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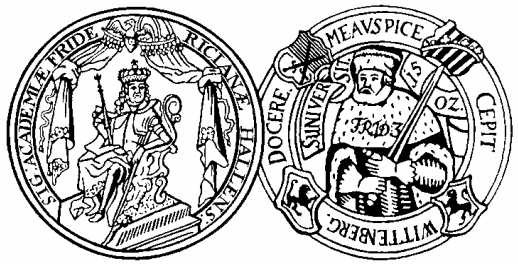

„Wirkungen einer PPARα-Aktivierung auf Parameter des Stoffwechsels von Lipiden, Schilddrüsenhormonen und Carnitin bei Schwein und Ratte als Modelltieren“

Dissertation

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Inhaltsverzeichnis

Abbildungsverzeichnis

Abkürzungsverzeichnis

13-HPODE	13-hydroperoxyoctadecadienic acid
ACC	acetyl-CoA carboxylase
ACO	acyl-CoA oxidase
Apo	Apolipoprotein
CPT	carnitine-palmitoyltransferase
CYP7	cholesterol-7α-hydroxylase
DNA	desoxyribonucleic acid
Duox2	dual oxidase-2
FAS	fatty acid synthase
H_2O_2	Wasserstoffperoxid
HDL	high density lipoprotein
HMG-CoA-Reduktase	β-Hydroxy-β-methylglutaryl-CoA-Reduktase
HMG-CoA-Synthase	β-Hydroxy-β-methylglutaryl-CoA-Synthase
Insig	insulin-induced gene
JVS	juvenile viscerale steatosis
LDL	low density lipoprotein
LPL	lipoprotein lipase
mRNA	messenger ribonucleic acid
OCTN	novel organic cation transporter
PP	Peroxisomen-Proliferator
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PUFA	polyunsaturated fatty acid; mehrfach ungesättigte Fettsäure
RXR	retinoic X receptor
SLC	solute carrier family
SREBP	sterol regulatory element binding protein
T_3	Trijodothyronin
T_4	Thyroxin
TSH	thyroid stimulating hormone; Thyrotropin
UGT	UDP-glucuronosyltransferase
VLDL	very low density lipoprotein

1. Einleitung

1. 1 Molekulare Grundlagen und Funktion des PPARα

Der *peroxisome proliferator-activated receptor-α* (PPARα) gehört zur Familie der nuklearen Transkriptionsfaktoren und wird durch eine Reihe natürlicher Liganden, wie freie Fettsäuren, mehrfach ungesättigte Fettsäuren (PUFA) und Eicosanoide, sowie durch Fibrate als synthetische Liganden aktiviert. Der funktionelle Einfluss des PPARα hinsichtlich des Lipoproteinmetabolismus, der zellulären Fettsäureaufnahme, des intrazellulären Fettsäuretransportes, der mitochondrialen und peroxisomalen Fettsäureoxidation, aber auch der Lipogenese, Ketogenese, Gluconeogenese und des Aminosäuremetabolismus weisen auf seine zentrale Bedeutung in der Regulation des Energie- und Lipidstoffwechsels im menschlichen Organismus hin (Mandard et al., 2004). In Bezug auf die PPARs sind drei Isoformen bekannt: PPARα (NR1C1), PPARβ/δ (NR1C2) und PPARγ (NR1C3) (Kliewer et al., 1994; Mukherjee et al., 1994). Für die Promoterregion des humanen PPARα konnten außerdem sechs alternative Splicingvarianten charakterisiert werden (Chew et al., 2003). Neben der Leber als zentralem Stoffwechselorgan, weisen bei der Ratte und beim Menschen vor allem Organe mit einer hohen Umsatzrate bezüglich des Fettsäurekatabolismus, wie Skelettmuskel, Niere, Herz und Dünndarm eine höhere Expression des PPARα auf (Mukherjee et al., 1994; Braissant et al., 1996; Auboeuf et al., 1996; Loviscach et al., 2000; Escher et al., 2001; Nishimura et al., 2004).

Nach liganden-induzierter Aktivierung bildet der PPARα mit dem Retinsäure-Rezeptor (RXR) als obligatem Partner ein Heterodimer, woraufhin der Komplex an sogenannte *peroxisome proliferator response elements* (PPREs) binden kann, welche sich in der Promoterregion der spezifischen Zielgene befinden (Abb. 1). Charakteristisch für diese PPREs ist eine direkte Wiederholung des hexameren Motives AGGTCA, auch DR1 genannt, wobei die Motive durch ein einzelnes Nukleotid getrennt sind (Kliewer et al., 1992). Durch Bindung des PPARα-RXR-Komplexes an dieses Motiv in der Promoterregion wird die Transkription des betreffenden Zielgenes reguliert. Dabei wird die Expression der Zielgene bei Bindung des PPARα-RXR-Komplexes zumeist erhöht, während für eine Repression spezifischer Gene meist weitere Faktoren erforderlich sind oder unabhängig einer DNA-Bindung z.B. durch eine Protein-Protein-Interaktion oder Konkurrenz mit anderen Faktoren inhibiert werden (IJpenberg et al., 1997; Desvergne und Wahli, 1999; Kersten et al., 2000; Wahli, 2002; Mandard et al., 2004). Weiterhin konnte für den humanen PPARα eine dominant negative Isoform des Rezeptors beschrieben werden, welche in Zellkulturstudien

nach Transfektion die transkriptionelle Funktion und Signaltransduktion des PPARα unterdrückte (Semple et al., 2005).

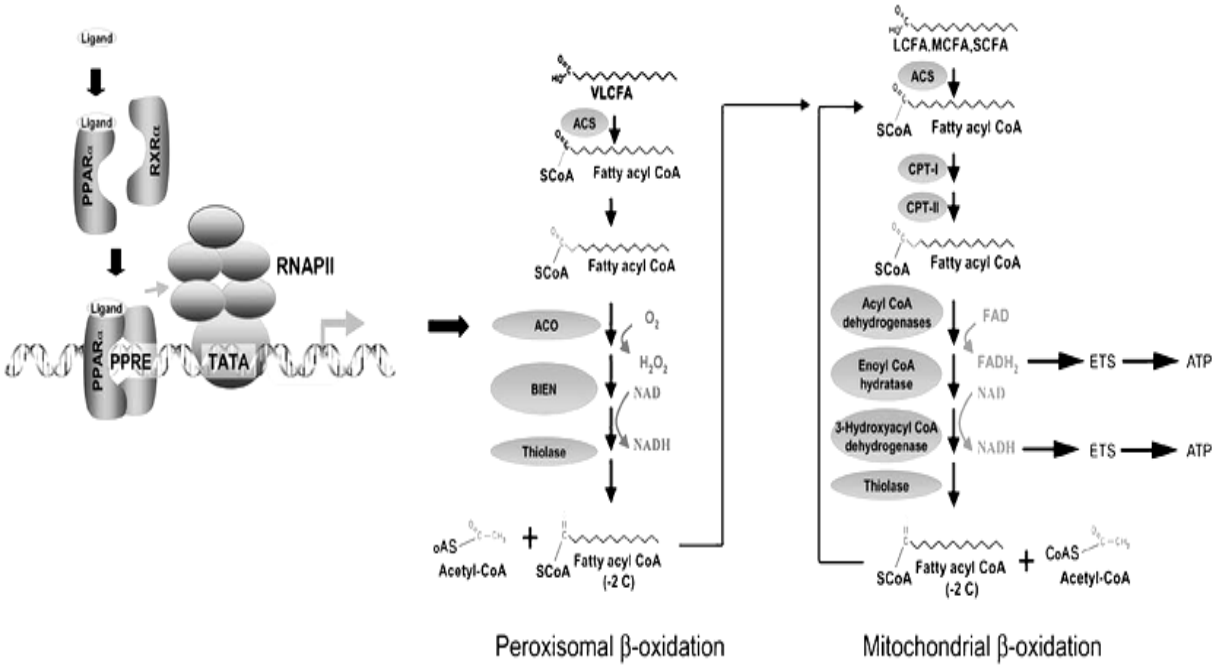

Abb. 1: Mechanismus der PPARα-vermittelten transkriptionellen Regulation von Genen der Fettsäureoxidation (grau unterlegt). (aus Peters et al., 2005)

1.2 PPARα-Aktivierung durch natürliche Liganden

Das Fasten stellt einen natürlichen physiologischen Zustand dar, in dem der PPARα vermehrt aktiviert wird. In dieser Situation werden Fettsäuren aus dem Fettgewebe freigesetzt, zirkulieren vermehrt im Blutkreislauf und stellen nach Aufnahme der freien Fettsäuren in den Geweben natürliche Liganden des PPARα dar (Kersten et al., 1999; Leone et al., 1999). Die Aufrechterhaltung des Energiehaushaltes durch eine PPARα-Aktivierung während des Fastens erklärt sich unter anderem in der Induktion von Proteinen, welche zu einer Erhöhung der Fettsäureoxidation führen (Abb. 1) und so konnte für die *acyl-CoA oxidase* (ACO), welche an der peroxisomalen β-Oxidation beteiligt ist, als erstes Gen ein PPRE nachgewiesen werden (Dreyer et al., 1992). Weiterhin konnten für die *carnitine palmitoyl transferasen* (CPTs), welche carnitinabhängig Fettsäuren für die β-Oxidation über die Membranen der Mitochondrien transportieren, PPREs in der Promoterregion identifiziert werden (Brandt et al., 1998; Barrero et al., 2003). Es konnte gezeigt werden, dass die Verfügbarkeit von Acyl-

CoA in den Mitochondrien und nicht der Energiestatus der Zelle bestimmend für die Aktivität der an der β-Oxidation beteiligten Enzyme ist, so dass die Aktivität der CPTs eine Schlüsselfunktion in der Regulation der mitochondrialen β-Oxidation einnehmen, welche in der Folge direkt durch den PPARα reguliert wird (Bremer und Wojtczak, 1972; Berry et al., 1983; Krauss et al., 1996; Spurway et al., 1997). In diesem Zusammenhang konnte bei Ratten sowohl im Fastenzustand (McGarry et al., 1975), als auch nach Verabreichung von Clofibrat (Mannaerts et al., 1978; Paul et al., 1986) eine Erhöhung der Carnitinkonzentrationen in der Leber beobachtet werden. Dass dabei ein direkter Zusammenhang zur Aktivierung des PPARα besteht, konnte anhand eines Fastenversuches bei Mäusen gezeigt werden, in dem dieser Effekt einer hepatischen Carnitinerhöhung bei Wildtyp-Mäusen, aber nicht bei PPARα-Null-Mäusen auftrat (Hashimoto et al., 1999). Allerdings ist bisher nicht bekannt, welche Mechanismen bei einer vermehrten PPARα-Aktivierung zu diesen erhöhten Carnitinkonzentrationen im Lebergewebe führen und welche systemischen Effekte natürliche oder synthetische PPARα-Liganden auf den Carnitinstoffwechsel haben.

Während im Fastenzustand die freien Fettsäuren aus körpereigenen Depots als Liganden dienen, kann der PPARα auch durch PUFAs, welche über die Nahrung aufgenommen werden, aktiviert werden (Ren et al., 1996; Lin et al., 1999; Jump et al., 2005). Dies ist aus ernährungswissenschaftlicher und medizinischer Sicht von großem Interesse, da dieser Aspekt einen präventiven Ansatz bietet, durch den verstärkten Einsatz von natürlichen Aktivatoren in der Humanernährung die hypolipidämische Wirkung einer PPARα-Aktivierung zur Prävention von Hyperlipidämien infolge ernährungsbedingten Fehlverhaltens zu nutzen. Neben den PUFAs konnte in Tierversuchen an Ratten weiterhin gezeigt werden, dass auch die Aufnahme oxidierter Fette, wie sie in der westlichen Ernährung häufig vorkommen, zu einer PPARα-Aktivierung und Modulation des Lipidstoffwechsels führt (Chao et al., 2001, 2004, 2005; Sülzle et al., 2004). Die Oxidation von Nahrungsfetten kann in moderater Form bei Lagerung und Verarbeitung von Lebensmitteln auftreten, während die wiederholte Erhitzung bei hohen Temperaturen, z.B. beim Frittieren, zu einer starken Oxidation der verwendeten Fette führt (Cohn, 2002). Dabei entstehen primäre und sekundäre Oxidationsprodukte der Fettsäuren, wie Hydroxide, Hydroperoxide, Aldehyde und Ketone, welche über Chylomikronen und *very low density lipoproteins* (VLDL) vom Körper aufgenommen werden und in den Blutkreislauf gelangen (Staprans et al., 1993, 1994, 1996; Suomela et al., 2004, 2005). Neben den modulierenden Eigenschaften hinsichtlich des Lipidstoffwechsels, konnten bei Schweinen (Eder und Stangl, 2000) und Ratten (Eder et al., 2002) erhöhte Plasmakonzentrationen des Schilddrüsenhormons Thyroxin (T_4) ohne

Veränderungen der Plasmakonzentrationen des Thyrotropins (TSH) nach Verfütterung eines oxidierten Fettes beobachtet werden. Außerdem führte die Aufnahme bei Ratten zu Veränderungen der Morphologie der Schilddrüse und der Expression schilddrüsenspezifischer Gene (Skufca et al., 2003). Allerdings ist bisher unklar, ob auch in diesem Zusammenhang eine Aktivierung des PPARα eine Rolle spielt, oder inwieweit oxidierte Fettsäuren einen direkten Einfluss auf den Hormonstoffwechsel in der Schilddrüse ausüben.
Bisher ist auch nicht bekannt, ob oxidierte Fette bei anderen Spezies als der Ratte oder beim Menschen eine PPARα-Aktivierung hervorrufen können, oder ob beim Menschen durch Aufnahme oxidierter Fette die Schilddrüsenfunktion beeinflusst wird. Deshalb sind weitere Untersuchungen zwingend nötig, um eine Einschätzung der Relevanz dieser Beobachtungen für die Humanernährung vornehmen zu können. In diesem Zusammenhang sollten dabei die Eigenschaften oxidierter Fette, Lipidperoxidation in Zellmembranen, oxidative Veränderungen der Lipoproteinstrukturen und oxidativen Stress in Geweben zu induzieren (Srinivasan und Pugalendi, 2000; Ammouche et al., 2002; Eder et al., 2003; Brandsch et al., 2004), kritisch bei der Bewertung der Rolle oxidierter Fette in der menschlichen Ernährung berücksichtigt werden. Dabei hat sich neben der Verfütterung oxidierter Fette in Tierstudien der Einsatz von definierten Lipidperoxidationsprodukten in Zellkulturstudien bewährt, um isolierte Effekte oxidierter Fettsäuren auf zellulärer Ebene untersuchen zu können (Friedrichs et al., 1999; Meilhac et al., 2000; König und Eder, 2006).

1.3 Effekte synthetischer PPARα -Liganden auf den Lipidstoffwechsel

Die hypolipidämischen Effekte einer PPARα-Aktivierung wurden pharmakologisch schon lange bevor man den Rezeptor Anfang der 1990er Jahre entdeckte, eingesetzt, nachdem man in den 1960er Jahren die hypotriglyzeridämischen und hypocholesterämischen Wirkungen nach Einnahme von Clofibrat beobachten konnte (Duncan et al., 1968). Die pharmakologische Gruppe der Fibrate beinhaltet verschiedene Clofibrinsäure-Analoga, welche seit inzwischen mehreren Jahrzehnten erfolgreich zur Senkung der Blutfettwerte beim Menschen eingesetzt werden. Ein Grossteil der existierenden Erkenntnisse über die Funktionen und Mechanismen hinsichtlich des PPARα stammen aus experimentellen Ansätzen, in denen diese synthetischen Liganden eingesetzt wurden. Inzwischen sind fünf Hauptmechanismen etabliert, welche den Lipidstoffwechsel durch Fibrate modulieren und diese sind von Staels et al. (1998) wie folgt zusammengefasst worden: vermehrte Lipolyse triglyzeridreicher Lipoproteine durch erhöhte Aktivität der Lipoproteinlipase (LPL) bzw.

erniedrigten Konzentrationen des ApolipoproteinC-III (ApoC-III); Erhöhung der Fettsäureaufnahme und β-Oxidation in der Leber; Erhöhung der hepatischen Rückaufnahme von *low density lipoproteins* (LDL); Reduzierung des Austausches neutraler Lipide zwischen VLDL und *high density lipoproteins* (HDL) und Erhöhung des reversen Cholesteroltransportes des HDL durch vermehrte Produktion von ApoA-I und ApoA-II.
Neben den indirekten anti-atherogenen Eigenschaften, die aus diesen Mechanismen hervorgehen, konnte anhand von Untersuchungen mit Endothelgefäßzellen und glatten Muskelzellen gezeigt werden, dass eine Aktivierung des PPARα zu einer Repression von Signalwegen und Mechanismen führte, welche zur Entstehung obstruktiver Gefäßveränderungen im Rahmen der Atherosklerose beitragen (Seki et al., 2005; Gizard et al., 2005). Weiterhin konnte im Fettgewebe bei übergewichtigen Mäusen gezeigt werden, dass die Verabreichung eines PPARα-Agonisten neben einer erhöhten Insulin-Sensitivität zu einer Abnahme von Entzündungsparametern führte (Tsuchida et al., 2005). So stellen die anti-atherogenen und anti-inflammatorischen Eigenschaften des PPARα weitere interessante Aspekte dar, bei denen durch den therapeutischen Einsatz von synthetischen Liganden, aber auch durch natürliche Liganden über die Ernährung in die Entstehung pathologischer Prozesse Einfluss genommen werden kann.

1.4 Weitere Effekte synthetischer PPARα-Liganden und Speziesunterschiede

Die Fibrate zählen zu den sogenannten Peroxisomen-Proliferatoren (PPs), einer Gruppe unterschiedlicher chemischer Verbindungen, welche in Ratten und Mäusen PPARα-vermittelt zu einer Peroxisomenproliferation, Hyperplasie, Hypertrophie und langfristig zu Leberkrebs führen (Reddy et al. 1976, 1980). Die Ursachen für die Krebsentstehung werden in Veränderungen der Regulation von Apoptose und Zellzyklus und in oxidativem Stress als Folge der erhöhten Fettsäureoxidation in der Leber vermutet (Gonzalez et al., 1998; Peters et al., 2005). So konnte bei Mäusen gezeigt werden, dass die Gabe von Fibrat zu einer erhöhten hepatischen Konzentration an Wasserstoffperoxid (H_2O_2) führte (Arnaiz et al., 1995). H_2O_2 entsteht als Nebenprodukt der ACO und anderer peroxisomaler Oxidasen und wird unter normalen Umständen von der ebenfalls peroxisomal lokalisierten Katalase rasch abgebaut, da es ein Zellgift darstellt. Die Imbalanz zwischen einer starken PPARα-induzierten Erhöhung der ACO-Aktivität und daraus resultierenden vermehrten Produktion von H_2O_2 und die inadäquate Anpassung detoxifizierender Enzyme wird als Grund einer vermehrten Akkumulation angesehen (Yeldandi et al., 2000; Peters et al., 2005). So konnten in Ratten

Schädigungen der DNA durch PPs beobachtet werden (Conway et al., 1989; Kasai et al., 1989; Takagi et al., 1990), die in Verbindung einer verminderten Apoptose und gleichzeitig gesteigertem Zellzyklus zur Krebsentstehung führen können (Roberts, 1996; Peters, 1997). Weiterhin konnte die Induktion einer Peroxisomenproliferation auch nach der Verfütterung eines oxidierten Fettes bei Ratten beobachtet werden (Chao et al., 2005). Dass für diese Mechanismen der Krebsentstehung der PPARα eine zentrale Rolle einnimmt, erschließt sich aus Untersuchungen mit PPARα-null-Mäusen, bei denen trotz langfristiger Gabe von PPs keine Veränderungen, welche auf eine Krebsentstehung deuten könnten, zu beobachten waren (Peters et al., 1997; Roberts et al., 2000).

Dabei ist seit längerem bekannt, dass dieser Effekt einer Peroxisomenproliferation bei der Ratte und der Maus nach Gabe von PPs, welcher letztlich zur Namensgebung dieser Rezeptorenfamilie beigetragen hat, beim Menschen und anderen Spezies, wie Primaten oder dem Schwein nicht oder nur in moderater Form zu beobachten ist (Orton et al., 1984; Eacho et al., 1986; Hoivik et al., 2004). Worin die Unterschiede zwischen sogenannten proliferierenden (Ratte, Maus) und nicht-proliferierenden Spezies (Mensch, Schwein, Primaten usw.) im Detail bestehen, ist bis dato nicht restlos aufgeklärt. Aus den bisherigen Studien sind sowohl quantitative, als auch funktionelle Unterschiede des PPARα zwischen den verschiedenen Spezies bekannt. So konnte anhand der Quantifizierung der hepatischen mRNA des PPARα in Gewebeproben (Tugwood et al., 1998) und auch in primären Zellkulturen und Zelllinien (Ammerschlaeger et al., 2004) eine bis zu zehnfach höhere Konzentration bei Ratte und Maus gegenüber humanen Hepatozyten gezeigt werden. In neueren Untersuchungen an Mäusen, welchen humaner PPARα in das Lebergewebe transfiziert wurde, konnte nach Fibratgabe eine deutliche Verringerung der Inzidenz von Lebertumoren und eine Erhöhung der Genexpression für p53 beobachtet werden, was auf strukturelle Unterschiede zurückgeführt wurde (Morimura et al., 2006). Weiterhin konnte hinsichtlich funktioneller Unterschiede gezeigt werden, dass die PPREs der ACO bei Primaten und beim Menschen keinen Response bei einer Aktivierung des PPARα zeigen (Kane et al., 2006). Bei einer Untersuchung des Lebergewebes von Patienten, welche Fibrate einnahmen, konnte eine moderate Peroxisomenproliferation beobachtet werden (Hanefeld et al., 1983), während in anderen Studien dieser Effekt nicht auftrat (De La Iglesia et al., 1982; Blumcke et al., 1983). Aufgrund der Tatsache, dass Fibrate seit inzwischen mehreren Jahrzehnten als Lipidsenker von Menschen eingenommen werden, stellt die Frage, inwieweit die Einnahme von Fibraten auch beim Menschen langfristig Leberkrebs verursachen kann, eine nach wie vor hochaktuelle und praxisbezogene Thematik dar. Durch die begrenzte

Übertragbarkeit von Studien mit Ratten und Mäusen auf den Menschen, die sich durch die Speziesunterschiede ergeben, ist es notwendig, in weiteren Untersuchungen zu dieser Thematik alternative Tiermodelle mit größerer Homologie zum Menschen einzusetzen.
Neben den modulierenden Eigenschaften hinsichtlich des antioxidativen Status und der Regulation der Apoptose und des Zellzyklus, ist aus vielen Untersuchungen bekannt, dass PPs die Aktivitäten mikrosomaler Enzyme modulieren, welche zu den Proteinen der Phase-II Biotransformation gehören und so zur Deaktivierung und Eliminierung endogener und exogener Metabolite in der Leber und extrahepatischen Geweben beitragen. Dazu gehören die *UDP-Glucuronyltransferasen* (UGTs), welche als UGT1- und UGT2-Isoformen im endoplasmatischen Retikulum lokalisiert sind und unter Verwendung von UDP-Glucuronsäure mit überlappender Substratspezifität unterschiedliche Stoffe glucuronidieren und so die renale Exkretion ermöglichen (Mackenzie et al., 1997; Wells et al., 2004; Shelby und Klaassen, 2006). Die Schilddrüsenhormone *Thyroxin* (T_4) und *Triiodthyronin* (T_3) stellen Substrate der UGTs dar und in Versuchen mit Ratten konnte beobachtet werden, dass PPs eine Absenkung der Plasmaspiegel von Schilddrüsenhormonen durch eine erhöhte hepatische Glucuronidierung induzieren und zu Hypertrophie und Hyperplasie in der Schilddrüse führen (Beetstra et al., 1991; Saito et al., 1991; Visser et al., 1993; Barter und Klaassen, 1992, 1994). Dabei wird die Beeinflussung des Schilddrüsenstoffwechsels durch die Induktion mikrosomaler Enzyme kritisch hinsichtlich neoplastischer Veränderungen und der Tumorenstehung in der Schilddrüse betrachtet (Klaassen und Hood, 2001). Untersuchungen, in denen für die Isoformen UGT1A9 und UGT2B4 jeweils funktionelle PPREs nachgewiesen werden konnten (Barbier et al., 2003a, 2003b), lassen auch in diesem Zusammenhang eine zentrale Rolle des PPARα vermuten. Die Tatsache, dass die für Ratten beobachteten Veränderungen des Schilddrüsenstoffwechsels in Mäusen nach Clofibratgabe nicht auftreten (Viollon-Abadie et al., 1999), zeigen auch hinsichtlich der Effekte auf den Fremdstoffmetabolismus Unterschiede zwischen verschiedenen Spezies auf. Allerdings sind bisher keine Untersuchungen an sogenannten nicht-proliferierenden Spezies bekannt, welche Rückschlüsse auf die Relevanz dieser Effekte bei der Einnahme von Fibraten beim Menschen zulassen könnten.

1.5 Molekulare Grundlagen und Funktion der SREBPs im Lipidstoffwechsel

Neben dem PPARα nehmen die *sterol regulatory element binding proteins* (SREBPs) eine zentrale Rolle in der transkriptionellen Regulation des hepatischen Lipidstoffwechsels ein.

Während SREBP-1c vor allem die Expression von Proteinen der Biosynthese von Fettsäuren reguliert, werden die Gene für Proteine der Cholesterolbiosynthese durch SREBP-2 aktiviert (Horton et al., 2002; Abb. 2). Beide Transkriptionsfaktoren sind als inaktive Vorstufen in der Membran des ER gebunden und müssen an die beiden Proteasen site-1 und site-2 Protease im Golgiapparat gebunden werden, um aktiviert zu werden. Die folgende Abspaltung der bHLH-Zip-containing Domäne ermöglicht die Translokation in den Zellkern, worauf hin die Transkription der Zielgene aktiviert wird. Durch die intrazelluläre Akkumulation von Cholesterol durch endogene Synthese oder Aufnahme von exogenem Cholesterol in die Zelle, wird die Aktivität der SREBPs in der Leber inhibiert, was einen wichtigen Feedback-Mechanismus zur Gewährleistung der Lipidhomöostase darstellt (Brown und Goldstein, 1999; Goldstein et al., 2006). In verschiedenen Studien konnte gezeigt werden, dass eine Aktivierung des PPARα einen Einfluss auf die Expression und Aktivität der SREBPs hat (Patel et al., 2001; Guo et al., 2001; Knight et al., 2005; König et al., 2007). In welchem Umfang die aus diesen Studien hervorgegangenen Erkenntnisse über die Interaktionen dieser Faktoren auf die Regulation des Lipidstoffwechsels in anderen Spezies übertragbar sind oder ob sich auch in diesem Zusammenhang weitere Speziesunterschiede aufzeigen, sollte in weiteren Studien detailliert untersucht werden.

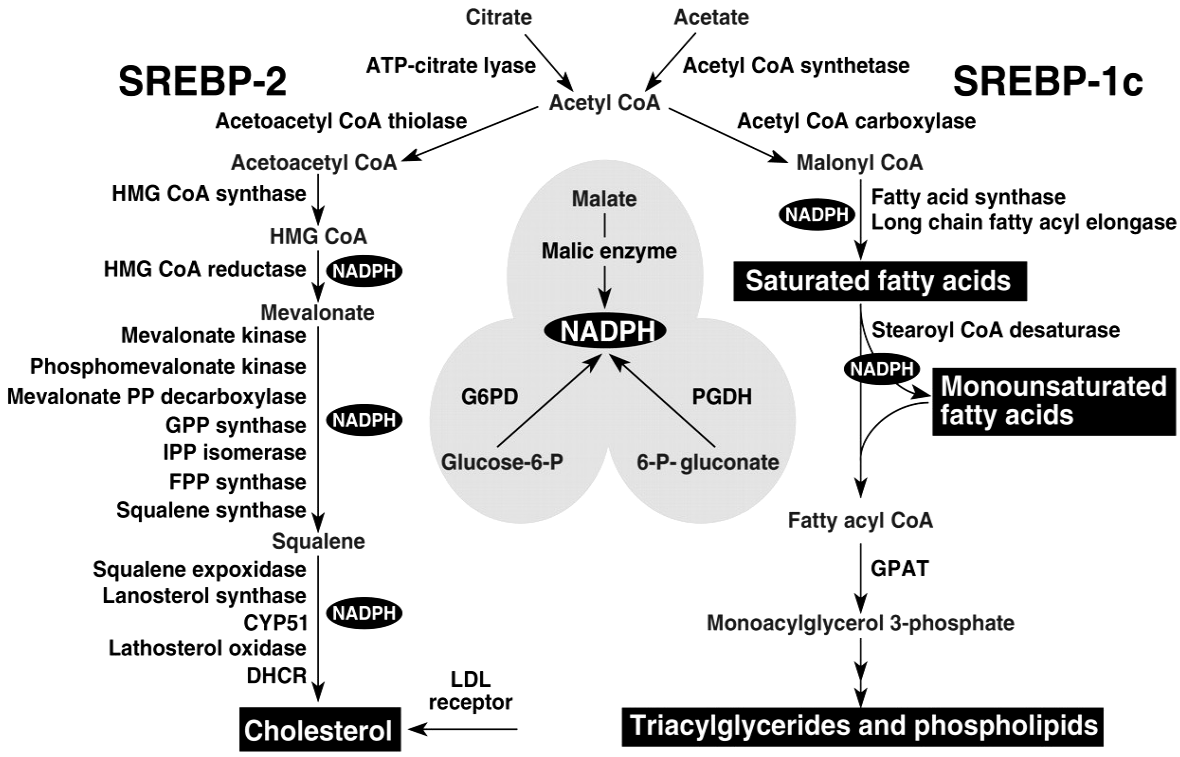

Abb. 2: Schematische Darstellung von Intermediärprodukten und Genen der Fettsäure- und Cholesterolsynthese, welche durch die SREBPs reguliert werden. (aus Horton et al., 2002)

2. Zielstellung

2.1 Zielstellung der Untersuchungen am Modelltier Schwein

Der Erkenntnisgewinn um die Funktion des PPARα hinsichtlich verschiedener Stoffwechselprozesse und Regulationsmechanismen stellt sowohl aus ernährungsphysiologischer, als auch aus pharmakologischer und toxikologischer Sicht eine nach wie vor hochaktuelle Thematik für die Gesundheit des Menschen dar. Um die Effekte von synthetischen und natürlichen PPARα-Agonisten *in vivo* auf verschiedene Parameter des Lipidstoffwechsels, der Zellzyklusregulation, der Phase-II Biotransformation und des antioxidativen Systems intensiv untersuchen zu können, wurde als Grundlage ein Fütterungsversuch mit Schweinen durchgeführt. Das Modelltier Schwein (*sus scrofa*) weist eine hohe Ähnlichkeit in Bezug auf den Lipidstoffwechsel (Carey, 1993), den Fremdstoffmetabolismus (Zuber et al., 2002) und grundlegende physiologische Eigenschaften des Menschen (Lunney, 2007) auf. Hinsichtlich des PPARα zählt das Schwein, wie auch der Mensch, zu den nicht-proliferierenden Spezies (Cheon et al., 2005). Es ist deswegen als Modelltier sehr gut geeignet, um in diesem Zusammenhang mögliche Rückschlüsse auf die Humanphysiologie ziehen zu können. Im Fütterungsversuch erhielt die Kontrollgruppe eine adäquate Standarddiät, eine Behandlungsgruppe ein moderat oxidiertes Fett als natürlichen PPARα-Agonisten zur Standarddiät (Sülzle et al., 2004) und eine weitere Behandlungsgruppe erhielt zusätzlich zur Standarddiät Clofibrat als etablierten synthetischen PPARα-Agonisten (Miller und Ntambi, 1996; Baker et al., 2004).

2.1.1 Untersuchungen zum Einfluss einer Clofibrat-induzierten PPARα-Aktivierung auf den Lipidstoffwechsel beim Schwein

Die erste Zielstellung des Fütterungsversuches war, zu untersuchen, welche Modifikationen nach oraler Gabe des synthetischen PPARα-Agonisten Clofibrat beim Schwein in Bezug auf den Lipidstoffwechsel und die Ketogenese zu beobachten waren. Dazu wurden die Lipid- und Ketonkörperkonzentrationen im Plasma und die Expression der mitochondrialen β-Hydroxy-β-methylglutaryl (HMG)-CoA-Synthase als Schlüsselenzym der Ketogenese in der Leber gemessen. Neben Veränderungen der Expression bekannter PPARα-Zielgene sollte im Rahmen dieser Zielstellung besonders berücksichtigt werden, inwieweit eine Aktivierung des PPARα die Expression der Gene, welche für die SREBPs und deren Zielgene kodieren, beim Schwein beeinflusst. Im Zusammenhang der SREBP-vermittelten Lipidsynthese stellen die Insig-Proteine durch ihre inhibierende Funktion auf die SREBP wichtige Faktoren in der

Regulation der Cholesterol- und Triglyzerid-Homöostase dar (Engelking et al., 2005). Erst kürzlich konnten die Gene für Insig-1 und Insig-2 für das Schwein beschrieben werden (Qiu et al., 2005a, 2005b), allerdings sind bisher keine weiteren Untersuchungen bekannt, welche die Übertragbarkeit der Erkenntnisse der Interaktionen zwischen den SREBPs und Insigs auf das Schwein zulassen. Im Rahmen dieses Versuches sollte deshalb untersucht werden, ob eine Clofibrat-induzierte PPARα-Aktivierung beim Schwein die Expression der Insigs beeinflusst und auf diesem Wege modulierend auf den Lipidstoffwechsel eingreifen könnte. Während die Lipogenese bei der Ratte hauptsächlich in der Leber stattfindet (Gandemier et al., 1982), konnte in Untersuchungen gezeigt werden, dass Schlüsselenzyme der Lipogenese, wie SREBP-1c und die Fettsäuresynthase (FAS), beim Schwein im Fettgewebe höher exprimiert sind, als in der Leber (Ding et al., 1999, 2000). Deshalb wurde neben der Leber als Zielgewebe das weiße Fettgewebe in die Expressionsanalysen einbezogen. Weiterhin sollte die Quantifizierung der mRNA des PPARα in verschiedenen Geweben und Spezies Rückschlüsse auf Speziesunterschiede hinsichtlich einer PPARα-Aktivierung geben. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Luci S, Giemsa B, Kluge H, Eder K (2007) Clofibrate causes an up-regulation of PPAR {alpha} target genes but does not alter expression of SREBP target genes in liver and adipose tissue of pigs. **Am J Physiol Regul Integr Comp Physiol.** (online published)

2.1.2 Untersuchungen zum Einfluss von Clofibrat auf den Schilddrüsenstoffwechsel beim Schwein

Im Rahmen der zweiten Zielstellung sollte untersucht werden, welchen Einfluss eine Clofibrat-induzierte PPARα-Aktivierung auf die hepatische Phase-II Biotransformation speziell der Schilddrüsenhormone und daraus resultierend auf deren Plasmakonzentrationen beim Schwein hat. Bisher sind für das Schwein und andere nicht-proliferierende Spezies keine *in vivo*-Studien bekannt, welche den Einfluss synthetischer PPARα-Liganden auf die Glucuronidierung von Schilddrüsenhormonen beschreiben. Um Auswirkungen einer möglichen Modifikation des Schilddrüsenstoffwechsels auf die Schilddrüse näher zu charakterisieren, wurden Untersuchungen hinsichtlich der Schilddrüsenmorphologie und Expressionsanalysen schilddrüsenspezifischer Gene in die Zielstellung einbezogen. Weiterhin sollte anhand von Expressionsanalysen untersucht werden, ob die Clofibratgabe beim

Schwein einen Einfluss auf die Signaltransduktion (Thyroidhormon-Rezeptor α_1) und die Konvertierung (Typ I-Dejodase) von Schilddrüsenhormonen in der Leber und auf die Plasmatransportproteine (Albumin, Transthyretin und Thyroxin bindendes Globulin) für Schilddrüsenhormone in der Leber hat. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Luci S, Kluge H, Hirche F, Eder K (2006) Clofibrate increases hepatic triiodothyronine (T_3)- and thyroxine (T_4)- glucuronosyltransferase activities and lowers plasma T_3 and T_4 concentrations in pigs. **Drug Metab Dispos** 34:1887-92.

2.1.3 Untersuchungen zum Einfluss von Clofibrat auf verschiedene Parameter des antioxidativen Status und der Regulation von Apoptose und Zellzyklus in der Leber beim Schwein

In einer dritten Zielstellung sollte der Frage nachgegangen werden, inwieweit die Gabe von Clofibrat beim Schwein zu pathologischen Veränderungen des hepatischen Gewebes führen kann und so möglicherweise zur Krebsentstehung, wie es bei chronischer Applikation von Fibraten bei Nagern gezeigt worden ist, beitragen könnte. Bisher sind keine Untersuchungen beim Schwein bezüglich dieser Aspekte durchgeführt worden. Eine zentrale Rolle stellt dabei vor allem oxidativer Stress dar, welcher durch Induktion der β-Oxidation in der Leber entstehen kann und dadurch möglicherweise die Aktivität von Enzymen der antioxidativen Abwehr, die Konzentrationen antioxidativ wirksamer Substanzen und die Konzentrationen von Lipidperoxidationsprodukten modifizieren kann. Außerdem sollte untersucht werden, inwieweit die Expression von Genen für Proteine, welche an der Regulation des Zellzyklus und der Apoptose beteiligt sind, durch den synthetischen Agonisten beeinflusst werden. Die Induktion einer Peroxisomenproliferation stellt einen weiteren interessanten Aspekt diesbezüglich dar und sollte in diesem Zusammenhang untersucht werden. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Luci S, Giemsa B, Hause G, Kluge H, Eder K (2007) Clofibrate treatment in pigs: Effects on parameters critical with respect to peroxisome proliferator-induced hepatocarcinogenesis in rodents. **BMC Pharmacology** 7:6.

2.1.4 Untersuchungen einer PPARα-Aktivierung durch moderat oxidiertes Fett beim Schwein

Als vierte Zielstellung sollte untersucht werden, ob die Verfütterung eines moderat oxidierten Fettes eine PPARα-Aktivierung in der Leber bei Schweinen, als Vertreter nichtproliferierender Spezies, induzieren kann. In diesem Zusammenhang sollten Parameter des Lipidstoffwechsels, der Ketogenese, des antioxidativen Status und der Lipidperoxidation, sowie eine mögliche Induktion einer Peroxisomenproliferation untersucht werden. Neben der Modulation bekannter hepatischer PPARα-Zielgene sollte auch untersucht werden, inwieweit die Expression der SREBPs und deren Zielgene sowie die Expression der Insigs durch Verfütterung eines moderat oxidierten Fettes als potentieller natürlicher PPARα-Agonist in der Leber bei Schweinen beeinflusst werden kann. Zusätzlich wurden Expressionsanalysen in den Enterozyten des Dünndarms in die Untersuchungen einbezogen, um Aussagen zu einer möglichen Aktivierung des PPARα bereits im Dünndarm treffen zu können. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Luci S, König B, Giemsa B, Huber S, Hause G, Kluge H, Stangl GI, Eder K (2007) Feeding of deep-fried fat causes PPARα activation in the liver of pigs as a non-proliferating species. **Br J Nutr** 7:1-11.

2.1.5 Untersuchungen zum Einfluss oxidierter Linolsäure auf Parameter des Schilddrüsenstoffwechsel in primären Thyreozyten vom Schwein

Im Rahmen einer fünften Zielstellung sollte anhand des *in vitro*-Modelles primärer Schilddrüsenkulturen vom Schwein untersucht werden, ob Lipidperoxidationsprodukte *per se* einen Einfluss auf den Schilddrüsenstoffwechsel ausüben. Zu diesem Zweck wurde die 13-Hydroperoxy-9,11-octadecadiensäure (13-HPODE) als primäres Oxidationsprodukt der Linolsäure (18:2n-6) und etablierter Vertreter von primären Lipidperoxidationsprodukten in verschiedenen Zellkulturstudien (Friedrichs et al., 1999; Meilhac et al., 2000; König und Eder, 2006), für diese Zielstellung eingesetzt. Es sollte untersucht werden, ob 13-HPODE die Expression schilddrüsenspezifischer Gene, die Iodid-Aufnahme und die Produktion von Wasserstoffperoxid (H_2O_2) in den Schilddrüsenzellen beeinflusst. Gleichzeitig sollte in diesem Zusammenhang ermittelt werden, welche Veränderungen sich durch das 13-HPODE

hinsichtlich der Aktivität antioxidativ wirksamer Enzyme und der intrazellulären Konzentration konjugierter Diene ergeben. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Luci S, Bettzieche A, Brandsch B, Eder K (2006) Effects of 13-HPODE on expression of genes involved in thyroid hormone synthesis, iodide uptake and formation of hydrogen peroxide in porcine thyrocytes. **Int J Vitam Nutr Res** 76:398-406.

2.2 Zielstellung der Untersuchungen am Modelltier Ratte

Für die Bearbeitung verschiedener Fragestellungen hinsichtlich des Einflusses einer PPARα-Aktivierung auf den Carnitinstoffwechsel wurden für weitere *in vivo*-Studien Sprague-Dawley-Ratten eingesetzt, welche als Modelltier bereits erfolgreich in verschiedenen Studien für Untersuchungen des Carnitinstoffwechsels verwendet worden sind (Brandsch und Eder, 2002; Davis und Monroe, 2005). Das vollständig entschlüsselte Genom der Ratte und bereits existierende Arbeiten hinsichtlich der Expressionsanalyse von Enzymen der Carnitinbiosynthese (Davis und Monroe, 2005) und verschiedener Carnitintransporter (Wu et al., 1999, 2000) stellen einen eindeutigen Vorteil dieses Modelltieres gegenüber dem Schwein dar, für das zum Zeitpunkt der Planung und Durchführung der Experimente keine Sequenzen dieser Gene verfügbar waren. Ein besonderes Interesse im Rahmen der Untersuchungen galt dabei den *novel organic cation transporters* (OCTNs), von denen vor allem der OCTN2 (*SLC22A5*) mit hoher Affinität und natriumabhängig Carnitin aus dem Plasma in die Zelle transportiert (Tamai et al., 1998) und für die intestinale Absorption und renale Rückresorption des Carnitins verantwortlich ist (Slitt et al., 2002).

2.2.1 Untersuchungen zum Einfluss einer Clofibrat-induzierten PPARα-Aktivierung auf den Carnitinstoffwechsel bei der Ratte

In der sechsten Zielstellung sollte grundlegend untersucht werden, welchen Einfluss eine PPARα-Aktivierung auf die Expression von Genen, welche für Enzyme der Carnitinbiosynthese und des transmembranösen Carnitintransportes in der Leber kodieren und auf die Carnitinkonzentrationen in Plasma und verschiedenen Geweben *in vivo* hat. Zu diesem

Zweck wurde männlichen Ratten Clofibrat als synthetischen PPARα-Agonisten zur Standarddiät verabreicht. Zusätzlich wurden Zellkulturexperimente mit der hepatischen Rattenzelllinie FAO und dem synthetischen PPARα-Agonisten WY-14,643 durchgeführt, um die Effekte einer PPARα-Aktivierung auf den Carnitinstoffwechsel am isolierten Zellkulturmodell untersuchen zu können. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Luci S, Geissler S, König B, Koch A, Stangl GI, Hirche F, Eder K (2006) PPARα agonists up-regulate organic cation transporters in rat liver cells. **Biochem Biophys Res Commun** 350:704-708.

2.2.2 Untersuchungen zum Einfluss einer PPARα-Aktivierung durch oxidiertes Fett auf den Carnitinstoffwechsel bei der Ratte

In einer siebten Zielstellung sollte der Frage nachgegangen werden, ob die Aufnahme eines oxidierten Fettes als natürlicher PPARα-Agonist zu Veränderungen der Expression von Genen der Enzyme der Carnitinbiosynthese, des Carnitintransportes und carnitin-abhängiger Transferasen in der Leber und zu Veränderungen der Carnitinkonzentrationen in Plasma und verschiedenen Geweben führt. Weiterhin sollte in diesem Versuchsansatz untersucht werden, ob durch eine PPARα-Aktivierung durch oxidiertes Fett im Dünndarm die Expression von Transportern, welche für die Carnitinaufnahme aus dem Darmlumen verantwortlich sind, beeinflusst werden. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Koch A, König B, Luci S, Stangl GI, Eder K (2007) Dietary oxidised fat up-regulates expression of organic cation transporters in liver and small intestine and alters carnitine concentrations in liver, muscle and plasma of rats. **Br J Nutr** 25:1-8.

2.2.3 Untersuchungen des Carnitinstoffwechsels in unterschiedlichen Fastenzuständen bei der Ratte

Im Rahmen der achten Zielstellung sollten mögliche systemische Veränderungen des Carnitinstoffwechsels beim Fasten untersucht werden. Im physiologischen Zustand des Fastens wird der PPARα durch freie Fettsäuren, welche vom Fettgewebe freigesetzt werden

und so vermehrt im Blut zirkulieren, aktiviert. Für diese Fragestellung wurden Ratten über 10 Tage mit 70 bzw. 40% Standarddiät, ausgehend vom Grundbedarf der Tiere, gefüttert, während der Kontrollgruppe das Futter *ad libitum* zur Verfügung stand. Zusätzlich wurde für die Simulation eines kurzfristigen Fastenzustandes eine Behandlungsgruppe für 24 h einer vollständigen Nahrungskarenz ausgesetzt. Im Anschluss sollten freies Carnitin, Carnitinester und Vorstufen des Carnitins (γ-Butyrobetain, Trimethyllysin) in verschiedenen Geweben untersucht werden. Einen weiteren Schwerpunkt dieses Versuches sollten die Expressionsanalysen hinsichtlich der Enzyme der Carnitinbiosynthese, des Carnitintransportes und carnitin-abhängiger Transferasen in verschiedenen Geweben darstellen, um möglicherweise Rückschlüsse über die Ursachen beobachteter Veränderungen von Plasma- und Gewebekonzentrationen von Carnitin, Carnitinestern und Vorstufen ziehen zu können. Als Positivkontrolle einer fasten-induzierten PPARα-Aktivierung sollten die freien Fettsäuren im Plasma, sowie die Expression der *acyl-CoA-oxidase* (ACO) als klassisches PPARα-Zielgen und Enzym der β-Oxidation gemessen werden. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Luci S, Hirche F, Eder K (2007) Effects of fasting or caloric restriction on mRNA concentration of organic cation transporter-2 and carnitine concentrations in tissues of rats. (eingereicht im **Br J Nutr**)

Am J Physiol Regul Integr Comp Physiol 293: R000–R000, 2007.
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Clofibrate causes an upregulation of PPAR-α target genes but does not alter expression of SREBP target genes in liver and adipose tissue of pigs

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Luci S, Giemsa B, Kluge H, Eder K. Clofibrate causes an upregulation of PPAR-α target genes but does not alter expression of SREBP target genes in liver and adipose tissue of pigs. *Am J Physiol Regul Integr Comp Physiol* 293: R000–R000, 2007. First published March 15, 2007; doi:10.1152/ajpregu.00603.2006.—This study investigated the effect of clofibrate treatment on expression of target genes of peroxisome proliferator-activated receptor (PPAR)-α and various genes of the lipid metabolism in liver and adipose tissue of pigs. An experiment with 18 pigs was performed in which pigs were fed either a control diet or the same diet supplemented with 5 g clofibrate/kg for 28 days. Pigs treated with clofibrate had heavier livers, moderately increased mRNA concentrations of various PPAR-α target genes in liver and adipose tissue, a higher concentration of 3-hydroxybutyrate, and markedly lower concentrations of triglycerides and cholesterol in plasma and lipoproteins than control pigs ($P < 0.05$). mRNA concentrations of sterol regulatory element-binding proteins (SREBP)-1 and -2, insulin-induced genes (*Insig*)*-1* and *Insig-2*, and the SREBP target genes acetyl-CoA carboxylase, 3-methyl-3-hydroxyglutaryl-CoA reductase, and low-density lipoprotein receptor in liver and adipose tissue and mRNA concentrations of apolipoproteins A-I, A-II, and C-III in the liver were not different between both groups of pigs. In conclusion, this study shows that clofibrate treatment activates PPAR-α in liver and adipose tissue and has a strong hypotriglyceridemic and hypocholesterolemic effect in pigs. The finding that mRNA concentrations of some proteins responsible for the hypolipidemic action of fibrates in humans were not altered suggests that there were certain differences in the mode of action compared with humans. It is also shown that PPAR-α activation by clofibrate does not affect hepatic expression of SREBP target genes involved in synthesis of triglycerides and cholesterol homeostasis in liver and adipose tissue of pigs.

peroxisome proliferator-activated receptor-α; cholesterol; triglycerides

FIBRATES ARE A GROUP OF HYPOLIPIDEMIC agents that have been in clinical use for several decades in humans (46). It is well established that these agents act as synthetic agonists of peroxisome proliferator-activating receptor-α (PPAR-α), a nuclear receptor also activated by natural ligands such as free fatty acids or some eicosanoids. PPAR-α is an important regulator of cellular fatty acid uptake and intracellular fatty acid transport, mitochondrial and peroxisomal fatty acid oxidation, ketogenesis, and gluconeogenesis (48). In humans, the most pronounced effect of fibrates is a decrease in plasma triglyceride-rich lipoproteins. Concentrations of low-density lipoprotein (LDL) cholesterol generally decrease in individuals with elevated baseline plasma concentrations, and plasma high-density lipoprotein (HDL) cholesterol concentrations are usually increased when baseline concentrations are low (46). Effects of PPAR-α activation have been mostly studied in rodents, which exhibit a strong expression of PPAR-α in liver and show peroxisome proliferation in the liver in response to PPAR-α activation (36). Expression of PPAR-α and sensitivity to peroxisomal induction by PPAR-α agonists, however, vary greatly among species (19, 21). In contrast to rats and mice, which are highly sensitive to induction by peroxisome proliferators, guinea pigs, monkeys, pigs, and humans are relatively insensitive (21, 32, 39, 48). In these nonproliferating species, expression of PPAR-α in the liver is much lower and the response of many genes to PPAR-α activation is weaker than in proliferating species (8).

In contrast to rodents, there is little information to date about the effects of PPAR-α agonists on lipid metabolism in pigs, which are not only of agricultural importance but are also a valuable model for studying the lipid metabolism because of their close relationship to humans (6). It has been shown that treatment of pigs with clofibrate stimulates mitochondrial and peroxisomal β-oxidation in liver, muscle, and kidney (34, 56). Moreover, it was found that pigs express functional PPAR-α in the liver, and several target genes induced in the liver by PPAR-α activation have been identified (8). However, effects of PPAR-α activation on lipid concentrations in plasma and liver of pigs have not yet been investigated. In contrast to rats, mice, or humans in which PPAR-α is predominant in liver (11), pigs exhibit also a high expression of PPAR-α in adipose tissue, and it has been suggested that pigs have a considerable capacity for β-oxidation in adipose tissue (13). The effect of PPAR-α agonists on gene expression of PPAR-α target genes in adipose tissue of pigs, however, has not yet been investigated.

Recent studies (17, 23, 25, 33) in rodents suggested that activation of PPAR-α influences hepatic triglyceride synthesis and cholesterol homeostasis by interacting with gene expression or proteolytic activation of sterol regulatory element-binding proteins (SREBPs), key regulators of lipid synthesis and homeostasis. SREBP-1 preferentially activates genes required for fatty acid synthesis, whereas SREBP-2 preferentially activates the LDL receptor gene and various genes required for cholesterol synthesis (22). SREBPs are synthesized as inactive precursors bound to the endoplasmatic reticulum membranes. For activation to occur, membranes have to be cleaved by two resident proteases within the Golgi, which sequentially cleave the SREBPs and release the amino-terminal bHLH-Zip-containing domain from the membrane, allowing it to translocate to the nucleus and activate transcription of target

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genes. Insulin-induced genes (*Insig*)-*1* and *Insig-2* are modulators of SREBP activity (53, 55). They block the proteolytic cleavage and transcriptional activation of SREBP. In pigs, in contrast to rodents in which lipogenesis takes place primarily in the liver (15, 44), adipose tissue is the major site of lipogenesis. It has been shown that SREBP-1 and its target gene fatty acid synthase, one of the key enzymes of de novo fatty acid synthesis, are expressed at a higher level in pig adipose tissue than in pig liver (12, 13). Whether a link exists also in pigs between PPAR-α activation and gene expression or proteolytic activation of SREBPs in liver or adipose tissue, which in turn could influence lipid synthesis, is presently unknown.

The objective of the present study was to investigate the effects of clofibrate treatment on expression of genes involved in lipid metabolism in liver and adipose tissue of pigs. We were particularly interested in to what extent clofibrate upregulates PPAR-α target genes in liver and adipose tissue of pigs and whether there is an interaction between PPAR-α activation and gene expression or proteolytic activation of SREBPs in these tissues. Therefore, we performed an experiment in which pigs were treated with clofibrate. We used relatively young pigs with a body weight slightly in excess of 10 kg because it has been recently shown that such young pigs express a functional PPAR-α in the liver (8). To characterize hepatic PPAR-α expression in the pig model, we compared mRNA concentration of PPAR-α in pig liver with those of rat and human livers. To assess PPAR-α expression in pig adipose tissue, we compared PPAR-α mRNA concentration in pig liver and pig adipose tissue. To examine PPAR-α activation in this animal model by clofibrate, we determined mRNA concentrations of several PPAR-α target genes in liver and adipose tissue and also plasma concentration of 3-hydroxybutyrate because it is known that PPAR-α activation leads to stimulation of ketogenesis (28). To find out whether activation of PPAR-α by clofibrate treatment also affects expression or proteolytic processing of SREBPs in pig liver or adipose tissue, we determined gene expression of SREBP-1, SREBP-2, *Insigs*, and target genes of SREBP-1 [acetyl-CoA carboxylase (ACC)] and SREBP-2 [3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and LDL receptor] in these tissues. To explore the molecular basis of alterations of plasma lipoprotein concentrations, we also determined genes involved in lipoprotein metabolism such as apolipoproteins (apo)A-I, apoA-II, and apoC-III and microsomal triglyceride transfer protein (MTP).

MATERIALS AND METHODS

Animals and treatments. Eighteen male 8-wk-old crossbred pigs [(German Landrace × Large White) × Pietrain] were kept in a room under controlled temperature at 23 ± 2°C and 55 ± 5% relative humidity with lights on from 0600 to 1800. One day before the beginning of the experimental feeding period, the pigs were weighed and randomly allocated to two groups, with body weights of 12.0 ± 0.4 kg in the control group and 11.9 ± 0.2 kg in the treatment group (means ± SE). Both groups of pigs received a nutritionally adequate diet (31) for growing pigs, which contained (in g/kg) 400 wheat, 230 soybean meal, 150 wheat bran, 100 barley, and 90 sunflower oil, as well as a mineral premix that included L-lysine, DL-methionine, and L-threonine (30). This diet contained 14.4 MJ metabolizable energy and 185 g crude protein/kg. The diet of the treatment group was supplemented with 5 g clofibrate/kg diet. Diet intake was controlled, and each animal in the experiment was offered an identical amount of diet per day. The amount of diet administered was ~15% below that consumed ad libitum by pigs of a similar weight (as assessed in a previous study). Therefore, the diet offered was completely taken in by all pigs in the experiment. During the feeding period, the amount of diet offered each day was increased continuously from 400 to 1,200 g. The pigs had free access to water via nipple drinking systems. The experimental diets were administered for 28 days. All experimental procedures described followed established guidelines for the care and use of laboratory animals and were approved by the local veterinary office.

Sample collection. After completion of the feeding period, the animals were killed under light anesthesia. Four hours before euthanasia, each pig was fed its respective diet. After death, blood was collected into heparinized polyethylene tubes. Plasma was obtained by centrifugation of the blood (1,100 *g* at 4°C for 10 min). Plasma lipoproteins were separated by step-wise ultracentrifugation (Mikro-Ultrazentrifuge; Sorvall Products, Bad Homburg, Germany) at 900,000 *g* at 4°C for 1.5 h. Plasma densities were adjusted by sodium chloride and potassium bromide, and the lipoprotein fractions $\delta <$ 1.006 kg/l [very low-density lipoproteins (VLDL) plus chylomicrons], 1.006 kg/l $< \delta <$ 1.063 kg/l (LDL), and $\delta >$ 1.063 kg/l (HDL) were removed by suction. The liver was dissected and weighed, and samples of liver, skeletal muscle (longissimus dorsal muscle), and subcutaneous adipose tissue (backfat, at the level of the 13th/14th rib) were stored at −80°C until analysis. For comparison of PPAR-α expression in rats, humans, and pigs, liver samples of three male adult rats (362 ± 25 g) and three male adult humans (collected during a resection of a test sample for a histopathological evaluation) and liver and adipose tissue of three randomly selected piglets of the control group were used.

Lipid analysis. Lipids from liver were extracted with a mixture of *n*-hexane and isopropanol (3:2, vol/vol) (18). After lipid extracts were dried, aliquots were dissolved with Triton X-100 (10). The concentrations of cholesterol and triglycerides in the lipoprotein fractions, plasma, and liver were determined with enzymatic kits (no. 113009990314 for cholesterol and no. 157609990314 for triglycerides, Ecoline S^+; DiaSys, Holzheim, Germany).

Determination of 3-hydroxybutyrate. Concentration of 3-hydroxybutyrate in plasma was determined with an enzymatic assay (no. 10907979035; R-BIOPHARM, Darmstadt, Germany).

RT-PCR analysis. Total RNAs from liver tissue, skeletal muscle, and adipose tissue were isolated by a tissue lyser (Qiagen, Hilden, Germany) using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from optical densities at 260 and 280 nm (SpectraFluor Plus; Tecan, Crailsheim, Germany). The quality of all RNA samples was furthermore assessed by agarose gel electrophoresis. Total RNA (1.2 μg) was used for cDNA synthesis as described previously (24). The mRNA concentration of genes was measured by real-time PCR, using SYBR green I and an MJ Research Opticon system (Biozym Diagnostik, Oldendorf, Germany). Real-time PCR was performed with 1.25 U of *Taq* DNA polymerase, 500 μM dNTPs, and 26.7 pmol of the specific primers. Amplification efficiencies for all primer pairs were determined by template dilution series. Calculation of the relative mRNA concentration was made with the amplification efficiencies and the threshold cycle values (37). The housekeeping gene *GAPDH* was used for normalization. The PCR primers used for real-time RT-PCR were obtained from Operon (Köln, Germany) and Roth (Karlsruhe, Germany), respectively, and are listed in Table 1.

Quantification of PPAR-α mRNA. For the quantification of copy numbers of PPAR-α mRNA, isolation of total RNA and cDNA synthesis from rat, pig, and human liver tissues and pig white adipose tissue were performed as described above. Real-time PCR was carried out with specific primers (Table 1) as described above; afterward, an aliquot of 10 μl per PCR product was submitted to agarose gel electrophoresis to create standard templates. After dissection from

Table 1. *Characteristics of the specific primers used for RT-PCR*

Gene	Forward Primer (from 5′ to 3′)	Reverse Primer (from 5′ to 3′)	bp	Annealing Temperature	GenBank No.
ACC	CTCCAGGACAGCACAGATCA	GCCGAAACATCTCTGGGATA	170	60°C	AF175308
ACO	CTCGCAGACCCAGATGAAAT	TCCAAGCCTCGAAGATGAGT	218	60°C	AF185048
apoA-I	CGATCAAAGACAGTGGCAGA	GCTGCACCTTCTTCTTCACC	234	60°C	NM_214398
apoA-II	GGAAGGAAGGAAGGACGAAC	TCCCAGAAGTCGGTGAACTT	156	60°C	AJ564196
apoC-III	GACACCTCCCTTCTGGACAA	TCCCAGAAGTCGGTGAACTT	185	60°C	NM_001002801
CPT-I	GCATTTGTCCCATCTTTCGT	GCACTGGTCCTTCTGGGATA	198	60°C	AF288789
CYP7	TATAGGGCACGATGCACAGA	ACCTGACCAGTTCCGAGATG	200	60°C	NM_001005352
GAPDH	AGGGGCTCTCCAGAACATCATCC	TCGCGTGCTCTTGCTGGGGTTGG	446	60°C	AF017079
HMG-CoA-R	GGTCAGGATGCGGCACAGAACG	GCCCCACGGTCCCGATCTCTATG	127	65°C	S79678
Insig-1	AGAGGGAGTGGGCCAGTGTGATGC	ACGGGAGCCAGGAGCGGATGTAG	276	65°C	AY336601
Insig-2	AAATCACGCCAGCGCTAAAGTG	TCCTACTCCAAGGCCAAAACCAC	127	60°C	AY585269
LDL-R	TGCGAAGATATCGACGAGTG	TACGGTCCAGGGTCATCTTC	196	62°C	AF118147
L-FABP	TTCGGTGCATGTCTAAGCTG	TGAGAGGGAGAGGATGAGGA	200	60°C	DQ182323
LPL	TGGACGGTGACAGGAATGTA	AAGGCTGTATCCCAGGAGGT	237	60°C	NM_214286
mHMG-CoA-S	GGACCAAACAGACCTGGAGA	ATGGTCTCAGTGCCCACTTC	198	62°C	U90884
MTP	CAGGACGGCAAAGAAAGAAGG	ATGGGAAGCAAAACCACAAGG	199	60°C	AY217034
PPAR-α, rat	CCCTCTCTCCAGCTTCCAGCCC	CCACAAGCGTCTTCTCAGCCATG	555	65°C	NM_013196
PPARα, human	TGTGGCTGCTATCATTTGCTGTGG	CTCCCCCGTCTCCTTTGTAGTGC	344	60°C	NM_0010019
PPARα, pig	CAGCCTCCAGCCCCTCGTC	GCGGTCTCGGCATCTTCTAGG	381	60°C	DQ437887
SCD	ACGTTGTGCCAGTGAGTCAG	GTCTTGGCCTCTTGTGCTTC	206	62°C	NM_213781
SREBP-1	CCTCTGTCTCTCCTGCACC	ACAAAGAGAAGCGCCAAGAA	213	62°C	NM_214157
SREBP-2	CGCTCGCGAATCCTGCTGTG	GGTGCGGGTCCGTGTCGTG	103	65°C	DQ020476

ACO, acyl-CoA oxidase; ACC, acetyl-CoA carboxylase; apo, apolipoprotein; CPT-1, carnitine palmitoyltransferase 1; CYP7, cholesterol-7α-hydroxylase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; lnsig-1 and -2; insulin-induced genes 1 and 2; LDL-R, low-density lipoprotein receptor; LPL, lipoprotein lipase; L-FABP, liver fatty acid binding protein; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal triglyceride transfer protein; PPAR-α, peroxisome proliferator-activated receptor α; SREBP-1 and -2, sterol regulatory element-binding protein 1 and 2; SCD, stearoyl-CoA desaturase. *For semiquantitative PCR.

ethidium bromide-stained gel, probes from each species and tissue were pooled and eluted with the peqGOLD gel extraction kit (PeqLab Biotechnologie, Erlangen, Germany). For the generation of standard curves, the concentration of double-stranded cDNA was measured by a Pico green double-stranded DNA quantitation kit (Molecular Probes, Leiden, The Netherlands). The calculated molecular weight of each PCR product was converted into copy numbers by using Avogadro's number (1 mol = 6.022×10^{23} molecules). The threshold cycles measured by real-time PCR were plotted vs. the copy numbers of diluted standard templates to create a standard curve. On the basis of the respective standard curve, the threshold cycle of each probe was used to calculate the copy number of PPAR-α mRNA and was normalized to 1 ng of total RNA.

Statistics. The results were analyzed with Minitab (State College, PA) statistical software (release 13). Statistical significances of differences between control group and treatment group were evaluated with Student's *t*-test. Mean values were considered significantly different at $P < 0.05$. Data in the text are presented as means ± SE.

RESULTS

Comparison of mRNA concentrations of PPAR-α in human, rat, and pig liver and in pig adipose tissue. To characterize PPAR-α gene expression in the pig model used, we determined mRNA concentrations of PPAR-α in liver and adipose tissue of three control pigs and compared them with those in livers of three adult rats and three male human subjects. PPAR-α mRNA concentrations, corrected for total RNA concentration, were similar in human and in pig liver; PPAR-α mRNA concentrations in human or pig liver were, however, ~10-fold lower than concentrations in rat liver (Fig. 1). In pigs, mRNA concentrations of PPAR-α were similar in adipose tissue and in liver (Fig. 1).

Food intake and body and liver weights in control pigs and pigs treated with clofibrate. Because we used a controlled feeding system, food intake throughout the feeding period was the same for each pig in the experiment, averaging 696 ± 2 g/day. Body weight after the 28-day experiment period did not differ between control pigs and pigs treated with clofibrate (Table 2). Relative liver weights, expressed per kilogram of body weight, were higher in pigs treated with clofibrate than in control pigs ($P < 0.05$; Table 2).

Gene expression in the liver of control pigs and pigs treated with clofibrate. mRNA concentration of PPAR-α in liver did not differ between both groups of pigs (control: 1.00 ± 0.13; clofibrate: 0.92 ± 0.07; $n = 9$), whereas relative mRNA concentrations of acyl-CoA oxidase (ACO), carnitine palmitoyltransferase (CPT) I, liver fatty acid binding protein (L-FABP), mitochondrial HMG-CoA synthase, and stearoyl-CoA desaturase (SCD) in the liver were moderately increased (1.7-

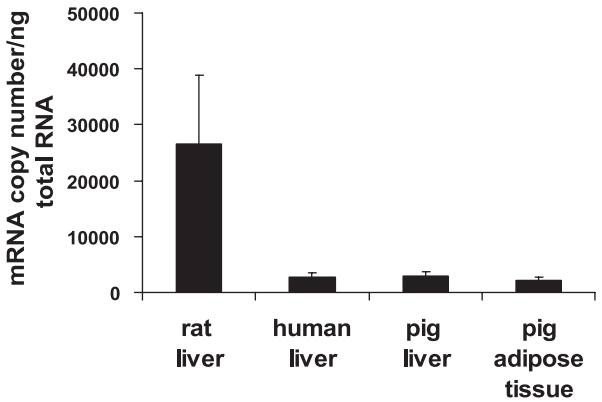

Fig. 1. mRNA concentrations of peroxisome proliferator-activated receptor (PPAR)-α in liver tissue of rats, humans, and control pigs and in adipose tissue of control pigs. mRNA concentrations were determined by real-time quantitative PCR and normalized to total RNA concentration. Data are means ± SE of 3 samples per species.

Table 2. *Body and liver weights and concentrations of triglycerides and cholesterol in plasma, lipoprotein fractions, and liver tissue in pigs fed a control diet or a diet supplemented with 5 g clofibrate/kg diet for 28 days*

	Control	Clofibrate
Initial body weight, kg	12.0±0.4	11.9±0.2
Final body weight, kg	26.0±0.5	25.2±0.4
Liver weight, g/kg body wt	25.9±0.8	30.9±0.9*
Triglycerides		
Plasma, mmol/l	1.09±0.06	0.78±0.08*
VLDL + chylomicrons, mmol/l	0.93±0.06	0.67±0.07*
Liver, μmol/g	90.8±6.7	82.7±8.1
Cholesterol		
Plasma, mmol/l	2.83±0.08	0.92±0.13*
LDL, mmol/l	0.97±0.05	0.38±0.08*
HDL, mmol/l	1.13±0.04	0.31±0.08*
Liver, μmol/g	69.0±3.6	73.1±3.0

Values are means ± SE with 9 animals per group. VLDL, very low-density lipoprotein; HDL, high-density lipoprotein. *$P < 0.05$ compared with control group.

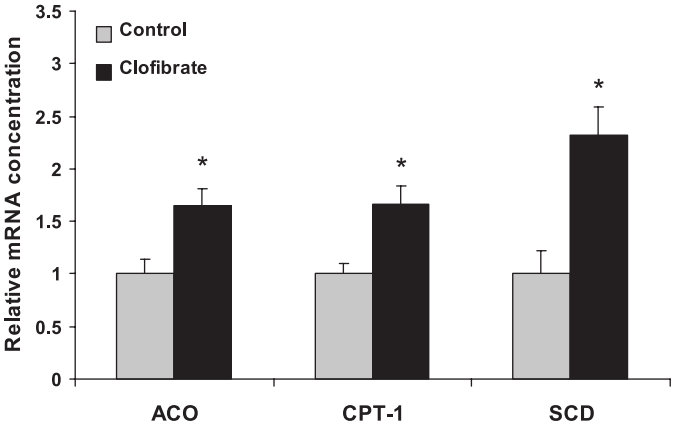

Fig. 3. Relative mRNA concentrations of ACO, CPT-1, and SCD in the adipose tissue of pigs fed a control diet or a diet supplemented with 5 g clofibrate/kg diet for 28 days. mRNA concentrations were determined by real-time RT-PCR and normalized to GAPDH. Data are means ± SE of 9 animals per group and are expressed relative to mRNA concentrations of control pigs (=1). *Significantly different from control group ($P < 0.05$).

to 2.5-fold) in pigs treated with clofibrate compared with control pigs ($P < 0.05$; Fig. 2). Relative mRNA concentrations of MTP and apoA-I, apoA-II, and apoC-III were not different between the two groups of pigs (relative mRNA concentrations in control pigs and pigs treated with clofibrate were 1.00 ± 0.10 vs. 0.90 ± 0.14 for MPT, 1.00 ± 0.15 vs. 1.10 ± 0.10 for apoA-I, 1.00 ± 0.17 vs. 0.86 ± 0.11 for apoA-II, and 1.00 ± 0.19 vs. 0.85 ± 0.18 for apoC-III, respectively; $n = 9$ for each group). Relative mRNA concentrations of SREBP-1, SREBP-2, *Insig-1*, and *Insig-2* in the liver did also not differ between both groups of pigs (relative mRNA concentrations in control pigs and pigs treated with clofibrate were 1.00 ± 0.10 vs. 0.93 ± 0.13 for SREBP-1, 1.00 ± 0.20 vs. 1.16 ± 0.27 for SREBP-2, 1.00 ± 0.16 vs. 0.97 ± 0.15 for *Insig-1*, and 1.00 ± 0.20 vs. 1.18 ± 0.21 for *Insig-2*, respectively; $n = 9$ for each group). Hepatic mRNA concentrations of ACC, a target gene of SREBP-1, and HMG-CoA reductase as well as LDL receptor, target genes of SREBP-2, were also not different between both groups (relative mRNA concentrations in control pigs and pigs treated with clofibrate were 1.00 ± 0.09 vs. 0.82 ± 0.14 for ACC, 1.00 ± 0.12 vs. 0.84 ± 0.10 for HMG-CoA reductase, and 1.00 ± 0.11 vs. 0.88 ± 0.12 for LDL receptor, respectively; $n = 9$ for each group). mRNA concentrations of cholesterol-7α-hydroxylase (CYP7) in liver (control: 1.00 ± 0.09; clofibrate: 1.03 ± 0.13; $n = 9$) and of lipoprotein lipase in muscle (control: 1.00 ± 0.13; clofibrate: 1.13 ± 0.15; $n = 9$) were also not different between both groups of pigs. mRNA concentrations of lipoprotein lipase in the liver were not detectable by mRNA analysis in pigs of both groups.

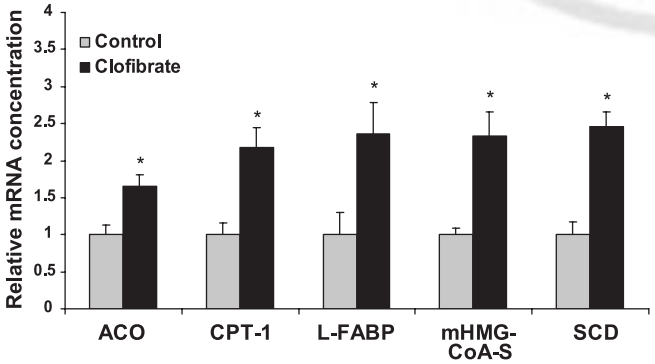

Fig. 2. Relative mRNA concentrations of acyl-CoA oxidase (ACO), carnitine palmitoyltransferase I (CPT-1), liver fatty acid binding protein (L-FABP), mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA-S), and stearoyl-CoA desaturase (SCD) in the liver of pigs fed a control diet or a diet supplemented with 5 g clofibrate/kg diet for 28 days. mRNA concentrations were determined by real-time RT-PCR and normalized to GAPDH. Data are means ± SE of 9 animals per group and are expressed relative to mRNA concentrations of control pigs (=1). *Significantly different from control group ($P < 0.05$).

Gene expression in adipose tissue of control pigs and pigs treated with clofibrate. mRNA concentration of PPAR-α in adipose tissue did not differ between both groups of pigs (control: 1.00 ± 0.17; clofibrate: 1.12 ± 0.16; $n = 9$), whereas pigs treated with clofibrate had moderately increased mRNA concentrations of the PPAR-α target genes ACO, CPT-1, and SCD ($P < 0.05$; Fig. 3). mRNA concentration of lipoprotein lipase, another PPAR-α target gene, in adipose tissue was not different between both groups (control: 1.00 ± 0.11; clofibrate: 0.90 ± 0.17; $n = 9$). mRNA concentrations of SREBP-1, SREBP-2, *Insig-1*, and *Insig-2*, and SREBP downstream genes ACC, HMG-CoA reductase, and LDL receptor in adipose tissue did not differ between both groups of pigs (relative mRNA concentrations in control pigs and pigs treated with clofibrate were 1.00 ± 0.06 vs. 1.10 ± 0.08 for SREBP-1, 1.00 ± 0.14 vs. 0.87 ± 0.16 for SREBP-2, 1.00 ± 0.18 vs. 0.94 ± 0.13 for *Insig-1*, 1.00 ± 0.20 vs. 0.92 ± 0.16 for *Insig-2*, 1.00 ± 0.19 vs. 1.19 ± 0.18 for ACC, 1.00 ± 0.10 vs. 1.10 ± 0.14 for HMG-CoA reductase, and 1.00 ± 0.08 vs. 1.07 ± 0.07 for LDL receptor, respectively; $n = 9$ for each group).

Concentration of 7β-hydroxybutyrate in plasma of control pigs and pigs treated with clofibrate. Pigs treated with clofibrate had a higher concentration of 7β-hydroxybutyrate in plasma than control pigs (control: 0.52 ± 0.09 mmol/l; clofibrate: 2.17 ± 0.18 mmol/l; $n = 9$, $P < 0.05$).

Concentrations of triglycerides and cholesterol in liver plasma and lipoproteins of control pigs and pigs treated with clofibrate. Pigs treated with clofibrate had lower concentrations of triglycerides in plasma and triglyceride rich-lipoproteins

(VLDL + chylomicrons) than control pigs ($P < 0.05$); triglyceride concentrations in the liver did not differ between both groups of pigs (Table 2). Pigs treated with clofibrate also had lower concentrations of total cholesterol in plasma and lower LDL and HDL levels than control pigs ($P < 0.05$); cholesterol concentrations in liver, however, did not differ between the two groups of pigs (Table 2).

DISCUSSION

To study the effect of clofibrate treatment on lipid metabolism and gene expression in pigs, we performed an experiment with young pigs. As in other studies dealing with the effects of clofibrate on metabolism in experimental animals, we added clofibrate to the diet. The concentration of clofibrate in the diet of 5 g/kg diet was adopted from other studies with pigs (8, 34, 56), resulting in a daily dose of 220 mg/kg body wt. This dose is relatively high compared with doses used in humans for treatment of hyperlipidemia, which are usually in the range between 25 and 30 mg/kg body wt.

The present study confirms that expression of PPAR-α in pig liver is much lower than that in rat liver. In the young control pigs, PPAR-α abundance in the liver was ~10-fold lower than that in the rat liver. The finding that hepatic PPAR-α abundance was similar in the liver of the pigs used in this study as in liver of adult humans indicates that young pigs may be a useful model for studying the response of PPAR-α activation. The finding that PPAR-α mRNA concentrations in human livers are much lower than concentrations in rat liver also agrees with literature data. In the study of Tugwood et al. (50), the abundance of PPAR-α transcript in human liver vs. that in rat liver was 1:5, which is close to the ratio of 1:10 found in the present study.

The finding that mRNA concentrations of the PPAR-α target genes ACO, CPT-1, L-FABP, mitochondrial HMG-CoA synthase, and SCD in the liver were increased by 50–150% compared with results shown in control animals clearly indicates that clofibrate treatment caused PPAR-α activation in the liver of the pigs. This is confirmed by an increased concentration of 3-hydroxybutyric acid, indicative of a stimulation of hepatic ketogenesis, which is a typical response of PPAR-α activation (28). The finding that clofibrate causes a moderate upregulation of PPAR-α target genes agrees well with recent studies conducted in piglets that were treated with clofibrate. In these studies, mRNA concentrations and activities of ACO and CPT-1 were two to four times higher in livers of piglets treated with clofibrate in doses similar to those used in the present study than in untreated piglets (34, 56). Interestingly, in our study, clofibrate treatment caused a significant increase in liver weights of pigs by ~15%, indicative of moderate peroxisome proliferation. Therefore, the present study suggests that clofibrate not only upregulated PPAR-α target genes in the liver but also caused a moderate peroxisome proliferation in the pigs. It should be noted that upregulation of PPAR-α target genes was much lower than that shown in rodents, where treatment with PPAR-α agonists typically increases mRNA concentrations of ACO 10- to 20-fold compared with untreated controls (14, 20, 23, 25). The reason for the comparatively low upregulation of these enzymes in pigs by clofibrate might be the lower hepatic PPAR-α expression in pigs compared with rodents. Furthermore, the presence of an alternative spliced PPAR-α isoform, which lacks the ligand-binding domain, could contribute to the lower responsiveness of the pig to clofibrate (49).

It has been found that pigs, in contrast to humans or rodents, have a high concentration of PPAR-α in adipose tissue. Ding et al. (12, 13) found that PPAR-α mRNA concentration, corrected for 18S ribosomal RNA, was three to four times higher in subcutaneous adipose tissue than in liver of young pigs with a body weight of 30 kg. In the young pigs used in the present study, expression of PPAR-α in adipose tissue, corrected for total mRNA content, was at a level in subcutaneous adipose tissue similar to that shown in liver. This also confirms that pig adipose tissue has a comparatively high expression of PPAR-α. To our knowledge, this is the first study that investigated the effect of treatment with a PPAR-α agonist on expression of PPAR-α target genes in adipose tissue of pigs. Our study shows that the PPAR-α target genes ACO, CPT-1, and SCD are indeed significantly upregulated by clofibrate, although only to a moderate extent. Although we did not perform a direct activation assay, we conclude that PPAR-α in adipose tissue is functional and is activated by PPAR-α agonists. In this study, we did not directly determine fatty acid oxidation in adipose tissue. The finding that genes involved in mitochondrial and peroxisomal were upregulated by clofibrate suggests that PPAR-α agonists indeed could stimulate β-oxidation in pig adipose tissue, which should be investigated in future studies.

To elucidate a possible link between PPAR-α activation and SREBP-mediated lipid homeostasis in pigs, we determined relative mRNA concentrations of SREBP-1 and -2, *Insig-1* and *-2*, and some target genes of SREBP-1 (ACC) and SREBP-2 (HMG-CoA reductase, LDL receptor) in the liver and in the adipose tissue, which is the major site of lipogenesis in the pig. We found that mRNA concentrations of SREBP-1 and -2, *Insig-1* and *-2*, and target genes of SREBP-1 and -2 in liver and adipose tissue were not different in clofibrate-treated and control pigs. This indicates that activation of PPAR-α by clofibrate did not influence expression and activity of SREBP-1 and SREBP-2 in both tissues. SCD is another target gene of SREBP-1 involved in lipogenesis, which also has a PPAR response element in its promoter and is upregulated by PPAR-α activation (30). We assume that an upregulation of SCD in liver and adipose tissue of pigs treated with clofibrate was probably due to PPAR-α activation. An upregulation of SCD in the liver of pigs by treatment with clofibrate has also been observed by Cheon et al. (8). SREBP-1 controls fatty acid and triglyceride synthesis, and SREBP-2 controls cholesterol synthesis and cholesterol uptake in cells via the LDL receptor (22). It is assumed that these SREBP-controlled processes were not altered by PPAR-α activation in liver and adipose tissue. This assumption is in contrast to recent findings in mice in which WY-14,643, a synthetic PPAR-α agonist, stimulated SREBP-1-mediated fatty acid synthesis in the liver (23) and findings in rats and hamsters in which fibrates decreased SREBP-2-mediated expression of HMG-CoA reductase and LDL receptor (17, 25).

The present study shows for the first time that clofibrate treatment strongly reduces triglyceride concentrations in plasma and triglyceride-rich lipoproteins in pigs. These results agree with observations in humans and rodents (2, 16). Studies in humans and rodents have shown that this effect is in part due to increased oxidation of fatty acids in the liver, leading to

reduced triglyceride synthesis and secretion, and in part due to increased lipolysis caused by induction of lipoprotein lipase activity and repressed transcription of apoC-III in the liver (14, 17, 41, 54). The observation that the PPAR-α target genes L-FABP, ACO, and CPT-1 were increased indicates that clofibrate increased the uptake of fatty acids into hepatocytes and stimulated both peroxisomal and mitochondrial β-oxidation in pigs. To find out whether clofibrate stimulates lipolysis, we determined mRNA concentrations of lipoprotein lipase in liver, adipose tissue, and muscle, extrahepatic tissues that also express comparatively high levels of PPAR-α (4), and mRNA concentration of apoC-III, an inhibitor of lipoprotein lipase. The observation that lipoprotein lipase mRNA could not be detected in liver of pigs of both groups suggests that the pig has generally a low expression of lipoprotein lipase in the liver. This suggestion is confirmed by recent studies that also showed a very low gene expression of lipoprotein lipase in liver of pigs (27, 44). The finding that clofibrate does not reduce apoC-III agrees with observations in monkeys (42) but disagrees with observations in human and rat hepatocytes and livers of hamsters in which apoC-III expression was downregulated by fibrates (9, 17, 26). Because lipoprotein lipase in adipose tissue and muscle was also not upregulated, the data of this study do not give any indication of increased lipolysis in pigs treated with clofibrate. It has recently been shown in monkeys that administration of a PPAR-α agonist increases serum levels of apoA-V, which has recently been recognized as a key regulator of serum triglyceride concentrations (42). Overexpression of apoA-V in mice caused a strong reduction of serum triglyceride concentrations (35, 51), and upregulation of apoA-V could be involved in the hypotriglyceridemic effect of PPAR-α agonists. For technical reasons, we were unable to determine gene expression of apoA-V, but it is possible that clofibrate reduced triglyceride concentrations in pigs by upregulation of apoA-V.

In this study, we also determined hepatic mRNA concentrations of MTP, the rate-limiting protein for assembly and secretion of VLDL in the liver, which has recently been demonstrated in mice to be a PPAR-α target gene (1). Our study shows that activation of PPAR-α by clofibrate does not stimulate gene expression of MTP in pigs, in contrast to rats, and probably does not stimulate secretion of lipids from the liver into the blood via VLDL. This could help to explain the observation that concentrations of triglycerides and cholesterol in the liver remained unchanged in pigs after clofibrate treatment.

In humans, treatment with fibrates usually reduces plasma and LDL cholesterol concentrations and increases HDL cholesterol (46). The present study shows that clofibrate reduces plasma and LDL cholesterol concentrations in pigs, as also shown in humans. The effect of clofibrate on HDL cholesterol, however, is opposite to that observed in humans. The elevation of HDL cholesterol by fibrate treatment in humans is caused primarily by increased gene expression of apoA-I and apoA-II in the liver (3, 52). In contrast to humans, fibrates lower plasma HDL concentrations in rats and hamsters because of a decrease of liver apoA-I and apoA-II gene expression (17, 45, 47). The finding that mRNA concentrations of apoA-I and apoA-II in the liver did not differ between both groups of pigs suggests that clofibrate reduced HDL cholesterol by a mechanism other than HDL reduction.

It has been shown in mice that activation of PPAR-α leads to downregulation of CYP7, the key enzyme of hepatic bile acid formation, which is probably because of reduced availability of hepatic nuclear factor-4, a transcription factor involved in the basal expression of CYP7 (29, 38). The present study shows that activation of PPAR-α by clofibrate does not alter hepatic mRNA concentrations of CYP7, which, along with LDL receptor and HMG-CoA reductase, is a key factor of hepatic cholesterol homeostasis (40). The finding that mRNA concentrations of these three genes were not altered by clofibrate treatment agrees with a recent study in which pigs were treated with clofibrate (34) and is in accordance with the observation that hepatic cholesterol concentrations were also unchanged in pigs treated with clofibrate compared with control pigs. These data also suggest that the greatly reduced concentration of LDL cholesterol is not because of a lowered cholesterol synthesis, enhanced elimination of cholesterol from the liver via bile acid formation, or upregulation of LDL receptor. It has been shown in humans that fibrate treatment enhances LDL uptake via the LDL receptor, not as a result of increased LDL receptor expression but as a result of the formation of LDL particles with a higher affinity to the LDL receptor (5, 7). It is possible that a similar effect is responsible for the strongly reduced LDL levels in pigs treated with clofibrate. This should be investigated further in future studies.

In conclusion, this study shows that clofibrate treatment causes an increase in liver weights, indicative of peroxisome proliferation, and moderate upregulation of PPAR-α target genes involved in liver and adipose tissue. Clofibrate treatment causes a strong reduction of triglyceride and cholesterol concentrations in plasma and lipoproteins, which agrees with findings in rodents. Gene expression analysis of lipoprotein lipase, apoA-I, apoA-II, and apoC-III in the liver suggests that biochemical mechanisms underlying these effects might be in part different from those in humans or rodents. In pigs, unlike rodents, hepatic concentrations of triglycerides and cholesterol are not altered by clofibrate. We also showed that PPAR-α activation by clofibrate does not affect expression of SREBP target genes, which are involved in synthesis of triglycerides and cholesterol, in liver and adipose tissue of pigs.

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Clofibrate Increases Hepatic Triiodothyronine (T_3)- and Thyroxine (T_4)-Glucuronosyltransferase Activities and Lowers Plasma T_3 and T_4 Concentrations in Pigs

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ABSTRACT:

In rats, clofibrate acts as a microsomal enzyme inducer and disrupts the metabolism of thyroid hormones by increasing hepatic glucuronidation of thyroxine. Whether similar effects occur in the pig has not yet been investigated. This study was performed to investigate the effect of clofibrate treatment on metabolism of thyroid hormones in pigs. To this end, an experiment with 18 pigs, which were assigned to two groups, was performed. One group received a control diet, and the other group was fed the same diet supplemented with 5 g of clofibrate/kg for 28 days. Pigs treated with clofibrate had higher hepatic activities of T_3- and T_4-UDP glucuronosyltransferases (UGT) and lower concentrations of total and free T_4 and total T_3 in plasma than control pigs ($P < 0.05$). Weights and histology of the thyroid gland (epithelial height, follicle lumen diameter) did not differ between the two groups, but pigs treated with clofibrate had higher mRNA concentrations of various genes in the thyroid responsive to thyroid-stimulating hormone (TSH) such as TSH receptor, sodium iodine symporter, thyroid peroxidase, and cathepsin B than control pigs ($P < 0.05$). Pigs treated with clofibrate also had lower hepatic mRNA concentrations of proteins involved in plasma thyroid hormone transport [thyroxine-binding globulin ($P < 0.10$), transthyretin ($P < 0.05$), and albumin ($P < 0.05$)] and thyroid hormone receptor α_1 ($P < 0.05$) than control pigs. In conclusion, this study shows that clofibrate treatment induces a strong activation of T_3- and T_4-UGT in pigs, leading to increased glucuronidation and markedly reduced plasma concentrations of these hormones, accompanied by a moderate stimulation of thyroid function.

Fibrates are synthetic agonists of peroxisome proliferator-activated receptor-α (PPARα), a nuclear receptor also activated by natural ligands like free fatty acids or some eicosanoids. Activation of PPARα leads to up-regulation of transcription of several genes involved mainly in mitochondrial and peroxisomal β-oxidation, ketogenesis, and gluconeogenesis (Mandard et al., 2004). Fibrates have been in clinical use as hypolipidemic agents for several decades. Several studies in rodents and cell culture systems have shown that fibrates, like many other drugs (e.g., phenobarbital, 3-methylcholantrene, polychlorinated biphenyl, tetrachlorobiphenyl, pregnenolone-16α-carbonitrile, or dexamethasone), induce UDP glucuronosyltransferases (UGT) (Beetstra et al., 1991; Saito et al., 1991; Barter and Klaassen, 1992a,b, 1994; Visser et al., 1993a,b; Jemnitz et al., 2000; Viollon-Abadie et al., 2000; Vansell and Klaassen, 2002). UGT, consisting of UGT1 and UGT2 isoforms, are localized in the endoplasmatic reticulum of hepatocytes and extrahepatic tissue and belong to the enzymes of phase II metabolism. With broad and overlapping substrate specificities, the UGT isoenzymes catalyze the glucuronidation of differential functional groups, using UDP-glucuronic acid as the cofactor (Miners and Mackenzie, 1991; Mackenzie et al., 1997). Thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) are substrates of hepatic UGT, and glucuronidation of these hormones is the main metabolic pathway for deactivating them (Jemnitz et al., 2000). In rats, several of the drugs acting as inducers of microsomal enzymes have been shown to produce hypertrophy and hyperplasia of thyroid follicular cells, most probably through increased deactivation of thyroid hormones by UGT, leading to a reduction of serum T_4 and possibly T_3 (Beetstra et al., 1991; Saito et al., 1991; Barter and Klaassen 1992a, 1994). In mice, in contrast to rats, clofibrate treatment did not alter T_3- and T_4-UGT activities and plasma concentrations of thyroid hormones (Viollon-Abadie et al., 1999). These studies show species-specific differences in the effects of clofibrate on hepatic thyroid hormone metabolism (i.e., glucuronidation of thyroid hormones).

In rodents, PPARα agonists not only induce many genes involved in various metabolic pathways such as β-oxidation, ketogenesis, and gluconeogenesis but also cause severe peroxisome proliferation in the liver, hepatomegaly, and hepatocarcinogenesis (Peters et al., 2005). In contrast to rodents, PPARα agonists do not induce peroxisome proliferation or tumor in the liver of many other species, such as guinea pigs, swine, monkeys, and humans, although they retain a hypotriglyceridemic effect in these species (Holden and Tugwood, 1999). In nonproliferating species, expression of PPARα in the liver is much lower, and the response of many genes to PPARα activation is weaker than in proliferating species (Cheon et al., 2005). It is known that

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ABBREVIATIONS: PPARα, peroxisome proliferator-activated receptor α; UGT, UDP glucuronosyltransferase(s); T_4, thyroxine; T_3, 3,3′,5-triiodothyronine; TSH, thyroid-stimulating hormone; pNP, *p*-nitrophenol; UDPGA, UDP-glucuronic acid; RT-PCR, reverse transcriptase polymerase chain reaction; GAPDH, glycerinaldehyde-3-phosphate dehydrogenase; ACO, acyl CoA oxidase; CPT-1, carnitine palmitoyl transferase 1.

TABLE 1

Sequences of primers used for semiquantitative RT-PCR

Gene (NCBI GenBank)	Forward Primer	Reverse Primer	Size/Annealing Temperature
			bp/°C
Acyl CoA oxidase (AF185048)	CTCGCAGACCCAGATGAAAT	TCCAAGCCTCGAAGATGAGT	218/60
Albumin (X12422)	GCACGAGAAGACACCAGTGA	CGAGTGCAGTTTGCTTCTTG	200/62
CPT-I (AF288789)	GCATTTGTCCCATCTTTCGT	GCACTGGTCCTTCTGGGATA	198/60
Cathepsin B (AJ315560)	GGCCTCTATGACTCGCATGT	GCAAGTTCCCCTCAAGTCTG	198/60
Dual oxidase 2 (AF547267)	GACCCAGCGGCAGTTTGAATGG	AGGGCCGCAGCTGAACACTCC	295/64
GAPDH (AF017079)	AGGGGCTCTCCAGAACATCATCC	TCGCGTGCTCTTGCTGGGGTTGG	446/60
Sodium iodide symporter (AJ487855)	AGTCATCAGCGGCCCCCTCCTC	ACCGATGCCGTCTGCCGTGTG	456/60
Thyroglobulin (AF165610)	CAGTAAGGGCTTCCGTCTTG	GGAGCTGCACTGAGGAATGT	198/60
Thyroid hormone receptor α_1 (AJ005797)	CCAGATGGAAAGCGAAAAAG	TGGGATGGAGATTCTTCTGG	199/60
Thyroid peroxidase (X04645)	CTGGGCGCCGTGCTCGTCTG	ACGCGGGTGGCATCTGACTCTGAC	287/65
Thyroxine-binding globulin (NM214058)	GTGGCTTCTTGGGCATGTAT	GAACCTCCGGTACAGGTTGA	206/62
Transthyretin (X87846)	ATGGTCAAAGTCCTGGATGC	TGCCTTCCAGTAGGATTTGG	207/60
TSH receptor (NM214297)	GCCTGCCCATGGACACTGAGAC	CTGACCCCGGTATGCCTGAGC	422/60
Type I iodothyronine deiodinase (AY533206)	CTCTGGGTGCTCTTTCAGGT	ATCGGACCTTCAGCACAAAC	199/62
Type II iodothyronine deiodinase (NM001001626)	CTCGGTCATTCTCCTCAAGC	TGCTTCCTTCAGGATTGGAG	200/60

PPARα activation can modulate metabolizing enzymes of phase I and II biotransformation (Rushmore and Kong, 2002; Zhou et al., 2005). Moreover, it has been shown that some UGT isoforms (UGT1A9, UGT2B4) are PPARα target genes (Barbier et al., 2003a,b). Therefore, nonproliferating species could respond differently from proliferating species to clofibrate with respect to induction of UGT (i.e., UGT involved in glucuronidation of thyroid hormones). To our knowledge, the effect of clofibrate on the hepatic thyroid hormone metabolism has not yet been investigated in vivo in a nonproliferating species.

The aim of our study was to investigate the effects of clofibrate treatment on hepatic thyroid hormone metabolism (i.e., activities of T_3- and T_4-UGT in pigs, representing a nonproliferating species). Therefore, as well as determining hepatic activities of T_3- and T_4-UGT, we also measured plasma concentrations of thyroid hormones, thyroid weights, thyroidal epithelial cell height, and follicle lumen diameter and gene expression levels of several thyroidal genes involved in thyroid hormone biosynthesis [thyroid-stimulating hormone (TSH) receptor, sodium iodide symporter, thyroid peroxidase, dual oxidase 2, thyroglobulin, cathepsin B, and type II iodothyronine deiodinase]. We also investigated the effect of clofibrate on mRNA expression of genes involved in thyroid hormone transport (transthyretin, thyroxine-binding globulin, and albumin), peripheral conversion of thyroid hormones (type I iodothyronine deiodinase), and thyroid hormone signaling (thyroid hormone receptor α_1) in the liver.

Materials and Methods

Chemicals. Bilirubin, Brij 56, clofibrate, dithiothreitol, *p*-nitrophenol (pNP), 6-propyl-2-thiouracil, cholic acid, T_3, T_4, Triton X-100, and UDP-glucuronic acid (UDPGA) were obtained from Sigma (Deisenhofen, Germany); ^{125}I-T_3 (3076 μCi/μg) and ^{125}I-T_4 (1500 μCi/μg) were obtained from Amersham Biosciences (Freiburg, Germany); and bicinchoninic acid protein assay reagent was from Interchim (Montelucon, France).

Animals and Treatments. Eighteen male 8-week-old crossbred [(German Landrace × Large White) × Pietrain] pigs, bred in the local animal facility, were used. They weighed between 11.0 and 13.5 kg. They were individually housed in a room maintained at 23°C and 50 to 60% relative humidity with light from 6:00 AM to 6:00 PM. On the day before the start of the experimental feeding period, all the pigs were weighed and assigned to two groups with body weights of 12.0 ± 1.1 (S.D.) kg (control group) and 11.9 ± 0.6 (S.D.) kg (treatment group). Both groups of pigs received a nutritionally adequate diet (National Research Council, 1998) for growing pigs containing wheat (400 g/kg), soybean meal (230 g/kg), wheat bran (150 g/kg), barley (100 g/kg), sunflower oil (90 g/kg), and mineral premix including L-lysine, DL-methionine, and L-threonine (30). This diet contained 14.4 MJ metabolizable energy and 185 g of crude protein/kg. The diet of the treatment group was supplemented with 5 g of clofibrate/kg. To standardize feed intake, each pig within the experiment received 700 g of the diet daily, which was completely consumed by all the animals in the experiment. The clofibrate dosage in the treated pigs was 220 mg/kg b.wt./day. The pigs had free access to water via nipple drinking systems. The experimental diets were administered for 28 days. All the experimental procedures described followed established guidelines for the care and use of laboratory animals and were approved by the local veterinary office.

Sample Collection. After completion of the feeding period, the animals were killed under a light anesthesia. Blood was collected into heparinized polyethylene tubes. Liver and thyroid gland were dissected and weighed. Plasma was obtained by centrifugation of the blood (1100*g*; 10 min). All the samples were stored at −80°C pending analysis.

Total RNA Preparation and cDNA Synthesis. Total RNA from liver and thyroid tissue was isolated by TRIzol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol, resuspended in diethyl pyrocarbonate-treated water, and stored at −80°C until use. The concentration of total RNA was determined by ultraviolet absorbance at 260 nm. The quality of all the RNA samples was assessed by agarose gel electrophoresis. cDNA was prepared from total RNA (1.2 μg) by reverse transcription using M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany) and oligo(dT)$_{18}$ primers (Operon, Cologne, Germany).

Semiquantitative Polymerase Chain Reaction. Expression analysis for semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard. cDNA templates (2 μl) were used in a final volume of 20 μl containing 0.2 μM concentration of the corresponding primers (Roth, Karlsruhe, Germany) (see Table 1), 1.5 mM magnesium chloride, 1× PCR buffer, 1 U *Taq* polymerase (Gene Craft, Luedinghausen, Germany), and 0.2 mM deoxyribonucleoside triphosphates (Roth). Each PCR cycle comprised denaturation for 30 s at 94°C, annealing for 30 s at 60 to 64°C (see Table 1), and elongation for 1 min at 72°C, followed by a final extension period for 10 min at 72°C. Number of cycles for each primer pair was tested previously. Cycle number was as follows: genes of thyroid gland: GAPDH, 23; sodium iodide symporter, 25; dual oxidase 2, 42; cathepsin B, 35; TSH receptor, 28; type II iodothyronine deiodinase, 40; and thyroglobulin, 33; hepatic genes: GAPDH, 32; acyl CoA oxidase (ACO), 32; carnitine palmitoyl transferase 1 (CPT-1), 32; albumin, 20; thyroid hormone receptor α_1, 35; thyroxine-binding globulin, 42; transthyretin, 30; and type I iodothyronine deiodinase, 40. A water control was included in all the PCRs for detection of contamination, and dilutions of the isolated total RNA corresponding to the cDNA synthesis were used as template to exclude impurities caused by genomic DNA. A volume of 10 μl per PCR was submitted to agarose gel electrophoresis (1.5%). Ethidium bromide-stained gels were digitized for quantification (apparatus and software from Syngene, Cambridge, UK).

Preparation of Hepatic Microsomes. One gram of liver was homogenized in a medium (10 ml) containing 0.25 M sucrose and 0.1 M phosphate buffer

(pH 7.4) using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 1000*g* for 10 min at 4°C, and the supernatant was centrifuged at 15,000*g* for 15 min. The microsomal pellet was obtained by centrifugation of the 15,000*g* supernatant at 105,000*g* for 60 min. Microsomal pellets were suspended in the homogenization medium, and the protein concentration was determined with the bicinchoninic acid reagent according to the supplier's protocol using bovine serum albumin as standard.

Enzyme Assays. The activity of pNP-UGT in hepatic microsomes was assayed by the method of Thurman et al. (1981). The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.4), 0.2 mg of bovine serum albumin/ml, 1 mM magnesium chloride, 0.5 mM dithiothreitol, 0.5 mg of Triton X-100/ml, 0.2 mM pNP, 0.2 mM UDPGA, and 0.25 mg of microsomal protein/ml. The final volume of the assay was 200 μl. A blank was incubated without UDPGA. The reaction was stopped after 30 min at 37°C by addition of 1 ml of 0.1 M sodium hydroxide solution. The pNP glucuronidation was quantified by measuring the decrease of absorbance at 400 nm. The concentration of pNP glucuronide was calculated using a molar extinction coefficient of 18,300/cm.

Activity of bilirubin-UGT in hepatic microsomes was measured in an assay mixture containing 0.1 M Tris-hydrochloride (pH 7.8), 0.1 mM bilirubin, and 5 mM UDPGA in a final volume of 200 μl. Suspended microsomes were preincubated with 20 mg/ml sodium cholate (1:1, v/v) for 10 min at 4°C. The reaction was started by addition of 1 mg of microsomal protein/ml for 60 min at 37°C. The amount of bilirubin glucuronide formed during the incubation was quantified with a commercial kit (DiaSys Diagnostic Systems, Holzheim, Germany).

T_3- and T_4-UGT activities were determined in separate assays using a modified version of the method of Beetstra et al. (1991) by incubating 1 μM T_3 or T_4, respectively, and 0.1 μCi of ^{125}I-labeled T_3 or T_4 in reaction mixture containing 75 mM Tris-hydrochloride (pH 7.8), 7.5 mM magnesium chloride, 0.25 mg of Brij 56/ml, 5 mM UDPGA, and 1 mM 6-propyl-2-thiouracil. The final volume of the assay was 200 μl. Reactions were started by adding 0.5 mg of microsomal protein/ml at 37°C. Blanks were performed in the absence of UDPGA. After 30 min, reactions were terminated by addition of 200 μl of ice-cold methanol, and the mixtures were centrifuged at 3500*g* for 8 min. Fifty microliters of the supernatants was injected into a high-performance liquid chromatograph for separation of T_3 or T_4 glucuronides formed during the incubation by a modified version of the method of Jemnitz et al. (2000). The high-performance liquid chromatography equipment consisted of a 1100 series pump (isocratic), an autosampler, a LiChrospher 100 RP 18e column (125 × 4 mm, 5-μm particle size) with matching guard column (4 × 4 mm; Agilent Technologies, Waldbronn, Germany). The mobile phase consisted of 50 mM potassium dihydrogen phosphate and methanol (43:57, v/v, pH 7.0). For separation of T_3-glucuronide, the flow rate was 0.8 ml/min. For separation of T_4-glucuronide, the flow rate was 1.25 ml/min. Fractions containing T_3- or T_4-glucuronide, respectively, were collected with a fraction collector 203 (Gilson International, Bad Camberg, Germany). The radioactivity of the fractions was counted to calculate T_3- and T_4-UGT activities.

Histology of Thyroid Gland. Samples of thyroid glands were fixed by immersion in 10% neutral buffered formalin, processed for embedding into paraffin wax, and cut into 4-μm sections. For light microscopy, the sections were stained with hemalum and eosin. The epithelial cell height was measured using 4 cells per follicle in 100 follicles of each thyroid. The lumen diameter was measured in 10 sections for 10 follicles per section of each thyroid. All the pictures were digitized, and the parameters were measured using the Lucia G (Nikon, Düsseldorf, Germany) software (release 4.81).

Analysis of Plasma Hormones. The plasma concentrations of free and total T_4 and total T_3 were measured with radioimmunoassay kits (MP Biomedicals, Eschwege, Germany).

Statistics. The results were analyzed using Minitab (State College, PA) statistical software (release 13). Statistical significance of differences between control group and treatment group was evaluated using Student's *t* test. Mean values were considered significantly different for $P < 0.05$.

Results

Initial and final body weights after an experimental period of 28 days were similar in both groups of pigs (Table 2). Animals treated with clofibrate had heavier livers ($P < 0.05$) and higher concentrations of microsomal protein in the liver ($P < 0.05$) than control pigs (Table 2). Relative hepatic mRNA concentration of the PPARα target genes ACO and CPT-1 was higher ($P < 0.05$) in pigs treated with clofibrate than in control pigs (ACO: 1.39 ± 0.27 versus 1.00 ± 0.35; CPT-1: 1.60 ± 0.13 versus 1.00 ± 0.12; mean ± S.D., $n = 9$ for each group). Moreover, concentrations of total and free T_4 and total T_3 in plasma were markedly lower in pigs treated with clofibrate than in control pigs ($P < 0.05$), whereas the T_4/T_3 ratio did not differ between both groups of pigs (Table 2).

TABLE 2

Body and liver weights, microsomal protein in the liver, and plasma thyroid hormone concentrations in pigs fed a control diet or a diet supplemented with 5 g of clofibrate/kg for 28 days

Data are reported as mean ± S.D. with nine animals per group.

	Control	Clofibrate
Initial body weight (kg)	12.0 ± 1.1	11.9 ± 0.6
Final body weight (kg)	26.0 ± 1.5	25.2 ± 1.2
Liver weight (g)	673 ± 63	779 ± 63[a]
Hepatic microsomal proteins (mg/g liver)	12.4 ± 1.8	15.4 ± 2.4[a]
Plasma thyroid hormones		
Total thyroxine (T_4, nM)	45.2 ± 13.1	29.6 ± 7.1[a]
Free thyroxine (pM)	12.6 ± 3.7	8.6 ± 2.2[a]
Total triiodothyronine (T_3, nM)	1.20 ± 0.52	0.64 ± 0.11[a]
T_4/T_3 ratio	41.1 ± 13.2	40.3 ± 10.7

[a] $P < 0.05$ compared with control group.

Pigs treated with clofibrate had a higher activity of bilirubin-UGT in the liver than control pigs (1.08 ± 0.05 versus 0.44 ± 0.02 nmol/min/mg; mean ± S.D., $n = 9$ for each group; $P < 0.05$). The activity of hepatic pNP-UGT was lower in pigs treated with clofibrate than in control pigs (44 ± 5 versus 70 ± 8 nmol/min/mg; mean ± S.D., $n = 9$ for each group; $P < 0.05$). Activities of hepatic T_3- and T_4-UGT were higher in pigs treated with clofibrate than in control pigs ($P < 0.05$) (Fig. 1).

Weights of thyroids, diameter of follicle lumen, and thyroid epithelial cell height did not differ between both groups of pigs (Table 3). Relative mRNA concentrations of TSH receptor, sodium iodide symporter, thyroid peroxidase, and cathepsin B were higher in thyroids of

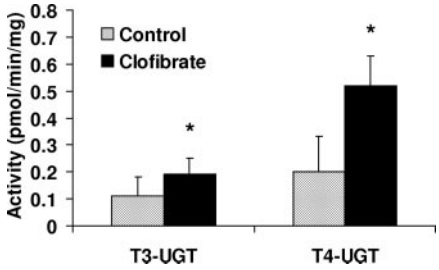

FIG. 1. Activities of T_3- and T_4-UGT in the liver of pigs fed a control diet or a diet supplemented with 5 g of clofibrate/kg for 28 days. Data are reported as mean ± S.D. with nine animals per group. *, significantly different to control group ($P < 0.05$).

TABLE 3

Thyroid weight, follicle lumen diameter, and epithelial cell height in thyroid of pigs fed a control diet or a diet supplemented with 5 g of clofibrate/kg for 28 days

Data are reported as mean ± S.D. with nine animals per group.

	Control	Clofibrate
Thyroid weight (g)	2.17 ± 0.19	2.39 ± 0.55
Follicle lumen diameter (μm)	84.9 ± 10.9	88.8 ± 14.2
Epithelial cell height (μm)	7.53 ± 0.14	7.64 ± 0.67

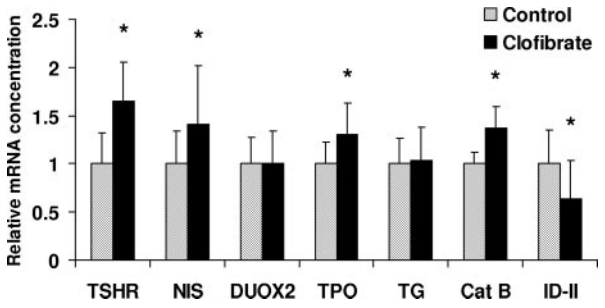

FIG. 2. Relative mRNA concentrations of TSH receptor (TSHR), sodium iodide symporter (NIS), dual oxidase 2 (DUOX2), thyroid peroxidase (TPO), thyroglobulin (TG), cathepsin B (Cat B), and type II iodothyronine deiodinase (ID-II) in the thyroid of pigs fed a control diet or a diet supplemented with 5 g of clofibrate/kg for 28 days. All the mRNA concentrations were determined by semiquantitative RT-PCR and normalized by GAPDH. Data are reported as mean ± S.D. with nine animals per group. Data are expressed relative to mRNA concentrations of control pigs (control = 1). *, significantly different to control group ($P < 0.05$).

pigs treated with clofibrate than in thyroids of control pigs ($P < 0.05$) (Fig. 2). mRNA concentration of type II iodothyronine deiodinase was lower in thyroids of pigs treated with clofibrate than in thyroids of control pigs ($P < 0.05$); those of dual oxidase 2 and thyroglobulin did not differ between both groups of pigs (Fig. 2).

Pigs treated with clofibrate had lower hepatic mRNA concentrations of transthyretin, albumin, and thyroid hormone receptor α_1 than control pigs ($P < 0.05$) (Table 4). Hepatic mRNA concentration of thyroxine-binding globulin tended to be lower in pigs treated with clofibrate compared with control pigs ($P < 0.10$), whereas hepatic mRNA concentration of type I iodothyronine deiodinase did not differ between both groups of pigs.

TABLE 4

Relative hepatic mRNA concentrations of transthyretin, thyroxine-binding globulin, albumin, thyroid hormone receptor α_1, and type I iodothyronine deiodinase in pigs fed a control diet or a diet supplemented with 5 g of clofibrate/kg for 28 days

Data are reported as mean ± S.D. with nine animals per group. All the mRNA concentrations were determined by semiquantitative RT-PCR and normalized by GAPDH. Data are expressed relative to mRNA concentrations of control pigs (control = 1).

	Control	Clofibrate
Transthyretin	1.00 ± 0.14	0.87 ± 0.12[a]
Thyroxine-binding globulin	1.00 ± 0.14	0.89 ± 0.18[b]
Albumin	1.00 ± 0.11	0.87 ± 0.11[a]
Thyroid hormone receptor α_1	1.00 ± 0.14	0.84 ± 0.10[a]
Type I iodothyronine deiodinase	1.00 ± 0.07	0.98 ± 0.07

[a] $P < 0.05$ compared with control group.
[b] $P < 0.10$ compared with control group.

Discussion

To our knowledge, this is the first study to investigate the effect of clofibrate on the hepatic metabolism of thyroid hormones in the pig. It is well known that pigs are a nonproliferating species, meaning that treatment with PPARα agonist causes no or only weak peroxisome proliferation in the liver. Interestingly, in this study clofibrate treatment caused a significant increase in liver weights of pigs, by about 15%. This is in disagreement with a recent study in which pigs did not show significantly increased liver weights after a 1-week treatment with a dose of clofibrate similar to those used in our study (Cheon et al., 2005). The difference in these results could be because of the longer treatment period in our study compared with that in the study of Cheon et al. (2005). Moreover, we observed a moderate up-regulation of the PPARα target genes ACO and CPT-1 in the liver of pigs treated with clofibrate, which indicates that clofibrate treatment caused PPARα activation in these pigs. Nevertheless, increases in liver weights and hepatic ACO and CPT-1 mRNA concentration were much lower than those observed in rodents treated with clofibrate. In rats and mice, feeding PPARα agonists increases liver weights by 50% or more and mRNA concentrations of ACO 5- to 10-fold compared with untreated controls (Kawashima et al., 1990; He et al., 2002; Frederiksen et al., 2004; Li et al., 2004).

Several families of UGT enzymes are expressed in the liver. To study the effect of clofibrate treatment on the induction of microsomal enzymes, we determined the activities of bilirubin- and pNP-UGT. The finding that clofibrate treatment strongly increases bilirubin-UGT is in accordance with studies in rats and mice (Visser et al., 1993a; Viollon-Abadie et al., 1999). The finding that clofibrate reduces the activity of pNP-UGT is also in accordance with a study in which clofibrate significantly reduced the activity of pNP-UGT in the liver of Wistar rats (Visser et al., 1993a). These observations suggest that clofibrate stimulated the microsomal enzyme system in pigs in a similar way as in rats. This study also shows that clofibrate treatment strongly increases the activity of T_3- and T_4-UGT in the liver, which in turn leads to a dramatic reduction of plasma T_3 and T_4 concentrations. Increased activity of T_4-UGT had previously been observed in Wistar rats but not in mice treated with clofibrate (Visser et al., 1993a; Viollon-Abadie et al., 1999). The increased activity of T_3-UGT in pigs treated with clofibrate, however, is in strong contrast to rats, in which clofibrate treatment did not increase T_3-UGT activity (Visser et al., 1993a).

In rats, T_4 is accepted as a substrate by hepatic bilirubin-UGT (UGT1A1) and phenol-UGT (UGT1A6), and it was shown that increased activities of these enzymes were associated with increased glucuronidation of T_4 in the liver (Beetstra et al., 1991; Magdalou et al., 1993; Visser et al., 1993a,b; Viollon-Abadie et al., 2000; Vansell and Klaassen, 2002). The enzymes involved in glucuronidation of thyroid hormones in pigs have not yet been identified. The fact that activities of both bilirubin- and T4-UGT were increased suggests that in pig liver T_4 was also glucuronidated by bilirubin-UGT, as happens in rats. It is probable that enzymes other than UGT1A1 and UGT1A6 can also be induced by clofibrate in rats and are involved in T4 conjugation (Jemnitz et al., 2000). In rat liver, glucuronidation of T_3, unlike glucuronidation of T_4, is catalyzed by androsterone-UGT (Beetstra et al., 1991; Visser et al., 1993b). The increased T_3-UGT activity in pigs treated with clofibrate could therefore also have been caused by an increased activity of androsterone-UGT, although this was not assayed in this study. The UGT in pig liver have been less extensively investigated and have not yet been phenotyped. Therefore, it remains unknown which specific UGT were responsive for the increased glucuronidation of T_3 and T_4 in pigs treated with clofibrate.

It has been shown that activation of PPARα leads to transcriptional up-regulation of the CYP4A genes, which are also constituents of the microsomal biotransformation system in both proliferating and nonproliferating species (Lawrence et al., 2001; Cheon et al., 2005). It has further been shown that some UGT isoforms (UGT1A9, UGT2B4) are PPARα target genes (Barbier et al., 2003a,b). PPARα is naturally activated during fasting, and Visser et al. (1996) showed that food restriction resulted in increased bilirubin and thyroid hormone UGT activities in rats. These findings suggest that UGT catalyzing the glucuronidation of thyroid hormones may be transcriptionally up-regulated by activation of PPARα. It is well known that expression of PPARα in the liver is much lower in nonproliferating species and that the response of many genes to PPARα activation is weaker than in proliferating species. This is also true for up-regulation of microsomal CYP4A genes by treatment with PPARα agonists (Lawrence et al., 2001; Cheon et al., 2005). If PPARα plays a crucial role in the

activation of UGT catalyzing thyroid hormone glucuronidation, the effect of clofibrate on up-regulation of these enzymes in pigs would be expected to be much lower than in proliferating species such as rats or mice. But activation of T_3- and T_4-UGT by clofibrate was even stronger in pigs than reported for rats or mice (Visser et al., 1993a; Viollon-Abadie et al., 1999). These findings suggest that activation of PPARα does not play a key role in clofibrate-induced up-regulation of thyroid hormone UGT. Nevertheless, the role of PPARα in the regulation of thyroid hormone glucuronidation should be further investigated.

The increased activities of T_3- and T_4-UGT make it highly probable that the markedly reduced plasma concentrations of T_3 and T_4 in pigs treated with clofibrate are mainly caused by increased glucuronidation of these hormones in the liver. Because most T_3 is generated in peripheral tissues, mainly the liver, by deiodination of T_4, a reduced T_3 concentration could potentially be caused by an inhibition of type I iodothyronine deiodinase. Indeed, in the study of Visser et al. (1993a), clofibrate treatment of rats reduced the activity of that enzyme, which might be responsible for the reduced concentration of T_3 observed in their study. We did not determine the activity but only the mRNA concentration of that enzyme in the liver, which was not influenced by clofibrate treatment. Interestingly, in contrast to hepatic type I iodothyronine deiodinase, type II deiodinase in the thyroid showed a reduced mRNA concentration in pigs treated with clofibrate compared with control pigs. A reduced activity of type II deiodinase, which converts T_4 to T_3 in the thyroid, may play some role in the reduced T_3 concentration in plasma. However, because the thyroid produces less than 20% of total T_3 (Findlay et al., 2000), a reduced activity of type II deiodinase most probably plays a minor role in the reduced plasma concentration of T_3. The reduction of plasma concentrations of total T_3 (by 47% versus control), free T_4 (by 32% versus control), and total T_4 (by 35%) concentrations by clofibrate are also stronger than those observed in Wistar rats. In Wistar rats, a dose of 800 mg of clofibrate/kg b.wt./day reduced plasma T_3 concentration by 27% but did not reduce plasma total and free T_4 (Visser et al., 1993a). In mice, a dose of 300 mg of clofibrate/kg b.wt./day reduced plasma free T_4 concentration by 13% but did not significantly reduce plasma concentration of free T_3 (Viollon-Abadie et al., 1999). It is clear that different studies cannot be directly compared with each other, but these data nevertheless suggest that pigs could be even more sensitive to disruptions of thyroid hormone metabolism by clofibrate than rodents.

Reduced plasma concentrations of T_3 and T_4 are expected to increase the release of TSH from the pituitary gland. It has indeed been shown that microsomal enzyme inducers elevate TSH plasma concentrations in rodents, which in turn stimulates proliferation of epithelial cells in thyroid tissue as a result of increased glucuronidation of thyroid hormones (e.g., Curran and DeGroot, 1991; De Sandro et al., 1991; Saito et al., 1991; Liu et al., 1995). As no assay was available for measuring TSH concentration in plasma of the pigs, we determined mRNA concentrations of various genes in the thyroid that are responsive to TSH treatment. The finding that mRNA concentrations of TSH receptor, sodium iodide symporter, thyroid peroxidase, and cathepsin B, all genes responsive to TSH, were moderately increased by 40 to 70% suggests that the thyroid was stimulated by the increased plasma concentration of TSH. This suggestion is confirmed by a study that showed that TSH plasma concentrations are increased by microsomal enzyme inducers, which stimulate the glucuronidation of T_3 (Klaassen and Hood, 2001). Our study further shows that expression levels of dual oxidase 2, a hydrogen peroxide-generating system that constitutes the rate-limiting step of thyroid hormone synthesis, and of thyroglobulin, a protein involved in thyroid hormone synthesis and storage, are not altered by clofibrate treatment.

The finding that thyroid weights, epithelial cells, and follicle lumen diameter were not increased by clofibrate was unexpected and suggests that stimulation of the thyroid was moderate, only increasing gene expression of TSH-responsive genes in the thyroid, whereas histological alterations (i.e., increased epithelial cell height) may take longer than 4 weeks to become evident.

The action of thyroid hormones like T_3 is mediated by thyroid hormone receptors that belong to the family of nuclear hormone receptors. The present study shows that clofibrate treatment reduces gene expression of thyroid hormone receptor α_1 in the liver of rats. This finding agrees with a recent study in which bezafibrate down-regulated thyroid hormone receptors in rat liver (Bonilla et al., 2001). That study further showed that down-regulation of thyroid hormone receptors was caused by activation of PPARα. Therefore, it is likely that in our study down-regulation of thyroid hormone receptor α_1 in pigs treated with PPARα was also caused by PPARα activation by clofibrate. Down-regulation of thyroid hormone receptor implies that the biological activity of T_3 may have been reduced in pigs treated with clofibrate.

Thyroxine-binding globulin, transthyretin, and albumin are the major plasma transport proteins in pigs (Janssen et al., 2002). These proteins are synthesized in the liver. We found in our study that gene expression of these proteins in the liver was reduced by clofibrate treatment of pigs. In studies by Motojima et al. (1992, 1997), the same effect of clofibrate on expression of transthyretin was observed in rats and several mouse strains, whereas there was no effect in PPARα-null mice. This suggests that down-regulation of transthyretin expression was induced by PPARα activation. Consequently, down-regulation of transthyretin and possibly also of thyroxine-binding globulin and albumin could be the result of PPARα activation by clofibrate. Our data suggest, although we did not measure concentrations of these proteins in blood, that clofibrate treatment lowers not only concentrations of thyroid hormones in plasma but also could reduce the transport capacity for thyroid hormones.

In conclusion, this study shows for the first time that clofibrate treatment induces a strong activation of T_3- and T_4-UGT in pigs, leading to increased glucuronidation and markedly reduced plasma concentrations of these hormones. These alterations were accompanied by moderately increased mRNA concentrations of various TSH-responsive enzymes in the thyroid gland, reduced hepatic mRNA concentrations of proteins involved in thyroid hormone transport, and thyroid hormone receptors. Because the pig represents a species that does not respond with peroxisome proliferation to treatment with PPARα agonists, the study shows that clofibrate treatment also disrupts the metabolism of thyroid hormones in nonproliferating species.

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BMC Pharmacology

Research article

Clofibrate treatment in pigs: Effects on parameters critical with respect to peroxisome proliferator-induced hepatocarcinogenesis in rodents

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Abstract

Background: In rodents treatment with fibrates causes hepatocarcinogenesis, probably as a result of oxidative stress and an impaired balance between apoptosis and cell proliferation in the liver. There is some debate whether fibrates could also induce liver cancer in species not responsive to peroxisome proliferation. In this study the effect of clofibrate treatment on peroxisome proliferation, production of oxidative stress, gene expression of pro- and anti-apoptotic genes and proto-oncogenes was investigated in the liver of pigs, a non-proliferating species.

Results: Pigs treated with clofibrate had heavier livers (+16%), higher peroxisome counts (+61%), higher mRNA concentration of acyl-CoA oxidase (+66%), a higher activity of catalase (+41%) but lower concentrations of hydrogen peroxide (-32%) in the liver than control pigs ($P < 0.05$); concentrations of lipid peroxidation products (thiobarbituric acid-reactive substances, conjugated dienes) and total and reduced glutathione in the liver did not differ between both groups. Clofibrate treated pigs also had higher hepatic mRNA concentrations of bax and the proto-oncogenes c-myc and c-jun and a lower mRNA concentration of bcl-X_L than control pigs ($P < 0.05$).

Conclusion: The data of this study show that clofibrate treatment induces moderate peroxisome proliferation but does not cause oxidative stress in the liver of pigs. Gene expression analysis indicates that clofibrate treatment did not inhibit but rather stimulated apoptosis in the liver of these animals. It is also shown that clofibrate increases the expression of the proto-oncogenes c-myc and c-jun in the liver, an event which could be critical with respect to carcinogenesis. As the extent of peroxisome proliferation by clofibrate was similar to that observed in humans, the pig can be regarded as a useful model for investigating the effects of peroxisome proliferators on liver function and hepatocarcinogenesis.

Background

Peroxisome proliferators (PPs) comprise a diverse group of chemicals, including pharmaceuticals, industrial chemicals, endogenous fatty acids and eicosanoids. They bind to and activate the peroxisome proliferator-activated receptor (PPAR)-α, a transcription factor belonging to the nuclear hormone receptor superfamily [1]. Activation of PPARα causes an increase in the transcription of genes related to fatty acid transport across the cell membrane, intracellular lipid trafficking, mitochondrial and peroxisomal fatty acid uptake, and both mitochondrial and peroxisomal fatty acid β-oxidation [2]. Administration of PPs to rats and mice typically causes hepatic peroxisome proliferation, hypertrophy, hyperplasia, and hepatocarcinogenesis [3,4]. PPARα-induced hepatocarcinogenesis in rats and mice may be mainly due to an increased oxidative stress caused by peroxisome proliferation and an alteration of the balance between apoptosis and cell proliferation [5,6]. Treatment with PPs such as fibrates causes a 15 to 20-fold up-regulation of acyl-CoA oxidase (ACO) and other peroxisomal oxidases that lead to the production of hydrogen peroxide (H_2O_2) which under normal non-induced circumstances can be detoxified by catalase [7,8]. Catalase induction increases only approximately twofold in response to PPARα agonists in rodents, and the activity of glutathione peroxidase is often depressed following long term administration of PPs [9,10]. The capacity of the H_2O_2-degrading enzymes therefore may be insufficient to detoxify the large increase in H_2O_2. Increased cellular H_2O_2 could also react with metals and generate highly reactive hydroxyl radicals that could damage DNA, proteins or lipids [7]. Indeed, oxidatively damaged DNA and peroxide-modified lipids have been found in hepatocytes of rats treated with PPs [11-13]. Activation of PPARα also leads to increases in hepatocellular proliferation and inhibition of apoptosis, and when this occurs in DNA-damaged cells, it is thought to lead to proliferation of initiated cells progressing to liver tumour [14,15]. That PPARα is required to mediate hepatocarcinogenesis by PPs has been demonstrated in studies with PPARα-null mice that are refractory to this in response to long term administration of PPs [15,16].

It is well known that non-human primates and humans are only weakly responsive to peroxisome proliferation in comparison to rodents [17,18]. Nevertheless, there is considerable controversy as to whether the administration of drugs which are ligands for PPARα to humans causes liver cancer. This is significant because PPARα agonists such as fibrates have been in clinical use for the treatment of hyperlipidaemias for many years. The data regarding the ability of fibrates to cause peroxisome proliferation in humans are diverse. Examination of liver biopsy samples from patients receiving therapeutic doses of PPARα agonists showed a slight increase in peroxisome counts [19], while others showed no increase [20,21]. In non-human primates, administration of clofibrate induced a moderate, dose-dependent peroxisome proliferation [22-24]. It is therefore conceivable that cellular events induced by PPs related to hepatocarcinogenesis in rodents could occur also in non-proliferating species, albeit probably less pronounced than in rodents.

Pigs, like humans and non-human primates, are a non-proliferating species [25]. They may therefore be a valuable model for investigating the effects of PPs on peroxisome proliferation and related processes. To our knowledge, the effect of fibrates on parameters related to hepatocarcinogenesis has not yet been investigated in pigs. We therefore treated pigs with clofibrate and determined hepatic weight, number of peroxisomes and ACO expression to provide information about the potency of fibrates to induce peroxisome proliferation in pigs. We also considered the antioxidant status of the pigs (mRNA concentrations and activities of various antioxidant enzymes including catalase, generation of H_2O_2 in the liver, concentrations of lipid peroxidation products) to find out whether clofibrate causes oxidative stress in pig liver. In order to ascertain whether clofibrate treatment could affect the balance between cell proliferation and apoptosis we determined mRNA concentrations of pro- and anti-apoptotic genes, namely bax, bcl-X_L and p53 tumour suppressor gene. It has been shown that the nuclear factor κB (NF-κB) pathway is important in the activation of genes that regulate cell proliferation and apoptosis in various cell types [26,27]. It was shown recently that NF-κB contributes to the proliferative and apoptotic changes that occur in liver in response to fibrates [28]. More recently, it has been demonstrated that the p50 subunit of the NF-KB family is necessary for the promotion of hepatocarcinogenesis by PPs [29]. To find out whether clofibrate treatment activated NF-KB in pig liver, we determined the mRNA concentration of tumor necrosis factor α (TNFα), a target gene of NF-KB which has also been identified as a suppressor of apoptosis and an inducer of DNA synthesis [30,31]. In rat liver and in mouse liver epithelial cells, treatment with the PPARα agonist WY-14,643 strongly up-regulated gene expression of various proto-oncogenes including c-fos, c-jun and c-myc [32-35]. In mouse liver cells these changes were followed by enhanced DNA synthesis, and it has been concluded that this could play an important role in tumour promotion by PPs [33]. Whether PPs stimulate expression of proto-oncogenes in pigs has not yet been investigated. We therefore also determined gene expression of c-myc, c-jun and c-fos in liver of pigs treated with clofibrate, which could be critical with respect to hepatocarcinogenesis.

Results

Due to the controlled feeding system, diet intake during the whole experimental period was identical in both groups of pigs, being 696 ± 7 g/d in average of the whole period. Final body weights of the pigs on day 29 did not differ between the control group and the group treated with clofibrate (26.0 ± 1.5 kg for control pigs *vs*. 25.2 ± 1.2 kg for pigs treated with clofibrate, n = 9 in each group). Pigs treated with clofibrate had higher liver weights (+16% in absolute terms, +19% in relative terms, expressed per kg body weight), higher peroxisome counts in the liver (+61%) ($P < 0.05$, Table 1). Relative mRNA concentration of PPARα in liver did not differ between both groups of pigs (control: 1.00 ± 0.38; clofibrate: 0.92 ± 0.20, n = 9, means ± SD) but pigs treated with clofibrate had a higher relative mRNA concentration of ACO in the liver (+66%) than control pigs ($P < 0.05$, Table 1).

As reference values for the expression of enzyme activities, we determined concentrations of protein in liver homogenate and liver cytosol. Protein concentration in liver homogenate did not differ between both groups of pigs (control: 20.8 ± 5.9 mg/g liver; clofibrate: 21.1 ± 5.8 mg/g liver, n = 9, means ± SD); protein concentration in liver cytosol was higher in pigs treated with clofibrate than in control pigs (control: 27.2 ± 2.3 mg/g liver; clofibrate: 30.4 ± 3.0 mg/g liver, n = 9, means ± SD, $P < 0.05$). Pigs treated with clofibrate had higher mRNA concentrations and activities of superoxide dismutase (SOD) (+77% and +128%, respectively) and catalase (+72% and +41%, respectively) in the liver than control pigs ($P < 0.05$, Table 2). In contrast, mRNA concentration and activity of glutathione peroxidase (GSH-Px) in liver were reduced by 26% and 15%, respectively, in pigs treated with clofibrate compared to control pigs ($P < 0.05$, Table 2). mRNA concentration and activity of glutathione S-transferase (GST) in liver cytosol and concentrations of total and reduced glutathione in liver homogenate did not differ between both groups of pigs (Table 2). However, the concentration of α-tocopherol, both in absolute terms and in relative terms, expressed per mmol of triglycerides + total cholesterol, was lower in the liver of pigs treated with clofibrate than in the liver of control pigs (-40%, $P < 0.05$) (Table 2).

The concentration of H_2O_2 in the liver was about 32% lower in pigs treated with clofibrate than in control pigs ($P < 0.05$, Table 3). Concentrations of lipid peroxidation products, thiobarbituric acid-reactive substances (TBARS) and conjugated dienes did not differ between both groups of pigs, both in absolute terms and in relative terms, expressed per mmol of triglycerides + total cholesterol (Table 3).

Hepatic mRNA concentrations of p53 and c-fos did not differ between pigs treated with clofibrate and control pigs whereas mRNA concentrations of bax, c-jun and c-myc were higher in pigs treated with clofibrate than in control pigs ($P < 0.05$, Fig. 1). Hepatic mRNA concentrations of bcl-X_L and TNFα were lower in pigs treated with clofibrate than in control pigs ($P < 0.05$, Fig. 1).

Discussion

To our knowledge, this is the first study to investigate the effect of clofibrate treatment on peroxisome proliferation and parameters that may be related to hepatocarcinogenesis in pigs, a non-proliferating species. As in many other studies dealing with the effects of clofibrate on metabolism in experimental animals, we added clofibrate to the diet. The concentration of clofibrate in the diet of 5 g per kg diet was adopted from other studies with pigs [25,36,37]. The resulting daily dose of 220 mg per kg body weight was relatively high compared with doses used in humans for treatment of hyperlipidaemia, which are usually in the range between 25 and 30 mg per kg body weight.

Analysis of liver weights and number of peroxisomes showed that treatment with clofibrate caused moderate peroxisome proliferation in pigs. The increase in the number of peroxisomes (+62%) observed in pigs treated with clofibrate is of a similar order of magnitude as the 50% increase in liver peroxisome counts observed in humans treated with clofibrate [19]. The extent of the change in peroxisome counts in pigs is modest when compared with that reported for rodents. In rodents, administration of a dose of 200 mg clofibrate per kg body weight, comparable with that used in this study in pigs, resulted in a three- to five-fold increase in peroxisome counts compared with controls [38]. The current study also shows that gene expression of ACO is moderately increased by clofibrate in pigs, which is in close accord with the moderate effect of clofibrate on the peroxisome count. The present study confirms other studies [25,36,37] which have also shown that clofibrate causes only a relatively weak up-regulation of PPARα target genes in pig liver. The moderate effect of clofibrate on ACO in pigs is in strong contrast to rodents where treatment with PPs causes a 10 to 20-fold increase in ACO expression [35,39]. Up-regulation of ACO in the liver is critical because it leads to increased production of H_2O_2 which causes oxidative stress within the cell. In contrast to observations in rodents, pigs treated with clofibrate in this study had not a higher but a lower concentration of H_2O_2 in the liver. We assume that this is due to up-regulation of catalase, the key enzyme involved in H_2O_2 detoxification. In our study both ACO and catalase were up-regulated by clofibrate to a similar extent. Catalase has a high H_2O_2-detoxifying activity and is the rate-limiting enzyme for inhibiting H_2O_2 leakage from peroxisomes [40]. The finding that the concentration of H_2O_2 was reduced even though the activ-

Table 1: Liver weights, number of peroxisomes and relative acyl-CoA oxidase mRNA concentration in the liver of pigs fed a control diet or a diet supplemented with 5 g clofibrate per kg for 28 days

Treatment	Control (*n* = 9)	Clofibrate (*n* = 9)
Liver weight (g)	673 ± 63	779 ± 63*
Liver weight (g/kg body weight)	25.9 ± 2.2	30.9 ± 2.6*
Number of peroxisomes (n/1,000 print)	366 ± 67	590 ± 116*
Acyl-CoA oxidase mRNA	1.00 ± 0.35	1.66 ± 0.41*

Results are means ± SD.
*Significantly different from control group (P < 0.05)

ity of GSH-Px, another H_2O_2-detoxifying enzyme localised in cytosol was reduced as well, suggests that the up-regulation of catalase was sufficient to eliminate all of the H_2O_2 produced in peroxisomes. The finding of a reduced activity of GSH-Px in liver of pigs treated with clofibrate agrees with findings in rodents treated with PPs which also showed a lower activity of that enzyme in the liver [11,41].

Unsaturated fatty acids are susceptible to reactive oxygen species and undergo oxidation. The determination of lipid peroxides such as TBARS or conjugated dienes is therefore a sensible method to detect oxidative stress. Indeed, in hepatocytes of rats treated with PPs, concentrations of lipid peroxidation products were increased due to oxidative stress induced by peroxisome proliferation [11,42]. The fact that concentrations of TBARS and conjugated dienes were not increased in the liver of pigs treated with clofibrate indicates that the moderate peroxisome proliferation was not accompanied by oxidative stress. This indication is supported by the observation that the concentration of reduced glutathione in the liver was also not altered in pigs treated with clofibrate when compared with control pigs. Glutathione plays a pivotal role in protecting cells against the noxious effects of oxidant agents, and oxidative stress leads to enhanced oxidation of glutathione, which in turn causes a lower concentration of reduced glutathione and a lower ratio of reduced to oxidized glutathione [43]. Likewise, activity of GST, an enzyme belonging to the phase II enzymes whose reactions include the addition of glutathione to electrophilic molecules as well as the detoxification of organic hydroperoxides, was not changed in pigs treated with clofibrate. This is in contrast to rodents treated with fibrates or other PPs that have a strongly reduced activity of GST in the liver [44,45]. As it has been suggested that the reduced GST activity in rodents treated with PPs is the consequence of oxidative stress due to peroxisome proliferation, the finding of an unchanged activity of that enzyme is another indication that oxidative stress did not occur in pigs treated with clofibrate. This indication agrees with a recent study which investigated the effect of high doses of

Table 2: Relative mRNA concentrations and activities of antioxidant enzymes and concentrations of some antioxidants in the liver of pigs fed a control diet or a diet supplemented with 5 g clofibrate per kg for 28 days

Treatment	Control (*n* = 9)	Clofibrate (*n* = 9)
Catalase		
mRNA concentration	1.00 ± 0.49	1.72 ± 0.58*
Activity (U/mg protein)	0.75 ± 0.14	1.06 ± 0.16*
Glutathione peroxidase		
mRNA concentration	1.00 ± 0.17	0.74 ± 0.18*
Activity (U/mg protein)	4.7 ± 0.8	4.0 ± 0.4*
Glutathione S-transferase		
mRNA concentration	1.00 ± 0.41	0.90 ± 0.32
Activity (U/mg protein)	0.76 ± 0.26	0.76 ± 0.18
Superoxide dismutase		
mRNA concentration	1.00 ± 0.42	1.77 ± 0.40*
Activity (U/mg protein)	43 ± 8	97 ± 13*
Glutathione, total (nmol/mg protein)	2.13 ± 0.51	1.97 ± 0.90
Glutathione, reduced (nmol/mg protein)	1.70 ± 0.50	1.67 ± 1.02
α-tocopherol		
(nmol/g)	14.5 ± 2.5	8.7 ± 2.9*
[nmol/(mmol triglycerides + cholesterol)]	2.32 ± 0.40	1.36 ± 0.45*

Results are means ± SD.
*Significantly different from control group (P < 0.05)
#Relative to control (= 1.00)

Table 3: Concentration of hydrogen peroxide and lipid peroxidation products in the liver of pigs fed a control diet or a diet supplemented with 5 g clofibrate per kg for 28 days

Treatment	Control (*n* = 9)	Clofibrate (*n* = 9)
Hydrogen peroxide (fluorescence/g liver)	29,437 ± 8,361	20,078 ± 7,225*
Thiobarbituric acid-reactive substances		
(nmol/g liver)	7.2 ± 1.6	7.7 ± 2.7
[nmol/(mmol triglycerides + cholesterol)]	1.15 ± 0.26	1.20 ± 0.42
Conjugated dienes (nmol/g)		
(nmol/g liver)	16 ± 2	16 ± 2
[nmol/(mmol triglycerides + cholesterol)]	2.56 ± 0.32	2.49 ± 0.31

Results are means ± SD.
*Significantly different from control group (P < 0.05)

fenofibrate and ciprofibrate in cynomolgus monkeys [24]. In that study, clofibrate treatment induced moderate hepatic peroxisome proliferation, similar to that observed in the pigs of the present study, but there was also minimal indication of oxidative stress. It is therefore concluded that even high doses of fibrates cause little oxidative stress in the liver of non-proliferating species.

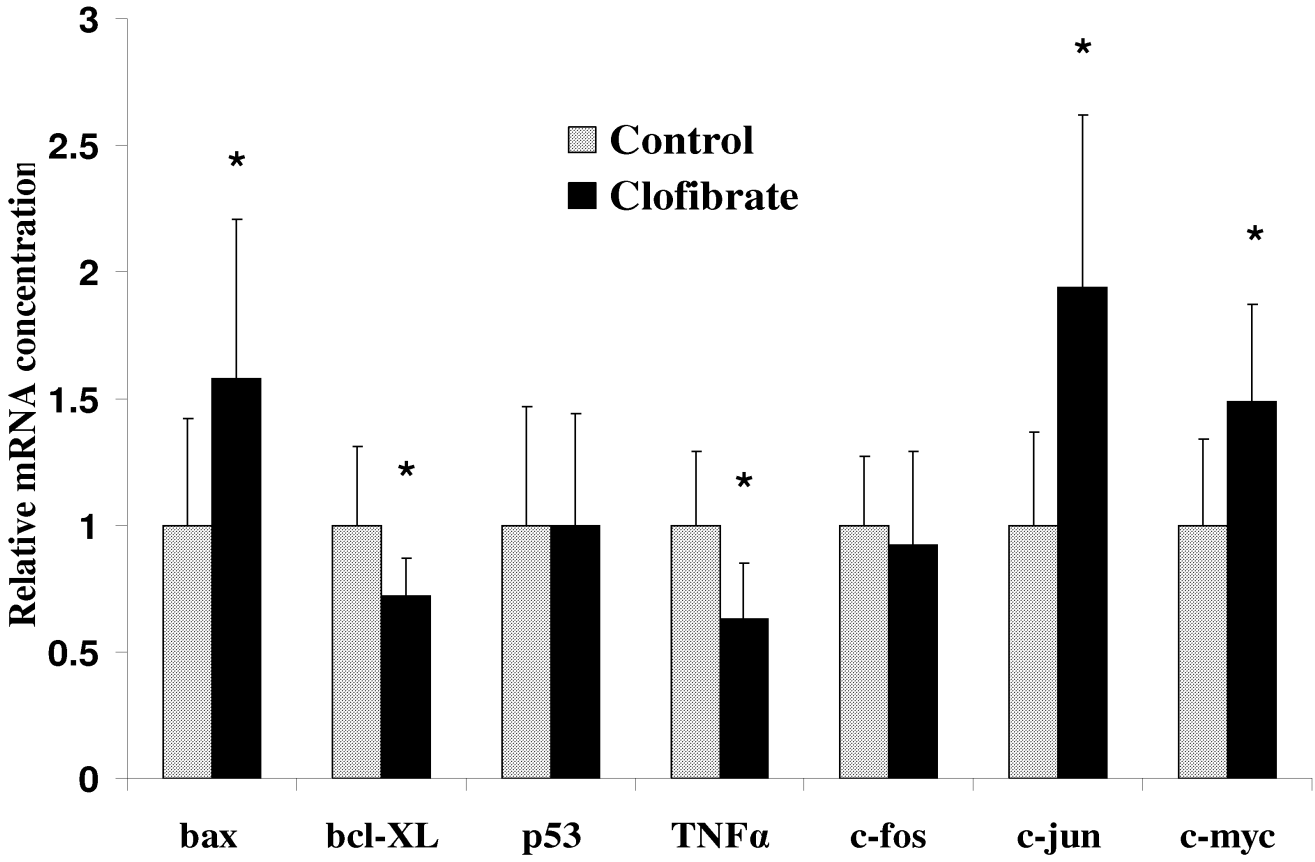

Figure 1
Relative mRNA concentrations of pro- and anti-apoptotic genes (bax, bcl-X_L, p53, TNFα) and proto-oncogenes (c-myc, c-jun, c-fos) in livers of pigs fed a control diet or a diet supplemented with 5 g clofibrate per kg for 28 days. All mRNA concentrations were determined by real-time quantitative PCR and normalized to GAPDH. Data are reported as means ± SD with nine animals per group. Data are expressed relative to mRNA concentrations of control pigs (control = 1). * Significantly different to control group (P < 0.05).

In the current study, pigs treated with clofibrate had increased hepatic mRNA concentration and activity of SOD, an enzyme that converts superoxide anions into H_2O_2 and therefore contributes to increased H_2O_2 production. The finding that H_2O_2 concentration in the liver was reduced in spite of the increased activity of SOD is another indication that pigs treated with fibrate had a high hepatic H_2O_2-detoxifying capacity. Results from other studies dealing with the effects of PPs on hepatic SOD are contradictory. In some studies, treatment of rodents with PPs lowered hepatic activity of SOD [41,46]; in others treatment with PPs increased the activity of hepatic SOD [47,48]. Moreover, it has been shown that different PPs can have different effects on hepatic activity of SOD, and that there are also differences between mice and hamsters in the effect of PPs on SOD activity [48]. The reason for these contradictory results is unclear and should be investigated further. As SOD is an important constituent of the hepatic antioxidant system, an increased activity of that enzyme observed in pigs treated with clofibrate could contribute to a high antioxidant capacity in the liver of these pigs.

The finding that pigs treated with clofibrate had a reduced concentration of α-tocopherol in the liver agrees with those of several other studies in which rodents were treated with PPs [11,48,49]. It has been shown that the tocopherol-lowering effect of PPs is stronger in rats than in hamsters, suggesting that there is a correlation with the degree of peroxisome proliferation [48]. It has been suggested that the reduction of hepatic tocopherol concentration is not primarily due to oxidative stress produced by PPs but rather to their hypolipidaemic effect [48]. Tocopherols are transported by lipoproteins within the body and as hypolipidaemic drugs reduce the number of lipoprotein particles in blood, they could also impair vitamin E transport in the body, i.e. transport of vitamin E from the intestine to the liver by chylomicrons. The fact that α-tocopherol concentration was reduced in the liver of pigs treated with clofibrate although there were no signs of oxidative stress confirms the suggestion that PPs do reduce hepatic vitamin E concentrations independent of oxidative stress.

It has been shown that peroxisome proliferation leads to hepatocellular proliferation and to inhibition of apoptosis, and these processes may contribute to hepatomegaly and to hepatocarcinogenesis observed in rodents treated with PPs [14,15]. The present study shows that clofibrate treatment of pigs increases gene expression of the proto-oncogenes c-jun and c-myc, which are required for entry into the S phase of the cell cycle. These findings are in agreement with recent studies in rodent livers and liver cells in which treatment with WY-14,643 strongly up-regulated gene expression of proto-oncogenes [32-34]. Up-regulation of proto-oncogenes in mouse liver cells was followed by enhanced progression, and it has been suggested that this could play a role in tumour promotion of PPs [32]. Although up-regulation of proto-oncogenes was smaller in pigs treated with fibrates than in rodents [32-34], increased levels of c-jun and c-myc could have enhanced cell proliferation, which might explain the increased liver mass in pigs treated with clofibrate. Up-regulation of proto-oncogenes could be a critical event with respect to tumorigenesis. On the other hand, c-myc can collaborate with other proteins to induce apoptosis and sensitize cells to a variety of apoptotic triggers [50,51]. In order to find out whether clofibrate treatment in pigs could have altered apoptosis, we determined mRNA concentrations of bax, bcl-X_L and p53 in the liver. Genes of the bcl-2 family have anti-apoptotic effects, which is antagonized by bax. The bcl-2/bax ratio is a key factor for determining apoptosis. When bcl-2 is expressed excessively, bcl-2-bax heterogenous dimer predominates, thus inhibiting apoptosis [52]. When bax is expressed excessively, bax-bax homogenous dimer or monomer predominates, thus promoting apoptosis. p53 is a tumour suppressor which exerts control over cell cycling by controlling the progression through the G1 phase [53]. Genotoxic stress or DNA damage leads to nuclear accumulation of p53, which in turn activates the transcription of several genes involved in DNA repair or apoptosis, including bcl-2 [54]. We did not directly determine apoptosis in the liver of piglets but the finding that expression of bax was up-regulated in pigs treated with clofibrate while expression of bcl-X_L was reduced, together with the observation of unchanged p53 expression suggests that apoptosis was not inhibited but could instead have been increased in these pigs. This suggestion agrees with recent studies which have shown that clofibrate induces apoptosis in human and rat hepatoma cells [55-59]. In agreement with our study, a recent study showed that treatment with WY 14,643 up-regulates pro-apoptotic genes and down regulates anti-apoptotic genes in the liver of mice, an effect which did not occur in PPARα-null mice [60]. In that study, it was also demonstrated that PPARα activation increases the sensitivity of liver towards apoptosis by Jo-2, an inducer of hepatic apoptosis. Therefore, it has been suggested that PPARα could serve as a pharmacological target in diseases where apoptosis is a contributing feature [60,61].

In a recent study in rats it was found that NF-KB is activated by WY-14,643, probably due to oxidative stress caused by peroxisome proliferation [62]. It has been demonstrated that NF-KB is essential for inducing cell proliferation and hepatocarcinogenesis in rodents treated with fibrates [28,29]. The finding that mRNA concentration of TNFα, a target gene of NF-κB, was reduced in liver of pigs treated with clofibrate compared to control pigs indicates

that clofibrate did not activate the NF-KB pathway in these animals. This finding may be related to the observation that clofibrate treatment did not cause oxidative stress in pigs, in contrast to rats. The finding that WY-14,643 did not activate the NF-KB pathway in hamsters either [62] suggests that PPs activate NF-KB only in species that are responsive to PP induced hepatocarcinogenesis.

Conclusion

Treatment with clofibrate at doses higher than those used for hypolipidaemic treatment in humans causes moderate peroxisome proliferation in the liver of pigs. Determination of the concentration of H_2O_2 and lipid peroxidation products indicates that this did not produce oxidative stress. Determination of mRNA concentrations of pro- and anti-apoptotic genes in the liver indicates that clofibrate treatment did also not inhibit but rather stimulated apoptosis in these animals. Up-regulation of the proto-oncogenes c-myc and c-jun in the liver, however, could be a critical event with respect to carcinogenesis, which deserves further investigation in future studies. As the extent of peroxisome proliferation by clofibrate was similar to that observed in humans, the pig can be regarded as a useful model for the investigating the effects of PPs on liver function and hepatocarcinogenesis.

Methods

Animals and treatments

Eighteen male 8-week-old crossbred pigs [(German Landrace × Large White) × Pietrain] were kept in a room under controlled conditions at 23 ± 2°C and 55 ± 5% relative humidity with light from 0600 to 1800 h. One day before the start of the experimental feeding period the pigs were weighed and randomly assigned to two groups with body weights of 12.0 ± 1.1 kg in the control group and 11.9 ± 0.6 kg in the treatment group. Both groups of pigs received a nutritionally adequate dry diet for growing pigs (according to [63]) containing (in g/kg) wheat (400), soybean meal (230), wheat bran (150), barley (100), sunflower oil (90) and mineral premix including L-lysine, DL-methionine and L-threonine (30). This diet contained 14.4 MJ metabolizable energy and 185 g crude protein per kg. The whole daily amount of diet was administered once at 8.00 h. Diet intake was controlled, and each animal in the experiment was offered an identical amount of diet per day. The amount of diet administered was about 15% below that consumed ad-libitum by pigs of a similar weight (as assessed in a previous study). Therefore, the diet offered was completely taken up by all pigs in the experiment. During feeding period, the amount of diet offered each day was increased continuously from 400 to 1,200 g. Pigs of both groups received the same diet. However, pigs of the treatment group were given additionally 5 g clofibrate per kg diet which was freshly given onto the diet on each day. The pigs had free access to water via nipple drinking systems. The experimental diets were administered for 28 d. All experimental procedures described followed established guidelines for the care and use of laboratory animals and were approved by the local veterinary office.

Sample collection

After completion of the feeding period the piglets were captive-bolt stunned and exsanguinated. Four hours before euthanasia each pig was fed its respective diet. After killing, blood was collected into heparinized polyethylene tubes. Plasma was obtained by centrifugation of the blood (1,100 × g; 10 min; 4°C). The liver was dissected and weighed and samples were stored at -80°C until analysis. For preparation of liver homogenate, one g of liver was homogenized in 10 mL of 0.1 M phosphate buffer, pH 7.4, containing 0.25 M sucrose using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 1,000 × g for 10 min at 4°C and the supernatant was centrifuged at 15,000 × g for 15 min again. The supernatant of that centrifugation was collected and centrifuged at 105,000 × g for 60 min to yield the cytosolic fraction. Liver homogenates and cytosolic fraction were stored at -20°C for further analysis. Protein concentrations of liver homogenates and cytosol were determined with the bicinchoninic acid reagent according to the supplier's protocol (Interchim, Montelucon, France) using bovine serum albumin as the standard.

RT-PCR analysis

Total RNA from liver tissue was isolated by TRIzol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol, resuspended in diethyl pyrocarbonate-treated water and stored at -80°C until use. The concentration and purity of total RNA was determined by ultraviolet absorbance at 260 and 280 nm (SpectraFluor Plus; Tecan, Crailsheim, Germany). The quality of all RNA samples was assessed by agarose gel electrophoresis. cDNA was prepared from total RNA (1.2 μg) by reverse transcription using M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany) and oligo$(dT)_{18}$ primers (Operon Biotechnologies, Cologne, Germany). The mRNA concentration of genes was measured by realtime detection PCR using SYBR® Green I and a MJ Research Opticon system (Biozym Diagnostik GmbH, Oldendorf, Germany). Realtime detection PCR was performed with 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 4). Annealing temperature for all primers was 60°C. Amplification efficiencies for all primer pairs were determined by template dilution series. Calculation of the relative mRNA concentration was made using the amplification efficiencies and the C_t values [64]. Glyceraldehyde 3-phosphate dehydro-

genase (GAPDH) was used as housekeeping gene for normalization.

Enzyme assays

SOD in liver cytosol was determined with pyrogallol as the substrate [65]. One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. The activity of GSH-Px in liver cytosol was determined with t-butyl hydroperoxide as substrate [66]. One unit of GSH-Px activity is defined as one μmol reduced β-nicotinamide adenine dinucleotide phosphate oxidized per min. The activity of GST was determined using 1-chloro-2,4-dinitrobenzene as substrate [67]. One unit of GST is defined as one nmol substrate consumed per min. Catalase activity in liver homogenate was determined using hydrogen peroxide as substrate [68]. One unit of catalase activity is defined as the amount consuming 1 mmol hydrogen peroxide per min. GSH concentration in liver homogenates was determined according to Griffith [69].

Determination of conjugated dienes, TBARS, α-tocopherol, cholesterol and triglycerides in the liver

Lipids from liver were extracted using a mixture of n-hexane and isopropanol (3:2, v/v). After drying the lipid extracts, 1 mg of extract was dissolved in 1 ml n-hexane. The concentrations of conjugated dienes were calculated by using the molar extinction coefficient for conjugated dienes at 234 nm ($\varepsilon = 29{,}500\ mol \times cm^{-1}$). The concentrations of TBARS were measured with thiobarbituric acid as reagent in a fluorimetric assay [70]. Concentration of α-tocopherol in liver tissue was determined by HPLC with fluorescence detection [70]. For determination of triglycerides and total cholesterol, an aliquot of the lipid extract was dried, and the dried lipids were dissolved with Triton X-100 [71]. The concentrations of cholesterol and triglycerides were determined using enzymatic kits (Cat.-No. 113009990314 for cholesterol and Cat.-No. 157609990314 for triglycerides, Ecoline S+, DiaSys, Holzheim, Germany).

Determination of H_2O_2

To determine the H_2O_2 content in liver homogenates, a method [72] described for cell culture systems was modified, using dihydrorhodamine 123 (DHR) as substrate. Homogenates were incubated with 27.5 μM DHR for 1 h at 37°C in a final volume of 400 μl. After incubation, the fluorescence of rhodamine 123, the oxidation product of DHR, was measured (excitation wavelength: 485 nm, emission wavelength: 538 nm). As previously shown [73], this test is specific for H_2O_2 as DHR is specifically oxidized by H_2O_2.

Transmission electron microscopy

Small liver segments of three animals per group were fixed immediately after dissection of the liver with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB, pH 7.2) for 3 hours at room temperature, washed with SCB, postfixed with 1% osmiumtetroxide in SCB, dehydrated in a graded ethanol series, and embedded in epoxy resin [74]. The material was sectioned with an ultramicrotome S (Leica, Bensheim, Germany). Ultrathin sections (80 nm) were transferred to coated copper grids and poststained with uranyl acetate and lead citrate. The sections were observed with an EM 900 transmission electron microscope (Zeiss SMT, Oberkochen, Germany) at an acceleration voltage of 80 kV. Electron micrographs were taken with a slow scan camera (TRS, Dünzelbach, Germany).

Table 4: Sequences of specific primers used for RT-PCR

Gene (NCBI Genbank)	Forward Primer	Reverse Primer	Size, bp
ACO (AF185048)	CTCGCAGACCCAGATGAAAT	TCCAAGCCTCGAAGATGAGT	218
bax (AJ606301)	CGAACTGATCAGGACCATCA	ACAGCCCATCTTCTTCCAGA	190
bcl-X_L (NM_214285)	GAAACCCCTAGTGCCATCAA	GGGACGTCAGGTCACTGAAT	196
Catalase (NM_214301)	CAGCTTTAGTGCTCCCGAAC	AGATGACCCGCAATGTTCTC	180
c-fos (Y14808)	CTGACACACTCCAAGCGGTA	CTTCTCCTTCAGGTTGG	209
c-jun (NM_213880)	CAGAGCATGACCCTGAACCT	TTCTTGGGGCATAGGAACTG	200
c-myc (NM_001005154)	AATGTCTTGGAACGCCAGAG	CAACTGTTCTCGCCTCTTCC	204
GAPDH (AF017079)	AGGGGCTCTCCAGAACATCA TCC	TCGCGTGCTCTTGCTGGGGT TGG	446
GSH-Px (NM_214201)	CAAGAATGGGGAGATCCTGA	GATAAACTTGGGGTCGGTCA	190
GST (NM_214300)	TTTTTGCCAACCCAGAAGAC	GGGGTGTCAAATACGCAATC	246
p53 (NM_214145)	GCGAGTATTTCACCCTCCAG	TCAGGCCCTTCTCTCTTGAA	199
PPARα (DQ437887)	CAGCCTCCAGCCCCTCGTC	GCGGTCTCGGCATCTTCTAG G	381
SOD (AF396674)	TCCATGTCCATCAGTTTGGA	CTGCCCAAGTCATCTGGTTT	250
TNFα (X57321)	CCCCTGTCCATCCCTTTATT	AAGCCCCAGTTCCAATTCTT	200

Abbreviations: ACO, acyl-CoA oxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH-Px, glutathione peroxidase; GST, glutathione S-transferase; SOD, superoxide dismutase; TNFα, tumor necrosis factor α.

Peroxisomes were counted in 1,000 different regions per liver sample for each animal at a screen (12,000-fold magnification).

Statistics

The results were analyzed using Minitab (State College, Pa, USA) statistical software (release 13). Statistical significance of differences between control group and treatment group was evaluated using Student's t-test. Mean values were considered significantly different for $P < 0.05$.

List of abbreviations

ACO, acyl-CoA oxidase; DHR, dihydrorhodamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH-Px, glutathione peroxidase; GST, glutathione S-transferase; NF-KB, nuclear factor KB; PPARα, peroxisome proliferator-activated receptor α; PPs, peroxisome proliferators; SCB, sodium cacodylate buffer; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TNFα, tumor necrosis factor α.

Authors' contributions

SL and BG carried out the feeding experiments, performed the analyses and helped to draft the manuscript.

GH determined peroxisome count in the liver by transmission electrone microscopy.

HK participated in the design of the study and in the interpretation of the results and supervised the animal experiment.

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

All authors read and approved the final manuscript.

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Feeding of a deep-fried fat causes PPARα activation in the liver of pigs as a non-proliferating species

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Recent studies have shown that dietary oxidised fats influence the lipid metabolism in rats by activation of PPARα. In this study, we investigated whether a mildly oxidised fat causes activation of PPARα in pigs which are non-proliferators like man. Eighteen pigs were assigned to two groups and received either a diet containing 90 g/kg of a fresh fat or the same diet with 90 g/kg of an oxidised fat prepared by heating for 24 h at 180°C in a deep fryer. Pigs fed the oxidised fat had a higher peroxisome count, a higher activity of catalase and a higher mRNA concentration of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in the liver and a higher concentration of 3-hydroxybutyrate in plasma than pigs fed the fresh fat ($P<0{\cdot}05$). Hepatic mRNA concentrations of acyl-CoA oxidase and carnitine palmitoyltransferase-1 tended to be increased in pigs fed the oxidised fat compared to pigs fed the fresh fat ($P<0{\cdot}10$). Pigs fed the oxidised fat, moreover, had higher mRNA concentrations of sterol regulatory element-binding protein (SREBP)-1 and its target genes acetyl-CoA carboxylase and stearoyl-CoA desaturase in the liver and higher mRNA concentrations of SREBP-2 and its target genes 3-hydroxy-3-methylglutary-CoA reductase and LDL receptor in liver and small intestine. In conclusion, this study shows that even a mildly oxidised fat causes activation of PPARα in the liver of pigs. Up-regulation of SREBP and its target genes in liver and small intestine suggests that the oxidised fat could stimulate synthesis of cholesterol and TAG in these tissues.

Oxidised fat: Pig: PPARα: Cholesterol: Triacylglycerols

The typical western diet contains large quantities of PUFA that are heated or processed to varying degrees. In fast-food restaurants fat is heated in fryers for up to 18 h daily, at temperatures close to 180°C (Frankel *et al.* 1984). Several studies with animals have been performed to investigate the effects of oxidised fats on the metabolism (reviewed in Cohn, 2002). Recently, it has been shown in rats that oxidised fats are able to influence the lipid metabolism by activation of PPARα (Chao *et al.* 2001, 2004, 2005; Sülzle *et al.* 2004), a transcription factor belonging to the nuclear hormone receptor superfamily (Schoonjans *et al.* 1996). This is probably due to the occurrence of hydroxy- and hydroperoxy fatty acids such as hydroxy octadecadienoic acid and hydroperoxy octadecadienoic acid which are potent activators of PPARα (Delerive *et al.* 2000; Mishra *et al.* 2004; König & Eder, 2006). Activation of PPARα leads to an increase in the transcription of genes related to fatty acid transport across the cell membrane, intracellular lipid trafficking, mitochondrial and peroxisomal fatty acid uptake, and both mitochondrial and peroxisomal fatty acid β-oxidation, gluconeogenesis and ketogenesis (Mandard *et al.* 2004). Recently, it has been shown that PPARα activation influences also the expression or the proteolytic activation of sterol regulatory element-binding proteins (SREBP), transcription factors which control fatty acid synthesis and cholesterol homeostasis (Patel *et al.* 2001; Guo *et al.* 2001; Knight *et al.* 2005; König *et al.* 2006). Therefore, PPARα activation stimulates not only the degradation of fatty acids by enhancing β-oxidation but affects also the synthesis of cholesterol and TAG. Reduced liver and plasma concentrations of TAG and cholesterol are typical effects observed in animals treated with PPARα agonists, and such effects have been also observed in rats administered oxidised fats (Huang *et al.* 1988; Eder & Kirchgessner, 1998; Eder, 1999; Chao *et al.* 2001, 2004, 2005; Sülzle *et al.* 2004).

Regarding the expression of PPARα in tissues and the effects of PPARα activation on transcription of its target genes, there are great differences between various species. In rodents, PPARα is highly expressed, and activation of PPARα not only induces many genes involved in various metabolic pathways such as β-oxidation, ketogenesis and

Abbreviations: ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; CPT-1, carnitine palmitoyltransferase-1; CYP7, cholesterol 7α-hydroxylase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; L-FABP, liver fatty acid binding protein; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal TAG transfer protein; SCD, stearoyl-CoA desaturase; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein; TBARS, thiobarbituric acid-reactive substances.

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gluconeogenesis but also causes severe peroxisome proliferation in the liver (Peters *et al.* 2005). In contrast to rodents, PPARα agonists do not induce peroxisome proliferation in the liver of many other species, such as guinea pigs, swine, monkeys and man (Holden & Tugwood, 1999). These non-proliferating species have a lower expression of PPARα in the liver and the response of many genes to PPARα activation is much weaker than in proliferating species. For that reason, effects related to PPARα activation observed in rodents cannot be directly applied for non-proliferating species such as man. Therefore, it remains unknown whether oxidised fats are able to cause PPARα activation also in non-proliferating species.

The aim of the present study was to investigate whether a dietary oxidised fat, prepared by heating sunflower oil under usual deep-frying conditions (180°C) for 24 h in a deep fryer, is able to activate PPARα and to cause peroxisome proliferation in pigs. Pigs have been chosen as a model since they belong – like man – to the non-proliferating species (Yu *et al.* 2001; Peffer *et al.* 2005) and since pig liver cells show a similarity to human liver cells in the gene response to PPARα agonists (Cheon *et al.* 2005). We focused our analyses on liver and small intestine as both tissues exhibit a high expression of PPARα (Braissant *et al.* 1996; Lemberger *et al.* 1996). Moreover, both tissues play an important role in whole body lipid homeostasis, i.e. synthesis and secretion of lipoproteins rich in TAG and cholesterol (Lindsay & Wilson, 1965; Dietschy *et al.* 1993). We examined the expression of various genes involved in lipid metabolism which have been already shown to be influenced by PPARα activation. Furthermore, in both tissues we determined gene expression of SREBP and important SREBP target genes involved in fatty acid synthesis and cholesterol uptake and synthesis.

Materials and methods

Animals

For the experiment, eighteen male 8-week-old crossbred pigs ((German Landrace × Large White) × Pietrain) were kept in a room under controlled temperature at 23 ± 2°C and 55 ± 5 % relative humidity with light from 06.00 to 18.00 hours. One day before the beginning of the experimental feeding period, the pigs were weighed and randomly allocated to two groups with body weights of 12·0 (SD 1·1) kg in the control group and 12·2 (SD 0·9) kg in the treatment group. All experimental procedures described followed established guidelines for the care and use of laboratory animals and were approved by the local veterinary office.

Diets and feeding

Both groups of pigs received a nutritionally adequate diet for growing pigs containing (in g/kg) wheat (400), soyabean meal (230), wheat bran (150), barley (100), sunflower oil or test oil (90), and mineral premix including L-lysine, DL-methionine and L-threonine (30). This diet contained 14·4 MJ metabolisable energy and 185 g crude protein/kg. Diet intake was controlled, and each animal in the experiment was offered an identical amount of diet per day. During the feeding period, the amount of diet offered each day was increased continuously from 400 to 1200 g. The pigs had free access to water via nipple drinking systems. The experimental diets were administered for 28 d.

Preparation of the test fats

To prepare the oxidised fat, sunflower oil obtained from a local supermarket was heated at a temperature of 180°C for 24 h in a deep fryer. This treatment caused a loss of PUFA and tocopherols. The major fatty acids in the fresh and the oxidised fat, respectively, were (g/100 g total fatty acids): palmitic acid (16 : 0), 6·30 *v.* 6·70; stearic acid (18 : 0), 4·0 *v.* 4·2; oleic acid (18 : 1*n*-9), 22·8 *v.* 23·8; linoleic acid (18 : 2*n*-6), 63·6 *v.* 59·9. Other fatty acids were present only in small amounts (< 0·5 g/100 g fatty acids). To equalise the fatty acid composition of the fresh and the oxidised fat, the fresh fat was composed of a mixture of sunflower oil and palm oil (93 : 7, w/w). To adjust dietary vitamin E concentrations, we analysed the native concentrations of tocopherols in the fresh fat and in the oxidised fats after the thermal treatment. With consideration of the native tocopherol concentrations of the dietary fats, the 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). The final vitamin E concentration was 620 mg α-tocopherol equivalents/kg in both fats. Concentrations of lipid peroxidation products were determined after the fats have been already included into the diets. Therefore, lipids of the diets were extracted by *n*-hexane and isopropanol (3 : 2, v/v; Hara & Radin, 1978). Concentration of thiobarbituric acid-reactive substances (TBARS; Sidwell *et al.* 1954), conjugated dienes (Recknagel & Glende, 1984), peroxide value (Deutsche Gesellschaft für Fettwissenschaft, 1994), acid value (Deutsche Gesellschaft für Fettwissenschaft, 1994) and concentration of total carbonyls (Endo *et al.* 2001) were determined in the extracted fat.

Sample collection

After completion of the feeding period the animals were killed under light anaesthesia. Each pig was fed its respective diet 4 h before being killed. After killing, blood was collected into heparinised polyethylene tubes. Plasma was obtained by centrifugation of the blood (1100 ***g***, 10 min, 4°C). Plasma lipoproteins were separated by step-wise ultracentrifugation (Mikro-Ultrazentrifuge; Sorvall Products, Bad Homburg, Germany) at 900 000 ***g*** at 4°C for 1·5 h. Plasma densities were adjusted by sodium chloride and potassium bromide and the lipoprotein fractions $\delta < 1{\cdot}006$ kg/l VLDL plus chylomicrons, $1{\cdot}006 < \delta < 1{\cdot}063$ kg/l LDL and $\delta > 1{\cdot}063$ kg/l HDL were removed by suction. The liver was dissected and weighted and samples were stored at −80°C until analysis. For preparation of liver homogenate, 1 g liver tissue was homogenised in PBS by TissueLyser (Qiagen, Haan, Germany), centrifuged at 600 ***g*** for 10 min at 4°C and the supernatant was stored at −20°C until analysis. For isolation of intestinal epithelial cells, the abdomen was immediately opened after killing, and a 35 cm intestinal segment was dissected starting at 30 cm distal to the pyloric sphincter, and flushed twice with ice-cold wash buffer (PBS containing 0·2 mM-phenylmethylsulphonyl fluoride and 0·5 mM-dithiothreitol, pH 7·4).

The isolation of porcine intestinal epithelial cells was performed by the modified distended intestinal sac technique according to Fan *et al.* (2004). In brief, the intestinal segments were filled with 100 ml preincubation buffer (PBS containing 27 mM-sodium citrate, 0·2 mM-phenylmethylsulphonyl fluoride and 0·5 mM-dithiothreitol, 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 pre-incubation buffer was discarded, and the intestinal segments were filled with isolation buffer (PBS containing 1·5 mM-Na_2EDTA, 0·2 mM-phenylmethylsulphonyl fluoride, 0·5 mM-dithiothreitol 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 twice with ice-cold PBS. Afterwards, cells were retained by centrifugation (400 ***g***, 4 min, 4°C) and immediately frozen at − 80°C. For further analysis, we used the crypt cell fraction as it has been shown that these cells have a 6–8-fold higher capacity of lipid synthesis than villus cells (Shakir *et al.* 1978).

Lipid analysis

Lipids from liver were extracted with a mixture of *n*-hexane and isopropanol (3 : 2, v/v; Hara & Radin, 1978). For determination of the concentrations of lipids in liver, aliquots of the lipid extracts were dried and the lipids were dissolved using Triton X-100 (De Hoff *et al.* 1978). Concentrations of TAG and cholesterol in plasma and lipoproteins and those of liver were determined using enzymatic reagent kits (cat. no. 113009990314 for cholesterol and cat. no. 157609990314 for TAG; Ecoline S^+, DiaSys, Holzheim, Germany).

Preparation of liver microsomal and cytosolic fractions

Liver (1 g) was homogenised in 10 ml 0·1 M-phosphate buffer, pH 7·4, containing 0·25 M-sucrose using a Potter-Elvehjem homogeniser. Homogenates were centrifuged at 1000 ***g*** for 10 min at 4°C, and the supernatant was centrifuged at 15 000 ***g*** for a further 15 min. The microsomal pellet was obtained by centrifugation of the 15 000 ***g*** supernatant at 105 000 ***g*** for 60 min. The resulting cytosolic fraction in the supernatant was separated, microsomal pellets were resuspended in the homogenisation buffer and all samples were stored at − 20°C for further analysis. The protein concentrations of cytosolic and microsomal fractions were determined with the BCA reagent according to the protocol of the supplier (Interchim, Montelucon, France) using bovine serum albumin as standard.

RT–PCR analysis

Total RNA from liver tissue and enterocytes, respectively, was isolated by the TissueLyser (Qiagen) 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). The quality of all RNA samples was furthermore assessed by agarose gel electrophoresis. Total RNA (1·2 μg) was used for cDNA synthesis as described previously (König & Eder, 2006). The mRNA concentration of genes was measured by real-time detection PCR using SYBR® Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). Real-time detection PCR was performed with 1·25 U Taq DNA polymerase, 500 μM-dNTP and 26·7 pmol of the specific primers. For determination of mRNA concentration a threshold cycle (C_t) and amplification efficiency was obtained from each amplification curve using the software RotorGene 4·6 (Corbett Research). Calculation of the relative mRNA concentration was made using the $\Delta\Delta C_t$ method as previously described (Pfaffl, 2001). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for normalisation. The PCR primers used for real-time RT–PCR were obtained from Operon (Köln, Germany) and Roth (Karlsruhe, Germany), respectively, and are listed in Table 1.

Enzyme assays

Superoxide dismutase (SOD) activity in liver cytosol was determined according to the method of Marklund & Marklund (1974) with pyrogallol as the substrate. One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50 %. The activity of glutathione peroxidase in liver cytosol was determined with *t*-butyl hydroperoxide as substrate according to the method of Paglia & Valentine (1967). One unit of glutathione peroxidase activity is defined as 1 μmol reduced β-nicotinamide adenine dinucleotide phosphate oxidised/min. The activity of glutathione *S*-transferase was determined using 1-chloro-2,4-dinitrobenzene as substrate as described by Habig *et al.* (1974). One unit of glutathione *S*-transferase is defined as one nmol substrate consumed/min. Catalase activity in liver homogenate was determined using H_2O_2 as substrate according to the method of Aebi (1986). One unit of catalase activity is defined as the amount consuming 1 mmol H_2O_2/min.

Determination of conjugated dienes, thiobarbituric acid-reactive substances and α-tocopherol

Lipids from liver were extracted using a mixture of *n*-hexane and isopropanol (3 : 2, v/v; Hara & Radin, 1978). After drying the lipid extracts, 1 mg extract was dissolved in 1 ml *n*-hexane. The concentrations of conjugated dienes were calculated by using the molar extinction coefficient for conjugated dienes at 234 nm ($\epsilon = 29\,500$ mol/cm). The concentrations of TBARS were measured in liver homogenates as described (Brandsch *et al.* 2002). The concentration of α-tocopherol in liver tissue was determined by HPLC (Brandsch *et al.* 2002).

Determination of H_2O_2

To determine the H_2O_2 content in liver homogenates, the method for cell culture systems described by Royall & Ischiropoulos (1993) was modified, using dihydrorhodamine 123 as substrate. Homogenates were incubated with 27·5 μM-dihydrorhodamine 123 for 1 h at 37°C in a final volume of 400 μl. After incubation, the fluorescence of rhodamine 123, the oxidation product of dihydrorhodamine 123, was measured (excitation wavelength 485 nm, emission wavelength 538 nm).

Table 1. Characteristics of the specific primers used for RT–PCR analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	bp	Annealing temp. (°C)	NCBI GenBank
ACC	CTCCAGGACAGCACAGATCA	GCCGAAACATCTCTGGGATA	170	60	AF175308
ACO	CTCGCAGACCCAGATGAAAT	TCCAAGCCTCGAAGATGAGT	218	60	AF185048
apo CIII	GACACCTCCCTTCTGGACAA	TCCCAGAAGTCGGTGAACTT	185	60	NM_001002801
CPT-1	GCATTTGTCCCATCTTTCGT	GCACTGGTCCTTCTGGGATA	198	60	AF288789
CYP7	TATAGGGCACGATGCACAGA	ACCTGACCAGTTCCGAGATG	200	60	NM_001005352
FAS	GAACACGGCCTAGAAGTGGA	ATCTGGATCCTGCAGATGG	199	62	NM_213839
FATP	GGTTCCAGCCTGTTGAATGT	AACAAAACCTTGGTGCTTGG	275	60	DQ192231
FDPS	GAAAGGCAGGATTTCATCCA	AGAAGGCTTGGAGCAGTTCA	259	60	AY609787
GAPDH	AGGGGCTCTCCAGAACATCATCC	TCGCGTGCTCTTGCTGGGGTTGG	446	60	AF017079
Glutathione peroxidase	CAAGAATGGGGAGATCCTGA	GATAAACTTGGGGTCGGTCA	190	60	NM_214201
Glutathione *S*-transferase	TTTTTGCCAACCCAGAAGAC	GGGGTGTCAAATACGCAATC	246	60	NM_214300
HMG-CoA-R	GGTCAGGATGCGGCACAGAACG	GCCCCACGGTCCCGATCTCTATG	127	65	S79678
I-FABP	TACAGCCTCGCAGACGGAACTG	TGCTTGATGAGGAGAGGAAAACAG	276	59	AY960624
Insig-1	AGAGGGAGTGGGCCAGTGTGATGC	ACGGGAGCCAGGAGCGGATGTAG	276	65	AY336601
Insig-2	AAATCACGCCAGCGCTAAAGTG	TCCTACTCCAAGGCCAAAACCAC	127	60	AY585269
LDL receptor	AGAACTGGCGGCTGAAGAGCATC	GAGGGGTAGGTGTAGCCGTCCTG	115	60	AF118147
L-FABP	TTCGGTGCATGTCTAAGCTG	TGAGAGGGAGAGGATGAGGA	200	60	DQ182323
mAAT	TATGTCACCGTGCAGACCAT	CTCCTTCCACTGCTCAGGAC	309	60	M11732
mHMG-CoA-S	GGACCAAACAGACCTGGAGA	ATGGTCTCAGTGCCCACTTC	198	62	U90884
MTP	CAGGACGGCAAAGAAAGAAGG	ATGGGAAGCAAAACCACAAGG	199	60	AY217034
NPC1	ACGCGGTATCTTTGGTCAAC	AGTGGCTCCCAGCAAGACTA	266	60	AF169635
NPC2	GGAGGGGAGGAGAAATCAAG	ATTCGGGTCTTGTCTGGTTG	267	60	NM_214206
PPARα	CAGCCTCCAGCCCCTCGTC	GCGGTCTCGGCATCTTCTAGG	382	58	DQ437887
SCD	ACGTTGTGCCAGTGAGTCAG	GTCTTGGCCTCTTGTGCTTC	206	62	NM_213781
SOD	TCCATGTCCATCAGTTTGGA	CTGCCCAAGTCATCTGGTTT	250	60	AF396674
SREBP-1	CCTCTGTCTCTCCTGCACC	ACAAAGAGAAGCGCCAAGAA	213	62	NM_214157
SREBP-2	CGCTCGCGAATCCTGCTGTG	GGTGCGGGTCCGTGTCGTG	103	65	DQ020476

ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; CPT-1, carnitine palmitoyltransferase-1; CYP7, cholesterol 7α-hydroxylase; FAS, fatty acid synthase; FATP, fatty acid transport protein; FDPS, farnesyl diphosphate synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; I-FABP, intestinal fatty acid binding protein; Insig, insulin-induced gene; L-FABP, liver fatty acid binding protein; mAAT, mitochondrial aspartate aminotransferase; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal TAG transfer protein; NPC, Niemann-Pick type C; SCD, stearoyl-CoA desaturase; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein.

As previously shown by Walrand *et al.* (2003), dihydrorhodamine 123 is specifically oxidised by H_2O_2.

Determination of 3-hydroxybutyrate

Concentration of 3-hydroxybutyrate in plasma was determined using an enzymatic assay (cat. no. 10907979035; R-Biopharm AG, Darmstadt, Germany).

Transmission electron microscopy

Liver tissues were fixed in 3 % sodium cacodylate-buffered glutaraldehyde (pH 7·2) and post-fixed with 1 % osmium tetroxide. After washing three times, probes were dehydrated in an ethanol series and embedded in Spurr's epoxy resin. For observations with an EM 900 transmission electron microscope (Carl Zeiss SMT, Oberkochen, Germany), ultrathin sections (80 nm) were mounted on copper grids. Catalase is known to be located in peroxisomes specifically and was marked for a better visualisation of peroxisomes. For immunohistochemistry, ultrathin sections were blocked for 30 min with 1 % bovine serum albumin and 0·1 % Tween in PBS and incubated overnight with sheep polyclonal anti-catalase serum (1 : 50; Biotrend, Köln, Germany). For detection of primary antibody, sections were incubated for 1 h with a gold-marked donkey–anti-sheep antibody (1 : 25; Biotrend) and finally stained with uranyl acetate/lead citrate. Peroxisomes were counted in 1000 different prints per liver sample for each animal with a magnification of 12 000×.

Statistics

The results were analysed using Minitab (State College, PA, USA) statistical software (release 13). Statistical significance of differences of the mean values of the two groups of pigs was evaluated using Student's *t* test. Mean values were considered significantly different for $P<0·05$.

Results

Fatty acid composition and concentration of lipid peroxidation products in the dietary fats

Palmitic, stearic, oleic and linoleic acid were the major fatty acids in the dietary fats. The sum of these fatty acids accounted for about 95 g/100 g total fatty acids in the fats (Table 2). Amounts of stearic, oleic and linoleic acid were nearly identical in both fats; the amount of stearic acid was slightly higher in the fresh fat than in the oxidised fat. Peroxide value, acid value and concentration of conjugated dienes were 4–5-fold higher in the oxidised than in the fresh fat included in the diet (Table 2). The concentration of total carbonyls was 10-fold higher and that of TBARS was 30-fold higher in the oxidised than in the fresh fat (Table 2).

Body weights, antioxidant status and concentrations of lipid peroxidation products in the liver

Body weights of the pigs at the end of the experiment on day 28 did not differ between the two groups (25·6 (SD 1·4) *v.* 26·0 (SD 1·5) kg in pigs fed the oxidised fat *v.* pigs fed the fresh fat; nine pigs per group). Pigs fed the oxidised fat had a higher mRNA concentration and a higher activity of SOD and a lower activity of microsomal glutathione *S*-transferase in the liver than pigs fed the fresh fat ($P<0·05$; Table 3). Activities of glutathione peroxidase and cytosolic glutathione *S*-transferase as well as mRNA concentrations of these enzymes in the liver did not differ between both groups of pigs (Table 3). Concentrations of total, reduced and oxidised glutathione in the liver also did not differ between the two groups of pigs whereas the concentration of α-tocopherol was lower in pigs fed the oxidised fat than in pigs fed the fresh fat ($P<0·05$; Table 3). Concentration of TBARS in the liver did not differ between the two groups of pigs whereas the concentration of conjugated dienes was slightly but significantly higher in pigs fed the oxidised fat than in pigs fed the fresh fat ($P<0·05$; Table 3).

Table 2. Major fatty acids and concentrations of some lipid peroxidation products in the fresh and the oxidised fat after inclusion into the diet

	Fresh fat	Oxidised fat
Major fatty acids (g/100 g fatty acids)		
16 : 0	9·0	6·7
18 : 0	4·1	4·2
18 : 1*n*-9	23·7	23·8
18 : 2*n*-6	59·8	59·9
Peroxidation products		
Conjugated dienes (mmol/kg)	22·7	89·1
TBARS (μmol/kg)	9	271
Peroxide value (mEq O_2/kg)	2·5	10·0
Acid value (g KOH/kg)	1·6	8·0
Total carbonyls (mmol/kg)	2·5	24·5

TBARS, thiobarbituric acid-reactive substances.

Indices of peroxisome proliferation

Liver weights of the pigs were not different between the two groups but pigs fed the oxidised fat had a higher peroxisome count and a higher activity of catalase in the liver than pigs fed the fresh fat ($P<0·05$; Table 4). Relative mRNA concentration of acyl-CoA oxidase (ACO), a peroxisomal enzyme, in the liver, was 34 % higher in pigs fed the oxidised fat than in control animals ($P=0·062$; Table 4). The concentration of H_2O_2 which is mainly released from peroxisomal oxidases was not different between the two groups of pigs (Table 4).

mRNA concentrations of genes in liver and intestine

In liver, mRNA concentrations of PPARα and genes involved in fatty acid transport and oxidation [liver fatty acid binding protein (L-FABP), carnitine palmitoyltransferase-1 (CPT-1)], fatty acid and cholesterol synthesis [SREBP-1 and -2, insulin-induced gene-1 and -2, fatty acid synthase, acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase (SCD), 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA-R)], cholesterol uptake (LDL receptor), bile acid synthesis [cholesterol 7α-hydroxylase (CYP7)], lipoprotein assembly and secretion [microsomal TAG transfer protein (MTP)], inhibition of lipoprotein lipase (apo CIII) and ketogenesis [mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA-S)]

Table 3. mRNA concentrations and activities of antioxidant enzymes and concentrations of antioxidants and lipid peroxidation products in livers of pigs fed a diet with a fresh fat or an oxidised fat

(Mean values and standard deviations)

	Fresh fat (*n* 9)		Oxidised fat (*n* 9)	
	Mean	SD	Mean	SD
Superoxide dismutase				
mRNA concentration (relative)	1·00	0·22	1·24*	0·14
Activity (U/mg protein)	42·7	8·4	58·8*	6·0
Glutathione *S*-transferase	202	42	144*	13
mRNA concentration (relative)	1·00	0·31	1·27	0·23
Activity in microsomes (U/mg protein)	202	42	144*	13
Activity in cytosol (U/mg protein)	760	262	761	186
Glutathione peroxidase				
mRNA concentration (relative)	1·00	0·13	1·14	0·22
Activity (U/mg protein)	4·72	0·77	5·14	0·75
Glutathione, total (nmol/mg)	2·13	0·51	2·17	0·37
Glutathione, reduced (nmol/mg)	1·70	0·50	1·84	0·55
Glutathione, oxidised (nmol/mg)	0·21	0·12	0·17	0·10
α-Tocopherol (nmol/g)	14·5	2·5	11·9*	1·8
Conjugated dienes (μmol/mg protein)	16	1	18*	3
TBARS (mmol/g)	7·2	1·6	7·3	2·8

TBARS, thiobarbituric acid-reactive substances.
Mean values were significantly different from those of the fresh fat group: *$P<0·05$.

were determined (Fig. 1). Pigs fed the oxidised fat had significantly higher mRNA concentrations of mHMG-CoA-S, a classical PPARα target gene, SREBP-1 and its target genes ACC and SCD, and SREBP-2 and its target genes HMG-CoA-R and LDL receptor than control pigs fed the fresh fat ($P<0·05$). mRNA concentration of CYP7 was lower in pigs fed the oxidised fat than in pigs fed the fresh fat ($P<0·05$). mRNA concentrations of CPT-1 and MTP, two other PPARα target genes, tended to be increased in pigs fed the oxidised fat ($P=0·074$ and $P=0·065$, respectively) compared to pigs fed the fresh fat whereas mRNA concentrations of PPARα, L-FABP, apo CIII, insulin-induced gene-1 and -2, and fatty acid synthase were not different between pigs fed the fresh fat and those fed the oxidised fat (Fig. 1).

In enterocytes, relative mRNA concentrations of PPARα and of proteins involved in fatty acid uptake (L-FABP, intestinal fatty acid binding protein, fatty acid transport protein, mitochondrial aspartate aminotransferase), fatty acid oxidation (ACO, CPT-1), intracellular trafficking of cholesterol (Niemann-Pick type C1 and 2) and fatty acid synthesis (SREBP-1, fatty acid synthase) were not different between pigs fed the oxidised fat and those fed the fresh fat (Fig. 2). However, mRNA concentration of SREBP-2 and its target genes HMG-CoA-R and LDL receptor, involved in cholesterol synthesis and uptake, were higher in pigs fed the oxidised fat than in pigs fed the fresh fat ($P<0·05$; Fig. 2). mRNA concentration of farnesyl diphosphate synthase did not differ between the two groups of pigs (Fig. 2).

Concentrations of TAG and cholesterol in liver, plasma and lipoproteins

Concentrations of TAG in liver, plasma and TAG-rich lipoproteins did not differ between pigs fed the fresh fat and those fed the oxidised fat. Concentrations in pigs fed the oxidised fat *v*. pigs fed the fresh were (nine pigs per group): liver, 88 (SD 20) *v*. 91 (SD 19) μmol/g; plasma, 0·96 (SD 0·26) *v*. 1·09 (SD 0·17) mmol/l; chylomicrons + VLDL, 0·80 (SD 0·25) *v*. 0·93 (SD 0·16) mmol/l. Concentrations of cholesterol in liver, plasma, LDL and HDL were also not different between the two groups of pigs. Concentrations in pigs fed the oxidised fat *v*. pigs fed the fresh were: liver, 73 (SD 14) *v*. 69 (SD 10) μmol/g; plasma, 2·63 (SD 0·32)

Table 4. Indices of peroxisome proliferation in livers of pigs fed a diet with a fresh fat or an oxidised fat

(Mean values and standard deviations)

	Fresh fat (*n* 9)		Oxidised fat (*n* 9)	
	Mean	SD	Mean	SD
Liver weight (g)	673	63	700	64
Peroxisome count (number/print)	366	67	515*	91
Acyl-CoA oxidase mRNA concentration (relative)	1·00	0·33	1·34†	0·37
Catalase (U/mg protein)	0·75	0·14	0·89*	0·13
H_2O_2 (fluorescence/g liver)	29 372	12 343	29 437	8361

Mean values were significantly different from those of the fresh fat group: †$P<0·1$; *$P<0·05$.

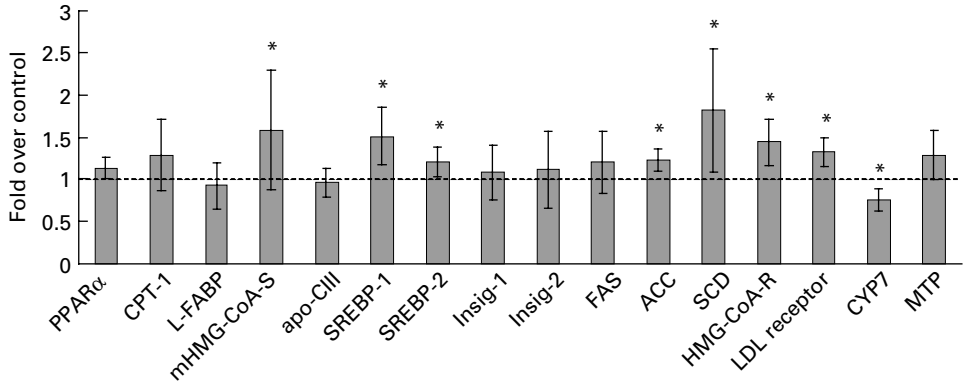

Fig. 1. Relative mRNA concentrations (--- represents 1·00) of various genes involved in hepatic lipid metabolism in enterocytes of pigs fed a diet with a fresh fat or an oxidised fat. Values were determined by real-time detection RT–PCR using the mRNA concentration of glyceraldehyde-3-phosphate dehydrogenase for normalisation Values are means with their standard deviations depicted by vertical bars (n 9) obtained for the pigs fed the oxidised fat relative to the values of the control group fed fresh fat. ACC, acetyl-CoA carboxylase; CPT-1, carnitine palmitoyltransferase-1; CYP7, cholesterol 7α-hydroxylase; FAS, fatty acid synthase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; Insig, insulin-induced gene; L-FABP, liver fatty acid binding protein; mHMG-CoA-S, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MTP, microsomal TAG transfer protein; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein. Mean values were significantly different from those of the fresh fat group: $^*P < 0{\cdot}05$.

v. 2·83 (SD 0·22) mmol/l; LDL, 0·96 (SD 0·16) *v*. 0·97 (SD 0·15) mmol/l; HDL, 1·02 (SD 0·18) *v*. 1·13 (SD 0·11) mmol/l.

Concentration of 3-hydroxybutyrate in plasma

Pigs fed the oxidised fat had a higher concentration of 3-hydroxybutyrate in plasma than pigs fed the fresh fat (1·23 (SD 0·58) *v*. 0·52 (SD 0·27) mmol/l; $P < 0{\cdot}05$).

Discussion

In the present study, pigs were fed a diet containing an oxidised fat prepared under usual deep-frying conditions. The relatively low concentrations of lipid peroxidation products (conjugated dienes, TBARS, peroxides and carbonyls) in the oxidised fat indicate that this fat was mildly oxidised. Concentrations of peroxidation products in this fat were indeed even lower than in soyabean oil or hydrogenated animal–vegetable oil blends used for frying of potatoes at 190°C over a period of 24 h (Frankel, 1998). The reason for the relatively low degree of oxidation is that we did not add foodstuffs to be fried during the preparation of the oil as we wanted to avoid contamination of the oil with food ingredients. It is well known that ingredients of foodstuffs, i.e. metal ions, enhance the lipid peroxidation process during frying of fats (Kubow, 1992). The concentration of conjugated dienes which include the potent PPARα activators hydroxy- and hydroperoxy fatty acids (Delerive *et al.* 2000; König & Eder, 2006) was approximately four times higher in the oxidised fat than in the fresh fat. The finding of an increased activity of SOD and a slightly elevated concentration of conjugated dienes, together with the observation of a slightly reduced concentration of α-tocopherol, indicates that the oxidised fat produced oxidative stress in the liver of the pigs. It has been demonstrated that under

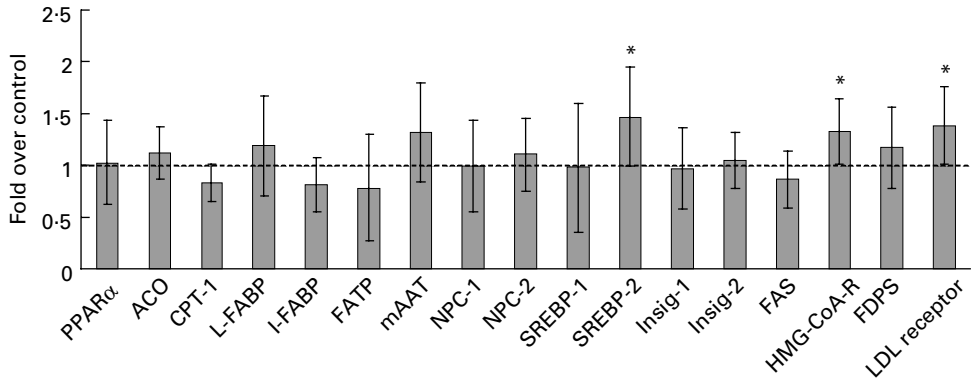

Fig. 2. Relative mRNA concentrations (--- represents 1·00) of various genes involved in intestinal lipid metabolism in livers of pigs fed a diet with a fresh fat or an oxidised fat. Values were determined by real-time detection RT–PCR using the mRNA concentration of glyceraldehyde-3-phosphate dehydrogenase for normalisation. Values are means with their standard deviations depicted by vertical bars (n 9) obtained for the pigs fed the oxidised fat relative to the values of the control group fed fresh fat. ACO, acyl-CoA oxidase; CPT-1, carnitine palmitoyltransferase-1; FAS, fatty acid synthase; FATP, fatty acid transport protein; FDPS, farnesyl diphosphate synthase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl-CoA reductase; I-FABP, intestinal fatty acid binding protein; Insig, insulin-induced gene; L-FABP, liver fatty acid binding protein; mAAT, mitochondrial aspartate aminotransferase; NPC, Niemann-Pick type C; SREBP, sterol regulatory element-binding protein. Mean values were significantly different from those of the fresh fat group: $^*P < 0{\cdot}05$.

oxidative stress, expression of SOD is stimulated and concentration of α-tocopherol is reduced due to an enhanced consumption (Liu & Huang, 1995; Ruiz-Gutierrez *et al.* 1999; Atalay *et al.* 2000). However, the oxidative stress produced by the oxidised fat was very moderate as concentrations of TBARS and glutathione remained completely unchanged. In rodents treated with PPARα agonists such as fibrates or WY-14,643, production of H_2O_2 is largely increased due to a strong up-regulation of peroxisomal oxidases, and this causes oxidative stress and contributes to hepatocarcinogenesis in these species (Peters *et al.* 2005). In the present study, feeding the oxidised fat did not increase the concentration of H_2O_2 in the liver. This was probably due to two reasons: first, there was only a slight increase in the mRNA concentration of ACO, one of the enzymes producing H_2O_2; second, activity of catalase, the key enzyme of decomposition of H_2O_2 in peroxisomes was increased. Therefore, generation of H_2O_2 did not contribute to oxidative stress in animals treated with oxidised fat. The reason for the moderate oxidative stress may be that a part of the dietary lipid peroxidation products is absorbed in the intestine and reaches the liver via lipoproteins (Staprans *et al.* 2005). Production of oxidative stress by intake of strongly oxidised fats has been shown several times in rodents (Yoshida & Kajimoto, 1989; Liu & Huang, 1996; Liu & Lee, 1998; Ammouche *et al.* 2002; Eder *et al.* 2004; Keller *et al.* 2004*a*, *b*). The present study shows for the first time that even a mildly oxidised fat, as used in human nutrition, can induce moderate oxidative stress in pigs as a non-proliferating species.

To find out whether the mildly oxidised fat caused activation of PPARα in the liver of pigs, we determined mRNA concentrations of the classical PPARα target genes ACO, CPT-1 and mHMG-CoA-S as well as peroxisome count, activity of catalase and plasma concentration of 3-hydroxybutyrate. Recent studies in pigs have shown that activation of PPARα in pigs, by either treatment with clofibrate or by fasting, leads to an increased expression of these PPARα target genes, and in turn stimulates mitochondrial and peroxisomal β-oxidation and ketogenesis (Yu *et al.* 2001; Peffer *et al.* 2005; Cheon *et al.* 2005). The finding of an increased peroxisome count together with increased activity of catalase, a peroxisomal enzyme, a significantly increased mRNA concentration of mHMG-CoA-S and an increased plasma concentration of 3-hydroxybutyrate strongly indicate that the oxidised fat caused PPARα activation in the liver of the pigs. The finding that mRNA concentrations of ACO and CPT-1, two other classical PPARα target genes were also increased by 34 and 29 %, although not significantly different to control, supports the assumption that the oxidised fat induced hepatic PPARα activation in the pigs. It has been shown that these two enzymes are only moderately up-regulated in pig liver by PPARα agonists. For instance, in pigs treated with clofibrate, a strong PPARα agonist, hepatic gene expression of CPT-1 and ACO was only 1·89- and 1·42-fold, respectively, increased over control while gene expression of mHMG-CoA-S was increased 3·32-fold (Cheon *et al.* 2005). This presents an explanation for the observations that mHMG-CoA-S was significantly increased in pigs treated with oxidised fat and that ACO and CPT-1 were only slightly increased. The finding that mRNA concentration of MTP, a gene recently shown to be up-regulated by PPARα activation (Ameen *et al.* 2005), tended to be increased in the liver of pigs fed the oxidised fat also indicates that the oxidised fat caused PPARα activation in the liver. Recently, studies in rats have already shown that oxidised fats are able to activate PPARα in the liver (Huang *et al.* 1988; Chao *et al.* 2001; Sülzle *et al.* 2004). In these rat studies, up-regulation of PPARα target genes in the liver was much stronger than in pigs of the present study. This may have two different reasons: first, most PPARα target genes respond stronger to PPARα activation in rats than in non-proliferating species such as pigs or man; second, fats used in the rat studies were more strongly oxidised than the mildly oxidised fat used in the present study. The present study shows for the first time that even a mildly oxidised fat causes activation of PPARα in pigs which are, as man, less sensitive to PPARα agonists than rodents.

To study whether the oxidised fat caused PPARα activation in small intestine, we considered in addition to the classical PPARα target genes ACO and CPT-1, several genes involved in fatty acid transport (L-FABP, intestinal fatty acid binding protein, fatty acid transport protein and mitochondrial aspartate aminotransferase) and cholesterol trafficking (Niemann-Pick type C1 and 2) in intestinal tissue. All these genes have been shown to be up-regulated by PPARα activation (Darimont *et al.* 1998; Motojima *et al.* 1998; Mochizuki *et al.* 2001; Chinetti-Gbaguidi *et al.* 2005). The finding that none of these genes was up-regulated in cells of small intestine indicates that oxidised fat caused no or even weak PPARα activation and does not influence intestinal fatty acid transport and cholesterol trafficking.

Synthesis of lipids in mammalian cells is controlled by a network involving the action of insulin-induced genes and SREBP, and it has been recently shown in several studies that this network is influenced by PPARα activation (Guo *et al.* 2001; Patel *et al.* 2001; Knight *et al.* 2005; König *et al.* 2006). The present study shows that feeding a mildly oxidised fat increased the mRNA concentration of SREBP-1 and its target genes ACC and SCD, two key enzymes of *de novo* fatty acid synthesis, in the liver. These alterations may be caused by activation of PPARα in the liver. Knight *et al.* (2005) found that treatment with WY 14,643, a synthetic PPARα agonist, causes a strong up-regulation of enzymes involved in hepatic fatty acid synthesis and stimulates fatty acid synthesis in wild-type mice but not in PPARα null mice. Knight *et al.* (2005) suggest that up-regulation of hepatic fatty acid synthesis is a compensatory response on the increased fatty acid oxidation to maintain a constant cellular TAG level. The finding that TAG levels in liver and plasma were not reduced in pigs fed the oxidised fat compared to control pigs indeed suggests that an increased β-oxidation of fatty acids was compensated by an increased fatty acid synthesis. As there is no evidence for a direct action of PPARα on the promoter regions of SREBP-1 and ACC genes, it is likely that the increased mRNA concentrations of these genes are an indirect result of PPARα activation. In contrast, SCD is not only dependent on SREBP-1 but has also a PPAR response element in its promoter (Miller & Ntambi, 1996). Therefore, its transcription may have been in part directly stimulated by PPARα activation. An up-regulation of SCD which catalyses the formation of MUFA from SFA has also been observed in pigs treated with clofibrate (Cheon *et al.* 2005). These findings of the effects of the oxidised fat on gene expression of lipogenic enzymes observed in pigs are opposite to those observed in rats in which a dietary oxidised fat causes a down-regulation of lipogenic enzymes and a strong reduction of liver and plasma TAG (Eder & Kirchgessner, 1998; Eder *et al.* 2003).

It is moreover shown that feeding the mildly oxidised fat led to a moderate but significant up-regulation of SREBP-2, and its target genes HMG-CoA-R and LDL receptor, in both liver and small intestine. The present findings suggest that the oxidised fat could have stimulated synthesis and uptake of cholesterol in these tissues. As this effect occurs not only in the liver but also in the small intestine where no PPARα activation was found in pigs fed the oxidised fat, it is questionable whether these effects are linked to PPARα activation. The finding that hepatic genes involved in cholesterol synthesis were not altered in pigs treated with clofibrate indeed suggests that PPARα activation does not influence SREBP-2 controlled transcription of genes involved in cholesterol homeostasis (Cheon *et al.* 2005). On the other hand, treatment with the PPARα agonist WY 14,643 caused an up-regulation of genes involved in hepatic cholesterol synthesis in wild-type mice but not in PPARα null mice, indicating that PPARα activation indeed could directly stimulate cholesterol synthesis (Knight *et al.* 2005). It should be noted, however, that there is also another study that found a suppression of gene expression and proteolytic activation of SREBP-2, and a strong down-regulation of its target genes accompanied by reduced cholesterol synthesis in rats (König *et al.* 2006). The effect of PPARα activation on SREBP-2-dependent cholesterol synthesis is not yet clear and may also be different between various species. Besides an up-regulation of genes involved in synthesis and uptake of cholesterol, we found a down-regulation of CYP7, the key enzyme of bile acid formation, in the liver. It has been shown in human and rat liver cells that PPARα agonists lower CYP7 expression probably by reducing the availability of hepatic nuclear factor 4α which is required for binding to a DR-1 in CYP7 promoter (Marrapodi & Chiang, 2000; Patel *et al.* 2000). Therefore, we assume that down-regulation of CYP7 in the liver of pigs fed the oxidised fat was caused by PPARα activation induced by the oxidised fat. Increased hepatic cholesterol synthesis and uptake of cholesterol into the liver, together with a decreased bile acid synthesis, is expected to increase hepatic cholesterol concentration. In contradiction to this, liver and plasma cholesterol concentrations were unchanged in pigs fed the oxidised fat compared to pigs fed the fresh fat. We assume that the changes in gene expression were too small to induce phenotypical alterations of cholesterol concentrations.

In conclusion, the present study shows that a mildly oxidised fat causes PPARα activation in the liver of pigs as indicated by an increased peroxisome count, a moderate up-regulation of PPARα target genes and a stimulation of ketogenesis. Moreover, the oxidised fat led to an up-regulation of the expression of SREBP-1 and SREBP-2 and their target genes involved in TAG and cholesterol synthesis, suggesting a stimulation of lipid synthesis. As the fat used in the present study was even less oxidised than fats used for deep-frying of foods, and as there exists a similarity in the gene response to PPARα agonists between pig and human liver cells, deep-fried fats could exert similar effects in man.

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Research Paper Effects of 13-HPODE on Expression of Genes Involved in Thyroid Hormone Synthesis, Iodide Uptake and Formation of Hydrogen Peroxide in Porcine Thyrocytes

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Abstract: It has been shown that dietary oxidized fats influence thyroid function in rats and pigs. Mechanisms underlying this phenomenon are unknown. This study was performed to investigate whether 13-hydroperoxy-9,11-octadecadienic acid (13-HPODE), a primary oxidation product of linoleic acid, affects expression of genes involved in thyroid hormone synthesis and formation of hydrogen peroxide in primary porcine thyrocytes. Thyrocytes were treated with 13-HPODE in concentrations between 20 and 100 μM. Cells treated with vehicle alone ("control cells") or with equivalent concentrations of linoleic acid were considered as controls. Treatment of cells with 13-HPODE did not affect cell viability but increased the activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase ($p < 0.05$) compared to control cells or cells treated with linoleic acid. Relative mRNA concentrations of genes involved in thyroid hormone synthesis like sodium iodide symporter, thyrotropin receptor, and thyroid peroxidase, as well as iodide uptake, did not differ between cells treated with 13-HPODE and control cells or cells treated with linoleic acid. Treatment of cells with 13-HPODE, however, reduced the relative mRNA concentrations of dual oxidase-2 and the formation of hydrogen peroxide compared to control cells or cells treated with linoleic acid ($p < 0.05$). Because the production of hydrogen peroxide is rate-limiting for the synthesis of thyroid hormones, it is suggested that 13-HPODE could have an impact on the formation of thyroid hormones in the thyroid gland.

Key words: 13-HPODE, thyrocytes, thyroid hormones, pig

Introduction

Oxidized fats are generated during processing and storage of foods and constitute an important portion of Western diets. Increased intake of oxidized fats has been linked to an enhanced incidence of coronary heart disease, endothelial dysfunction, and cancer in humans [1, 2]. Several studies examined the physiological effects of oxidized dietary fats in animal models [3–6]. In rats and pigs, increased concentrations of free and total thyroxine in plasma were observed after feeding a diet rich in oxidized fats [7, 8]. In rats, a dietary oxidized fat, moreover, led to alterations in the morphology of the thyroid gland and in the expression of genes involved in thyroid hormone synthesis. Rats fed oxidized fat exhibited an increased height of epithelial cells while the diameter of follicles was reduced; gene expression of sodium iodide symporter (NIS) was reduced and that of thyroid peroxidase (TPO) increased in the thyroid glands of these rats [9]. These alterations suggest that dietary oxidized fat affects the function of the thyroid gland, i.e. activities of proteins involved in the formation of thyroid hormones. The components responsible for the effects of oxidized fat and the mechanisms by which they influence thyroid function are unknown.

Oxidized fats include a mixture of primary and secondary lipid peroxidation products depending on their thermal treatment. Heating fats for a long period at low temperature without catalysts produces mainly primary lipid peroxidation products such as hydroxy or hydroperoxy fatty acids. Since primary lipoxy radicals are unstable, fats treated at high temperature or in the presence of catalysts contain predominantly secondary lipid peroxidation products such as carbonyls or dimeric, trimeric, polymeric, and cyclic fatty acids [10]. In our previous studies, feeding fats that had been treated at a low temperature caused a stronger increase of plasma thyroxine concentrations in rats than feeding a fat treated at a high temperature [7]. Therefore, we assume that primary lipid peroxidation products may be responsible for the effects of oxidized fat on thyroid hormone metabolism. A number of studies have shown that lipid oxidation products are readily absorbed, incorporated into chylomicrons as well as very-low (VLDL) and low-density lipoproteins (LDL), and are taken up in body cells [11–14]. 13-hydroperoxy-9,11-octadecadienic acid (13-HPODE) is the primary autoxidation product of linoleic acid [15]. It originates not only from the diet but is also formed in the body by radical-driven non-enzymatic processes and by the action of 15-lipoxygenase (15-LOX), an enzyme that occurs in many human tissues [16]. Lipid hydroperoxides such as 13-HPODE have a strong impact on the metabolism of cells. As a component of cells, oxidized fatty acids produce oxidative stress, exert cytotoxic effects [17], and, as natural ligands of the alpha and gamma peroxisome proliferator activated receptors (PPARα and PPARγ) they are able to influence lipid metabolism and cell differentiation in several ways [18, 19]. Recently, it has been shown that 13-HPODE activates the pro-inflammatory NF-κB pathway in vascular smooth muscle cells [20, 21]. As the quantitatively most important lipid oxidation product in oxidized LDL, 13-HPODE is also involved in the process of atherosclerosis [22]. However, potential effects of 13-HPODE in thyrocytes have not yet been studied.

The aim of this study was to investigate whether 13-HPODE affects the function of thyrocytes, i.e. metabolic steps involved in the formation of thyroid hormones. Iodide required for thyroid hormone synthesis enters the thyrocyte through an active process mediated by NIS located in the basal membrane [23]. TPO synthesizes thyroid hormone residues on thyroglobulin by successively catalyzing the iodination of tyrosyl residues and the coupling of iodotyrosine pairs into iodothyronines. These reactions take place on the outer side of the apical plasma membrane of thyrocytes in the presence of hydrogen peroxide as an electron acceptor [24]. The hydrogen peroxide-generating system which constitutes the rate-limiting step of thyroid hormone synthesis [25] is a Ca^{2+}-dependent NADPH oxidase. This enzyme contains two integral membrane flavoproteins called dual oxidases (DUOX) 1 and 2 [26]. Recent studies suggested that DUOX2 is the major generator of hydrogen peroxide in thyrocytes, while it has been questioned whether DUOX1 is involved in thyroid hormone generation [27]. The catalytic activity of NADPH oxidase is essentially triggered by the Ca^{2+}-phosphatidylinositol cascade [28]. All steps of thyroid hormone synthesis are stimulated by thyrotropin (TSH), which binds to the TSH-receptor and increases the expression of NIS, TPO, and DUOX genes through the cAMP or IP_3 pathway, respectively [28–30]. In this study we determined the effects of 13-HPODE on gene expression of NIS, TPO, DUOX2, and TSH-receptor, on iodide uptake, and on formation of hydrogen peroxide in primary porcine thyrocytes, used as a model of thyroid cells that expresses all important genes of thyroid hormone synthesis.

Oxidized fatty acids are able to cause oxidative stress in cells by producing superoxide anions, which may affect the cellular antioxidant system [31]. To study whether 13-HPODE affects the antioxidant status of thyrocytes, we determined the activities of the most important cellular antioxidant enzymes, namely superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase. The effects of 13-HPODE were compared with those of linoleic acid (LA) or vehicle alone.

Materials and Methods

Isolation and treatment of primary porcine thyrocytes

Thyroid glands from 10-week-old pigs used in training programs of the Faculty of Medicine were transported on ice and processed within one hour of death. Thyrocytes were isolated from glands as described previously [32]. Briefly, the glands were stripped of connective tissue and cut into ~2 mm pieces. These fragments were washed in Hank's buffered salt solution (HBSS) and sedimented at 100 × g for 2 minutes. The pellets were resuspended and incubated in HBSS containing 2% collagenase (Biochrom AG, Berlin, Germany) for 30 minutes at 37° C. The digest was dissociated by repeated pipetting, filtered through nylon mesh, and pelleted at 100 × g for 1 minute. The pellet was washed 6 times in Dulbecco's modified Eagle medium (DMEM) supplemented with gentamicin (0.5%). The final pellet was resuspended in DMEM supplemented with gentamicin (0.5%) and fetal calf serum (FCS) (10%), plated in cell culture vessels, and incubated at 37°C in an incubator with a humidified atmosphere and 5% CO_2 for 4 days without further passage. Medium was changed after 24 hours (complete medium) and 72 hours (FCS-free medium). Thyrocytes were incubated for 2 hours or 24 hours in FCS-free medium containing either 20, 40, or 100 µmoles 13-HPODE or LA or ethanol as vehicle alone (control cells).

Preparation of 13-HPODE

Stock solution of linoleic acid (Sigma-Aldrich GmbH, Taufkirchen, Germany) was prepared in absolute ethanol. The linoleic acid was oxidized to 13-HPODE with immobilized soybean lipoxygenase (100 U/mL, Biochrom AG, Berlin, Germany) at 37° C for 1 hour. The formation of 13-HPODE was monitored spectrophotometrically by scanning the absorption between 200 and 300 nm using phosphate buffered saline as a reference [33]. Under these conditions, the conversion into 13-HPODE is observed as an increase in absorbance at an optical density of 234 nm. Usually, more than 90% conversion of linoleic acid to 13-HPODE was achieved as determined by the molar extinction coefficient of conjugated dienes (E = 29,500 × mol^{-1} × cm^{-1}). 13-HPODE was extracted with n-hexane, dried under vacuum, and resuspended in ethanol. Concentration of 13-HPODE in the alcohol stock solution was checked by measuring the absorbance at 234 nm. Various dilutions of the stock solutions were used for the incubations.

Viability and iodide uptake

The viability of the cells was determined following treatment with either 13-HPODE or LA in a concentration range from 20 to 100 µM for 2 hours or 24 hours by MTT assay.

Iodide uptake was measured in cells seeded into 6 well plates. After treatment of the cells with 20 µmoles of either 13-HPODE or LA or vehicle alone for 24 hours the medium was removed and 2.0 mL of HBSS containing $Na^{125}I$ (0.3 µCi/mL) were added to each well. For estimation of specific uptake by NIS, sodium perchlorate was added to the incubation medium at a final concentration of 1 mM. Cells were incubated at 37° C for 5 or 30 minutes. The incubation was terminated by aspiration of the medium. After rinsing 3 times with ice-cold Hank's buffered salt solution (HBSS), cells were lysed with 1 mL Igepal-lysis buffer [50 mM Tris, 140 mM NaCl, 1.5 mM $MgSO_4 \times 7\ H_2O$, 0.5% (v/v) Igepal, pH 8.0] and scraped from each well for ^{125}I counting.

Determination of hydrogen peroxide

The generation of hydrogen peroxide was quantified by measuring the oxidation of dihydrorhodamine 123 (DHR) into the fluorescent product rhodamine 123 (excitation wavelength: 485 nm, emission wavelength: 538 nm) [34]. After treatment of cells seeded into 24-well plates, DHR solution was added either to the cells with the incubation medium for analysis of total hydrogen peroxide concentration or after exchange of the incubation medium by phosphate-buffered saline (PBS) for analysis of the intracellular hydrogen peroxide concentration. The final concentration of DHR was 27.5 µM. The amount of hydrogen peroxide secreted into the medium was calculated by subtracting the intracellular from the total concentration. It has been shown that DHR is specifically oxidized by hydrogen peroxide [34]. There was no interference of the determination of hydrogen peroxide with 13-HPODE added to the medium. Cell protein was assayed by the Bicinchoninic Acid (BCA) protein assay method. Values are given as fluorescence intensity per mg cell protein.

Measurement of activities of SOD, GSH-Px, and catalase

For measurement of enzyme activities after treatment, cells were dislodged from culture vessels by scraping. Cells were pelleted, resuspended in PBS, and disintegrated with ultrasound 2 times for 30 seconds each. SOD activity was determined according to the method of Marklund and Marklund [35] with pyrogallol as the substrate. The activity of GSH-Px was determined with t-butyl hy-

droperoxide at 25° C according to the method of Paglia and Valentine [36]. Catalase activity was determined at 25°C using hydrogen peroxide as substrate according to the method of Aebi [37]. All enzyme activities were related to the protein concentration of the analyzed cells.

Determination of conjugated dienes

Total lipids of cells seeded into 6-well plates were extracted with 2 mL hexane. The absorbance of the extract was measured at a wavelength of 234 nm and the concentration of dienes was related to the protein concentration of the analyzed cells. Concentration of conjugated dienes was calculated by using the molar extinction coefficient for conjugated dienes at 234 nm ($E = 29{,}500 \times mol^{-1} \times cm^{-1}$).

Isolation of RNA and semi-quantitative RT-PCR

Total RNA from thyrocytes was isolated by using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturers protocol. The relative mRNA quantities of NIS, TPO, TSH receptor, and DUOX2, related to the reference gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA, were determined by means of reverse-transcriptase polymerase chain reaction (RT-PCR). First strand cDNA synthesis was performed from 1.2 µg total RNA by reverse transcription using the RevertAid™ M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany) and oligo dT primers (Operon, Köln, Germany). cDNA was amplified in a 20 µL reaction containing 2 µL RT-mixture, 0.2 µL Biotherm™ DNA polymerase, 2 µL 10X PCR buffer, 0.4 µL DNA polymerization mix (all from Genecraft, Lüdinghausen, Germany), and gene-specific primers obtained from Carl Roth (Karlsruhe, Germany). The primer sequences used were as follows: 5'-AGT-CAT-CAG-CGG-CCC-CCT-CCT-C-3' (forward) and 5'-ACC-GAT-GCC-GTC-TGC-CGT-GTG-3' (reverse) for pig NIS; 5'-CTG-GGC-GCC-GTG-CTC-GTC-TG-3' (forward) and 5'-ACG-CGG-GTG-GCA-TCT-GAC-TCT-GAC-3' (reverse) for pig TPO; 5'-GCC-TGC-CCA-TGG-ACA-CTG-AGA-C-3' (forward) and 5'-CTG-ACC-CCG-GTA-TGC-CTG-AGC-3' (reverse) for pig TSH receptor; 5'-GAC-CCA-GCG-GCA-GTT-TGA-ATG-G-3' (forward) and 5'-AGG-GCC-GCA-GCT-GAA-CAC-TCC-3' (reverse) for pig DUOX2 and 5'-AGG-GGC-TCT-CCA-GAA-CAT-CAT-CC-3' (forward) and 5'-TCG-CGT-GCT-CTT-GCT-GGG-GTT-GG-3' (reverse) for pig GAPDH.

Statistical analysis

Means of the treatments (13-HPODE, LA) and control were compared by Student's *t*-test using the Minitab Statistical Software (Minitab, State College, PA, USA). Data were considered significantly different at $p < 0.05$.

Results

Viability and iodide uptake into thyroid cells

Viability of primary porcine thyrocytes was not influenced by treatment with LA or 13-HPODE up to concentrations of 100 µM. When examined under the light microscope, the cells appeared normal during the whole incubation period of 24 hours. Radioiodide was taken up by the cells in a time-dependent manner and this uptake was partially inhibited by perchlorate, indicating active and specific uptake of iodide via NIS. However, there was no difference in total iodide uptake and iodide uptake by NIS between control cells and cells treated with 20 µM of 13-HPODE or LA for 24 hours (Figure 1).

Concentration of hydrogen peroxide

Hydrogen peroxide was measurable in the cells as well as in the cell medium. Cells treated for 24 hours with 40 µmoles of 13-HPODE released less ($p < 0.05$) hydrogen

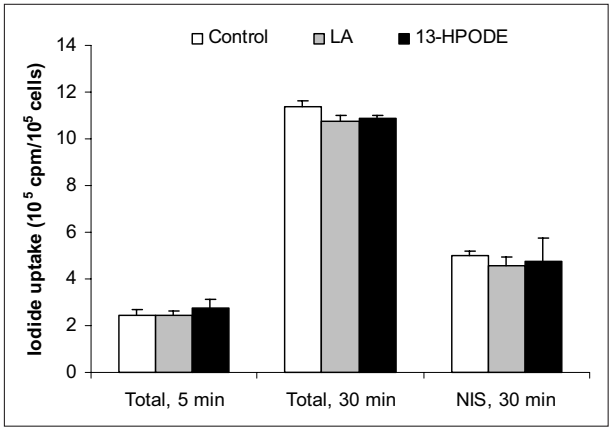

Figure 1: Effect of 13-HPODE on uptake of ^{125}I by primary porcine thyrocytes. Cells were treated with 20 µmoles of 13-HPODE or linoleic acid (LA) or with vehicle alone (control) for 24 hours. Thereafter, cells were incubated at 37° C for 5 or 30 minutes with $Na^{125}I$ alone (Total) or together with sodium perchlorate (1 mM) for determination of specific iodide uptake by sodium iodide symporter (NIS). Values are means ± SD (n = 2).

peroxide into the medium during the incubation than cells treated with 40 µmoles of LA or control cells [Fluorescence intensity (AU * 10^3)/mg protein: Control, 37.0 ± 11.5; cells treated with 40 µmoles of LA, 28.1 ± 7.0; cells treated with 40 µmoles of 13-HPODE, 20.0 ± 8.1, means ± SD, n = 8 for each treatment]. The intracellular concentration of hydrogen peroxide tended ($p < 0.15$) also to be lower in cells treated with 13-HPODE than in cells treated with LA or in control cells [Fluorescence intensity (AU * 10^3)/mg protein: Control, 4.6 ± 1.3; cells treated with 40 µmoles of LA, 4.4 ± 0.9; cells treated with 40 µmoles of 13-HPODE, 3.9 ± 1.2, means ± SD, n = 8 for each treatment].

Activities of antioxidant enzymes and concentrations of conjugated dienes

Cells treated for 24 hours with 40 µmoles of 13-HPODE had higher activities of SOD and GSH-Px than cells treated with 40 µmoles of LA or control cells ($p < 0.05$, Figure 2). The activity of catalase did not differ between cells treated for 24 hours with 40 µmoles of LA or 13-HPODE and control cells (Figure 2). The concentration of conjugated dienes was measured in cells treated with 40 µmoles of LA and in cells treated with 40 µmoles of 13-HPODE, for 24 hours. It was significantly higher in cells treated with 13-HPODE than in cells treated with LA (11.1 ± 0.8 vs. 2.6 ± 0.8 µmol/mg protein, n = 3 for each treatment, $p < 0.05$).

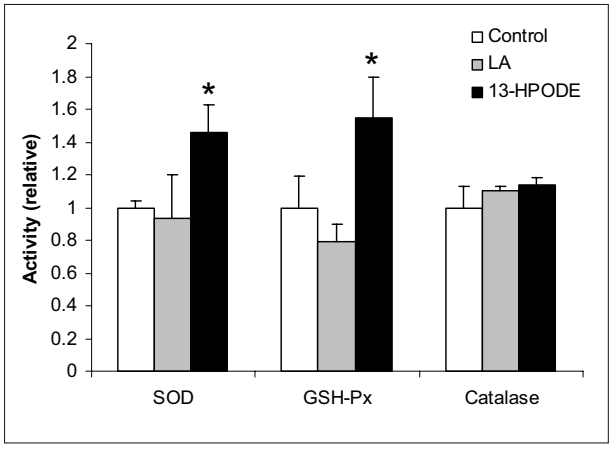

Figure 2: Effect of 13-HPODE on activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase in primary porcine thyrocytes. Cells were treated with 40 µmoles of 13-HPODE or linoleic acid (LA) or with vehicle alone (control) for 24 hours. Enzyme activities in the cells were measured by spectrophotometric assays. Activities were related to the values of the control group. Values are means ± SD (n = 3). *Significantly different from control cells, $p < 0.05$.

Relative mRNA levels of proteins involved in thyroid hormone synthesis

Treatments were done with 100 µmoles of LA or 13-HPODE and with vehicle alone (control cells) for 2 and 24 hours. Treatment with both LA or 13-HPODE did not change the relative mRNA level of NIS, TPO, and TSH receptor relative to control cells treated with vehicle alone (Figure 3). Relative mRNA level of DUOX2 was not changed by treatment with LA or 13-HPODE for 2 hours (Figure 3). However, cells treated for 24 hours with 100 µmoles of 13-HPODE lowered the relative mRNA level of DUOX2 compared with cells treated with 100 µmoles of LA or control cells ($p < 0.05$, Figure 3).

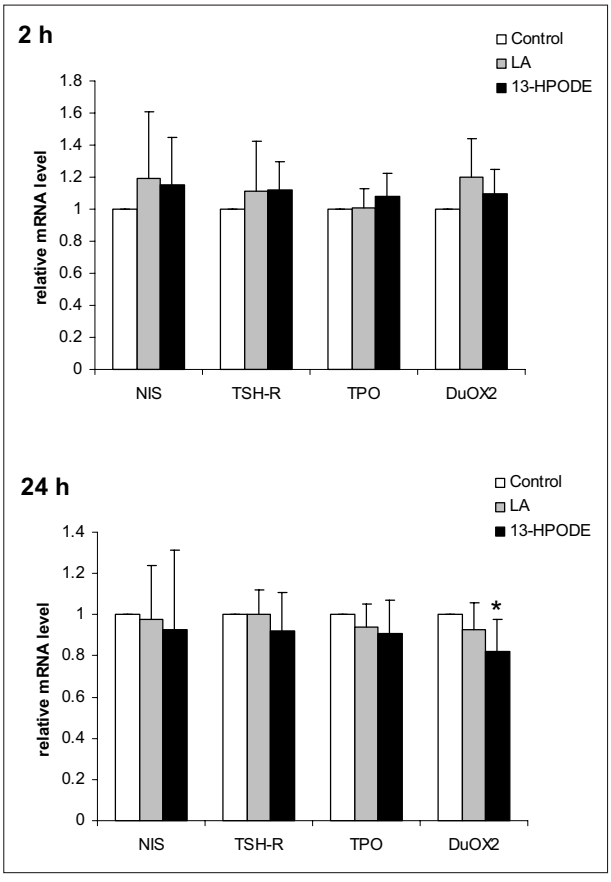

Figure 3: Effect of 13-HPODE on relative mRNA concentrations of sodium iodide symporter (NIS), thyrotropin receptor (TSH-R), thyroid peroxidase (TPO), and dual oxidase-2 (DUOX2) in primary porcine thyrocytes. Cells were treated either with 100 µmoles of 13-HPODE or linoleic acid (LA) or with vehicle alone (control) for 2 hours (upper panel), or for 24 hours (lower panel). Relative mRNA concentrations were determined by RT-PCR using GAPDH mRNA for normalization. Concentrations were related to the values of the control group. Values are means ± SD (n = 4). *Significantly different from control cells, $p < 0.05$.

Discussion

Previous studies have shown that dietary oxidized fats influence the thyroid function in rats and pigs [6–9]. In this study we treated porcine thyrocytes with 13-HPODE, an oxidation product of LA. We are aware of the fact that oxidized fats contain many products which are formed during the initial stage of lipid peroxidation such as hydroxyl, keto, and epoxy linoleic acids as well as end-products such as aldehydes, which may have complex effects on thyrocyte metabolism [10]. Therefore, 13-HPODE alone surely cannot resemble the effects of an oxidized fat. Nevertheless, 13-HPODE is widely used to study effects of oxidized fatty acids on function of various cell types as it is quantitatively the most important primary oxidation product of LA [19–22, 38–42]. Therefore, treatment with 13-HPODE should provide preliminary data on whether oxidized fatty acids could influence the function of thyrocytes.

In most of the studies dealing with the effects of 13-HPODE on cellular function, limited concentrations of 5 to 50 μmol/L were used, as higher concentrations could be cytotoxic [38, 39, 42]. According to this caveat, in the initial experiments dealing with the effects of 13-HPODE on iodide uptake, hydrogen peroxide production, and activities of antioxidant enzymes, we incubated cells with 20 or 40 μmol/L. As the MTT test showed that cell function is not affected even by 100 μmol/L, in the experiments investigating the expression of genes involved in thyroid hormone synthesis, we used concentrations of 100 μmol/L, which can be considered as very high relative to those commonly used in other studies. To date there is less information about the uptake and metabolism of 13-HPODE in thyrocytes. It has been shown that oxidized fatty acids are generally poorly taken up by cells compared to unoxidized free fatty acids [38]. The mechanism of uptake of oxidized fatty acids is different from unoxidized fatty acids and may not involve cellular expression of CD 36. Uptake of oxidized fatty acids depends on specific cell type, and is not known for thyrocytes [38]. *In vivo*, a large portion of oxidized fatty acids may enter the cell as a component of oxidized LDL [22]. Nevertheless, in most studies cells are treated directly with 13-HPODE rather than with oxidized LDL because the application of complex lipid systems could exert some effects on cell function that are not caused by oxidized fatty acids but by other lipid components. Within the cell, oxidized fatty acids are poorly used by microsomal acyltransferases and are therefore incorporated less efficiently into cell lipids [38]. Most of the biological effects of 13-HPODE and other oxidized fatty acids are probably caused by interaction with cell-surface components [38]. In Caco-2 cells, 13-HPODE is almost completely reduced by GSH-Px to 13-HODE [43]. The fate of 13-HPODE administered to thyrocytes has not yet been investigated. Therefore, the possibility exists that in thyrocytes a large part of 13-HPODE was also reduced to 13-HODE by the action of GSH-Px.

The current study reveals that incubation of porcine thyrocytes with 13-HPODE increases the activities of SOD and GSH-Px, and we assume that the increased activities of these antioxidant enzymes may be the consequence of oxidative stress caused by 13-HPODE. It has been shown that 13-HPODE induces oxidative stress by stimulating the generation of superoxide radicals or hydrogen peroxide [18, 44], and that oxidative stress stimulates gene expression and activities of SOD and GSH-Px in order to protect the cells against reactive oxygen species [45–48]. Concentrations of conjugated dienes are considered as a marker of lipid peroxidation [33]. Measurement of the absorbance at 234 nm shows that cells treated with 13-HPODE had much higher concentrations of conjugated dienes than cells treated with LA, indicative of increased absolute concentrations of oxidized fatty acids. Increased concentrations of conjugated fatty acids can occur for two different reasons. First, 13-HPODE and its decomposition product 13-HODE themselves have conjugated diene structures, and their uptake into the cell contributes to increased cellular concentrations of dienes. Second, the 13-HPODE that enters the cell could further propagate oxidation of membrane-bound fatty acids that contribute to increased levels of conjugated dienes. In this respect, it is also likely that oxidized fatty acids from arachidonic acid, such as hydroxyeicosatetraenoic acids (HETEs) or hydroperoxytetraenoic acids (HPETEs), are formed. These fatty acids have been reported to exert many biologic effects in various cell types. For example, they act chemotactically and cytotoxically, induce cell proliferation, and activate various signal transduction pathways in various cell types [49–51]. Although no information about the effects of HETEs and HPETEs on thyrocyte function is available, it is likely that these fatty acids could contribute to the effects observed in cells treated with 13-HPODE. It is well known that peroxidation of lipids can be prevented by antioxidants [33]. To assess whether oxidation products of polyunsaturated fatty acids (PUFA) formed during incubation are involved in the effects of 13-HPODE, further studies should consider the interaction between 13-HPODE and the supply of cells with antioxidants.

The present study shows that incubation of porcine thyrocytes with 13-HPODE does not lead to alterations in gene expression of NIS and TPO or iodide uptake, even in nonphysiologic high concentrations of 100 μM over a relatively long period of 24 hours. Gene expression of NIS and TPO is primarily regulated by TSH. Binding of TSH to the TSH receptor leads to the release of cAMP, which in turn enhances gene expression of NIS and TPO [29, 30].

The observation that gene expression of the TSH receptor was also not influenced by 13-HPODE suggests that it also did not influence the effect of TSH on the function of porcine thyrocytes. Gene expression of NIS, however, is not only regulated by TSH through the cAMP pathway but also by tumor necrosis factor-alpha (TNFα), a cytokine whose release is stimulated by activation of NF-κB [52, 53]. Recently, it has been shown that 13-HPODE leads to an activation of NF-κB in vascular smooth muscle cells [20, 21]. It has been observed that NF-κB activity is also present in thyrocytes [54, 55]. The results of the present study indirectly suggest that 13-HPODE did not activate NF-κB in porcine thyrocytes, because an activation of this nuclear factor would have led to a down-regulation of NIS expression.

The present study shows that high concentrations of 13-HPODE lead to a down-regulation of the DUOX2 gene and to a reduced release of hydrogen peroxide. We did not measure the activity of NADPH oxidase but we assume that a reduced gene expression of DUOX2 might have been associated with a reduced activity of this enzyme. The mechanism by which high concentrations of 13-HPODE reduce gene expression of DUOX2 remains to be elucidated.

The reduced concentrations of hydrogen peroxide in the cells and in the cell medium could be due to the increased activity of GSH-Px observed in cells treated with 13-HPODE. GSH-Px protects thyrocytes against a high intracellular concentration of hydrogen peroxide, which for instance can lead to apoptosis [56]. It has been postulated that GSH-Px in thyrocytes acts as a regulator of thyroid hormone biosynthesis by controlling the concentration of hydrogen peroxide available for thyroid hormone synthesis [57].

As the concentration of hydrogen peroxide in thyrocytes is the rate-limiting factor of thyroid hormone synthesis [25], it can be suggested that high concentrations of 13-HPODE could lead to a reduced formation of thyroid hormones. Recently, it has been shown that generation of reactive oxygen species inhibit the formation of thyroid hormones in cultured thyroid cells [58]. It cannot be excluded that the effects observed on DUOX2 expression and release of hydrogen peroxide by 13-HPODE are due, at least in part, to reactive oxygen species produced or to lipid oxidation products formed during incubation in the cell. It is possible that a reduction of the release of hydrogen peroxide could lead to a reduced formation of thyroid hormones in thyrocytes. Whether 13-HPODE has an effect on the release of hydrogen peroxide in the thyroid and the formation of thyroid hormones *in vivo* remains to be elucidated.

Previously, we have observed that feeding a dietary oxidized fat leads to a reduced gene expression of NIS and an increased gene expression of TPO in the thyroid gland and an increased concentration of thyroxine in the blood of rats [7, 9]. The present study reveals that these effects probably are not caused by 13-HPODE, the quantitatively most important oxidation product of LA, or by other secondary lipid oxidation products that are formed in the cell during incubation with 13-HPODE.

In conclusion, this study shows that incubation of porcine thyrocytes with 13-HPODE does not change gene expression of NIS, TPO, and TSH receptor, or iodide uptake into the cell. High concentrations of 13-HPODE, however, reduced gene expression of DUOX2 and production of hydrogen peroxide. Because the production of hydrogen peroxide is rate-limiting for the synthesis of thyroid hormones, it cannot be excluded that oxidized fatty acids could have an impact on the formation of thyroid hormones in the thyroid gland.

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PPARα agonists up-regulate organic cation transporters in rat liver cells

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Abstract

It has been shown that clofibrate treatment increases the carnitine concentration in the liver of rats. However, the molecular mechanism is still unknown. In this study, we observed for the first time that treatment of rats with the peroxisome proliferator activated receptor (PPAR)-α agonist clofibrate increases hepatic mRNA concentrations of organic cation transporters (OCTNs)-1 and -2 which act as transporters of carnitine into the cell. In rat hepatoma (Fao) cells, treatment with WY-14,643 also increased the mRNA concentration of OCTN-2. mRNA concentrations of enzymes involved in carnitine biosynthesis were not altered by treatment with the PPARα agonists in livers of rats and in Fao cells. We conclude that PPARα agonists increase carnitine concentrations in livers of rats and cells by an increased uptake of carnitine into the cell but not by an increased carnitine biosynthesis.

Keywords: Carnitine; Peroxisome proliferator activated receptor α; Rat; Organic cation transporter

Carnitine (L-3-hydroxy-4-*N*-*N*-*N*-trimethylaminobutyrate) is an essential metabolite, which has a number of indispensable functions in intermediary metabolism. The most prominent function lies in its role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix where β-oxidation takes place. Other functions of carnitine include the transfer of products of peroxisomal β-oxidation to the mitochondria for oxidation in the citrate cycle, the modulation of the acyl-CoA/CoA-ratio, and the storage of energy as acetylcarnitine [1–3].

All tissues that use fatty acids as a fuel source require carnitine for normal function. Carnitine is derived from dietary sources and endogenous biosynthesis [4]. Carnitine biosynthesis involves a complex series of reactions involving several tissues [5]. Lysine provides the carbon backbone of carnitine. Lysine in protein peptide linkages undergoes methylation of the ε-amino group to yield trimethyllysine (TML), which is released upon protein degradation. Muscle is the major source of TML. The released TML is further oxidized to butyrobetaine by the action of trimethyllysine dioxygenase (TMLD), 3-hydroxy-*N*-TML aldolase, and 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH). Butyrobetaine is hydroxylated by γ-butyrobetaine dioxygenase (BBD) to form carnitine. The last reaction which is rate-limiting for carnitine synthesis occurs primarily in liver and kidney [6].

Distribution within the body and intracellular homeostasis of carnitine are controlled by membrane transporters. The organic cation transporters (OCTNs), in particular OCTN-2, physiologically the most important one, operate on the intestinal absorption and renal reabsorption of carnitine and play a major role in tissue distribution by catalyzing the uptake of carnitine into body cells. In most tissues, carnitine concentrations are much higher than in plasma, and the high tissue-to-plasma concentrations (up to about 100:1 in muscle) are maintained by carnitine transporters [7]. The fact that inborn or acquired defects of OCTNs lead to primary or secondary systemic carnitine deficiency demonstrates their essential role in carnitine homeostasis [8].

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It has been shown that treatment of rats with clofibrate increases the concentration of carnitine in the liver [9], and it was suggested that this effect is caused by increased hepatic carnitine synthesis due to an increased availability of TML from muscle [10]. Clofibrate belongs to a group of hypolipidemic compounds which exert their effects by activation of peroxisome proliferator activated receptor (PPAR)-α, a transcription factor belonging to the nuclear hormone receptor superfamily [11]. Activation of PPARα causes an up-regulation of carnitine palmitoyltransferase (CPT)-1 and CPT-2 in the liver to enhance β-oxidation of fatty acids [12,13]. Since carnitine is a cofactor of these enzymes, activation of PPARα should increase the need for carnitine which could be met either by an increased de novo carnitine synthesis in the liver or by an increased uptake of carnitine from blood into the liver by membrane carnitine transporters, OCTN-1 and OCTN-2 [14]. The possibility that the clofibrate induced increase in hepatic carnitine concentration could have been mediated by an activation of PPARα has not yet been investigated. We hypothesized that activation of PPARα causes either up-regulation of enzymes involved in hepatic carnitine biosynthesis or increases carnitine uptake into liver cells by an up-regulation of OCTNs. The hypothesis that activation of PPARα could be involved in clofibrate induced increase of hepatic carnitine concentration is supported by the finding that hepatic carnitine concentration is also increased during starvation [15,16], a state in which PPARα is activated by increased hepatic concentrations of unesterified fatty acids [17]. In order to investigate this hypothesis, we performed experiments with rats and Fao rat hepatoma cells. In the first experiment, we treated rats with the synthetic PPARα agonist clofibrate and determined hepatic mRNA concentrations of the carnitine transporters OCTN-1 and -2, and enzymes involved in carnitine biosynthesis (TMLD, TMABA-DH, BBD). In order to explore whether effects of PPARα agonists on carnitine concentration are dependent on other tissues (e.g., muscle which provides TML for hepatic carnitine biosynthesis) or not, the second experiment was performed with rat hepatoma Fao cells which were treated with WY-14,643, another synthetic PPARα agonist.

Materials and methods

Animal experiment. Male Sprague–Dawley rats, with an average initial body weight of 366 g (±28; SD), were randomly assigned to two groups ($n = 8$) and kept individually in Macrolon cages in a room controlled for temperature (22 ± 2 °C), relative humidity (50–60%), and light (12 h light/dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. The animals were treated with 250 mg/kg of clofibrate (Fluka Chemie GmbH, Buchs, Switzerland) in 1 mL sunflower oil or with an equal volume of the vehicle sunflower oil by gavage once a day 2 h after beginning of the light cycle. All rats were fed a commercial standard basal diet (''altromin 1324'', Altromin GmbH, Lage, Germany). To standardize food intake, the diets were fed daily in restricted amounts of 18 g per day. Water was available *ad libitum* from nipple drinkers during the whole experiment. At day 4 of treatment, animals received the last dose of clofibrate or vehicle alone and 9 g of the diet and were killed 4 h later by decapitation under light anesthesia with diethyl ether. Blood was collected into heparinized polyethylene tubes. Liver and gastrocnemius muscles were quickly removed, frozen with liquid nitrogen, and stored at −80 °C pending further analysis. Plasma was obtained by centrifugation of the blood (1100*g*, 10 min, 4 °C) and stored at −20 °C.

Cell culture experiment. Fao rat hepatoma cells (ECACC, Salisbury, UK) were cultured in Ham-F12 medium supplemented with 10% FCS and 0.05 mg/mL gentamycin (Invitrogen, Karlsruhe, Germany). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, Fao cells were seeded in 6-well culture plates at a density of 1.06×10^6 cells per well and used prior reaching confluence (usually 3 days after seeding). The cells were then stimulated for 6 and 20 h with 50 μM WY-14,643 (Sigma–Aldrich, Steinheim, Germany). WY-14,643 was added to the medium from a stock solution in DMSO. Final DMSO concentration did not exceed 0.1% (v/v). Cells treated with the appropriate vehicle concentration were used as a control. Cell viability after treatment with WY-14,643 was assessed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MTT) [18]. Cell viability of Fao cells was not reduced by treatment with WY-14,643 (data not shown).

Carnitine analysis. Concentrations of free carnitine and acetyl carnitine in rat liver, muscle, plasma, and Fao cells were analyzed using tandem mass spectrometry according to Vernez et al. [19]. Quantitative analysis was achieved by use of stable isotope-labeled internal standard carnitine-d_3 (Larodan Fine Chemicals, Malmö, Sweden). Plasma or cells (50 μL) were added with methanol containing the internal standard; freeze-dried liver and muscle tissues (50 mg) were added with a water:methanol mixture (2:1, v/v) containing the internal standard. For extraction, the specimen was first sonified in an ultrasound bath for 20 min, then shaken in a water bath for 30 min at a temperature of 50 °C and finally centrifuged at 13,000*g* for 10 min at 4 °C. The supernatant was used for further analysis. A 1100-er series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a Kromasil 100 column (5 μm particle size, 125 mm length, 2 mm internal diameter, CS-Chromatographie Service, Langerwehe, Germany) and an API 2000 LC-MS/MS-System (Applied Biosystems, Darmstadt, Germany) were used for quantification of free carnitine and acetyl carnitine. For detection, the analytes were ionized by positive ion (5500 V) electrospray. As eluents, methanol and a methanol:water:acetonitrile mixture (50:45:5) were used.

RT-PCR analysis. Total RNA was isolated from Fao cells and rat livers, respectively, by TRIZOL™ reagent (Sigma–Aldrich, Steinheim, Germany) according to the manufacturer's protocol. cDNA synthesis was carried out as described [20]. The mRNA concentration of genes was measured by realtime detection PCR using SYBR® Green I and a MJ Research Opticon system (Biozym Diagnostik GmbH, Oldendorf, Germany). Realtime detection PCR was performed with 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 1). Annealing temperature for all primers was 60 °C. Amplification efficiencies for all primer pairs were determined by template dilution series. Calculation of the relative mRNA concentration was made using the amplification efficiencies and the C_t values [21]. The housekeeping gene β-actin was used for normalization.

Statistical analysis. Means of treatments and control were compared by Student's t test using the Minitab Statistical Software (Minitab, State College, PA, USA). Differences with $P < 0.05$ were considered to be significant.

Results

Carnitine concentrations in rat liver, plasma, and muscle and in Fao cells

Rats treated with clofibrate had a higher concentration of free carnitine in the liver than control rats ($P < 0.05$,

Table 1
Characteristics of the specific primers used for RT-PCR analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	bp	NCBI GenBank
β-Actin	ATCGTGCGTGACATTAAAGAGAAG	GGACAGTGAGGCCAGGATACAG	429	BC063166
CPT-1	GGAGACAGACACCATCCAACATA	AGGTGATGGACTTGTCAAACC	416	NM_031559
CPT-2	TCCTCGATCAAGATGGGAAC	GATCCTTCATCGGGAAGTCA	237	NM_012930
OCTN-1	AGCATTTGTCCTGGGAACAG	ACTCAGGGATGAACCACCAG	200	NM_022270
OCTN-2	CCTCTCTGGCCTGATTGAAG	CTCCGCTGTGAAGACGTACA	226	NM_012930
TMLD	GCCCTGTGGCATTCAAGTAT	GGTCCAACCCCTATCATGTG	201	AF374406
TMABA-DH	TTTGAGACTGAAGCCGAGGT	CACCGGGCTGACGTTATAGT	156	NM_022273
BBD	ATTCTGCAAAAGCTCGGAAA	CTCCTTGGAGTCCTGCTCTG	183	NM_022629

Table 2
Concentrations of total carnitine in liver, plasma, and gastrocnemius muscle of control rats and rats treated with clofibrate

	Control	Clofibrate
Liver		
Free carnitine (nmol/g)	282 ± 39	900 ± 145*
Acetyl carnitine (nmol/g)	12 ± 6	3 ± 1*
Plasma		
Free carnitine (μmol/L)	55 ± 8	28 ± 4*
Acetyl carnitine (μmol/L)	20 ± 4	8 ± 2*
Gastrocnemius		
Free carnitine (nmol/g)	631 ± 71	637 ± 63
Acetyl carnitine (nmol/g)	208 ± 52	144 ± 36*

Values are means ± SD ($n = 8$). An asterisk (*) indicates a significant difference from control rats ($P < 0.05$).

Table 2). The concentration of acetyl carnitine which made up however only a very small percentage of total carnitine was lower in rats treated with clofibrate than in control rats ($P < 0.05$, Table 2). Concentrations of free and acetyl carnitine in plasma were both lower in rats treated with clofibrate than in control rats ($P < 0.05$, Table 2). In gastrocnemius muscle, concentrations of free carnitine did not differ between both groups of rats whereas the concentration of acetyl carnitine was lower in rats treated with clofibrate than in control rats ($P < 0.05$, Table 2).

Fao cells treated with 50 μM WY-14,643 for either 6 or 20 h had higher concentrations of free carnitine than control cells treated with medium alone ($P < 0.05$, Table 3). Moreover, cells treated with WY-14,643 for 20 h had a higher concentration of acetyl carnitine than control cells ($P < 0.05$, Table 3). After 6 h incubation, the concentration of acetyl carnitine did not differ between cells treated with WY-14,643 and control cells (Table 3).

Table 3
Concentrations of free carnitine and acetyl carnitine in Fao cells treated either with vehicle alone (control) or with WY-14,643 for 6 or 20 h

	Control	WY-14,643
Six hours incubation		
Free carnitine (pmol/mg protein)	23 ± 8	33 ± 7*
Acetyl carnitine (pmol/mg protein)	87 ± 20	81 ± 17
Twenty hours incubation		
Free carnitine (pmol/mg protein)	40 ± 7	63 ± 25*
Acetyl carnitine (pmol/mg protein)	78 ± 20	97 ± 30*

Values are means ± SD ($n = 3$). An asterisk (*) indicates a significant difference from control cells ($P < 0.05$).

Relative mRNA concentrations of CPTs, OCTNs, and enzymes involved in carnitine biosynthesis in rat liver and Fao cells

Rats treated with clofibrate had higher relative mRNA concentrations of CPT-1, CPT-2, OCTN-1, and OCTN-2 in the liver than control rats ($P < 0.05$) whereas relative mRNA concentrations of genes encoding enzymes of hepatic carnitine synthesis (TMLD, TMABA-DH, BBD) did not differ between both groups of rats (Fig. 1).

Fao cells treated with 50 μM WY-14,643 for either 6 or 20 h had also higher relative mRNA concentrations of CPT-1, CPT-2, and OCTN-2 than control cells ($P < 0.05$, Fig. 2). The extent of up-regulation of expression of these genes by WY-14,643 was similar in cells treated for 6 h and those treated for 20 h, indicating that a period of 6 h was already sufficient for maximum up-regulation. Relative mRNA concentrations of OCTN-1 and of genes encoding enzymes of hepatic carnitine synthesis (TMLD, TMABA-DH, BBD) did not differ

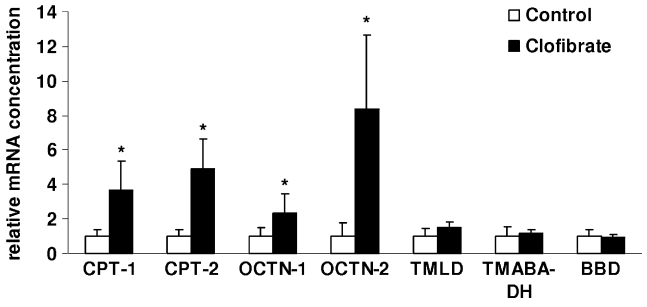

Fig. 1. Effect of clofibrate on the relative mRNA concentrations of carnitine palmitoyltransferases (CPT-1, CPT-2), organic cation transporters (OCTN-1, OCTN-2), and enzymes of carnitine biosynthesis (trimethyllysine dioxygenase, TMLD; 4-*N*-trimethylaminobutyraldehyde dehydrogenase, TMABA-DH; γ-butyrobetaine dioxygenase, BBD) in the liver of rats. Rats were treated orally with 250 mg/kg of clofibrate for 4 days. Control animals obtained the appropriate volume of the vehicle sunflower oil. Total RNA was extracted from rat livers and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalization. Values are means ± SD ($n = 8$). An asterisk (*) indicates a significant difference from control rats ($P < 0.05$).

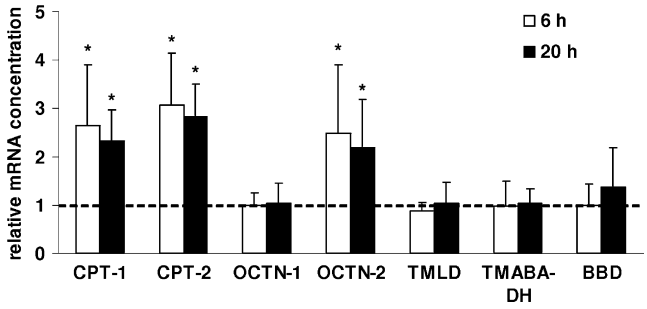

Fig. 2. Effect of WY-14,643 treatment for either 6 or 20 h on the relative mRNA concentrations of carnitine palmitoyltransferases (CPT-1, CPT-2), organic cation transporters (OCTN-1, OCTN-2), and enzymes of carnitine biosynthesis (trimethyllysine dioxygenase, TMLD; 4-*N*-trimethylaminobutyraldehyde dehydrogenase, TMABA-DH; γ-butyrobetaine dioxygenase, BBD) in Fao cells. Fao cells were grown in culture medium until subconfluent state and were then incubated with 50 μM of the PPARα agonist WY-14,643 for 6 and 20 h, respectively. Control cells were incubated with low-serum medium containing vehicle alone. Total RNA was extracted from cells and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalization. mRNA concentrations of the genes in the treated cells are shown relative to control cells (=1.00; dotted line) treated for 6 and 20 h, respectively. An asterisk (*) indicates a significant difference from control cells ($P < 0.05$).

between cells treated with WY-14,643 and control cells (Fig. 2).

Discussion

In this study, rats and Fao cells were treated with the PPARα agonists clofibrate and WY-14,643, respectively. CPT-1 and CPT-2 have been identified as PPARα target genes [22–24], and the observation that expression of these genes was strongly up-regulated in the liver of rats and in Fao cells by treatment with the agonists indicated PPARα activation. The rat study confirms the previous observation that clofibrate treatment increases the concentration of carnitine in the liver of rats [9,10]. The novel finding of this study is that clofibrate treatment caused a strong up-regulation of OCTN-2 (8.4-fold) and a less strong up-regulation of OCTN-1 (2.4-fold) whereas mRNA concentrations of genes encoding enzymes of hepatic carnitine biosynthesis remained unchanged. These observations strongly indicate that the increased carnitine concentration in the liver of rats treated with clofibrate was rather due to an increased uptake of carnitine from the blood into the liver than to an increased synthesis of carnitine. This indication accords with the observation that carnitine concentrations in plasma were reduced by clofibrate treatment, probably due to an increased uptake into cells. More than 95% of the total carnitine in the body is localized in the muscle which serves as a carnitine storage. When plasma carnitine concentrations are lowered such as by treatment with pivalate, carnitine can be mobilized from the muscle in order to normalize plasma carnitine concentrations [25]. The finding that the concentration of acetyl carnitine, the storage form of carnitine, was reduced in gastrocnemius of rats treated with clofibrate indeed indicates that carnitine might have been mobilized from muscle.

The in vitro study in which Fao cells were treated with WY-14,643 confirms most of the observations of the rat study. It is shown that treatment of cells with WY-14,643 increased gene expression of OCTN-2 and increased the intracellular concentration of free carnitine whereas gene expression of enzymes of hepatic carnitine biosynthesis was not altered, too. The only disagreement between the cell and the rat study was that expression of OCTN-1 was not up-regulated by the PPARα agonist in Fao cells. This may have two reasons: first, the effect of WY-14,643 in Fao cells was generally weaker than the effect of clofibrate in rats on the respective parameters; second, OCTN-1 was generally less responsive to PPARα agonists than OCTN-2. As gene expression of OCTN-1 remained unchanged in Fao cells treated with WY-14,643, it can be concluded that the increased concentration of free carnitine was exclusively the result of an increased carnitine uptake into the cell by OCTN-2.

The cell culture study disproves the hypothesis that the increase of the carnitine concentration in livers of rats treated with clofibrate is caused by an increased hepatic carnitine synthesis due to an increased availability of TML derived from muscle [10]. Carnitine concentration in Fao cells was increased by WY-14,643 although concentrations of TML in the media of treated and control cells were identical. This means that carnitine concentrations in Fao cells were increased by WY-14,643 independent of the availability of TML from muscle or other tissues.

The observation that OCTN-2 expression was up-regulated and that carnitine concentration was increased in liver and cells treated with two different PPARα agonists indicates that these effects were caused by PPARα activation. This indication provides also an explanation for the observation of increased hepatic carnitine concentrations in fasted rats [15,16]. During fasting, non-esterified fatty acids are liberated from adipose tissue and act as activators of PPARα when they have entered the liver. Activation of PPARα up-regulates many genes involved in hepatic mitochondrial and peroxisomal β-oxidation of fatty acids to supply acetyl-CoA used for the generation of ATP via citrate cycle and for the generation of ketone bodies, an important fuel for the brain during fasting [17,26]. These metabolic adaptations during fasting triggered by PPARα aim to minimize the use of protein and carbohydrates as fuel and allow mammals to survive long periods of energy deprivation. CPTs are rate-limiting for β-oxidation of fatty acids [22,24]. The up-regulation of CPTs, which is essential for the metabolic adaptations occurring during fasting, might increase the demand of carnitine in liver cells. We postulate that up-regulation of OCTNs by PPARα activation is a means to supply liver cells with sufficient carnitine required for transport of excessive amounts of fatty acids into the mitochondrion, and therefore plays an important role in the adaptive response of liver metabolism to fasting.

In conclusion, the present study shows for the first time that treatment of rats and rat Fao hepatoma cells with PPARα agonists clofibrate and WY-14,643, respectively, causes an up-regulation of OCTNs whereas expression of genes encoding enzymes involved in hepatic carnitine biosynthesis remain unchanged. Up-regulation of OCTNs may enhance carnitine uptake from blood or medium, respectively, into liver cells and this may be the reason for the increased carnitine concentrations in livers of rats treated with clofibrate.

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Dietary oxidised fat up regulates the expression of organic cation transporters in liver and small intestine and alters carnitine concentrations in liver, muscle and plasma of rats

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It has been shown that treatment of rats with clofibrate, a synthetic agonist of PPARα, increases mRNA concentration of organic cation transporters (OCTN)-1 and -2 and concentration of carnitine in the liver. Since oxidised fats have been demonstrated in rats to activate hepatic PPARα, we tested the hypothesis that they also up regulate OCTN. Eighteen rats were orally administered either sunflower-seed oil (control group) or an oxidised fat prepared by heating sunflower-seed oil, for 6 d. Rats administered the oxidised fat had higher mRNA concentrations of typical PPARα target genes such as acyl-CoA oxidase, cytochrome P450 4A1 and carnitine palmitoyltransferases-1A and -2 in liver and small intestine than control rats ($P<0{\cdot}05$). Furthermore, rats treated with oxidised fat had higher hepatic mRNA concentrations of OCTN1 (1·5-fold) and OCTN2 (3·1-fold), a higher carnitine concentration in the liver and lower carnitine concentrations in plasma, gastrocnemius and heart muscle than control rats ($P<0{\cdot}05$). Moreover, rats administered oxidised fat had a higher mRNA concentration of OCTN2 in small intestine (2·4-fold; $P<0{\cdot}05$) than control rats. In conclusion, the present study shows that an oxidised fat causes an up regulation of OCTN in the liver and small intestine. An increased hepatic carnitine concentration in rats treated with the oxidised fat is probably at least in part due to an increased uptake of carnitine into the liver which in turn leads to reduced plasma and muscle carnitine concentrations. The present study supports the hypothesis that nutrients acting as PPARα agonists influence whole-body carnitine homeostasis.

Carnitine: Oxidised fat: Peroxisome proliferator-activated receptor-α: Organic cation transporters

Carnitine (L-3-hydroxy-4-*N*-*N*-*N*-trimethylaminobutyrate) is an essential metabolite that has a number of indispensable functions in intermediary metabolism. The most prominent function lies in its role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix where β-oxidation takes place[1–3]. All tissues that use fatty acids as a fuel source require carnitine for normal function. Carnitine is derived from dietary sources and endogenous biosynthesis[4]. Carnitine biosynthesis involves a complex series of reactions involving several tissues[5]. Lysine provides the carbon backbone of carnitine. Lysine in protein peptide linkages undergoes methylation of the ϵ-amino group to yield trimethyllysine, which is released upon protein degradation. Muscle is the major source of trimethyllysine. The released trimethyllysine is further oxidised to butyrobetaine by the action of trimethyllysine dioxygenase, 3-hydroxy-*N*-trimethyllysine aldolase and 4-*N*-trimethylaminobutyraldehyde dehydrogenase. Butyrobetaine is hydroxylated by γ-butyrobetaine dioxygenase to form carnitine. The last reaction which is rate-limiting for carnitine synthesis occurs primarily in the liver and kidneys[6] (see Fig. 1).

Distribution of carnitine within the body and intracellular homeostasis of carnitine are controlled by organic cation transporters (OCTN) which belong to the solute carrier (SLC) 22A family, localised to the apical membrane of cells[7,8]. Three OCTN have been identified so far: OCTN1, OCTN2 and OCTN3[9–11]. OCTN are polyspecific; they transport several cations and L-carnitine[12,13]. Carnitine transport by OCTN1 and OCTN2 is Na dependent whereas that by OCTN3 is Na independent[11]. OCTN1 and OCTN2 are expressed in several tissues such as kidney, intestine, skeletal muscle, heart, liver and brain[11,14,15]. In contrast, OCTN3 is expressed exclusively in the testes and kidneys[11]. Among the three OCTN, OCTN3 has the highest specificity for carnitine; OCTN1 has the lowest one[11]. OCTN operate on the intestinal absorption and renal reabsorption of carnitine and play a major role in tissue distribution by catalysing the uptake of carnitine into body cells. Due to its high binding affinity for carnitine and its wide expression, OCTN2 seems to be the most physiologically important carnitine transporter. OCTN1 contributes less to carnitine transport than OCTN2 due to its low carnitine transport activity. OCTN3 may be important for carnitine uptake into the testes, and may contribute to the reabsorption of carnitine in the kidneys[11]. The fact that inborn or acquired defects of OCTN lead to primary or secondary systemic carnitine deficiency demonstrates their essential role in carnitine homeostasis[8].

Abbreviations: CPT, carnitine palmitoyltransferase; Cyp, cytochrome P450; OCTN, organic cation transporter.

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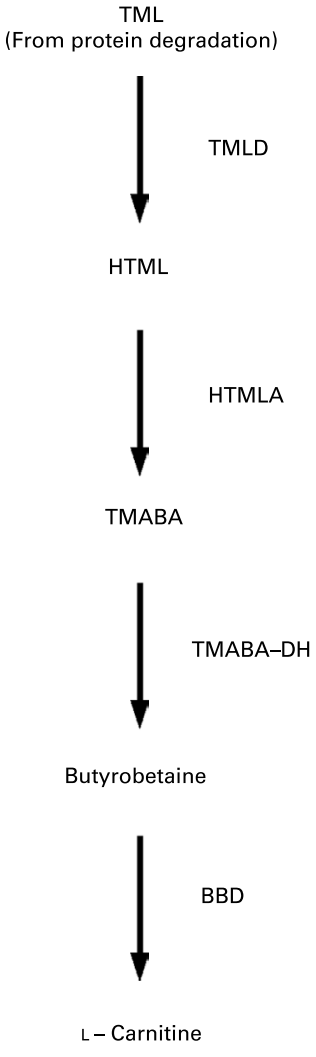

Fig. 1. Schematic diagram of carnitine biosynthesis from trimethyllysine (TML) (according to Vaz & Wanders[6]). TML is oxidised to butyrobetaine by trimethyllysine dioxygenase (TMLD), 3-hydroxy-*N*-trimethyllysine aldolase (HTMLA) and 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH). In the last rate-limiting step, butyrobetaine is hydroxylated to L-carnitine by γ-butyrobetaine dioxygenase (BBD). HTML, 3-hydroxy-*N*-trimethyllysine; TMABA, 4-*N*-trimethylaminobutyraldehyde.

It has been shown previously that starvation or treatment of rats with clofibrate increases the concentration of carnitine in the liver[16–18]. Both starvation and clofibrate treatment lead to an activation of PPARα, a transcription factor belonging to the nuclear hormone receptor superfamily[19]. We have recently shown that activation of PPARα by clofibrate treatment causes an up regulation of OCTN1 and OCTN2 in rat liver[20]. These results strongly indicated that increased carnitine concentrations in livers of rats starved or treated with clofibrate were due to increased uptake of carnitine from blood into the liver. Indeed, plasma carnitine concentrations were reduced in rats treated with clofibrate which may be caused by an increased uptake into the liver[20].

In addition to synthetic agonists, several naturally occurring compounds are able to activate PPARα *in vivo*. Recently, we and others have shown that dietary oxidised fats prepared by the heating of vegetable oils activate hepatic PPARα in rats and pigs[21–25]. In the present study, we tested the hypothesis that oxidised fats are also able to up regulate the expression of OCTN (OCTN1, OCTN2) in the liver due to their ability to activate PPARα and thereby increase hepatic carnitine concentration. For this end, we performed an experiment with growing rats as an animal model, according to a previous study dealing with the effects of an oxidised oil on PPARα activation[24].

More than 95 % of the total carnitine in the body is localised in the muscle which serves as a carnitine storage[2]. When plasma carnitine concentrations are lowered, such as by treatment with pivalate, carnitine is mobilised from the muscle in order to normalise plasma carnitine concentrations[26]. Therefore, an increased uptake of carnitine from the blood into the liver by up regulation of hepatic OCTN should lead to a mobilisation of carnitine storage in the muscle. To investigate this, we also determined carnitine concentrations in skeletal muscle and heart of the rats.

OCTN1 and OCTN2 are also highly expressed in the intestine and particularly OCTN2 plays an important role in the absorption of L-carnitine from the diet[15,27,28]. As the small intestine also has a high expression of PPARα, it seems possible that an oxidised fat could increase the gene expression of OCTN also in the small intestine via an activation of PPARα. Besides OCTN, the amino acid transporter ATB^{0+} is involved in the intestinal absorption of carnitine from the diet[27,29]. In order to obtain information whether PPARα activation by synthetic or native agonists could influence intestinal carnitine absorption, we also determined mRNA concentration of ATB^{0+} in small intestine.

Materials and methods

Animal experiment

Male Sprague–Dawley rats, aged 5 weeks old, supplied by Charles River (Sulzfeld, Germany) with an average initial body weight of 115 (SD 14) g were randomly assigned to two groups of nine rats each. They were kept individually in Macrolon cages in a room controlled for temperature (22 ± 2°C), relative humidity (50–60 %) and light (12 h light–dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. The animals received either 2 ml fresh sunflower-seed oil (control group) or oxidised sunflower-seed oil (see Preparation of the oxidised fat) by oral administration once per d 2 h after the beginning of the light cycle. Afterwards, they obtained their daily food ration. All rats were fed a commercial standard basal diet (Altromin 1324; Altromin GmbH, Lage, Germany). Concentration of total carnitine in the basal diet was 22 μmol/kg. To standardise food intake, diet intake was controlled. Each rat in the experiment received 12 g diet/d. This amount of diet which is approximately 20 % below the amount of diet rats would consume *ad libitum* was completely ingested by all rats. Thus, the diet intake was identical in all the rats within this experiment. Water was available *ad libitum* from nipple drinkers during the whole experiment. At day 6 of treatment, rats received the last dose of fresh or oxidised fat and 9 g diet and were killed 4 h later by decapitation under light anaesthesia with diethyl ether. Blood was collected into heparinised polyethylene tubes. Liver, heart

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and gastrocnemius muscles were quickly removed, frozen with liquid N_2 and stored at −80°C pending further analysis. Plasma was obtained by centrifugation of the blood (1100 ***g***; 10 min; 4°C) and stored at −20°C. Liver samples for RNA isolation and lipid extraction were snap-frozen in liquid N_2 and stored at −80°C. The small intestine was rapidly excised, washed with cold 0·9 % NaCl (w/v) and mucosal scrapings were obtained from the jejunum (defined by length), snap-frozen and stored at −80°C for RNA extraction.

Preparation of the oxidised fat

The thermoxidised oil was prepared by heating sunflower-seed oil obtained from a local supermarket in an electric fryer (Saro Gastro-Products GmbH, Emmerich, Germany) for 25 d at 60°C. Throughout the heating process, air was continuously bubbled into the fat. The extent of lipid peroxidation was determined by assaying the peroxide value[30], concentration of thiobarbituric acid-reactive substances[31] and conjugated dienes[32], acid values[30], the percentage of total polar compounds[33] and the concentration of total carbonyls[34]. The oxidised fat had much higher concentrations of peroxides (126-fold), conjugated dienes (>2740-fold), thiobarbituric acid-reactive substances (12-fold), total carbonyls (33-fold), polar compounds (5-fold) and a higher acid value (15-fold) than the fresh fat (Table 1).

Carnitine analysis

Carnitine was determined as [^{3}H]acetyl-carnitine after the esterification of non-esterified carnitine by carnitine acyltransferase according to McGarry & Foster[35] with modifications proposed by Parvin & Pande[36] and Christiansen & Bremer[37]. Plasma samples were used directly for the determination of the total carnitine after alkaline hydrolysation as described for the tissue samples below. Tissue samples were freeze dried and milled. Then 100 mg liver or 50 mg muscle powder were sonificated in 5 ml water for 15 min. Samples were centrifuged (12 000 ***g***; 5 min) and non-esterified carnitine in the supernatant fraction was measured. For the determination of the total carnitine the samples were hydrolysed before the centrifugation. For this, 10 ml 0·2 M-potassium hydroxide were added, the samples were incubated at 30°C for 1 h and then neutralised by the addition of 0·2 M-HCl. Carnitine esterification was done in a final volume of 1 ml containing 0·1 M-HEPES (pH 7·4), 2 mM-*N*-ethylmaleimide, 1·25 mM-EDTA, 25 μM-[^{3}H]acetyl-CoA (29·4 MBq/mmol; GE Healthcare, Buckinghamshire, UK) and 1 U carnitine acyltransferase (Roche Diagnostic, Mannheim, Germany) for 30 min at room temperature. [^{3}H]acetyl-CoA not consumed by the reaction was bound to Dowex 1-X 8 and separated by centrifugation. Carnitine concentration was calculated using the radioactivity of the supernatant fraction measured in a liquid scintillation counter and corrected for non-specific radioactivity.

Table 1. Concentrations of various lipid oxidation products in the fats*

Oxidation product	Fresh fat	Oxidised fat
Peroxide value (mEq O_2/kg)	3·0	378·6
Conjugated dienes (mmol/kg)	<0·1	273·6
TBARS (mmol/kg)	1·1	13·1
Total carbonyls (mmol/kg)	2·9	96·9
Total polar compounds (%)	5·1	27·8
Acid value (g KOH/kg)	0·4	5·8

TBARS, thiobarbituric acid-reactive substances.
* Data are the results of single measurements.

Reverse transcriptase polymerase chain reaction analysis

Total RNA was isolated from rat livers and mucosa scrapings, respectively, by TRIZOL™ reagent (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's protocol. cDNA synthesis was carried out as described[38]. The mRNA concentration of genes was measured by real-time detection PCR using SYBR® Green I and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). Real-time detection PCR was performed with 1·25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 μM-dNTP and 26·7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 2). Annealing temperature for all primers was 60°C. For determination of mRNA concentration a threshold cycle (C_t) and amplification efficiency was obtained from each amplification curve using the software RotorGene 4·6 (Corbett Research). Calculation of the relative mRNA concentration was made using the $\Delta\Delta C_t$ method as previously described[39]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for normalisation. mRNA concentration of glyceraldehyde-3-phosphate dehydrogenase was not influenced by the treatment of rats with oxidised fat.

Statistical analysis

Means of the treatment and control groups were compared by an unpaired *t* test using the Minitab Statistical Software (Minitab, State College, PA, USA). Differences with $P<0·05$ were considered to be significant.

Results

Final weights and body-weight gains of the rats

Final body weights of rats treated with the oxidised fat (133 (SD 14) g) were not significantly different from the control rats (144 (SD 14) g) (nine rats for each group). However, rats treated with the oxidised fat had a lower body-weight gain (17·5 (SD 6·4) g) over the feeding period than the control rats (29·7 (SD 4·5) g) (nine rats for each group; $P<0·05$).

mRNA concentrations of acyl-CoA oxidase, cytochrome P450-4A1, carnitine palmitoyltransferases-1A and -2, organic cation transporters-1 and -2 and enzymes involved in hepatic carnitine synthesis (trimethyllysine dioxygenase, 4-N-trimethylaminobutyraldehyde dehydrogenase and γ-butyrobetaine dioxygenase) in the liver

Rats treated with the oxidised fat had higher mRNA concentrations of acyl-CoA oxidase, cytochrome P450 (Cyp)-4A1, carnitine palmitoyltransferase (CPT)-2, OCTN1 and OCTN2 in the liver than control rats ($P<0·05$); mRNA concentration of CPT1A, however, was not different in the rats treated with oxidised fat from the control rats (Fig. 2). Rats treated with the oxidised fat had a higher mRNA concentration of trimethyllysine dioxygenase in the liver than control

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Table 2. Characteristics of the primers used in reverse transcriptase polymerase chain reaction analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	bp	NCBI GenBank
ACO	CTTTCTTGCTTGCCTTCCTTCTCC	GCCGTTTCACCGCCTCGTA	415	NM_017340
ATB^{0+}	ATCCGGAAGCACTAGCTCAA	CCCAGTAAATTCCAGCCTGA	237	NM_001037544
BBD	ATTCTGCAAAAGCTCGGAAA	CTCCTTGGAGTCCTGCTCTG	183	NM_022629
Cyp4A1	CAGAATGGAGAATGGGGACAGC	TGAGAAGGGCAGGAATGAGTGG	460	NM_175837
CPT1A	GGAGACAGACACCATCCAACATA	AGGTGATGGACTTGTCAAACC	416	NM_031559
CPT2	TCCTCGATCAAGATGGGAAC	GATCCTTCATCGGGAAGTCA	237	NM_012930
GAPDH	GCATGGCCTTCCGTGTTCC	GGGTGGTCCAGGGTTTCTTACTC	337	BC059110
OCTN1	AGCATTTGTCCTGGGAACAG	ACTCAGGGATGAACCACCAG	200	NM_022270
OCTN2	CCTCTCTGGCCTGATTGAAG	CTCCGCTGTGAAGACGTACA	226	NM_012930
TMLD	GCCCTGTGGCATTCAAGTAT	GGTCCAACCCCTATCATGTG	201	AF374406
TMABA-DH	TTTGAGACTGAAGCCGAGGT	CACCGGGCTGACGTTATAGT	156	NM_022273

ACO, acyl-CoA oxidase; BBD, γ-butyrobetaine dioxygenase; Cyp, cytochrome P450; CPT, carnitine palmitoyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OCTN, organic cation transporter; TMLD, trimethyllysine dioxygenase; TMABA-DH, 4-*N*-trimethylaminobutyraldehyde dehydrogenase.

rats ($P<0{\cdot}05$; Fig. 2). mRNA concentrations of 4-*N*-trimethylaminobutyraldehyde dehydrogenase and γ-butyrobetaine dioxygenase in the liver, however, did not differ between the two groups of rats (Fig. 2).

mRNA concentrations of acyl-CoA oxidase, cytochrome P450-4A1, carnitine palmitoyltransferases-1A and -2, organic cation transporters-1 and -2 and ATB^{0+} in the small intestine

Rats treated with oxidised fat had higher mRNA concentrations of acyl-CoA oxidase, Cyp4A1, CPT1A, CPT2 and OCTN2 ($P<0{\cdot}05$), and they tended to have a higher mRNA concentration of OCTN1 ($P=0{\cdot}066$) in the small intestine compared with control rats (Fig. 3). mRNA concentration of ATB^{0+} in the small intestine was reduced in the rats fed the oxidised fat compared with those fed the fresh fat ($P<0{\cdot}05$; Fig. 3).

Carnitine concentrations in liver, plasma and muscle

Rats treated with the oxidised fat had a higher carnitine concentration in the liver than control rats ($P<0{\cdot}05$, Fig. 4). Plasma carnitine concentration was lower in the rats treated with oxidised fat (18·8 (SD 3·1) μmol/l) than in the control group (28·4 (SD 4·3) μmol/l) (nine rats for each group; $P<0{\cdot}05$). Rats treated with oxidised fat also had lower carnitine concentrations in gastrocnemius and heart muscle than control rats ($P<0{\cdot}05$; Fig. 4).

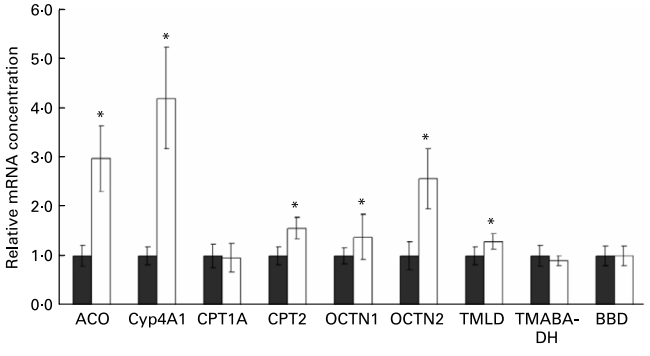

Fig. 2. Effect of an oxidised fat on the relative mRNA concentrations of acyl-CoA oxidase (ACO), cytochrome P450 (Cyp)-4A1, carnitine palmitoyltransferases (CPT)-1A and -2, organic cation transporters (OCTN)-1 and -2, trimethyllysine dioxygenase (TMLD), 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH) and γ-butyrobetaine dioxygenase (BBD) in the liver of rats. Rats were treated orally with 2 ml oxidised fat (□) or fresh fat (■; control = 1·00) for 6 d. Total RNA was extracted from rat livers and mRNA concentrations were determined by real-time detection RT-PCR analysis using glyceraldehyde-3-phosphate dehydrogenase mRNA concentration for normalisation. Values are means, with standard deviations represented by vertical bars (*n* 9). *Mean value was significantly different from that of the control rats ($P<0{\cdot}05$).

Discussion

We have recently found that treatment with clofibrate causes a strong up regulation of OCTN2, and a less strong up regulation of OCTN1, in the liver of rats which was accompanied by an increased hepatic carnitine concentration[20]. This effect was probably caused by PPARα activation. In the present study, we investigated the hypothesis that oxidised fats are able to exert similar effects due to their ability to activate PPARα. Hydroxy- and hydroperoxy fatty acids such as hydroxyoctadecadienoic and hydroperoxyoctadecadienoic acid occurring in oxidised fats are very potent PPARα agonists[38,40,41]. These fatty acids are produced during the early stage of lipid peroxidation. Since they are unstable and decompose at high temperatures, fats treated at low temperature have much higher concentrations of these primary lipid

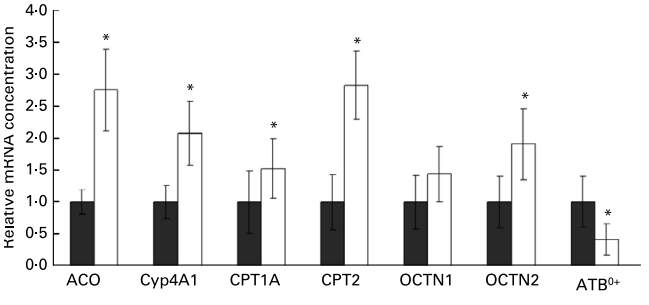

Fig. 3. Effect of an oxidised fat on the relative mRNA concentrations of acyl-CoA oxidase (ACO), cytochrome P450 (Cyp)-4A1, carnitine palmitoyltransferases (CPT)-1A and -2, organic cation transporters (OCTN)-1 and -2 and amino acid transporter ATB^{0+} in the small intestine of rats. Rats were treated orally with 2 ml oxidised fat (□) or fresh fat (■; control = 1·00) for 6 d. Total RNA was extracted from mucosal scrapings and mRNA concentrations were determined by real-time detection RT-PCR analysis using glyceraldehyde-3-phosphate dehydrogenase mRNA concentration for normalisation. Values are means, with standard deviations represented by vertical bars (*n* 9). *Mean value was significantly different from that of the control rats ($P<0{\cdot}05$).

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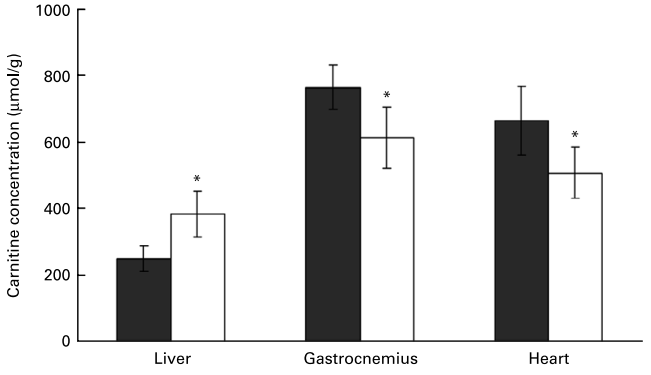

Fig. 4. Effect of an oxidised fat on the concentrations of total carnitine in liver, gastrocnemius and heart. Rats were treated orally with 2 ml oxidised fat (□) or fresh fat (■; control) for 6 d. Values are means, with standard deviations represented by vertical bars (n 9). *Mean value was significantly different from that of the control rats ($P<0{\cdot}05$).

peroxidation products than fats treated at high temperature[24]. This is the reason why we used a fat treated at a relatively low temperature for a long period. The high peroxide value and the high concentration of conjugated dienes indicate that this fat indeed had high concentrations of hydroxy- and hydroperoxy fatty acids which may be particularly responsible for the PPARα-activating effects of oxidised fats. To ensure that all rats obtained the same dose of oxidised fat, it was administered orally. The oxidised and fresh fat, respectively, accounted for about 25 % of total energy of the total daily feed. Since it was observed in a previous rat study that even short-term application of a PPARα agonist led to the typical changes known for PPARα activation such as up regulation of classical target genes involved in β-oxidation and reduction of TAG concentration[38], we decided to give the oxidised fat over a relatively short time of 6 d. It has been shown that the intake of oxidised fats could cause a reduction of the food intake in rats which could cause secondary effects which interact with the effect of treatment[42,43]. To ensure an identical food intake in both groups of rats, we used a controlled feeding system in which each rat consumed 12 g diet/d. This amount of diet is slightly below that that rats would consume *ad libitum* but in clear excess of that necessary to meet the maintenance energy requirement (which is approximately 6 g/d[44]) and ensures an adequate growth of the rats. It is known that fasting causes an activation of PPARα due to the release of NEFA from the adipose tissue[45]. To avoid PPARα activation due to an insufficient supply of energy, all the rats received their last portion of diet 4 h before decapitation. Therefore, we can exclude the possibility that PPARα was also activated in the control group fed the fresh fat. The finding that rats fed the oxidised fat gained less weight during the experimental period than those fed the fresh fat although both groups received an identical amount of diet indicates that the oxidised fat impaired the feed conversion ratio. This finding agrees with other reports which also showed that feeding of oxidised fats impairs the growth of rats[46–49]. We did not investigate the reason for this. Previous studies, however, have shown that oxidised fats lower the digestibility of nutrients[46,50] and this may be the reason for the reduced body-weight gains of the rats fed the oxidised fat observed in the present study. However, as rats fed the oxidised fat appeared quite normal, we assume that the oxidised fat did not cause general toxicity.

The finding of increased mRNA concentrations of the typical PPARα downstream genes acyl-CoA oxidase, Cyp4A1, CPT1A and CPT2 (for a review, see Mandard *et al.*[45]) in liver and intestine indeed indicates that the oxidised fat caused an activation of PPARα in both liver and intestine of the rats. This indication agrees with recent studies in rats and pigs which also showed that intake of oxidised fats leads to an activation of PPARα in the liver[21–25].

The present study shows further that treatment of rats with an oxidised fat caused the same alterations as observed for clofibrate[20], namely increased hepatic mRNA concentrations of OCTN1 and OCTN2 and an increased hepatic carnitine concentration. Considering that a similar up regulation of OCTN1 and OCTN2 was observed in the liver of rats treated with the synthetic PPARα agonist clofibrate and in rat hepatoma cells treated with the more potent and selective PPARα agonist WY 14,643[20], we propose that the oxidised fat up regulated OCTN in the liver also by PPARα activation.

In rat liver, OCTN1 and OCTN2 are highly expressed[15]. Both of them are able to transport carnitine into the liver cell[51,52]. However, it has been shown that OCTN2 has a higher carnitine transport activity than OCTN1[11]. For that reason and as mRNA concentration of OCTN2 was more strongly increased by the oxidised fat than that of OCTN1, we assume that increased hepatic carnitine concentrations in rats treated with oxidised fat were caused mainly by an increased uptake of carnitine via OCTN2. Plasma carnitine concentrations are regulated by several events, namely intestinal absorption from the diet, renal excretion, endogenous synthesis in the liver and kidneys and movement of carnitine between plasma and tissues[53]. We have not studied the pharmacokinetics of carnitine but it seems plausible that reduced plasma concentrations of carnitine in rats fed the oxidised fat may at least in part be due to an enhanced uptake into the liver. We measured mRNA concentrations of OCTN only in liver and small intestine; however, it is possible that they were increased also in other tissues in rats fed the oxidised fat. Therefore, an increased uptake of carnitine into other tissues besides liver could also contribute to the reduced plasma carnitine concentrations. In the kidney, OCTN2 functions to reabsorb carnitine from the urine[13,54]. An up regulation of OCTN2 in kidney would be expected to reduce urinary excretion of carnitine which in turn results in an increased plasma carnitine concentration. However, the effect of oxidised fats on the gene expression of OCTN in those tissues and their consequences on whole-body carnitine homeostasis should be determined in future studies.

In the present study we also determined mRNA concentrations of various enzymes involved in hepatic carnitine biosynthesis in the liver which belongs like the kidney to the tissues being able to synthesise carnitine[6]. It was found that oxidised fat treatment led to a moderate up regulation of trimethyllysine dioxygenase while mRNA concentrations of 4-*N*-trimethylaminobutyraldehyde dehydrogenase and γ-butyrobetaine dioxygenase, the rate-limiting enzyme of carnitine biosynthesis[6], remained unchanged by the treatment. This finding shows that PPARα activation by the oxidised oil does not up regulate the gene expression of enzymes involved in hepatic carnitine synthesis. Nevertheless, it is

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possible that carnitine hepatic biosynthesis was increased in rats treated with oxidised fat. The liver has a high capacity to convert γ-butyrobetaine into carnitine[6]. As OCTN2 has a high affinity for γ-butyrobetaine[10,11] it is likely that an increased expression of OCTN2 may have led to an increased uptake of γ-butyrobetaine from plasma into the liver which in term may have stimulated synthesis of carnitine in the liver. This assumption, however, has to be proven in further studies.

Muscle contains more than 95 % of whole-body carnitine and serves as a carnitine storage[2]. When plasma carnitine concentrations are lowered, such as by treatment with pivalate, carnitine can be mobilised from the muscle in order to normalise plasma carnitine concentrations[26]. Therefore, we expected that a reduced plasma carnitine concentration may lead to a reduction of the carnitine concentration in muscle. The finding that the concentration of carnitine was reduced in gastrocnemius and heart muscle of rats treated with oxidised fat indeed suggests that carnitine might have been mobilised from muscle. In rats treated with clofibrate, a reduction of muscle carnitine concentration has also been found[20]. A reduced carnitine concentration in muscle could also be due to a reduced uptake of carnitine due to a decreased activity of OCTN, which, however, is unlikely with respect to the finding that OCTN in liver were up regulated in rats fed the oxidised fat. As muscle also has a high expression of PPARα, we expect that the expression of OCTN in muscle was increased rather than reduced by the dietary oxidised fat.

The present study further shows that a dietary oxidised fat leads to an up regulation of OCTN2 in the small intestine. As PPARα target genes (acyl-CoA oxidase, CYP4A1, CPT1a, CPT2) in the intestine were also up regulated in rats fed the oxidised fat, we assume that the increased expression of OCTN in intestine was also caused by activation of PPARα. As intestinal OCTN localised in the apical membrane of mucosa cells are able to transport carnitine from the diet into the cell[27,28], an increased expression of these transporters may enhance their capacity to absorb carnitine. However, as ATB^{0+}, another transporter involved in the intestinal absorption of carnitine[27], was down regulated in rats fed the oxidised fat, it is difficult to draw conclusions about the whole intestinal absorption of carnitine from the diet. Nevertheless, the observed up regulation of intestinal OCTN may be relevant because they are polyspecific and do not only transport carnitine from the intestinal lumen into the mucosa cell but are also able to bind various drugs such as verapamil, spironolactone or mildronate and other monovalent cations[14,28,55–58]. As oxidised fats increase the gene expression of OCTN in the small intestine, it is possible that these fats also increase the absorption of various drugs from the intestine.

The hypothesis that the up regulation of OCTN was caused by PPARα activation provides also an explanation for the observed increased hepatic carnitine concentrations in fasted rats[16,17]. During fasting, NEFA are liberated from adipose tissue and act as activators of PPARα when they have entered the liver. Activation of PPARα up regulates many genes involved in hepatic mitochondrial and peroxisomal β-oxidation of fatty acids to supply acetyl-CoA used for the generation of ATP via the citrate cycle and for the generation of ketone bodies, an important fuel for the brain during fasting[59,60]. These metabolic adaptations during fasting triggered by PPARα aim to minimise the use of protein and carbohydrates as fuel and allow mammals to survive long periods of energy deprivation. CPT are rate limiting for β-oxidation of fatty acids[61,62]. The up regulation of CPT, which is essential for the metabolic adaptations occurring during fasting, might increase the demand for carnitine in liver cells. We postulate that up regulation of OCTN by PPARα activation is a means to supply liver cells with sufficient carnitine required for the transport of excessive amounts of fatty acids into the mitochondrion, and therefore plays an important role in the adaptive response of liver metabolism to fasting.

In conclusion, the present study shows that an oxidised fat causes an up regulation of OCTN2 in the liver and small intestine of rats. As OCTN2 catalyses the uptake of carnitine into cells, these fats influence whole-body carnitine homeostasis. An increased hepatic carnitine concentration in rats treated with oxidised fat may be at least in part due to an increased uptake of carnitine from blood into the liver. Since OCTN2 binds not only carnitine but also various drugs, the possibility exists that increased OCTN2 expression in the small intestine may improve the absorption of various drugs.

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Effects of fasting or caloric restriction on mRNA concentration of organic cation transporter-2 and carnitine concentrations in tissues of rats

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Running title: Caloric restriction and carnitine homeostasis

Key words: Carnitine: Fasting: caloric restriction: PPARα: Organic cation transporter

Abbreviations: ACO, acyl-CoA oxidase; BB, γ-butyrobetaine; BBD, γ-butyrobetaine dioxygenase; CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; CPT, carnitine palmitoyltransferase; NEFA, non-esterified fatty acids; OCTN, organic cation transporter; TMABA-DH, 4-N-trimethylaminobutyraldehyde dehydrogenase; TML, trimethyllysine; TMLD, trimethyllysine dioxygenase.

Abstract

We tested the hypothesis that fasting or caloric restriction up-regulates gene expression of organic cation transporter (OCTN)-2 and thereby influences carnitine concentrations in rat tissues. Three groups of rats received the diet either *ad libitum* (control rats) or 10.5 g diet/d (70% of energy requirement for maintenance, E70 rats) or 6 g diet/d (40% of energy requirement for maintenance, E40 rats) for 10 days. A fourth group received the diet *ad-libitum* for nine days and was then fasted for 24 h (fasted rats). Fasted and caloric restricted rats had increased mRNA concentrations of acyl-CoA oxidase (ACO) in liver, heart and kidney compared to control rats ($P<0.05$) indicative of activation of PPARα in these tissues. E70 rats had increased OCTN2 mRNA concentrations in liver (2.59-fold) and kidney (1.49-fold) and increased total carnitine concentrations in these tissues compared to control rats. E40 rats had increased OCTN2 mRNA concentration in liver (3.29-fold), skeletal muscle (2.23-fold), heart (2.30-fold) and kidney (3.52-fold) and increased total carnitine concentrations in these four tissues compared to control rats. Fasted rats had increased OCTN2 mRNA concentrations in liver (4.01-fold), heart (2.05-fold) and kidney (2.03-fold) and increased total carnitine concentrations in these three tissues ($P<0.05$). The present study shows for the first time that both fasting and caloric restriction lead to an up-regulation of OCTN2 in several tissues, probably mediated by activation of PPARα. Increased tissue carnitine concentrations in fasted and caloric restricted rats might be due to increased uptake of carnitine from blood into tissues by OCTN2.

Introduction

Carnitine (L-3-hydroxy-4-N-N-N-trimethylaminobutyrate) is an essential metabolite, which has a number of indispensable functions in intermediary metabolism. The most prominent function lies in its role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix where β-oxidation takes place[1-4]. All tissues that use fatty acids as a fuel source require carnitine for normal function. Carnitine is derived from dietary sources and endogenous biosynthesis[4,5]. Carnitine biosynthesis involves a complex series of reactions[6]. Lysine provides the carbon backbone of carnitine. Lysine in protein peptide linkages undergoes methylation of the ε-amino group to yield trimethyllysine (TML), which is released upon protein degradation. The released TML is further oxidised to butyrobetaine by the action of trimethyllysine dioxygenase (TMLD), 3-hydroxy-N-trimethyllysine aldolase and 4-N-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH). γ-Butyrobetaine (BB) is hydroxylated by γ-butyrobetaine dioxygenase (BBD) to form carnitine. In rats, a considerable activity of that enzyme which is rate-limiting for carnitine synthesis has been found only in the liver[7]. From tissues which lack BBD, BB is excreted and transported via the circulation to the liver, where it is converted into carnitine[6].

Distribution of carnitine within the body and intracellular homeostasis of carnitine are controlled by organic cation transporters (OCTN) which belong to the solute carrier 22A family, localised on the apical membrane of cells[8,9]. Three OCTN have been identified so far, OCTN1, OCTN2 and OCTN3[10-12]. OCTN are polyspecific; they transport several cations and L-carnitine[13,14]. Carnitine transport by OCTN1 and OCTN2 is sodium dependent whereas that by OCTN3 is sodium independent[12]. OCTN2 is the most important carnitine transporter as it is expressed in several tissues such as kidney, intestine, skeletal muscle, heart, liver and brain[12,15,16]. OCTN2 operates in the reabsorption of carnitine from the urine, plays a major role in tissue distribution of carnitine and is also the key transporter involved in intestinal absorption of carnitine. OCTN2 also transports BB from plasma into liver where it is used for carnitine synthesis[12]. The fact that inborn or acquired defects of OCTN2 lead to primary or secondary systemic carnitine deficiency demonstrates the essential role of these transporters in carnitine homeostasis[9].

We have recently shown that activation of PPARα by clofibrate, a synthetic agonist, causes an up-regulation of OCTN2 in the liver of rats[17]. Increased expression of OCTN2 leads to an increased uptake of carnitine into the liver and this may provide an explanation for increased carnitine concentrations in the liver of rats treated with clofibrate[17]. The recent finding that feeding of an oxidised fat, which is known to activate PPARα[18-20], also causes an up-regulation of gene expression of OCTN2 in liver and intestine and increases tissue carnitine concentrations confirms a role of PPARα in gene expression of OCTN2 and carnitine homeostasis[21]. Previous studies have shown that fasting also leads to an increase of hepatic carnitine concentrations in rats[22,23]. The reason for this, however, has not yet been elucidated. It has been well established that fasting or caloric restriction causes also an activation of PPARα due to the release of non-esterified fatty acids (NEFA) from adipose tissue taken up into other tissues where they act as agonists of PPARα[24]. This prompted us to the hypothesis that fasting or caloric restriction may also influence gene expression of OCTN2 due to activation of PPARα, which in turn may influence tissue concentrations of carnitine. This hypothesis is strengthened by a previous study which showed that fasting for 48 or 72 h leads to an increased hepatic carnitine concentration in wild type mice but not in mice deficient in PPARα[25]. To test our hypothesis, we performed an experiment with rats which were either fasted for 24 h or set on energy deficiency, receiving either 40 or 70% of energy requirement for maintenance for 10 days. To study the effect of fasting or caloric restriction on carnitine homeostasis, we determined mRNA concentration of OCTN2 and carnitine concentrations in liver, skeletal muscle, heart, kidney and small intestine. Liver was

considered due to its high capacity of β-oxidation which requires carnitine as a cofactor of carnitine palmitoyltransferase (CPT), the rate limiting enzyme of β-oxidation[26]; skeletal muscle and heart were considered as muscle acts as a carnitine storage which contains more than 95% of whole body carnitine[2]. Small intestine and kidney were considered because of the important role of OCTN2 in absorption of carnitine from the diet and reabsorption from the urine, respectively in these tissues[12,27]. To make conclusions about carnitine biosynthesis, we also considered mRNA concentrations of enzymes involved in carnitine biosynthesis in the liver and tissue concentrations of TML and BB which are precursors for carnitine synthesis in the liver.

Within cells, carnitine acts as a substrate for carnitine acyltransferases, enzymes which catalyse equilibria between acyl-CoA esters and the respective carnitine esters[28]. There are three types of carnitine acyltransferases. These are carnitine acetyltransferase (CAT), carnitine octanoyltransferase (COT), and the CPT which are further divided into those inhibited by malonyl-CoA (CPT-1) and those which are not (CPT-2)[28]. CTP-1 moreover exists in at least two isoforms, namely the liver type (L-CPT-1) and the muscle type (M-CPT-1)[28]. It has been well established that CPT are PPARα target genes and that they are up-regulated by fasting or treatment with PPARα agonists[29]. In contrast, less is known about the transcriptional regulation of CAT and COT[28]. To assess whether transcription of these enzymes is also influenced by fasting or energy restriction, physiological conditions leading to PPARα activation, we determined the mRNA concentration of these enzyme in rat tissues.

Materials and methods

Animal experiment

Female Sprague-Dawley rats, with an average initial body weight of 267 ± 32 g were randomly assigned to four groups of 9 rats each and kept individually in Macrolon cages in a room controlled for temperature (22 ± 2°C), relative humidity (50-60%) and light (12 h light/ dark cycle). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt. All animals were fed a commercial standard diet for rats ("altromin 1324", Altromin GmbH, Lage, Germany). According to the declaration of the producer, the content of metabolisable energy of this diet was 11.9 MJ/kg. The analysed concentration of total carnitine in this diet was 22 μmol/kg. The diet was fed for 10 days. Group 1 ("control rats") received the diet *ad libitum*. Group 2 ("E70 rats") received 10.5 g of diet per day according to 70% of the energy requirement for maintenance; group 3 ("E40 rats") received 6 g of diet per day according to 40% of the energy requirement for maintenance. Group 4 ("fasted rats") received the diet *ad libitum* for 9 days and was then fasted for 24 h. Energy requirement for maintenance of the rats was calculated according to National Research Council[30]. After 10 days, animals were killed by decapitation under light anesthesia with diethyl ether. Blood was collected into heparinised polyethylene tubes. Liver, kidney, skeletal muscle (M. *longissimus dorsi*) and heart were quickly removed, frozen with liquid nitrogen, and stored at -80°C until further analysis. For collecting samples of small intestine, abdomen was immediately opened after killing and intestinal segment was dissected starting at 15 cm from distal to the pyloric sphincter and washed twice with ice-cold phosphate buffered saline (pH 7.4). After opening the segment, enterocytes were scraped from tissue with a thin plate, frozen with liquid nitrogen and stored at -80°C until analysis. Plasma was obtained by centrifugation of the blood (1100*g*, 10 min, 4°C) and stored at -20°C.

Carnitine analysis

Concentrations of free carnitine, acetyl carnitine, TML and BB in plasma and tissues were determined by tandem mass spectrometry using deuterated carnitine-d_3 (Larodane Fine Chemicals, Malmö, Sweden) as internal standard[31]. 50 mg of freeze-dried tissues were extracted with 0.5 mL methanol:water (2:1, v/v) by homogenization (Tissue Lyzer, Qiagen, Hilden, Germany), followed by sonification for 20 min and incubation at 50 °C for 30 min in a shaker. After centrifugation (13000*g*, 10 min) 20 µL of the supernatant were added with 100 µL methanol containing the internal standard, mixed, incubated for 10 min and centrifuged (13000*g*, 10 min). Plasma samples were handled at 4 °C in the same manner as the supernatant after tissue extraction. The final supernatants were used for quantification of the compounds by a 1100-er series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a Kromasil 100 column (125 mm x 2 mm, 5 µm particle size, CS-Chromatographie Service Langerwehe, Germany) and an API 2000 LC-MS/MS-System (Applied Biosystems, Darmstadt, Germany). The analytes were ionised by positive ion (5500 V) electrospray. As eluents, methanol and a methanol:water:acetonitrile:acetic acid mixture (100:90:9:1, v/v/v/v) were used.

RT-PCR analysis

Total RNA of liver, kidney, heart, skeletal muscle and small intestine tissues was isolated by TRIzol reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer´s protocol, resuspended in diethyl pyrocarbonate-treated water and stored at -80°C until use. The concentration and purity of total RNA was determined by ultraviolet absorbance at 260 and 280 nm (SpectraFluor Plus; Tecan, Crailsheim, Germany). The quality of all RNA samples was assessed by agarose gel electrophoresis. cDNA was prepared from total RNA (1.2 µg) by reverse transcription using M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany) and oligo$(dT)_{18}$ primers (Operon Biotechnologies, Cologne, Germany). The mRNA concentrations were measured by realtime detection PCR using SYBR® Green I and a MJ Research Opticon system (Biozym Diagnostik GmbH, Oldendorf, Germany). Realtime detection PCR was performed with 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 µM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies, Cologne, Germany; Table 1). For CTP-1, two different primers encoding for liver type isoform (L-CPT-1) and for muscle type isoform (M-CPT-1) were used. L-CPT-1 primers were used for analysis in liver, kidney and small intestine; M-CPT-1 primers were used for analysis in skeletal muscle and heart. Annealing temperature for all primer pairs was 60 °C. Amplification efficiencies for all primer pairs were determined by template dilution series. Calculation of the relative mRNA concentration was made using the amplification efficiencies and the C_t values[32]. For normalisation, β-actin was used as housekeeping gene.

Plasma NEFA concentration

Plasma NEFA concentrations were measured using a commercial kit (WAKO Chemicals GmbH, Neuss, Germany).

Statistical analysis

Treatment effects were evaluated by one-factorial ANOVA. For significant F values ($P<0.05$), means of the treatments (fasted, E40, E70) were compared pairwise with the control group by Student´s *t* test. Means were considered significantly different for $P<0.05$. Values in the text are given as means ± SD.

Table 1. Characteristics of the specific primers used for RT-PCR analysis

Gene (NCBI Genbank)	Forward Primer	Reverse Primer	Product length, bp
ACO (NM_017340)	CTTTCTTGCTTGCTTTCCTTCTCC	GCCGTTTCACCGCCTCGTA	415
BBD (NM_022629)	ATTCTGCAAAAGCTCGGAAA	CTCCTTGGAGTCCTGCTCTG	183
CAT (NM_001004085)	CCAAGCAGGACTTCATGGAT	TGTGTGGGTGGTTTCTTTGA	232
COT (NM_031987)	GACACCCAGTCCACATGCAAC	GAACCCTTCCATCTCCCTTC	230
CPT-2 (NM_012930)	TCCTCGATCAAGATGGGAAC	GATCCTTCATCGGGAAGTCA	237
L-CPT-1 (NM_031559)	GGAGACAGACACCATCCAACATA	AGGTGATGGACTTGTCAAACC	416
M-CPT-1 (NM_013200)	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG	180
OCTN-1 (NM_012930)	CCTCTCTGGCCTGATTGAAG	CTCCGCTGTGAAGACGTACA	226
OCTN-2 (NM_022270)	AGCATTTGTCCTGGGAACAG	ACTCAGGGATGAACCACCAG	200
TMABA-DH (NM_022273)	TTTGAGACTGAAGCCGAGGT	CACCGGGCTGACGTTATAGT	156
TMLD (AF374406)	GCCCTGTGGCATTCAAGTAT	GGTCCAACCCCTATCATGTG	201
β-Actin (BC063166)	ATCGTGCGTGACATTAAAGAGAAG	GGACAGTGAGGCCAGGATACAG	429

Abbreviations: ACO, acyl-CoA oxidase; BBD, γ-butyrobetaine dioxygenase; CPT, carnitine palmitoyltransferase (L-CPT, liver isoform; M-CPT, muscle isoform); OCTN, novel organic cation/carnitine transporter; TMABA-DH, 4-*N*-trimethylaminobutyraldehyde dehydrogenase; TMLD, 3- hydroxy-*N*-trimethyllysine dioxygenase

Results

Diet intake and weight change of the rats

In the control group, the daily food intake in average of the 10 days was 20.2 ± 1.4 g (means ± SD, n=9). These rats had no change in body weight during the feeding period (initial body weight: 268 ± 37 g, final body weight: 274 ± 35 g, n=9). E70 rats consumed daily 10.5 ± 0 g of food; they lost 28 g of body weight during the feeding period (initial body weight: 265 ± 34 g, final body weight: 237 ± 31 g, n=9); E40 rats consumed daily 6.0 ± 0 g of food; they lost 51 g of body weight (initial body weight: 265 ± 28 g, final body weight: 214 ± 25 g, n=9). In the forth group of rats which was fed the diet *ad libitum* for 9 days and then fasted at the tenth day, the average daily food intake during the first 9 days was 20.5 ± 1.8 g. In these rats, body weight at the end of the experimental period was similar to the initial body weight (initial body weight: 269 ± 34 g, final body weight: 269 ± 28 g, n=9).

Concentrations of NEFA in plasma

E40 rats, E70 rats and fasted rats had higher plasma concentration of NEFA than control rats (control: 13.8 ± 2.5 µmol/L; E70: 23.7* ± 3.4; E40: 23.5* ± 4.1; fasted: 19.8* ± 5.9, n=9 for each group, means ± SD; *P<0.05 vs. control)

Tissue mRNA concentrations of ACO

E40 rats, E70 rats and fasted rats had a higher mRNA concentration of ACO in liver, heart and kidney than control rats (P<0.05, Figure 1). Fasted rats had also an increased mRNA concentration of ACO in skeletal muscle compared to control rats (P<0.05, Figure 1); E40 rats and E70 rats did not differ in ACO mRNA concentration in skeletal muscle from control rats (Figure 1). ACO mRNA concentration in intestinal mucosa was reduced in E40 rats, E70 rats and fasted rats compared to control rats (P<0.05, Figure 1).

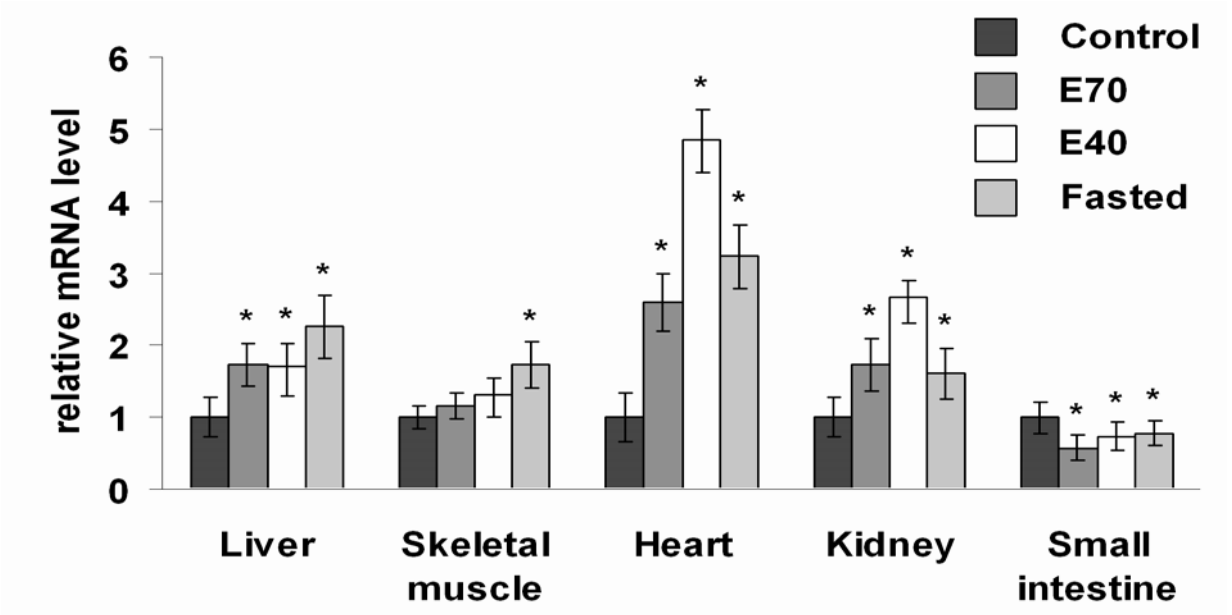

Fig. 1. Effect of caloric restriction or fasting on mRNA concentration of acyl-CoA oxidase in liver, skeletal muscle, heart, kidney and small intestine of rats. Rats were fed *ad libitum* (control) or obtained 10.5 or 6.0 g of diet/d according to 70% (E70) and 40% (E40), respectively, of the energy requirement for maintenance for 10 days. Fasted rats were fed *ad libitum* for 9 days and then fasted for 24 h. Total RNA was extracted from tissues and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalisation. Values are means ± SD (n=9). *Significantly different from control rats (P<0.05).

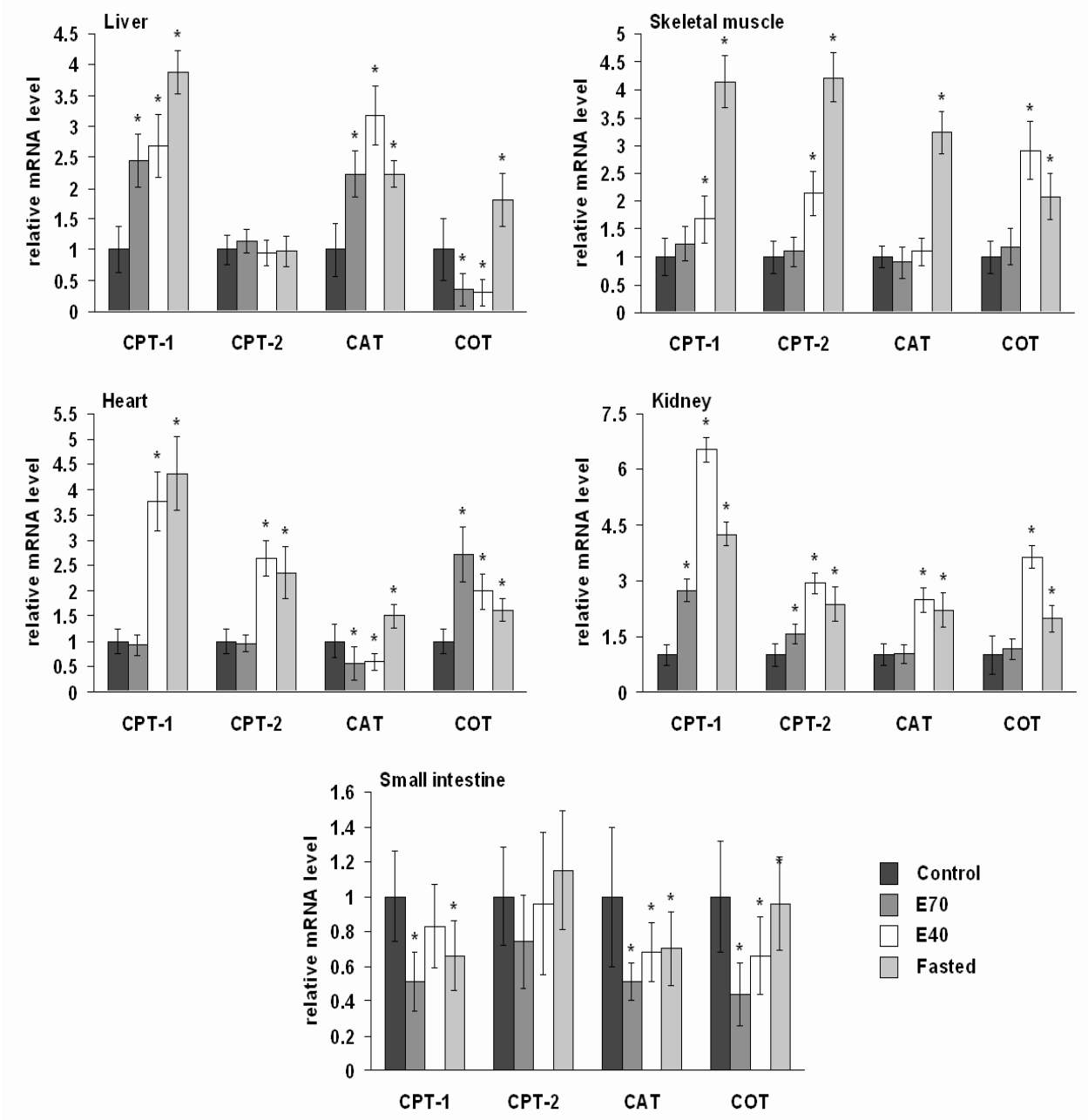

Fig. 2. Effect of caloric restriction or fasting on mRNA concentration of carnitine palmitoyltransferases (CPT)-1 and -2, carnitine acetyltransferase (CAT) and carnitine octanoyltransferase (COT) in liver, skeletal muscle, heart, kidney and small intestine of rats. Rats were fed *ad libitum* (control) or obtained 10.5 or 6.0 g of diet/d according to 70% (E70) and 40% (E40), respectively, of the energy requirement for maintenance for 10 days. Fasted rats were fed *ad libitum* for 9 days and then fasted for 24 h. Total RNA was extracted from tissues and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalisation. Values are means ± SD (n=9). *Significantly different from control rats (P<0.05).

Relative mRNA concentration of carnitine acyltransferases (CPT-1, CPT-2, CAT, COT) in tissues.

Liver: E40 rats and E70 rats had higher mRNA concentrations of CPT-1 and CAT and a lower mRNA concentration of COT than control rats (P<0.05, Figure 2). In fasted rats, mRNA concentrations of CPT-1, CAT and COT were higher in the liver than in control rats (P<0.05, Figure 2). mRNA concentration of CPT-2 did not differ between the four groups of rats.

Skeletal muscle: E70 rats did not differ in mRNA concentrations of CPT-1, CPT-2, CAT and COT in skeletal muscle from control rats (Figure 2). E40 rats had higher mRNA concentrations of CPT-1, CPT-2 and COT in skeletal muscle than control rats (P<0.05, Figure 2); mRNA concentration of CAT was unchanged in E70 rats compared to control rats. Fasted rats had higher mRNA concentrations of CPT-1, CPT-2, CAT and COT in skeletal muscle than control rats (P<0.05, Figure 2).
Heart: In E70 rats, mRNA concentration of CAT in heart was lower and that of COT was higher than in control rats (P<0.05, Figure 2); mRNA concentrations of CPT-1 and CPT-2 did not differ between E70 rats and control rats (Figure 2). In E40 rats, mRNA concentrations of CPT-1, CPT-2 and COT in heart were increased and that of CAT was decreased compared to control rats (P<0.05, Figure 2). In fasted rats, mRNA concentrations of all these genes (CPT-1, CPT-2, COT, CAT) in heart were increased compared to control rats (P<0.05, Figure 2).
Kidney: In E70 rats, mRNA concentrations of CPT-1 and CPT-2 in kidney were higher than in control rats (P<0.05); mRNA concentrations of CAT and COT in kidney of E70 rats did not differ from those of control rats (Figure 2). In E40 rats, mRNA concentrations of all these genes (CPT-1, CPT-2, COT, CAT) were increased compared to control rats (P<0.05).
Small intestine: E70 rats had lower mRNA concentrations of CPT-1, CAT and COT in small intestine than control rats (P<0.05); mRNA concentration of CPT-2 in small intestine was unchanged in E70 rats compared to control rats (Figure 2). In E40 rats, mRNA concentrations of CAT and COT were reduced compared to control rats (P<0.05) whereas mRNA concentrations of CPT-1 and CPT-2 were unchanged (Figure 2). In fasted rats, mRNA concentrations of CPT-1 and CAT were reduced (P<0.05), those if CPT-2 and COT were unchanged compared to control rats (Figure 2).

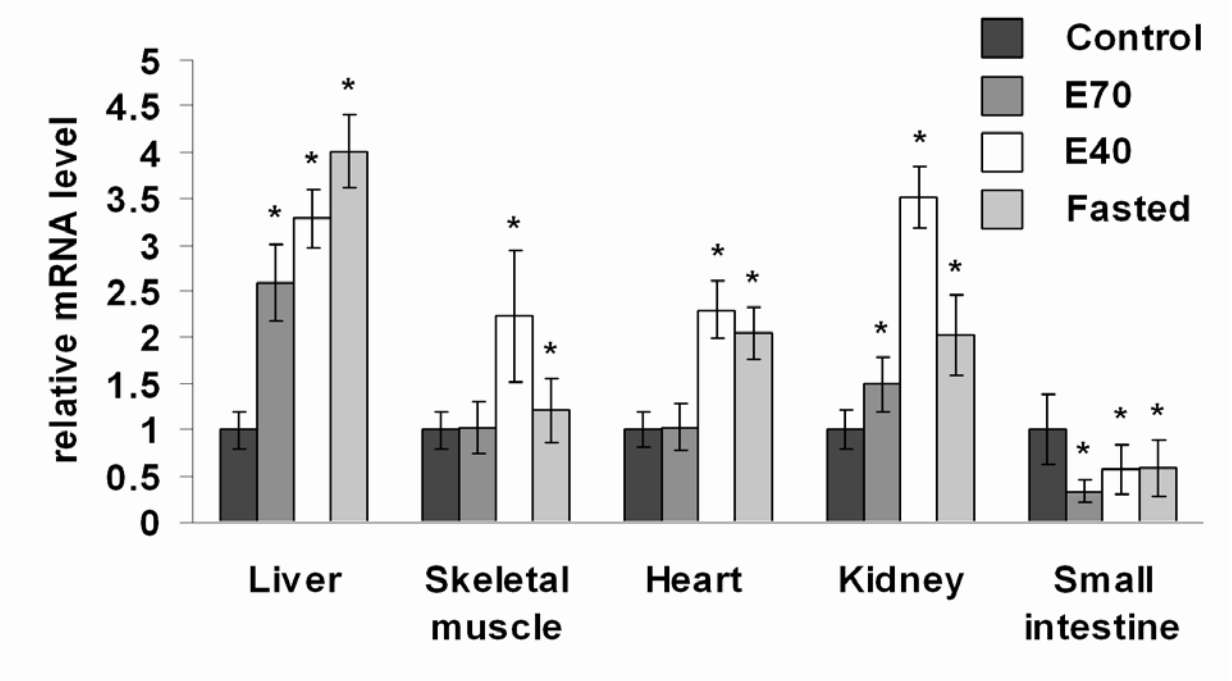

Fig. 3. Effect of caloric restriction or fasting on mRNA concentration of organic cation transporter (OCTN)-2 in liver, skeletal muscle, heart, kidney and small intestine of rats. Rats were fed *ad libitum* (control) or obtained 10.5 or 6.0 g of diet/d according to 70% (E70) and 40% (E40), respectively, of the energy requirement for maintenance for 10 days. Fasted rats were fed *ad libitum* for 9 days and then fasted for 24 h. Total RNA was extracted from tissues and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalisation. Values are means ± SD (n=9). *Significantly different from control rats (P<0.05).

Relative mRNA concentration of OCTN2 in tissues

E70 rats had an increased mRNA concentration of OCTN2 in liver and kidney and a reduced mRNA concentration in small intestine compared to control rats ($P<0.05$, Figure 3); OCTN2 mRNA concentration in skeletal muscle and heart and was not different between these two groups (Figure 3). E40 rats had a higher mRNA concentration of OCTN2 in liver, skeletal muscle, heart and kidney and a lower concentration in small intestine than control rats ($P<0.05$, Figure 3). Fasted rats had a higher mRNA concentration of OCTN2 in liver, heart and kidney than control rats ($P<0.05$); mRNA concentration of OCTN2 in small intestine was lower in fasted rats than in control rats ($P<0.05$) whereas that in skeletal muscle did not differ between both groups (Figure 3).

Concentrations of carnitine, BB and TML in plasma and tissues

Plasma: E70 rats, E40 rats and fasted rats had lower concentrations of free carnitine and higher concentrations of acetyl carnitine in plasma than control rats ($P<0.05$, Table 2). Accordingly, the ratio between free and acetyl carnitine in plasma was lower in E70 rats, E40 rats and fasted rats than in control rats ($P<0.05$, Table 2). Concentration of total carnitine in plasma was not different between the four groups of rats (Table 2). E70 and E40 rats had lower concentrations of BB in plasma than control rats ($P<0.05$); fasted rats did not differ in plasma BB concentration from control rats (Table 2). Plasma concentration of TML did not differ between the four groups of rats (Table 2).

Liver: E70 rats, E40 rats and fasted rats had a higher concentration of free and total carnitine, a lower concentrations of acetyl carnitine and a higher ratio between free and acetyl carnitine in the liver than control rats ($P<0.05$, Table 2). E70 rats and E40 rats had a lower concentration of BB and a higher concentration of TML in the liver than control rats ($P<0.05$, Table 2). Fasted rats did not differ in hepatic BB and TML concentrations from control rats (Table 2).

Skeletal muscle: E70 rats, E40 rats and fasted rats had a higher concentration of free carnitine, a lower concentration of acetyl carnitine and a higher ratio between free and acetyl carnitine in skeletal muscle than control rats ($P<0.05$). E40 rats had also an increased concentration of total carnitine in skeletal muscle compared to control rats ($P<0.05$); E70 rats and fasted rats did not differ in total carnitine concentration in skeletal muscle from control rats (Table 2). Concentrations of BB and TML in skeletal muscle did not differ between the four groups of rats (Table 2).

Heart: E70, E40 and fasted rats had a higher concentration of free carnitine, a lower concentration of acetyl carnitine, a higher ratio between free carnitine and acetyl carnitine and a higher concentration of BB in heart than control rats ($P<0.05$, Table 2). E40 rats and fasted rats, but not E70 rats, had a higher concentration of total carnitine in heart than control rats. Concentration of TML in heart did not differ between the four groups of rats.

Kidney: E70 rats, E40 rats and fasted rats had higher concentrations of free carnitine and total carnitine and a lower concentration of TML in the kidney than control rats ($P<0.05$, Table 2). The concentrations of acetyl carnitine and BB in kidney did not differ between the four groups of rats (Table 2).

Relative mRNA concentration of hepatic enzymes involved in carnitine biosynthesis

In E40 rats and E70 rats, hepatic mRNA concentrations of TMLD and BBD were lower than in control rats ($P<0.05$); mRNA concentration of TMABA-DH in E40 rats and in E70 rats did not differ from that of control rats (Figure 4). In fasted rats, hepatic mRNA concentrations of TMLD, TMABA-DH and BBD did not differ from those of control rats (Figure 4).

Table 2. Concentrations of free carnitine, acetyl carnitine, total carnitine, BB and TML in plasma and tissues of control rats, rats receiving 70% (E70) or 40% (E40) of their energy requirement for maintenance and rats fasted for 24 h

Treatment	Control		E70		E40		Fasted	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Plasma (μmol/L)								
Free carnitine	22.4	4.1	15.7*	4.7	15.1*	2.0	17.8*	4.4
Acetyl carnitine	11.4	4.5	16.3*	6.2	18.2*	3.2	15.5*	4.1
Total carnitine	31.5	10.7	33.2	14.4	35.2	6.7	33.6	8.2
Free:acetyl ratio[#]	1.7	0.4	1.0*	0.2	0.9*	0.1	1.2*	0.1
BB	0.39	0.08	0.32*	0.07	0.31*	0.06	0.43	0.11
TML	0.91	0.16	1.06	0.21	1.01	0.18	0.93	0.30
Liver (nmol/g)								
Free carnitine	318	30	395*	47	442*	43	382*	48
Acetyl carnitine	2.9	1.0	1.1*	0.3	1.2*	0.3	1.5*	0.4
Total carnitine	322	30	390*	43	445*	43	385*	38
Free:acetyl ratio[#]	163	69	349*	95	444*	104	256*	25
BB	4.32	0.66	3.41*	0.53	3.44*	0.40	4.70	0.93
TML	1.94	0.37	3.53*	1.01	3.42*	0.66	2.43	0.47
Skeletal muscle (nmol/g)								
Free carnitine	591	128	713*	71	774*	139	740*	104
Acetyl carnitine	192	50	137*	37	145*	42	134*	47
Total carnitine	777	170	849	84	920*	157	876	115
Free:acetyl ratio[#]	3.3	0.7	5.3*	1.4	5.7*	2.0	6.0*	1.7
BB	12.0	2.8	10.5	2.9	10.8	4.5	12.7	2.7
TML	16.5	3.7	18.1	4.4	18.7	3.9	17.0	4.2
Heart (nmol/g)								
Free carnitine	637	132	761*	106	798*	81	923*	85
Acetyl carnitine	147	24	91*	44	107*	31	54*	25
Total carnitine	785	159	820	97	907*	91	979*	63
Free:acetyl ratio[#]	4.4	1.0	10.5*	4.3	7.7	1.8	19.2*	7.3
BB	12.5	2.6	15.7*	3.7	16.0*	2.6	17.6*	3.3
TML	9.4	2.1	10.5	3.0	10.5	2.8	11.1	2.6
Kidney (nmol/g)								
Free carnitine	293	61	374*	54	405*	43	377*	46
Acetyl carnitine	2.9	0.6	2.9*	0.9	3.0	0.5	2.6	0.5
Total carnitine	297	53	377*	87	408*	43	380*	46
Free:acetyl ratio[#]	104	19	133	40	140	28	150*	43
BB	16.8	2.7	16.5	2.2	16.6	2.1	17.0	3.7
TML	27.9	9.7	20.4*	5.7	17.7*	6.0	19.1*	5.5

* Significantly different from control ($P<0.05$). n=9 for each group.
[#] mol/mol

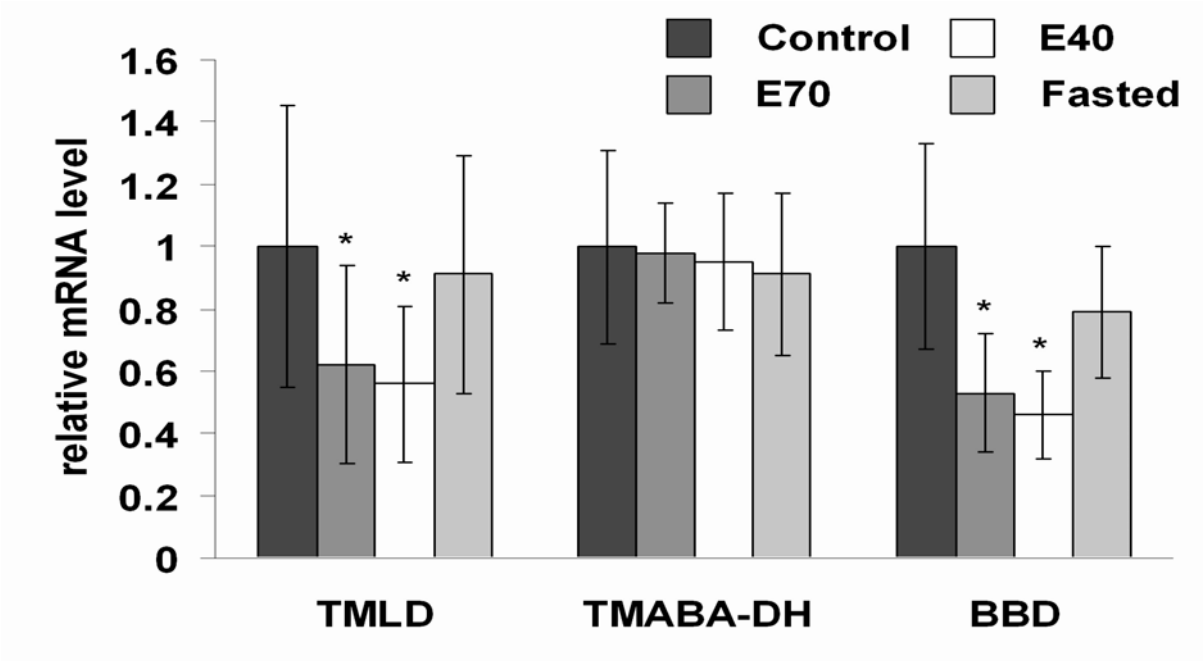

Fig. 4. Effect of caloric restriction or fasting on mRNA concentration of trimethyllysine dioxygenase (TMLD), 4-N-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH) and γ-butyrobetaine dioxygenase (BBD) in the liver of rats. Rats were fed *ad libitum* (control) or obtained 10.5 or 6.0 g of diet/d according to 70% (E70) and 40% (E40), respectively, of the energy requirement for maintenance for 10 days. Fasted rats were fed *ad libitum* for 9 days and then fasted for 24 h. Total RNA was extracted from tissues and mRNA concentrations were determined by realtime detection RT-PCR analysis using β-actin mRNA concentration for normalisation. Values are means ± SD (n=9). *Significantly different from control rats (P<0.05).

Discussion

This study was performed to investigate the effect of energy deficiency or fasting on gene expression of OCTN2 in tissues and carnitine homeostasis in rats. For this purpose, rats received diets providing either 70 or 40% of their energy demand for maintenance for 10 days or were fasted for 24 h. As expected, energy restriction to 70 or 40%, respectively, of the requirement of energy for maintenance led to a considerable loss of body weight. Mobilisation of TAG from adipose tissue by energy restriction or fasting moreover caused an increase in the concentrations of NEFA in plasma. To study whether increased concentrations of NEFA in plasma caused activation of PPARα, we determined mRNA concentration of ACO, a classical PPARα target gene[29], in liver, skeletal muscle, heart, kidney and in the mucosa of small intestine. The finding that ACO was up-regulated in liver, skeletal muscle, heart and kidney in energy restricted and in fasted rats, confirms activation of PPARα in these tissues by free fatty acids released from adipose tissues. It was thus not surprising that CPT-1, another PPARα target gene[29], was also up-regulated in tissues in energy restricted and fasted rats. The finding that transcription of CPT-2 was less up-regulated by fasting or energy restriction than CPT-1 is due to the fact that PPARα up-regulates CPT-2 less strong than CPT-1[33]. To our knowledge, less is known about the regulation of CAT and COT. The present study shows that both of these carnitine acyltransferases are up-regulated by fasting in tissues in which PPARα activation occurred, as indicated by up-regulation of ACO. However, in energy restricted rats, there was no uniform effect on mRNA concentrations of CAT and COT in various tissues. In some tissues, COT or CAT were up-regulated, in others they were down-regulated. As ACO was up-regulated in all tissues of caloric restricted rats, indicative of activation of PPARα, it is suggested that up-regulation of COT and CAT is not mediated by

PPARα. This suggestion is confirmed by another study in which mice were fasted for either 48 or 72 h[25]. In that study, mRNA concentrations in CAT and COT did not increase in kidney although mRNA concentration of ACO was markedly increased.

The present study shows for the first time that fasting or caloric restriction leads to an up-regulation of OCTN2 in liver, heart, kidney, and in rats with strong caloric restriction (E40 rats) additionally in skeletal muscle. These are the tissues in which fasting or caloric restriction caused also activation of PPARα as indicated by up-regulation of ACO. Recently, we have observed that treatment of rats with PPARα agonists such as clofibrate or oxidised fats causes an up-regulation of OCTN2 in tissues of rats[17, 21]. The present findings in energy restricted and fasted rats strengthen the hypothesis that up-regulation of gene expression of OCTN2 in tissues is mediated by PPARα activation.

The present study moreover shows for the first time that strong caloric restriction increases total carnitine concentrations not only in liver but also in other tissues such as skeletal muscle, heart and kidney. As OCTN2 catalyses the transport of carnitine into cells, we suggest that increased carnitine concentrations in these tissues are at least partially due to the up-regulation of OCTN2. An increased uptake of carnitine from plasma into tissues should normally lead to a reduced plasma carnitine concentration as recently observed in rats treated with clofibrate or oxidised fat[17, 21]. We assume that plasma carnitine concentration was not lowered in fasted and energy restricted rats in spite of an increased uptake into cells because re-absorption of carnitine from the urine may have been increased due to an up-regulation of OCTN2 in kidney.

Our data show that OCTN2 in scraped mucosa of small intestine was in opposite to other tissues not up-regulated but even down-regulated in caloric restricted and in fasted rats. The fact that ACO was also down-regulated suggests that those rats had even a lower PPARα activity in small intestine than control rats. Under the assumption that expression of OCTN2 is controlled by PPARα, a lower activity of PPARα would explain the lower mRNA concentration of OCTN2 in small intestine of caloric restricted and fasted rats. It should be noted, however, that other studies in opposite to the present one found a slight up-regulation of PPARα target genes in intestinal mucosa of fasted rats[34,35]. The contradiction between our study and those studies in this respect cannot be explained. Nevertheless, as OCTN2 is responsible for the dietary absorption of carnitine, a down-regulation of this transporter indicates that absorption of carnitine from the diet could have been reduced in these rats. As the diet used in this study had a very low carnitine concentration, a potential impairment of intestinal absorption of carnitine in caloric restricted rats probably had less impact for total carnitine homeostasis. Nevertheless, the data of this study open the possibility that the utilisation of carnitine from the diet could be impaired under caloric restriction.

Another finding of this study was that fasting or energy restriction leads to an increase of the ratio between free carnitine and acetyl carnitine in most of the tissues analysed. Esterification of carnitine with acetate by CAT and release of acetate from the ester in the mitochondrion regulates the concentration of acetyl-CoA[36]. We assume that the amount of acetyl-CoA in the mitochondrion available for esterification of free carnitine was reduced in fasted or energy restricted rats which could be an explanation for the observation that tissue concentrations of carntine were increased and those of acetyl carnitine were reduced. It is unclear, however, why there was an opposite situation in plasma where the concentration of free carnitine was reduced and that of acetyl carnitine was increased. OCTN2 is able to transport both, free carnitine and acetyl carnitine, and it has even a higher affinity for acetyl carnitine than for free carnitine[12,14,37]. If the concentration of free carnitine in plasma was reduced due to an increased uptake into tissues, a similar effect would have been expected for acetyl carnitine. In contrast to this study, treatment of rats with clofibrate caused a reduced concentration of both, free carnitine and acetyl carnitine. It was suggested that this effect is due to an increased uptake of free carnitine and acetyl carnitine by up-regulation of OCTN2 in

tissues[17]. Therefore, it is suggested that the increased concentration of acetyl carnitine in plasma of fasted and energy restricted rats is not primarily due to up-regulation of OCTN2 but to the low energy status of the rats.

TML is the first metabolite of carnitine biosynthesis pathway which is generated by lysosomal degradation of proteins containing trimethylated lysine residues (such as calmodulin, histones, actin and myosin)[6]. As energy restriction or fasting stimulates protein breakdown, we expected increased concentrations of TML in plasma and tissues of fasted and energy restricted rats. However, our analyses revealed increased TML concentrations only in liver of energy restricted rats but not in plasma or any other tissue. TML is converted in tissues into BB which is transported to the liver where it acts as a precursor of carnitine. In energy restricted rats, concentration of BB was also not increased in most tissues with the only exception of heart. This suggests that supply of the liver with BB from extrahepatic tissues was not markedly increased during fasting or energy restriction. The fact that BB concentrations in liver of energy restricted rats were even reduced compared to control rats opens the possibility that more BB was converted into carnitine in the liver which could contribute to the increased hepatic total carnitine concentration observed in these rats. Conversion of BB into carnitine is catalysed by BBD. The finding that mRNA concentrations of that enzyme as well as that of TMLD were reduced in liver of E70 rats and in E40 rats however suggests that carnitine synthesis in the liver was rather reduced than increased by energy restriction. However, we are aware that mRNA concentration of enzymes involved in carnitine synthesis must not necessarily reflect their activities. The reduced concentration of BB in plasma of energy restricted rats could be due to an increased uptake of BB into tissues by OCTN2 which was up-regulated in most tissues of these rats, including liver. Interestingly, the effect of short-term fasting on plasma and liver BB concentrations and mRNA concentrations of hepatic enzymes involved in carnitine synthesis in fasted rats was different from that of energy restriction over ten days. The finding that BB concentrations in plasma and liver and mRNA concentrations of enzymes involved in carnitine synthesis were not altered in fasted rats suggests that carnitine biosynthesis was not altered in these rats. The effect of PPARα activation on hepatic carnitine biosynthesis has not yet been clarified. Paul et al.[37] proposed that an increase of hepatic carnitine concentration observed in rats was due to an increased rate of hepatic carnitine biosynthesis. In contrast, in another study[38] there was no increased activity of enzymes of carnitine biosynthesis in the liver of rats treated with phytol, a natural PPARα agonist, although those rats had elevated hepatic carnitine concentrations.

In conclusion, this study shows for the first time that fasting or energy restriction leads to an up-regulation of OCTN2 in various tissues such as liver, muscle, heart and kidney. It is proposed that this effect is mediated by PPARα activation in these tissues. It is also shown that strong energy restriction causes an increase of total carnitine concentrations in liver, skeletal muscle and heart. This may be due to an increased uptake of carnitine from plasma into these tissues by OCTN2 and to an increased capacity of the kidney to reabsorb carnitine from the urine.

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4. Diskussion

4.1 Effekte einer PPARα-Aktivierung beim Schwein

Einen zentralen Ausgangspunkt der vorliegenden Untersuchungen stellen die bekannten Speziesunterschiede zwischen proliferierenden und nicht-proliferierenden Spezies dar, welche die Übertragbarkeit der Ergebnisse aus Studien mit Ratten und Mäusen auf die physiologischen Wirkungen des PPARα auf den Menschen nur begrenzt zulassen. Das Schwein zählt wie der Mensch zu den nicht-proliferierenden Spezies und wurde wegen seiner hohen Ähnlichkeit zum Menschen als Modelltier für Untersuchungen ernährungsbedingter pathologischer Prozesse, wie Diabetes, Adipositas und Atherosklerose bereits erfolgreich in mehreren Studien eingesetzt (Gerrity et al., 2001; Johansen et al., 2001; Roehe et al., 2003; Xi et al., 2004; Kim et al., 2004; Sebert et al., 2005). Hinsichtlich des PPARα sind bisher jedoch nur wenige Untersuchungen für das Schwein bekannt (Ding et al., 2000; Sundvold et al., 2001; Peffer et al., 2005; Cheon et al., 2005). In den vorliegenden Studien sollten die Effekte einer PPARα-Aktivierung durch synthetische und natürliche Liganden beim Schwein ausführlich untersucht werden, um dieses Modelltier näher zu charakterisieren und mögliche Rückschlüsse auf die Vergleichbarkeit der Beobachtungen auf die Humanphysiologie ziehen zu können.

4.1.1 Effekte synthetischer und natürlicher Liganden des PPARα beim Schwein

Um die Effekte einer PPARα-Aktivierung beim Schwein zu untersuchen, wurde den Tieren über 28 Tage täglich 5 g Clofibrat pro kg Diät oral verabreicht, was im Versuch einer Dosis von 220 mg pro kg Körpergewicht entsprach. Diese Dosierung wurde bereits in anderen Studien eingesetzt und stellt in etwa das Zehnfache der Menge dar, wie sie von Patienten als hypolipidämisches Medikament eingenommen wird.

Anhand der Quantifizierung des PPARα im Lebergewebe konnte in Studie 1 gezeigt werden, dass unter anderem die quantitativen Unterschiede der zellulären Konzentration des Rezeptors zwischen proliferierenden und nicht-proliferierenden Spezies für die unterschiedliche Aktivierbarkeit und Stärke der Effekte des PPARα verantwortlich sind (Tugwood et al., 1998; Ammerschlaeger et al., 2004). Im Gegensatz zur Ratte und dem Menschen (Gandemier et al., 1982; Semenkovich et al., 1995) war im vorliegenden Versuch der PPARα in Leber und Fettgewebe gleich stark exprimiert. Dies deutet darauf hin, dass das Fettgewebe beim

Schwein eine bedeutende Rolle im Lipidstoffwechsel besitzt (Ding et al., 1999, 2000; Wang et al., 2006). Durch die Erhöhung der mRNA-Konzentrationen bekannter Zielgene des PPARα sowohl in der Leber als auch im Fettgewebe nach der Verabreichung von Clofibrat, konnte erstmals gezeigt werden, dass beim Schwein auch das Fettgewebe in der Vermittlung einer PPARα-induzierten Modulation des Lipidstoffwechsels involviert ist und die Steigerung der Fettsäureoxidation in diesem Gewebe möglicherweise zur Senkung der Triglyzeride beigeträgt. Eine vermehrte Aufnahme triglyzeridreicher Lipoproteine durch eine erhöhte LPL-Aktivität, beziehungsweise durch eine verminderte Konzentration des ApoC-III, welches die LPL hemmt (Staels et al., 1998), konnte anhand der Expressionsanalysen der vorliegenden Untersuchungen nicht beobachtet werden. Daraus kann geschlussfolgert werden, dass vor allem die Erhöhung der Fettsäureoxidation in der Leber und nicht die vermehrte Lipolyse von triglyzeridreichen Lipoproteinen im Versuch zu erniedrigten Konzentrationen der Triglyzeride im Plasma führte. Eine Erhöhung des HDL-Cholesterols, vermutlich durch eine gesteigerte hepatische Synthese von ApoA-I und ApoA-II durch Fibrate beim Menschen (Caslake et al., 1993; Vu-Dac et al., 1995; Berthou et al., 1996), konnte nicht beobachtet werden. Vielmehr ist die Senkung des LDL- und des HDL-Cholesterols mit den Ergebnissen aus Studien an Hamstern und Ratten vergleichbar (Guo et al., 2001; König et al., 2007). So stimmen die Effekte einer Fibrat-induzierten PPARα-Aktivierung auf die Plasmalipide beim Schwein im großen Umfang mit denen anderer Modelltiere und dem Menschen überein. Hinsichtlich der mechanistischen Hintergründe, welche für die Senkung der Plasmalipide verantwortlich sind, sowie funktioneller Aspekte des PPARα, scheinen beim Schwein jedoch Unterschiede im Vergleich zu anderen Spezies zu existieren.

Um einen detaillierten Erklärungsansatz der hypolipidämischen Wirkung des Clofibrat zu erhalten, sollte im Rahmen der Untersuchungen am Schwein in Betracht gezogen werden, dass die lipidsenkenden Eigenschaften des PPARα möglicherweise durch eine Beeinflussung weiterer Transkriptionsfaktoren und Proteine, welche an der Regulation der Homöostase des Lipidstoffwechsels beteiligt sind, moduliert werden. Aus mehreren Studien sind bereits direkte Interaktionen zwischen dem PPARα und dem SREBP-1c bekannt (Patel et al., 2001; Sandberg et al., 2005; Neess et al., 2006; Hsu und Huang, 2006). Außerdem konnten Interaktionen mit dem SREBP-2 beschrieben werden (Guo et al., 2001). In einer aktuellen Studie konnten König et al. (2007) zeigen, dass die Gabe von Clofibrat bei Ratten in der Leber neben der verminderten Expression und verringerten nuklearen Konzentrationen des SREBP-2, zu einer Erhöhung der Expression für die Proteine der *insulin-induced genes* (Insigs) führte. Insig-1 und Insig-2 blockieren die proteolytische Spaltung und die

transkriptionelle Aktivität der SREBPs und sind so an der Modulation des zellulären Lipidstoffwechsels beteiligt (Goldstein et al., 2006).

In den vorliegenden Untersuchungen konnte in der Leber und dem Fettgewebe beim Schwein kein Einfluss einer PPARα-Aktivierung auf die Expression der SREBPs, dessen Zielgenen oder der Insigs beobachtet werden. Dies stimmt mit einer Studie an Mäusen überein, bei denen nach Verabreichung des synthetischen PPARα-Liganden WY-14643 ebenfalls kein Einfluss auf die hepatische Expression der SREBPs und Insigs beobachtet werden konnte (Knight et al., 2005). So kann aus den vorliegenden Untersuchungen geschlussfolgert werden, dass die SREBPs und Insigs nicht an der Vermittlung der hypolipidämischen Wirkung des PPARα beim Schwein involviert sind. Dies stellt somit einen weiteren Unterschied zwischen dem Schwein, als nicht-proliferierende Spezies, und der Ratte dar. In neueren Untersuchungen konnte bei Patienten nach der Einnahme verschiedener Fibratpräparate ein cholesterolsenkender Effekt gezeigt werden, ohne dass es jedoch zu Veränderungen der hepatischen Expression des SREBP-2 oder der HMG-CoA-Reduktase kam (Roglans et al., 2004). Zusammenfassend mit der moderaten Induktion des PPARα sind diese Beobachtungen als Indizien dafür zu bewerten, dass das Schwein, im Vergleich zu Ratte und Maus, als Modelltier hinsichtlich der Effekte synthetischer Liganden des PPARα eine bessere Übertragbarkeit auf den Menschen gewährleistet.

Diese Vermutung wird durch die Untersuchungen der Studie 3 bekräftigt. Dabei konnten die Mechanismen, welche für die Enstehung eines Fibrat-induzierten Leberkrebses bei Nagern vermutet werden (Peters et al., 2005), beim Schwein nicht beobachtet werden. Dies bestätigt die Annahme, dass nicht-proliferierende Spezies refraktär hinsichtlich dieser Effekte sind (Lake et al., 1989, 2000; Makowska et al., 1992). Zwar konnten ein erhöhtes Lebergewicht und eine Peroxisomenproliferation festgestellt werden, jedoch scheint wegen der nur moderat zu beobachtenden Effekte durch Clofibrat das Schwein auch in diesem Zusammenhang eine hohe Ähnlichkeit mit dem Menschen zu besitzen. So ist das Schwein als alternatives Tiermodell anscheinend besser geeignet, die Effekte von Fibraten und weiteren sogenannten Peroxisomen-Proliferatoren (PPs) (Hess et al., 1965; Reddy und Krishnakantha, 1975; Reddy et al., 1980) im Hinblick auf die Risikoabschätzung für den Menschen zu untersuchen. Allerdings kann in den vorliegenden Untersuchungen anhand der gesteigerten hepatischen Expression von Proto-Onkogenen ein Risiko bei langfristiger Fibratgabe für nicht-proliferierende Spezies nicht restlos ausgeschlossen werden. Aufgrund der Tatsache, dass die Fibrate seit Jahrzehnten als hypolipidämische Medikamente in der Humanmedizin verabreicht

werden, sollte dieser Aspekt mit besonderer Relevanz für den Menschen in weiteren Studien berücksichtigt und ausführlicher untersucht werden.

Ausgehend von der offensichtlich hohen Ähnlichkeit zwischen dem Schwein und dem Menschen sollte in Studie 4 der Frage nachgegangen werden, in welchem Umfang die Aufnahme thermisch oxidierter Fette über die Nahrung eine Aktivierung des PPARα bei nicht-proliferierenden Spezies bewirkt, wie es bei Ratten bereits gezeigt werden konnte (Chao et al., 2001, 2004, 2005; Sülzle et al., 2004). Zu diesem Zweck wurde ein Fett in Anlehnung an thermisch behandeltes Fett, wie es in der westlichen Humanernährung häufig vorkommt, eingesetzt. Die moderate Oxidation des eingesetzten Fettes führt dazu, dass dieses vor allem Hydroxid- und Hydroperoxidverbindungen als primäre Oxidationsprodukte von Fettsäuren enthält, welche bekannte Aktivatoren des PPARα darstellen (Delerive et al., 2000; Mishra et al., 2004; König und Eder, 2006). In den vorliegenden Untersuchungen konnte dabei erstmals gezeigt werden, dass ein thermisch oxidiertes Fett auch beim Schwein, als Vertreter nicht-proliferierender Spezies, zu einer moderaten PPARα-Aktivierung führt. Dies war begleitet von einer moderaten Peroxisomenproliferation. Allerdings konnten die triglyzeridsenkenden Eigenschaften einer PPARα-Aktivierung durch oxidierte Fette, wie sie für Ratten beschrieben wurden (Eder und Kirchgessner, 1998; Chao et al., 2001; Eder et al., 2003), beim Schwein nicht beobachtet werden. Natürlich kann damit argumentiert werden, dass ein stärker oxidiertes Fett womöglich stärkere Effekte hervorrufen könnte. Allerdings scheinen auch hier wieder vor allem Speziesunterschiede hinsichtlich des PPARα ausschlaggebend für die unterschiedlichen Beobachtungen zu sein, wie es bereits in Zellkulturstudien gezeigt werden konnte (König und Eder, 2006). Eine stärkere Oxidation des Fettes könnte zudem auch zu einer stärkeren Induktion oxidativen Stresses führen. Fasst man also die untergeordnete Rolle einer PPARα-Aktivierung auf die Plasmalipide, die Induktion oxidativen Stresses und eine mögliche induzierte Lipogenese aus Studie 3 zusammen, so ist die Aufnahme oxidierter Fette bei nicht-proliferierenden Spezies, auch in Bezug auf die Humanernährung eher als kritisch einzuschätzen. Dabei sollte auch die mögliche Beteiligung an der Entstehung atherosklerotischer Prozesse berücksichtigt werden (Staprans et al., 1999, 2005; Obinata et al., 2005).

4.1.2 Effekte synthetischer und natürlicher Liganden des PPARα auf den Schilddrüsenstoffwechsel

Wie in Studie 2 ausführlich belegt werden konnte, führte die Gabe von Clofibrat beim Schwein zu Modifikationen des Schilddrüsenstoffwechsels. Dabei konnte gezeigt werden, dass die Clofibrat-induzierte Erhöhung der UGT-Aktivitäten in der Leber für die starke Senkung von T_3 und T_4 im Plasma verantwortlich ist. Aus den Ergebnissen der Expressionsanalysen in der Schilddrüse kann auf eine vermehrte hypophysäre *thyroid stimulating hormone* (TSH)-Ausschüttung geschlossen werden, welche jedoch nicht in der Lage war, die aus hepatischer Eliminierung resultierende Senkung der Plasmakonzentrationen zu kompensieren. Damit konnte erstmals *in vivo* gezeigt werden, dass Clofibrat bei einer nicht-proliferierenden Spezies zu einer Störung der thyroidalen Hormonachse führt.

Der Einfluss auf die Schilddrüsenfunktion durch die Induktion hepatischer UGT-Aktivitäten für T_3 und T_4 ist für verschiedene chemische Verbindungen gezeigt worden (Visser et al., 1993). Dabei erhöhten jedoch nur jene Substanzen den TSH-Spiegel im Plasma, welche die Glucuronidierung von T_3 erhöhten (Hood und Klaassen, 2000), wie es im vorliegenden Versuch auch für Clofibrat gezeigt wurde. Weiterhin konnten durch relativ moderate Erhöhungen des TSH eine vermehrte Zellproliferation der Schilddrüsenfollikel beobachtet werden, woraufhin Klaassen und Hood (2001) vermuteten, dass diese Störung des Schilddrüsenstoffwechsels langfristig zur Krebsentstehung in der Schilddrüse führen können. Dies unterstützt die Vermutung der gesteigerten Schilddrüsenfunktion durch erhöhte TSH-Spiegel beim Schwein in Studie 2. Zwar konnten im Versuch über einen Zeitraum von 28 Tagen beim Schwein keine morphologischen Veränderungen der Schilddrüse festgestellt werden. Allerdings ist nicht auszuschließen, dass diese Veränderungen erst bei längerfristiger Fibratgabe auftreten und dadurch ein Risiko für die Entstehung von Erkrankungen der Schilddrüse darstellen.

Dabei ist noch immer unklar, welche spezifischen Isoformen der hepatischen UGT für die Glucuronidierung von T_3 und T_4 verantwortlich sind. Auch war bisher unklar, in welchem Rahmen der PPARα an der Induktion der UGT-Aktivitäten beteiligt ist. Funktionelle PPREs konnten bereits für UGT1A9 und UGT2B4 nachgewiesen werden (Barbier et al., 2003a; 2003b). In einer aktuellen Studie konnte eine Induktion des humanen UGT1 Locus in transgenen Mäusen durch PPARα-Agonisten in Leber und Darm gezeigt werden. Außerdem konnten in HepG2-Zellen für mehrere UGT1-Isoformen funktionelle PPREs identifiziert werden (Senekeo-Effenberger et al., 2007). Werden diese neueren Erkenntnisse in

Verbindung mit Studie 2 berücksichtigt, ist es durchaus denkbar, dass eine PPARα-Aktivierung direkt für die Glucuronidierung der Schilddrüsenhormone verantwortlich ist. Warum jedoch in dem Zusammenhang der Effekt beim Schwein im Vergleich zur Ratte, als proliferierende Spezies, stärker ausfällt (Visser et al., 1993), muss in weiteren Studien untersucht werden. Da im Gegensatz zur Ratte (Visser et al., 1993) zusätzlich erhöhte UGT-Aktivitäten für T_3 festgestellt werden konnten, muss außerdem in Betracht gezogen werden, dass hinsichtlich der Substratspezifität der Isoformen oder aber auch für die Isoformen selbst, Unterschiede zwischen verschiedenen Spezies existieren können (Li und Wu, 2007). Darüber hinaus sollte in weiteren Studien berücksichtigt werden, dass möglicherweise durch die Glucuronidierung der Fibrate selbst, also PPARα-unabhängig, die Aktivität von UGT-Isoformen gesteigert wird, welche möglicherweise auch die Schilddrüsenhormone als Substrat akzeptieren.

Aus bisherigen Humanstudien ist außerdem nicht ersichtlich, in welchem Umfang diese Beobachtungen für das Schwein auf den Menschen übertragbar sind, da in solchen Studien vor allem die Veränderungen der Plasmalipide im Mittelpunkt der Untersuchungen standen. Dass solche Effekte durch Fibrate durchaus auch bei nicht-proliferierenden Spezies beobachtet werden können, sollte jedoch kritisch in zukünftigen Studien berücksichtigt werden. Letztlich könnten vor allem Humanstudien darüber Aufschluss geben, zumal sich die Konzentrationen für T_3, T_4 und TSH im Plasma als Zielparameter problemlos und schnell bestimmen lassen, wie es ja auch für die Plasmalipide der Fall ist. In welchem Umfang diese Effekte beim Schwein auch bei einer Dosierung, welche der Medikamentation von Fibraten beim Menschen gleicht, zu beobachten sind, sollte als weiterer wichtiger Aspekt in diesem Zusammenhang in zukünftigen Studien untersucht werden. Interessant wäre zudem, ob diese Effekte beim Schwein auch im Fastenzustand zu beobachten sind.

Im Rahmen des Fütterungsversuches mit Schweinen sollte weiterhin untersucht werden, ob durch die Verabreichung des moderat oxidierten Fettes der Schilddrüsenstoffwechsel beeinflusst wird. In Studien mit Ratten und Schweinen konnte gezeigt werden, dass die Aufnahme von thermisch oxidierten Fetten zu einer Erhöhung der Plasmakonzentrationen des freien und gesamten T_4 führte (Eder und Stangl, 2000; Eder et al., 2002; Skufca et al., 2003). Da keine signifikant erhöhten TSH-Spiegel, jedoch Veränderungen in der Expression spezifischer Proteine der Hormonsynthese und morphologische Veränderungen in der Schilddrüse beobachtet werden konnten, wurde ein direkter Effekt des oxidierten Fettes auf die Schilddrüse diskutiert (Skufca et al., 2003). Allerdings konnten im vorliegenden Versuch keine Effekte nach Verabreichung des moderat oxidierten Fettes auf die

Plasmakonzentrationen der Hormone und die Morphologie der Schilddrüse beim Schwein festgestellt werden (Daten nicht publiziert). Weiterhin konnte, wie aus Studie 4 ersichtlich, keine Verminderung der Cholesterolkonzentrationen im Plasma festgestellt werden, wie von Eder und Stangl (2000) für das Schwein berichtet. Auf Grundlage dieser Beobachtungen kann geschlussfolgert werden, dass das oxidierte Fett im vorliegenden Versuch keinen Einfluss auf die Funktion der Schilddrüse beim Schwein hatte. Dadurch erübrigten sich auch weitere Untersuchungen im Rahmen dieses Versuches, welche zur Aufklärung der Ursachen erhöhter T_4-Konzentrationen in den vorangegangenen Studien hätten beitragen können.

Um die vermutete direkte Wirkung oxidierter Fette auf die Schilddrüse *in vitro* näher zu untersuchen, wurden dafür in Studie 5 primäre Schilddrüsenkulturen vom Schwein angelegt. Als Vertreter primärer Oxidationsprodukte wurde das 13-HPODE eingesetzt. Dass dadurch oxidativer Stress induziert wurde, stimmt mit den Beobachtungen in der Leber in Studie 4 überein. Dabei sind neben der zellulären Aufnahme des 13-HPODE auch Interaktionen mit der Zelloberfläche nicht auszuschließen (Auge et al., 1999). Eine Erhöhung der Iodid-Aufnahme oder der Expression schilddrüsenspezifischer Proteine konnte allerdings nicht beobachtet werden. Im Gegenteil deuten die Untersuchungen sogar auf einen hemmenden Effekt des 13-HPODE auf die Funktion der Thyreozyten hin, was gegen eine direkte steigernde Wirkung oxidierter Fette oder Fettsäuren auf die Schilddrüsenfunktion spricht.

So sind anscheinend andere Ursachen für die Erhöhung des T_4 in den vorangegangenen Studien verantwortlich. Während gezeigt werden konnte, dass oxidierte Fette bei der Ratte zu einer Peroxisomenproliferation führen (Chao et al., 2005), ist bisher völlig unklar, in welchem Umfang oxidierte Fette die Aktivitäten hepatischer UGTs modulieren, wie es zum Beispiel für verschiedene PPs bekannt ist (Visser et al., 1993). Auch eine verminderte Aufnahme peripherer Gewebe und einer daraus resultierenden Anreicherung des T_4 im Plasma kann nicht ausgeschlossen werden. Die Diskrepanzen in den Beobachtungen des vorliegenden Versuches und der Studie von Eder und Stangl (2000) könnten ursächlich aus Unterschieden der verwendeten Fette resultieren und schließen eine Störung des Schilddrüsenstoffwechsels durch stärker oxidierte Fette beim Schwein nicht aus. Gerade weil auch in diesem Zusammenhang eine vermittelnde Rolle des PPARα vermutet werden könnte, werden weitere Studien vor allem an nicht-proliferierenden Spezies nötig sein, um eine bessere Übertragbarkeit der gewonnenen Erkenntnisse auf den menschlichen Organismus zu gewährleisten. Ausgehend von den Studien 2 und 4 der vorliegenden Arbeit, scheint auch für diese Fragestellung das Schwein als hervorragendes Modelltier geeignet.

4.1.3 Fazit der Versuche am Modelltier Schwein

In den vorliegenden Studien wurde das Schwein als alternatives Modelltier eingesetzt. Anhand dieser *in vivo* Experimente konnten erfolgreich ernährungsphysiologische, pharmakologische und toxikologische Fragestellungen bearbeitet werden. Im Rahmen der Untersuchungen konnte gezeigt werden, dass das Schwein bezüglich des PPARα eine hohe Ähnlichkeit zum Menschen aufweist und wie beim Menschen im Vergleich zu Studien mit Ratten und Mäusen große Unterschiede existieren. Da der PPARα eine zentrale Rolle in den vorgestellten Arbeiten darstellt, scheinen vor allem die bekannten Speziesunterschiede des Rezeptors die Ursache für diese Unterschiede zu sein. Dabei stellt die Eigenschaft des Schweins als nicht-proliferierende Spezies den wichtigsten Vorteil dieses Modelltieres dar, um durch die Untersuchungen der Funktion des PPARα auch Rückschlüsse auf die Humanphysiologie ziehen zu können. Außerdem scheint dieses Modelltier hervorragend dazu geeignet, die Übertragbarkeit der aus Studien mit Ratten und Mäusen gewonnenen Erkenntnisse hinsichtlich der PPARs auf nicht-proliferierende Spezies und letztlich den Menschen zu überprüfen (Ajuwon et al., 2003; Xu et al., 2005, 2006).
Weiterhin konnte in Studien eine hohe Ähnlichkeit zwischen Schwein und Mensch in Bezug auf den Lipidstoffwechsel (Gerrity et al., 2001; Johansen et al., 2001; Rohe et al., 2003), den Fremdstoffmetabolismus (Pollock et al., 2007), der Tumorgenese (Adam et al., 2007) und der Aufnahme nutritiver und pharmakologischer Substanzen (Gu et al., 2006; Petri et al., 2006) aufgezeigt werden. Die starke genetische Homologie und weitere, von Lunney (2007) zusammengefasste Gemeinsamkeiten lassen dieses Modelltier für die wissenschaftliche Forschung immer interessanter erscheinen. Dazu tragen auch mehrere ehrgeizige Projekte bei, welche das Ziel der vollständigen Sequenzierung des Genoms für das Schwein verfolgen (Wernersson et al., 2005; Kim et al., 2006; Uenishi et al., 2007; Ruan et al., 2007). Dies ist ein bedeutender Fortschritt, da die Verfügbarkeit bestimmter Sequenzen bisher einen limitierenden Faktor für Analysen beim Schwein darstellt. Wie aus den vorliegenden Studien hervorgeht, ist das Schwein außerdem sehr gut geeignet, ernährungsphysiologische Fragestellungen und Abschätzungen der Toxizität und pathogener Eigenschaften bestimmter Substanzen in engem Bezug auf den Menschen zu untersuchen. So kann die Verwendung dieses Modelltieres in zukünftigen Studien maßgeblich dazu beitragen, regulatorische Mechanismen und Zusammenhänge mit starker Relevanz für die Humanphysiologie aufzuzeigen und näher zu charakterisieren.

4.2 Einfluss einer PPARα-Aktivierung auf den Carnitinstoffwechsel bei der Ratte

In Studien mit Ratten konnten im Fastenzustand oder nach Fibratgabe erhöhte Carnitinkonzentrationen im Lebergewebe festgestellt werden (McGarry et al., 1975; Mannaerts et al., 1978; Paul et al., 1986). Dieser Effekt wird durch eine Aktivierung des PPARα hervorgerufen (Cook et al., 2000). Dabei scheint durch die Steigerung der Aktivitäten PPARα-induzierter carnitinabhängiger Transferasen (Brandt et al., 1998; Yamazaki et al., 2002; Barrero et al., 2003; Vanden Heuvel et al., 2003; Seo et al., 2004) ein vermehrter Bedarf für das Carnitin in den Hepatozyten zu bestehen. Welche Mechanismen allerdings zu einer Akkumulation des Carnitins führen, ist bisher nicht bekannt. Wie aus Studie 1 ersichtlich, führte die Fibrat-induzierte PPARα-Aktivierung auch beim Schwein zu einer gesteigerten Expression der CPT-I. Außerdem konnte beobachtet werden, dass eine Erhöhung der hepatischen Carnitinkonzentration durch eine Aktivierung des PPARα in dieser Studie auch bei nicht-proliferierenden Spezies stattfand (Daten nicht publiziert). Bedingt durch fehlende Sequenzen für Expressionsanalysen von transmembranösen Transportern und Syntheseproteinen des Carnitins für das Schwein, wurde die Ratte als Modelltier für weitere Untersuchungen in den Experimenten verwendet.

So konnten in den Studien 6, 7 und 8 durch grundlegende Untersuchungen erstmals gezeigt werden, dass durch synthetische und natürliche PPARα-Liganden bei Ratten die hepatische Expression der OCTNs gesteigert wird, welche den Carnitintransport in die Zelle vermitteln (Tamai et al., 1998; Wu et al., 1999, 2000). Durch weitere Arbeiten an der Zelllinie FAO konnte vor allem für den OCTN2 (*SLC22A5*) eine positive Regulation über den PPARα nachgewiesen werden. Aufgrund der Studien kann geschlussfolgert werden, dass ein Mehrbedarf an Carnitin in der Zelle durch die Aufnahme aus dem Plasma über spezifische membranständige Transportmechanismen gedeckt wird, welche im vorliegenden Fall vermutlich durch natürliche und synthetische Agonisten des PPARα auf transkriptioneller Ebene reguliert werden.

Weiterhin kann vermutet werden, dass durch die gleichzeitige Steigerung der β-Oxidation und der Carnitinaufnahme, ein limitierender Einfluss der Verfügbarkeit des Carnitins auf die Energiegewinnung durch Fettsäureoxidation vermieden wird. In Studie 7 konnte außerdem gezeigt werden, dass oxidierte Fette bei der Ratte bereits im Dünndarm zu einer Aktivierung des PPARα führen und so die Expression der CPTs und OCTNs steigern. Dies steht zwar im Gegensatz zu den Untersuchungen am Schwein in Studie 4, könnte jedoch darauf hindeuten, dass natürliche Liganden des PPARα als Bestandteil der Nahrung zu einer vermehrten

Absorption von Carnitin führen können. Dies könnte außerdem einen Einfluss auf weitere Substanzen haben, welche Substrate des OCTN2 darstellen (Grube et al., 2006; Kato et al., 2006). Aus Studie 8 kann geschlussfolgert werden, dass bei der Ratte die Carnitinversorgung des Organismus beim Fasten und kalorischer Restriktion über eine vermehrte Rückresorption und nicht durch eine erhöhte Resorption im Darm gewährleistet wird. Diese regulatorischen Mechanismen erscheinen aus dem Grund sinnvoll, weil damit ein exogener Einfluss, zum Beispiel durch die verminderte Carnitinverfügbarkeit aus der Nahrung, auf die Energiebereitstellung über die vermehrte Fettsäureoxidation weitgehend vermieden werden kann. Daneben kann trotz marginaler Effekte eine Beteiligung der körpereigenen Synthese in allen drei Studien nicht ausgeschlossen werden (Davis und Monroe, 2005).

Die vorliegenden Studien liefern starke Indizien dafür, dass der OCTN2 ein direktes Zielgen des PPARα darstellt. Den endgültigen Beweis dazu kann die Identifizierung eines oder mehrerer PPREs in der Promoterregion des OCTN2 in künftigen Untersuchungen darstellen. Weiterhin sollte auch im Rahmen dieser Thematik auf mögliche Speziesunterschiede hinsichtlich des PPARα eingegangen werden. Aufgrund der erhöhten hepatischen Konzentrationen des Carnitins beim Schwein kann zumindest indirekt vermutet werden, dass eine Positivregulation des OCTN2 durch den PPARα nach Fibratgabe auch bei nicht-proliferierenden Spezies stattfindet. Eine signifikant gesteigerte Expression des OCTN2 konnte allerdings auch in zusätzlichen experimentellen Ansätzen mit der humanen Hepatozytenzelllinie HepG2 durch den synthetischen PPARα-Agonisten WY-14,643 beobachtet werden (Daten nicht publiziert). Diese Fakten sind ein starkes Indiz für die Annahme, dass die Übertragbarkeit der Ergebnisse der vorliegenden Studien auf nicht-proliferierende Spezies und letztlich den Menschen gegeben ist.

Die vorliegenden Studien lassen außerdem vermuten, dass in allen Situationen eines vermehrten Bedarfes an Carnitin, einschließlich des Fastens, eine ausreichende Verfügbarkeit vorgelegen hat. Brandsch und Eder (2002) konnten bereits in einer ähnlichen Studie zeigen, dass eine zusätzliche Carnitinaufnahme bei hypokalorischer Ernährung keinen Einfluss auf die Reduzierung der Fett- und Körpermasse hat und schlussfolgernd die Regulation des endogenen Carnitinstoffwechsels ausreichend sei. Weitere Untersuchungen bestätigen diese Aussage (Wächter et al., 2002; Saldanha Aoki et al., 2004; Melton et al., 2005). Einer aktuellen Studie zufolge führte die erhöhte orale Carnitinaufnahme bei Wildtyp-Mäusen zu keiner Veränderung der Triglyzeride im Plasma und auch ein additiver Effekt nach Fibratgabe konnte nicht nachgewiesen werden (Asai et al., 2006). Eine OCTN2-vermittelte Erhöhung der Carnitinkonzentrationen im Skelettmuskel, bei gleichzeitig verminderten Konzentrationen im

Plasma, konnte in Humanstudien auch für das Insulin gezeigt werden (Stephens et al., 2006) und ist direkt abhängig von der Höhe der Plasmakonzentrationen des Insulins (Stephens et al., 2007). Diese Fakten machen auch deutlich, dass im intakten Organismus nicht eine vermehrte Verfügbarkeit des Carnitins ausschlaggebend für die Veränderung der Oxidation von Fettsäuren ist, sondern wie am Beispiel des PPARα und des Insulins bis auf transkriptioneller Ebene einer präzisen und komplexen Regulation verschiedener Faktoren unterliegt. Außerdem konnte in den vorliegenden Studien 6-8 gezeigt werden, dass die zelluläre Aufnahme des Carnitins unter den beschriebenen Versuchsbedingungen eng an Prozesse, welche den zellulären Bedarf des Carnitins verändern, gekoppelt ist.

Dass hingegen die Fibrat-induzierte Aktivierung des PPARα ein großes therapeutisches Potential besitzt, wurde bereits in einer Studie an *juvenile visceral steatosis* (JVS)-Mäusen demonstriert. Dabei führte die Fibratgabe zu einer Verbesserung des kardialen und systemischen Lipidstoffwechsels und zu einer gesteigerten Überlebensrate der Tiere (Asai et al., 2006). Die JVS-Maus stellt ein hervorragendes Modell des pathologischen Krankheitsbildes des systemischen Carnitinmangels beim Menschen dar. Der Grund dieses Krankheitsbildes sind Mutationen im Gen *SLC22A5*, welche zu einem Funktionsverlust des OCTN2 und somit zum Verlust der hochaffinen zellulären Carnitinaufnahme führen (Nezu et al., 1999; Wang et al., 1999; Yokogawa et al., 1999; Mayatepek et al., 2000). In Studie 6 und 7 konnte neben der starken Erhöhung der hepatischen OCTN2-Expression, eine gesteigerte Expression des OCTN1 (*SLC22A4*) gezeigt werden. Weiterhin konnte eine erhöhte Expression des OCTN1 im Darm in Studie 7 beobachtet werden. Somit ist möglicherweise die gesteigerte Aktivität des OCTN1 in Darm und Geweben für die Kompensation des funktionellen Verlustes des OCTN2 verantwortlich und vermittelt die positiven Effekte der Fibratgabe bei JVS-Mäusen (Asai et al., 2006). Darüber hinaus kann eine gesteigerte Absorption im Darm durch eine Induktion des OCTN3 nicht ausgeschlossen werden (Duran et al., 2005). So stellt die positive Regulation weiterer Proteine des Carnitintransportes einen interessanten Ansatz der pharmakologischen Nutzung synthetischer und natürlicher PPARα-Liganden in Zuständen eines Carnitinmangels pathologischen Ursprungs dar. Darüber hinaus könnte die aufgezeigte Rezeptorabhängigkeit der OCTNs möglicherweise auch im Zusammenhang der therapeutischen Maßnahmen bei Typ-1 Diabetes (Santiago et al., 2006) und Colitis (D´Argenio et al., 2006) an Bedeutung gewinnen. Unter Berücksichtigung aufgezeigter Speziesunterschiede (Hilgendorf et al., 2007), sollte das therapeutische Potential dieses Aspektes hinsichtlich verschiedener Ansatzpunkte in weiteren Studien intensiv untersucht werden.

5. Zusammenfassung

Der *peroxisome proliferator-activated receptor-α* (PPARα) ist als Vertreter der Familie nuklearer Transkriptionsfaktoren maßgeblich an der Regulation des Lipidstoffwechsels, der Ketogenese, des Glucosestoffwechsels und inflammatorischer Prozesse beteiligt. Eicosanoide, freie und mehrfach ungesättigte Fettsäuren stellen natürliche Liganden des PPARα dar. In Versuchen mit Ratten konnte außerdem gezeigt werden, dass die Aufnahme oxidierter Fette, wie sie in der westlichen Ernährung häufig vorkommen, zu einer Aktivierung des PPARα führen. Die lipidsenkenden Eigenschaften einer PPARα-Aktivierung werden seit mehreren Jahrzehnten beim Menschen durch den Einsatz von Fibraten genutzt, welche synthetische Liganden des Rezeptors darstellen. Aus Studien an Ratten und Mäusen ist bekannt, dass die Aufnahme von Fibraten und oxidierten Fetten eine Peroxisomenproliferation und oxidativen Stress in der Leber induziert und eine langfristige Fibratgabe zur Enstehung von Leberkrebs bei sogenannten proliferierenden Spezies führt. Im Gegensatz dazu treten diese Effekte bei nicht-proliferierenden Spezies, wie Mensch, Primaten und Schwein, nicht auf. Als Gründe für diese Speziesunterschiede werden vor allem quantitative und strukturelle Unterschiede hinsichtlich des PPARα vermutet. Dies wirkt sich limitierend auf die Übertragbarkeit der Studien mit proliferierenden Spezies auf den Menschen aus. Deshalb sollten im ersten Abschnitt der vorliegenden Arbeit die Effekte synthetischer und natürlicher Liganden des PPARα beim Schwein, als Vertreter nicht-proliferierender Spezies, untersucht werden.

Dafür wurde ein Versuch mit 18 Schweinen durchgeführt, welche entweder eine Kontrolldiät oder zusätzlich 5 g Clofibrat/kg zur Kontrolldiät über 28 Tage erhielten. In ***Studie 1*** sollten die Effekte auf den Lipidstoffwechsel untersucht werden. Die Gabe des synthetischen PPARα-Agonisten Clofibrat führte zu erhöhten Konzentrationen von Ketonkörpern im Plasma, zu einer Senkung der Triglyzeride und des Cholesterols im Plasma und Lipoproteinen und zu moderat erhöhten relativen mRNA-Konzentrationen bekannter Zielgene des PPARα in Leber und Fettgewebe im Vergleich zur Kontrollgruppe ($P < 0,05$). In beiden Geweben konnte kein Einfluss auf die Expression von *sterol regulatory element binding protein-1* (SREBP-1), SREBP-2, *insulin-induced gene-1* (Insig-1), Insig-2, *Acetyl-CoA Carboxylase* (ACC), LDL-Rezeptor und *3-Methyl-3-hydroxyglutaryl* (HMG)-CoA Reduktase durch Clofibrat festgestellt werden. Weiterhin waren die relativen mRNA-Konzentrationen der Apolipoproteine A-I, A-II und C-III, der Cholesterol-7α-Hydroxylase und des mikrosomalen Triglyzerid-Transferproteins in der Leber, sowie der Lipoproteinlipase im Skelettmuskel beim Schwein durch Clofibrat nicht verändert. Die hepatischen mRNA-Konzentrationen des

PPARα waren beim Schwein und beim Menschen gleich, jedoch im Vergleich zur Leber der Ratte zehnfach niedriger. Die mRNA-Konzentrationen des PPARα waren beim Schwein in Leber und Fettgewebe gleich. Die Effekte des Clofibrat auf die Plasmalipide beim Schwein stimmen in großem Umfang mit denen anderer Modelltiere und dem Menschen überein. Hinsichtlich der mechanistischen Ursachen, welche für die Senkung der Plasmalipide verantwortlich sind, scheinen beim Schwein jedoch Unterschiede zu anderen Spezies zu existieren.

In ***Studie 2*** konnte erstmals gezeigt werden, dass die hepatischen Aktivitäten der T_3- und T_4-UDP-Glucuronyltransferasen (UGT) durch Clofibrat beim Schwein erhöht und die Konzentrationen des freien und gesamten T_4 und des gesamten T_3 im Plasma im Vergleich zur Kontrollgruppe erniedrigt waren ($P < 0,05$). Während das Gewicht und histologische Parameter der Schilddrüse unverändert waren, konnten gesteigerte relative mRNA-Konzentrationen Thyrotropin (TSH)-abhängiger Gene (Natrium-Iodid-Symporter, Kathepsin B, TSH-Rezeptor, Thyroidperoxidase) nach Gabe von Clofibrat festgestellt werden ($P < 0,05$). Relative mRNA-Konzentrationen von Transportproteinen der Schilddrüsenhormone im Plasma (Transthyretin, Albumin) und des Schilddrüsenhormon-Rezeptor α_1 in der Leber waren beim Schwein durch Clofibrat vermindert ($P < 0,05$). Die erhöhte Expression von Proteinen der Hormonsynthese in der Schilddrüse lässt auf eine Störung des Schilddrüsenstoffwechsels schlussfolgern. Diese Beobachtungen sollten in Bezug auf die Humanphysiologie als kritische Parameter betrachtet werden.

Das Ziel der ***Studie 3*** war es, die verschiedenen Effekte, welche für die Entstehung eines Fibrat-induzierten Leberkrebses bei proliferierenden Spezies vermutet werden, beim Schwein zu untersuchen. Die Untersuchungen ergaben ein erhöhtes Lebergewicht und eine Zunahme der Peroxisomenzahl in der Leber durch Clofibrat beim Schwein ($P < 0,05$). Im Vergleich zur Kontrollgruppe konnte beim Schwein eine gesteigerte relative mRNA-Konzentration der *Acyl-CoA Oxidase* (ACO), eine gesteigerte Aktivität der Katalase und eine erniedrigte Konzentration des Wasserstoffperoxides in der Leber nach der Einnahme von Clofibrat beobachtet werden ($P < 0,05$). Ein Einfluss von Clofibrat auf die hepatischen Konzentrationen des gesamten und reduzierten Glutathion und von Lipidperoxidationsprodukten konnte beim Schwein nicht festgestellt werden. Im Vergleich zur Kontrolle waren die relativen mRNA-Konzentrationen von bax, c-jun und c-myc erhöht und von bcl-xl durch Clofibrat in der Leber beim Schwein erniedrigt ($P < 0,05$). Die Erhöhung der hepatischen Expression von Proto-Onkogenen im Versuch sollte als kritisch betrachtet werden. Im Zusammenhang der

Einnahme von Fibraten als hypolipidämische Medikamente beim Menschen, sollte dieser Aspekt in zukünftigen Studien ausführlicher untersucht werden.

In ***Studie 4*** sollte untersucht werden, ob die Aufnahme eines moderat oxidierten Fettes auch bei einer nicht-proliferierenden Spezies zu einer Aktivierung des PPARα führt. Es wurde ein Fütterungsversuch mit 18 Schweinen über 28 Tage durchgeführt, welche entweder 90 g/kg eines frischen Fettes oder 90 g/kg eines moderat oxidierten Fettes (24 h bei 180°C) zur gleichen Diät erhielten. Nach Aufnahme des oxidierten Fettes konnten, im Vergleich zum frischen Fett, eine erhöhte relative mRNA-Konzentration der mitochondrialen HMG-CoA Synthetase in der Leber und erhöhte Plasmakonzentrationen von Ketonkörpern beim Schwein beobachtet werden ($P < 0,05$). Die hepatischen relativen mRNA-Konzentrationen der ACO und der *Carnitin-Palmitoyltransferase-I* (CPT-I) waren durch die Aufnahme des oxidierten Fettes in der Tendenz erhöht ($P < 0,10$). Die Plasmalipide waren unverändert. Die Aktivitäten der Katalase, die Konzentration konjugierter Diene und die Peroxisomenzahl waren in der Leber beim Schwein, im Vergleich zur Gruppe mit frischem Fett, durch oxidiertes Fett erhöht und die Konzentration des α-Tocopherol vermindert ($P < 0,05$). Beim Schwein führte die Aufnahme des oxidierten Fettes zu erhöhten relativen mRNA-Konzentrationen des SREBP-1 und dessen Zielgenen in der Leber und des SREBP-2 und dessen Zielgenen in Leber und Dünndarm ($P < 0,05$). Schlussfolgernd konnte erstmals gezeigt werden, dass ein moderat oxidiertes Fett beim Schwein den PPARα in der Leber aktiviert und möglicherweise die Lipogenese in Leber und Dünndarm induziert.

Einen stimulierenden Einfluss des moderat oxidierten Fettes auf den Stoffwechsel der Schilddrüse, wie aus Studien mit Ratten und Schweinen bekannt, konnte im vorliegenden Versuch mit Schweinen nicht festgestellt werden. Um einen direkten Einfluss oxidierter Fettsäuren auf die Funktion der Schilddrüse zu untersuchen, wurden in ***Studie 5*** Primärkulturen von Thyreozyten des Schweins angelegt und die *13-Hydroperoxy-9,11-octadecadiensäure* (13-HPODE) als primäres Oxidationsprodukt der Linolsäure (18:2n-6) eingesetzt. Eine Erhöhung der Aktivitäten der Superoxiddismutase und Glutathionperoxidase wurde nach Inkubation mit 13-HPODE gegenüber der Kontrolle beobachtet ($P < 0,05$). Ein Effekt auf die relativen mRNA-Konzentrationen des Natrium-Iodid-Symporters, des TSH-Rezeptors und der Thyroidperoxidase, die Zellviabilität und die zelluläre Iodidaufnahme der Thyreozyten konnte nicht festgestellt werden, jedoch war die relative mRNA-Konzentration der Dual-Oxidase-II und die Produktion von Wasserstoffperoxid durch 13-HPODE gegenüber der Kontrolle vermindert ($P < 0,05$). Da die Bildung von Wasserstoffperoxid einen

limitierenden Faktor in der Synthese der Schildrüsenhormone darstellt, kann eine hemmende Wirkung des 13-HPODE auf die Funktion der Thyreozyten vermutet werden.

Im zweiten Teil der vorliegenden Arbeit sollte grundlegend untersucht werden, welche Ursachen für die erhöhten hepatischen Konzentrationen des Carnitins verantwortlich sind, wie sie bei Tieren nach Fibratgabe oder im Fastenzustand beobachtet wurden. In ***Studie 6*** wurden 16 männlichen Ratten 250 mg/kg Clofibrat in Sonnenblumenöl oder nur Sonnenblumenöl in der Kontrollgruppe zu einer kommerziellen Standarddiät für 4 Tage verabreicht. Die Gabe des synthetischen PPARα-Agonisten Clofibrat führte zu erhöhten relativen mRNA-Konzentrationen der *novel organic cation transporter-1* (OCTN1), OCTN2, CPT-I und CPT-II und zu einer erhöhten Konzentration des Carnitins im Lebergewebe ($P < 0,05$). In der Leberzelllinie FAO der Ratte wurde nach Inkubation mit dem synthetischen PPARα-Agonisten WY-14,643 eine gesteigerte relative mRNA-Konzentration des OCTN2 und eine erhöhte zelluläre Konzentration gegenüber der Kontrolle festgestellt ($P < 0,05$). Bei der Ratte und in FAO-Zellen konnte kein Effekt auf die Expression von Enzymen der Carnitinsynthese durch synthetische PPARα-Agonisten beobachtet werden. In *Studie 6* konnte erstmals gezeigt werden, dass durch synthetische PPARα-Liganden die hepatische Expression der OCTNs, welche für die zelluläre Aufnahme des Carnitins verantwortlich sind, gesteigert wurde. Die erhöhten Konzentrationen des Carnitins in der Leber und die erniedrigten Konzentrationen im Plasma machen einen direkten Zusammenhang zu einer gesteigerten Transportaktivität deutlich. Dabei scheint vor allem die Steigerung des hochaffinen OCTN2 (*SLC22A5*) für die erhöhten Konzentrationen im Gewebe verantwortlich zu sein.

In ***Studie 7*** wurden 18 Ratten entweder thermisch oxidiertes Sonnenblumenöl oder frisches Sonnenblumenöl in der Kontrollgruppe zu einer kommerziellen Standarddiät für 6 Tage verabreicht. Die Aufnahme des oxidierten Fettes führte bei Ratten zu erhöhten relativen mRNA-Konzentrationen der bekannten PPARα-Zielgene ACO, CPT-I und CPT-II in Leber und Dünndarm im Vergleich zur Kontrolle ($P < 0,05$). Die relativen mRNA-Konzentrationen von OCTN1 und OCTN2 und der Carnitinkonzentration in der Leber und von OCTN2 im Dünndarm waren bei Ratten nach Aufnahme des oxidierten Fettes erhöht ($P < 0,05$). Weiterhin konnte eine Verminderung der Carnitinkonzentrationen im Plasma, Skelettmuskel und im Herz durch das oxidierte Fett, im Vergleich zur Kontrollgruppe mit frischem Fett, festgestellt werden ($P < 0,05$). Im Rahmen der *Studie 7* konnte erstmals gezeigt werden, dass auch durch Aufnahme eines oxidierten Fettes die hepatische Expression der OCTNs und die Carnitinkonzentration in der Leber der Ratte erhöht wird. Nach Verabreichung des oxidierten

Fettes konnte zusätzlich eine PPARα-Aktivierung und gesteigerte Expression der OCTNs im Dünndarm beobachtet werden, was auf eine vermehrte Absorption deutet.

In ***Studie 8*** sollte untersucht werden, ob auch beim Fasten und kalorischer Restriktion, als physiologische Zustände einer PPARα-Aktivierung, ein Einfluss auf die Konzentrationen des Carnitins und Expression der OCTNs beobachtet werden kann. Im Versuch mit 36 Ratten wurden die Tiere entweder *ad libitum* (Kontrollgruppe), mit 10,5 g/d oder 6 g/d einer kommerziellen Standarddiät, was 70% beziehungsweise 40% des täglichen Energiebedarfes darstellt, für 10 Tage gefüttert. In einer vierten Gruppe erhielten die Tiere 9 Tage die Diät *ad libitum* und wurden dann für 24 h gefastet. Im Fastenzustand und kalorischer Restriktion waren die relativen mRNA-Konzentrationen der ACO, als bekanntes Zielgen des PPARα, in Leber, Niere und Herz im Vergleich zu den Ratten der Kontrollgruppe erhöht ($P < 0,05$). Bei Ratten, welche 70% des täglichen Energiebedarfes erhielten, waren die relativen mRNA-Konzentrationen des OCTN2 und die Konzentrationen des Carnitins in Leber und Niere im Vergleich zur Kontrollgruppe erhöht ($P < 0,05$). Nach Aufnahme von 40% des täglichen Energiebedarfes, waren bei Ratten die relativen mRNA-Konzentrationen des OCTN2 und die Konzentrationen des Carnitins in Leber, Niere, Herz und Skelettmuskel im Vergleich zur Kontrollgruppe erhöht ($P < 0,05$). Bei gefasteten Ratten konnte eine gesteigerte relative mRNA-Konzentration des OCTN2 und eine erhöhte Konzentration des Carnitins in Leber, Niere und Herz im Vergleich zur Kontrollgruppe beobachtet werden ($P < 0,05$). In diesen Zuständen scheint eine vermehrte renale Rückresorption als Mechanismus für die Aufrechterhaltung der Carnitinversorgung verantwortlich zu sein.

Anhand der *Studien 6-8* konnte demonstriert werden, dass in Zuständen eines vermehrten zellulären Carnitinbedarfes die Verfügbarkeit infolge der Regulation der OCTNs auf transkriptioneller Ebene gewährleistet wird. In den Studien kann allerdings eine Beteiligung von Enzymen der Eigensynthese durch erhöhte Aktivitäten nicht ausgeschlossen werden. Aufgrund der vorliegenden Arbeiten kann weiterhin vermutet werden, dass der OCTN2 ein direktes Zielgen des PPARα darstellt. Die Identifizierung eines funktionellen PPRE in der Promoterregion des OCTN2 würde einen eindeutigen Beweis dafür liefern und sollte deshalb Gegenstand weiterer Studien sein.

6. Summary

The *peroxisome proliferator-activated receptor-α* (PPARα) is a member of the family of nuclear transcription factors and plays a crucial role in the regulation of lipid metabolism, ketogenesis, carbohydrate metabolism and inflammatory processes. Eicosanoids, free and polyunsaturated fatty acids are natural ligands of PPARα. Furthermore, dietary oxidized fat has been shown to activate PPARα in studies with rats. The lipid lowering properties of PPARα activation are used since several decades by application of fibrates, which are known to act as synthetic ligands of the receptor. It is well established, that fibrates and oxidized fatty acids induce peroxisome proliferation and oxidative stress in liver of rats and mice. In these proliferating species, long-term administration of fibrates cause hepatocarcinogenesis. In contrast, non-proliferating species, like human, primates and pig, are refractory for these effects. Several quantitative and structural differences have been suggested as reasons for these species differences. This fact is limiting in the assignment of the findings from studies with rodents for humans.The aim of the first part of the present work was to investigate the effects of synthetic and natural ligands of PPARα in the pig, representing a non-proliferating species.

Therefore, we performed an experiment in which 18 pigs received a control diet or control diet supplemented with 5 g clofibrate/kg diet for 28 days. The aim of ***study 1*** was to investigate the effects on lipid metabolism. The administration of synthetic PPARα-agonist clofibrate increased plasma concentration of ketone bodies, decreased triglyceride and cholesterol concentrations in plasma and lipoproteins and moderatly increased the relative mRNA concentration of known target genes of PPARα in liver and adipose tissue, compared to control pigs ($P < 0.05$). In both tissues, the expression of *sterol regulatory element binding protein-1* (SREBP-1), SREBP-2, *insulin-induced gene-1* (Insig-1), Insig-2, *acetyl-CoA carboxylase* (ACC), LDL receptor and *3-hydroxy-3-methylglutaryl* (HMG)-CoA reductase was not affected by clofibrate. Furthermore, relative mRNA concentrations of apolipoproteins A-I, A-II and C-III, cholesterol-7α-hydroxylase and microsomal triglyceride transfer protein in liver and of lipoprotein lipase in skeletal muscle of pigs were unchanged by clofibrate treatment. The mRNA concentrations of PPARα were similar in pig and human liver, but 10-fold lower than concentrations in rat liver. In pigs, the mRNA concentrations of PPARα were similar in liver and adipose tissue. In conclusion, the effects of clofibrate on plasma lipids in pigs are consistent to a great extent with other animal models and humans. However, the

biochemical mechanisms which are responsible for the hypolipidemic effects, might be in part different from those in other species.

In ***study 2*** it was shown for the first time, that clofibrate treatment increased the hepatic glucuronidation of thyroid hormones in pigs as non-proliferating species, resulting in decreased plasma concentrations of T_3 and T_4 compared to control pigs ($P < 0.05$). Thyroid weight and histological parameters of thyroid gland did not differ between control pigs and pigs treated with clofibrate, but increased relative mRNA concentrations of TSH-responsive genes (sodium iodide symporter, cathepsin B, TSH receptor, thyroid peroxidase) were observed in thyroid of pigs treated with clofibrate ($P < 0.05$). In liver, relative mRNA concentrations of proteins involved in plasma thyroid hormone transport (transthyretin, albumin) and of thyroid hormone receptor α_1 were decreased after clofibrate treatment ($P < 0.05$). In conclusion, it can be suggested that clofibrate acts as a disruptor of thyroid hormone axis in pigs. These disturbances of thyroid function should be seen critical with respect to humans.

The aim of ***study 3*** was to investigate several effects in pigs which are suggested to be responsible for the fibrate-induced development of hepatocarcinogenesis in rodents. Liver weights and number of peroxisomes in liver were increased in pigs treated with clofibrate ($P < 0.05$). The relative mRNA concentration of *acyl-CoA oxidase* (ACO) and catalase activity were increased and concentration of hydrogen peroxide was decreased in liver of pigs after clofibrate treatment ($P < 0.05$). Hepatic concentrations of total and reduced glutathione and lipid peroxidation products did not differ between control pigs and pigs treated with clofibrate, but relative mRNA concentrations of bax, c-jun and c-myc were increased and relative mRNA concentration of bcl-xl was decreased in liver after clofibrate treatment ($P < 0.05$). However, the increased hepatic expression of proto-oncogenes should be seen critical. Because fibrates are used as hypolipidaemic drugs in humans, this aspect should be examined more detailed in further studies.

Whether the dietary intake of a moderat oxidised fat causes an activation of PPARα in non-proliferating species, should be investigated in ***study 4***. Therefore, an experiment was performed with 18 pigs which were fed either a diet containing 90 g/kg diet of a fresh fat or the same diet with 90 g/kg diet of a moderat oxidised fat (24 h; 180°C) for 28 days. In pigs fed the moderat oxidised fat, the hepatic relative mRNA concentration of mitochondrial HMG-CoA synthase and plasma concentrations of ketone bodies were increased compared to pigs fed the fresh fat ($P < 0.05$). In liver, relative mRNA concentrations of ACO and *carnitine-palmitoyl transferase-1* (CPT-I) tended to be higher in pigs received the moderat

oxidised fat ($P < 0.10$), but failed to decrease lipid concentrations in plasma. In pigs fed the moderat oxidised fat, the activity of catalase, the concentration of conjugated dienes and the number of peroxisomes were increased and concentration of α-tocopherol was decreased in liver compared to pigs fed the fresh fat ($P < 0.05$). The relative mRNA concentrations of SREBP-1 and its target genes in liver and of SREBP-2 and its target genes in small intestine were increased in pigs recieved the moderat oxidised fat ($P < 0.05$). Taken together, it could be shown for the first time that the dietary intake of a moderat oxidised fat activates PPARα in non-proliferating species and might induce lipogenesis in liver and small intestine of pigs. Furthermore, no influence on thyroid function or plasma thyroid hormone concentrations could be observed after dietary intake of a moderat oxidised fat, as it was shown in previous studies for rats and pigs. To investigate a possible direct influence of oxidised fatty acids on thyroid function, experiments with primary thyroid cells from pigs were performed in ***study 5***, using 13-hydroperoxy-9,11-octadecadienic acid (13-HPODE) as primary oxidation product of linoleic acid (18:2n-6). The activities of superoxide dismutase and glutathione peroxidase were increased after incubation of 13-HPODE compared to control ($P < 0.05$). The relative mRNA concentrations of sodium iodide symporter, TSH receptor and thyroid peroxidase, as well as cell viability and iodide upake were unchanged, but the relative mRNA concentration of dual oxidase-2 and hydrogen peroxide production were decreased in thyrocytes treated with 13-HPODE compared to control cells ($P < 0.05$). Because the formation of hydrogen peroxide is rate-limiting in thyroid hormone synthesis, an inhibitory effect of 13-HPODE on thyroid function might be suggested.

The aim of the second part of the present work was to investigate the basic mechanisms, which are responsible for the increased hepatic concentrations of carnitin, as it was observed for animals after fibrate administration or fasting in previous studies. In ***study 6***, an experiment was performed with 16 rats which recieved 250 mg clofibrate/kg in sunflower oil or only sunflower oil in control group additionally to commercial standard diet for 4 days. Treatment of synthetic PPARα agonist clofibrate increased the relative mRNA concentrations of *novel organic cation transporter-1* (OCTN1), OCTN2, CPT-I and CPT-II and the carnitine concentrations in liver of rats ($P < 0.05$). Furthermore, incubation of FAO hepatoma cells with synthetic PPARα agonist WY-14,643 resulted in increased relative mRNA concentration of OCTN2 and increased cellular carnitine concentrations ($P < 0.05$). In liver of rats and in FAO hepatoma cells, relative mRNA concentrations of proteins involved in carnitne synthesis were unchanged by treatment of synthetic PPARα agonists. In conclusion, we could

demonstrate for the first time, that activation of PPARα was accompanied by strong increased hepatic expression of OCTNs, which are responsible for the cellular carnitine uptake. The findings, that concentrations of carnitine were increased in the liver and decreased in plasma of rats are indicative for elevated activities of carnitine transporters. Primarily the increase of OCTN2 (*SLC22A5*), which possesses high affinity for carnitine, might be responsible for increased tissue concentrations.

In ***study 7***, an experiment was performed with 18 rats either fed a commercial standard diet with oxidised sunflower oil or fresh sunflower oil in control group for 6 days. Relative mRNA concentrations of the known PPARα target genes ACO, CPT-I and CPT-II in liver and small intestine and relative mRNA concentrations of OCTN1 and OCTN2 in liver and of OCTN2 in small intestine were increased in rats administered the oxidised fat compared to control group ($P < 0.05$). Furthermore, increased carnitine concentrations in liver and decreased carnitine concentrations in plasma, skeletal muscle and heart were observed in rats fed oxidised fat ($P < 0.05$). In conclusion, these results demonstrate for the first time, that dietary oxidised fat increases hepatic expression of OCTNs and carnitine concentrations in the liver of rats. The increased expression of OCTN2 in small intestine might suggest an increased absorption of carnitine.

The aim of ***study 8*** was to investigate the effects of fasting or caloric restriction, both physiological conditions of PPARα activation, on carnitine concentrations and the expression of OCTNs. Therefore, an experiment was performed for 10 days with 36 rats either recieved *ad libitum* (control group), 10.5 g/d (70% of energy requirement for maintenance) or 6 g/d (40% of energy requirement for maintenance) of a commercial standard diet. A fourth group received the diet *ad libitum* for nine days and was then fasted for 24 h. Fasting and caloric restriction resulted in increased relative mRNA concentrations of PPARα target gene ACO in liver, kidney and heart of rats compared to control group ($P < 0.05$). In rats recieving 70% of energy requirement for maintenance, relative mRNA concentrations of OCTN2 and carnitine concentrations were higher in liver and kidney than in control rats ($P < 0.05$). In animals recieving 40% of energy requirement for maintenance, relative mRNA concentrations of OCTN2 and carnitine concentrations were increased in liver, kidney, heart and skeletal muscle compared to control group ($P < 0.05$). Fasted rats had higher relative mRNA concentrations of OCTN2 and carnitine concentrations in liver, kidney and heart than control rats ($P < 0.05$). The increase of OCTN2 in kidney might be an evidence for elevated resorption as an mechanism for the maintenance of carnitine in these conditions.

As a result of the *studies 6-8*, it can be concluded that maintenance of carnitine is ensured by transcriptional regulation of transporters in situations of increased cellular requirement. Whether there was only a marginal effect on the expression in these studies, however, an increased activity of enzymes involved in carnitine synthesis can not be excluded completely. Furthermore, these studies support strong evidence particularly for OCTN2 to represent a direct target gene of PPARα. This assumption could be verified by the identification of a functional PPRE in the promoter region of OCTN2 and should be investigated further studies.

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Erklärung

Hiermit versichere ich an Eides Statt, dass ich die eingereichte Dissertation „Wirkungen einer PPARα-Aktivierung auf Parameter des Stoffwechsels von Lipiden, Schilddrüsenhormonen und Carnitin bei Schwein und Ratte als Modelltieren“ selbständig angefertigt und diese nicht bereits für eine Promotion oder ähnliche Zwecke an einer anderen Universität eingereicht habe. Weiterhin versichere ich, dass ich die zur Erstellung der Dissertationsschrift verwendeten wissenschaftlichen Arbeiten und Hilfsmittel genau und vollständig angegeben habe.

Des Weiteren erkläre ich, dass keine Strafverfahren gegen mich anhängig sind.

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