, 168, **A1**].

Obwohl eine direkte Extrapolation dieser Zellkulturbefunde auf die *in vivo*-Situation nicht möglich ist, lässt sich aus den eigenen Untersuchungen an Aorta-Endothelzellen schlussfolgern, dass die antiatherogene Wirkung von CLA vermutlich nicht über eine Reduzierung der Monozytenadhäsion am Gefäßendothel, welche als wichtiger Schritt in der Frühphase der Atherosklerose gilt, erklärt werden kann. Die Beobachtung aus einer Humanstudie, dass Supplementierung mit einer CLA-Mischung keine Veränderung der Serumkonzentration an zirkulierenden Adhäsionsmolekülen im Serum bewirkte [169], unterstützt diese Vermutung. Allerdings legt dies die Vermutung nahe, dass CLA ihre tierexperimentell ermittelten antiatherogenen Effekte über andere Mechanismen oder während der Spätphase der Atherosklerose ausüben.

3.1.2 Wirkungen in Macrophagen

Macrophagen und cholesterinbeladene Macrophagen-Schaumzellen sind kritische und zugleich dominierende zelluläre Bestandteile früher und fortgeschrittener atherosklerotischer Läsionen. Diese Zellen sezernieren eine Vielzahl chemotaktischer Substanzen und beschleunigen damit die Einwanderung weiterer Leukozyten, Monozyten und inflammatorischer Lymphozyten in die Gefäßwand, was die atherosklerotische Läsionsentwicklung fördert. In den eigenen Arbeiten zur Wirkung von CLA-Isomeren auf atheroskleroseassoziierte Prozesse in Macrophagen wurden humane PMA-differenzierte THP-1-Zellen und murine RAW264.7-Macrophagen verwendet. Beide Zelltypen sind weithin akzeptierte und häufig genutzte Macrophagenmodelle zur Untersuchung der Wirkung von nutritiven und pharmakologischen Substanzen auf atherosklerose relevante Prozesse [170].

Die eigenen Untersuchungen an acLDL-transformierten RAW264.7-Macrophagen-Schaumzellen konnten deutlich zeigen, dass CLA-Isomere die Cholesterinakkumulierung in Macrophagen-Schaumzellen vermindern und gleichzeitig den Cholesterinefflux auf extra-

zelluläre Akzeptoren erhöhen [**A5**]. Die CLA-induzierte zelluläre Cholesterinverminderung war mit signifikanten Veränderungen in den mRNA- bzw. Protein-Konzentrationen verschiedener in die Cholesterinhomöostase involvierter Gene verbunden [**A5**]. Die infolge der Behandlung mit CLA beobachtete Induzierung von NPC-1 und NPC-2, die den intrazellulären Cholesterintransport vom späten Endosom bzw. reifen Lysosom zur Plasmamembran vermitteln [171, 172], durch CLA-Isomere trägt vermutlich über einen gesteigerten Cholesterintransport zur Plasmamembran zu einer erhöhten Verfügbarkeit von Cholesterin für Effluxsysteme wie ABCA1 bei [19]. Die Induktion von ABCA1 durch CLA-Isomere erklärt sich vermutlich über deren stimulierende Wirkung auf die Expression des LXRα [**A5**], welcher als positiver Regulator der ABCA1-Gentranskription wirkt [108, 173, 174]. Da trotz Induktion des Scavengerrezeptors CD36, welcher die zelluläre Aufnahme modifizierter LDL-Partikel und damit den Cholesterininflux vermittelt, die Cholesterinakkumulierung durch CLA vermindert war [**A5**], ist zu vermuten, dass beide CLA-Isomere über die Stimulierung der Cholesterinexportwege (NPC-1, NPC-2, ABCA1) einen Nettoefflux an Cholesterin bewirkten. Ein erhöhter Cholesterinefflux aus den CLA-behandelten Macrophagen-Schaumzellen konnte in der Tat in einem *in vitro*-Efflux-Assay unter Verwendung von HDL als extrazellulärem Cholesterinakzeptor nachgewiesen werden [**A5**]. Insgesamt lassen die eigenen Befunde den Schluss zu, dass CLA-Isomere ihre Wirkung auf die Cholesterinhomöostase in Macrophagen-Schaumzellen primär auf transkriptioneller Ebene ausüben. Da die Verminderung der Cholesterinakkumulierung und die Erhöhung des Cholesterinefflux in Macrophagen-Schaumzellen durch pharmakologische PPARα- oder PPARγ-Agonisten über ähnliche Mechanismen erfolgt [108, 110], ist zu vermuten, dass die durch CLA-Isomere verursachten Effekte ebenfalls über eine Aktivierung von PPARs zustande kommt. Allerdings kann auf Basis der durchgeführten Experimente und des verwendeten Zellmodells nicht beantwortet werden, ob beide PPAR-Subtypen oder ein spezifischer Subtyp für die Vermittlung dieser CLA-Effekte verantwortlich sind.

Im Gegensatz zu diesen potenten Effekten von CLA auf die Cholesterinhomöostase in RAW264.7-Macrophagen-Schaumzellen, zeigten die CLA-Isomere keine Wirkung auf die mRNA-Expression und enzymatische Aktivität der Matrixmetalloproteinasen (MMP) MMP-2 und MMP-9 in humanen PMA-differenzierten THP-1-Macrophagen [**A6**]. Auch die Gentranskription der MMP-Inhibitoren TIMP-1 und TIMP-2, die entscheidend für die Regulation der MMP-Aktivität in Macrophagen sind [175, 176], wurde durch Behandlung mit CLA-Isomeren nicht beeinflusst [**A6**]. MMP sind proteolytische Enzyme, die zu einem Abbau extrazellulärer Matrixproteine führen und damit den vaskulären und inflammatorischen Zellen eine Invasion und Gewebsdurchquerung erlauben [177, 178]. Innerhalb der atherosklerotischen Plaque gilt die

MMP-Freisetzung als zentraler Schritt, der zur Ausdünnung der schützenden fibrösen Kappe der Plaque beiträgt und somit die Plaqueruptur fördert [179]. Die vollständige Plaqueruptur oder die Ablösung einzelner Plaqueteile kann zur Embolisierung von Gefäßen oder über die Aktivierung des Gerinnungssystems zur Thrombosierung mit nachfolgender Okklusion des Gefäßes führen. *In vitro*-Studien konnten zeigen, dass Behandlung von Macrophagen mit pharmakologischen PPARγ-Aktivatoren die Freisetzung und enzymatische Aktivität von MMP-9 hemmt [112, 180, 181], was in den eigenen Untersuchungen unter Verwendung von Troglitazon bestätigt werden konnte [**A6**]. Da PPARγ vor allem im Schulterbereich atherosklerotischer Läsionen durch Monozyten und Macrophagen exprimiert wird [112, 180], einer Lokalisation, an der die Plaque häufig rupturiert, gelten PPARγ-Aktivatoren auch als vielversprechende Substanzen zur Stabilisierung atherosklerotischer Läsionen [112]. Die ebenfalls in der eigenen Arbeit beobachtete erhöhte PPARγ-Transaktivierung und gleichzeitig reduzierte DNA-Bindungsaktivität des NF-κB durch Troglitazon stellen vermutlich die molekulare Grundlage für diesen Effekt dar [**A6**], der auch von anderen Arbeitsgruppen beschrieben wurde [180, 181]. Da keines der untersuchten CLA-Isomere weder auf die DNA-Bindungsaktivität des PPARγ noch auf die des NF-κB einen Effekt hatte, ist zu vermuten, dass dies der Grund für den fehlenden Effekt auf die MMP-Genexpression und -Aktivität in PMA-differenzierten THP-1-Macrophagen ist. Neben den bereits angeführten potenziellen Ursachen für den ausbleibenden Effekt [**A6**], ist ferner nicht auszuschliessen, dass CLA in diesem Zellmodell Einfluss auf die Rekrutierung und Freisetzung von Coaktivatoren bzw. Corepressoren der PPARs nehmen. Diese Cofaktoren sind neben der Ligandenverfügbarkeit ebenfalls entscheidend für die transkriptionelle Aktivität der PPARs [182-185]. Die Bedeutung der PPAR-Cofaktoren lässt sich auch daran ermessen, dass deren zellspezifische Expression auch für die zell- bzw. gewebespezifische PPAR-Antwort mitverantwortlich ist. Allerdings ist ein zellspezifischer Mangel an PPAR-Cofaktoren in dem verwendeten Zellsystem nicht als Ursache für den ausbleibenden Effekt anzunehmen. Es wäre eher denkbar, dass CLA-Isomere in dem verwendeten Zellsystem über eine Aktivierung alternativer Signalwege und Transkriptionsfaktoren eine Kompetition um transkriptionelle Cofaktoren bewirken, mit der Folge, dass die PPAR-Transaktivierung verhindert oder abgeschwächt wird. Deshalb müssen zukünftige Studien klären, ob CLA-Isomere möglicherweise die MMP-Freisetzung aus anderen Zellen wie beispielsweise Gefäßmuskelzellen beeinflussen. In Gefäßmuskelzellen, deren Funktion in entscheidender Weise durch CLA-Isomere modulierbar ist [**A1**, **A2**, **A3**], wird die MMP-Expression und -Aktivität durch synthetische PPARγ-Aktivatoren gehemmt [186]. Diese Wirkung hemmt auch die Gefäßmuskelzellmigration [186], da diese aufgrund der verminderten MMP-Aktivität das sie

umgebende Matrixgeflecht nicht durchbrechen können. Gleichzeitig wird die migrationshemmende Wirkung hochaffiner PPARγ-Liganden vermutlich dadurch verstärkt, dass diese eine antiproliferative Effektivität besitzen [157, 158]. *In vivo* verhindert eine MMP-Hemmung die Durchquerung der die Gefäßmedia und Intima trennenden Lamina elastica interna und Invasion der Intima durch die Gefäßmuskelzellen und inhibiert somit die Plaqueprogression.

Insgesamt zeigen die eigenen Untersuchungen an Macrophagen, dass CLA-Isomere in Abhängigkeit des Zellmodells und der untersuchten Parameter differenzielle Effekte ausüben. Die in RAW264.7-Macrophagen-Schaumzellen beobachteten Effekte auf die Cholesterinhomöostase sind als potenziell günstig im Hinblick auf die Atheroskleroseprävention anzusehen, da ein erhöhter Cholesterinefflux der Schaumzellbildung und der damit verbundenen Cholesterinakkumulierung in der Gefäßwand entgegenwirkt. Daher könnte dieser zelluläre Effekt der CLA zumindest teilweise einen Erklärungsansatz für die *in vivo* beobachtete antiatherogene Wirkung der CLA liefern. Dagegen deuten die eigenen Untersuchungen in THP-1-Macrophagen daraufhin, dass CLA-Isomere im Gegensatz zu pharmakologischen PPARγ-Aktivatoren nicht in der Lage sind, die MMP-vermittelte Degradation extrazellulärer Matrixproteine in der Gefäßwand, welche die Ruptur fortgeschrittener atherosklerotischer Läsionen und die Migration glatter Muskelzellen in der Gefäßwand begünstigt, zu beeinflussen.

3.1.3 Wirkungen in Hepatozyten

Neben direkten Effekten von CLA auf die Gefäßwand, werden die vaskuloprotektiven Wirkungen der CLA möglicherweise auch indirekt über eine günstige Beeinflussung atherogener Risikofaktoren wie die Absenkung des Gesamt- und LDL-Cholesterins vermittelt. Hierauf deuten Ergebnisse aus tierexperimentellen und Humanstudien hin [187-191]. Von zentraler Bedeutung für die Gesamt- und LDL-Cholesterinkonzentration im Plasma ist die Leber, da diese über den hepatischen LDL-Rezeptor, der etwa 2/3 des Gesamtkörper-LDL-Rezeptorpools ausmacht, maßgeblich an der Eliminierung der LDL-Partikel aus dem Plasma beteiligt ist. Patienten mit genetischem Defekt im LDL-Rezeptorgen weisen entsprechend massiv erhöhte Plasmacholesterinspiegel auf und versterben unbehandelt oftmals innerhalb der ersten beiden Lebensjahrzehnte an den Folgen einer frühzeitigen Koronar- oder Zerebralsklerose. Die eigenen Untersuchungen an der immortalisierten Leberzelllinie HepG2 zeigen, dass CLA eine aktivierende Wirkung auf Gene der Cholesterinhomöostase (LDL-Rezeptor, HMG-CoA-Reduktase) und der SREBPs (1 und 2) entfalten [**A7**]. So führte Behandlung mit t10c12-CLA zu einer erhöhten LDL-Rezeptorpromotoraktivität und erhöhten Transkriptkonzentrationen von HMG-CoA-Reduktase, SREBP-1 und SREBP-2. Darüber hinaus war der

^{14}C-Acetat-Einbau in das zelluläre Cholesterin durch t10c12-CLA erhöht, was auf eine stimulierte Cholesterinsynthese hinweist. Obgleich eine Beeinflussung der Prozessierung der SREBPs durch CLA nicht untersucht wurde, deuten die erhöhten Transkriptkonzentrationen der SREBP-Zielgene (HMG-CoA-Reduktase, LDL-Rezeptor) auf eine erhöhte Ausreifung der inaktiven SREBP-Vorstufen zu den transkriptionell aktiven nukleären SREBPs hin. Mittels RNA-Interferenz konnte ferner gezeigt werden, dass der Effekt von t10c12-CLA auf die LDL-Rezeptor- und HMG-CoA-Reduktase-Transkriptkonzentrationen abhängig von der Induzierung der SREBPs ist [**A7**]. Zusätzlich konnte eine Induzierung der LDL-Rezeptorexpression über SREBP-unabhängige Regulationsmechanismen wie dem AP-1-Signalweg als Wirkmechanismus ausgeschlossen werden [**A7**]. Ähnliche Effekte von CLA auf die Genexpression von SREBP-regulierten Genen wurden auch von anderen Arbeitsgruppen in HepG2-Zellen [192], Macrophagen [193] und in der Leber von Mäusen beschrieben [194]. Legt man die potente Bindung und Aktivierung von PPARs durch CLA [27, 28, 195] und die zelluläre Expression der PPARs in HepG2-Zellen zugrunde [196-199], so könnten die durch CLA beobachteten Effekte auf die SREBP-regulierte Gentranskription in HepG2-Zellen ebenfalls in Zusammenhang mit einer Aktivierung von PPARs stehen. Diese Annahme wird durch zahlreiche Studien gestützt, die eine transkriptionelle Aktivierung der SREBPs und eine Stimulierung der SREBP-abhängigen Cholesterinsynthese in der Leber oder kultivierten Leberzellen durch PPARα-Agonisten nachweisen [115-120, 200, 201]. Ähnliche Befunde wie mit PPARα-Aktivatoren wurden auch mit PPARγ-Liganden erzielt. Diese bewirken in gleicher Weise wie PPARα-Agonisten eine Induktion von SREBP-2-Zielgenen in Macrophagen [202] und eine erhöhte Genexpression und Aktivität des LDL-Rezeptors in HepG2-Zellen [203]. Auch wenn diesbezüglich gegenteilige Effekte beschrieben wurden [204], deuten die Befunde aus Zellkulturstudien mehrheitlich daraufhin, dass PPARγ-Aktivierung ebenfalls eine transkriptionelle Aktivierung der SREBPs bewirkt. Ob die Aktivierung des PPARα- oder PPARγ-Signalweges oder beider Signalwege für die Vermittlung des beobachteten Effektes der CLA verantwortlich ist, kann allerdings aus den eigenen Untersuchungen nicht abgeleitet werden. Zukünftige Studien unter Verwendung von PPAR-Subtyp-spezifischen Knockdown-Zellen sollten hierüber Auskunft geben.

Da der hepatische LDL-Rezeptor von herausragender Bedeutung für die Eliminierung von VLDL- und LDL-Partikeln aus dem Plasma ist, könnte die t10c12-CLA-induzierte Hochregulation des hepatischen LDL-Rezeptors einen Erklärungsansatz für die in einigen tierexperimentellen und Humanstudien beobachtete Absenkung des Gesamt- und LDL-Cholesterins durch CLA liefern [187-191, 205-207]. Allerdings bleibt unklar, ob eine Reduzierung der Plasmacholesterinkonzentration tatsächlich zur antiatherogenen Wirkung der CLA beiträgt. Während in

einigen tierexperimentellen Studien die Absenkung der Plasmacholesterinkonzentration durch CLA mit einer Inhibierung der atherosklerotischen Läsionsentwicklung korrelierte [190, 205, 207], war in anderen Studien die antiatherogene Wirkung mit unveränderten [208] oder sogar erhöhten [209] Cholesterinkonzentrationen im Plasma (Gesamt-C, LDL-C) verbunden.

3.2 Metabolisierung von CLA-Isomeren in Zellen der Gefäßwand

Aus Arbeiten der eigenen Arbeitsgruppe war bereits bekannt, dass CLA-Isomere in die Zelllipide humaner Blutgefäßzellen dosisabhängig inkorporiert werden [122, **A1**, **A2**, **A3**, **A4**]. Ähnlich hohe CLA-Konzentrationen in den Zelllipiden von Endothelzellen und Gefäßmuskelzellen lassen den Schluss zu, dass sich Blutgefäßzellen unterschiedlicher Gefäßwandabschnitte (Initima, Media) nur marginal in ihrer Aufnahmekapazität für CLA unterscheiden. Auch die Art des Blutgefäßes (Aorta vs. Koronararterie), aus dem die Zellen stammen, hat hierauf nur geringen Einfluss [**A2**]. Im Gegensatz dazu weisen andere Zelltypen wie Leberzellen oder Macrophagen selbst bei höheren Inkubationskonzentrationen deutlich geringere CLA-Konzentrationen in den Zelllipiden nach Behandlung mit CLA auf [123, 210]. Dies deutet insgesamt auf eine hohe Aufnahmekapazität der Blutgefäßzellen für CLA im Vergleich zu anderen Zelltypen hin. Gleichzeitig zeigt dies eine gute nutritive Modulierbarkeit der Fettsäurezusammensetzung von Blutgefäßzellen an. In Bezug auf die Metabolisierung von CLA-Isomeren in Blutgefäßzellen standen allerdings bislang keine Literaturdaten zur Verfügung. Gleichwohl ist bekannt, dass spezifische CLA-Metabolite eine hohe biologische Aktivität besitzen [64, 65] und somit auch als potenzielle Mediatoren antiatherogener Effekte in Betracht gezogen werden müssen. So konnte bereits nachgewiesen werden, dass ein konjugierter C20:2-Metabolit der t10c12-CLA die Aktivität der Lipoproteinlipase, ein Enzym des Kapillarendothels, *in vitro* hemmt [64]. Dies deutet auf die Möglichkeit einer Beeinflussung der Endothelfunktion durch CLA-Metabolite hin. Auch die Beobachtung, dass COX-Enzyme nicht nur durch CLA-Isomere [65, 129], sondern auch durch CLA-Metabolite wirkungsvoll inhibiert werden [65], unterstützt diese Annahme. COX-Enzyme spielen eine wichtige Rolle im enzymatischen Arachidonsäuremetabolismus zu biologisch hochaktiven Substanzen und beeinflussen daher entscheidend die Endothelfunktion, die Gefäßhomöostase und damit die Entstehung atherosklerotischer Gefäßveränderungen [139, 211]. Die im Rahmen dieser Arbeit vorgenommenen isomerspezifischen und dosisabhängigen Untersuchungen zur Bildung von CLA-Metaboliten in Endothelzellen und Gefäßmuskelzellen konnten zeigen, dass unterschiedliche CLA-Isomere zu mittel- (C16) und langkettigen (C20, C22) Fettsäuren unter Beibehaltung der charakteristischen konjugierten Doppelbindung umgesetzt werden [**A8**, **A9**, **A10**]. Diese Beobachtungen decken

sich mit Befunden in anderen Zellsystemen bzw. tierischen Geweben [64, 212-215] und deuten daraufhin, dass CLA in Blutgefäßzellen offensichtlich in ähnlicherweise metabolisiert werden wie in anderen tierischen Geweben. Die in Endothelzellen [**A8**] und Gefäßmuskelzellen [**A9**] nachgewiesenen konjugierten C16:2-Metabolite der CLA sind nach Ansicht der meisten Untersucher in der Literatur ein peroxisomales β-Oxidationsprodukt der CLA [213]. Interessanterweise liess sich in den eigenen Studien beobachten, dass diese konjugierten C16:2-Metabolite zwar in den mit CLA behandelten Blutgefäßzellen nachzuweisen sind, nicht jedoch der entsprechende C16:2-Metabolit der Linolsäure in den mit Linolsäure behandelten Zellen. Diese offensichtliche Akkumulierung von konjugierten C16:2-Metaboliten in CLA-behandelten Blutgefäßzellen deutet möglicherweise daraufhin, dass die weitere β-Oxidation der CLA durch CLA oder deren Metabolite gehemmt wird [**A8**, **A9**]. Im Unterschied zu CLA-behandelten Leukämiezellen und Lebergewebe CLA-gefütterter Ratten [212-215] konnten in Blutgefäßzellen allerdings keine konjugierten C20:3- und C20:4-Metabolite der CLA-Isomere nachgewiesen werden. Dieser Gegensatz ist vermutlich auf die äußerst geringe Kapazität der Zellen der Blutgefäßwand zur enzymatischen Desaturation von ungesättigten Fettsäuren im Vergleich zur Leber zurückzuführen [216-218]. Allerdings konnten in Blutgefäßzellen konjugierte C22:2-Elongationsprodukte der CLA nachgewiesen werden [**A8**, **A9**], die bislang nicht in der Literatur beschrieben wurden. Dieser Befund bestätigt frühere Beobachtungen, dass Gefäßmuskelzellen einerseits eine geringe enzymatische Kapazität zur Desaturation, andererseits eine hohe enzymatische Kapazität zur Elongation mehrfach ungesättigter Fettsäuren besitzen [217-219]. Darüber hinaus zeigen die Untersuchungen an Blutgefäßzellen, dass die Bildung der konjugierten C22:2-Metabolite CLA-Isomer-spezifisch erfolgt [**A8**, **A9**].

Insgesamt deuten die in den eigenen Untersuchungen nachgewiesenen CLA-Metabolite daraufhin, dass CLA-Isomere in der Gefäßwand über bekannte Wege des Fettsäurenstoffwechsels (β-Oxidation, Elongation) metabolisiert werden. Im Unterschied zur Leber werden diese Fettsäuren in der Gefäßwand aber entweder nicht oder in nur sehr begrenztem Ausmaß zu höher ungesättigten Fettsäuren desaturiert. Berücksichtigt man die potenten biologischen Wirkungen spezifischer CLA-Metabolite wie CD20:2t12c14 in zellulären Systemen [64], so ist nicht auszuschliessen, dass verschiedene gefäßprotektive Effekte von CLA in Blutgefäßzellen nicht nur über die CLA-Isomere selbst, sondern auch über deren Metabolite verursacht werden. Zukünftige Studien unter Verwendung isolierter CLA-Metabolite sollten hierüber Auskunft geben.

3.3 Beeinflussung einer alkoholischen Steatose durch CLA und oxidiertes Fett

Die Steatose (Lipidspeicherung) ist der früheste histologisch erkennbare pathologische Leberbefund bei Alkoholikern und findet sich bei über 90% der chronischen Alkoholiker. Bei Steatose der Leber machen Lipide bis zu 50% des Lebergewichtes aus. Je nach morphologischem Typ der Lipidspeicherung enthält die Leberzelle einen großen Fetttropfen, der den Zellkern an den Rand drängt, oder das Zytoplasma ist von multiplen kleinen Fetttropfen ohne Verdrängung des Kernes angefüllt. Der Grad der Steatose kann von einzelnen disseminierten verfetteten Leberzellen bis zu einer diffusen Verfettung des Leberparenchyms variieren [220]. Die Gefahr der Steatose besteht darin, dass sich bei ca. 20-40% der Alkoholiker daraus eine alkoholische Steatohepatitis entwickelt, die häufig in eine irreversible Leberzirrhose fortschreitet [221, 222]. Daher sind Strategien zur Behandlung der Steatose von großer Relevanz. Auf molekularer Ebene lässt sich die alkoholische Fettleber vor allem auf eine ethanolinduzierte Blockierung des PPARα und nachfolgend beeinträchtigtem Fettsäurenkatabolismus sowie eine Aktivierung der SREBP-1c-regulierten Lipogenese zurückführen [223, 224]. Aus der Eigenschaft oxidierten Fettes bzw. von CLA, den hepatischen PPARα und nachfolgend den Fettsäurenkatabolismus in der Leber zu aktivieren, leitete sich die Fragestellung ab, ob derartige Fette in der Lage sind, der Entstehung einer ethanolinduzierten Fettleber entgegenzuwirken. Als Untersuchungsmodell wurde ein für derartige Fragestellungen häufig genutztes Nagermodell verwendet [225]. Die Verabreichung des Ethanols erfolgte über die etablierte Lieber-DeCarli-Flüssigdiät [226]. Mit dieser Diät lässt sich unter Umgehung der natürlichen Aversion des Nagers gegenüber Ethanol eine ausreichend hohe Ethanolaufnahme erzielen, um typische Kennzeichen einer alkoholischen Lebererkrankung wie beispielsweise eine Steatose zu induzieren [225].

Die eigenen Untersuchungen konnten zeigen, dass eine ethanolinduzierte Triglyzeridakkumulierung in der Leber durch gleichzeitige Fütterung oxidierten Fettes im Vergleich zu frischem Fett deutlich vermindert wird [**A11**]. Dies deutet in der Tat daraufhin, dass oxidiertes Fett die Entstehung einer alkoholischen Fettleber verhindern kann und damit ernährungspräventives bzw. ernährungstherapeutisches Potenzial besitzt. Auf molekularer Ebene zeigen die eigenen Untersuchungen, dass oxidiertes Fett auch bei gleichzeitiger Gabe von Ethanol eine erhöhte Transkription typischer PPARα-Zielgene, die im Lipidkatabolismus eine wichtige Rolle spielen, bewirkt, während die ethanolinduzierte SREBP-1c-abhängige Lipogenese durch oxidiertes Fett nicht beeinflusst wird [**A11**]. Auch wenn in den eigenen Untersuchungen eine

mögliche Beeinflussung der hepatischen Sekretionsrate triglyzeridreicher Lipoproteine nicht berücksichtigt wurde, deuten die vorliegenden Ergebnisse daraufhin, dass der wesentliche Wirkmechanismus des oxidierten Fettes auf der Aufhebung der ethanolinduzierten Blockierung des PPARα beruht. Ein ähnlicher Wirkmechanismus konnte auch für synthetische PPARα-Aktivatoren, die ebenfalls die Entstehung einer alkoholinduzierten Steatose verhindern, identifiziert werden [80, 81]. Im Unterschied zum oxidierten Fett bewirkte CLA keine Verminderung der ethanolinduzierten Triglyzeridakkumulierung in der Leber [**A11**]. Allerdings führte Behandlung mit CLA in der vorliegenden Studie zu keiner Aktivierung des hepatischen PPARα. Obwohl dies im Widerspruch zu früheren Studien steht [27, 195, 227, **A14**], die eine hepatische PPARα-Aktivierung durch CLA nachweisen konnten, existieren auch Studien, die keine PPARα-Aktivierung in der Leber durch CLA zeigen konnten [siehe hierzu **A11**]. Diese insgesamt sehr inkonsistente Datenlage bezüglich der Wirkung von CLA auf die PPARα-Antwort im Rattenmodell ist vermutlich auf die heterogene Zusammensetzung der verwendeten CLA-Präparate zurückzuführen. Zahlreiche Studien belegen in der Tat, dass die Isomerenverteilung des CLA-Gemisches entscheidenden Einfluss auf die biologische Wirkung des untersuchten CLA-Präparates hat [193, 227-229]. Daher sollte in zukünftigen Studien auch der Einfluss verschiedener CLA-Präparate bzw. von CLA-Isomeren auf die Entstehung einer alkoholischen Steatose untersucht werden.

Mit Blick auf die beobachtete Hemmung der alkoholischen Steatoseentstehung durch oxidiertes Fett lässt sich schlussfolgern, dass oxidiertes Fett ernährungspräventive Wirkungen aufweist. Es ist zu vermuten, dass oxidiertes Fettes über diesen Effekt auch eine präventive Wirkung auf Folgeerkrankungen der alkoholischen Steatose wie Alkoholhepatitis und Leberfibrose ausübt. Diese Vermutung stützt sich darauf, dass eine Steatose die Leber anfälliger gegenüber schädigenden Agenzien wie Medikamenten und Toxinen macht [230], welche in der Pathogenese dieser Erkrankungen eine wichtige Rolle spielen [231, 232]. Ob sich diese am Nagermodell ermittelte ernährungspräventive Wirkung des oxidierten Fettes auch am Menschen nachvollziehen lässt, bleibt zu klären. Bezüglich einer Effektivität von PPARα-Aktivatoren für die Behandlung einer Steatose beim Menschen sind bislang kaum Daten aus der Literatur bekannt. Zumindest eine am Menschen durchgeführte Pilotstudie zeigte, dass durch Gabe des PPARα-Aktivators Fenofibrat die Serumtriglyzeridspiegel von Patienten mit alkoholischer Fettleber signifikant abnahmen, obwohl die Patienten während der Intervention weiter Alkohol aufnahmen (>80 g/Tag) [81]. Obwohl dieser Studie keine Angaben über das Ausmaß der Fettleber nach der Fenofibrat-Intervention zu entnehmen sind, lassen diese Ergebnisse den Schluss zu, dass PPARα-Aktivatoren zur Behandlung einer alkoholbedingten

Hypertriglyzeridämie und möglicherweise auch zur Behandlung einer alkoholischen Steatose beim Menschen in Frage kommen. Diese Beobachtung ist umso bedeutsamer als der Mensch ebenso wie Schwein und Affe aufgrund einer im Vergleich zum Nager geringeren hepatischen PPARα-Expression eine schwächere Reaktion auf PPARα-Aktivatoren zeigt [233-236]. Nichtsdestotrotz konnte eine kürzlich publizierte Studie auch beim Schwein eine durch oxidiertes Fett hervorgerufene PPARα-Aktivierung in der Leber nachweisen [237], was die Bedeutung von oxidiertem Fett als Quelle nutritiver PPARα-Agonisten für den Menschen deutlich macht. Somit besitzen die am Nagermodell ermittelten Befunde möglicherweise auch für die Behandlung einer alkoholischen Fettleber beim Menschen eine Relevanz.

3.4 Beeinflussung PPARγ-regulierter Prozesse in Darmepithelzellen durch oxidiertes Fett

Obwohl in einer Vielzahl an Studien die Aktivierung des PPARα durch oxidiertes Fett dokumentiert ist [79, 237-239, **A11**, **A13**], fehlten bislang entsprechende Studien zur Beeinflussung anderer PPAR-Isoformen durch oxidiertes Fett. Gleichwohl ist bekannt, dass spezifische Substanzen oxidierter Fette wie Hydroxy- und Hydroperoxyfettsäuren (9-HODE, 13-HODE, 13-HPODE) als Liganden und potente Aktivatoren des PPARγ fungieren [29, 240, 241]. Um deshalb zu klären, ob oxidierte Fette auch *in vivo* den PPARγ-Signalweg aktivieren, wurde eine Studie initiiert, in der die Wirkung von oral verabreichtem oxidiertem Fett auf die PPARγ-Transaktivierung und die Beeinflussung des NF-κB-Signalweges in Darmepithelzellen untersucht wurde. Darmepithelzellen stellten hierfür ein geeignetes Untersuchungsobjekt dar, da diese im Vergleich zu anderen Zellen und Geweben ein hohes PPARγ-Expressionsniveau aufweisen und PPARγ die dominante PPAR-Isoform in Darmepithelzellen darstellt [242]. Darüber hinaus nehmen Enterozyten, im Gegensatz zu anderen Zelltypen [243], die für die PPARγ-Aktivierung relevanten Substanzen des oxidierten Fettes mit hoher Rate auf [244]. Darmepithelzellen überziehen als Einzelzellschicht die gesamte Oberfläche der Darmschleimhaut. Studien der jüngeren Vergangenheit machen zunehmend deutlich, dass das Darmepithel nicht nur als inerte und mechanische Barriere zwischen Darmbakterien und Körperinnerem fungiert, sondern auch integraler und essentieller Bestandteil des mukosalen Immunsystems ist. So wird durch das dynamische Zusammenspiel bakterieller und wirtseigener Signale am Darmepithel das Gleichgewicht zwischen Induktion und Hemmung der Darmepithelzellaktivität (z.B. Zellproliferation, Differenzierung, Produktion von Entzündungsmediatoren) reguliert. Dadurch wird vermutlich auch Einfluss auf die Fähigkeit des Darm-assoziierten Immunsystems ausgeübt, zwischen harmlosen Bakterien und Infektionserregern in der Darmflora zu unter-

scheiden [245]. Die Folgen einer Aktivierung von Darmepithelzellen, die auf molekularer Ebene über den Nachweis aktivierter Transkriptionsfaktoren wie NF-κB erkennbar ist, sind entweder lokal und zeitlich begrenzte moderate Entzündungsprozesse im gesunden Darm („physiologische Entzündung“) oder pathologisch-chronische Entzündungsprozesse wie sie bei Patienten mit chronisch entzündlichen Darmererkrankungen zu beobachten sind. Zu den regulatorischen Signalkaskaden, die die intestinale Epithelzellaktivierung beeinflussen, zählt der PPARγ-Signalweg. Dieser wirkt hemmend auf Schlüsseltranskriptionsfaktoren der Entzündungsantwort wie NF-κB und damit negativ auf die intestinale Epithelzellaktivierung, was wiederum zur Abschwächung bzw. Inhibierung inflammatorischer Prozesse im Darm führt. Daher ist die Betrachtung der Expression NF-κB-abhängiger Entzündungsmediatoren (NO, IL-6, TNFα) im Darmepithel geeignet, eine Aussage über die Modulierung PPARγ-regulierter Prozesse durch oxidiertes Fett zu treffen.

Im Rahmen der eigenen Untersuchungen konnte festgestellt werden, dass die Fütterung oxidierten Fettes eine moderate Aktivierung der DNA-Bindungsaktivität des PPARγ in Darmepithelzellen zur Folge hat [**A12**], was in der Tat zeigt, dass oxidiertes Fett zu einer Modulierung der transkriptionellen Aktivität des PPARγ *in vivo* führt. Die lediglich moderate PPARγ-Transaktivierung liegt vermutlich in der nur mäßigen oxidativen Modifizierung des thermisch behandelten Fettes begründet, was anhand der ermittelten Fettkennzahlen des oxidierten Fett abgeschätzt werden kann. Nichtsdestotrotz ist das eingesetzte Fett für die Humanernährung von Relevanz, da es unter Frittierbedingungen hergestellt wurde und somit die physiologische Wirkung eines Frittierfetts reflektiert. Allerdings konnte als Folge der Aktivierung des PPARγ-Signalweges in Darmepithelzellen keine Inhibierung der NF-κB-DNA-Bindungsaktivität und der Expression NF-κB-abhängiger Entzündungsmediatoren beobachtet werden [**A12**], was im Gegensatz zur Wirkung pharmakologischer PPARγ-Liganden ist [246-250]. Auch nach Gabe von CLA konnte, im Unterschied zu oxidiertem Fett, in den eigenen Untersuchungen [**A12**], eine PPARγ-abhängige Hemmung inflammatorischer Prozesse im Darm nachgewiesen werden [141, 251, 252]. Obgleich für den ausbleibenden Effekt des oxidierten Fettes auf den inflammatorischen Prozess im Darmepithel mehrere Ursachen in Frage kommen [siehe hierzu **A12**], könnte der geringe basale Entzündungsstatus eines intakten intestinalen Epithels hierfür ursächlich sein. Dieser lässt sich vermutlich durch Gabe entzündungshemmend wirkender Substanzen nur marginal verbessern. Deshalb sollte in zukünftigen Studien auch die Wirkung oxidierter Fette auf akute inflammatorische Prozesse im Darm, z.B. in einem chemisch oder bakteriell induzierten Enterokolitismodell, untersucht werden. Ferner ist zu berücksichtigen, dass in den eigenen Untersuchungen Indizien für die Induzierung von oxidativem Stress im

Darmepithel (Depletierung nicht-enzymatischer und enzymatischer Antioxidanzien, Bildung von Lipidperoxidationsprodukten) durch oxidiertes Fett gefunden wurden [**A12**]. Diese Wirkung oxidierten Fettes konnte auch schon in früheren Studien in anderen Geweben beobachtet werden [69, 253-255]. Die im Rahmen von oxidativem Stress gebildeten reaktiven Sauerstoffspezies (ROS) wirken als Stimulus für die Aktivierung redoxsensitiver Transkriptionsfaktoren wie NF-κB [256, 257]. Daher besteht die Möglichkeit, dass die Generierung von ROS durch oxidiertes Fett als proinflammatorischer Stimulus wirkt und die hemmende Wirkung einer PPARγ-Aktivierung auf den NF-κB-Signalweg im Darmepithel antagonisiert. Frühere Beobachtungen, dass 13-HPODE, welche sowohl den PPARγ aktiviert als auch die ROS-Bildung induziert, zu einer Aktivierung der NF-κB-abhängigen Expression inflammatorischer Gene führt [258], stützen diese Vermutung. Die entscheidende Rolle des oxidativen Stresses für diesen Effekt wird dadurch ersichtlich, dass in Anwesenheit des Antioxidans *N*-Acetylcystein die proinflammatorische Wirkung von 13-HPODE aufgehoben wird [258]. Die Ergebnisse dieser Studie [258] legen sogar die Vermutung nahe, dass die durch 13-HPODE induzierte ROS-Bildung einen dominanteren Einfluss auf die NF-κB-Aktivität ausübt als die 13-HPODE induzierte PPARγ-Aktivierung. Diese und die eigenen Untersuchungen machen deutlich, dass oxidiertes Fett bzw. Bestandteile oxidierter Fette wie 13-HPODE vermutlich sowohl stimulierende als auch inhibierende Effekte auf die Aktivität des Transkriptionsfaktors NF-κB ausüben, die je nach Dominanz des einen oder anderen Effekts zu einer inhibierten, aktivierten oder unveränderten Aktivität des NF-κB führen.

Insgesamt zeigen die eigenen Untersuchungen [**A12**], dass ein unter Frittierbedingungen hergestelltes oxidiertes Fett eine moderate Aktivierung des PPARγ-Signalweges in Darmepithelzellen bewirkt, die allerdings keine Auswirkung auf die NF-κB-abhängige Expression von Entzündungsmediatoren hat. Dies zeigt, dass oxidierte Fette, ähnlich wie oxidierte Fettsäuren, einen aktivierenden Einfluss auf den PPARγ ausüben. Darüber hinaus machen diese Untersuchungen deutlich, dass oxidiertes Fett im Darmepithel oxidativen Stress induziert und eine Beeinträchtigung des enzymatischen und nicht-enzymatischen antioxidativen Schutzsystems bewirkt. Allerdings haben beide Effekte oxidierten Fettes (moderate PPARγ-Transaktivierung, Induktion von oxidativem Stress) keinen Einfluss auf die Aktivität des NF-κB, welcher als Schlüsseltranskriptionsfaktor physiologischer als auch chronisch-pathologischer Entzündungsprozesse im Darmepithel fungiert. Da das Darmepithel integraler Bestandteil der Barriere- und Abwehrfunktion des Darmes ist und als Nahtstelle zwischen Darmbakterien und Signalen des Immunsystems einen entscheidenden Beitrag zur Regulation der Darmhomöostase leistet, ist davon auszugehen, dass die Aufnahme oxidierten Fettes keinen negativen

Einfluss auf die mukosale Barriere- und Immunfunktion sowie die Darmhomöostase hat.

3.5 Transplazentare Aktivierung des PPARα durch oxidiertes Fett

Während bereits Hinweise existierten, die für einen transplazentaren Übergang von CLA-Isomeren sprechen [259], fehlten bislang Hinweise bezüglich eines transplazentaren Transports von Bestandteilen oxidierter Fette. Ausgehend von Befunden, die auf eine transplazentare PPARα-Aktivierung durch maternale Gabe von synthetischen PPARα-Aktivatoren hindeuten [260-263], wurde untersucht, ob maternal verabreichtes oxidiertes Fett ebenfalls zu einer PPARα-Aktivierung in der fetalen Leber führt. Die Relevanz dieser Fragestellung besteht vor allem darin, dass die Behandlung trächtiger Tiere mit PPARα-Aktivatoren zu einem beeinträchtigten Wachstum der Feten und pathologischen Veränderungen der Neugeborenen führt [264, 265]. Eine jüngere Studie konnte sogar nachweisen, dass die Gabe des PPARα-Aktivators WY-14643 während der frühen Trächtigkeitsphase eine letale Wirkung auf die Embryos entfaltet [266]. Ferner konnte gezeigt werden, dass PPARα-Agonisten den konstitutiven Androstan-Rezeptor, den Pregnan-X-Rezeptor und die Expression steroidmetabolisierender Enzyme in der Leber von Rattenfeten aktivieren [267]. Dies deutet auf die Beeinflussung des fetalen Stoffwechsels durch PPARα-Agonisten hin. Eine Beeinflussung des Stoffwechsels des Feten wird vor allem im Hinblick auf langfristige Adaptationsmechanismen im Fetus (*fetal programming*) kritisch gesehen, da diese Einfluss auf das Mortalitäts- und Morbiditätsrisiko im späteren Leben nehmen können [92-97]. Zudem wird eine PPARα-Aktivierung vor dem Hintergrund der Hepatokarzinogenese kritisch gesehen, die zumindest beim Nager als Folge der Gabe von Peroxisomenproliferatoren auftritt [268-270].

Die eigenen Untersuchungen zur transplazentaren Wirkung maternal verabreichter oxidierter Fette wurden am Rattenmodell durchgeführt [**A13**]. Dieses stellt ein geeignetes Trächtigkeitsmodell für den Menschen dar, da die Ratte im Gegensatz zu anderen Spezies (z.B. Kaninchen, Rhesusaffe) ähnliche Veränderungen im Lipidstoffwechsel während Trächtigkeit aber auch während Laktation zeigt wie der Mensch [271-273]. Darüber hinaus weist der histologische Aufbau der Plazenta, die als selektive Diffusionsbarriere wirkt, große Ähnlichkeiten zwischen Ratte und Mensch auf [274-276]. Bei beiden Spezies liegt eine hämochoriale Diffusionsbarriere vor, bei der die Nährstoffe aus den maternalen Spiralarterien im intervillösen Raum durch die Trophoblastschichten, die Basalmembranen, das Zottenstroma und das fetale Kapillarendothel hindurchtreten. Diese Ähnlichkeit im Aufbau der fetoplazentaren Einheit zwischen Ratte und Mensch bedingt ähnliche gute Permeabilitätsverhältnisse und hohe Transportkapazitäten für Fettsäuren und erlaubt deshalb mit gewissen Einschränkungen eine

Extrapolation von Beobachtungen beim Nager auf den Menschen. Spezies mit epitheliochorialer Plazenta wie beispielsweise Wiederkäuer, Pferd und Schwein haben dagegen nur geringe transplazentare Transportkapazitäten für Fettsäuren und sind daher als Modell für den Menschen für derartige Fragestellungen eher ungeeignet [277]. Deshalb schwankt der fetale Fettgehalt zwischen unterschiedlichen Spezies in Abhängigkeit zum fetalen Lipidtransfer sehr stark und liegt beispielsweise beim menschlichen Neugeborenen bei ca. 18% des Körpergewichtes und beim Lamm nur bei etwa 3%.

Die eigenen Untersuchungen konnten erstmalig zeigen, dass maternal verabreichtes oxidiertes Fett nicht nur in der Leber der trächtigen Tiere, sondern auch in der fetalen Leber eine Aktivierung des PPARα und eine Verminderung der Triglyzeridkonzentrationen bewirkt [**A13**]. Auch wenn die PPARα-Aktivierung in der Leber der Feten nicht so stark ausgeprägt war wie nach Gabe von Clofibrat, deuten diese Befunde an, dass offensichtlich nicht nur pharmakologische PPARα-Ativatoren eine transplazentare PPARα-Aktivierung in der Fetalleber hervorrufen, sondern auch oxidierte Fette. Die in der Fetalleber durch oxidiertes Fett beobachtete Reduktion der Triglyzeridkonzentrationen ist vermutlich Folge der Induzierung von PPARα-regulierten Genen der peroxisomalen und mitochondrialen Fettsäurenoxidation [**A13**], was eine Steigerung der Fettsäurenoxidationskapazität der Leber und damit des Fettsäurenkatabolismus zur Folge hat. Damit zeigen diese Befunde ferner, dass über die transplazentare PPARα-Aktivierung durch oxidiertes Fett auch der Stoffwechsel des Feten beeinflusst wird. Obgleich die eigenen Untersuchungen keine kausalen Rückschlüsse auf die ursächlichen Substanzen des oxidierten Fettes zulassen, kommen hierfür vermutlich vor allem verschiedene oxidierte Fettsäuren und zyklische Fettsäuremonomere in Betracht. Diese werden während der thermischen Behandlung von Speiseölen in hohem Umfang gebildet [42-45] und wirken als potente PPARα-Aktivatoren [23, 25, 29].

Bislang wurde der Transport dieser nutritiven PPARα-Aktivatoren über die plazentare Diffusionsbarriere nicht charakterisiert. Trotzdem liefert die beobachtete Induktion der PPARα-Zielgene in der fetalen Leber nach maternaler Gabe oxidierter Fette einen indirekten Hinweis auf einen effizienten transplazentaren Transport dieser Substanzen [**A13**]. Vom transplazentaren Transport nicht-oxidierter Fettsäuren ist bekannt, dass dieser durch eine hohe Selektivität (Bevorzugung langkettiger polyungesättigter Fettsäuren wie Docosahexaensäure und Arachidonsäure) gekennzeichnet ist [278-281]. Diese Selektivität stellt eine auf die fetalen Bedürfnisse (v.a. Gehirn- und Retinaentwicklung) optimierte Versorgung mit spezifischen Fettsäuren sicher. Der transplazentare Fettsäurentransport erfolgt in einem mehrstufigen Prozess der Aufnahme und intrazellulären Translokation von Fettsäuren über verschiedene membran-

ständige (FABPpm, FAT/CD36, FATPs) und zytosolische (cFABPs) fettsäurebindende Proteine [282, 283]. Diese Transportproteine binden die Fettsäuren reversibel und nichtkovalent, so dass ein Transportfluss über diese Diffusionsbarriere möglich ist. Bislang wurde angenommen, dass für die beobachtete Transportselektivität die hohe Affinität und Bindungskapazität des Plazenta-FABPpm für langkettige polyungesättigte Fettsäuren allein entscheidend ist [282, 284]. Dieses wird nur auf der maternalen mikrovillösen Membranseite exprimiert und vermittelt so einen unidirektionalen Fluss der langkettigen polyungesättigten Fettsäuren von der maternalen Seite zum Fetus [278, 285]. Jüngere Studien der Arbeitsgruppe Koletzko weisen jedoch auch auf eine Schlüsselbedeutung der FATPs, insbesondere FATP-4, für den selektiven Docosahexaensäure-Transport über die Plazentaschranke hin [286, 287]. Diese Annahme stützt sich darauf, dass die FATP-4-mRNA-Expression in der Plazenta positiv mit den Docosahexaensäure-Konzentrationen der maternalen und fetalen Plasmalipide korreliert. Zudem existieren Hinweise darauf, dass die Transportselektivität von Docosahexaensäure und Arachidonsäure auch durch ein erhöhtes Angebot an langkettigen polyungesättigten Fettsäuren in der nichtveresterten Fettsäurefraktion des intervillösen Raums der Plazenta zustande kommt [288]. Dies erklärt sich vermutlich dadurch, dass die Plazenta-LPL bevorzugt Fettsäuren an der *sn*-2-Position der Triglyzeride, an der üblicherweise langkettige polyungesättigte Fettsäuren gebunden sind, hydrolysiert [289, 290]. Da Bindungsstudien eine hohe Affinität zytosolischer FABPs wie H-FABP und L-FABP, die auch im Syncytiotrophoblasten der Plazenta exprimiert sind [282, 284], für oxidierte Fettsäuren wie 13-HODE im Vergleich zu ungesättigten Fettsäuren wie C18:2 oder C18:1 dokumentieren [291], lässt sich mutmaßen, dass dies auch für membranständige FABPs wie Plazenta-FABPpm zutrifft. Unterstützt wird diese Annahme dadurch, dass zytosolische und membranständige FABPs hinsichtlich ihrer Tertiärstruktur, die maßgeblich die Bindungsaffinität zum Liganden (Fettsäure) bestimmt, eine hohe Übereinstimmung aufweisen [292]. Die Beobachtung aus den eigenen Untersuchungen, dass zumindest im Kurzzeit-Experiment die Induktion der PPARα-Zielgene durch das oxidierte Fett in der fetalen Leber stärker ausgeprägt war als in der Leber der trächtigen Tiere [**A13**], könnte tatsächlich ein Indiz für einen bevorzugten Transport von oxidierten Fettsäuren oder zyklischen Fettsäuremonomeren über die Plazentaschranke sein.

Bezüglich CLA, die wie oxidierte Fettsäuren als potente PPAR-Liganden fungieren, existieren mehrere Hinweise, die für einen transplazentaren Transport dieser Fettsäuren sprechen [259, 293]. Im Gegensatz zu Bestandteilen oxidierter Fette deutet allerdings die Beobachtung, dass die CLA-Anteile in den Plasmalipiden des Neonatalblutes nur etwa 60-70% derjenigen des maternalen Blutes ausmachen [293], daraufhin, dass CLA im Vergleich zu langkettigen

polyungesättigten und möglicherweise auch oxidierten Fettsäuren vermutlich mit geringerer Präferenz über die Plazentaschranke transportiert werden. Dies könnte einen Erklärungsansatz dafür liefern, dass in eigenen Arbeiten die maternale Gabe von CLA zu keiner PPARα-Transaktivierung in der Leber der Föten führte [nicht-publizierte Daten]. Ein verminderter transplazentarer Transport von CLA könnte darin begründet liegen, dass CLA-Isomere vornehmlich an der *sn*-1- und der *sn*-3-Position der Plasmatriglyzeride gebunden sind und damit in geringerem Umfang durch die plazentaeigene LPL hydrolysiert werden.

Insgesamt zeigen die Untersuchungen am Rattenmodell, dass maternal verabreichte oxidierte Fette zu einer PPARα-Aktivierung in der Leber der Feten führen und den fetalen Lipidstoffwechsel beeinflussen. Dies lässt auf einen effizienten Transport von Substanzen des oxidierten Fettes, die als PPARα-Agonisten wirken, über die plazentare Diffusionsbarriere schliessen. Ob hierüber eine langfristige Prägung des Stoffwechsels im Sinne eines *fetal programmings* erfolgt, muss in zukünftigen Studien gezeigt werden. Da die fetoplazentare Einheit des Nagers hinsichtlich des histologischen Aufbaus der Diffusionsbarriere (hämochorial) der des Menschen ähnlich ist, sind diese Befunde auch für Schwangere relevant, die in höherem Maße oxidierte Fette verzehren.

3.6 Beeinflussung der Milchfettsynthese durch CLA und oxidiertes Fett unter besonderer Berücksichtigung des PPARα

Bei laktierenden Tieren bzw. stillenden Frauen konnte in vorangegangenen Studien eine Absenkung des Gehalts an Triglyzeriden und Energie in der Milch durch Gabe von CLA oder thermisch behandelten Fetten beobachtet werden [82-88]. Da die Milch für den säugenden Nachkommen die einzige Nahrungs- und Energiequelle darstellt und damit entscheidend das postnatale Wachstum beeinflusst, ist dieser Effekt kritisch zu betrachten. Um die molekularen Ursachen für diesen Effekt oxidierter Fette bzw. von CLA aufzuklären, wurden zwei Studien am Rattenmodell durchgeführt [**A14**, **A15**].

Die eigenen Arbeiten lassen den Schluss zu, dass der reduzierte Milchfettgehalt bei Fütterung von CLA zum einen das Ergebnis einer beeinträchtigten *de novo*-Fettsäurensynthese in der Milchdrüse ist [**A14**], wie dies auch von anderen Untersuchern an Milchkühen und laktierenden Mäusen beobachtet wurde [84, 86]. Zum anderen deuten die Reduktion der LPL-mRNA-Konzentration in der Milchdrüse und der Konzentration an langkettigen Fettsäuren in der Milch an, dass CLA auch über eine verminderte Aufnahme an Fettsäuren aus triglyzeridreichen Lipoproteinen in die Milchdrüse den Milchfettgehalt reduziert [**A14**]. Allerdings weist ein ernied-

rigtes Verhältnis von mittelkettigen Fettsäuren, die überwiegend der Eigensynthese im Milchdrüsenepithel entstammen [295], zu langkettigen Fettsäuren, die überwiegend aus triglyzeridreichen Lipoproteinen stammen [294, 295], in der Milch CLA-gefütterter Ratten daraufhin, dass die Inhibierung der *de novo*-Fettsäurensynthese durch CLA einen dominanteren Einfluss auf die Reduzierung des Milchfettgehalts hat. Im Gegensatz dazu konnten bei Fütterung oxidierter Fette an laktierende Ratten keine Anzeichen für eine reduzierte *de novo*-Fettsäurensynthese in der Milchdrüse gefunden werden [**A15**], was Ergebnisse früherer Untersuchungen bestätigt [85]. Reduzierte Transkriptkonzentrationen der LPL und membranständiger Fettsäuretransporter (FAT/CD36, FABPpm, FATP) in der Milchdrüse und verminderte Konzentrationen an langkettigen Fettsäuren in der Milch deuten vielmehr auf eine Einschränkung der Aufnahme an langkettigen Fettsäuren aus triglyzeridreichen Lipoproteinen bzw. an albumingebundenen freien Fettsäuren durch Fütterung oxidierter Fette hin. Verstärkt wird der milchfettsenkende Effekt des oxidierten Fettes vermutlich dadurch, dass oxidiertes Fett über die Aktivierung des PPARα in der Leber und nachfolgend erhöhtem Fettsäurenkatabolismus in der Leber auch die Ausschleusung von triglyzeridreichen Lipoproteinen (VLDL) in die Zirkulation vermindert [**A15**]. Durch dieses verminderte Angebot an VLDL-Triglyzeriden wird möglicherweise zusätzlich die Milchfettsynthese in der Milchdrüse vermindert. Ein weiterer Effekt, der sich als Folge der Aktivierung des hepatischen PPARα durch oxidiertes Fett erstmalig beobachten liess, ist die deutliche Induzierung der hepatischen Lipase und von Fettsäuretransportern (FAT/CD36, FABPpm, FATP) in der Leber der Ratten. Fettsäuretransporter weisen funktionelle PPREs in ihrer Promotorregion auf und werden durch PPAR-Liganden in ihrer Expression induziert [296-298]. Daher ist zu vermuten, dass die Induktion dieser Gene in der Leber vermutlich Folge der Aktivierung des hepatischen PPARα in der Leber durch oxidiertes Fett ist. Dieser gegensätzliche Effekt des oxidierten Fettes auf hepatische Lipase bzw. LPL und Fettsäuretransporter in der Leber im Vergleich zur Milchdrüse fördert vermutlich einen zur Leber gerichteten Transport an freien Fettsäuren. Physiologischerweise werden freie Fettsäuren während der Laktation als Folge einer gesteigerten Aktivität der hormonsensitiven Lipase lipolytisch aus dem Fettgewebe freigesetzt [299], um Substrat für die Milchfettsynthese bereitzustellen. Ferner begünstigt eine bereits während der späten Trächtigkeit einsetzende Verminderung der LPL-Aktivität im Fettgewebe und Erhöhung der LPL-Aktivität in der Milchdrüse eine verstärkte Aufnahme von Fettsäuren aus triglyzeridreichen Lipoproteinen hepatischen Ursprungs in die Milchdrüse [300, 301]. Ein als Folge der hepatischen PPARα-Aktivierung erhöhter Fettsäureeinstrom in die Leber reduziert vermutlich den Strom an freien Fettsäuren in die Milchdrüse.

Dort kann vermutlich der erhöhte Substratbedarf der laktierenden Milchdrüse für die Triglyzeridsynthese nicht mehr gedeckt werden, so dass der Milchfettgehalt abnimmt.

In den Untersuchungen zur Wirkung von CLA auf den laktierenden Organimus wurde ein Effekt von CLA auf die Expression von Fettsäuretransportern in der Leber und in der Milchdrüse nicht untersucht. Allerdings ist nicht auszuschliessen, dass als Folge der CLA-induzierten Aktivierung des PPARα in der Leber auch eine Induzierung der hepatischen Lipase und Fettsäuretransporter in der Leber erfolgte [**A14**]. Aufgrund der Bedeutung dieser Transportmechanismen in der Leber für die Substratbereitstellung in der Milchdrüse, sollten zukünftige Studien auch den Einfluss von CLA auf diese Mechanismen untersuchen.

Insgesamt ist zu vermuten, dass durch Fütterung oxidierter Fette während der Laktation die normalen metabolischen Anpassungsmechanismen, die auf eine erhöhte Utilisierung metabolischer Substrate (Fettsäuren, Glukose) in der Milchdrüse für die Milchsynthese auf Kosten einer reduzierten Substratoxidation in anderen Geweben (Leber, Skelettmuskulatur) ausgelegt sind [91], durch den PPARα-aktivierenden Effekt oxidierter Fette in der Leber empfindlich gestört werden. In ähnlicher Weise liess sich zeigen, dass die mit fortschreitendem Laktationsverlauf zunehmende Verminderung in der Expression des Entkopplungsproteins UCP-3 des Skelettmuskels in Mäusen durch Injektion von verschiedenen PPARα-Aktivatoren wieder aufgehoben wird [302]. Durch diese Aufhebung der durch die Laktation ausgelösten Minderexpression des UCP-3, das ebenso wie die Fettsäuretransporter funktionell aktive PPREs im Promotorbereich aufweist [303], wird eine Senkung der zitterfreien Thermogenese im Skelettmuskel und damit des Energieverbrauchs des Organismus während der Laktation verhindert. Auch dies deutet daraufhin, dass die mit fortschreitender Laktation zunehmend einsetzenden Energiesparmechanismen des Organismus (reduzierte Wärmebildung im Skelettmuskel [304], funktionelle Atrophie des braunen Fettgewebes [304-306]) durch gleichzeitige Gabe von PPARα-Aktivatoren zu Lasten der Milch(fett)synthese beeinträchtigt werden. Im Unterschied zum Nager existieren beim Menschen kaum Hinweise für derartige Adaptationen im Energiehaushalt (Grundumsatz, nahrungsinduzierte Thermogenese) während der Laktation bzw. Stillzeit [307]. Allerdings wird bei optimal ernährten stillenden Frauen der Energiemehrbedarf für die Laktation normalerweise durch eine erhöhte Nahrungsenergie-aufnahme (2,7 MJ/Tag bis zum 4. Monat post partum, 2,2 MJ/Tag nach dem 4. Monat) gedeckt, sodass keine Energiesparmechanismen erforderlich sind. Lediglich unter ungünstigen Ernährungsbedingungen (Energiemangel) konnte bei stillenden Frauen eine Reduktion der nahrungsinduzierten Thermogenese festgestellt werden [308], was auch die Bedeutung der adaptiven Thermogenese beim Menschen während der Stillzeit belegt. Daher ist zu vermuten,

dass sich eine hepatische PPARα-Aktivierung während der Stillzeit lediglich bei energetisch mangelernährten Frauen auf die Milchfettsynthese auswirkt.

Im Unterschied zum oxidierten Fett [**A15**] wurde das CLA-Präparat [**A14**] in den eigenen Untersuchungen bereits während der Trächtigkeit verabreicht. Während dieser Phase werden unter der synergistischen Kontrolle von Östrogenen, Progesteron, Prolaktin und plazentarem Laktogen Wachstums- und Differenzierungsprozesse eingeleitet, die zur Umwandlung der nicht-laktierenden Milchdrüse in die laktierende Milchdrüse führen. Hierbei wandeln sich die wenig verzweigten tubulusähnlichen Endstücke (Tubuloalveoli) der Drüsenläppchen in weitlumige, dicht nebeneinander liegende, milchsezerniernde alveoläre Endstücke (laktierende Alveoli) um. Eine kürzlich publizierte Studie konnte eindrucksvoll zeigen, dass eine PPARα-Aktivierung während der Trächtigkeit zu schwerwiegenden Defekten in der lobuloalveolären Entwicklung der laktierenden Milchdrüse in einer transgenen Mauslinie führt, die mit einem schweren Laktationsdefekt und einer erhöhten Sterblichkeit der Nachkommen verbunden ist [309]. Ähnliche Effekte auf die lobuloalveoläre Entwicklung und die Sterblichkeit der Nachkommen wurden auch in Wildtyp-Mäusen nach Gabe synthetischer PPARα-Aktivatoren (WY-14643) nachgewiesen [266]. Die Beobachtung, dass dieser Effekt in PPARα-Knockout-Mäusen nicht auftritt, deutet daraufhin, dass die gestörte lobuloalveoläre Entwicklung Folge einer PPARα-Aktivierung in der Milchdrüse ist. Da in der eigenen Arbeit weder die transkriptionelle Aktivität des PPARα in der Milchdrüse untersucht wurde noch eine histologische Untersuchung der Milchdrüse erfolgte [**A14**], kann somit nicht ausgeschlossen werden, dass für die drastische Senkung des Milchfettgehalts durch CLA auch eine PPARα-vermittelte Störung der lobuloalveolären Entwicklung der laktierenden Milchdrüse mitverantwortlich ist. Dies muss in zukünftigen Studien geklärt werden.

Insgesamt zeigen die eigenen Untersuchungen, dass die Gabe oxidierter Fette bzw. von CLA während der Laktation bzw. während Trächtigkeit und Laktation zu einer Senkung des Fett- und Energiegehalts der Milch und zu einem beeinträchtigten postnatalen Wachstums bzw. einer erhöhten Sterblichkeit der Nachkommen führt [**A14**, **A15**]. Ob für diese Wirkung die Aktivierung des PPARα in der Leber bzw. Milchdrüse verantwortlich ist, muss in weiteren Studien an geeigneten Versuchsmodellen (z.B. PPARα-Knockout-Maus) geklärt werden. Jüngere tierexperimentelle Befunde, die eine Beeinträchtigung der Milchdrüsenentwicklung und damit verbundenem Laktationsdefekt durch Gabe pharmakologischer PPARα-Liganden während der Trächtigkeit in Wildtyp-Mäusen, nicht jedoch in PPARα-Knockout-Mäusen, dokumentieren, deuten auf eine kausale Beteiligung des PPARα hin. Auch wenn die am Nagermodell beobachteten Effekte nicht direkt auf den Menschen extrapoliert werden können,

lassen diese Ergebnisse den Schluss zu, dass sowohl die Aufnahme kommerziell erhältlicher CLA-Supplemente als auch ein erhöhter Konsum thermisch behandelter Fette (z.B. Frittierfette) für Schwangere und Stillende ungünstig ist.

4 Zusammenfassung

Die in der vorliegenden Arbeit vorgestellten Untersuchungen beschäftigten sich schwerpunktmäßig mit der Aufklärung physiologischer und pathophysiologischer Wirkungen von CLA und oxidierten Fetten in zellulären Systemen und im Tiermodell. Aufgrund der Aktivierbarkeit von PPARs durch diese Nahrungsfette/-fettsäuren und der zentralen Rolle von PPARs in der Vermittlung von physiologischen und pathophysiologischen Wirkungen, standen solche Wirkungen im Vordergrund, die durch PPARs beeinflusst werden.

Zur Beantwortung der Frage, ob CLA PPARα- bzw. PPARγ-regulierte Prozesse in Zellen, die an atherosklerotischen Gefäßveränderungen beteiligt sind, beeinflussen, wurden Untersuchungen an Gefäßmuskelzellen, Endothelzellen, Macrophagen und Hepatozyten durchgeführt. In Gefäßmuskelzellen konnten durch Behandlung mit CLA-Isomeren inhibitorische Wirkungen auf die basale und zytokininduzierte Eicosanoidfreisetzung und auf die mitogeninduzierte Kollagenbiosynthese festgestellt werden. Als Wirkmechanismus für die Hemmung der zytokininduzierten Eicosanoidfreisetzung und der mitogeninduzierten Kollagenbiosynthese durch CLA-Isomere konnte ein PPARγ-abhängiger Mechanismus identifiziert werden. In RAW264.7-Macrophagen-Schaumzellen bewirkten CLA-Isomere eine Reduzierung der zellulären Cholesterinakkumulierung und eine Erhöhung des HDL-abhängigen Cholesterinefflux. Diese Effekte waren bei Behandlung mit CLA-Isomeren mit signifikant erhöhten Transkriptkonzentrationen an CD36, ABCA1, LXRα, NPC-1 und NPC-2 verbunden. In HepG2-Zellen führte Behandlung mit t10c12-CLA, nicht jedoch c9t11-CLA, zu einer signifikanten Erhöhung der Transkriptkonzentrationen an SREBP-1 und -2, LDL-Rezeptor und HMG-CoA-Reduktase sowie zu einer erhöhten Cholesterinsynthese. Mittels siRNA-vermitteltem Knockdown von SREBP-1 bzw. SREBP-2 konnte gezeigt werden, dass der stimulierende Effekt von t10c12-CLA auf die Expression von LDL-Rezeptor und HMG-CoA-Reduktase abhängig von der Induktion von SREBP-1 und -2 ist. In Aorta-Endothelzellen und PMA-differenzierten THP-1-Zellen konnten dagegen keine Wirkungen von CLA-Isomeren auf die zytokininduzierte Adhäsionsmolekülexpression, U937-Monozytenadhäsion und Chemokinsynthese bzw. auf die Expression und Aktivität von Matrixmetalloproteinasen (MMP-2, MMP-9) festgestellt werden.

Bezüglich der Frage, ob CLA-Metabolite in CLA-behandelten Zellen der Gefäßwand nachweisbar sind, konnte gezeigt werden, dass durch Behandlung von Gefäßmuskelzellen und Endothelzellen mit CLA-Isomeren (c9t11-CLA, t10c12-CLA) verschiedene β-Oxidationsprodukte wie CD16:2c7t9 und CD16:2t8c10 und Elongationsprodukte wie CD20:2c11t13,

CD20:2t12c14 sowie CD22:2c13t15 und CD22:2t14c16 gebildet werden. Desaturationsprodukte der CLA-Isomere konnten dagegen nicht in Endothelzellen oder Gefäßmuskelzellen nachgewiesen werden.

Die Untersuchungen zur Frage, ob CLA bzw. oxidiertes Fett in der Lage sind, über die Aktivierung des hepatischen PPARα der Entstehung einer alkoholischen Steatose entgegenzuwirken, ergaben, dass oxidiertes Fett eine ethanolinduzierte Triglyzeridakkumulierung in der Leber von Ratten durch Induzierung von hepatischen Genen des Fettsäurenkatabolismus (ACO, CYP4A1, L-CPT I, M-CPT I, MCAD, LCAD) reduzieren kann. Dagegen konnten durch Fütterung von CLA keine Effekte auf die hepatischen Trigylzeridkonzentrationen und die Transkriptkonzentrationen von ACO, CYP4A1, L-CPT I, M-CPT I, MCAD und LCAD in der Leber ethanolgefütterter Ratten im Verleich zu Kontrollratten hervorgerufen werden.

In Bezug auf die Frage, ob oxidierte Fette inflammatorische Prozesse im Darmepithel über die Aktivierung des PPARγ modulieren, liess sich zeigen, dass Fütterung von oxidiertem Fett im Vergleich zu frischem Fett zu einer moderaten, aber nicht signifikanten Aktivierung des PPARγ in Epithelzellen des Schweinedarmes führt. Ferner zeigte sich, dass Fütterung oxidierten Fettes eine Erhöhung der Konzentration an Lipidperoxidationsprodukten (TBARS) und eine erniedrigte Konzentration an α-Tocopherol sowie erniedrigte Aktivitäten von Enzymen des antioxidativen Schutzsystems (Cu/Zn-SOD, GSH-Px, Katalase) im Vergleich zu frischem Fett bewirkt. Keine Effekte wurden als Folge der Fütterung oxidierten Fettes auf die DNA-Bindungsaktivität des NF-κB und die Expression NF-κB-abhängiger inflammatorischer Gene wie iNOS, COX-2 und IL-6 in Darmepithelzellen beobachtet.

Bezüglich der Frage, ob maternal verabreichte oxidierte Fette eine transplazentare Aktivierung des PPARα im Fetus bewirken, konnte gezeigt werden, dass die Gabe von oxidiertem Fett an trächtige Ratten zu signifikant erhöhten Transkriptkonzentrationen typischer PPARα-Zielgene wie ACO, CYP4A1, MCAD und LCAD in der Leber der Feten führte. Ferner konnte nachgewiesen werden, dass diese Feten erniedrigte Triglyzeridkonzentrationen in der Leber aufwiesen, sofern das oxidierte Fett über die gesamte Trächtigkeit an die Muttertiere verabreicht wurde. Keine Veränderung der hepatischen Triglyzeridkonzentrationen zeigte sich, wenn das oxidierte Fett nur während der späten Trächtigkeitsphase gefüttert wurde.

Zur Aufklärung des milchfettsenkenden Effektes von CLA bzw. oxidiertem Fett im laktierenden Organismus wurden zwei Untersuchungen mit Ratten durchgeführt. Während bei Fütterung von CLA eine Verminderung der *de novo*-Synthese an Fettsäuren in der Milchdrüse

(verminderte Konzentrationen an mittelkettigen Fettsäuren in der Milch, reduzierte Transkriptkonzentrationen an SREBP-1c und FAS) festgestellt werden konnte, konnte dies bei Fütterung oxidierten Fettes ausgeschlossen werden (unveränderte Konzentrationen an mittelkettigen Fettsäuren in der Milch und unveränderte Transkriptkonzentrationen an SREBP-1c, FAS, IDH-1 und SCD). Allerdings zeigten sich nach Gabe oxidierten Fettes im Vergleich zu frischem Fett erniedrigte Serumkonzentrationen an Triglyzeriden und nichtveresterten Fettsäuren sowie erniedrigte mRNA-Konzentrationen der Lipoproteinlipase und von Fettsäuretransportern (FAT/CD36, FATP, FABPpm) in der Milchdrüse, während die mRNA-Konzentrationen der hepatischen Lipase und der Fettsäuretransporter in der Leber durch Fütterung oxidierten Fettes erhöht waren. Diese Befunde deuten daraufhin, dass oxidierte Fette den Milchfettgehalt über eine reduzierte Aufnahme an freien Fettsäuren und Fettsäuren aus triglyzeridreichen Lipoproteinen in die Milchdrüse vermindern.

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Eidesstattliche Erklärung

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