Einfluss von Selen auf ausgewählte Funktionen des Intermediärstoffwechsels und die differenzielle Regulation von Phase-II-Enzymen sowie die antagonistische und synergistische Wirkung von Methionin und Glucoraphanin auf diese Prozesse

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der Martin-Luther-Universität Halle-Wittenberg

vorgelegt von
M.Sc. Nicole Michaela Blum, geb. Wolf
Geb. am 05.10.1981 in Heidelberg

Gutachter: Prof. Dr. G. Stangl
Prof. Dr. K. Eder
Dr. habil. A. Müller

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Abkürzungsverzeichnis

ABCG8	ATP-*Binding Cassette Transporter* G8
AhR	*Aryl Hydrocarbon Receptor*
apoB	Apolipoprotein B
ARE	*Antioxidant Response Element*
BHMT	Betain-Homocystein Methyltransferase
b-Zip	basischer Leucinzipper
CAR	*Constitutive Androstane Receptor*
CBS	Cystathionin-β-Synthase
CNC	*Cap 'N' Collar*
COX	Cyclooxygenase
CREB	cAMP *Response Element-BindingProtein*
Cyp1a1	*Cytochrome* P450 *Member* 1a1
Cyp7a1	Cholesterol-7-alpha-Hydroxylase
DIO	Deiodinase
DTT	Dithiotreitol
EFSec	*Slenocysteine-Specific Elongation Factor*
FAS	Fettsäuresynthase
GCL	Glutamatcysteinligase
GCLC	katalytische Untereinheit der GCL
GCLM	modulatorische Untereinheit der GCL
GNMT	Glycin-N-Methyltransferase
GPx	Glutathionperoxidase
GR	Glutathionreduktase
GSH	Glutathion
GST	Glutathion-S-Transferase
HDL	*High Density Lipoprotein*
HMGCoAR	3-Hydroxy-3-Methylglutaryl Coenzym A Reduktase
HO1	Hämoxygenase1
KEAP1	Kelch-*Like* ECH-*Associated Protein* 1
LDL	*Low Density Lipoprotein*
LDLR	LDL-Rezeptor
LXRα	Leber-X-Rezeptor α

MRP4	*Multidrug Resistance Associated Protein* 4
MTR	5-Methyltetrahydrofolat-Homocystein Methyltransferase
Neh	Nrf2-ECH *Homology*
NF-Y	*Nuclear Factor* Y
NFκB	*Nuclear Factor kappa* B
NQO1	NAD(P)H-Quinonoxidoreduktase
Nrf2	*Nuclear Factor Erythroid 2-Related Factor 2*
PDX1	*Pancreatic Duodenal Homeobox* 1
PPARα	P*eroxisome Proliferator-Activated Receptor* α
PTP1B	Proteintyrosinphosphatase 1B
PXR	P*regnane X Receptor*
RPL13a	ribosomales Protein L13a
RPL30	ribosomales Protein L30
SBP2	SECIS *Binding Protein* 2
SCAP	SREBP *Cleavage-Activating Protein*
Sec	Selenocystein
SECIS	*Selenocysteine Insertion Sequence*
Secp43	*tRNA selenocysteine 1 associated protein*
SPS2	Selenophosphatsynthetase 2
SREBP1c	S*terol Regulatory Element Binding Protein* 1c
TrxR	Thioredoxinreduktase
UGT	UDP-Glucuronosyltransferase
Wnt	*Wingless*/Int-1

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1. Einleitung

1.1 Selen

Das Element Selen wurde 1817 vom schwedischen Chemiker Jöns Jakob Berzelius entdeckt und galt lange Zeit als toxisch. Erst seit 1957, nach einer Veröffentlichung von Schwarz und Foltz (1957), in der eine positive Funktion von Selen in Bezug auf die Prävention der Lebernekrose nachgewiesen wurde, gilt es als essentielles Spurenelement, das im menschlichen Organismus wichtige physiologische Funktionen erfüllt. Der Schätzwert für die tägliche Selenzufuhr für Erwachsene in Deutschland liegt bei 30 µg bis 70 µg (D-A-CH 2000). Die tatsächliche Selenaufnahme in Europa beträgt durchschnittlich 40 µg pro Tag und in den USA zwischen 93 µg (Frauen) und 134 µg (Männer) pro Tag (Rayman 2012). Eine mangelnde Versorgung mit Selen kann zum Verlust der Immunkompetenz, einer erhöhten Anfälligkeit gegenüber viralen Erkrankungen (z.B. HIV) sowie zur männlichen Infertilität führen (Kupka et al. 2004, Foresta et al. 2002, Parnham et al. 1983). Des Weiteren kann Selenmangel auch zur Entstehung der Keshan-Krankheit beitragen. Hierbei handelt es sich um eine Kardiomyopathie, die vor allem in stark selenarmen Regionen Chinas auftritt. Eine weitere Erkrankung, die in Zusammenhang mit einem Selenmangel steht, ist die Kaschin-Beck-Krankheit, eine Osteoarthropathie. Im Gegensatz hierzu haben schon 600 µg Selen pro Tag eine toxische Wirkung und führen zu Müdigkeit, Fingernagel- und Haarverlust oder Depressionen. Der „*Tolerable upper intake level*" von Selen liegt in Europa bei 300 µg pro Tag (SCF 2000). Somit gilt Selen als ein Spurenelement mit einer sehr geringen therapeutischen Breite. Das bedeutet, dass die Spanne zwischen Essentialität und Toxizität, welche auf den massiven prooxidativen Effekten von Selen beruht, sehr niedrig ist (Mueller et al. 2009). Bei einer Zufuhr im empfohlenen Bereich werden dem Selen hauptsächlich antioxidative, antiinflammatorische, antivirale sowie chemopräventive Eigenschaften zugeschrieben, die es vor allem in Form von Selenoproteinen ausübt (Weeks et al. 2012, Papp et al. 2007).

1.1.1 Selenoproteine

Biosynthese

Im Gegensatz zu anderen Mineralstoffen und Spurenelementen, die als Cofaktoren katalytisch aktiv sind, wird Selen cotranslational in Form der 21. proteinogenen Aminosäure Selenocystein (Sec) in die Polypeptidkette der funktionellen Selenoproteine eingebaut (Papp et al. 2007). Sec ist in der mRNA der Selenoproteine durch die Sequenz UGA codiert. Da dieses Basentriplett in der Regel als Stoppcodon fungiert, dient eine sekundäre RNA-

Haarnadelstruktur zur Umcodierung. Diese befindet sich in der 3'-untranslatierten mRNA-Region der funktionellen Selenoproteine und wird als *Selenocysteine Insertion Sequence* (SECIS)-Element bezeichnet. Im Gegensatz zu anderen Aminosäuren wird Sec an einer besonderen tRNA synthetisiert ($tRNA^{[Ser]Sec}$). Dazu wird die spezielle tRNA ($tRNA^{sec}$) zunächst mittels der Seryl-tRNA-Synthetase mit Serin beladen und reagiert anschließend mit Selenophosphat zur Selenocysteinyl-$tRNA^{[Ser]Sec}$. Selenophosphat wird aus Selenid in einer ATP-abhängigen Reaktion durch die Selenophosphatsynthetase 2 (SPS2) synthetisiert. Dabei ist bemerkenswert, dass die SPS2 selbst ein Selenoprotein ist. Neben der Selenocysteinyl-$tRNA^{[Ser]Sec}$ und dem SECIS-Element sind weitere Faktoren am Einbau von Sec in die Proteinkette beteiligt. Das SECIS *Binding Protein* 2 (SBP2) bindet spezifisch an das SECIS-Element und transportiert die mRNA zu den Ribosomen, während der *Selenocysteine-Specific Elongation Factor* (EFsec) die Selenocysteinyl-$tRNA^{[Ser]Sec}$ zu den Ribosomen bringt. Der Einbau von Sec wird außerdem durch weitere Faktoren, wie das *tRNA selenocysteine 1 associated protein 1* (Secp43) und das ribosomale Protein L30 (RPL30), reguliert (Papp et al. 2007, Hoffmann und Berry 2005).

Funktionen verschiedener Selenoproteine

Beim Menschen wurden bisher 25 funktionelle Selenoproteine charakterisiert. Bei Ratten und Mäusen dagegen existieren nur 24 Selenoproteine, da die Glutathionperoxidase 6 (GPx6) Cystein anstelle von Selenocystein enthält (Kryukov et al. 2003). Zu den am besten charakterisierten Selenoenzymen zählen die GPx, die Thioredoxinreduktasen (TrxR) sowie die Deiodinasen (DIO). Die Funktionen sowie die zelluläre Lokalisation der verschiedenen Selenoproteine sind in **Tabelle 1** dargestellt.

Tabelle 1: Funktionen und zelluläre Lokalisation humaner Selenoproteine (nach Papp et al. 2007, Reeves und Hoffmann 2009, Moghadaszadeh und Beggs 2006)

	Hauptfunktion	**Vorkommen**
Glutathionperoxidasen (GPx)		
GPx1	Antioxidativ, Reduktion von Hydroperoxiden	Ubiquitär, zytosolisch
GPx2	Antioxidativ, Reduktion von Hydroperoxiden, mukosale Homöostase	Vor allem im Gastrointestinaltrakt, zytosolisch
GPx3	Antioxidativ, Reduktion von Hydroperoxiden	Plasma, sezerniert aus der Niere
GPx4	Reduktion von Phospholipid- und Cholesterinhydroperoxiden Antioxidatives Strukturprotein in Spermien	Ubiquitär, mitochondrial, zytosolisch, nukleär
GPx6	Antioxidativ	Olfaktorisches Epithel

Thioredoxinreduktasen (TrxR)		
TrxR1	Reduktion von oxidiertem Thioredoxin, dadurch Aufrechterhaltung der Ribonukleotid Reduktase Aktivität und somit der DNA-Synthese	Ubiquitär, zytosolisch, nukleär
TrxR2	Reduktion von oxidiertem Thioredoxin	Ubiquitär, mitochondrial
TrxR3	Reduktion von oxidiertem Thioredoxin, Glutathionreduktase- und Glutaredoxinreduktase-Aktivität	Testes, zytosolisch
Deiodinasen (DIO)		
DIO1	Umwandlung von T4 zu bioaktivem T3, Inaktivierung von T3	Schilddrüse, Leber, Niere, Hypophyse, Plasmamembran
DIO2	Umwandlung von T4 zu bioaktivem T3	Schilddrüse, Gehirn, Herz, Darm, Skelettmuskulatur, ER-Membran
DIO3	Inaktivierung von T3 und T4	Gehirn, Plazenta, Skelettmuskulatur, Plasmamembran
SPS2	Beteiligung an der Selenoproteinsynthese	Ubiquitär, zytosolisch
Selenoprotein H	Redox-reguliertes DNA-Bindeprotein	Ubiquitär, nukleär
Selenoprotein I	Mögliche Beteiligung an der Phospholipidbiosynthese	Ubiquitär
Selenoprotein K	Schutz vor ROS in Kardiomyozyten	Ubiquitär, ER-Membran
Selenoprotein M	Proteinfaltung	ER
Selenoprotein N	Embryonale Muskelentwicklung, Calciummobilisierung	Ubiquitär, ER-Membran
Selenoprotein O	Unbekannt	Ubiquitär
Selenoprotein P	Selentransport, antioxidativ, GPx-Aktivität, Komplexierung von Schwermetallionen	Ubiquitär, sezerniert
Selenoprotein R	Methionin-Sulfoxid-Reduktase-Funktion	Ubiquitär, zytosolisch
Selenoprotein S	Moduliert durch Glukose und ER-Stress, Regulation inflammatorischer Zytokine	Ubiquitär, ER
Selenoprotein T	Calciummobilisierung	Ubiquitär, ER
Selenoprotein V	Testesspezifisch, Funktion unbekannt	Testes
Selenoprotein W	Vermutlich antioxidative Wirkung, Muskelwachstum und -differenzierung	Ubiquitär, zytosolisch
Selenoprotein 15	Proteinfaltung im ER, Regulation der Apoptose	Ubiquitär, ER

Beim Menschen sind bisher acht Glutathionperoxidasen bekannt. Allerdings besitzen die epididymal vorkommende GPx5, die GPx7 und die GPx8 kein Selenocystein, sondern nur Cystein (Brigelius-Flohé und Maiorino 2013). Zu den fünf selenocysteinhaltigen GPx zählen: (1) zytosolische GPx (GPx1), (2) gastrointestinale GPX (GPx2), (3) plasmatische GPx

(GPx3), (4) Phospholipidhydroperoxid GPx (GPx4), und (5) olfaktorische GPx (GPx6) (Kryukov et al. 2003). GPx reduzieren unter Oxidation von Glutathion verschiedene Peroxide zu ihren jeweiligen Alkoholen und Wasser (R-OOH + 2 GSH →R-OH + H_2O + GSSG) (Gromer et al. 2005). Obwohl die katalysierte Reaktion für alle GPx gleich ist, unterscheiden sie sich in ihrer Substratspezifität, Lokalisierung, transkriptionellen Regulation und Funktion (Brigelius-Flohé 2006, 1999). Die GPx1 bis 3 katalysieren die Reduktion von Wasserstoffperoxid und organischen Hydroperoxiden, während die GPx4 Phospholipid- und Cholesterinhydroperoxide reduziert (Lu und Holmgren 2009). Die verschiedenen Selenoproteine reagieren unterschiedlich auf Selenmangel und unterliegen daher einer Hierarchie. Während bei einigen Selenoenzymen im Selenmangel die Aktivität schnell abnimmt, bleibt die Aktivität anderer Selenoenzyme während eines moderaten Selenmangels stabil und sinkt erst bei langanhaltendem, stark ausgeprägtem Selenmangel. Die zuletzt genannten Selenoproteine werden bei erneuter Selensupplementation schneller regeneriert als die im Selenmangel instabileren Selenoproteine. Die Selenoproteine die langsamer auf ein Selendefizit reagieren, werden weiter oben in der Hierarchie eingeordnet, als die schnell auf Selenmangel reagierenden Selenoproteine. Die abnehmende Proteinsynthese während eines Selendefizits geht mit einer Reduktion der mRNA-Konzentration einher. Diese ist nicht auf eine geringere Transkription, sondern auf einen Stabilitätsverlust zurück zu führen (Brigelius-Flohé 1999). Die mRNA der GPx1 wird bei Selenrestriktion sehr schnell abgebaut, während die mRNA der GPx4 stabil bleibt und die GPx2-mRNA-Konzentration in einigen Zellen sogar zunimmt. Aus einer Zellkulturstudie ergab sich für die GPx folgende Hierarchie: GPx2 > GPx4 >> GPx3 = GPx1 (Wingler et al. 1999).

Die GPx1 wurde erstmals 1957 von Mills (1957) in Erythrozyten entdeckt. 1973 konnten Flohé et al. (1973) und Rotruck et al. (1973) zeigen, dass es sich um ein selenabhängiges Enzym handelt. Die GPx1, ist ein homotetrameres zytosolisches Enzym, das ubiquitär exprimiert wird und vor allem für die Reduktion von oxidativem Stress verantwortlich ist. GPx1-Knockout-Mäuse sind in der Regel gesund, fertil und zeigen keine erhöhte Empfindlichkeit gegenüber einer Hyperoxie (Ho et al. 1997). Allerdings führt starker oxidativer Stress, zum Beispiel in Form des Redoxcyclers Paraquat oder in Form von Wasserstoffperoxid bei GPx1-Knockout-Mäusen zu erhöhter Morbidität und Mortalität (Cheng et al. 1998, de Haan et al. 1998). Eine Überexpression der GPx1 fördert bei Mäusen die Entstehung von Übergewicht, Hyperinsulinämie, Insulinresistenz und Hyperglykämie (McClung et al. 2004).

Da die GPx2 vor allem im mukosalen Epithel des Gastrointestinaltraktes stark exprimiert ist, wurde ihr zuerst eine Funktion als Barriere gegen Hydroperoxide aus der Nahrung zugesprochen (Wingler et al. 2000, Chu et al. 1993). Im Ileum wird die GPx2 vor allem in den Panethzellen, im Kolon und im Rektum überwiegend im Kryptengrund exprimiert (Florian et al. 2001). Heute wird der GPx2 auf Grund ihrer Lokalisation im Kryptengrund und ihrer Regulation über den Wnt (*Wingless*/Int-1) -Signalweg sowie über p63 eine wichtige Funktion bei der Zellproliferation zugeschrieben (Florian et al. 2010). Ebenso wie bei der GPx1 führt auch bei der GPx2 ein Knockout nicht zu phänotypischen Veränderungen (Esworthy et al. 2000). An GPx2-Knockout-Mäusen konnte gezeigt werden, dass das Fehlen der GPx2 durch eine Hochregulation der GPx1 kompensiert werden kann (Florian et al. 2010). Ein Doppelknockout von GPx1 und GPx2 führt allerdings zur Entwicklung einer Kolitis und zu intestinalen Tumoren (Chu et al. 2004, Esworthy et al. 2001). Dies lässt auf antiinflammatorische und antikanzerogene Effekte der GPx2 schließen. Die antiinflammatorische Wirkung der GPx2 beruht auf einer Hemmung der Cyclooxygenase 2 (COX2), die vermutlich durch eine Verminderung der Hydroperoxidlevel zu Stande kommt, da die COX2 Hydroperoxide für ihre Aktivität benötigt (Banning et al. 2008, Kulmacz et al. 2005). Die GPx2 weist in ihrer Promotorregion ein funktionelles *Antioxidant Response Element* (ARE) auf und wird daher auch über den Transkriptionsfaktor *Nuclear Factor Erythroid 2-Related Factor 2* (Nrf2) reguliert.

1.1.2 Pathophysiologische Wirkungen von Selen

Insulinresistenz, Typ 2 Diabetes und Hyperlipidämien

Die Rolle von Selen als Antidiabetikum und Insulinmimetikum ist umstritten und wurde vor allem in den letzten Jahren stark diskutiert. Während dem Selen lange Zeit eine positive Wirkung in Bezug auf Insulinresistenz, Typ 2 Diabetes und Hyperlipidämien zugesprochen wurde, konnten aktuelle Untersuchungen zeigen, dass sowohl die Supplementation hoher Selenkonzentrationen, als auch ein hoher Selenstatus, negativ mit diesen Erkrankungen korrelieren.

In verschiedenen Fall-Kontroll-Studien wurde ein inverser Zusammenhang zwischen dem Selenstatus und der Diabetesprävalenz beobachtet (Park et al. 2012, Kljai und Runje 2011, Akbaraly et al. 2010, Navarro-Alarcón et al. 1999). Allerdings existieren im Humanbereich nahezu keine Plazebo-kontrollierten Interventionsstudien zur präventiven sowie zur therapeutischen Wirkung von Selen in Bezug auf Insulinresistenz und Typ II Diabetes (Mueller et al. 2009). Vor allem in zwei Tierversuchen mit db/db-Mäusen (als Modell für Typ

2 Diabetes) konnten positive Effekte von Selen bezüglich Insulinresistenz und Typ 2 Diabetes gezeigt werden. Diese äußerten sich in einem verbesserten Glukosestoffwechsel sowie in einer Reduktion der Insulinresistenz (Mueller und Pallauf 2006, Mueller et al. 2003). Die positiven Wirkungen konnten jedoch nur unter Verwendung sehr hoher Selendosierungen in Form von Natriumselenat (Selenoxidationsstufe +VI) erzielt werden. In Humanstudien ist die Verwendung solch hoher Selenkonzentrationen auf Grund möglicher toxischer Effekte nicht praktikabel.

Vor allem in den letzten Jahren lieferten Tier- und Humanstudien wiederholt Hinweise auf einen Zusammenhang zwischen einer langfristig hohen Selensupplementierung beziehungsweise einem hohen Selenstatus und der Entstehung von Insulinresistenz und Typ 2 Diabetes (Lippman et al. 2009, Bleys et al. 2007, Stranges et al. 2007, Czernichow et al. 2006). Weiterhin konnte in Tierversuchen gezeigt werden, dass eine längerfristige Selensupplementierung (Mueller et al. 2009) sowie eine permanent hohe GPx1-Aktivität (McClung et al. 2004) zur Entstehung von Insulinresistenz und Typ 2 Diabetes führen können (Mueller et al. 2009, Übersicht zu Tier- und Humanstudien mit negativer Wirkung von Selen auf Insulinresistenz und Typ 2 Diabetes).

Neben der Entstehung von Insulinresistenz und Typ 2 Diabetes wurde auch der Zusammenhang zwischen Selen und der Entstehung von Hyperlipidämien untersucht. Auch hier ist die Studienlage uneinheitlich. In der amerikanischen NHANES III-Studie (1988-1994), einer Querschnittsuntersuchung, wiesen die Probanden der höchsten Serumselen-Quintile 10 % höhere Plasma-Triglyceridkonzentrationen auf als die Probanden der niedrigsten Quintile. Auch die Gehalte an Gesamt-, *Low Density Lipoprotein* (LDL)- und *High Density Lipoprotein* (HDL)-Cholesterin sowie verschiedener Apolipoproteine im Serum korrelierten positiv mit der Serumselenkonzentration (Bleys et al. 2008). Diese Ergebnisse wurden auch für einen späteren Beobachtungszeitraum (2003-2004) bestätigt (Laclaustra et al. 2010). In einer Untersuchung mit britischen Probanden konnte ebenfalls ein Zusammenhang zwischen hohen Plasmaselenkonzentrationen und erhöhtem Gesamt- und nicht-HDL-Cholesterin festgestellt werden. Die Studienlage zum Einfluss einer Selensupplementierung auf Parameter des Lipidmetabolismus ist dennoch uneinheitlich. Während zwei Untersuchungen keinen Effekt von Selensupplementation fanden (Yu et al. 1990, Luoma et al. 1984), konnte an älteren Probanden gezeigt werden, dass die Supplementation von 100 µg sowie 200 µg Selen pro Tag in Form einer selenhaltigen Hefe zu einer Senkung des Gesamtserumcholesterins und des nicht-HDL-Cholesterins führt. Die Verabreichung von 300 µg Selen pro Tag bewirkte keine signifikanten Effekte auf das Gesamt- und nicht-HDL-

Cholesterin, erhöhte aber das HDL-Cholesterin signifikant (Rayman et al. 2011). Die Bewertung dieser Humanstudien ist jedoch schwierig, da es sich überwiegend um Untersuchungen mit geringer Teilnehmerzahl und kurzer Studiendauer (Luoma et al. 1984, Yu et al. 1990), oder um Kombinationen mit anderen Vitaminen und Mineralstoffen handelt (Zhang et al. 2006a, Hercberg et al. 2005).

Die zu Grunde liegenden Mechanismen, die den Zusammenhang von Selensupplementen beziehungsweise einem hohen Selenstatus mit der Entstehung von Insulinresistenz, Typ 2 Diabetes und Hyperlipidämien erklären, sind größtenteils unbekannt. Einen möglichen Mechanismus stellt die selenabhängige Regulation der Proteintyrosinphosphatase 1B (PTP1B) dar (Mueller et al. 2009). Die PTP1B ist ein zytosolisches Enzym, das in der Insulinsignalkaskade an der Balancierung des Insulinsignals über die Dephosphorylierung der β-Untereinheit des Insulinrezeptors und des Insulinrezeptorsubstrats 1 beteiligt ist (Koren und Fantus 2007; Tonks 2003). Darüber hinaus beeinflusst die PTP1B über die Aktivierung des lipogenen Transkriptionsfaktors *Sterol Regulatory Element Binding Protein 1c* (SREBP1c) und dessen Zielgen Fettsäuresynthase (FAS) auch den Fettstoffwechsel (Shimizu et al. 2003). Die physiologische Hemmung der PTP1B verläuft über die reversible Oxidation der –SH-Gruppe des katalytisch aktiven Cysteinrests 215 zur Sulfensäure und die folgende Addition von Glutathion (Glutathionylierung) mittels Glutathion-S-Transferasen (GST) (Townsend et al. 2006, Barrett et al. 1999). Einen weiteren möglichen Mechanismus bei der Entstehung von Insulinresistenz, Typ 2 Diabetes und Hyperlipidämien durch Selen stellt das *Pancreatic and Duodenal Homeobox1* (PDX1) –Protein dar. Mäuse mit einer Überexpression der GPx1 wiesen im Vergleich zu Wildtypmäusen eine Hyperinsulinämie mit der einhergehenden Erhöhung des PDX1-Proteins auf, welches an der β-Zelldifferenzierung sowie an der Genexpression und Synthese des Insulins beteiligt ist (Wang et al. 2008). Ein weiterer möglicher Regulator für die Entstehung von Hyperlipidämien ist der Transkriptionsfaktor Nrf2. In einer Untersuchung mit Mäusen bewirkte der Knockout von Nrf2 eine erhöhte Konzentration an Gesamtlipiden, gesättigten sowie mehrfach ungesättigten Fettsäuren in der Leber und damit letztendlich eine Steatohepatitis (Chowdhry et al. 2010).

Krebs

Es ist bekannt, dass ein niedriger Selenstatus mit einem signifikant erhöhten Krebsrisiko einhergeht (Shamberger und Frost. 1969). Verschiedene prospektive Studien deuten auf einen positiven Zusammenhang zwischen der Selenaufnahme respektive dem Selenstatus und dem

Risiko für Lungen-, Blasen-, Kolorektal-, Leber-, Ösophagus-, Schilddrüsen- und Prostatakrebs hin (Übersicht in Rayman 2012). In der NPC-Studie hatte die Supplementation von 200 µg Selen pro Tag in Form von Selenomethionin über 4,5 Jahre keinen Effekt auf den primären Endpunkt Nicht-Melanom-Hautkrebs. Allerdings führte die Selensupplementation zu einer signifikanten Abnahme der Mortalität sowie der Inzidenz von Prostata- (52 %), Lungen- (26 %) und Kolorektalkrebs (54 %) (Duffield-Lillico et al. 2002). Insbesondere für den Zusammenhang zwischen dem Selenstatus und dem Kolorektalkrebsrisiko sind die Ergebnisse klinischer Studien uneinheitlich (Übersicht in Méplan und Hesketh 2012). In einem Mausmodell für die Kolonkarzinogenese konnte die Selensupplementierung (1 ppm) der Azoxymethan-induzierten Bildung von aberranten Kryptenfoci und der Tumorbildung vorbeugen (Hu et al. 2008). Für den zu Grunde liegenden Mechanismus des präventiven Effekts von Selen wird vor allem den Selenoproteinen eine große Rolle zugeschrieben, da diese die Kanzerogenese über verschiedene Wege, wie zum Beispiel die Redoxregulation sowie die Proteinfaltung, beeinflussen können. Neben der Rolle von Selen und Selenoproteinen bezüglich oxidativem Stress und ER Stress konnten noch weitere Stoffwechselwege aufgezeigt werden, die im Kolon von Mäusen durch die Selenaufnahme beeinflusst werden. Dazu zählen der Wnt-Signalweg, die Proteinsynthese, die Regulation von eiF4E und der p70s6 Kinase, ribosomale Proteine, *Nuclear Factor Kappa* B (NFkB)- sowie Nrf2-regulierte Signalwege (Müller et al. 2010, Kipp et al. 2009).

Kardiovaskuläre Erkrankungen und Homocysteinstoffwechsel

Die Forschungsergebnisse zum Zusammenhang von Selen und der Entstehung von kardiovaskulären Erkrankungen sind kontrovers. Mögliche protektive Mechanismen des Selens in Bezug auf kardiovaskuläre Erkrankungen bestehen im Schutz vor oxidativen Lipidmodifikationen, in der Hemmung der Blutplättchenaggregation sowie in den antiinflammatorischen Eigenschaften (Rayman 2012). In einer Meta-Analyse wurde eine inverse Assoziation zwischen der Selenkonzentration und dem Risiko für koronare Herzkrankheiten gezeigt (Übersicht in Flores-Mateo et al. 2006). Allerdings konnten randomisierte Untersuchungen, wie die NPC- und die SELECT-Studie, keine signifikant protektiven Effekte von Selen in Bezug auf kardiovaskuläre Erkrankungen nachweisen (Lippman et al. 2009, Stranges et al. 2006). Im Gegensatz dazu wird eine permanent hohe Selensupplementation beziehungsweise ein hoher Selenstatus auch als negativ für die Entstehung von Typ 2 Diabetes und Hyperlipidämien angesehen (Stranges et al. 2010a, Lippman et al. 2009, Bleys et al. 2007, Stranges et al. 2007, Czernichow et al. 2006), welche

ihrerseits Risikofaktoren für kardiovaskuläre Erkrankungen darstellen. Ein weiterer Risikofaktor für die Entstehung von kardiovaskulären Erkrankungen ist das Homocystein (Zhou und Austin 2009). Allerdings sind auch hier die Ergebnisse aus der Literatur bezüglich des Einflusses von Selen widersprüchlich. In humanen Querschnittsstudien wurde eine inverse Korrelation des Selenstatus mit der Plasmahomocysteinkonzentration nachgewiesen (Kłapcińska et al. 2005, González et al. 2004, Bates et al. 2002). Im Gegensatz dazu konnte in Tierversuchen mit Hühnern, Mäusen und Ratten gezeigt werden, dass eine selenarme Ernährung im Vergleich zu adäquater oder leicht supranutritiver Selenversorgung zu einer Abnahme der Plasmahomocysteinkonzentration führt (Uthus und Ross 2009, 2007, Uthus et al. 2002, Halpin und Baker 1984).

Homocystein ist ein Metabolit des Methioninstoffwechsels. Es kann über zwei verschiedene Wege umgesetzt werden. Zum einen kann es durch die Transsulfurierung zu Cystein metabolisiert werden, welches für die Glutathionbiosynthese mittels der GCL benötigt wird. Zum anderen kann eine Remethylierung durch die Methioninsynthase oder die Betain-Homocystein Methyltransferase (BHMT) erfolgen. Die in den Tierexperimenten gezeigte Reduktion des Homocysteins, welche mit einer Erhöhung der Glutathionkonzentration einherging, wird durch eine vermehrte Aktivierung des Transsulfurierungsweges begründet (Uthus et al. 2002). Neben Selen bewirkt auch die Supplementierung mit Methionin einen Anstieg der Homocysteinkonzentration im Plasma von Ratten (Hirche et al. 2006a, b). Auch in einer Humanstudie konnte ein Methionin-bedingter Anstieg der Plasmahomocysteinwerte nachgewiesen werden (Ward et al. 2001). Allerdings gibt es unterschiedliche Aussagen bezüglich der kritischen Methioninkonzentration.

1.2 Glucoraphanin und Sulforaphan

Das Glucoraphanin zählt zu den Glucosinolaten. Hierbei handelt es sich um eine große Anzahl von schwefelhaltigen Verbindungen, die vor allem in Kreuzblütengewächsen (*Brassicaceae*), wie zum Beispiel Brokkoli, Blumenkohl und Weißkohl enthalten sind (Johnson 2002, Verhoeven et al. 1996). Chemisch bestehen die Glucosinolate aus einer β-D-Thioglukosegruppe, einer sulfonierten Oximgruppe sowie einer variablen Seitenkette. Die Glucosinolate können in Abhängigkeit von ihrer Seitengruppe in aliphatische, aromatische, indolische oder allylische Glucosinolate unterteilt werden. Das dominierende Glucosinolat in Brokkoli ist das aliphatische Glucoraphanin. Die Hydrolyse von Glucosinolaten erfolgt durch das Enzym Myrosinase (ß-Thioglucosidase). Dabei wird Glukose abgespalten und es entsteht das entsprechende Aglykon. Dieses ist jedoch instabil und wird spontan durch Abgabe eines

Sulfats in ein Isothiocyanat umgewandelt (Lossen-Reaktion) (siehe **Abbildung 1**). Neben Isothiocyanaten können auch Thiocyanate oder bei niedrigem pH-Wert Nitril und elementarer Schwefel entstehen. In Pflanzen ist die Myrosinase kompartimentell von den Glucosinolaten getrennt und wird beim Kauen oder Zerkleinern freigesetzt. Die Myrosinase kann durch Hitze inaktiviert werden. Glucosinolate aus hitzebehandelten Pflanzen oder Nahrungsergänzungsmitteln können im Zäkum und Kolon durch bakterielle ß-Glucosidasen zu Isothiocyanaten hydrolysiert werden (Brigelius-Flohé und Banning 2006). Das bioaktive Hydrolyseprodukt des Glucoraphanins ist das Isothiocyanat Sulforaphan (1-Isothiocyanato-4R-Methylsulfinyl-Butan) (Fahey und Talalay 1999).

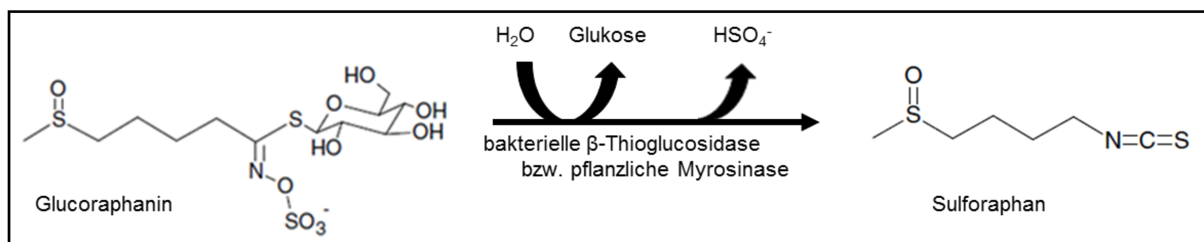

Abbildung 1: Hydrolyse von Glucoraphanin zu Sulforaphan durch Myrosinase oder bakterielle ß-Thioglucosidasen (modifiziert nach Clarke et al. 2008)

Sulforaphan besitzt eine hohe Bioverfügbarkeit. In Humanstudien sowie Untersuchungen an Ratten sind 60 % bis 75 % des verabreichten Sulforaphans in Form von Sulforaphan und dessen Metaboliten (z.B. Dithiocarbamate, Sulforaphan-Cystein und Sulforaphan-*N*-Acetylcystein) im Urin nachweisbar (Veeranki et al. 2013, Egner et al. 2011, Zhang et al. 2006b, Kassahun et al. 1997). Die Aufnahme in die Enterozyten des Dünndarms erfolgt mittels passiver Diffusion. Im Enterozyten wird Sulforaphan durch eine GST-abhängige Reaktion mit Glutathion konjugiert. Das entstandene Dithiocarbamat kann entweder über P-Glykoprotein wieder ins Lumen transportiert werden oder über Transporter wie MRP1 in das Blutsystem gelangen (Petri et al. 2003). Verschiedene Human- und Nagetierstudien konnten, gemäß der hohen Lipophilie und der kleinen Molekülgröße, eine schnelle Absorption des Sulforaphans nachweisen. So zeigte sich bereits 1-3 Stunden nach Sulforaphanaufnahme eine maximale Plasmakonzentration an Sulforaphan und der entsprechenden Metabolite (Hanlon et al. 2008; Clarke et al. 2011). Das glutathionylierte Sulforaphan wird schrittweise über den Mercaptursäureweg zu N-Acetylcysteinyl-Sulforaphan abgebaut, welches schließlich über den Urin ausgeschieden wird (Fahey et al. 2012). Nicht-hydrolysiertes Glucoraphanin kann entweder intakt absorbiert werden und in den enterohepatischen Kreislauf gelangen, oder im

Zäkum sowie im Kolon teilweise durch bakterielle Myrosinasen hydrolysiert werden (Bheemreddy und Jeffery 2007). Die Isothiocyanate weisen eine circa 6-mal höhere Bioverfügbarkeit als die Glucosinolate auf (Shapiro et al. 2001).

Dem Sulforaphan werden vor allem chemopräventive Wirkungen zugesprochen. Zahlreiche Untersuchungen konnten zeigen, dass Sulforaphan das Risiko für verschiedene Krebsarten, wie Kolon-, Prostata-, Lungen-, Blasen- und Brustkrebs reduziert (Li et al. 2010, Keum et al. 2009, Munday et al. 2008, Shen et al. 2007, Conaway et al. 2005, Chung et al. 2000). Dieser Effekt beruht vermutlich zu einem hohen Anteil auf der Sulforaphan-bedingten Induktion von Phase-II-Enzymen. Weitere Mechanismen, die der antikarzinogenen Wirkung von Sulforaphan zu Grunde liegen, sind: (1) die Hemmung von Phase-I-Enzymen, (2) antioxidative Funktionen durch erhöhte Glutathionspiegel, (3) die Apoptose-Induktion, (4) die Induktion des Zellzyklusarrests, (5) antiinflammatorische Eigenschaften und (6) die Hemmung der Angiogenese (Juge et al. 2007).

1.3 KEAP1/Nrf2/ARE-System

Der Transkriptionsfaktor Nrf2 ist von Bedeutung für die Entgiftung von reaktiven Elektrophilen und Oxidanzien, die andernfalls zur Entwicklung von Mutationen und der Entstehung von Krebs führen können (Slocum und Kensle 2011).

Nrf2 zählt zur Familie der basischen Leucin-Zipper (b-Zip)-Transkriptionsfaktoren und reguliert über die Bindung an das ARE (auch als *Electrophilic Response Element* bezeichnet) die Expression von verschiedenen Genen des antioxidativen Schutzsystems sowie des Fremdstoffmetabolismus (Itoh et al. 1997). Das Nrf2 Protein besteht aus 6 hochkonservierten Regionen, die als Nrf2-ECH *Homology* Domänen (Neh1-6) bezeichnet werden. Neh1 enthält die b-Zip- sowie die *Cap 'N' Collar* (CNC) -Region und ist somit für die Dimerisierung mit kleinen Maf-Proteinen und auch für die DNA-Bindung verantwortlich. Die Domäne Neh2 vermittelt die negative Regulation von Nrf2 durch *Kelch-like ECH-Associated Protein 1* (KEAP1) (Baird und Dinkova-Kostova 2011, Itoh et al. 1999). KEAP1 ist ein zytoplasmatisches Protein, das bei Mäusen 25 und beim Mensch 27 Cysteinreste aufweist (Baird und Dinkova-Kostova 2011, Wakabayashi et al. 2004). Unter basalen Bedingungen bildet dimeres Keap1 mit monomerem Nrf2 einen Komplex. Dabei erfolgt die Bindung an die Neh2-Domäne zum einen über ein ETGE-Motiv mit hoher Affinität und zum anderen über ein DLG-Motiv mit niedriger Affinität (Li und Kong 2009). Keap1 hält Nrf2 durch Bindung an Aktin im Zytoplasma. Die Verbindung von Keap1 und Nrf2 führt außerdem über die Cullin3-Rbx1 E3 Ubiquitinligase zur Ubiquitinierung sowie dem proteasomalen Abbau von Nrf2.

Oxidativer Stress oder elektrophile Verbindungen führen zur Modifikation der Sulfhydrylgruppen verschiedener Cysteinreste im Keap1 (Baird und Dinkova-Kostova 2011, Li und Kong 2009). Vor allem die Cysteingruppen C151, C273 und C288 sind für die Funktion von Keap1 von Bedeutung (Baird und Dinkova-Kostova 2011). Durch Modifikation der Cysteingruppen und die folgende Konformationsänderung des Keap1 wird die Bindung am DLG-Motiv getrennt. Die Ubiquitinbindungsstellen sind nicht mehr zugänglich. Dadurch werden die Ubiquitinierung und der proteasomale Abbau von Nrf2 gehemmt. Die Proteintranslation findet vermehrt statt und der Pool an freiem Nrf2 nimmt zu. Freies Nrf2 kann in den Zellkern translozieren. (Li und Kong 2009). Dort bildet es ein Heterodimer mit kleinen Maf-Proteinen. Dieses Heterodimer bindet an das ARE in der Promotorregion verschiedener Gene und verstärkt deren Transkription (Itoh et a. 1997).

Zu den Nrf2 regulierten Genen gehören neben den beiden Selenoproteinen GPx2 und TrxR1 zahlreiche weitere Gene. Dazu zählen vor allem antioxidative sowie detoxifizierende Enzyme, wie die Hämoxygenase 1 (HO1), GST, UDP-Glucuronosyltransferasen (UGT) und die NAD(P)H-Quinonoxidoreduktase 1 (NQO1). Des Weiteren werden auch Transportproteine für Fremdstoffe und deren Metabolite, wie zum Beispiel S*olute Carrier*- und ATP-*Binding Cassette* Transporter sowie Enzyme, die an der Synthese und Regeneration von Glutathion beteiligt sind (zum Beispiel GCL, Glutathionreduktase (GR)), über den Nrf2/ARE-Weg reguliert. Die Substanzen, die das Nrf2-ARE-System induzieren, können in zehn chemische Klassen eingeteilt werden: (1) oxidierbare Diphenole und Phenylendiamine, (2) Michael-Akzeptoren, (3) Isothiocyanate (z.B. Sulforaphan) und Sulfoxythiocarbamate, (4) Thiocarbamate, (5) Dithiolethione, (6) konjugierte Polyene, (7) Hydroperoxide (z.B. bei Selenmangel), (8) trivalente Arsenverbindungen, (9) Schwermetalle und (10) vicinale Dimercaptane (Baird und Dinkova-Kostova 2011).

2. Zielstellung

Das Spurenelement Selen wird derzeit kritisch im Hinblick auf die Entstehung unterschiedlicher Stoffwechselerkrankungen diskutiert. Vor allem bezüglich der zu Grunde liegenden zellulären Mechanismen besteht Forschungsbedarf.

Das Ziel der vorliegenden Arbeit war es daher, den Einfluss verschiedener Selenkonzentrationen auf unterschiedliche Stoffwechselwege zu prüfen und einen Einblick in die zu Grunde liegenden Mechanismen zu erhalten. Dabei wurden auch Wechselwirkungen des Selens mit Methionin sowie mit Glucoraphanin beziehungsweise Sulforaphan untersucht.

Selen wird vor allem in aktuellen Humanstudien mit der Entstehung von Hyperlipidämien in Zusammenhang gebracht. Auf Grund der physiologischen PTP1B-Regulation durch H_2O_2 sowie Lipidhydroperoxide mittels Glutathionylierung und der wichtigen Rolle des Selenoproteins GPx im Peroxidstoffwechsel sollte in ***Studie 1*** der Einfluss verschiedener Selenkonzentrationen auf die Regulation der PTP1B sowie daraus resultierende Effekte auf den Fettstoffwechsel untersucht werden. Hierzu wurden wachsende männliche Ratten mit Diäten gefüttert, die drei verschiedene Selenkonzentrationen enthielten. Eine Gruppe erhielt eine selenarme Diät (<20 µg Selen pro kg Diät), während das Futter der beiden anderen Gruppen 75 µg beziehungsweise 150 µg Selen pro kg Diät enthielt. Nach Versuchsende wurden der Gesamtfettgehalt sowie die Konzentrationen an Triglyceriden, Phospholipiden und Lipidperoxiden in der Leber bestimmt. Außerdem wurde die Aktivität sowie die Expression verschiedener GPx und der TrxR1 ermittelt. Um den Einfluss der unterschiedlichen Selenkonzentrationen auf die PTP1B zu ermitteln, wurden deren Aktivität, Proteinexpression und Glutathionylierung analysiert. Weiterhin wurde die relative mRNA-Konzentration des Transkritpionsfaktors SREBP1c sowie dessen Zielgen FAS bestimmt. Die verwendeten Methoden sowie die Ergebnisse und deren Diskussion sind ersichtlich in:

Studie 1 (S1):

Mueller AS, Klomann SD, **Wolf NM**, Schneider S, Schmidt R, Spielmann J, Stangl G, Eder K, Pallauf J. Redox regulation of protein tyrosine phosphatase 1B by manipulation of dietary selenium affects the triglyceride concentration in rat liver. J Nutr. 2008; 138: 2328-2336.

Homocystein, welches als Risikofaktor für kardiovaskuläre Erkrankungen diskutiert wird, kann sowohl durch Selen- als auch durch Methioninsupplementation beeinflusst werden. Das Ziel der ***Studie 2*** war es, die Wirkungen und Wechselwirkungen verschiedener Selen- und Methioninkonzentrationen auf den Homocystein- und Cholesterinstoffwechsel zu untersuchen. Hierfür wurden männliche wachsende Ratten mit vier verschiedenen

Selenkonzentrationen (von selenarm bis zum Dreifachen der Empfehlung) sowie zwei unterschiedlichen Methioninkonzentrationen (in Höhe der Empfehlung sowie dem Fünffachen der Empfehlung) gefüttert. Nach Versuchsende wurde die Konzentration des Gesamtglutathions sowie des Homocysteins im Plasma und der Leber bestimmt. Um Aufschluss über die Enzyme zu erlangen, die durch Selen beziehungsweise Methionin beeinflusst werden, wurde die relative mRNA-Konzentration verschiedener Gene des Methionin-, Homocystein- und Glutathionstoffwechsels mittels *real-time* RT-PCR ermittelt. Dies waren im Einzelnen die Glycin-N-Methyltransferase (GNMT), die Cystathionin-β-Synthase (CBS), die S-Adenosylmethionin-Decarboxylase (SAMDC), die Betainhydroxymethyltransferase (BHMT), die katalytische und modulatorische Einheit der GCL (GCLC und GCLM) sowie die Glutathionsynthase (GS). Zur Ermittlung der Effekte auf den Cholesterinstoffwechsel wurde die Cholesterinkonzentration in der Leber und im Plasma sowie die relative mRNA-Konzentration von SREBP2, dem LDL-Rezeptor (LDLR), der 3-Hydroxy-3-Methylglutaryl Coenzym A-Reduktase (HMGCoAR) sowie des ATP-*binding cassette transporter* G8 (ABCG8) analysiert. Weitere Details zu den Methoden, sowie den Ergebnissen und deren Diskussion sind dargestellt in:

Studie 2 (S2):

Wolf NM, Mueller K, Hirche F, Most E, Pallauf J, Mueller AS. Study of molecular targets influencing homocysteine and cholesterol metabolism in growing rats by manipulation of dietary selenium and methionine concentrations. Br J Nutr. 2010; 104: 520-532.

Die Ergebnisse der ***Studie 2*** zeigten eindeutig erhöhte Plasmahomocysteinkonzentrationen in adäquat beziehungsweise leicht supranutritiv mit Selen versorgten Ratten im Vergleich zu Ratten die eine selenarme Diät erhielten. Dies war vor allem auf eine geringere Glutathionbiosynthese zurückzuführen. Die an der Glutathionbiosynthese beteiligten Enzyme GS und GCL werden durch den Transkriptionsfaktor Nrf2 reguliert. Daher sollte in ***Studie 3*** die Frage beantwortet werden, ob die Fütterung mit Glucoraphanin, dessen Hydrolyseprodukt Sulforaphan ein Induktor des Nrf2-Weges ist, die Homocysteinwerte durch Aktivierung von Glutathionbiosyntheseenzymen in selenversorgten Ratten senken kann. Dazu wurden männliche wachsende Ratten mit Diäten gefüttert, welche die gleichen Selenkonzentrationen wie in ***Studie 2*** enthielten. Die Hälfte der Diäten war zusätzlich mit Glucoraphanin (700 µmol pro kg Diät) angereichert. Nach Versuchsende wurde die Glutathion- sowie die Homocysteinkonzentration sowohl in der Leber als auch im Plasma bestimmt. Außerdem wurden die relativen mRNA-Konzentrationen verschiedener Gene, die an der Regulation der

Glutathionbiosynthese sowie des Homocysteinstoffwechsels beteiligt sind, gemessen. Darüber hinaus wurden auch die relativen mRNA-Konzentrationen von Keap1, Nrf2 sowie verschiedener Nrf2-regulierter Gene wie HO1, *Cytochrom P450 Member* 1a1 (Cyp1a1), NQO1 und *Multidrug Resistance Associated Protein* 4 (Mrp4) bestimmt. Eine detaillierte Darstellung der verwendeten Methoden, sowie der Ergebnisse und deren Diskussion findet sich in:

Studie 3 (S3):

Blum NM, Mueller K, Hirche F, Lippmann D, Most E, Pallauf J, Linn T, Mueller AS. Glucoraphanin does not reduce plasma homocysteine in rats with sufficient Se supply via the induction of liver ARE-regulated glutathione biosynthesis enzymes. Food Funct. 2011; 2: 654-664.

Sowohl das Isothiocyanat Sulforaphan als auch Selen können über den Transkriptionsfaktor Nrf2 die Expression von Xenobiotika-metabolisierenden und von antioxidativen Enzymen beeinflussen. Dies wird vor allem im Zusammenhang mit der antikanzerogenen Wirkung von Selen und Sulforaphan in Verbindung gebracht. Das Ziel der ***Studie 4*** bestand in der Untersuchung des Einflusses von Selen und Glucoraphanin auf Nrf2-regulierte antioxidative und Xenobiotika-metabolisierende Enzyme. Da Glucoraphanin vornehmlich im Zäkum und Kolon zu Sulforaphan hydrolysiert wird, wurde sowohl die Wirkung im Jejunum (vor der Hydrolyse) als auch im Kolon untersucht. Ein weiteres Ziel stellte die Untersuchung der systemischen Effekte beider Supplemente in der Leber dar. Hierfür wurden wachsende männliche Ratten mit drei verschiedenen Selenkonzentration gefüttert. Zusätzliche Diäten mit der gleichen Abstufung des Selens waren mit Glucoraphanin (700 µmol pro kg Diät) angereichert. Nach Versuchsende wurde die relative mRNA-Konzentration eines breiten Spektrums von Nrf2-regulierten antioxidativen Enzymen und Phase-II-Enzymen sowie von Nrf2 und Keap1 mittels *real-time* RT-PCR bestimmt. Außerdem wurden die Aktivitäten der GSTA, der GSTP sowie der GPx und der NQO1 enzymatisch ermittelt. Des Weiteren wurde Nrf2 auf Ebene der Proteinexpression mittels Immunoblot analysiert. Weitere detaillierte Angaben zu den Methoden sowie die Ergebnisse und deren Diskussion finden sich in:

Studie 4 (S4):

Blum NM, Mueller K, Lippmann D, Metges CC, Linn T, Pallauf J, Mueller AS. Feeding of Selenium Alone or in Combination with Glucoraphanin Differentially Affects Intestinal and Hepatic Antioxidant and Phase II Enzymes in Growing Rats. Biol Trace Elem Res. 2013; 151: 384-399.

Während in ***Studie 4*** der Einfluss des Glucosinolates Glucoraphanin auf die Regulation von antioxidativen Enzymen und von Phase-II-Enzymen in unterschiedlichen Darmabschnitten beziehungsweise der Leber von wachsenden Ratten untersucht wurde, sollte in ***Studie 5 (S5)*** die direkte Wirkung des Isothiocyanates Sulforaphan bestimmt werden. Ein weiteres Ziel war es, die Wirkung von Sulforaphan verglichen mit einem selenarmen Zustand, auf den Fettstoffwechsel zu ermitteln. In den ***Studien 1*** und ***2*** konnte gezeigt werden, dass selenarm gefütterte Ratten niedrigere hepatische Triglycerid- und Cholesterinkonzentrationen als die Kontrolltiere aufwiesen. Aus einer Untersuchung mit Hamstern geht hervor, dass auch Sulforaphan positiv auf den Cholesterinstoffwechsel wirken kann (Rodríguez-Cantú et al. 2011). Ziel der ***Studie 5*** war es daher zu untersuchen, ob das Isothiocyanat Sulforaphan bei wachsenden Ratten eine vergleichbare Wirkung auf den Fettstoffwechsel hat, wie eine selenarme Fütterung. Dazu wurden Ratten in vier Gruppen eingeteilt. Eine Gruppe erhielt über 6 Wochen eine selenarme Diät, während die drei anderen Gruppen Selen in Höhe der Empfehlung (150 µg/kg Diät) erhielten. Zwei Tage vor dem Versuchsende wurde zwei der adäquat mit Selen versorgten Gruppen 50 µmol beziehungsweise 100 µmol Sulforaphan oral verabreicht. Anschließend wurde sowohl die Aktivität und Expression von antioxidativen und Phase-II-Enzymen als auch die Wirkung auf Parameter des Lipidstoffwechsels ermittelt. Die Ergebnisse sind bisher unveröffentlicht und unter ***3. Originalarbeiten*** dargestellt.

3. Originalarbeiten

3.1 Studie 1: *Mueller AS, Klomann SD, **Wolf NM**, Schneider S, Schmidt R, Spielmann J, Stangl G, Eder K, Pallauf J. Redox regulation of protein tyrosine phosphatase 1B by manipulation of dietary selenium affects the triglyceride concentration in rat liver. J Nutr. 2008; 138: 2328-2336.*

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Redox Regulation of Protein Tyrosine Phosphatase 1B by Manipulation of Dietary Selenium Affects the Triglyceride Concentration in Rat Liver[1,2]

Andreas S. Mueller,[3,4]* Sandra D. Klomann,[5] Nicole M. Wolf,[5] Sandra Schneider,[6] Rupert Schmidt,[6] Julia Spielmann,[3] Gabriele Stangl,[3] Klaus Eder,[3] and Josef Pallauf[5]

[3]Institute of Agricultural and Nutritional Sciences, and [4]Preventive Nutrition Group, Martin Luther University Halle Wittenberg, D-06120 Halle/Saale, Germany, [5]Interdisciplinary Research Centre, Department of Animal Nutrition and Nutritional Physiology, and [6]Biotechnology Center, Justus Liebig University Giessen, D-35392 Giessen, Germany

Abstract

Protein tyrosine phosphatase 1B (PTP1B) is a key enzyme in the counter-regulation of insulin signaling and in the stimulation of fatty acid synthesis. Selenium (Se), via the activities of glutathione peroxidase (GPx) and thioredoxin reductase (TrxR), is involved in the removal of H_2O_2 and organic peroxides, which are critical compounds in the modulation of PTP1B activity via glutathionylation. Our study with growing rats investigated how the manipulation of dietary Se concentration influences the regulation of PTP1B and lipogenic effects mediated by PTP1B. Weanling albino rats were divided into 3 groups of 10. The negative control group (NC) was fed a Se-deficient diet for 8 wk. Rats in groups Se75 and Se150 received diets supplemented with 75 or 150 μg Se/kg. Se supplementation of the rats strongly influenced expression and activity of the selenoenzymes cytosolic GPx, plasma GPx, phospholipidhydroperoxide GPx, and cytosolic TrxR, and liver PTP1B. Liver PTP1B activity was significantly higher in groups Se75 and Se150 than in the NC group and this was attributed to a lowered inhibition of the enzyme by glutathionylation. The increased liver PTP1B activity in groups Se75 and Se150 resulted in 1.1- and 1.4-fold higher liver triglyceride concentrations than in the NC rats. The upregulation of the sterol regulatory element binding protein-1c and of fatty acid synthase, 2 PTP1B targets, provided a possible explanation for the lipogenic effect of PTP1B due to the manipulation of dietary Se. We therefore conclude that redox-regulated proteins, such as PTP1B, represent important interfaces between dietary antioxidants such as Se and the regulation of metabolic processes. J. Nutr. 138: 2328–2336, 2008.

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Introduction

In recent years, many reports have focused on protein tyrosine phosphatase 1B (PTP1B)[7] as an important target enzyme for the treatment of obesity and diabetes accompanied by insulin resistance (1,2). PTP1B belongs to the cysteine-based phosphatases and antagonizes insulin signaling, because it is capable of dephosphorylating the β-subunit of the insulin receptor and insulin receptor substrate 1 (3,4). In studies with humans (5) and in animal models, a low PTP1B activity, obtained by RNA interference (6–9) or biochemical enzyme inhibition through selenate and vanadium compounds (10–13), protected against obesity, insulin resistance, and diabetes, whereas high PTP1B activities accelerated the development of these diseases. The involvement of PTP1B in fatty acid metabolism is another important physiological function of the enzyme. Feeding fructose-rich diets to rats elevated PTP1B expression and activity, resulting in the induction of fatty acid synthase (FAS) and in an increased liver triglyceride concentration (14,15). The lipogenic mechanism of PTP1B involves the activation of protein phosphatase 2A and subsequently of the sterol regulatory element-binding protein-1c (SREBP-1c) (16,17). This acts as a transcription factor for FAS and other lipogenic enzymes (18,19).

In contrast to PTP1B regulation by exogenously applied inhibitors or RNA interference, the enzyme undergoes a physiological inhibition via oxidation of its active site cysteine residue, Cys-215. In the presence of H_2O_2, a reversibly oxidized sulphenic

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[7] Abbreviations used: GPx, glutathione peroxidase; GPx1, cytosolic glutathione peroxidase; GPx3, plasma glutathione peroxidase, GPx4, phospholipidhydroperoxide glautathione peroxidase; FAS, fatty acid synthase; NC, negative control group (<30 μg Se/kg diet); PTP1B, protein tyrosine phosphatase 1B; Se75, group fed a diet containing 75 μg selenium/kg; Se150, group fed a diet containing 150 μg selenium/kg; SREBP-1c, sterol regulatory element-binding protein-1c; TrxR1, cytosolic thioredoxin reductase 1.

* To whom correspondence should be addressed. Email: andreas.mueller@landw.uni-halle.de.

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acid intermediate (PTP1B-SOH) is initially formed (20,21). Its further oxidation can be prevented by the formation of a cyclic sulphenyl amide (22,23), followed by the reaction with reduced and oxidized glutathione (GSH/GSSG) to a mixed disulfide with Cys-215, known as glutathionylation (24). Glutathionylated PTP1B thus represents a catalytically inactive form of the enzyme. Protein glutathionylation of PTP1B and of other enzymes can also take place in the presence of lipid hydroperoxides (25–27). The activity of reversibly oxidized PTP1B and of the glutathionylated enzyme can be partially recovered by the addition of dithiothreitol (DTT) or thiol-transferases (22–24). The direct reaction of the reduced Cys-215 sulfhydryl group in the presence of high oxidized glutathione concentrations (>25 mmol/L) may also lead to glutathionylated PTP1B (24). Besides being a by-product of the respiratory chain and being generated by the activity of some oxidoreductases, H_2O_2 is also produced in mammalian tissues after insulin binding to the insulin receptor, presumably for the differential regulation of PTP activity (28,29). This demonstrates the role of reactive oxygen species, including H_2O_2, as "second messengers" involved in signal transduction processes (30,31). When taken up at the recommended level (rats, 0.15–0.30 mg Se/kg dietary dry matter; humans, 50–100 μg Se daily), the trace element Se performs its physiological functions in the human and animal body in the form of a catalytically active selenocysteine residue in functional selenoproteins (32). In particular, glutathione peroxidases (GPx) and thioredoxin reductases (TrxR) largely contribute to the maintenance of cellular antioxidant defense. GPx efficiently detoxify H_2O_2 and organic hydroperoxides (33–36). Besides their important role in the ribonucleotide reductase cycle, TrxR have an unusually wide substrate spectrum. This includes inorganic selenium compounds, dehydroascorbate, and proteins as well as lipid hydroperoxides (37–40). Within the GPx family, cellular GPx (GPx1) exhibits the highest activity and is expressed in all mammalian tissues. Under conditions of dietary Se deficiency, GPx1 as well as plasma GPx (GPx3) undergo a severe loss of enzyme activity accompanied by a distinct down-regulation of their mRNA. Gastrointestinal GPx and phospholipidhydroperoxide GPx (GPx4) are much more resistant to dietary Se deficiency and therefore rank high in the hierarchy of GPx (35,41,42). Within the TrxR, the cytosolic TrxR (TrxR1) is more sensitive to changes in dietary Se supply compared with the mitochondrial enzyme TrxR2 (43). In addition to the microsomal enzyme catalase, which has a major function in H_2O_2 removal, the selenoperoxidases and TrxR1 are other important enzymes in the reduction of H_2O_2 and lipid hydroperoxides (33–40,44). In view of the crosslink between physiological PTP1B regulation by H_2O_2 and lipid hydroperoxides via glutathionylation on the one hand, and the important roles of GPx and TrxR in peroxide metabolism on the other hand, our trial with growing rats examined the following question:

Does a manipulation of dietary Se concentration influence the regulation of PTP1B and PTP1B-dependent lipogenic mechanisms?

Materials and Methods

Rats and diets. Thirty healthy growing male albino rats (initial body weight, 78.6 ± 2.69 g) from the Interdisciplinary Research Centre, Department of Animal Nutrition and Nutritional Physiology's own strain HK51 were randomly assigned to 3 experimental groups of 10. The rats of the Se-deficient negative control group (NC) received a diet based on Torula yeast and Se-deficient wheat (**Table 1**). The diets for groups Se75 and Se150 were supplemented with sodium selenate to obtain final Se concentrations of 75 μg/kg diet (one-half of the recommended level) and of 150 μg Se/kg diet, representing the recommended level (45–47). The rats were kept individually and had free access to the diet and bidistilled water. After 8 wk, the rats were decapitated under CO_2 anesthesia and the livers were excised and prepared for further analysis. The protocol of the animal study was approved by the Regional Council of Giessen.

TABLE 1 Composition of the experimental diets

Ingredient	g/kg diet
Torula yeast (Attisholz)	250.00
Se-deficient wheat (Germany)	100.00
Cellulose BWW 40 (Rettenmaier)	50.00
Glucose (Sigma-Aldrich)	50.00
Sucrose (Suedzucker)	50.00
Soybean oil (Heess)	50.00
DL-Methionine[1] (DEGUSSA)	6.00
L-Tryptophan[2] (Sigma-Aldrich)	0.50
Mineral premix[3] (Salts from Sigma-Aldrich)	35.00
Vitamin premix[4] (Vitamins from Roche)	10.00
Choline chloride (BASF)	2.00
Corn starch (Roquette)	396.50
Selenium premix[5]	0.00
Total	1000.00

[1] Added according to the recommendations of the NRC (47).
[2] Added according to the recommendations of the NRC (47).
[3] Prepared according to the AIN-93G formulation (45,46), with the exception of Se.
[4] Prepared according to the AIN-93G formulation (45,46).
[5] In diets of groups Se75 and Se150 12.5 g and 25.0 g corn starch were replaced by equal amounts of selenium premix containing 6 mg Se/kg as sodium selenate.

Liver total fat, triglycerides, phospholipids, and lipid peroxides. Liver lipids were measured as described previously (11).

Two individual crude fat extracts per rat liver were prepared using a hexane:isopropanol (3:2) mixture containing 0.005% butylated hydroxytoluene. Subsequently, the triglyceride concentration in the lipid extracts was determined in triplicate with a test kit from Biocon (Fluitest TG). The concentration of phospholipids in liver lipid extracts was measured with a test kit after digestion of the samples and liberation of the phospholipid-phosphorus in a mixture of 70% perchloric acid and 30% H_2O_2. The concentration of liver lipid hydroperoxides was measured using the FOX assay (48). In contrast to the original method, 0.3 g of rat liver were homogenized in ice-cold, HPLC-grade methanol instead of 3.0 g of muscle tissue.

Se assay. Se concentration in the diets and livers was measured by hydride generation atomic absorption spectrometry (Unicam PU 9400 X; PU 3960 X) as reported previously (49). Certified samples from the National Institute of Standard and Technology (soft winter wheat flour, no. 8438 and bovine liver, no. 1577 b) served as reference material for Se determination in the different matrices.

Liver GPx1 and GPx4 and plasma GPx3. The activities of GPx1, GPx3, and GPx4 were measured spectrophotometrically (Beckmann DU 50) recording glutathione-dependent peroxide reduction coupled to glutathione reductase and NADPH oxidation (50). For GPx1 activity, the diluted cytosolic supernatants of rat liver homogenates served as sample material, and undiluted rat plasma was used for GPx3. For GPx4 activity, crude homogenates of livers were prepared in a sucrose buffer (51,52). H_2O_2 served as the substrate for GPx1 and GPx3 determination, whereas freshly synthesized phosphatidylcholine hydroperoxide was used for measurement of GPx4 activity (50–52). One unit of GPx1, GPx3, and GPx4 activity was defined as 1 μmol NADPH oxidized per minute and normalized to 1 mg protein.

Liver TrxR1. We determined the activity of TrxR spectrophotometrically in the 10,000 × g; 30 min at 4°C cytosolic supernatant of rat liver

homogenates according to the 5,5′-dithiobis-2-nitrobenzoic acid reduction assay (53). The time-dependent increase in absorption at 412 nm (Beckmann DU 50) based on the formation of 5′-thio-2-nitrobenzoic acid was recorded for 3 min. After subtraction of the absorption obtained in the presence of the TrxR inhibitor aurothioglucose (54), 1 unit of TrxR activity was calculated and defined as 1 μmol 5′-thio-2-nitrobenzoic acid formed per minute and normalized to 1 mg protein.

Liver PTP. Differentiated measurement of PTP was carried out using a modified protocol based on paranitrophenyl phosphate hydrolysis (55,56). For the analysis of PTP activity, 1:5 (wt:v) liver homogenates were prepared under nitrogen gassing in a nonreducing HEPES buffer (50 mmol/L HEPES, 50 mmol/L NaCl, 1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulphonylfluoride, pH = 7.4). Cytosol was obtained by centrifugation at 10,000 × *g*; 30 min at 2°C and brought to a final dilution of 1:25 (wt:v). A total of 10 μL of diluted liver cytosol was preincubated at 25°C in 240 μL of HEPES buffer, containing no reducing agents, for 3 min. After the addition of 250 μL HEPES buffer containing 20 mmol/L of the substrate paranitrophenyl phosphate, samples were incubated for a further 5 min before the reaction was terminated by the addition of 500 μL 2 mol/L NaOH and the absorption was read in a spectrophotometer (Beckmann DU 50) at 410 nm. A blank without cytosol was carried out. Native PTP activity (nonreducing conditions) was calculated using an extinction coefficient of $(1.66 \times 10\ \text{mmol/L})^{-1}\cdot\text{cm}^{-1}$ for the paranitrophenolate ion and normalized to 1 mg protein. To determine maximum PTP activity as well as the percentage of glutathionylation (reversible by DTT), enzymatic measurement was repeated as described, but HEPES buffer containing an additional 2.5 mmol/L DTT was used. The percentage of glutathionylated PTP enzyme was calculated from the difference in enzyme activities measured under reducing and nonreducing conditions.

mRNA expression of liver GPx1, GPx4, TrxR1, SREBP-1c, FAS, and Cyp18. RNA isolation was carried out using the acid guanidine thiocyanate phenol chloroform method (57). RT of RNA, followed by PCR for the examination of GPx1, GPx4, TrxR1, SREBP-1c, FAS, and Cyp18 expression in the liver was carried out as described in detail previously (11,32). The gene bank accession numbers and the primers used in PCR were as follows: GPx1 (*NM0308261*): primer forward (5′→3′): TCA TTG AGA ATG TCG CGT CT, primer reverse (5′→3′): CCC ACC AGG AAC TTC TCA AA, amplicon length (bp): 388; GPx4 (*NM0171165*), primer forward (5′→3′): ATG CAC GAA TTC TCA GCC AAG, primer reverse (5′→3′): GGC AGG TCC TTC TCT AT, amplicon length (bp): 461; TrxR1 (*NM031614*): primer forward (5′→3′): CCT ATG TGC CCT TGG AAT GT, primer reverse (5′→3′): TGT AAG GCA CAT TGG TCT GC, amplicon length (bp): 390; SREBP-1c (*BE632748*): primer forward (5′→3′): GGA GCC ATG GAT TGC ACA TT, primer reverse (5′→3′): AGG AAG GCT TCC AGA GAG GA, amplicon length (bp): 158; FAS (*NM017332*): primer forward (5′→3′): GGC ATC ATT GGG CAC TCC TT, primer reverse (5′→3′): ACC AAC AGC TGC CAT GGA TC, amplicon length (bp): 147; Cyp18 (*XM345915*): primer forward (5′→3′): AGC ACT GGG GAG AA, primer reverse (5′→3′): AGC CAC TCA GTC TT, amplicon length (bp): 101.

The amplified DNA sequences were visualized with a UV imager (Syngene) after electrophoresis in 1.5% agarose gels containing 0.02 g ethidium bromide/L gel and evaluated in relation to Cyp18 expression using the software Gene Tools from Syngene.

Western blot analysis of PTP1B glutathionylation. For analysis of PTP1B glutathionylation, 1:10 (wt:v) liver homogenates were prepared in a nonreducing RIPA lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulphonylfluoride, 1 mmol/L EDTA, 1.0% sodium desoxycholate, 0.1% SDS, and 1% TritonX-100, pH = 7.4). After centrifugation (10,000 × *g*; 30 min at 2°C), the cytosol was diluted to 1:50 (wt:v). A total of 40 μg of protein was separated according to the standard method (58) but under nonreducing conditions on 15% SDS-polyacrylamide gels (50 mA, 4°C, 2 h). Separated proteins were transferred onto a polyvinylidene difluoride membrane (PALL Biotrace 0.45 μm) by semidry blotting [25 min at constant 6V (~60 mA)]. After blocking membranes overnight at 4°C in Tris-buffered saline and Tween (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween 20, pH = 7.6) containing 5% nonfat dry milk and 0.2% bovine serum albumin, the analysis was continued with a 2-h incubation with the monoclonal anti-glutathione antibody (Virogen 101-A-100) in Tris-buffered saline (1:1500) buffer and a 1-h incubation with the secondary antibody (1:3000) linked to alkaline phosphatase (goat anti-mouse IgG-h+I). Membranes were stained in a reaction buffer (0.1 mol/L Tris, 0.1 mol/L NaCl, 0.05 mol/L $MgCl_2$) containing 0.00375% nitro-blue tetrazolium and 0.0025% 5-bromo-4-chloro-3-indoylphosphate. Optical density of the ~50-kDa PTP1B-band was evaluated (Gene Tools, Syngene) on scanned membranes (CanoScan LiDe 500F).

Immunohistochemistry and fluorescence microscopy of liver PTP1B. Small cubic sections (~75 mm^3) from the left lobe of freshly dissected livers were embedded in stainless steel wells in a tissue freezing medium (Jung) and frozen in isoamyl alcohol, which had been previously cooled in liquid nitrogen (59). The frozen blocks were stored at −80°C until use in fluorescence microscopy. For immunofluorescence, 9-μm sections were prepared using a microtom cryostat (HM 500, Microm) and fixed on slides coated with chrome alaun gelatin (60). The fixed sections were then incubated for 5 min in 70% ethanol, washed for 5 min in PBS, and permeabilized for 10 min in PBST (PBS, 0.1% Tween 20). After 3 washing steps with PBS, the slides were incubated for 1 h with the monoclonal first antibody (anti-rat-PTP1B, BD Biosciences Pharmingen) and diluted 1:200 in PBS. After being washed 3 times with PBST (PBS, 0.02% Tween), the slides were incubated with the second fluorescence antibody (Alexa Fluor 488 goat anti-rat IgG, Molecular Probes) for 1 h. The excess second antibody was removed by 3 washes with PBST and 3 washes with PBS. Subsequently, dyeing of the nuclei was carried out by incubating the sections for 30 s in DAPI solution (2 g 4′,6-Diamidino-2- phenylindoldihydrochloride/L PBS). Finally, the sections were covered with Dabco (25 g Dabco/L PBS) and analyzed using an automated fluorescence microscope (Olympus AX 70) equipped with the software "Analysis." For fluorescence detection of nuclei, an exposure time of 50 ms was chosen. For visualization of PTP1B expression, the exposure time was 1000 ms. All images were taken using a 40 × 2.5 objective, providing an enlargement of 1:100.

Protein concentration of samples. The protein concentration of liver cytosol, including samples for Western blotting, was determined using a standard method (61).

Statistical analysis. Data are given as means ± SD. Each variable was analyzed in 3 replications. Statistical differences were analyzed with SPSS 14.0 for Windows using the 1-way ANOVA after ascertaining the normality of distribution (Shapiro Wilk test and Kolmogorov Smirnov test) and the homogeneity of variance (Levene test). If the variances were homogenous, the Least Significant Difference test was used to examine significant differences between means. If not, the Games Howell test was used. Differences between means were considered significant at an error probability <5%. For selected variables, an error probability <10% is indicated as a statistical trend. Two-tailed Pearson correlation coefficients and the significance of correlation were analyzed using the correlation mode in SPSS 14.0. Linear regression equations were calculated using Microsoft Excel.

Results

Diets, animal performance, and body weight. The expected dietary Se concentrations were confirmed by Se analysis (NC, < detection limit of 20; Se75, 78.0 ± 1.57; Se150, 153 ± 1.83 μg Se/kg diet). Calorimetric analysis and protein analysis of the diets revealed that all experimental diets were isoenergetic and isonitrogenous (NC, 17.5 ± 0.19 MJ/kg, 14.3 ± 0.51% protein; Se75, 17.8 ± 0.11 MJ/kg, 14.1 ± 0.18% protein; Se150, 17.6 ± 0.12 MJ/kg, 14.5 ± 0.29% protein). At the beginning of the experiment body weight did not differ among

the NC (78.6 ± 2.46 g), Se75 (78.6 ± 2.91 g), and Se150 (78.6 ± 2.72 g) groups. Total feed intake (TFI) over 8 wk and feed efficiency ratio (FER; g feed intake:g body weight gain) in group NC (TFI, 945 ± 59.0 g; FER, 4.56 ± 0.26) were significantly impaired compared with the Se75 (TFI, 1066 ± 63.8 g; FER, 4.22 ± 0.23) and Se150 (TFI, 1014 ± 48.6 g; FER, 4.33 ± 0.19) groups. Final body weight in Se75 (331 ± 23.4 g) and Se150 (314 ± 21.3 g) groups was significantly higher than in the NC group (287 ± 24.9 g).

Se status. The liver Se concentration in the NC was reduced to 2.2 and 1.7% of the concentrations in the Se75 and Se150 groups (**Table 2**). Rats in group Se150 had a liver Se concentration that was greater than that in Se75 rats. Eight weeks of Se deficiency led to the most distinct loss of activity of liver GPx1 and of GPx3. In the NC group, liver GPx1 activity was 99% lower than in the Se75 and Se150 groups and was accompanied by a ~5.5-fold downregulation of GPx1 mRNA. Raising the dietary Se concentration from 75 to 150 mg/kg tended to increase liver GPx1 activity ($P = 0.08$). The 98% reduction of GPx3 in the NC group compared with rats in the Se75 and Se150 groups was similar to that for liver GPx1. However, the mRNA abundance and the activity of liver GPx4 and liver TrxR1 were distinctly less affected by the lack of dietary Se. Compared with groups Se75 and Se150, GPx4 mRNA in the NC group was downregulated by 23 and 45% and resulted in a reduced GPx4 activity to 34% and 22%, respectively. The 1.8-fold and 2.0-fold downregulation of liver TrxR1 mRNA abundance resulted in reductions in enzyme activity to 40 and 30%, respectively, in the NC group compared with the Se75 and Se150 groups.

Regulation of liver PTP1B. The effective liver PTP activity, measured under native, nonreducing conditions (without DTT), was 1.3- higher in the Se75 group and 1.9-fold higher in the Se150 group compared with the NC group (**Fig. 1*A***). Measurement of PTP activity under reducing conditions (with DTT added) indicated the regeneration of enzyme activity inhibited by glutathionylation. Under reducing conditions, the increase in liver PTP activity in group NC (+105%) was greater than in the Se75 (+24%) and Se150 (+19%) groups. PTP inhibition by glutathionylation, calculated from the ratio of PTP activity under native and reducing conditions, was significantly higher in NC rats (50.1 ± 7.76%) than in Se75 (23.4 ± 8.17%) and Se150 (15.5 ± 10.7%) rats. Under reducing conditions, PTP activity was still significantly lower in NC rats (1.69 ± 0.37 U/mg protein) than in the Se75 (2.37 ± 0.49 U/mg protein) and Se150 (2.99 ± 0.42 U/mg protein) groups. However, the factors for activity difference between the NC group and the Se75 (0.4-fold) and Se150 (0.8-fold) groups were significantly lower than the native conditions (1.4- and 2.0-fold). The remaining differences in PTP activity between the experimental groups can therefore be explained by changes in PTP expression due to dietary Se. They could be visualized using immunofluorescence (Fig. 1*B*). PTP1B expression was distinctly higher in group Se150 than in the NC group. Post-transcriptional PTP1B regulation via glutathionylation could be depicted by Western blot analysis using an anti-glutathione antibody (Fig. 1*C*). In Western blot analysis (Fig. 1*C*), a significantly higher PTP1B glutathionylation was measured in NC rats than in Se 75 (1.9-fold) and Se 150 (2.3-fold) rats, demonstrating increased PTP1B inhibition by glutathionylation due to Se deficiency.

TABLE 2 Liver Se concentration, liver GPx1, GPx4, and TrxR1 activity and expression, and GPx3 activity of rats fed diets containing 0, 75, and 150 μg Se/kg for 8 wk[1]

	NC	Se75	Se150
Liver Se concentration, *nmol/g dry matter*	0.66 ± 0.11[c]	28.7 ± 1.83[b]	36.9 ± 2.71[a]
Liver GPx1 activity, *mU/mg protein*	8.73 ± 2.25[b]	722 ± 147[a]	831 ± 139[a]
mRNA, *fold of NC*	1.00 ± 0.17[b]	6.11 ± 0.44[a]	6.81 ± 0.82[a]
Liver GPx4 activity, *mU/mg protein*	1.41 ± 0.71[c]	4.16 ± 0.81[b]	6.31 ± 1.85[a]
mRNA, *fold of NC*	1.00 ± 0.09[c]	1.23 ± 0.11[b]	1.45 ± 0.20[a]
Liver TrxR1 activity, *mU/mg protein*	4.02 ± 0.45[c]	10.0 ± 0.87[b]	13.5 ± 2.81[a]
mRNA, *fold of NC*	1.00 ± 0.10[b]	2.82 ± 0.14[a]	3.04 ± 0.26[a]
GPx3 activity, *mU/mg protein*	0.62 ± 0.17[b]	25.0 ± 5.48[a]	31.0 ± 7.48[a]

[1] Values are means ± SD, $n = 10$. Means in a row without a common letter differ, $P < 0.05$.

Liver total fat, triglyceride, phospholipid, and lipid hydroperoxide concentrations. Total liver fat concentration was significantly higher in groups Se75 (14%) and Se150 (21%) than in the NC group (**Table 3**). Se150 rats tended to have a higher liver total fat concentration than Se75 rats ($P = 0.06$). Liver triglyceride concentrations were significantly higher in the Se75 (1.1-fold) and Se150 (1.4-fold) groups than in the NC group. Se150 rats had a significantly higher liver triglyceride concentration than Se75 rats. This effect also remained uninfluenced when liver triglyceride concentration was related to 1 g of final body weight. In contrast, the liver phospholipid concentration was uninfluenced by dietary Se (NC, 192 ± 19.1; Se75, 183 ± 16.9; Se150, 193 ± 14.2 μmol/g dry matter). The livers of NC rats had a significantly higher lipid hydroperoxide concentration than those of the Se75 (0.7-fold) and Se150 (2.0-fold) rats.

mRNA expression of SREBP-1c and FAS. To further explain the chain of cause and effect linking Se-dependent PTP1B redox regulation to triglyceride metabolism, we subsequently investigated the expression of 2 key genes involved in FAS. We analyzed a significantly higher expression of liver SREBP-1c in the Se75 (1.2-fold) and Se150 (1.5-fold) groups than in the NC group (**Fig. 2**). FAS, a target of the transcription factor SREBP-1c, was expressed significantly higher in livers of Se75 (0.6-fold) and Se150 (0.9-fold) rats than in NC rats (Fig. 2).

Correlation and regression analyses between dietary Se, antioxidant selenoenzymes, PTP1B regulation, and liver triglyceride concentration. PTP1B is an accepted molecular trigger of triglyceride synthesis via SREBP-1c and FAS. Because our present data showed a distinct regulation of PTP1B due to a manipulation of dietary Se and Se-dependent antioxidant enzymes, we consequently examined possible molecular links between dietary Se supply, the resulting activity of antioxidant selenoenzymes, the redox regulation of PTP1B, and liver triglyceride concentration by correlation and regression analyses.

Liver Se concentration (**Fig. 3*A***) as well as the activity of liver GPx1, GPx4, TrxR1, and of GPx3 (**Table 4**) were positively correlated ($P < 0.01$) with native liver PTP activity and liver triglyceride concentration. Whereas the native PTP activity correlated positively with liver triglyceride concentration ($P <$

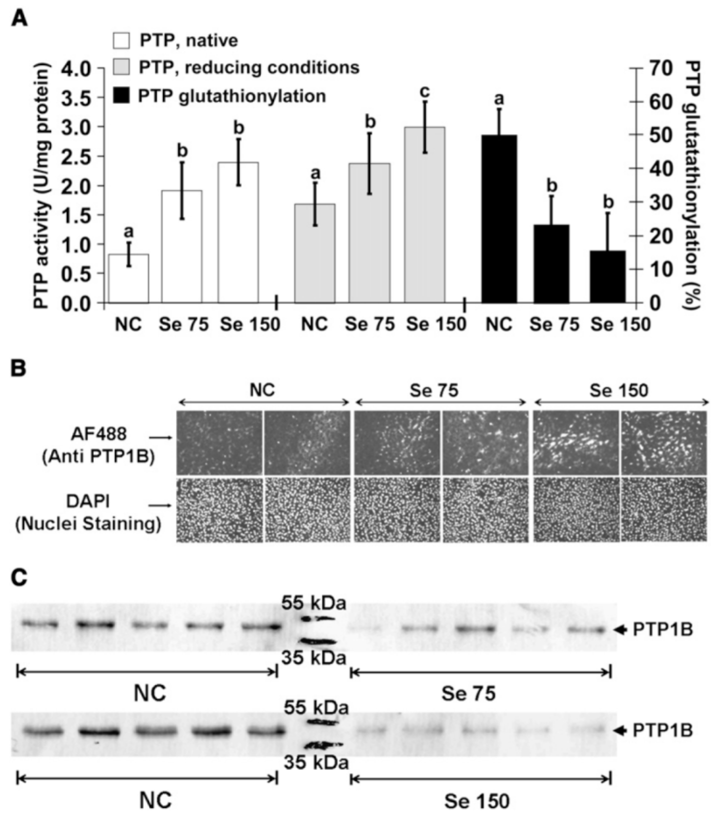

FIGURE 1 Regulation of liver PTP1B of rats fed diets containing 0, 75, and 150 µg Se/kg for 8 wk (*A*). Activity of PTP under native PTP and reducing conditions and PTP glutathionylation, calculated from the activity ratio under both conditions. Values are means ± SD, $n = 10$. For each variable, means without a common letter differ, $P < 0.05$. (*B*) Immunofluorescence imaging of PTP1B expression and of the hepatocyte nuclei in 9-µm liver section planes. Two representative images of PTP1B immunofluorescence from 2 rats per experimental group are shown. (*C*) PTP1B glutathionylation detected by Western blotting. Five protein pools with 2 rats per pool were prepared for each experimental group. Each band shows glutathionylation of 1 individual protein pool.

0.01), PTP glutathionylation, indicating PTP inhibition, and liver triglyceride concentration were negatively correlated ($P < 0.01$) (Fig. 3*B*). All interrelationships examined fit well to linear regression lines.

Discussion

Our current results on the regulation of the functional selenoproteins in rats are in accordance with data from the literature. The dramatic loss of GPx1 mRNA abundance and enzyme activity due to a lack of dietary Se supply indicates the low rank of GPx1 in the hierarchy of functional selenoproteins (62,63). GPx3 was also strongly affected by Se deficiency and therefore is the second-lowest ranking selenoperoxidase (35). GPx4 with a much lower activity in rat liver than GPx1 and TrxR1 are much less affected by Se deficiency with regard to mRNA abundance and enzyme activity (41,43,64,65). GPx4 and TrxR1 therefore have a high ranking in the selenoprotein hierarchy. Our data confirm that a Se supply of 150 µg/kg diet meets the requirements of growing rats for an abundant synthesis of GPx1, GPx3, GPx4, and of TrxR1 (43,62,63,65). Only a slight further increase in mRNA abundance and enzyme activity was achieved for all

TABLE 3 Liver total fat, triglycerides, and lipid hydroperoxides of rats fed diets containing 0, 75, and 150 µg Se/kg for 8 wk[1]

	NC	Se75	Se150
Total fat, *% in dry matter*	16.8 ± 3.19[b]	19.5 ± 2.39[a]	21.2 ± 2.43[a]
Triglycerides, *µmol/g dry matter*	52.7 ± 14.1[c]	111 ± 22.8[b]	127 ± 29.5[a]
Triglycerides, *µmol/g body weight*	0.16 ± 0.015[c]	0.33 ± 0.025[b]	0.41 ± 0.028[a]
Lipid hydroperoxides, *nmol/µmol liver* triglycerides	2.53 ± 0.64[a]	1.48 ± 0.20[b]	0.83 ± 0.29[c]

[1] Values are means ± SD, $n = 10$. Means in a row without a common letter differ, $P < 0.05$.

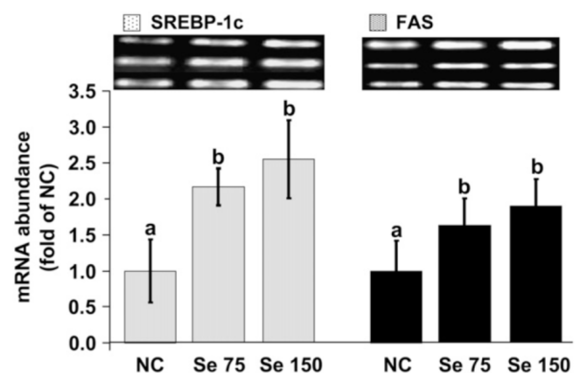

FIGURE 2 mRNA abundance of liver SREBP-1c and FAS of rats fed diets containing 0, 75, and 150 µg Se/kg for 8 wk. Values are means ± SD, $n = 10$. For each variable, means without a common letter differ, $P < 0.05$. Representative images of SREBP-1c- and FAS mRNA expression are shown above the bars for 3 rats per experimental group.

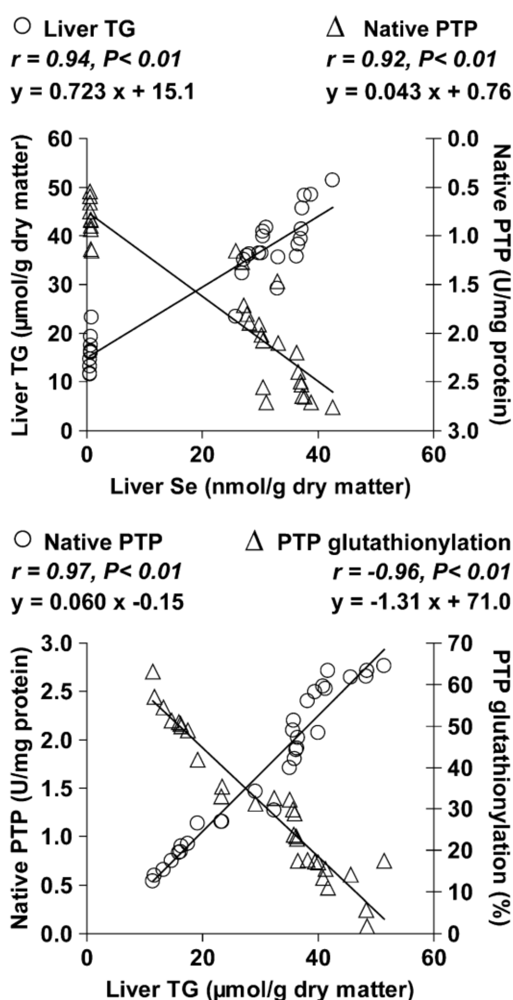

FIGURE 3 (*A*) Linear correlation and regression analyses investigating the interrelationship between liver Se concentration and liver triglyceride concentration and native PTP activity. (*B*) The interrelationship between liver triglyceride concentration and PTP activity and glutathionylation. Analyses were based on 30 rats fed diets containing 0, 75, and 150 μg Se/kg for 8 wk. All correlations were significant, $P < 0.01$.

selenoproteins investigated by raising the dietary Se concentration from 75 μg/kg diet to 150 μg/kg diet. Our results, moreover, demonstrate an additional role of Se and antioxidant selenoproteins in the regulation of metabolic processes, which exceeds a purely antioxidant function. The inhibition of PTP1B activity by H_2O_2 and lipid hydroperoxides followed by glutathionylation and a subsequent partial regeneration of PTP1B activity with DTT has, as yet, only been shown in vitro (20–24,27). These mechanisms are physiologically relevant and influenced by Se and the antioxidant selenoenzymes GPx1, GPx3, GPx4, and TrxR1. On the one hand, higher peroxide concentrations (Table 3) resulting from a low dietary Se concentration and lowered activities of the antioxidant selenoproteins lead to an increased inactivation of PTP1B via glutathionylation (Table 2; Fig. 1*A*,*C*). On the other hand, increased expression and optimized activities of GPx1, GPx3, GPx4, and TrxR1, which participate in the metabolism of H_2O_2 and organic peroxides, can be assumed to lower peroxide

TABLE 4 Linear correlation and regression analyses between selenoprotein activities and native liver PTP activity and liver triglyceride concentration[1]

Correlation investigated	Pearson coefficient	Regression equation
GPx1:native PTP	0.95	$y = 0.0019\ x + 0.74$
GPx3:native PTP	0.96	$y = 0.051\ x + 0.75$
GPx4:native PTP	0.92	$y = 0.203\ x + 0.79$
TrxR1:native PTP	0.93	$y = 0.166\ x + 0.18$
GPx1:liver TG	0.96	$y = 0.031\ x + 15.0$
GPx3:liver TG	0.97	$y = 0.832\ x + 15.4$
GPx4:liver TG	0.90	$y = 3.195\ x + 7.04$
TrxR1:liver TG	0.94	$y = 2.747\ x + 5.76$

[1] Analyses were based on $n = 30$ rats fed diets containing 0, 75, and 150 μg Se/kg for 8 wk. All correlations were significant, $P < 0.01$.

tone and disable PTP1B inhibition (33,34,39,66,67). A similar connection between a H_2O_2-detoxifying enzyme and PTP1B regulation, as suggested by our data, has also already been demonstrated for catalase. However, most of the experiments, in which the inactivation of PTP1B by reactive oxygen species could be prevented by catalase, were also carried out only in vitro (68–70). In addition to post-transcriptional regulation of PTP1B by glutathionylation, our results also indicate a changed expression of PTP1B due to dietary Se supply. This transcriptional regulation is reflected in the difference in PTP activity that still exists among the experimental groups after DTT treatment and in the protein expression examined by immunofluorescence (Fig. 1*A*,*B*). This finding of an increased PTP1B expression is in accordance with data from a mouse study in which the overexpression of catalase, another H_2O_2-detoxifying enzyme, led to a 3-fold elevated PTP1B expression (71). An altered PTP1B activity has a number of additional metabolic consequences. Lipid metabolism seems to represent one particular target of PTP1B. An upregulated PTP1B activity activates lipogenic mechanisms, involving the activation of SREBP-1c and of its target gene, FAS, as was shown originally for rats fed fructose-enriched diets (14–17). Moreover, PTP1B expression is a valuable indicator for the diagnosis of nonalcoholic-fatty-liver-disease, a disorder closely related to hepatic insulin resistance and metabolic syndrome (72–74). However, the manipulation of PTP1B by dietary Se powerfully influences liver lipid metabolism. Simultaneously, we could confirm that the higher liver triglyceride concentrations due to the Se-dependent upregulation of PTP1B are based on the activation of SREBP-1c and FAS (18,19), as was described for fructose feeding (14–17). The existence of a relationship between liver lipid metabolism and antioxidant selenoproteins was also demonstrated by an inverse experimental setup (75). In this study, the overexpression of SREBP-1c in fibroblasts and hepatocytes led to a manifold upregulation of GPx3. This result corresponds to the highest correlation between GPx3 and liver triglyceride concentration in our study (**Table 4**). Liver triglyceride metabolism represents a Se- and PTP1B-dependent target and does not mainly depend on changes in feed intake and body weight gain. Despite a higher feed intake and a higher body weight in the Se75 group compared with the Se150 group, liver triglyceride concentration was higher in the Se150 group and was accompanied by the most effective PTP activity. **Figure 4** summarizes our hypotheses regarding the influence of Se on liver triglyceride metabolism. Nevertheless, PTP1B regulation by dietary Se seems to influence metabolic processes variously. In a mouse trial, PTP1B-deficient mice had a significantly higher energy expenditure than wild-type mice (7).

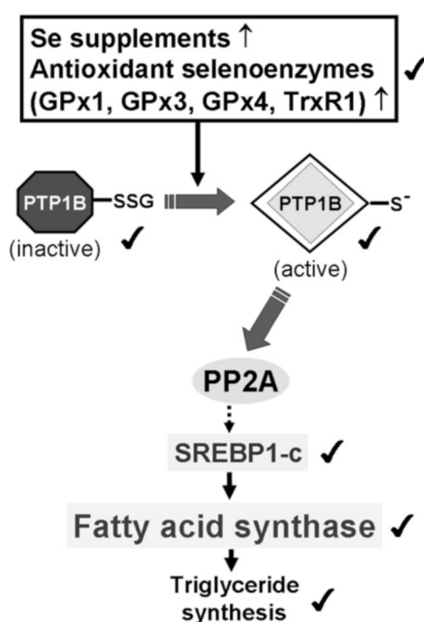

FIGURE 4 The possible molecular link between dietary Se and liver triglyceride metabolism involves the differential regulation of PTP1B. Checkmarks indicate checkpoints investigated in the present study.

In our trial, the feed conversion ratio (g feed:g body weight gain) was better in the Se-supplemented groups than in the Se-deficient rats of the NC group. The higher feed expense in the Se-deficient NC group may therefore be an indicator for a higher energy expenditure due to the reduced PTP1B activity. Data of a very recent study even suggest an important role for hypothalamic PTP1B in the reduction of feed intake and body fat stores (76). In human studies and in animal trials, PTP1B was demonstrated as one factor increasing body weight gain and the development of obesity (1,2,5–9). GPx1-overexpressing mice had a significantly higher body weight and body fat gain (77,78), whereas mice with a selenoprotein P knockout and the resulting lack of antioxidant selenoprotein synthesis were emaciated (79). Thus, our results regarding body weight are in line with both trials (77–79), demonstrating that Se supply and high activities of antioxidant selenoproteins seem to have a function in body weight development and in lipid metabolism.

Our findings, demonstrating a distinct influence of dietary Se supplementation on the redox regulation of the insulin-antagonistic PTP1B and on metabolic processes such as lipid metabolism, are of importance in assessing the benefits and risks of Se supplements. The mouse study, in which GPx1 overexpression led to the development of obesity and diabetes (77,78) as well as most recent data from epidemiological human studies in which a high Se status increased the diabetes risk and effected an elevation of serum lipids (80–83), highlight the ambivalent role of antioxidants such as Se in metabolic processes and in the development of metabolic disorders.

The influence of physical activity is also important in the context of antioxidant supplementation. In our rat trial, the lower body weight and liver triglyceride concentration in NC rats may have derived from the reduced PTP1B activity due to artificial oxidative stress caused by Se deficiency. In a trial with diet-induced obese rats, oxidative stress resulting from a single bout of exercise effectively reduced PTP1B and improved insulin sensitivity (84). Thus, reduced physical activity combined with a high antioxidant supplementation may explain even undesirable effects of antioxidants in the development of diseases of civilization.

In conclusion, it appears that redox-regulated proteins, such as PTP1B, which are involved in cellular signaling processes represent important interfaces between dietary antioxidants such as Se and metabolic processes (85). The involvement of PTP1B in the development of insulin resistance, obesity, and fatty liver disease on the one hand (1,2,6–9,72–74) and very recent critical observations regarding the relation between Se, obesity, elevated serum lipids, and diabetes (77,78,80–82) on the other strongly suggest the necessity for future research of regulatory functions of Se and other antioxidants in metabolic processes and metabolic disorders.

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Study of molecular targets influencing homocysteine and cholesterol metabolism in growing rats by manipulation of dietary selenium and methionine concentrations

Nicole M. Wolf[1], Kristin Mueller[1], Frank Hirche[1], Erika Most[2], Josef Pallauf[2] and Andreas S. Mueller[1]*

[1]*Institute of Agricultural and Nutritional Sciences, Preventive Nutrition Group, Martin Luther University Halle Wittenberg, Von-Danckelmann-Platz 2, D-06120 Halle/Saale, Germany*

[2]*Institute of Animal Nutrition and Nutritional Physiology, Interdisciplinary Research Centre, Justus Liebig University Giessen, Heinrich-Buff-Ring 26–32, D-35392 Giessen, Germany*

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Inconsistent results exist from human and animal studies for Se and methionine (Met) regarding their influence on homocysteine (HCys) and cholesterol (Chol) metabolism. To elucidate these contradictions, sixty-four weanling albino rats were divided into eight groups of 8, and were fed diets containing four different Se levels (15, 50, 150 and 450 μg/kg) either in combination with the recommended Met level of 3 g/kg (C15, C50, C150 and C450) or with an increased Met concentration of 15 g/kg (M15, M50, M150 and M450) for 8 weeks. Plasma HCys was twofold higher in the Se-supplemented C groups than in group C15. Met addition also doubled plasma HCys compared with the respective C groups. In contrast, the expression of the key enzymes of glutathione biosynthesis in the liver was significantly lowered by Se and in particular by Met. Liver Chol concentration was significantly higher in all the Se-supplemented C and M groups than in groups C15 and M15. Plasma Chol was, however, lowered. The uninfluenced expression of sterol-regulatory element-binding protein 2 and of hydroxymethyl-glutaryl-CoA reductase, the increased LDL receptor expression and the reduced expression of the hepatobiliary Chol exporter ATP-binding-cassette-transporter 8 (ABCG8) by Se and/or Met explain these findings. We conclude that the elevation of plasma HCys in rats by Se and Met results from a higher export into plasma. The fact that Se in particular combined with Met increases liver Chol but reduces plasma Chol should be addressed in future investigations focussing on the regulation of ABCG8, which is also selectively involved in the reverse transport of phytosterols in the small intestine.

Selenium: Methionine: Homocysteine: Cholesterol: Gene expression

Results obtained from human and animal studies suggest that an elevated plasma homocysteine (HCys) concentration may promote the early development of coronary artery disease and ischaemic stroke[1]. The generation of reactive oxygen species[2], subsequently leading to increased LDL oxidation[3], the generation of vascular injury via the increased expression and liberation of proinflammatory transcription factors and cytokines[4 6] as well as reduced vessel relaxation via decreased NO availability[7] are discussed as mechanisms underlying the proatherogenic effects of HCys. With the exception of inherited disorders in methionine (Met) and HCys metabolism such as cystathione-β-synthase (CBS) or Met synthase deficiency or polymorphisms in these genes, hyperhomocysteinaemia is assumed as being influenced by various nutritional components[8]. Currently, the influence of folic acid, vitamin B_{12} and vitamin B_6 status as well as the effectiveness of the supplementation of these vitamins in lowering HCys concentration and reducing the risk and recurrence of CVD and ischaemic stroke is the subject of controversial discussion[9 11]. Despite evidence and inconsistent data on the beneficial effects of vitamin B supplementation in CVD and ischaemic stroke, the American Heart and Stroke Association and the German, Austrian and Swiss Homocysteine Society advise treating patients with hyperhomocysteinaemia or with a history of transient ischaemic attacks or stroke daily with a standard multivitamin preparation in order to achieve the recommended daily amounts of 0·4 mg folic acid, 2·4 μg vitamin B_{12} and 1·7 mg vitamin B_6[12,13]. The results of the National Nutrition Survey II revealed a deficit in folic acid intake in the German population also, and subsequently, a fortification of selected foods with folic acid is under consideration[14]. Other important factors determining plasma HCys concentration are age, sex and renal status[15,16]. In recent years, contradictory results have also been reported with regard to the influence of the trace element Se on HCys metabolism. Whereas studies with chicks[17] and with

Abbreviations: ABCG8, ATP-binding-cassette-transporter 8; BHMT, betaine hydroxymethyltransferase; CBS, cystathione-β-synthase; Chol, cholesterol; GCLC, glutamate cysteine ligase, catalytic subunit; GCLM, glutamate cysteine ligase, modulatory subunit; GPx, glutathione peroxidase; GS, glutathione synthetase; HCys, homocysteine; HMGCoAR, hydroxymethyl-glutaryl CoA reductase; LDLR, LDL receptor; Met, methionine; PE, phosphatidylethanolamine; SREBP2, sterol-regulatory element-binding protein 2; T_a, annealing temperature.

* **Corresponding author:** Andreas S. Mueller, fax +49 345 55 27124, email andreas.mueller@landw.uni-halle.de

mice and rats[18 21] showed that Se-deficient animals had an impressively reduced plasma HCys concentration than their littermates supplemented with the recommended dietary Se amount or with supranutritional Se, human cross-sectional observational studies with elderly people from Spain[22], Upper Silesia[23] and the UK[24] uniquely showed an inverse correlation between Se status and plasma HCys concentration. Another study with a middle-aged Inuit population also demonstrated the inverse correlation between Se status and plasma HCys concentration[25]. Both in a 20-week intervention trial in New Zealand whereby 189 volunteers aged 18–64 years were supplemented with a placebo or 200 μg Se/d as selenomethionine[26] and in an intervention trial in the UK with 501 elderly people who were supplemented with a placebo or 100, 200 or 300 μg Se/d as high Se yeast for 6 months[27], Se supplementation had no influence on plasma HCys level. However, the results of a rat study demonstrated a significant reduction of plasma HCys concentration in Se-supplemented rats fed diets supplemented simultaneously with a diet containing a high folic acid concentration of 2 mg/kg[19]. Besides the feeding of Se-rich diets, the feeding of Met-rich diets to rats is accepted as a means of increasing their plasma HCys level[28,29]. Results of the impact of dietary Met on plasma HCys in human subjects vary with regard to the critical dietary Met amount. Whereas some reports suggest that about fivefold of the recommended Met amount is necessary to produce a considerable increase in plasma HCys concentration[30,31], another study with Dutch men found a distinct postprandial increase in plasma HCys concentration lasting about 6 h in healthy men given a high protein diet (21 % energy)[32]. Results from a Finish study with men suggest an association between a slightly increased daily Met intake between 25 and 40 mg/kg body weight and a 31 % higher risk of coronary problems[33]. Feeding Met-rich diets to rats and subjecting human subjects to a Met-loading test additionally effected an increase in plasma cholesterol (Chol) concentration, representing another risk factor for the accelerated development of atherosclerosis[28,29,34]. Moreover, data from two large cross-sectional observational trials from the USA and the UK found that a high Se status was positively associated with increased plasma values for total Chol and LDL Chol[35,36]. However, combined effects of dietary Se and Met on HCys and Chol metabolism have not been investigated to date. Consequently, we have designed a trial with growing rats to study the effects of different dietary Se concentrations (Se deficiency, half the recommended dietary amount, recommended dietary amount and three times the recommended dietary amount) and of two dietary Met concentrations (recommended dietary amount and five times the recommended dietary amount) on the HCys and Chol metabolism of these animals. Taking into consideration both the recommendations and species differences between human subjects and rats, we have chosen the Se and Met supplementation range in a way that allows transferability of the data.

Methods and materials

Rats and diets

The protocol of the rat nutrition study was approved by the Regional Council of Giessen and by the Animal Welfare Committee of the Justus Liebig University of Giessen (record token: V54-19c20/15cGI 19/3). Sixty-four healthy growing male rats (initial body weight, 64·2 (SE 0·25) g from the Interdisciplinary Research Center, Department of Animal Nutrition and Nutritional Physiology's own strain (HK 51) were randomly assigned to eight groups of 8. The Se-deficient basal diet of group C15 had an analysed Se concentration of approximately 15 μg/kg, and was based on Torula yeast and Se-deficient wheat (Table 1). The diets for groups C50 (one-third of the recommended dietary Se level), C150 (recommended dietary Se level) and C450 (three times the recommended dietary Se level) were supplemented with 50, 150 and 450 μg Se/kg diet as sodium selenate. The diets for groups C15, C50, C150 and C450 were supplemented with 3·0 g DL-Met/kg to adjust the Met level to the requirement of the growing rats. The high Met diets of groups M15, M50, M150 and M450 had dietary Se levels that were the same as those of the diets of the respective C groups, but were supplemented with fivefold Met amount (15 g/kg). With the exception of the variations in Se and Met concentrations, the diets were composed according to the American Institute of Nutrition-93G recommendations[37]. The rats were kept individually, and had *ad libitum* access to the diet and bidistilled water. After 8 weeks, the animals were decapitated under CO_2. The livers were excised, frozen in liquid N_2 and stored at −80°C until further analysis. Blood was collected in heparinised tubes, and was centrifuged for 20 min at 3000 *g* for plasma preparation. Plasma was stored at −80°C until analysis.

Table 1. Composition of the basal diet

Ingredient	g/kg diet
Torula yeast (Attisholz, Solothurn, Switzerland)	250·0
Se-deficient wheat (harvested in the Vogelsberg region in Hesse, Germany)	100·0
Cellulose BWW 40 (Rettenmaier, Rosenberg, Germany)	50·0
Glucose (Sigma-Aldrich, St Louis, MO, USA)	50·0
Sucrose (Suedzucker, Mannheim, Germany)	50·0
Soya bean oil (Hesse)	50·0
dl-Met (Degussa, Dusseldorf, Germany)*	3·00
l-Trp (Sigma-Aldrich)†	0·50
Mineral premix (all salts from Sigma-Aldrich)‡	35·0
Vitamin premix (all vitamins from Roche, Basel, Switzerland)§	10·0
Cholin chloride (BASF, Mannheim, Germany)	2·00
Maize starch (Roquette, Lestrem, France)	399·5
Se premix‖	0·00
Total	1000·0

* In the diets of groups M15, M50, M150 and M450, the amount of methionine in the basal diet was increased to 15 g/kg by replacing maize starch.

† Added according to the American Institute of Nutrition-93G recommendations[37].

‡ Considering the high natural concentrations of Ca, K, Mg P, Cu, Fe and Zn of Torula yeast, the following mineral amounts were added to 1 kg complete diet with 35 g/kg of the concentrated premix to achieve the recommended amounts[37]: Ca, 3·0 g ($CaCO_3$); Mg, 228 mg ($MgSO_4.7H_2O$); Cu, 2·50 mg ($CuSO_4.5H_2O$); Mn, 8·62 mg ($MnSO_4.H_2O$); Na, 1·02 g (NaCl); K, 741 mg ($K_3C_6H_5O_7.H_2O$); Cr, 1·00 mg ($KCR(SO_4)_2.12H_2O$); B, 0·51 mg (H_3BO_3); Ni, 0·49 mg ($NiSO_4.6H_2O$); F, 1·00 mg (NaF); Si, 5·00 mg ($Na_2SiO_3.9H_2O$); Li, 0·10 mg (LiCl); Mo, 0·16 mg ($H_{24}Mo_7N_6O_{24}.4H_2O$); I, 0·23 mg (KI); V, 0·09 mg (NH_4VO_3).

§ All vitamins were added according to the recommendations[37] without any corrections.

‖ In the Se-supplemented groups, maize starch was replaced with 8·33 g (C50 and M50), 25 g (C150 and M150), and 75 g (C450 and M450) of the Se premix containing 6 mg Se/kg as sodium selenate in order to adjust the final Se concentrations of 50, 150 and 450 μg/kg.

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Selenium assay

Se concentration in the diets and livers was measured by hydride generation atomic absorption spectrometry (Unicam PU 9400 X; PU 3960 X) as described previously[38]. Certified samples from the National Institute of Standard and Technology (soft winter wheat starch, no. 8438 and bovine liver, no. 1577 b) and from Medichem (control serum, Metalle S; Steinenbronn, Germany) served as the reference materials for Se determination in the different matrices.

Liver glutathione peroxidase 1 and plasma glutathione peroxidase 3

Glutathione peroxidase (GPx) 1 and GPx3 activity was measured spectrophotometrically (Carry 50; Varian, Darmstadt, Germany) using the assay method coupled to GSH reductase at 340 nm[39]. NADPH oxidation, which is proportional to GPx-dependent peroxide reduction, was recorded for 3 min. GPx1 activity was analysed in the rat liver cytosol diluted with 50 mmol/l phosphate buffer, and GPx3 activity was analysed in the undiluted plasma. For both enzymes, H_2O_2 served as the substrate. One unit of GPx1 and GPx3 activity was defined as 1 μmol NADPH oxidised per minute and normalised to 1 mg protein.

Protein concentration in the liver cytosol and in plasma

The protein concentration of the liver cytosol and of plasma was determined according to a standard protocol[40] in microtitre plates using the plate reader Tecan SpectraFluor Plus (Tecan, Grödig, Austria).

Total GSH and total homocysteine in the liver and plasma

Plasma and liver Hcys were measured with modifications of a procedure described earlier[41] using reversed-phase HPLC on a 100 RP-18e column (Agilent Technologies, Santa Clara, CA, USA). In brief, 10 μl of the internal standard mercaptopropionylglycine (200 μmol) per litre potassium borate buffer 1 (100 mM-trihydrogen borate and 2·0 mM-EDTA, pH 9·5) were added to 30 μl of undiluted plasma. The samples were mixed thoroughly. To reduce protein disulphide bonds and to liberate reduced thiols, 4·0 μl of a mixture of tri-*N*-butylphosphate–dimethylformamide (1:1, v/v) were added. After 30 min of incubation and centrifugation, protein precipitation was carried out by the addition of 25 μl of 0·6 M-perchloric acid containing 1·0 mM-EDTA. After 15 min of incubation at 20°C, the samples were centrifuged for 10 min at 10 000 *g*. Twenty microlitres of the supernatant were diluted with potassium borate buffer 2 (2·0 M-trihydrogen borate and 5 mM-EDTA, pH 10·5). Following thorough mixing, 20 μl of a 0·1 % (w/v) 7-fluorobenzofurazone-4-sulphonic acid ammonium salt solution in potassium borate buffer 3 (2·0 M-trihydrogen borate, pH 9·5) were added. After 60 min of incubation in a thermo mixer at 60°C, samples were cooled on ice, mixed and centrifuged for 10 min at 10 000 *g*. Sixty microlitres of the supernatant were pipetted into dark HPLC vials and put into the autosampler. The injection volume was 10 μl. The elution profile was as follows: 6·5 min eluent A (0·10 M-sodium acetate buffer containing 2 % (v/v) methanol, pH 4·0), 7·5 min eluent B (0·1 M-sodium phosphate buffer containing 5 % (v/v) methanol, pH 6·0), 3·0 min eluent B and 3·0 min eluent A. Fluorescence detection was carried out at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. A calibrator plasma with known HCys and GSH concentrations was carried along to calculate sample concentrations of total GSH and HCys. For GSH and HCys determination in the livers, 1:5 (w/v) homogenates were prepared in ice-cold PBS. The remaining procedure was the same as described above for the undiluted plasma.

Cholesterol concentration in plasma and liver

Total Chol concentration in the liver and plasma was determined using the commercial test kits Ecoline®S + from DiaSys Diagnostic Systems GmbH (Holzheim, Germany). Ten microlitres of undiluted plasma were used for Chol determination with the test kit. Before liver Chol analysis, total liver lipids were extracted with a hexane–isopropanol (3:2, v/v) mixture[42]. Fifty-microlitre aliquots of these lipid extracts were evaporated under N_2 gassing, and the remaining lipids were resolved in 50 μl of a Triton X-100–chloroform mixture (1:1, v/v)[43]. After evaporation of the chloroform, 10 μl of the Triton X-100 extracts were used for Chol determination with the test kit. Chol concentration in all the liver and plasma samples was measured in duplicate.

mRNA expression of glutathione peroxidase 1, glutathione S-transferase α 2, glycine-N-methyltransferase, cystathione-β-synthase, S-adenosylmethionine decarboxylase, betaine hydroxymethyltransferase, glutamate cysteine ligase, modulatory subunit, glutamate cysteine ligase, catalytic subunit and glutathione synthetase in the liver

Liver RNA was extracted with TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Following reverse transcription of 3·0 μg of total RNA using a commercial complementary DNA synthesis kit (RevertAid™ First Strand synthesis kit; Fermentas, Vilnius, Latvia), the complementary DNA solutions were diluted 2:3 (v/v) with diethyl pyrocarbonate-treated sterile water for use in real-time detection PCR. For the amplification of specific sequences of the genes investigated, the RotorGene 6000™ real-time detection PCR apparatus (Corbett Research, Mortlake, NSW, Australia) was used.

Amplification data were analysed with the RotorGene 6000™ series software using the amplification efficiencies and the C_t values[44]. The expression of the single genes was normalised to β-actin expression.

The primers used in PCR and their gene bank accession numbers are as follows: GPx1 (NM030826), primer forward (5′ → 3′): TCA TTG AGA ATG TCG CGT CT, primer reverse (5′ → 3′): CCC ACC AGG AAC TTC TCA AA (annealing temperature (T_a): 55°C); glycine-*N*-methyltransferase (NM017084), primer forward (5′ → 3′): CCA CCG CAA CTA CGA CTA CA, primer reverse (5′ → 3′): TCT TCT TGA GCA CGT GGA TG (T_a: 57°C); CBS (NM012522), primer forward (5′ → 3′): ATG CTG CAG AAA GGC TTC AT, primer reverse (5′ → 3′): GCG GTA TTG GAT CTG

CTC AT (T_a: 55°C); betaine hydroxymethyltransferase (BHMT; NM030850), primer forward (5′ → 3′): GCA CCA GCT TGC AGA CAA TA, primer reverse (5′ → 3′): TGT GCA TGT CCA AAC CAC TT (T_a: 55°C); *S*-adenosylmethionine decarboxylase (NM031011), primer forward (5′ → 3′): CCC AGC AGT TAT GGA CCA GT, primer reverse (5′ → 3′) TCC ATC CGA TTT CAT TCC AT (T_a: 55°C); glutathione synthetase (GS; NM012962), primer forward (5′ → 3′): AGA TGG CTA CAT GCC CAG TC, primer reverse (5′ → 3′): TGT CTT TCA GCT GCT CCA GA (T_a: 57°C); glutamate cysteine ligase, catalytic subunit (GCLC; NM012815), primer forward (5′ → 3′): CCA CAA ACT GGC AGA CAA TG, primer reverse (5′ → 3′): TCC TTC CCA TTG ATG ATG GT (T_a: 55°C); glutamate cysteine ligase, modulatory subunit (GCLM; NM017305), primer forward (5′ → 3′): AGG CAC CTC GGA TCT AGA CA, primer reverse (5′ → 3′): AAA TCT GGT GGC ATC ACA CA (T_a: 55°C); glutathione *S*-transferase α 2 (NM031509), primer forward (5′ → 3′): GGG GAG AAA GAG GCA AGT CT, primer reverse (5′ → 3′): CTT CAG CAG AGG GAA GTT GG (T_a: 57°C); sterol-regulatory element-binding protein 2 (SREBP2; NM001033694), primer forward (5′ → 3′): ATC CGC CCA CAC TCA CGC TCC TC, primer reverse (5′ → 3′): GGC CGC ATC CCT CGC ACT G (T_a: 65°C); hydroxymethyl-glutaryl CoA reductase (HMGCoAR; NM013134), primer forward (5′ → 3′): AAG GGG CGT GCA AAG ACA ATC, primer reverse (5′ → 3′): ACG GCA CGG AAA GAA CCA TAG T (T_a: 57°C); ATP-binding-cassette-transporter 8 (ABCG8; NM130414), primer forward (5′ → 3′): AGA CCC TCA CAC AGG ACA CC, primer reverse (5′ → 3′): CAG TCC GTC CTC CAG TTC AT (T_a: 59°C); LDL receptor (LDLR; NM175762), primer forward (5′ → 3′): AGA ACT GCG GGG GCC GAA GAC AC, primer reverse (5′ → 3′): AAA CCG CTG GGA CAT AGG CAC TCA (T_a: 60°C); β-actin (NM031144), primer forward (5′ → 3′): ATC GTG CGT GAC ATT AAA GAG AAG, primer reverse (5′ → 3′): GGA CAG TGA GGC CAG GAT AGA G (T_a: 60°C).

Relative mRNA expression levels are expressed as *x*-fold changes compared with group C15.

Statistical analysis

The data were analysed by one-way ANOVA using the SPSS Statistical Package 14.0 for Windows (Chicago, IL, USA). Homogeneity of variances was tested using the Levene test. If variances were homogenous, differences of means ($P<0·05$) were evaluated with the least significant difference test, if not the Games-Howell test was used.

Results

Diets and rat performance variables (diet consumption, live weight and feed efficiency)

The dietary Se concentrations scheduled in the experimental design were confirmed by Se analysis (C15, 14·8 (SE 2·94); C50, 49·7 (SE 3·73); C150, 156 (SE 7·12); C450, 488 (SE 9·51); M15, 16·2 (SE 4·73); M50, 47 (SE 4·02); M150, 149 (SE 2·27); M450, 493 (SE 14·65)). At the beginning of the experiment, body weight did not differ among the groups (Table 2).

Table 2. Performance variables of growing rats fed diets containing different selenium concentrations in combination with two dietary methionine levels for 8 weeks (*n* 8 animals are considered)*

(Mean values with their standard errors)

	C15		C50		C150		C450		M15		M50		M150		M450	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Initial body weight (g)	64·3	0·60	64·2	0·61	64·2	0·58	64·2	0·66	64·3	0·73	64·2	0·78	64·2	0·93	64·0	0·92
Final body weight (g)	318^{a}	3·60	371^{c}	2·99	359^{c}	5·23	379^{c}	4·38	284^{b}	6·89	365^{c}	3·19	370^{c}	2·82	362^{c}	4·81
Total weight gain (g)	254^{a}	3·27	307^{c}	2·61	295^{c}	4·56	315^{c}	4·28	220^{b}	6·25	301^{c}	3·78	306^{c}	2·69	298^{c}	3·74
Total feed intake (g)	1030^{a}	8·98	1123^{c,e}	7·35	1088^{c}	9·72	1140^{d,e}	9·58	907^{b}	6·47	1073^{a,f}	5·45	1099^{c,e,f}	5·72	1062^{a,f}	8·87
Feed conversion (g feed/g weight gain)	4·06^{a}	0·023	3·66^{b}	0·017	3·69^{b}	0·015	3·62^{b}	0·018	4·13^{a}	0·050	3·57^{b}	0·029	3·59^{b}	0·035	3·56^{b}	0·053

a,b,c,d,e,f Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* For details of diets and procedures, see Methods and materials.

For rats receiving the Se-deficient diets either in combination with the recommended Met concentration or with a high Met concentration (C15 and M15), the total feed intake over 8 weeks and feed efficiency were significantly impaired compared with their respective Se-supplemented companions. In nearly all cases, the mean total feed intake of the rats with the high dietary Met concentration was significantly lower than that of the groups with the recommended Met concentration (C15 *v.* M15, C50 *v.* M50 and C450 *v.* M450). Corresponding to the improved feed conversion, all Se-supplemented rats of both dietary Met levels had significantly higher final body weights than their controls. Whereas the final body weight in the high Met Se-deficient rats (M15) was significantly lower than that in their companions with the recommended Met concentration (C15), no differences in the final body weight existed between the matching Se-supplemented groups at the other dietary Se levels (50, 150 and 450 μg/kg).

Selenium status, variables of the selenium- and glutathione-dependent redox system in the liver and plasma, and homocysteine concentration in the liver and plasma

The liver Se concentration in the Se-deficient rats of groups C15 and M15 was reduced to 1·7 and 1·5 % of the concentrations in groups C450 and M450 (Table 3). At both dietary Met levels, the gradual increase in Se supplementation from 50 to 450 μg/kg increased the liver Se concentration approximately twofold in each case. With the exception of groups C150 and M150, the liver Se concentration in the other corresponding groups (C15 *v.* M15, C50 *v.* M50 and C450 *v.* M450) was significantly higher in the C groups with the recommended dietary Met concentration. At both Met levels with Se deficiency (C15 and M15), GPx1 mRNA expression was approximately fivefold lower than that in the Se-supplemented rats (C50, C150, C450, M50, M150 and M450). Rats supplemented with 50 μg Se/kg (C50 and M50) already had a GPx1 mRNA expression, which was comparably as high as that in rats supplemented with 150 and 450 μg Se/kg. No influence of the dietary Met level on GPx1 mRNA expression could be analysed. In contrast to GPx1 expression, the expression of the phase II enzyme glutathione *S*-transferase (glutathione *S*-transferase α 2), another sensitive indicator of Se deficiency, was 1·5- to 2·5-fold higher in Se-deficient rats than in their Se-supplemented companions. Liver GPx1 activity in C15 and M15 rats was reduced to approximately 1 % of the activity of groups C150, C450, M150 and M450. Liver GPx1 activity of rats of groups C50 and M50 was 40–60 % lower than that of their companions supplemented with the recommended Se amount (C150 and M150) or with slightly supranutritional Se supplementation (C450 and M450). As observed for the liver Se concentration, the liver GPx1 activity of rats with the high dietary Met supplementation was lower than the activity of their companions fed the diets with the recommended dietary Met concentration. However, with the exception of groups C50 and M50, this effect was not statistically significant. Plasma Se concentration in C15 and M15 rats was decreased to 2·5 and 4·3 % of the concentration in groups C450 and M450. Plasma Se concentration gradually and significantly increased when the dietary Se concentration was raised from 50 to 450 μg/kg. In contrast to the liver Se concentration, plasma Se concentration was not influenced by the dietary Met concentration. GPx3 activity in the Se-deficient groups C15 and M15 was reduced to approximately 3 % of the activity in groups C450 and M450. In groups C50 and M50, GPx3 reached an activity of approximately 70 % of that in the respective groups receiving diets containing the recommended Se concentration (C150 and M150) or a slightly supranutritional Se supplementation (C450 and M450). In the Se-deficient groups C15 and M15, plasma GPx3 activity dropped to 3·0–3·5 % of that in groups C150 and M150 supplemented at the recommended level. However, in contrast to liver GPx1 activity, plasma GPx3 activity in the Se-supplemented rats of groups M50 and M150 fed the high dietary Met concentration was significantly higher than that in their companions in groups C50 and C150 on diets with the recommended Met level. For groups M450 and C450, a distinct trend ($P=0·061$) existed with regard to this effect. At both dietary Met levels, total liver GSH concentration was significantly higher in C15 and M15 rats than in the respective Se-supplemented groups. Independent of the dietary Se concentration, rats receiving the diets with the high Met concentration had a significantly higher liver GSH concentration than the corresponding groups receiving diets with the recommended dietary Met level. Liver HCys concentration was 1·4- to 1·5-fold higher in Se-deficient rats of groups C15 and M15 than in their Se-supplemented companions. In contrast to the effect observed for GSH, the higher dietary Met concentration had no influence on liver HCys concentration with the exception of groups C50 and M50. Whereas both the liver GSH concentration and the liver HCys concentration decreased in a dose-dependent manner due to increasing Se supplementation at both dietary Met levels, a completely different regulation profile of the plasma concentration of both compounds could be observed. At both dietary Met levels, increasing the Se supplementation of the diets led to a reduction of total plasma GSH concentration in a dose-dependent manner. Thus, the lowest plasma GSH values could be measured in groups C450 and M450. Moreover, rats receiving the diets with the high Met concentration (groups M15, M50, M150 and M450) had in each case a significantly lower plasma GSH concentration than the respective C groups C15, C50, C150 and C450. In contrast, total plasma HCys concentration was the lowest in the Se-deficient group C15 with the recommended dietary Met level. Se addition to the diets approximately doubled plasma HCys levels. However, a dose-dependent effect of the different dietary Se levels on plasma HCys concentration could not be analysed. The addition of the high dietary Met concentration to the diets (rats of groups M15, M50, M150 and M450) again approximately doubled plasma HCys concentration in comparison to the groups C15, C50, C150 and C450. As analysed in the C groups, also in the M groups, a significant increase in plasma HCys concentration existed only between the Se-deficient group M15 and all groups with Se supplementation (M50, M150 and M450), whereas an increase in dietary Se supplementation did not lead to a further rise in plasma HCys level. On the contrary, in M450 rats, even a small but significant decline in plasma HCys concentration could be analysed compared with groups M50 and M150 with lower Se supplementation.

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Table 3. Effects of different dietary selenium concentrations in combination with two dietary methionine levels on liver and plasma selenium concentrations, total liver and plasma GSH and homocysteine (HCys) concentrations, and the activity as well as the expression of glutathione peroxidase (GPx) 1, GPx3 and glutathione *S*-transferase α 2 (GSTA2) of growing rats (*n* 8 animals are considered)*

(Mean values with their standard errors)

	C15		C50		C150		C450		M15		M50		M150		M450	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Liver																
Se (nmol/g fresh matter)	0·91[a]	0·13	26·1[c]	0·70	54·3[d]	1·93	98·2[f]	3·31	0·87[a]	0·18	22·6[b]	0·67	55·3[d]	0·88	86·4[e]	2·76
GPx1 activity (mU/mg prot.)	6·16[a]	0·27	323[c]	15·2	535[d]	37·3	511[d]	22·4	5·70[a]	0·41	205[b]	12·8	524[d]	30·7	469[d]	33·6
GPx1 mRNA (fold of C15)	1·00[a]	0·22	4·37[b]	0·36	5·48[b]	1·02	4·57[b]	0·57	1·03[a]	0·24	5·00[b]	0·35	5·42[b]	0·68	5·39[b]	0·85
GSTA2 mRNA (fold of C15)	1·00[a]	0·08	0·47[b,d]	0·03	0·68[c]	0·04	0·57[b,c]	0·03	0·91[a]	0·05	0·47[b,d]	0·04	0·57[b,c]	0·05	0·36[d]	0·05
Total GSH (nmol/mg prot.)	69·4[a,e]	2·86	64·3[a,b]	2·03	61·5[b]	3·35	61·4[b]	2·48	91·0[f]	5·94	82·2[c]	3·12	72·8[d,e]	3·52	72·6[d,e]	2·23
Total HCys (nmol/mg prot.)	91·0[a]	4·75	76·2[b]	3·94	58·4[c]	5·35	55·3[c]	9·85	85·5[a,b]	10·9	59·6[c]	4·31	54·5[c]	6·19	51·4[c]	3·86
Plasma																
Se (nmol/ml)	1·06[a]	0·12	26·9[c]	0·30	38·5[d]	0·65	52·4[e]	0·55	1·87[b]	0·09	27·6[c]	0·26	40·6[d]	0·73	52·8[e]	0·57
GPx3 activity (mU/mg prot.)	4·22[a]	0·46	88·2[b]	2·40	123[d]	3·26	132[d,e]	5·12	4·32[a]	0·68	101[c]	2·52	148[e]	3·82	159[e]	5·76
Total GSH (μmol/l)	65·9[a]	2·61	51·0[b]	1·90	48·9[b]	2·79	39·1[c,e]	1·03	53·0[b]	7·55	38·4[c,e]	2·18	35·3[c,e,d]	1·51	32·2[d]	1·44
Total GSH (nmol/mg prot.)	1·12[a]	0·06	0·87[b]	0·04	0·82[b]	0·05	0·68[c]	0·03	0·84[b]	0·18	0·62[c,e]	0·05	0·57[e,d]	0·04	0·54[d]	0·04
Total HCys (μmol/l)	6·78[a]	0·23	12·7[b]	0·61	14·0[b]	0·76	13·6[b]	0·46	12·0[b]	2·17	23·4[d]	1·68	23·2[d]	2·47	18·6[c]	1·61
Total HCys (nmol/mg prot.)	0·12[a]	0·01	0·22[b]	0·02	0·23[b]	0·03	0·24[b]	0·02	0·22[b]	0·08	0·38[d]	0·06	0·37[d]	0·07	0·31[c]	0·06
Liver + plasma																
GSH (nmol/mg prot.)	70·5[a]	1·46	65·2[b]	1·04	62·3[b]	1·70	62·1[b]	1·25	91·8[c]	3·06	82·8[d]	1·59	73·4[e]	1·78	73·1[e]	1·13
HCys (nmol/mg prot.)	91·1[a]	2·38	76·4[b]	1·98	58·6[c]	2·68	55·5[c]	4·93	85·7[a]	5·47	59·9[c]	2·17	54·9[c]	3·11	51·7[c]	1·94

prot., Protein.

a,b,c,d,e Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

*For details of diets and procedures, see Methods and materials.

mRNA expression of key enzymes of homocysteine and glutathione metabolism

To investigate the molecular causes underlying the present results for liver and plasma GSH and HCys concentrations, we analysed the mRNA expression of the key enzymes of GSH and HCys metabolism in the liver of the rats (Fig. 1(a)–(g)). Glycine-*N*-methyltransferase expression was affected neither by the dietary Se concentration nor by the dietary Met level (Fig. 1(a)). In the C groups with the recommended dietary Met concentration, liver CBS expression in the Se-supplemented rats was 38–43 % lower than that in the Se-deficient group. However, no significant differences in CBS gene expression occurred between rats with different dietary Se supplementations (C50, C150 and C450). The high Met concentration in the diet did not significantly change the CBS expression level in the Se-deficient group M15 compared with group C15. In Se-supplemented rats fed the high Met diet, CBS expression was further reduced to 22–35 % of that in C15 rats (17–30 % of that in M15 rats). Thus, Se-supplemented rats with high Met diets (M50, M150 and M450) had in each case a significantly lower liver CBS expression than groups C50, C150 and C450 (Fig. 1(b)). A very similar expression profile as analysed for CBS could also be observed for liver *S*-adenosylmethionine decarboxylase expression. *S*-adenosylmethionine decarboxylase expression in the Se-supplemented rats was reduced to approximately 40 % (C groups) and 5–20 % (M groups) of that measured in their respective Se-deficient companions in groups C15 and M15 (Fig. 1(c)). In the C groups, BHMT responsible for betaine-dependent remethylation of HCys to Met was increased in the Se-supplemented groups C50, C150 and C450 up to 1·6-fold compared with group C15. With the exception of groups C150 and M150, BHMT expression was slightly but not significantly higher in all M groups compared with their respective C groups (Fig. 1(d)). The key enzymes of the GSH biosynthesis pathway were also strongly influenced by the dietary Se and Met concentrations. The expression of the GCLM was reduced by Se supplementation to 30–60 % of that in the Se-deficient rats (C15 and M15), with the lowest expression in group M450

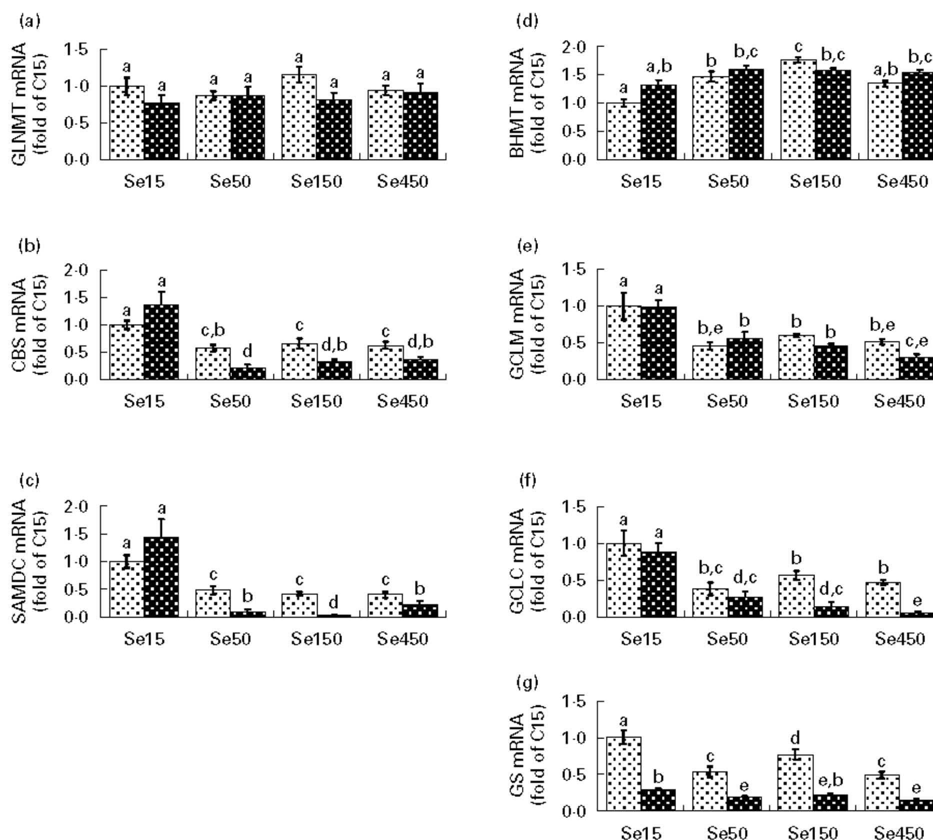

Fig. 1. (a)–(g) mRNA expression of the key enzymes of homocysteine metabolism ((a)–(d)) and GSH metabolism ((e)–(g)) of rats fed diets containing different dietary concentrations in combination with two dietary methionine levels (values are means with their standard errors). [a,b,c,d,e]Mean values with unlike letters were significantly different ($P<0·05$). *n* 8 animals are considered. For details of diets and procedures, see Methods and materials. GLNMT, glycine-*N*-methyltransferase; CBS, cystathione-β-synthase; SAMDC, *S*-adenosylmethionine decarboxylase; BHMT, betaine hydroxymethyltransferase; GCLM, glutamate cysteine ligase, modulatory subunit; GCLC, glutamate cysteine ligase, catalytic subunit; GS, glutathione synthetase. (a)–(g) ⬚, C groups; ■, M groups.

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(Fig. 1(e)). In contrast to the expression of GCLM, the expression of the GCLC was in addition strongly regulated by the dietary Met concentration. Whereas the expression of liver GCLC in the C groups was reduced to 40–55 % of that in Se-deficient C15 rats by Se supplementation at all the dietary levels investigated (C50, C150 and C450), the expression of the enzyme in the Se-supplemented groups with high dietary Met (M50, M150 and M450) was reduced to 4–27 % of that in C15 rats and to 5–30 % of that in M15 rats. Moreover, in Se-supplemented high Met rats of groups M50, M150 and M450, a significant influence of the dietary Se concentration on GCLC expression could be analysed (Fig. 1(f)). GS expression in the liver was the highest in the Se-deficient C15 rats. Independent of the Se concentration in groups C50, C150 and C450, a reduction of GS expression to 50–70 % of the level in group C15 was analysed. The addition of the higher Met level to the diets drastically reduced GS expression. Thus, in M15 rats, GS expression was only 30 % of that in C15 rats (Fig. 1(g)).

Cholesterol concentration in the liver and plasma, and mRNA expression of genes involved in the regulation of cholesterol metabolism

Both Se-deficient groups (C15 and M15) had a 18–27 % (C groups) and a 37–44 % (M groups) lower liver Chol concentration than the respective Se-supplemented groups C50, C150, C450, M50, M150 and M450 (Table 4). At both dietary Met concentrations, the rats with the highest dietary Se supplementation of 450 µg/kg (C450 and M450) had a slightly higher liver Chol concentration than the other Se-supplemented groups. However, at the same dietary Met level, no significant differences in the liver Chol level existed between the Se-supplemented groups. With the exception of the Se-deficient groups C15 and M15 and groups C50 and M50, the Se-supplemented groups M150 and M450 had a significantly higher liver Chol concentration than their companions in groups C150 and C450 receiving diets with the recommended dietary Met concentration. In contrast to the liver Chol concentration at both dietary Met levels, plasma Chol concentration in the Se-supplemented groups C50, C150, C450, M50, M150 and M450 was 15–25 % lower than that in the Se-deficient groups. The gene expression of enzymes involved in the regulation of whole body Chol metabolism showed a differential expression pattern (Table 4). No significant differences in gene expression could be analysed for the transcription factor SREBP2 between all the experimental groups. The expression of HMGCoAR was also not affected by the different dietary conditions. The expression of liver LDLR was the lowest in group C15 with Se deficiency and the recommended dietary Met level. All the Se-supplemented rats receiving diets with the recommended dietary Met concentration (C50, 150 and C450) had an approximately 2·5-fold higher expression of liver LDLR than the C15 rats. The rats of the M groups had a 1·5- to 2·0-fold higher expression of liver LDLR than their companions in the respective C groups (M15 *v.* C15, M50 *v.* C50, M150 *v.* C150 and M450 *v.* C450). The hepatobiliary Chol exporter ABCG8 showed the highest expression level in the Se-deficient M15 rats. The second highest expression was analysed in the corresponding Se-deficient C15 group fed

Table 4. Effects of different dietary selenium concentrations in combination with two dietary methionine levels on liver and plasma cholesterol (Chol) concentrations, and the expression of liver sterol-regulatory element-binding protein 2 (SREBP2), LDL receptor (LDLR), hydroxymethyl-glutaryl CoA reductase (HMGCoAR) and ATP-binding-cassette-transporter 8 (ABCG8) (*n* 8 animals are considered)*

(Mean values with their standard errors)

	C15		C50		C150		C450		M15		M50		M150		M450	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Liver																
Chol (nmol/mg prot.)	8·32^{a}	0·49	10·4^{b,c}	0·18	9·83^{a,b}	0·15	10·6^{b,c}	0·66	8·52^{a}	0·33	11·7^{c,d}	0·79	11·7^{c,d}	0·38	12·3^{d}	0·51
SREBP2 mRNA (fold of C15)	1·00^{a}	0·16	0·89^{a}	0·10	1·01^{a}	0·17	0·81^{a}	0·11	0·95^{a}	0·19	0·87^{a}	0·11	0·85^{a}	0·09	0·89^{a}	0·12
LDLR mRNA (fold of C15)	1·00^{a}	0·22	2·42^{b,d}	0·47	2·47^{b,d}	0·85	2·87^{b,d,e}	0·75	1·98^{a,d}	0·41	4·96^{c}	0·73	4·09^{c,e}	0·79	3·82^{c,e}	0·36
HMGCoAR mRNA (fold of C15)	1·00^{a}	0·12	0·87^{a}	0·09	0·93^{a}	0·07	0·91^{a}	0·08	0·99^{a}	0·12	0·97^{a}	0·11	1·08^{a}	0·13	0·99^{a}	0·14
ABCG8 mRNA (fold of C15)	1·00^{a}	0·08	0·49^{b}	0·08	0·60^{b}	0·12	0·56^{b}	0·11	1·62^{c}	0·25	0·51^{b}	0·10	0·36^{b}	0·14	0·59^{b}	0·11
Plasma																
Chol (nmol/mg prot.)	33·6^{a}	0·83	25·8^{b}	1·18	25·2^{b}	0·88	28·9^{b}	1·11	34·8^{a}	2·86	26·3^{b}	1·23	27·0^{b}	0·87	28·6^{b}	1·2

prot., Protein.

a,b,c,d,e Mean values within a row with unlike superscript letters were significant different ($P<0·05$).

* For details of diets and procedures, see Methods and materials.

diets containing the recommended dietary Met level. Se supplementation reduced the expression of ABCG8 to approximately 50 % (groups C50, C150 and C450) and 30 % (groups M50, M150 and M450) of the level in the corresponding Se-deficient groups C15 and M15.

Discussion

Rat performance parameters and selenium status

In the present study, we investigated for the first time the combined effect of four dietary Se concentrations and two Met concentrations on the metabolism of HCys and Chol in rats. In accordance with our previous studies with rats on the effects of Se on the regulation of protein tyrosine phosphatase-1B and TAG metabolism[45,46], our current data confirm a reduced feed conversion of Se-deficient rats at both dietary Met levels tested. With regard to Met and HCys metabolism, other dietary factors (e.g. folate, vitamin B_{12} and choline) influencing these pathways remained constant in the present experiment. The parameters of Se status (liver and plasma Se, liver GPx1 and plasma GPx3) confirm that the basal diets C15 and M15 produced a marked Se deficiency, and that the graded amounts of Se added to the other diets led to a stepwise increase in liver and plasma Se concentrations. However, the plateau in GPx1 expression and activity as well as in GPx3 activity was definitely reached with the recommended dietary Se level of 150 μg/kg[47,48]. The phase II enzyme glutathione *S*-transferase α 2, another sensitive indicator of Se deficiency, also indicated Se-deficient conditions of groups C15 and M15 by its higher expression in Se deficiency[48].

Influence of selenium and methionine on GSH and homocysteine metabolism

In accordance with studies on rats investigating the influence of Se status on HCys metabolism[18–20], we also found a doubling of total plasma HCys concentration in the Se-supplemented groups at both dietary Met levels compared with the respective Se-deficient groups C15 and M15. An interesting finding of the present study is that the addition of 15 g Met/kg diet (M15, M50, M150 and M450) again doubled plasma HCys concentration compared with the respective groups with the recommended dietary Met amount (C15, C50, C150 and C450). In this context, it is noteworthy that the HCys ratio between Se-deficient rats (C15 and M15) and Se-supplemented rats of the respective dietary Met level remained nearly constant at 1:2. The fact that dietary Met concentration is an important factor that influences plasma HCys concentration was reported for rats as well as for human subjects through an increased dietary Met intake by the consumption of a high protein diet, or subjected to oral Met loading[28,29,32,34]. The inclusion of two dietary Met levels in our experimental design, the measurement of liver and plasma HCys and GSH concentrations, and the measurement of the expression of an increased spectrum of the key enzymes of HCys and GSH homoeostasis contribute to an improved understanding of HCys metabolism in rats (Table 2 and Fig. 1). In these studies, the main conclusion explaining the higher plasma HCys concentration was deduced from the fact that Se-deficient rats had a higher expression and activity of the key enzymes of the GSH biosynthesis pathway in the liver (confirmed in the present study by GCLM, GCLC and GS expression) than their Se-supplemented companions, leading to a ‘pull’ on transsulphuration, and consequently, to lower plasma HCys levels in Se-deficient rats. However, in these studies, liver HCys concentration was not analysed[18,20,21]. Additional analysis of glycine-*N*-methyltransferase expression (not influenced by Se status and dietary Met level), CBS expression (strongly reduced in Se-supplemented C and M groups compared with the Se-deficient C15 and M15 rats) and BHMT expression (slightly increased in Se-supplemented C and M rats compared with their Se-deficient companions) and of the liver HCys concentration (reduced by Se supplementation in C and M rats) in the present study gives rise to some new hypotheses regarding the regulation of the HCys metabolism in rats. In comparison to previous studies on rat GSH or HCys metabolism, the present results suggest the following analogies and new aspects:

(1) In accordance with previous work on rat GSH and HCys metabolism[18–21,49–51], our data confirmed that both the plasma and liver GSH concentrations were increased in Se deficiency, and may result from a pull on the transsulphuration pathway (liver GSH) and a higher export into the plasma (plasma GSH). In these earlier studies, plasma GSH values in Se-deficient rats were nearly twofold increased compared with controls with adequate Se supply[49,50]. In the present experiment at both dietary Met concentrations, the reduction of plasma GSH values to half of that in the Se-deficient groups was obtained with a Se supplementation of 450 μg/kg (groups C450 and M450). The increased expression levels of CBS as well as of GCLC, GCLM and GS in Se deficiency support these findings.

(2) Contrary to expectations, the highest expression of CBS in the Se-deficient groups C15 and M15 and almost unchanged expression levels of glycine-*N*-methyltransferase and BHMT compared with the Se-supplemented groups produced the highest liver HCys concentrations. Therefore, this effect seems to be the consequence of a higher liver HCys disposal under Se-deficient conditions and an increased HCys export into the plasma by Se supplementation and particularly by a higher dietary Met intake rather than deriving from the reduction of the key enzymes of GSH biosynthesis. The dependency of HCys export into the plasma on dietary Met concentration was impressively demonstrated in a rat study[52].

(3) However, in rats, the Se-dependent manipulation of HCys export into the plasma only responds to switching over from Se deficiency to Se supply. A further increase in plasma HCys concentration due to an increase in dietary Se supplementation and plasma Se values cannot be observed. This particular aspect may explain the contradictory results for the interaction of Se status and plasma HCys level in rats[18–21] and human subjects[22–27], since in none of the human studies was a group with severe Se deficiency included. However, in the human studies, in which an inverse relationship between plasma Se and plasma HCys has been identified, the groups with an increased plasma HCys concentration

had very low plasma Se values (0·56–0·79 μmol/l)[22 25] than those in the studies in which Se status or Se supplementation remained without an effect on plasma HCys concentration (>1·0 μmol/l)[26,27]. In this context, it is of interest that the summation of plasma and liver HCys concentrations from the present experiment leads to an inverse relationship between Se status and the summated HCys concentration (Table 2). Moreover, independent of the dietary Met concentration, nearly identical summated HCys concentrations can be observed for the different dietary Se concentrations (C15 *v.* M15, C50 *v.* M50, C150 *v.* M150 and C450 *v.* M150) (Table 2). Thus, future investigations into the influence of Se, Met or other nutrients on HCys metabolism should consider species differences in hepatic HCys export, and investigate the influence of nutrients on specific carriers involved in HCys export[53].

(4) Another interesting finding of the present study is that it was not only liver HCys disposal that was reduced by Se and Met. The Se- and above all Met-dependent distinct down-regulation of the polyamine-synthesising enzyme *S*-adenosylmethionine decarboxylase seems to have a protective function for the organism since polyamines are discussed as being involved in the generation of a number of diseases from cancer and psoriasis to parasitic infections[54].

Influence of selenium and methionine on cholesterol metabolism

In the present study, we also aimed to elucidate the molecular links between the amount of dietary Se and Met supplementation and Chol metabolism. In the literature, there exists inconsistent information on the effects of both nutrients on Chol metabolism. Thus, for Se, a large recent US cross-sectional observational trial with 7129 participants reported a distinct positive correlation between serum Se concentration and serum Chol levels. In the present study, participants in the top quartile (serum Se >1·70 μmol/l) had 8 % higher serum Chol levels, 8 % higher LDL Chol levels and 5 % higher HDL Chol levels than participants in the bottom quartile (serum Se <1·43 μmol/l)[35]. The data of a very recent cross-sectional observational trial from the UK[36] including 1042 white female and male adults aged 19–64 years confirm the findings of the above-mentioned US study. Despite the overall somewhat lower plasma Se concentrations (bottom quartile: <0·98 μmol/l, top quartile: 1·20–2·79 μmol/l) compared with the US study, the participants of the top quartile had 11 % higher total plasma Chol values and 12 % higher LDL Chol values than those in the bottom quartile[35,36]. Another study from Lebanon confirms these results. A higher Se status correlated significantly and positively with a number of markers of the metabolic syndrome including plasma Chol[55]. In contrast, a study from Japan in pre- and post-menopausal women found a decrease of Se status and an increase in plasma Chol level after menopause, and suggested a beneficial effect of Se supplements in lowering Chol values[56]. Several studies with rats with a dietary induced hypercholesterolaemia found that Se supplementation with 1·0 mg/kg diet clearly reverses hypercholesterolaemia by lowering liver apo B and HMGCoAR expression and by increasing LDLR expression[57 59]. A similarly unclear effect as found for Se on Chol metabolism also exists for Met and HCys on Chol metabolism.

In this context, a hypothesis report[60] and a study with transgenic mice[61] suggest that a high dietary Met concentration increases the need for phosphatidylethanolamine (PE)-*N*-methyltransferase for the formation of HCys. Through this increased use of PE-*N*-methyltransferase for HCys metabolism, the reaction actually catalysed by the enzyme, namely the conversion of PE to phosphatidylcholine, is decelerated. Phosphatidylcholine is again an important phospholipid for the formation of VLDL particles. Therefore, high Met and HCys may increase lipid storage in the liver, and contribute to the development of non-alcoholic fatty liver disease. Moreover, high HCys concentrations may lower liver HDL synthesis, and therefore reduce reverse Chol transport[60]. A study with transgenic PE-*N*-methyltransferase −/− mice showed that these animals had a reduced liver VLDL secretion than their PE-*N*-methyltransferase +/+ companions[61]. Accordingly, Met loading in the elderly led to a fourfold increase in plasma HCys concentration and a 22·5 % increase in total Chol:HDL ratio in the plasma[34]. In a study with growing rats, the gradual increase of the dietary Met concentration from 2·6 to 6·0 g/kg diet led to the stepwise increase in plasma HCys and in both plasma and liver Chol. In the present study, the most distinct increase in plasma and liver Chol was analysed between rats consuming the low Met diet (2·6 g/kg) and rats fed the next higher dietary Met level of 3·5 Met/kg diet. A by far lower and NS increase in plasma and liver Chol was observed between rats consuming the other diets containing up to 6·0 g Met/kg diet. An increased expression of the SREBP2 and of HMGCoAR was analysed as the molecular cause for the increasing effect of Met/HCys on liver and plasma Chol. However, the expression of the liver LDLR, another target gene of SREBP2, showed no response to the different Met concentrations[29]. In another study with tissue cultures and mice, it was postulated that the increased liver Chol synthesis by HCys derives from the activation of lipogenic SREBP transcription factors and VLDL secretion by endoplasmatic reticulum stress[62]. In contrast to the above-mentioned studies, describing a Chol-increasing effect of Met supplementation to diets, Chol-lowering effects of Met have also been investigated. Thus, in a rat study, the addition of 3·0 g Met/kg to a 10 % soya protein diet effected a 40 % reduction of plasma Chol, whereas the Met addition to a 20 % soya protein diet effected a small and NS increase in plasma Chol. A significant reduction of liver HMGCoAR activity and a significant increase in Chol-7-α-hydroxylase activity, the key enzyme for bile acid synthesis, were analysed as the molecular causes for the Chol-lowering effect of Met[63]. In another study, rats with an implanted ascites hepatoma cell line developed severe hypercholesterolaemia, based on the reduction of bile acid excretion. Met addition to the diets restored Chol metabolism by increasing Chol excretion via the bile[64].

The comparison of our data with the data from the above-mentioned studies on the role of Se and Met in Chol metabolism indicates both analogies and inconsistencies. In accordance with the studies in which hypercholesterolaemia in rats was induced by feeding high Chol diets, our data

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confirm the up-regulation of the liver LDLR and the decrease of plasma Chol by Se. In contrast to these studies, we could not find a reduction of HMGCoAR expression by Se. This difference may result from a higher Chol loading of the livers in the cited studies by feeding of high Chol diets in combination with an up-regulated LDLR. However, liver Chol data were not provided in these studies[57 59]. When the free liver Chol concentration exceeds a critical value, the processing of the transcription factor SREBP2 by the SREBP cleavage-activating protein is blocked via an up-regulation of the insulin-induced genes 1 and 2 (Insig 1 and 2) which bind to SREBP cleavage-activating protein[65]. Despite significantly higher liver Chol concentrations in the Se-supplemented C and M groups than in the respective Se-deficient groups, the feeding of Chol-free diets in the present study obviously did not result in a liver Chol concentration switching on the Insig–SREBP cleavage-activating protein–SREBP2 system. In this context, it should be mentioned that we could not detect any differences in the expression of Insig 1 and 2 (data not presented). A further new result in the present study was the finding that the hepatobiliary Chol exporter ABCG8, which also plays an important role in the reverse transport of phytosterols into the lumen of the small intestine[66,67], was distinctly down-regulated in all Se-supplemented rats, and therefore provides a further explanation for the higher Chol concentration in the livers of Se-supplemented rats and for their lower plasma Chol levels. In contrast to the previous rat studies[57 59] and to the present results, recent human studies found a positive correlation between a high Se status and an increased plasma Chol concentration[35,36]. One possible explanation for this difference is the fact that in the rat studies, young and still growing animals were used, whereas the human data were collected from adults. This fact raises the question whether the long-term uptake of Se above the recommendations via permanent Chol accumulation in the liver would ultimately also increase plasma Chol values. Thus, long-term studies with rats or better with pigs, which have a lipoprotein metabolism which is more closely related to that of human subjects, are needed to confirm or confute the risk of Se supplementation for increased plasma Chol values. In accordance with previous rat studies[29,68], in the present study, a positive and significant correlation also existed between the dietary Met level and the liver Chol concentration. However, in contrast to these studies[29,68] and to the human study[34] examining the effect of Met loading on Chol metabolism, we could not find a significant increase in plasma Chol concentration by increasing the dietary Met concentration to fivefold of the recommended amount. Similarly as discussed for Se, it is conceivable that feeding adult rats a high Met diet in combination with Chol addition to the diet also increases plasma Chol concentration. However, the present results showing the up-regulation of the LDLR by Se and Met and the additional down-regulation of ABCG8 by Se suggest that the over-supplementation with both nutrients may rather increase the risk of the early development of non-alcoholic fatty liver disease. Another very interesting issue regarding ABCG8 regulation which should be examined in the future involves the function of this transporter in the small intestine where it is selectively involved in the reverse transport of phytosterols but not of Chol back into the lumen. In the case of a phytosterol-rich diet, this particular aspect would suggest a positive influence of a high Se status on plasma Chol level, since a reduced export of phytosterols back to the intestinal lumen would lead to a replacement of Chol by phytosterols.

Conclusions

The present rat study investigating the combined effects of Se and Met on HCys and Chol metabolism reveals the following new aspects:

(1) Se increases plasma HCys concentration only when Se-deficient conditions are compared with Se supplementation, independent of the dietary Met concentration. Already at a suboptimal Se supply of 50 μg/kg diet, the plasma HCys level reaches a plateau concentration. When compared with Se-deficient conditions, the elevation of plasma HCys level by dietary Se does not only result from the reduction of the liver key enzymes of GSH biosynthesis and the subsequent deceleration of the transsulphuration pathway. Se also reduces liver HCys disposal by an increased export into the plasma. Due to the fact that plasma HCys concentration can only be reduced by severe Se deficiency, which is not desirable in human subjects, in our opinion, it can be assumed that Se supplementation is not clearly a risk factor for homocysteinaemia in human subjects. Moreover, our rat model suggests that the summation of plasma and liver HCys concentration results in an inverse relationship between Se status and the HCys concentration, as was similarly reported from some human trials. A high dietary Met concentration, however, is a substantial risk factor for homocysteinaemia. In summary, the rat, in particular when fed diets with a high Met concentration and sufficient Se, may represent a useful model for further critical study of the controversial role of HCys as a risk factor for atherosclerosis, CVD and ischaemic stroke.

(2) Se increases liver Chol via two mechanisms, the concentration-dependent up-regulation of the liver LDLR and the down-regulation of the hepatobiliary Chol exporter ABCG8. A high dietary Met concentration amplifies the Se effects on liver Chol concentration. The present results do not support the findings from recent human trials in which a high Se status corresponded to elevated levels of total Chol and LDL Chol in plasma, but point rather to the development of non-alcoholic fatty liver disease by the long-term over-supplementation with Se and Met.

However, future investigations with animal models should examine the effects of Se supplementation on Chol metabolism when diets with a high energy and Chol concentration are consumed, as is the case in industrial nations. In this context, the role of phytosterols should also be addressed. Furthermore, the pig may represent a better animal model for future studies on Se and Chol metabolism, since its lipoprotein metabolism is more closely related to that of human subjects than to that of the rat.

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PAPER

Glucoraphanin does not reduce plasma homocysteine in rats with sufficient Se supply *via* the induction of liver ARE-regulated glutathione biosynthesis enzymes

Nicole M. Blum,[a] Kristin Mueller,[a] Frank Hirche,[a] Doris Lippmann,[a] Erika Most,[b] Josef Pallauf,[b] Thomas Linn[c] and Andreas S. Mueller*[a]

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Data from human and animal trials have revealed contradictory results regarding the influence of selenium (Se) status on homocysteine (HCys) metabolism. It was hypothesised that sufficient Se reduces the flux of HCys through the transsulphuration pathway by decreasing the expression of glutathione (GSH) synthesising enzymes. Glucoraphanin (GRA) is a potent inducer of genes regulated *via* an antioxidant response element (ARE), including those of GSH biosynthesis. We tested the hypothesis that GRA supplementation to rat diets lowers plasma HCys levels by increasing GSH synthesis. Therefore 96 weaned albino rats were assigned to 8 groups of 12 and fed diets containing four different Se levels (15, 50, 150 and 450 μg kg(diet)$^{-1}$), either without GRA (groups: C15, C50, C150 and C450) or in combination with 700 μmol GRA kg(diet)$^{-1}$ (groups G15, G50, G150 and G450). Rats fed the low Se diets C15 and G15 showed an impressive decrease of plasma HCys. Se supplementation increased plasma HCys and lowered GSH significantly by reducing the expression of GSH biosynthesis enzymes. As new molecular targets explaining these results, we found a significant down-regulation of the hepatic GSH exporter MRP4 and an up-regulation of the HCys exporter Slco1a4. In contrast to our hypothesis, GRA feeding did not reduce plasma HCys levels in Se supplemented rats (G50, G150 and 450) through inducing GSH biosynthesis enzymes and MRP4, but reduced their mRNA in some cases to a higher extent than Se alone. We conclude: 1. That the long-term supplementation of moderate GRA doses reduces ARE-driven gene expression in the liver by increasing the intestinal barrier against oxidative stress. 2. That the up-regulation of ARE-regulated genes in the liver largely depends on GRA cleavage to free sulforaphane and glucose by plant-derived myrosinase or bacterial β-glucosidases. As a consequence, higher dietary GRA concentrations should be used in future experiments to test if GRA or sulforaphane can be established as HCys lowering compounds.

Introduction

A large number of human and animal studies has associated a high level of total plasma homocysteine (tHCys) with an increased risk for the accelerated onset of coronary artery disease, myocardial infarction and ischemic stroke.[1–5] Several mechanisms, including the generation of reactive oxygen species, an increase in LDL oxidation, the liberation of pro-inflammatory transcription factors and cytokines, and a reduction of vessel relaxation *via* a decreased NO availability are discussed as molecular mechanisms underlying the hypertensive and proatherogenic effects of HCys.[5–9] Besides the inherited disorders cystathionine-β-synthase (CBS) deficiency and methionine synthase deficiency, a high dietary methionine intake and other factors, like age, sex and race, can trigger hyperhomocysteinemia.[10–14] Whereas a number of studies has shown an effective reduction of plasma tHCys by combined vitamin B6, B9 (folic acid) and B12 supplementation, benefits of these supplements on risk reduction, recurrence of associated diseases, and on CVD- and ischemic stroke-endpoints are recently in doubt.[15] Nevertheless, taking a standard multivitamin preparation to achieve the recommended vitamin B6, B9 and B12 amounts is advised to risk patients by the international professional societies.[16,17]

Conflicting results have also been reported with regard to the influence of selenium (Se) on HCys metabolism. Studies with chicks, mice and rats have shown that Se deficiency reduced plasma tHCys concentration impressively compared to animals

[a]*Institute of Agricultural and Nutritional Sciences, Preventive Nutrition Group, Martin Luther University Halle Wittenberg, Von Danckelmann Platz 2, D-06120 Halle (Saale), Germany. E-mail: andreas.mueller@landw.uni-halle.de*
[b]*Interdisciplinary Research Centre, Institute of Animal Nutrition and Nutritional Physiology, Justus Liebig University Giessen, Heinrich Buff Ring 26-32, D-35392 Giessen, Germany*
[c]*Medical Clinic for Internal Medicine, Endocrinology and Diabetes, Justus Liebig University Giessen, Rodthohl 6, D-35392 Giessen, Germany*

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with adequate or slightly supranutritive Se supply.[18–21] Quite in contrast, data collected from human cross-sectional observational studies in different countries uniquely demonstrated an inverse correlation between Se status and plasma tHCys concentration.[22–24] Accordingly, a recent U.S. cross sectional observational study clearly confirmed the above mentioned inverse relation between Se status and plasma tHCys.[25] Opposite to these studies, Se intervention in humans with 100–300 μg selenomethionine day^{-1} for 3 to 6 months produced no effects on plasma tHCys compared to the placebo treated controls.[26,27] Nevertheless, in a rat study plasma HCys concentration in Se supplemented rats could be lowered by the addition of a high folic acid concentration to the diet.[28] In a current study, no effects of high Se supplementation for 48 weeks could be measured on brachial artery occlusion parameters of healthy young men in comparison with placebo treated study participants. However, in the placebo group plasma tHCys concentration declined from 7.2 μmol L^{-1} to 6.4 μmol L^{-1}, whereas in the intervention group a minimal increase from 7.1 μmol L^{-1} to 7.2 μmol L^{-1} was observed.[29] These data confirm information from the rat trials and contradict data from the above mentioned human studies. In analogy to variations in Se status, another study with elderly people found a positive correlation between plasma vitamin C and plasma tHCys.[30] These findings were supported by the results of a study in which participants with a high Trolox Equivalent Antioxidant Capacity (TEAC) in plasma came along with increased plasma tHCys levels.[31]

An *in vitro* study with HepG2 cells showed that HCys flux through the transsulphuration pathway and subsequent GSH biosynthesis was stimulated by prooxidants, whereas the addition of various antioxidants to the culture media reduced GSH biosynthesis due to diminished HCys utilisation.[32] A similar hypothesis regarding HCys metabolism has been also postulated for Se: "The up-regulation of GSH biosynthesis in Se deficiency produces a pull on the transsulphuration pathway *via* an increased HCys utilization."[19] Both the GSH biosynthesis enzymes glutamate cysteine ligase (GCL) and glutathione synthase (GS), and a number of phase II enzymes, like glutathione-S-transferases (GSTs), nicotinamide-adenine-dinucleotide-(phosphate)-quinone-oxidase 1 (NQO1) and heme oxygenase 1 (HO1) share the existence of an antioxidant response element (ARE) in their DNA promoter region as a common feature.[33] Increased oxidative stress, as present in Se deficiency or under conditions of high Se supply,[34,35] and electrophilic isothiocyanates, like sulforaphane from cruciferous vegetables potently induce phase II-and GSH biosynthesis enzymes.[36,37] When cells are sufficiently protected against oxidative stress the transcription factor NF-E2-related factor 2 (Nrf2) is bound in the cytosol to the Kelch-like ECH-associated protein 1 (KEAP1), and it is presented for ubiquitination and proteasomal degradation. Both oxidative stress and electrophilic isothiocyanates modify KEAP1 sensor-SH-residues, effecting Nrf2 liberation, its nuclear translocation and its association with the DNA-ARE sequences of target genes. As a consequence, transcription and translation of GSH biosynthesis enzymes and of phase II enzymes increases.[36] Sulforaphane has been demonstrated to act as a potent inducer of phase II enzymes, in particular in the intestine.[36] Results of very recent studies, in which rat liver and lung slices were incubated with the sulforaphane glucosinolate precursor glucoraphanin (GRA), revealed that GRA has a comparable potential to induce ARE-regulated enzymes like pure sulforaphane.[38,39]

The aim of our study was to investigate if feeding a diet containing GRA can reduce the higher HCys levels as present under Se sufficiency or when Se is applied in slightly supranutritive concentrations *via* the induction of GSH biosynthesis enzymes (Fig. 1).

Experimental

Animals and diets

96 healthy weaned male albino rats (mean body weight: 71.9 ± 2.26 g) from the Interdisciplinary Research Center, Department of Animal Nutrition and Nutritional Physiology's own strain HK51 were randomly assigned to eight groups of 12 (C15, C50, C150, C450, G15, G50, G150 and G450).

The Se deficient basal diet of group C15 was based on Torula yeast and Se deficient wheat. Its composition has been previously described in detail.[12] The analysed Se concentration of the Se deficient basal diet was approximately 15 μg $kg(diet)^{-1}$. The diets of groups C50, C150 and C450 were supplemented with 50 μg Se $kg(diet)^{-1}$ (one third of the recommended level), 150 μg Se kg $(diet)^{-1}$ (recommended level) and 450 μg Se $kg(diet)^{-1}$ (three times the recommended level) as sodium selenate. Dietary Se concentrations of groups G15, G50, G150 and G450 were identical to those in the corresponding C groups. Broccoli extract (Jarrow-Formulas®) was additionally added to the diets of the G groups at a level of 3000 mg kg^{-1} diet, providing 300 mg GRA kg $(diet)^{-1}$ (=700 μmol GRA $kg(diet)^{-1}$). With the exception of Se and GRA, the diets were composed according to the American Institute of Nutrition-93G recommendations.[40] The rats were housed individually and had *ad libitum* access to their respective diets and water. After 8 weeks the animals were decapitated under CO_2 anesthesia. Liver samples for gene expression analyses and enzymatic determinations were immediately excised, transferred into snap tubes, frozen in liquid nitrogen, and stored at −80 °C until further analysis. Blood was collected in heparinised tubes and centrifuged for 20 min at 3000*g* for plasma preparation. Plasma was stored at −80 °C until analysis.

All experiments with live animals were performed according to the German Animal Welfare Act. The protocol of this rat nutrition study was approved by the Regional Council of Giessen and by the Animal Welfare Committee of the Justus Liebig University Giessen (Germany) [record token: V54-19c20/15cGI 19/3; 39-2008A].

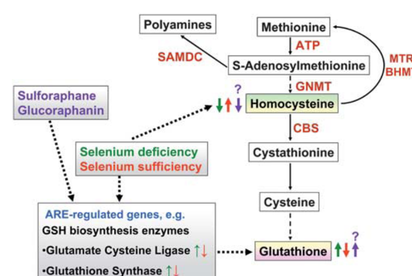

Fig. 1 We studied the hypothesis that GRA supplementation to rat diets lowers plasma HCys, which is high under Se sufficiency due to a reduced GSH synthesis.

Se assay

Se concentrations in diets and plasma were analyzed by hydride generation atomic absorption spectrometry (Unicam PU 9400 X; PU 3960 X), as described previously. Soft winter wheat starch (No. 8438, National Institute of Standard and Technology) and control serum (Metalle S, Medichem, Steinenbronn, Germany) were used as reference materials.[41]

Activity of liver glutathione peroxidase 1 and of plasma glutathione peroxidase 3

The activity of liver cytosolic glutathione peroxidase (GPx1) and of plasma glutathione peroxidase (GPx3) was measured spectrophotometrically (Ultrospec 3300 pro, Amersham Pharmacia Biotech, Freiburg, Germany) at 340 nm using the assay protocol coupled to glutathione reductase (GR) and NADPH.[42] NADPH oxidation, which is proportional to GPx-dependent peroxide reduction, was recorded for 3 min. For both enzymes H_2O_2 was used as substrate. One unit of GPx1 and GPx3 was defined as 1 µmol NADPH oxidized per minute and normalized to 1 mg protein.

Glutathione and homocysteine concentrations in liver and plasma

The concentration of total glutathione in the liver (tGSH) was analyzed using the spectrophotometrical standard protocol coupled to 5,5′-dithiobis-(2-nitrobenzoic acid) [DTNB] and GR.[43] GSH concentration of samples was calculated from a standard curve prepared with oxidized glutathione in the concentration range 0–0.066 µmol oxidised glutathione mL^{-1}. Total homocysteine concentration (tHCys) in plasma and liver and total plasma glutathione (tGSH) were analyzed using a modified standard protocol by reversed-phase HPLC, as described in detail previously.[12] Liver values for tHCys and tGSH were normalized to 1 mg protein.

Protein concentration of samples

Protein concentration in liver cytosol, plasma, and whole liver tissue lysates for Nrf2-immunoblotting was determined in microtitre plates according to a standard protocol using the plate reader Tecan SpectraFluor Plus (Tecan, Grödig, Austria).[44]

Real-time RT-PCR analysis

Liver RNA was extracted with TRIzol ® reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Total RNA concentration and RNA purity were determined spectrophotometrically at 260 and 280 nm. Following reverse transcription of 3.0 µg total RNA using a commercial cDNA synthesis kit (RevertAid™ First strand synthesis kit, MBI Fermentas,Vilnius, Latvia), RNA of two rats per group was pooled. For use in real-time RT-PCR the cDNA solutions were diluted 2.5-fold (v/v) with sterile diethyl pyrocarbonate (DEPC) treated water. Amplification of specific sequences of the genes investigated were performed in duplicate using the Rotor-Gene 6000™ real-time detection apparatus (Corbett Research, Mortlake, Australia). Information on the genes investigated and primer data are given in Table 1. Amplification data were analyzed with the Rotor-Gene 6000™ series software using the ΔΔCt method.[45] The expression of the genes investigated was normalized to β-Actin expression. Prior to this a ranking of expression stability was performed for different housekeeping genes and revealed β-Actin as being the most stable gene in the liver.[46] Relative mRNA expression levels are expressed as x-fold changes relative to group C15 = 1.0.

Immunoblot analysis of Nrf2

For analysis of Nrf2 protein expression in whole liver cell lysate 1 : 10 (w/v) liver homogenates were prepared in a non-reducing RIPA lysis buffer [50 mmol L^{-1} TRIS-HCl, 150 mmol L^{-1} NaCl, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM EDTA, 1.0% sodium desoxycholate, 0.1% sodiumdodecylsulphate (SDS) and 1% TritonX-100, pH = 7.4]. 60 µg of protein were separated according to the standard method[47] under non-reducing conditions on 10% SDS-polyacrylamide gels (50 mA, 4 °C, 2 h). Separated proteins were transferred onto a PVDF membrane (PALL Biotrace 0.45 µm™) by semi-dry blotting [25 min at constant 6 V (~60 mA)]. After blocking membranes overnight at 4 °C in TBST (20 mmol L^{-1} Tris-HCl, 150 mmol L^{-1} NaCl, 0.1% Tween 20, pH = 7.6) containing 5% non-fat dry milk and 0.2% bovine serum albumin (BSA) analysis was continued by a 12 h incubation with the monoclonal Nrf2 antibody (R&D systems, MAB3925) in TBS buffer (1 : 1000) followed by a 1 h incubation with the secondary antibody (1 : 3000) linked to alkaline phosphatase (Goat Anti-Mouse IgG-h + I). Membranes were stained in reaction buffer (0.1 mol L^{-1} TRIS, 0.1 mol L^{-1} NaCl, 0.05 mol L^{-1} $MgCl_2$) containing 0.00375% Nitro-Blue Tetrazolium (NBT)-and 0.0025% 5-bromo-4-chloro-3-indoylphosphate (BCIP). Optical density of the 67 kDA Nrf2-band was evaluated (Gene Tools, Syngene) on scanned membranes (CanoScan LiDe 500F). β-Actin protein expression was determined as control.

Statistical analysis

Data are given as means ± their standard error of the mean (S.E. M.). Statistical differences were analyzed with SPSS 19.0 for Windows (IBM, New York, USA) using one-way ANOVA after testing the normality of distribution (Kolmogorov Smirnov test and Shapiro Wilk test) and the homogeneity of variances (Levene test). If the variances were homogenous, the Least Significant Difference (LSD) test was used to analyze significant differences between means, if not the Games-Howell test was used. Differences between means were considered statistically significant at an error probability of $P < 0.05$.

Results

Performance parameters

At the beginning of the experiment body weight did not differ among groups (Table 2). Se deficient rats of groups C15 and G15 had a lower final mean body weight than all Se-supplemented rats. This difference was significant between group C15 and groups C150, G150, C450 and G450. Despite a higher final body weight in all Se and GRA supplemented groups (G50, G150 and G450), the weight differences were not statistically significant compared to the matching Se deficient G15 rats. The differences in final body weight were accompanied by a significantly reduced feed intake in Se deficient C15- and G15 rats compared to their Se supplemented companions of groups C50, G50, C150, G150,

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Table 1 Gene names with their abbreviations and gene bank accession numbers, and primer sequences of the genes investigated by real-time RT-PCR

Gene name and (abbreviation used)	Gene bank accession. number	Primer sequences (5′ → 3′) for = forward; rev = reverse; TA = annealing *T*/°C
Betaine hydroxymethyltransferase **(BHMT)**	NM_030850	for: 5′ GCACCAGCTTGCAGACAATA 3′ rev: 5′ TGTGCATGTCCAAACCACTT 3′ TA = 55
Cystathionine beta synthase **(CBS)**	NM_012522	for: 5′ ATGCTGCAGAAAGGCTTCAT 3′ rev: 5′ GCGGTATTGGATCTGCTCAT 3′ TA = 55
Glutamate-cysteine ligase modifier subunit **(GCLM)**	NM_017305	for: 5′ AGGCACCTCGGATCTAGACA 3′ rev: 5′ AAATCTGGTGGCATCACACA 3′ TA = 57
Glycine N-methyltransferase **(GNMT)**	NM_017084	for: 5′ CCACCGCAACTACGACTACA 3′ rev: 5′ TCTTCTTGAGCACGTGGATG 3′ TA = 55
Glutathione synthase **(GS)**	NM_012962	for: 5′ AGATGGCTACATGCCCAGTC 3′ rev: 5′ TGTCTTTCAGCTGCTCCAGA 3′ TA = 57
Kelch-like ECH-associated protein1 **(Keap1)**	NM_057152	for: 5′ GTGGCGGATGATTACACCAAT′ 3′ rev: 5′ GAAAAGTGTGGCCATCGTAGC 3′ TA = 57
Multidrug resistance associated protein 4 **(MRP4)**	NM_133411	for: 5′ CTGGATCCAATTTCAGTGTTG 3′ rev: 5′ GGCAAACTTCTCCCGGATTT 3′ TA = 56
5-methyltetrahydrofolate-homocysteine methyltransferase **(Mtr)**	NM_030864	for: 5′ CCTGCTTTGGGGTTGAAGAG 3′ rev: 5′ GGAGTTTGCGCAAGTCTGTG 3′ TA = 57
Nuclear factor (erythroid-derived 2)-like 2 **(Nrf2)**	NM_031789	for: 5′ CCAAGGAGCAATTCAACGAAG 3′ rev: 5′ CTCTTGGGAACAAGGAACACG 3′ TA = 57
S-adenosylmethionine decarboxylase **(SAMDC)**	NM_031011	for: 5′ CCCAGCAGTTATGGACCAGT 3′ rev: 5′ TCCATCCGATTTCATTCCAT 3′ TA = 55
Solute carrier organic anion transporter family, member 1a4 **(Slco1a4)**	NM_131906	for: 5′ GTCATCGGGAAACTCATCTGC 3′ rev: 5′ CCAAAGTAAATGGGTGCAGGA 3′ TA = 57
Cytochrome P450 member 1A1 **(Cyp1A1)**	NM_012540	for: 5′ CAGGAACTATGGGGTGATCCA 3′ rev: 5′ ATATCCACCTTCTCGCCTGGT 3′ TA = 60
NAD(P)H dehydrogenase [quinone] 1 **(NQO1)**	NM_017000	for: 5′ CGCAGAGAGGACATCATTCA 3′ rev: 5′ CGCCAGAGATGACTCAACAG 3′ TA = 57
Heme oxygenase 1 **(HO1)**	NM_012580	for: 5′ AGGCACTGCTGACAGAGGAAC 3′ rev: 5′ AGCGGTGTCTGGGATGAACTA 3′ TA = 61
β-actin	NM_031144	for: 5′ ATCGTGCGTGACATTAAAGAGAAG 3′ rev: 5′ GGACAGTGAGGCCAGGATAGAG 3′ TA = 60

C450 and G450. Independent of additional GRA supplementation the feed conversion ratio (g feed intake : g weight gain) did not differ between Se deficient and Se supplemented rats (Table 2).

Selenium status

Final plasma Se concentrations in Se deficient C15 and G15 rats were 96.4 and 94.3% lower than in rats of groups C150 and G150, fed the recommended dietary Se amount (Table 2). Independent of additional GRA supplementation, plasma Se concentration increased gradually and significantly by raising the dietary Se level from 15 to 450 μg kg^{-1}. Only in GRA supplemented Se deficient rats (G15) plasma was Se significantly (30.9%) higher than in group C15, whereas no differences in plasma Se existed between the Se supplemented G groups (G50, G150 and G450) and the matching C groups. Liver GPx1 activity was 65- to 70-fold higher in the Se-supplemented groups C150, C450, G150 and G450 than in the Se deficient groups C15 and G15 (Table 2). Rats fed one third of the recommended dietary Se level (C50 and G50) reached an intermediate liver GPx1 activity compared to their companions fed the recommended dietary Se concentration (C150 and G150). No significant differences in liver GPx1 activity were measured between GRA supplemented rats and their control littermates (C15 *vs.* G15, C50 *vs.* G50, C150 *vs.* G150 and C450 *vs.* G450). GPx3 activity was regulated in an analogous manner as described for liver GPx1. GSTA3 is a further sensitive indicator of oxidative stress. The highest GSTA3 expression was measured in Se deficient control rats (C15). The addition of only one third of the recommended dietary Se amount (group G50) significantly reduced both, the expression and the activity of GSTA3 compared to C15 rats. The addition of higher Se amounts to the diets (C150 and C450) caused no further changes in GSTA3 expression and activity. In Se deficient rats with a GRA supplement (G15), GSTA3 expression was reduced by 55% compared to C15 rats whereas the enzymes' activity was

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Table 2 Performance parameters (initial, final body weight, total feed intake and feed conversion) and parameters of selenium status in the liver (GPx1 activity and mRNA expression) and the plasma (selenium concentration and GPx3 activity) of growing rats fed diets supplemented with increasing dietary selenium concentrations (15 μg kg^{-1}, 50 μg kg^{-1}, 150 μg kg^{-1} or 450 μg kg^{-1}) either without (C15, C50, C150, C450) or with the addition of 700 μmol glucoraphanin kg(diet)$^{-1}$ (G15, G50, G150, G450)

	C15	C50	C150	C450	G15	G50	G150	G450
Performance parameters								
Initial body weight (g)	71.9 ± 2.29	71.9 ± 2.25	72.0 ± 2.22	72.0 ± 2.19	71.3 ± 2.54	72.2 ± 2.18	71.9 ± 2.26	72.0 ± 2.21
Final body weight (g)	328 ± 6.40^{a}	343 ± 6.32^{ab}	348 ± 9.24^{b}	354 ± 6.41^{b}	336 ± 6.28^{ab}	342 ± 6.49^{ab}	350 ± 4.28^{b}	351 ± 6.02^{b}
Total feed intake (g)	984 ± 5.59^{a}	1027 ± 12.7^{b}	1054 ± 16.0^{b}	1068 ± 12.9^{bc}	1011 ± 7.80^{a}	1036 ± 13.6^{b}	1042 ± 7.00^{b}	1076 ± 8.28^{c}
Feed conversion (g/g)	3.87 ± 0.11	3.81 ± 0.07	3.86 ± 0.13	3.80 ± 0.07	3.84 ± 0.09	3.85 ± 0.07	3.81 ± 0.07	3.87 ± 0.08
Daily feed intake (g)	17.5 ± 0.02^{a}	18.3 ± 0.22^{ab}	18.8 ± 0.28^{ab}	19.1 ± 0.23^{ab}	18.1 ± 0.14^{ab}	18.5 ± 0.24^{ab}	18.6 ± 0.13^{ab}	19.2 ± 0.14^{b}
Daily GRA intake per rat (μmol)	12.3 ± 0.06^{a}	12.8 ± 0.16^{ab}	13.2 ± 0.20^{ab}	13.4 ± 0.16^{ab}	12.6 ± 0.12^{ab}	13.0 ± 0.17^{ab}	13.0 ± 0.09^{ab}	13.4 ± 0.10^{b}
Selenium status								
Liver								
GPx1 (mU mg(prot.)$^{-1}$)	4.08 ± 0.46^{a}	137 ± 10.7^{b}	259 ± 15.6^{c}	282 ± 14.9^{c}	4.49 ± 0.26^{a}	168 ± 13.6^{b}	273 ± 20.0^{c}	314 ± 23.9^{c}
GPx1 expression (fold of C15)	1.00 ± 0.12^{a}	8.17 ± 0.86^{b}	9.56 ± 0.67^{b}	12.4 ± 1.09^{b}	2.07 ± 0.49^{a}	6.60 ± 1.36^{ab}	8.82 ± 1.09^{b}	9.63 ± 0.39^{b}
GSTA3 (U mg(prot.)$^{-1}$)	0.38 ± 0.01^{a}	0.25 ± 0.11^{c}	0.22 ± 0.01^{c}	0.24 ± 0.01^{c}	0.33 ± 0.01^{b}	0.25 ± 0.01^{c}	0.24 ± 0.01^{c}	0.27 ± 0.02^{c}
GSTA3 expression (fold of C15)	1.00 ± 0.06^{a}	0.54 ± 0.03^{b}	0.39 ± 0.02^{b}	0.41 ± 0.04^{b}	0.45 ± 0.01^{b}	0.26 ± 0.01^{c}	0.29 ± 0.03^{c}	0.35 ± 0.03^{bc}
Plasma								
Se (μg L^{-1})	20.7 ± 0.62^{a}	415 ± 11.8^{c}	572 ± 8.55^{d}	628 ± 10.9^{e}	27.1 ± 1.03^{b}	457 ± 11.4^{c}	577 ± 6.88^{d}	630 ± 10.4^{e}
GPx3 (mU mg(prot.)$^{-1}$)	0.18 ± 0.02^{a}	10.1 ± 0.53^{b}	16.0 ± 0.77^{c}	16.3 ± 1.19^{cd}	0.33 ± 0.04^{a}	11.7 ± 0.80^{bd}	15.9 ± 0.95^{cd}	16.7 ± 0.90^{c}

Unlike superscripts within a line indicate significant differences between means ($P < 0.05$). $n = 12$ rats per experimental group. Se and enzymatic analyses were performed in duplicate for each individual.

reduced only by 24%. GSTA3 expression in GRA- and Se-supplemented rats (G50, G150 and G450) was significantly lower than in their companions of the respective C groups. GSTA3 activity did not differ between Se supplemented C and G rats.

Glutathione and homocysteine concentrations in liver and plasma

Total liver glutathione (tGSH) was significantly higher in Se deficient control rats (C15) than in Se supplemented rats of groups C50 and C150. Liver tGSH in group C450 was also distinctly lower ($P = 0.22$) compared to group C15. GRA addition to the Se deficient diet (G15) lowered liver tGSH significantly compared to C15 rats. The highest liver tGSH concentration within all groups was measured in group G450. Significantly higher liver tGSH levels were also analysed in rats of the Se and GRA supplemented groups G50 and G450 compared to their Se deficient companions of group G15 and their matching C groups C50 and C450 (Fig. 2A). Neither Se nor GRA significantly influenced liver tHCys concentration. However, liver tHCys tended to be higher in all Se supplemented groups compared to the Se deficient groups C15 and G15 (effect size: $P = 0.15$) (Fig. 2B). tGSH in plasma was reduced by approximately 30% in all Se supplemented control groups (C50, C150 and C450) compared to the Se deficient group (C15). Both Se deficient and Se supplemented rats with dietary GRA (G15, G50, G150 and G450) had significantly lower plasma tGSH concentrations than Se deficient control rats (C15). Plasma tGSH concentration of Se- and GRA supplemented rats either tended to be lower (G150, $P = 0.10$ and G450, $P = 0.15$), or it was significantly lower (G50, $P = 0.045$) than in rats of the respective Se deficient group G15 (Fig. 2C). C15 rats had an about 2.4-fold lower plasma tHCys concentration than their Se supplemented companions (C50, C150 and C450). GRA did not influence plasma tHCys concentration at all dietary Se levels investigated (Fig. 2D).

mRNA expression of key enzymes and transporters of homocysteine and glutathione metabolism

In order to study the molecular mechanisms underlying the measured GSH and HCys concentrations in plasma and liver, the mRNA expression of key enzymes and of transport proteins involved in GSH and HCys metabolism was analyzed in the liver as the main organ of GSH and HCys synthesis and metabolism (Table 3). GCLM and GS, two important key enzymes of GSH biosynthesis, revealed the highest mRNA expression in the Se deficient groups C15 and G15. In the C groups C50, C150 and C450 Se supply at all levels investigated decreased GCLM mRNA to levels of 61 to 78% of that in group C15. GRA addition to the Se deficient diet (G15) had no influence on GCLM mRNA compared to C15 rats. GRA plus Se (G50, G150,

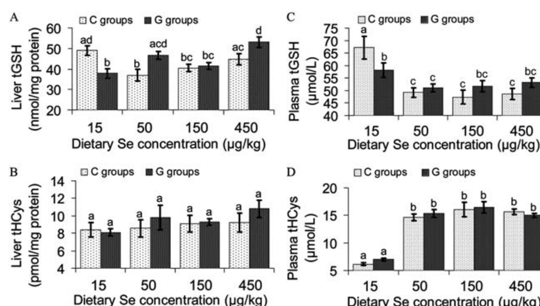

Fig. 2 Concentration of total glutathione (tGSH) and total homocysteine (tHCys) in the liver and the plasma of growing rats fed diets supplemented with increasing dietary selenium concentrations (15 μg kg^{-1}, 50 μg kg^{-1}, 150 μg kg^{-1} or 450 μg kg^{-1}) either without (C15, C50, C150, C450) or with the addition of 700 μmol glucoraphanin/kg diet (G15, G50, G150, G450). Unlike small letters in a figure indicate significant differences between means ($P < 0.05$). $n = 12$ rats per experimental group. Analyses were performed in duplicate for each individual.

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G450) reduced GCLM expression by 24 to76% in comparison with the respective Se deficient rats (G15). GS showed a different regulation profile. Se deficient rats with or without GRA supply (C15 and G15) had the highest GS mRNA. Independent of GRA, Se supplementation at all levels investigated reduced GS expression by 23 to 45% of that in Se deficient C15 rats. Independent of GRA, rats receiving the highest Se supplementation (C450 and G450), had the highest GS expression levels within all Se supplemented groups. GNMT, the first enzyme in HCys metabolism, converting *S*-adenosyl-methionine to *S*-adenosyl-homocysteine, was down-regulated in tendency by Se supplementation at the recommended (C150, $P = 0.10$) and at the supranutritive level (C450, $P = 0.08$) compared to Se deficient control rats (C15). GRA effected a general down-regulation of GNMT expression compared to C15 rats. The combination of Se and GRA (G50, G150, G450) reduced GNMT mRNA to a higher extent than Se alone. The mRNA of SAMDC, passing *S*-adenosyl-methionine into the polyamine pathway, was reduced by Se at all levels investigated by about 50% compared to Se deficiency (C15). Independent of the dietary Se level, GRA decreased SAMDC expression by about 60% compared to C15 rats. The mRNA level of Mtr, the key enzyme of folate and vitamin B12 dependent HCys remethylation, was neither affected by Se nor by GRA. In contrast, Se supplementation at all levels, reduced expression of BHMT, the second HCys remethylating enzyme, by 23% to 32% compared to Se deficiency (C15). GRA *per se* decreased BHMT expression by 36% to 47% compared to Se deficient controls (C15). BHMT mRNA was already reduced by GRA supply to Se deficient rats (G15) compared to Se deficient controls without GRA (C15). In relation to Se deficient controls (C15), the combined Se- and GRA-supplementation (G50, G15, G450) reduced BHMT mRNA to a comparable extent than Se alone (C50, C150, C450). The mRNA of CBS, the key enzyme of HCys utilization for cysteine production, was not influenced by variations in the dietary Se concentration in all C groups. At all dietary Se levels GRA supply reduced CBS mRNA compared to the C groups. This reduction was significant in Se- and GRA-supplemented rats of groups G50, G150 and G450 compared to Se deficient controls (C15). mRNA levels of MRP 4, the main GSH exporter into plasma, were 50 to 60% lower in all Se supplemented controls (C50, C150, C450) than in their Se deficient companion (C15). GRA supply reduced MRP4 mRNA already in Se deficiency by 34% compared to Se deficient controls (C15). MRP4 mRNA levels of GRA- and Se-supplemented rats (G50, G150 and G450) were comparable to those of their respective controls without GRA (G50. G15, G450). mRNA expression of Slco1a4, releasing HCys into the plasma, was higher with Se supplementation (C50, C150, C450, G50, G150 and G450) than in Se deficiency (C15 and G15). This raise was only significant in C450 and G150 rats compared to their respective Se deficient companions (C15 and G15).

mRNA expression of the phase I enzyme CYP1A1 and of the antioxidant phase II enzymes NQO1 and HO1

Se deficient control rats (C15) had an about 3-fold higher CYP1A1 expression than all their Se supplemented companions (C50, C150 and C450) (Fig. 3A). Independent of the Se effects, GRA supply reduced CYP1A1 mRNA by about 60% of that in Se deficient controls (C15). The mRNA of NQO1, preventing free radical formation by one electron reductions, was the highest in Se deficient control rats (C15). In Se deficiency GRA supply (G15) reduced NQO1 expression by 20%. Se supply alone (C50, C150, C450) or in combination with GRA (G50, G150, G450) further reduced NQO1 expression by 66 to 74% compared to the respective Se deficient groups (C15 and G15) (Fig. 3B). The antioxidant HO1 had the highest expression in the liver of Se deficient controls (C15). Se addition to the diets reduced HO1 mRNA dose dependently (C50, −10%), (C150, −37%), (C450, −49%). Irrespective of the dietary Se level, GRA reduced HO1 mRNA by 42 to 55% compared to Se deficient controls (Fig. 3C).

mRNA expression of KEAP 1 and Nrf2 and KEAP 1:Nrf2-ratio

In C rats Se supply reduced KEAP1 mRNA expression dose-dependently (relative expression: C50 = 0.74, C150 = 0.62,

Table 3 Expression of genes involved in glutathione and homocysteine metabolism in the liver of growing rats fed diets supplemented with increasing dietary selenium concentrations (15 μg kg $^{-1}$, 50 μg kg $^{-1}$, 150 μg kg $^{-1}$ or 450 μg kg $^{-1}$) either without (C15, C50, C150, C450) or with the addition of 700 μmol glucoraphanin kg(diet) $^{-1}$ (G15, G50, G150, G450)

	C15	C50	C150	C450	G15	G50	G150	G450
Glutathione biosynthesis								
GCLM (fold of C15)	1.00 ± 0.06^{a}	0.64 ± 0.04^{bd}	0.61 ± 0.03^{bd}	0.78 ± 0.03^{bc}	0.99 ± 0.13^{ac}	0.76 ± 0.06^{b}	0.53 ± 0.01^{d}	0.24 ± 0.09^{e}
GS (fold of C15)	1.00 ± 0.04^{a}	0.60 ± 0.05^{bc}	0.69 ± 0.7^{bc}	0.73 ± 0.05^{b}	0.94 ± 0.06^{a}	0.55 ± 0.04^{c}	0.57 ± 0.05^{bc}	0.67 ± 0.04^{bc}
S-adenosyl-methionine utilisation								
GNMT (fold of C15)	1.00 ± 0.08^{a}	0.96 ± 0.09^{ab}	0.78 ± 0.08^{abc}	0.86 ± 0.07^{abc}	0.74 ± 0.09^{abc}	0.72 ± 0.11^{bc}	0.69 ± 0.05^{bc}	0.66 ± 0.09^{c}
SAMDC (fold of C15)	1.00 ± 0.14^{a}	0.59 ± 0.07^{b}	0.47 ± 0.07^{b}	0.48 ± 0.05^{b}	0.38 ± 0.05^{b}	0.42 ± 0.09^{b}	0.39 ± 0.08^{b}	0.40 ± 0.06^{b}
HCys remethylation and utilisation of HCys for cysteine production								
Mtr (fold of C15)	1.00 ± 0.08^{a}	1.12 ± 0.12^{a}	1.17 ± 0.24^{a}	0.91 ± 0.05^{a}	0.91 ± 0.09^{a}	0.99 ± 0.10^{a}	0.87 ± 0.08^{a}	0.97 ± 0.08^{a}
BHMT (fold of C15)	1.00 ± 0.03^{a}	0.77 ± 0.09^{b}	0.66 ± 0.09^{bc}	0.68 ± 0.05^{bc}	0.64 ± 0.04^{bc}	0.56 ± 0.08^{bc}	0.53 ± 0.06^{c}	0.58 ± 0.07^{bc}
CBS (fold of C15)	1.00 ± 0.09^{a}	0.96 ± 0.08^{a}	1.00 ± 0.19^{ab}	1.05 ± 0.09^{a}	0.83 ± 0.07^{ab}	0.69 ± 0.01^{c}	0.77 ± 0.03^{bc}	0.71 ± 0.04^{c}
Glutathione and homocysteine exporters								
MRP4 (fold of C15)	1.00 ± 0.06^{a}	0.45 ± 0.03^{b}	0.49 ± 0.06^{b}	0.48 ± 0.01^{b}	0.76 ± 0.10^{c}	0.45 ± 0.03^{b}	0.39 ± 0.01^{b}	0.48 ± 0.04^{b}
Slco1a4 (fold of C15)	1.00 ± 0.09^{ac}	1.37 ± 0.18^{ad}	1.10 ± 0.10^{acd}	1.77 ± 0.11^{b}	0.93 ± 0.07^{c}	1.30 ± 0.06^{acd}	1.52 ± 0.20^{bd}	1.26 ± 0.10^{cd}

Unlike superscripts within a line indicate significant differences between means ($P < 0.05$). $n = 6$ cDNA pools of 2 rats per experimental group. Analyses were performed in duplicate for each pool.

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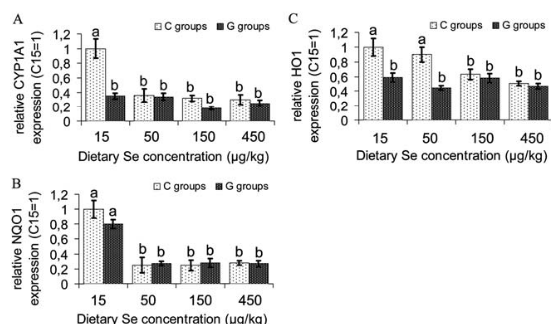

Fig. 3 Expression of the phase I cytochrome P450 oxidase CYP1A1, and of the phase II antioxidant enzymes NAD(P)H Quinone Reductase 1 (NQO1) and Heme Oxygenase 1 (HO1) in the liver of growing rats fed diets supplemented with increasing dietary selenium concentrations (15 µg kg^{-1}, 50 µg kg^{-1}, 150 µg kg^{-1} or 450 µg kg^{-1}) either without (C15, C50, C150, C450) or with the addition of 700 µmol glucoraphanin kg (diet)$^{-1}$ (G15, G50, G150, G450). Unlike superscripts in a figure indicate significant differences between means ($P < 0.05$). $n = 6$ cDNA pools of 2 rats per experimental group. Analyses were performed in duplicate for each pool.

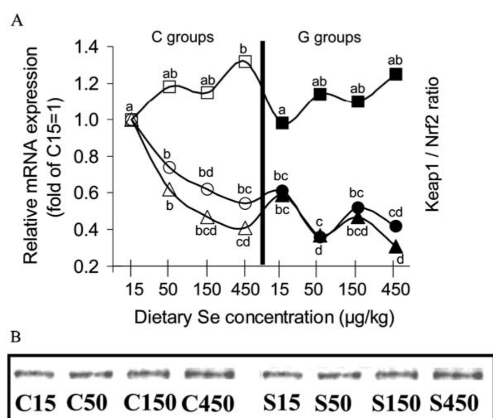

Fig. 4 (A) Expression of Keap1 and Nrf2 and Keap1:Nrf2-ratio in the liver of growing rats fed diets supplemented with increasing dietary selenium concentrations (15 µg kg^{-1}, 50 µg kg^{-1}, 150 µg kg^{-1} or 450 µg kg^{-1}) either without (C15, C50, C150, C450) or with the addition of 700 µmol glucoraphanin kg(diet)$^{-1}$ (G15, G50, G150, G450). Circles show the expression of Keap1 relative to group C15 = 1, in the left side of the figure empty circles (○) represent Keap1 mRNA expression of the C groups, in the right side of the figure black-filled circles (●) represent the Keap1 mRNA expression of the G groups. Squares show the expression of Nrf2 relative to group C15 = 1, in the left side of the figure empty squares (□) represent Nrf2 mRNA expression of the C groups, in the right side of the figure black-filled squares (■) represent the Nrf2 mRNA expression of the G groups. Triangles show the expression ratio of Keap1 : Nrf2 relative to group C15 = 1, in the left side of the figure empty triangles (△) represent the ratio of Keap1 : Nrf2 in the C groups, in the right side of the figure black-filled triangles (▲) represent the ratio of Keap1: Nrf2 in the C groups. Unlike superscripts in the figure indicate significant differences between means ($P < 0.05$). $n = 6$ cDNA pools of 2 rats per experimental group. Analyses were performed in duplicate for each pool. (B) Protein expression of Nrf2 in whole liver lysate of growing rats fed diets supplemented with increasing dietary selenium concentrations (15 µg kg^{-1}, 50 µg kg^{-1}, 150 µg kg^{-1} or 450 µg kg^{-1}) either without (C15, C50, C150, C450) or with the addition of 700 µmol glucoraphanin kg(diet)$^{-1}$ (G15, G50, G150, G450). The figure shows a representative immunoblot prepared from one rat per group, representing the mean body weight of its group.

C450 = 0.54) compared to Se deficient controls (C15, relative expression =1.00) (Fig. 4A). GRA *per se* significantly reduced KEAP1 mRNA already in Se deficiency (relative expression: G15 = 0.61). The lowest KEAP1 mRNA levels were measured in the Se supplemented G groups (relative expression: G50 = 0.36, G150 = 0.52, G450 = 0.42). Irrespective of GRA supply, Nrf2 expression was higher in Se supplemented rats (relative expression: C50 = 1.18, C150 = 1.15, C450 = 1.32, G50 = 1.14, G150 = 1.11, G450 = 1.25) than in the respective Se deficient rats (C15 and G15, relative expression: C15 = 1.00, G15 = 0.98). The results for slightly increased Nrf2 expression in Se supplemented rats could be confirmed by immunoblotting (Fig. 4B). As a consequence of these results the KEAP1:Nrf2-ratio decreased dose-dependently in Se supplemented C rats (C15 = 1.00, C50 = 0.62, C150 = 0.47, C450 = 0.41). Moreover the KEAP 1: Nrf2-ratio of GRA supplemented Se deficient rats (G15, 0.59) was significantly lower than that of their Se deficient companions without GRA (C15). Se in combination with GRA (G50, G150, G150) reduced the KEAP 1: Nrf2-ratio (Se treatment effect: $P = 0.041$) compared to their companions with Se deficiency (G15). The KEAP 1 : Nrf2-ratio of rats receiving diets with combined GRA- and Se-supply (G50 = 0.31, G150 = 0.47 and G450 = 0.31) tended to be lower than in their companions of the respective C groups (C50, C150, C450, GRA treatment effect: $P = 0.10$).

Discussion

Performance parameters

In accordance with prior research from our group and with other rat studies we could confirm that marginal Se supply (C15, G15) reduces feed intake and weight gain of growing rats.[48–50] Although final body weights of Se deficient C -and G-rats did not differ significantly, GRA influenced feed intake and weight gain slightly positively. Irrespective of GRA, no significant differences with regard to performance parameters could be observed between the Se supplemented groups (C50, C150, C450, G50, G150 and G450) (Table 2).

Se status

Se status, assayed by means of plasma Se concentration, plasma GPx3 activity, liver GPx1 activity- and expression, indicated a marked Se deficiency in groups C15 and G15 and a dose dependent increase in plasma Se concentration by raising the dietary Se level. The achievement of a plateau of GPx3 activity and of liver GPx1 activity- and expression by feeding the recommended dietary Se amount confirmed the well established regulation-hierarchy of functional selenoproteins, which was also the topic of numerous studies dealing with the determination of rats' Se requirement.[51,52] That the ARE-regulated GSTA3 was up-regulated under conditions of oxidative stress and in

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particular of Se deficiency was also observed in two earlier mouse studies and could be confirmed by our data.[53,54] That GSTA3 expression- and activity were drastically reduced by supplementing only one third of the recommended Se amount (group C50) indicate a massive decrease of oxidative stress already with this low Se dose. The reduction of GSTA3 expression and activity by GRA supply to Se deficient rats (G15) compared to C15 rats indicated a reduction of oxidative stress by GRA. Decreased oxidative stress by GRA supplementation was also reflected by distinctly lower GSTA3 mRNA levels in Se supplemented G rats (G50, G150, G450) compared to their corresponding C companions (C50, C150, C450) (Table 2).

Influence of various dietary Se levels on GSH and HCys metabolism

Studies with rats and humans have reported conflicting results with regard to the influence of Se status on plasma HCys level. Whereas rat studies clearly have demonstrated that a low Se status reduces plasma HCys and increases plasma GSH, a human study with subjects having a low Se status (plasma Se concentration range: 54–70 μg L^{-1})[23] found a significantly inverse relationship between Se status and plasma HCys. Contrariwise in populations with a higher baseline Se status (plasma Se concentration range: 80–100 μg L^{-1}), Se supplements (100 to 300 μg Se per day in form of Se enriched yeast) remained without an effect on plasma tHCys. In these trials participants of the intervention groups achieved final plasma Se levels of 150–250 μg L^{-1} after 2.5 and 6 months.[26,27] In our rat study plasma Se levels in the Se deficient groups C15 and G15 (Table 2) were even lower compared to the human study with a low basal Se status.[23] An increase in plasma tHCys by adding graded Se amounts to our rat diets could be observed only between the Se deficient groups C15 and G15 and the groups supplemented with the lowest Se concentration of 50 μg kg^{-1} diet (C50 and G50). Additional Se supply, up to slightly supranutritive levels (450 μg Se $kg(diet)^{-1}$), produced no further effects (Table 3). Our data rather support the results of the human supplementation trials, which have demonstrated the ineffectiveness of Se as a HCys lowering agent.[26,27]

Moreover our data confirm the hypothesis of prior investigations that only severe Se deficiency lowers plasma tHCys concentration by increasing GSH biosynthesis.[12,19–21] In addition to this generally accepted information our study revealed some interesting new results as to how a low Se status contributes to a decrease of plasma tHCys and an increase in plasma tGSH. The up-regulation of HCys remethylation by BHMT in Se deficiency (C15, Table 3) in combination with decreased HCys export (Slco1a4) and increased GSH export (MRP4) into the plasma provide additional information for the particular changes in liver and plasma tHCys-and tGSH levels. This new information is supported by the knowledge that both GSH biosynthesis enzymes (GCLM and GS) and MRP 4 are Nrf2 targets with ARE-containing promoters.[53–57]

Finally, it should be mentioned that positive effects of an adequate or high Se status may derive from the distinct down-regulation of SAMDC, involved in spermidine sythesis. Since spermidine is discussed as a cancer-promoting compound, the reduction of SAMDC expression by Se may represent an additional and novel mechanism explaining its cancer protective potential.[58]

Influence of GRA in combination with various dietary Se levels on GSH and HCys metabolism

The main hypothesis of our rat study was to investigate if feeding GRA could lower plasma tHCys concentration in Se sufficiency by increasing the expression of the ARE-regulated GSH biosynthesis enzymes. The up-regulation of ARE-regulated genes in the liver thereby was expected to deriving from GRA cleavage by bacterial β-glucosidases in the caecum followed by the release of sulforaphane into the blood.[59]

In contrast to our expectations at all Se levels investigated, GRA feeding had no mentionable influence on liver mRNA levels of GSH biosynthesis enzymes (Table 3).

In the Se deficient group G15 the GSH exporter MRP 4[56,57] was even down-regulated by additional GRA feeding, whereas no expression differences existed between the Se supplemented groups. This result was directly reflected by a significantly lower plasma tGSH concentration in GRA treated Se deficient rats (G15) compared to the Se deficient controls (C15) (Fig. 2C).

Another interesting result of GRA feeding could be made with regard to CBS expression. Whereas CBS mRNA was not influenced by Se (C groups), its expression was markedly down-regulated by GRA supplementation (G groups) (Table 3). This particular result may base on a more pronounced down-regulation of the antioxidant HO1 in the G groups (Fig. 3C). Beside sufficient vitamin B6, heme is a second major factor responsible for full CBS activity.[60] With GRA supply CBS activity may have reached a maximum activity followed by the reduction of its expression due to reduced heme degradation by HO1.

A further difference between C and G groups could be observed with regard to BHMT expression.[61] Se deficient C rats (C15) had the highest BHMT expression. Se supplementation and, in particular, additional GRA supply distinctly reduced BHMT mRNA.

The combination of reduced BHMT- and CBS expression by GRA feeding thus could be expected to cause an accumulation of liver HCys and higher plasma HCys values. However, liver and plasma tHCys concentration in G rats only tended to be higher than in C rats.

As observed for the Se supplemented C rats (C50, C150 and C450), additional GRA supply reduced SAMDC mRNA to a somewhat greater extent than Se alone and therefore its function as a cancer protective compound is supported.[58]

Finally, a conflicting result of our study should be addressed. We have measured the highest GNMT mRNA concentration in Se deficient control rats. Se supply, in particular in combination with GRA, led to a dose-dependent decline of GNMT mRNA (Table 3). This result would imply higher liver and plasma HCys concentrations in C15 rats than in Se supplemented groups. An explanation for the lacking effect of the high GNMT expression on liver and plasma HCys in C15 rats may be based on the dual function of the enzyme. GNMT acts as both a methylase and as the 4S Polycyclic Aromatic Hydrocarbon Receptor (4S-PAHR), which in turn is one important factor for CYP1A1 induction.[62] Concordant with these facts, C15 rats had the highest GNMT and the highest CYP1A1 expression. This implicates that GNMT

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under conditions of increased oxidative stress (Se deficiency) acts as both a methylase and as the 4S-PAHR. When oxidative stress is reduced by Se- and/or GRA-supply it works just as a methylase, which implies that changes in GNMT do not influence the particular changes of liver and plasma HCys.

We could not establish GRA as a HCys lowering dietary supplement under the conditions tested. Presumably the dietary GRA amount was too low for sufficient sulforaphane release, which induces ARE-regulated GSH biosynthesis enzymes in peripheral organs. This topic will be discussed in the following section.

Discussion of the lacking effect of GRA on the induction of ARE-regulated genes in the liver

Local effects of GRA on the induction of ARE-regulated genes. First it must be stated that the isothiocyanate concentrations for an effective induction of ARE-regulated genes in prior studies varied extremely.[63,64]

In contrast to earlier trials, which have studied the induction of phase II enzymes in cell culture models and in rats using pure sulforaphane, the purpose of our study was to investigate the effects of a broccoli extract with a guaranteed GRA concentration of 10% (w/w). Another intention of our study was to adapt the GRA supply of the rats to a realistic uptake of 500–1000 μmol GRA per day in humans. These concentrations can be realised by eating 250–400 g cooked broccoli with almost inactivated myrosinase or taking a dietary supplement.

The latest results from a rat study have shown that in the absence of plant-derived myrosinase, 10 to 15% of dietary GRA are cleaved by β-glucosidases from ceacal bacteria and that a peak value of free sulforaphane in mesenterial blood is reached 2–3 h after GRA ingestion. In this study a single dose of 150 μmol of GRA was applied to the rats for one time only.[65] In contrast, in our study the average daily GRA intake of G group rats was only about 13 μmol (~730 μmol during the whole experiment). That way the GRA intake in our trial was distinctly lower than in the above mentioned study and may provide one explanation for the lacking response on the induction of ARE-regulated liver enzymes. Whereas prior studies with tissue cultures and laboratory animals have postulated that only pure isothiocyanates (*e.g.* sulforaphane) induce ARE-regulated enzymes, the latest investigations have shown that GRA also potently up-regulates these enzymes.[38,39] Effective GRA concentrations for a maximum phase II enzyme induction varied between 1 μM and 25 μM, depending on the enzyme.[38,39] However, it must be remarked that the mentioned studies were carried out *in vitro* by incubating tissue slices with GRA. In our study we have measured a distinct induction of a broad panel of ARE-regulated genes, including different subclasses of GSTs, NQO1, epoxide hydrolase1, HO1, GPx2, and TrxR1, in the small and in the large intestine of the GRA fed rats (data are not shown and will be published separately). Transferring the *in vitro* data[38,39] to our study, it can be concluded that GRA, in the concentration used, rather has produced a local response in the gastrointestinal tract than systemic effects. Local concentrations in the intestine of our rats, having an average daily GRA intake of ~13 μmol per rat, correspond well to the mentioned *in vitro* incubation studies.[38,39] and to results from another trial in which 9 μmol pure sulforaphane potently induced ARE-regulated genes in the intestine of rats.[63]

Another explanation for the lacking response of GRA on ARE gene induction in the liver may consist of an increased intestinal barrier against oxidative stress,[66,67] which reduces oxidative stress in the organism and may produce quite contrary effects on ARE enzymes in peripheral organs.

Systemic effects of GRA on the induction of ARE-regulated genes. In contrast to local GRA effects the induction of ARE-regulated genes in the liver seems to depend largely on sufficient GRA cleavage by myrosinase or bacterial β-glucosidases followed by sulforaphane disposal into mesenterial blood.

This hypothesis is confirmed by data of a rat study. In this trial a distinct increase in the expression of a large number of liver phase II enzymes was induced by the application of 50 μmol pure sulforaphane per rat for two times.[64] In this study a 2.2-fold increase in liver CBS expression was also achieved by sulforaphane application. Potentially in this study tHCys values would have responded to the high sulforaphane dose.

A very recent study with human lung cells confirmed our results for the liver. The incubation of these cells with low doses of pure sulforaphane (1 and 2 μmol L^{-1}) caused a distinct up-regulation of NQO1 mRNA expression for 2 days. Interestingly, after 6 days NQO1 mRNA levels drastically dropped even below that in the DMSO treated control group, whereas the protein level was kept up-regulated.[68] In our study we could observe a similar effect for GSTA3 expression and activity in group G15. In accordance with the above mentioned study,[68] in our trial the mRNA levels of many ARE genes were also lower in GRA rats than in the respective C groups. These particular changes in ARE genes were most obvious when Se deficient C15 rats were compared to their GRA supplemented companions of group G15. Nevertheless, gene expression levels of some ARE-regulated genes (*e.g.* GCLM, GS, HO1) were also lower in the Se- and GRA-supplemented groups (G50, G150 and G450) than in the respective C groups.

The observed changes in ARE gene expression were also reflected by KEAP1 (Fig. 4A). Both Se supplementation and in particular GRA supplementation reduced KEAP1 expression. KEAP1 modification at sensitive –SH groups by oxidative stress or electrophilic compounds like GRA or sulforaphane[36,38,39] leads to Nrf2 liberation, its nuclear translocation and the induction of phase II enzymes.[37,56] Latest results have revealed that KEAP1 *per se* is regulated *via* an ARE.[55] Thus the reduced expression of KEAP1 by Se and long-term GRA may indicate that GRA treated rats (in particular C15 *vs.* G15) had reduced oxidative stress in their livers. Interestingly, Nrf2 expression responded with a nearly constant or a slightly increased mRNA- and protein-expression due to Se and GRA supplementation (Fig. 4A, B). This would implicate a higher nuclear Nrf2 translocation and contradicts the down-regulation of ARE genes by Se and GRA. Potentially, the up-regulation of Nrf2 due to reduced KEAP1 expression represents a counter regulatory mechanism. However, this aspect needs further intensive investigation with regard to nuclear Nrf2 translocation by immunoblotting or EMSA.

Balanced regulation of phase I, phase II and phase III enzymes by GRA. Besides phase II enzymes, a number of phase I and phase

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III enzymes are also regulated by the cellular redox status. A balanced regulation of all three phases of xenobiotic metabolism is necessary to prevent the organism from damage. In this context a controversial discussion about the influence of GRA or sulforaphane on the expression of the phase I cytochrome P450 oxygenases has come up. Whereas a number of studies have shown a reduction of phase I enzymes and an induction of phase II enzymes by GRA or SFN,[69–71] results of other investigators clearly demonstrated an up-regulation of phase I and II enzymes by these substances.[38,72–74] The latter constellation would have rather negative consequences for the organism, in particular when phase I enzymes are up-regulated to a larger extent than phase II enzymes.

In our study we could show that CYP1A1 was down-regulated by Se supplementation and even more by additional long-term GRA supplementation (Fig. 3A). As mentioned above, potentially the down-regulation of GNMT contributes to this effect.[62] Moreover we could measure a reduction of phase II and also of phase III enzymes (MRP4) by the applied dietary regimes. In our study CYP1A1 was down-regulated to a higher extent than some phase II and III enzymes (GCLM, GS, HO1,MRP4) (Table 3 and Fig. 3C) or down-regulation was nearly comparable (GSTA3, NQO1) (Table 2 and Fig. 3B). Therefore our results suggest that Se supplementation, in particular with combined long-term low dose GRA supply, reduces oxidative stress in the liver of rats, resulting in a balanced down-regulation of phase I, II and III enzymes.

Conclusions and perspectives

Conclusions

We have hypothesised that long-term GRA supplementation at a level, corresponding to a realistic uptake in humans, lowers plasma tHCys, which is higher in Se supplemented rats than in Se deficient. Moreover, we assumed that GRA reduces plasma tHCys by a pull on the transsulphuration pathway *via* the induction of ARE-regulated enzymes of GSH biosynthesis. As expected, Se impressively reduced the expression of GSH biosynthesis enzymes and that of other ARE-regulated genes. As a consequence, plasma tHCy levels were higher in Se supplemented rats than in Se deficient.

In contrast to our hypothesis GRA did not counteract the Se dependent mRNA decrease of ARE genes, but reduced their mRNA in some cases to a higher extent than Se alone. Under the conditions tested we could not establish GRA as a HCys lowering compound.

We conclude:

- That GRA at the dietary level tested (~13 μmol per rat and day) reduces oxidative stress in the liver *via* increasing the intestinal barrier against oxidative stress. As a consequence the organism responds rather with a down-regulation of ARE genes than with an up-regulation.
- That an up-regulation of ARE-regulated genes in peripheral organs, like the liver, largely seems to depend on GRA cleavage to free sulforaphane and glucose by plant-derived myrosinase or bacterial β-glucosidases.
- That the GRA amount tested in our study was too low for the production of a sulforaphane amount (~50 μmol per rat and day) sufficient for the up-regulation of ARE genes also in the liver. As a consequence the reduction of plasma tHCys failed.

Perspectives

- In future studies dose-response experiments starting with distinctly higher GRA concentrations should be carried out to examine if these higher GRA concentrations influence the mRNA of ARE genes also in the liver.
- In addition to the above mentioned dose-response, experiments with animal models with defects in HCys metabolism or with dietary-induced hyperhomocyteinemia should be used to test if GRA or sulforaphane can be established as complementary HCys lowering compounds, supporting the conventional therapy with vitamins B6, B9 and B12.

Abbreviations

BHMT	Betaine homocysteine methyl transferase
CBS	Cystathionine beta synthase
tGSH	total glutathione
tHCys	total homocysteine
GNMT	Glycine-*N*-methyl transferase
GPx1	Glutathione peroxidase 1
GPx3	Glutathione peroxidase 3
GCLM	Glutamate cysteine ligase (modifier subunit)
GS	Glutathione synthetase
KEAP1	Kelch-like ECH-associated protein 1
MRP4 = ABCG4	Multidrug resistance protein 4 = ATP-binding cassette transporter 4
MTR	5-methyltatrahydrofolate-homocysteine methyltransferase
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
Slco1a4	solute carrier organic anion transporter family (member 1a4)
Cyp1A1	Cytochrome P450 member 1A1
NQO1	NAD(P)H dehydrogenase [quinone] 1
HO1	Heme oxygenase 1

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Feeding of Selenium Alone or in Combination with Glucoraphanin Differentially Affects Intestinal and Hepatic Antioxidant and Phase II Enzymes in Growing Rats

Nicole M. Blum · Kristin Mueller · Doris Lippmann · Cornelia C. Metges · Thomas Linn · Josef Pallauf · Andreas S. Mueller

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Abstract The anti-carcinogenic effects of sulforaphane (SFN) are based on the up-regulation of antioxidant enzymes (AE) and phase II enzymes (PIIE) through the transcription factor Nrf2. Current knowledge on the roles of the SFN precursor glucoraphanin (GRA) on these processes is limited. Anti-carcinogenic effects of Se depending on glutathione peroxidase (GPx) activity have also been reported. We studied effects and possible synergisms of Se and GRA on the expression and activity of a broad spectrum of AE and PIIE in jejunum, colon and the liver of rats fed diets differing in Se and GRA concentration. In all organs, GPx1 mRNA expression was 70 % to 90 % lower in Se deficiency than in Se sufficiency. GPx2 expression increased in jejunum and liver under Se deficiency and decreased in the colon. Se deficiency increased most colonic AE and PIIE compared to Se adequacy. Adequate and in particular supranutritive Se combined with GRA increased colonic AE and PIIE expression up to 3.72-fold. In the liver Se deficiency raised the expression of AE and PIIE up to 4.49-fold. GRA attenuated liver AE and PIIE response in Se deficiency. Expression- and correlation analyses revealed that Keap1 mRNA better reflects AE and PIIE gene expression than Nrf2 mRNA. We conclude that: (1) GPx1 sensitively indicates Se deficiency; (2) the influence of Se and Nrf2/Keap1 on GPx2 expression depends on the organ; (3) GRA combined with supranutritive Se may effectively protect against inflammation and colon cancer; (4) future investigations on AE and PIIE expression should consider the role of Keap1 to a higher extent.

Keywords Selenium · Glucoraphanin · Antioxidant enzymes · Phase II enzymes · Cancer prevention · Inflammation markers

N. M. Blum · K. Mueller · A. S. Mueller (✉)
Institute of Agricultural and Nutritional Sciences,
Preventive Nutrition Group, Martin Luther University Halle Wittenberg, Von Danckelmann Platz 2,
06120 Halle (Saale), Germany
e-mail: andreas.mueller@landw.uni-halle.de

D. Lippmann
"Department Biochemistry of Micronutrients",
German Institute of Human Nutrition Potsdam-Rehbruecke,
Arthur-Scheunert-Allee 114-116,
14558 Nuthetal, Germany

C. C. Metges
Leibniz Institute for Farm Animal Biology, Research Unit Nutritional Physiology "Oskar Kellner", Wilhelm Stahl Allee 2,
18196 Dummerstorf, Germany

T. Linn
Medical Clinic and Policlinic 3, Endocrinology and Diabetes,
Justus Liebig University Giessen, Klinikstraße 33,
5392 Giessen, Germany

J. Pallauf
Interdisciplinary Research Centre, Institute of Animal Nutrition and Nutritional Physiology, Justus Liebig University Giessen,
Heinrich Buff Ring 26-32,
35392 Giessen, Germany

Abbreviations

AE	Antioxidant enzymes
ARE	Antioxidant response element
COX	Cyclooxygenase
EPHX1	Microsomal epoxide hydrolase
GPx	Glutathione peroxidase

GRA	Glucoraphanin
GST	Glutathione *S*-transferase
HO1	Heme oxygenase 1
iNOS	Inducible nitric oxide synthase
Keap1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharide
NQO1	NAD(P)H:quinine oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
PIIE	Phase II enzymes
Se	Selenium
SFN	Sulforaphane
TNFα	Tumor necrosis factor-alpha
UGT	UDP-glucuronosyltransferase
VCAM1	Vascular cell adhesion molecule 1

Introduction

A permanent exposure to exogenous and endogenous xenobiotics increases cancer risk. In particular, the intestine and the liver represent sites involved in the entry and the metabolism of xenobiotics [1]. The detoxification of xenobiotics is also referred to as biotransformation, which is divided into three phases. Cytochrome P450 oxidases represent one of the most important classes of phase I enzymes since they introduce oxygen-containing functional groups into xenobiotics [2–4], whereas the resulting products of the phase I reaction are inert metabolites in most cases, other metabolites represent highly reactive molecules (e.g., electrophiles like epoxides) [4]. Phase II of biotransformation primarily catalyses the conjugation of the phase I reaction products, and therefore it produces easily excretable molecules or inactive forms of pharmacological active substances [2, 3]. Finally, in phase III the excretion of the hydrophilic products from the phase II reactions is expedited by transport proteins, like members of the multidrug resistance family (MDR) which are located at excretion sites (bile and kidney). UDP-glucuronosyltransferases (UGT) represent one important family of phase II enzyme (PIIE). In humans, about 40–70 % of all pharmaceuticals are metabolised by glucuronidation reactions through UGT activity [2]. UGT1a6 is preferentially involved in the conjugation of complex phenols and primary amines [2]. Glutathione *S*-transferases (GST) belong to another very important class of PIIE. They facilitate xenobiotic excretion by conjugating them with the cysteine sulphur atom of reduced glutathione [2, 3]. In addition, GST possess antioxidative effects through unrolling a peroxidase activity towards organic hydroperoxides [3]. GST can be divided into three major families: (1) soluble cytosolic GST, (2) mitochondrial GST, and (3) structurally distinct membrane-bound microsomal GST [2, 5, 6]. Within GST, cytosolic GST represents the largest family which again can be divided into seven subclasses: (1) Alpha (A), (2) Mu (M), (3) Pi (P), (4) Sigma (S), (5) Theta (T), (6) Zeta (Z), and (7) Omega (O) [3, 7]. GSTK is the mammalian mitochondrial GST. Mice, rats and humans express only GSTK1 which is also a dimeric enzyme. GSTK1 has a high substrate affinity towards aryl halides like 1-chloro-2,4-dinitrobenzene (CDNB). Additionally, cumene hydroxide is a high-affinity substrate for GSTK1 [7]. Several polymorphisms in the genes of GST A, M, P, and T have been associated with an increased risk of developing colorectal, bladder, breast, head/neck and lung cancers [2]. Both UGT and a number of GST have a so-called antioxidant response element (ARE) in their genes' promoter region. For the transcriptional up-regulation of AE and PIIE the transcription factor "nuclear factor erythroid 2-related factor 2" (Nrf2) is needed [3, 8]. Under balanced pro- and-antioxidant conditions in cells, Nrf2 is bound to a dimer of Kelch-like ECH-associated protein 1 (Keap1) in the cytosol through a high affinity motif (ETGE) and a low affinity motif (DLG). Linkage of Nrf2 to Keap1 initiates rapid Nrf2 degradation via the ubiquitin–proteasome pathway. Prooxidants or electrophilic compounds are able to modify sensitive cysteine sulphydryl groups of Keap1. As a consequence, the DLG bond is disrupted and ubiquitination and proteasomal Nrf2 degradation are inhibited. Once Keap1 is saturated with undegradated Nrf2, Nrf2 protein translation increases. Hence, the pool of free Nrf2 expands, and Nrf2 can translocate into the nucleus and bind to the ARE sequence in the promoter of AE and PIIE [8, 9]. Besides the mentioned UGT and GST enzymes, the AE glutathione reductase, heme oxygenase 1 (HO1), microsomal epoxide hydrolase (EPHX1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) are further important ARE-containing and Nrf2-regulated genes [8]. The selenoenzyme glutathione peroxidase 2 (GPX2) is another and unusual Nrf2 target gene [10]. In recent years, the isothiocyanate sulforaphane (SFN) has been characterised as a potent inducer of Nrf2-regulated genes [11, 12]. Several cruciferous vegetables, but in particular broccoli sprouts, are a very rich source of SFN. However, in plants SFN is found in S-cells in form of its glucosinolate precursor glucoraphanin (GRA). GRA cleavage to SFN and glucose is catalysed by β-thioglucoside glucohydrolases (myrosinase). GRA cleavage is initiated when the myrosinase, also contained in cruciferous vegetables, is liberated from vacuoles of particular idioblasts of the plants due to chewing or chopping. GRA derived from cooked vegetables with almost heat inactivated myrosinase or GRA from dietary supplements can be hydrolysed by β-glucosidases of gut bacteria [13, 14]. Results of a recent study have shown that GRA is cleaved to SFN and glucose in the caecum of rats and that the liberated SFN was absorbed into blood by ceacal enterocytes [13]. Latest studies, in which liver and lung slices of rats were incubated

with GRA, revealed that the SFN precursor itself is a potent inducer of Nrf2-regulated enzymes [15, 16]. Moreover Nrf2-regulated AE and PIIE are also influenced by Se status. Several studies have shown that Se deficiency [17–21] as well as large doses of several Se compounds [22–24] induce Nrf2 target genes like GST, NQO1 and HO1.

Nrf2 does not only have an important impact on AE and PIIE, but it also possesses an influence on inflammatory processes. This particular effect results from the Nrf2-dependent down-regulation of inflammation mediators like tumor necrosis factor-alpha (TNFα), vascular cell adhesion molecule 1 (VCAM1), monocyte chemoattractant protein 1 (MCP1) and cyclooxygenase 2 (COX2) [25, 26]. COX is the rate-limiting enzyme in prostaglandin biosynthesis, and COX2, the inducible COX form, is up-regulated due to cell injury and inflammation [27]. In peritoneal macrophages with lipopolysaccharide (LPS) induced inflammation SFN treatment has been shown to reduce inflammation by decreasing COX2 and inducible nitric oxide synthase (iNOS) protein expression [28]. In a mouse study it has been shown that SFN and Se interact with regard to their anti-inflammatory properties. SFN unfolded proinflammatory effects under Se restriction, but it potently inhibited acute inflammation under Se adequate conditions [29].

Consequently, the first objective of our present study was to investigate the effects of feeding the SFN precursor GRA to rats on Nrf2 and Nrf2-regulated genes in different intestinal segments and in the liver. The second main goal of our study was to examine interactions between GRA and Se regarding the expression and activity of Nrf2-target genes. For this purpose diets with different Se concentrations with or without added GRA were fed to growing rats for 8 weeks.

Experimental

Animals and Diets

A total of 72 healthy weaned male albino rats (initial body weight: 71.9±0.26 g) from the Institute's own strain HK51 (Interdisciplinary Research Center Giessen University, Institute of Animal Nutrition and Nutrition Physiology) were randomly assigned to six groups of 12 (C150, G150, C15, G15, C450 and G450). The rats were fed diets differing in Se and GRA content. The Se-deficient basal diet of group C15 was based on Torula yeast and Se-deficient wheat as described previously [30]. The diets of two of the Se supplemented groups contained Se at the recommended level (150 µg Se/kg diet, group C150) or at a slightly supranutritive concentration (450 µg Se/kg, group C450). The diets of the remaining three experimental groups had identical Se supplements like the C groups, but they were additionally supplemented with 700 µmol GRA/kg diet (G15, G150 and G450). For Se addition to the diets, sodium selenate was used. The GRA supplementation was realised by adding a natural broccoli sprouts extract (Jarrow-Formulas®) to the diets which has a standardised GRA concentration of 10 % (w/w). The rats were kept individually and had ad libitum access to their diet and water. After 8 weeks of feeding the rats were decapitated under CO_2 anesthesia. Liver, jejunum and colon were excised, washed with sterile physiological sodium chloride solution, frozen in liquid nitrogen and stored at −80 °C until further analysis. All experiments with living rats were performed according to the German Animal Welfare Act. The protocol of this study was approved by the Regional Council of Giessen (Germany) and by the Animal Welfare Committee of the Justus Liebig University Giessen (Germany) [record token: V54-19c10/15cGI 19/3; 39-2008A].

Activity of Glutathione *S*-Transferase Alpha and Pi

The activity of glutathione *S*-transferase *Alpha (GSTA class)*, was analysed spectralphotometrically (Ultrospec 3300 pro; Amersham Pharmacia Biotech, Freiburg, Germany) in the cytosol of liver, jejunum and colon, using the specific substrate 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) following the method of Ricci et al. [31]. 1:5 (w/v) crude homogenates of liver, jejunum and colon were prepared in potassium phosphate buffer (0.1 M; pH 6.5) and centrifuged for 30 min at 13,000×*g* and 2 °C. The supernatant was dialysed over night in order to remove glutathione. 75 µl of the dialysate were used for the assay and mixed with 622.5 µl sodium acetate buffer (0.1 M; pH 5.0) containing 37.5 µl NBD-Cl (4 mM in ethanol) and 15 µl reduced glutathione (25 mM in potassium phosphate buffer). The increase in extinction, due to formation of the conjugation product 7-glutathionyl-4-nitrobenzo-2-oxa-1,3-diazole (NBD-SG) catalysed by GSTA class, was recorded at 419 nm for 2 min. One unit of GSTA activity was defined as 1 µmol NBD-SG formed per minute. GST A class activity was normalised to 1 mg protein.

The activity of GST P class was measured according to the protocol of Habig and Jakoby [32], using ethacrynic acid as the substrate. In a 96-well plate 140 µl potassium phosphate buffer (0.1 M; pH 6.5), 20 µl reduced glutathione (2.5 mM), 20 µl ethacrynic acid (2 mM in 96 % ethanol) and 20 µl dialysed cytosolic supernatant were mixed. The ethacrynate–glutathione conjugate formed by GST P activity was measured for 2 min at 270 nm using the plate reader Tecan SpectraFluor Plus (Tecan, Grödig, Austria). One unit of GSTP activity was defined as 1 µmol ethacrynate–glutathione conjugate per minute. GSTP activity was normalised to 1 mg protein.

Activity of GPx

Due to the lack of a specific substrate, the combined activity of GPx1 and 2 was analysed in jejunum, colon and liver according to the method of Lawrence and Burk [33] as described previously [34].

NQO1 Activity

NQO1 activity was analysed using the method originally described by Prochaska and Santamaria [35] with modifications [17], which is based on the NADPH-dependent menadione-mediated reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Therefore 1:20 (w/v) lysates of the tissues were prepared in a homogenisation buffer (100 mM Tris–HCl, 300 mM KCl, 0.01 % Triton X-100; pH 7.6). For the assay 3 µl lysate were mixed with 50 µl water or dicoumarol solution (0.3 mM dicoumarol, 0.5 % DMSO, 5 mM potassium phosphate buffer, pH 7.4) in a 96-well plate. Then 190 µl reaction buffer (25 mM Tris–HCl; pH 7.4, 0.665 mg/ml BSA, 0.01 % Tween 20, 5 µM FAD, 1 mM glucose-6-phosphate, 30 µM NADP, 0.72 mM MTT, 0.3 U/ml glucose-6-phosphate dehydrogenase, 50 µM menadione) were added. The reduction of MTT was measured for 5 min at 590 nm in a plate reader (Tecan SpectraFluor Plus; Tecan). NQO1 activity was calculated by subtracting the background activity, which was determined by addition of the specific NQO1-inhibitor dicoumarol. One unit of NQO1 activity was defined as 1 µmol reduced MTT per minute. NQO1 activity was normalised to 1 mg of protein.

Protein Concentration of Samples

Protein concentration of liver, jejunum and colon lysates was determined in microtiter plates according to a standard protocol [36] using a plate reader (Tecan SpectraFluor Plus; Tecan).

Real-Time RT-PCR Analysis of ARE-Regulated Antioxidant and PIIE in Liver, Jejunum and Colon of Rats, and of Colonic Inflammation Markers

RNA of liver, jejunum and colon was extracted using the acid guanidinium thiocyanate–phenol–chloroform extraction method [37]. Reverse transcription of 3.0 µg of total RNA and real-time RT-PCR were performed as described previously [34]. For real-time RT-PCR analysis the cDNA of two rats per group was pooled (6 cDNA pools per group). Gene bank accession numbers and primer sequences (5′→3′) are shown in Table 1. Amplification data were analysed with the Rotor-Gene 6000™ series software using the $\Delta\Delta$Ct method [38]. The expression of the single genes was normalised to β-actin expression. Prior to this, a ranking of expression stability was performed for different housekeeping genes [39] and revealed β-actin as being the most stable gene in liver, jejunum and colon. Relative mRNA expression levels are expressed as x-fold changes relative to group C150=1.0.

Immunoblot Analysis of Nrf2

For analysis of Nrf2 protein expression whole colon and liver tissue lysates (1:10 w/v) were prepared from six rats per group, representing the mean body weight of their group, in a non-reducing homogenisation buffer (TRIS 50 mM, NaCl 150 mM, phenylmethylsulfonylfluoride 0.5 mM; pH 7.4). 35 µg of protein were separated under non-reducing conditions on 10 % SDS-polyacrylamide gels following the standard method [40]. Blotting, blocking and treatment with antibodies [monoclonal Nrf2 antibody (R&D Systems; MAB3925); secondary antibody linked to alkaline phosphatase (Goat Anti-Mouse IgG-h+I)] were performed as described previously [34]. Optical density of the 70 kDa Nrf2 band was evaluated (Gene Tools, Syngene) on scanned membranes. The intensity of the Nrf2 bands was normalised to β-actin expression in the single samples. For the immunoblots colon and liver of six animals were analysed.

Statistical Analysis

Data are given as means±their standard error of mean (SEM). Statistical differences were analysed with SPSS 19.0 for Windows (IBM, Chicago, IL, USA) using the one-way ANOVA procedure after ascertaining the normality of distribution (Kolmogorov–Smirnov test and Shapiro–Wilk test) and the homogeneity of variances (Levene test). If the variances were homogenous, the least significant difference (LSD) test was used to analyse significant differences between means, if not the Games–Howell test was used. Differences between means were considered as significant at $p<0.05$. Pearson correlations and their significance level were also analysed with SPSS 19.0 for Windows using the correlation mode.

Results

mRNA Expression of Nrf2 and Keap1 in Jejunum, Colon and Liver and Protein Expression of Nrf2 in Colon and Liver

Compared with C150 rats jejunal Nrf2 mRNA was neither influenced by varying dietary Se concentration nor by GRA supply (Table 2). With the exception of group C450, jejunal Keap1 expression was significantly lower in all experimental groups (G150, C15, G15, G450) than in C150 rats.

Table 1 Primer sequences and gene bank accession numbers of the genes investigated by real-time RT-PCR

Gene name (abbreviation used)	Gene bank accession number	Primer sequences (5′ → 3′)
Chemokine (C–C motif) ligand 2 (Ccl2) (MCP1)	NM_031530	For: GTGCGACCCCAATAAGGAA Rev: TGAGGTGGTTGTGGAAAAGA
Epoxide hydrolase 1 (Ephx1)	NM_001034090	For: GGCTACTCAGAGGCATCCAG Rev: TTGGTGGCTTGGATGTGTAA
Glutathione *S*-transferase A3 (GSTA3)	NM_031509	For: For: GGGGAGAAAGAGGCAAGTCT Rev: CTTCAGCAGAGGGAAGTTGG
Glutathione *S*-transferase K1 (GSTK1)	NM_181371	For: GAGCATGGAGCAACCAGAGAT Rev: AGCTTGCTCTTCACCAGTTCG
Glutathione *S*-transferase M5 (GSTM5)	NM_172038	For: TCACCCAGAGTAACGCCATCT Rev: TACTGAGGCTTCAGGCTTTCG
Glutathione *S*-transferase O1 (GSTO1)	NM_001007602	For: TGCCGTCTCTGGTTACGAGTT Rev: GAGCTTGAGTTTTGGGGTGTG
Glutathione *S*-transferase P1 (GSTP1)	NM_012577	For: GAGGCAAAGCTTTCATTGTGG Rev: GTTGATGGGACGGTTCAAATG
Glutathione *S*-transferase T1 (GSTT1)	NM_053293	For: TGATGCATCCTGTAGGTGGTG Rev: TTTGCTTTATGACGGGGTCAG
Glutathione *S*-transferase T2 (GSTT2)	NM_012796	For: GAGGAAAAGGTGGAACGGAAC Rev: CGCCCCTCAAACAGATTACAG
Glutathione peroxidase 1 (GPx1)	NM_030826	For: TCATTGAGAATGTCGCGTCT Rev: CCCACCAGGAACTTCTCAAA
Glutathione peroxidase 2 (GPx2)	NM_183402	For: GTGTGATGTCAATGGGCAGAA Rev: ACGTTTGATGTCAGGCTCGAT
Heme oxygenase 1 (HO1)	NM_012580	For: AGGCACTGCTGACAGAGGAAC Rev: AGCGGTGTCTGGGATGAACTA
Kelch-like ECH-associated protein1 (Keap1)	NM_057152	For: GTGGCGGATGATTACACCAAT Rev: GAAAAGTGTGGCCATCGTAGC
NAD(P)H dehydrogenase [quinone] 1 (NQO1)	NM_017000	For: CGCAGAGAGGACATCATTCA Rev: CGCCAGAGATGACTCAACAG
Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)	NM_031789	For: CCAAGGAGCAATTCAACGAAG Rev: CTCTTGGGAACAAGGAACACG
Prostaglandin-endoperoxide synthase 1 (Ptgs1) (COX1)	NM_017043	For: CCATGGAATTCAACCACCTC Rev: AGTTCCTACCCCCACCAATC
Prostaglandin-endoperoxide synthase 2 (Ptgs2) (COX2)	NM_017232	For: GCTGTACAAGCAGTGGCAAA Rev: CCCCAAAGACAGCATCTGGA
UDP-glucuronosyltransferase 1A6 (UGT1A6)	NM_001039691	For: GTGGAGCACCTCAGTGAACG Rev: CAGCAAAGTGGTTGTTCCCA
β-Actin	NM_031144	For: ATCGTGCGTGACATTAAAGAGAAG Rev: GGACAGTGAGGCCAGGATAGAG

For forward, *Rev* reverse

In the colon, GRA increased Nrf2 mRNA abundance 1.48- to 2.80-fold compared to C150 rats independent from Se content. The effect of Se (groups C15 and C450) was indifferent and not significant. With the exception of group C450 colonic Nrf2 protein expression, as analysed by immunoblotting, approximately reflected the data on mRNA regulation (Fig. 1a). In the colon, Keap1 mRNA levels were not significantly affected by Se supplementation. GRA (groups G15, G150, G450) increased colonic Keap1 mRNA expression 1.27- to 1.87-fold compared to C150 rats.

Independent of GRA supply hepatic Nrf2 mRNA levels tended to be reduced by Se deficiency (C15 and G15) compared with Se supplementation at the recommended or at the slightly supranutritive level (C150 C450, G150, G450). These results could be confirmed by immunoblotting (Fig. 1b).

Table 2 Relative mRNA Expression of Nrf2, Keap1, GPx1, and ARE-regulated antioxidant and phase II enzymes in the jejunum of growing rats fed diets supplemented with three different dietary Se concentrations (50,150 and 450 μgkg^{-1}) either without or with addition of 700 μmol GRA kg (diet)$^{-1}$

	C 150	G150	C15	G15	C450	G450
Nrf2	1.00±0.07^{a}	0.99±0.09^{a}	0.94±0.06^{a}	0.86±0.14^{a}	1.18±0.17^{a}	0.94±0.07^{a}
Keap1	1.00±0.04^{a}	0.80±0.07^{b}	0.79±0.03^{b}	0.71±0.08^{b}	1.01±0.08^{a}	0.79±0.03^{b}
GPx1	1.00±0.14^{a}	0.33±0.05^{b}	0.34±0.05^{b}	0.23±0.03^{b}	1.09±0.16^{a}	1.42±0.36^{a}
GPx2	1.00±0.04^{ac}	1.30±0.13^{b}	1.35±0.08^{b}	0.77±0.08^{a}	1.14±0.18^{bc}	1.05±0.08^{ab}
EPHX	1.00±0.09^{ab}	0.71±0.08^{b}	1.24±0.09^{a}	0.79±0.10^{ab}	0.97±0.21^{ab}	0.82±0.03^{b}
HO1	1.00±0.06^{a}	0.91±0.05^{ab}	0.96±0.08^{a}	0.72±0.05^{b}	1.01±0.10^{a}	0.78±0.08^{ab}
NQO1	1.00±0.06^{a}	1.02±0.08^{a}	1.09±0.08^{a}	0.83±0.10^{a}	1.22±0.24^{a}	0.98±0.03^{a}
UGT1a6	1.00±0.08^{a}	0.77±0.04^{a}	0.97±0.08^{a}	0.96±0.19^{a}	0.95±0.19^{a}	0.82±0.06^{a}
GSTA3	1.00±0.05^{a}	0.71±0.04^{b}	0.63±0.12^{b}	0.68±0.09^{b}	1.02±0.09^{a}	0.81±0.09^{ab}
GSTK1	1.00±0.06^{ad}	0.75±0.06^{bc}	0.89±0.04^{ab}	0.68±0.07^{c}	1.02±0.12^{a}	0.82±0.01^{bcd}
GSTM5	1.00±0.09^{a}	0.98±0.09^{a}	1.01±0.06^{a}	0.75±0.13^{a}	1.02±0.23^{a}	0.87±0.03^{a}
GSTO1	1.00±0.06^{a}	0.86±0.06^{ab}	0.86±0.05^{ab}	0.68±0.07^{b}	0.91±0.09^{a}	0.80±0.02^{ab}
GSTP1	1.00±0.11^{ac}	1.61±0.16^{b}	0.92±0.07^{a}	0.95±0.10^{a}	1.15±0.22^{ac}	1.35±0.10^{bc}
GSTT1	1.00±0.05^{a}	0.94±0.05^{a}	0.95±0.04^{a}	0.78±0.10^{a}	1.09±0.17^{a}	0.64±0.15^{a}
GSTT2	1.00±0.09^{a}	0.74±0.04^{ab}	0.84±0.03^{ab}	0.55±0.07^{b}	0.94±0.17^{ab}	0.64±0.07^{ab}

Values are means±SEM and represent relative mRNA concentrations as n-fold of group C150=1. Different small letters in a row indicate significant differences between means ($p \leq 0.05$). n=6 cDNA pools of two rats per experimental group

Relative Keap1 mRNA concentration was the highest in the Se-deficient group C15, and it differed significantly from all other groups. Under Se deficiency, GRA significantly reduced hepatic Keap1 expression nearly to the level in C150 rats. Supranutritive Se supply in combination with dietary GRA (group 450) reduced Keap1 mRNA abundance ($p<0.10$) in tendency compared to the C150 control rats.

mRNA Expression of Various ARE Containing Antioxidant and PIIE in Jejunum, Colon and Liver

The expression patterns of the analysed relative mRNA concentrations of Nrf2-regulated genes differed among the tissues investigated (Tables 2, 3 and 4).

In groups C15 and G15 the strong down-regulation of jejunal cellular GPx1 mRNA by about 70 % sensitively indicated Se deficiency compared to C150 rats. Most interestingly, at the recommended dietary Se level the GRA supplement (G150) decreased GPx1 expression compared to C150 rats whereas GRA in combination with supranutritive Se effectuated an increase. In sharp contrast, jejunal gastrointestinal GPx2 expression in G150 rats was significantly higher than in group C150. Compared to C150 rats jejunal GPx2 expression was increased in Se-deficient C15 rats whereas the GRA supplement reduced the enzymes' mRNA. All other dietary regimes had no significant influence on jejunal GPx2 mRNA abundance.

In the jejunum, UGT1a6 mRNA was neither affected by Se nor GRA. Accordingly also the relative mRNA concentration of NQO1 was not affected by the different dietary regimes. HO1 was also not influenced by varying Se concentration. Additional GRA decreased the relative mRNA concentration in groups G15 (p=0.039) and G450 (p=0.068) by 22–28 % compared to their corresponding C groups (C15 and C450). The mRNA level of EPHX1 did not differ among the C groups, but the levels of all G groups were about 18–29 % lower than that of the corresponding C groups. This reduction was significant for groups G150 and G450. In the jejunum also the different GST classes showed an individual and partially heterogenous expression profile. The results can be summarised in brief as follows: Supranutritive Se (C450) had no influence on the mRNA

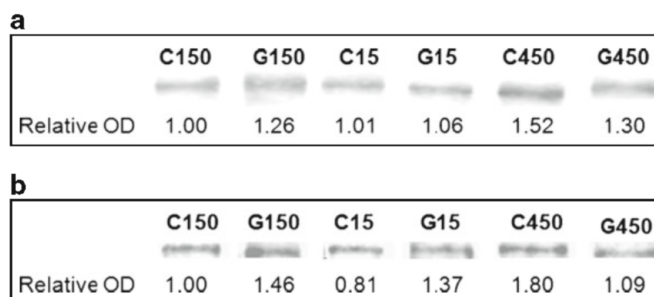

Fig. 1 Protein expression of Nrf2 in whole tissue lysates of colon (**a**) and liver (**b**). For immunoblot analysis, the liver and colon samples of six rats per group, representing the mean body weight of their group, were used. In this figure, one representative immunoblot is shown. The relative optical densities (*OD*) were normalised to β-actin

Table 3 Relative mRNA Expression of Nrf2, Keap1, GPx1, and ARE-regulated antioxidant and phase II enzymes in the colon of rats fed diets supplemented with three different dietary Se concentrations (50, 150 and 450 $\mu g kg^{-1}$) either without or with addition of 700 μmol GRA kg $(diet)^{-1}$

	C 150	G150	C15	G15	C450	G450
Nrf2	1.00 ± 0.12^{ac}	2.38 ± 0.16^{b}	0.78 ± 0.08^{a}	1.48 ± 0.10^{c}	1.28 ± 0.11^{ac}	2.80 ± 0.35^{b}
Keap1	1.00 ± 0.07^{a}	1.27 ± 0.09^{a}	1.08 ± 0.13^{a}	1.36 ± 0.22^{ab}	0.95 ± 0.10^{a}	1.87 ± 0.29^{b}
GPx1	1.00 ± 0.08^{a}	0.83 ± 0.08^{a}	0.16 ± 0.02^{b}	0.20 ± 0.02^{b}	0.85 ± 0.05^{a}	1.02 ± 0.08^{a}
GPx2	1.00 ± 0.14^{ab}	1.42 ± 0.22^{ac}	0.53 ± 0.14^{b}	0.95 ± 0.05^{ab}	1.17 ± 0.03^{ac}	1.72 ± 0.14^{c}
EPHX1	1.00 ± 0.21^{a}	2.03 ± 0.22^{b}	1.48 ± 0.11^{ab}	1.86 ± 0.22^{bc}	1.21 ± 0.14^{ac}	2.70 ± 0.30^{d}
HO1	1.00 ± 0.13^{a}	1.41 ± 0.35^{ab}	1.33 ± 0.24^{a}	1.46 ± 0.15^{ab}	0.82 ± 0.11^{a}	2.12 ± 0.24^{b}
NQO1	1.00 ± 0.16^{a}	2.22 ± 0.39^{b}	1.59 ± 0.30^{ab}	1.36 ± 0.22^{ab}	1.29 ± 0.25^{a}	3.40 ± 0.29^{c}
UGT1a6	1.00 ± 0.22^{ac}	2.53 ± 0.57^{bd}	2.26 ± 0.5^{ab}	1.32 ± 0.26^{abc}	0.81 ± 0.32^{c}	3.72 ± 0.70^{d}
GSTA3	1.00 ± 0.13^{a}	0.93 ± 0.10^{a}	1.28 ± 0.22^{a}	1.24 ± 0.16^{a}	0.89 ± 0.17^{a}	0.83 ± 0.21^{a}
GSTK1	1.00 ± 0.14^{a}	2.88 ± 0.29^{b}	1.30 ± 0.26^{ac}	1.90 ± 0.13^{c}	1.38 ± 0.15^{ac}	3.54 ± 0.32^{b}
GSTM5	1.00 ± 0.19^{a}	1.53 ± 0.11^{ab}	1.08 ± 0.21^{a}	1.11 ± 0.21^{a}	1.22 ± 0.13^{ab}	1.82 ± 0.25^{b}
GSTO1	1.00 ± 0.04^{a}	2.12 ± 0.24^{b}	1.32 ± 0.14^{a}	1.25 ± 0.14^{a}	0.94 ± 0.13^{a}	2.83 ± 0.26^{c}
GSTP1	1.00 ± 0.13^{a}	2.36 ± 0.29^{b}	1.13 ± 0.19^{a}	1.66 ± 0.11^{ab}	1.97 ± 0.33^{b}	3.36 ± 0.28^{c}
GSTT1	1.00 ± 0.16^{ac}	1.12 ± 0.15^{abc}	1.60 ± 0.19^{ab}	1.75 ± 0.39^{b}	0.83 ± 0.12^{c}	1.72 ± 0.26^{b}
GSTT2	1.00 ± 0.13^{a}	1.77 ± 0.24^{bc}	1.27 ± 0.20^{ab}	1.85 ± 0.31^{abc}	1.08 ± 0.08^{ab}	2.31 ± 0.40^{c}

Values are means±SEM and represent relative mRNA concentrations as *n*-fold of group C150=1. Different small letters in a row indicate significant differences between means ($p \leq 0.05$). *n*=6 cDNA pools of two rats per experimental group

expression of any GST. With the exception of GSTP1 mRNA which was up-regulated by GRA at the recommended dietary Se level and at the slightly supranutritive level compared to group C150 all other dietary regimes (G150, C15, G15, G450) led to a more or less pronounced down-regulation of the other GST classes.

As expected, in both Se-deficient groups C15 and G15 colonic GPx1 mRNA expression was reduced to about 20 % of the level in group C150. Both under adequate and supranutritive Se supply, GRA (G150 and G450) had no influence on colonic GPx1 expression. In Se-deficient rats (C15), the colonic GPx2 mRNA was about 50 % lower than that in their littermates of groups C150 and C450. Additional GRA supply to Se-deficient rats (G15) significantly increased colonic GPx2 mRNA compared to rats with an isolated Se deficiency (C15). Within the GRA-supplemented groups, colonic GPx2 mRNA level in group G15 was comparable to that of the control group C150. Increasing dietary Se (G150 and G450) effectuated a dose dependent increase in GPx2 expression.

The colonic mRNA expression pattern of ARE-regulated AE and PIIE considerably differed from that in the jejunum. GSTA3, a member of the GSTA class, was the only Nrf2 target gene that responded to none of the dietary regimes. Most strikingly, with the exception of GSTT1, the highest relative mRNA concentration of all ARE-regulated genes

Table 4 Relative mRNA Expression of Nrf2, Keap1, GPx1, and ARE-regulated antioxidant and phase II enzymes in the liver of rats fed diets supplemented with three different dietary Se concentrations (50, 150 and 450 $\mu g kg^{-1}$) either without or with addition of 700 μmol GRA kg $(diet)^{-1}$

	C150	G150	C15	G15	C450	G450
Nrf2	1.00 ± 0.11^{ab}	1.03 ± 0.06^{ab}	0.87 ± 0.06^{ac}	0.78 ± 0.07^{a}	1.20 ± 0.12^{b}	1.09 ± 0.07^{bc}
Keap1	1.00 ± 0.16^{a}	0.86 ± 0.12^{a}	1.60 ± 0.18^{b}	0.97 ± 0.13^{a}	0.94 ± 0.08^{a}	0.67 ± 0.06^{a}
GPx1	1.00 ± 0.06^{a}	0.92 ± 0.10^{a}	0.10 ± 0.01^{b}	0.26 ± 0.05^{b}	1.30 ± 0.10^{a}	1.01 ± 0.04^{a}
GPx2	1.00 ± 0.20^{a}	0.77 ± 0.13^{a}	8.49 ± 0.65^{b}	5.91 ± 0.98^{c}	1.02 ± 0.12^{a}	1.31 ± 0.02^{a}
EPHX1	1.00 ± 0.15^{a}	1.27 ± 0.09^{a}	4.90 ± 0.44^{b}	3.20 ± 0.11^{c}	1.27 ± 0.20^{a}	1.46 ± 0.14^{a}
HO1	1.00 ± 0.11^{a}	0.92 ± 0.10^{a}	1.45 ± 0.15^{b}	0.94 ± 0.09^{a}	0.82 ± 0.05^{a}	0.75 ± 0.06^{a}
NQO1	1.00 ± 0.10^{a}	1.14 ± 0.10^{a}	4.27 ± 0.29^{b}	3.47 ± 0.27^{c}	1.12 ± 0.05^{a}	1.09 ± 0.09^{a}
UGT1a6	1.00 ± 0.07^{a}	0.97 ± 0.07^{a}	4.22 ± 0.15^{b}	3.09 ± 0.29^{c}	0.96 ± 0.03^{a}	1.01 ± 0.05^{a}
GSTA3	1.00 ± 0.08^{a}	0.75 ± 0.09^{a}	2.58 ± 0.23^{b}	1.16 ± 0.07^{a}	1.05 ± 0.13^{a}	0.91 ± 0.11^{a}
GSTK1	1.00 ± 0.27^{a}	1.29 ± 0.10^{a}	2.09 ± 0.19^{b}	1.19 ± 0.15^{a}	1.35 ± 0.16^{a}	1.58 ± 0.16^{ab}
GSTM5	1.00 ± 0.07^{a}	0.94 ± 0.07^{a}	1.96 ± 0.25^{b}	1.07 ± 0.06^{a}	1.11 ± 0.11^{a}	0.85 ± 0.08^{a}
GSTO1	1.00 ± 0.11^{a}	0.86 ± 0.08^{a}	1.57 ± 0.19^{b}	1.18 ± 0.09^{a}	0.87 ± 0.05^{a}	0.89 ± 0.09^{a}
GSTP1	1.00 ± 0.12^{a}	0.90 ± 0.15^{a}	2.25 ± 0.20^{b}	1.29 ± 0.15^{a}	1.06 ± 0.13^{a}	1.09 ± 0.18^{a}
GSTT1	1.00 ± 0.13^{a}	1.60 ± 0.27^{ab}	4.49 ± 0.41^{c}	2.17 ± 0.18^{b}	1.56 ± 0.16^{ab}	1.63 ± 0.17^{ab}
GSTT2	1.00 ± 0.17^{a}	0.89 ± 0.11^{a}	1.96 ± 0.20^{b}	0.95 ± 0.08^{a}	0.91 ± 0.06^{a}	0.86 ± 0.07^{a}

Values are means±SEM and represent relative mRNA concentrations as *n*-fold of group C150=1. Different small letters in a row indicate significant differences between means ($p \leq 0.05$). *n*=6 cDNA pools of two rats per experimental group

investigated (EPHX1, HO1, NQO1, UGT1a6, GSTK1, GSTM5, GSTO1, GSTP1, GSTT1, GSTT2), could be measured in G450 rats receiving the diet containing GRA in combination with slightly supranutritive Se. With the exception of GSTT1, ARE regulated genes showed the second strongest response to dietary treatment with GRA in combination with Se at the recommended dietary level (G150). Slightly supranutritive Se alone (C450) only increased colonic GSTP1 mRNA compared to C150 rats. In Se-deficient rats (C15) colonic mRNA abundance of Nrf2 target genes was principally higher than in their companions with adequate Se supply (C150). In Se-deficient rats additional GRA supplementation (G15) influenced colonic mRNA concentration of ARE-regulated genes differently compared to rats with an isolated Se deficiency (C15). Whereas GRA reduced NQO1- and UGT1a6-expression it augmented EPHX1-, HO1-, GSTK1-, GSTP1-, GSTT1- and GSTT2-mRNA abundance.

Comparably to the colonic data, GPx1 mRNA expression in the liver of the Se-deficient groups was down-regulated by 74 % and 90 % compared with control rats of group C150. In the other groups with sufficient or slightly supranutritive Se supply (G150, C450, G450) no significant changes with regard to GPx1 expression could be observed compared to group C150.

With regard to all Nrf2 target genes in the liver another, very specific expression pattern, could be observed. Se deficiency alone increased the expression of AE (EPHX1, HO1, NQO1) and of the PIIE UGT1a6 and of all GST classes 1.45- to 4.90-fold compared to control rats with adequate Se supply (C150). With the exception of EPHX1, NQO1, UGT1a6 and GSTP1, GRA supply to Se-deficient rats (G15) strongly reduced the increase in mRNA expression due to Se deficiency. Only the mRNA levels of EPHX1, NQO1, UGT1a6 and GSTP1 remained above the Se adequate group C150. The selenoprotein GPx2 showed a regulation profile comparable to that of the above mentioned AE and PIIE. Within the GST enzymes only GSTT1 mRNA showed a small response to GRA (G150 and G450) and to supranutritive Se (C450) compared to C150 rats.

Activity of GPx, of GSTA Class, of GSTP Class and of NQO1 in Colon and Liver

In order to test if the changes found in gene expression were also reflected by the activity of selected ARE-regulated enzymes, we have measured the activities of GPx, of GSTA class, of GSTP class and of NQO1 in colon and liver (Table 5), the two most reactive tissues in gene expression analysis. Since in the intestine and the liver of rats, both GPx1 and GPx2 are expressed, and the measurement of a differentiated activity is not possible, we have determined total GPx activity. In the colon, Se deficiency in groups C15 and G15 was indicated by a 90 to 92 % lower GPx activity compared to control rats of group C150. Colonic GPx activity was slightly, but not significantly, higher in G15 rats than in C15 rats. Accordingly, the activity data for total GPx in G150 and G450 rats compared with C150 rats impressively reflected the potential of GRA to increase GPx2 expression and activity. With the exception of group G150, colonic GSTA class activity principally reflected the data of GSTA3 expression. In the Se-deficient groups, GSTA class activity was distinctly higher than in C150 rats. In rats receiving diets with supranutritive Se alone or in combination with GRA, GSTA class activity was similar to that in the control group C150. GSTP class activity turned out to be an insensitive enzyme to control the regulation of colonic phase II gene expression. Quite in contrast NQO1 activity sensitively reflected the changes in gene expression due to the different dietary conditions. Thus the enzymes'

Table 5 Enzyme activity of GPx, and of ARE-regulated antioxidant and phase II enzymes in the colon and the liver of rats fed diets supplemented with three different dietary Se concentrations (50, 150 and 450 μgkg^{-1}) either without or with addition of 700 μmol GRA kg (diet)$^{-1}$

	C150	G150	C15	G15	C450	G450
Colon						
GPx (mU/mg protein)	38.2±3.24^{a}	53.4±5.52^{b}	3.2±0.41^{c}	3.6±0.48^{c}	47.9±2.95^{b}	56.9±4.89^{b}
GSTA class (mU/mg protein)	22.9±1.05^{a}	26.6±0.83^{bc}	27.3±2.02^{b}	26.1±0.97^{ab}	23.8±0.79^{ac}	24.1±0.85^{ab}
GSTP class (mU/mg protein)	13.7±0.75^{a}	11.5±0.76^{b}	13.4±0.85^{ac}	13.8±0.49^{a}	13.2±0.52^{ab}	11.7±0.34^{bc}
NQO1 (mU/mg protein)	150±5.62^{a}	228±23.6^{b}	235±25.3^{b}	214±24.0^{bc}	144±8.07^{a}	168±11.9^{ac}
Liver						
GPx (mU/mg protein)	259.4±15.6^{a}	273.1±20.0^{a}	4.08±0.46^{b}	4.49±0.26^{b}	281.5±14.9^{a}	313.8±23.9^{a}
GSTA class (mU/mg protein)	223±14.2^{a}	241±8.90^{ac}	374±14.8^{b}	332±18.5^{b}	241±14.0^{ac}	280±22.5^{c}
GSTP class (mU/mg protein)	24.4±1.25^{a}	23.8±0.64^{a}	31.1±1.42^{b}	28.7±1.27^{bc}	24.8±0.99^{a}	26.1±1.03^{ac}
NQO1 (mU/mg protein)	343±18.4^{a}	352±20.1^{a}	594±29.4^{b}	515±14.9^{c}	395±20.4^{a}	387±14.5^{a}

Values are means±SEM. Different small letters in a row indicate significant differences between means ($p \leq 0.05$)

activity was significantly increased by Se deficiency (C15, G15) and by GRA treatment (G150, G450) compared to C150 rats.

In the liver, in which GPx1 is the predominant form of glutathione peroxidase, GPx activity in the Se-deficient groups (C15, G15) was decreased to 1.57 % and 1.70 % of that in C150 rats. Feeding supranutritive Se alone or in combination with GRA (C450, G450) did not further affect GPx activity compared to C150 rats. The highest activity of GSTA class in the liver of Se-deficient rats (C15) directly reflected the enzymes' mRNA expression level. Adding GRA to the Se-deficient diet lowered both GSTA class mRNA abundance and its activity. With the exception of group G450, GSTA class gene expression and activity also matched well for the remaining groups G150 and C450. As similarly observed for GSTA class, also the activity of GSTP class was the highest in the liver of Se-deficient C15 rats. Additional GRA (G15) reduced GSTP expression and activity. No differences in GSTP mRNA abundance and activity compared to control rats (C150) could be measured in their littermates of groups G150, C450 and G450. Liver NQO1 activity most sensitively reflected the changes in the mRNA expression as likely observed in the colon.

mRNA Expression of Genes Associated with Inflammation (COX1, COX2, MCP) in the Colon

Due to the strong effects of GRA on colonic AE and PIIE, we have measured additionally the mRNA expression of selected inflammation markers (Table 6).

In the colon, COX1 expression was the highest in the Se-deficient rats (C15) and in the Se adequate control rats of group C150. Supranutritive Se and GRA supply at all dietary Se levels decreased COX1 expression. Gene expression of the highly inducible COX2 was the highest in Se-deficient rats. Already in Se-deficient rats dietary GRA significantly reduced COX2 expression. The other dietary treatments (G450, C450 and G450) had no significant effect on COX2 expression compared to the Se adequate control group C150. Se-deficient rats (C15) showed also the highest expression of MCP1. In contrast, MCP1 expression was the lowest in rats with supranutritive Se in combination with GRA (G450).

Discussion

Se Status

GPx activity and the mRNA levels of GPx1 were analysed in all tissues investigated because they are accepted biomarkers for Se status [41]. Total GPx activity and relative GPx1 mRNA concentration confirmed a distinct Se deficiency in groups C15 and G15 in jejunum, colon and the liver. The decrease of GPx1 expression in the Se-deficient groups can be explained by a lowered mRNA stability [42]. In the colon, however, increasing the dietary Se concentrations from 150 to 450 μg Se/kg diet elevated total GPx activity whereas the mRNA levels of GPx1 and GPx2 remained uninfluenced. In agreement with the commonly accepted hierarchy of selenoproteins the increase in GPx activity is rather the result of an augmented GPx1 translation, since GPx2 reaches its plateau activity already under lower Se concentrations [42].

Effect of Different Dietary Se Concentrations on ARE Containing Genes

The transcription factor Nrf2 and the existence of an ARE sequence in the promoter are important factors modulating the gene expression and activity of numerous AE and PIIE. Nrf2 therefore seems to play an important role in the prevention of different cancers, but in particular of colon cancer [12, 43–45]. Se status has also been shown to influence Nrf2 target genes. Oxidative stress resulting from both, Se deficiency, or high doses of different Se compounds, is able to modify the critical cysteine residues of Keap1, the cytosolic adapter-protein of Nrf2 [17]. In this context, several studies have proven that both Se deficiency [18–21] and high doses of several Se compounds [22–24] induce Nrf2 target genes like GST, NQO1 and HO1.

Table 6 Effects of diets differing in Se concentration (50, 150 and 450 $\mu g\,kg^{-1}$) and/or GRA content on the relative mRNA concentrations of the inflammation markers COX1, COX2, and MCP1 in the colon of rats

	C150	G150	C15	G15	C450	G450
Colon						
COX1	1.00 ± 0.16^{a}	0.58 ± 0.09^{b}	1.01 ± 0.09^{a}	0.70 ± 0.10^{ab}	0.80 ± 0.07^{ab}	0.70 ± 0.10^{ab}
COX2	1.00 ± 0.20^{a}	1.29 ± 0.28^{a}	4.13 ± 0.94^{b}	1.77 ± 0.50^{a}	1.14 ± 0.18^{a}	1.58 ± 0.83^{a}
MCP1	1.00 ± 0.19^{ab}	1.23 ± 0.07^{ab}	1.47 ± 0.19^{a}	1.16 ± 0.20^{ab}	1.10 ± 0.20^{ab}	0.88 ± 0.13^{b}

Values are means±SEM and represent relative mRNA concentrations as *n*-fold of group C150=1. Different small letters in a row indicate significant differences between means ($p\leq0.05$). $n=6$ cDNA pools of two rats per experimental group

Therefore, the first aim of our study was to investigate the influence of different dietary Se concentrations on a broad spectrum of Nrf2 target genes in different tissues.

In contrast to cellular GPx1, gastrointestinal GPx2 is not only modulated by Se status, but it also represents a Nrf2 target [10]. GPx2 is predominantly expressed in the mucosal epithelium of the gastrointestinal tract and ranks high in the hierarchy of glutathione peroxidases [42, 46]. The regulation of GPx2 by oxidative stress and its resistance against Se deficiency have been demonstrated in a mouse study in which marginal Se deficiency led to an increase in duodenal GPx2 mRNA [17]. Our data confirm this effect of Se deficiency on jejunal GPx2 expression. Most interestingly, in the colon Se deficiency decreased GPx2 mRNA levels, suggesting that in this tissue Se deficiency is the dominating trigger of GPx2 expression [17]. In contrast to earlier studies, more recent investigations have shown that GPx2 is also expressed in rat liver [47–49]. Our current data have confirmed this hypothesis trough the impressive GPx2 regulation under oxidative stress. In Se deficiency, the distinct raise in hepatic GPx2 presumably counteracts oxidative stress deriving from the tremendous loss of GPx1 expression and activity. Moreover previous studies have shown that in addition to GPx2 a number of other Nrf2 target genes are up-regulated in Se deficiency. In this context, Burk et al. [21] reported on a 450-fold increase in the ARE-reporter enzyme “human placental alkaline phosphatase (hPAP)” in the livers of Se-deficient mice, and therefore they showed for the first time the activating effect of Se deficiency on the Nrf2/Keap1-ARE system. In the same study, Burk et al. [21] have demonstrated that the Nrf2 target genes NQO and HO1 were 2.30- and 7.70-fold up-regulated by Se deficiency. Our present data have confirmed the increase in the hepatic mRNA concentrations of EPHX1, HO1 and NQO1 under Se deficiency. Moreover our results suggest that within ARE-regulated enzymes changes in NQO1 activity reflect alterations in the enzymes’ mRNA expression most sensitive.

Our study has investigated the differential regulation of a broad spectrum of liver PIIE, including UGT1a6 and several GST classes due to changes in dietary Se supply. The distinct up-regulation of all GST mRNAs could be verified by a strong increase in the activity of GST-A and -P class. Within the GST classes, GSTT1 showed the strongest response to Se deficiency compared to Se adequate rats. In this context a previous rat study has shown that the catalytic GSTT1 subunit is highly overexpressed in Se-deficient rats. The increase in GSTT1 during Se deficiency is believed to be very important, because this transferase is involved in the activation of several chemical carcinogens [19]. The distinct increase in all GST classes in Se deficiency seems to compensate for the loss of selenoenzymes because many GST possess a Se independent peroxidase activity [50, 51]. In contrast to our present results in a mice study no effects of marginal Se deficiency (86 μg Se/kg diet) on liver GST mRNA expression could be observed. Presumably, a marginal Se deficiency is insufficient to raise GST expression [17]. This hypothesis is confirmed by the results of another rat trial of our group. In this trial we could show that GPx1 expression and activity of rats receiving a diet with only 50 μg/kg had reached already 50 % of the level in Se sufficient rats (150 μg/kg) [34]. In contrast, moderate supranutritive Se did not increase liver GPx1 expression and activity any further and had no effects on the regulation AE and PIIE. This result agrees with the outcome of a previous rat trial. In this study it has been shown that supranutritive Se had no influence on ARE-regulated genes until a toxic level of 5,000 μg/kg diet was reached [52]. In summary it can be assumed that the induction of hepatic GST by Se deficiency is mediated only in parts via the Nrf2–ARE system [21] since the biological mechanisms underlying the expression and regulation of the different GST isoforms are complex [53, 54].

Besides the ARE, the glucocorticoid response element (GRE) and the xenobiotic response element (XRE) have been reported to represent further regulation sites of GST. Potential further transcription factors involved in the regulation of PIIE are the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), the pregnane X receptor (PXR) and the peroxisome proliferator activated receptor α (PPARα) [53, 55]. In this context, our data have indicated that also GST classes without an ARE (GSTK1, GSTM5 and GSTO1) have strongly responded to Se deficiency. Therefore, it can be assumed that one of the mechanisms mentioned above was involved in the up-regulation of these GST enzymes.

With the exception of GPx2, and in contrast to hepatic mRNA expression manipulating the dietary, Se level had no noteworthy influence on the mRNA abundance of other Nrf2 targets in the small intestine. In our present study the mRNA abundance of colonic ARE-regulated AE (EPHX1, HO1, NQO1) and PIIE (UGT1a6, all GST classes) was distinctly, but not significantly, increased by Se deficiency (C15). The distinct up-regulation of AE and PIIE in our trial may base on the loss of GPx2 which is highly Se sensitive in the colon. Moreover in our trial supranutritive Se (C450) only increased colonic GSTP1 expression significantly. GSTP function is closely linked to cancer. GSTP suppression stimulates myeloproliferation which again triggers immunosuppression and angiogenesis of tumors [56, 57]. In rats, GSTP1 is the predominant GST form in the colon, and its colonic expression is even higher than in the liver [48, 58]. Therefore, it can be assumed that slightly supranutritive Se via GSTP1 may protect in particular against colon cancer [22–24].

Effect of Dietary GRA Combined with Different Se Concentrations on ARE Containing Genes

In contrast to a number of previous trials in which the effects of the free isothiocyanate SFN on AE and PIIE were examined in vitro and in vivo, we have studied the supplementation of its glucosinolate precursor GRA. We have done this deliberately because GRA is the predominant form of SFN both in nutritional supplements and in heated broccoli. In order to guarantee a constant dietary GRA concentration we have used a broccoli extract with a standardised GRA concentration of 10 % (w/w). The dietary GRA concentration of 700 μmol/kg diet was adapted to a feasible GRA intake of 500–1,000 μmol GRA/day in humans [34]. As the first target organ of GRA supplementation, we have chosen the jejunum in order to investigate local effects of GRA because our broccoli extract had no myrosinase activity. In this context, results of previous in vitro studies revealed that besides the isothiocyanate SFN also its precursor GRA can potently induce ARE-regulated PIIE. The effective GRA concentration used in these studies for the incubation of freshly isolated tissue slices ranged between 15 and 20 μmol/l [15, 16]. In contrast, in our present in vivo study jejunal mRNA expression of most of the analysed Nrf2 target genes was even slightly reduced by GRA supplementation. Therefore, we conclude that 700 μmol GRA per kg diet (approximately 13 μmol per rat and day) is not sufficient to induce Nrf2 target genes. Additionally, it must be considered that the total daily GRA amount was not ingested by a single bolus, but instead taken up by the rats over the whole day. Moreover, it can be speculated that humans either ingest GRA by taking a single supplement or by eating a larger broccoli serving as a side dish at a defined point of time. In this context, further studies are needed to investigate the effects of a higher dietary GRA concentration or of a bolus ingestion on PIIE in the small intestine. Finally, it is remarkable that in our study only jejunal GSTP1 was up-regulated due to GRA supplementation in combination with adequate or slightly supranutritive Se concentrations, but not under Se deficiency. GSTP1 has not only a pivotal role in the suppression of tumor growth, as discussed above, but it also interacts with the MAPK c-jun NH_2 terminal kinase (JNK). Oxidative stress initiates the dissociation of the GSTP–JNK complex and reverses the intrinsic JNK inhibition by GSTP. By its function as a sensor of intracellular redox status GSTP is important in the regulation of apoptosis [56].

The second target tissue investigated in our study was the colon. Uncleaved glucosinolates entering the large intestine are hydrolysed by bacterial β-glucosidases. In an in situ study, Lai et al. [13] have proven that GRA undergoes an efficient cleavage to SFN and glucose and that the liberated SFN transits the ceacal enterocytes for systemic absorption. The GRA dose tested in the above mentioned study was 150 μmol per kg body weight. This dose was able to effectively induce colonic NQO1 [13]. Despite adapting GRA supplementation to a realistic value for humans (~38 μmol per kg body weight) the applied GRA concentration in our study was adequate to increase the mRNA expression and the activity of a broad spectrum of colonic AE and PIIE significantly. A number of studies have demonstrated that SFN reduces the risk of developing several cancers including colon, prostate, lung and breast cancer [43, 45, 59]. Besides the cancer preventive effects of SFN, cancer protection has also been reported for Se [60–62]. Data of the Nutritional Prevention of Cancer Trial (NPC) and the follow-up data indicated that the daily supplementation of slightly supranutritive Se (200 μg Se/day) had no influence on the primary end point of non-melanoma skin cancer. However, Se supplementation significantly lowered both, total cancer mortality, and the incidence of prostate, colorectal and lung cancers [63]. The protective effect of Se may derive from its ability to reduce oxidative stress by specific selenoproteins like GPx [29, 62]. Because SFN as well as Se are able to induce the selenoproteins thioredoxin reductase 1 (TrxR1) and GPx2, it is assumed that SFN and Se act synergistically in the prevention of cancer [60]. The synergism has been proven in a previous study that tested the influence of different Se concentrations in combination with SFN in a mouse model of inflammation-associated colon carcinogenesis. The results of this study have shown that SFN acts anti-inflammatory under Se adequate conditions, but exhibits proinflammatory effects under Se deficiency. The authors concluded that SFN requires the full activity of another selenoprotein, sensitive to Se deficiency in order to develop its protective and anticarcinogenic potential. They further hypothesised that presumably GPx1, ranking low in the hierarchy of selenoproteins, is the most supposable candidate protein for these processes [29]. To the best of our knowledge, we could show for the first time that also GRA up-regulates a broad spectrum of colonic AE and PIIE and that this effect is boosted by adequate and in particular by slightly supranutritive Se supply.

We therefore deeply assume that the combination of GRA and slightly supranutritive Se can provide an optimum protection against colon cancer.

To investigate systemic effects of GRA on AE and PIIE, we have selected the liver as the third target tissue in our study. We could not detect an influence of GRA on the above mentioned parameters in the livers of rats fed diets with adequate or slightly supranutritive Se supply. In contrast, we have found a distinct effect of GRA under Se-deficient conditions. Whereas Se deficiency alone (C15) increased liver mRNA expression and enzyme activity of AE and PIIE 1.45- to 4.49-fold, GRA supply (G15) distinctly reduced the expression of all target genes investigated.

Moreover also the activities of NQO1, GSTA class and GSTP class were distinctly reduced in Se-deficient GRA supplied rats (G15) compared to their companions with an isolated Se deficiency. We assume that the down-regulation of Nrf2-target genes in the liver may result from an increased intestinal barrier against oxidative stress in the organism [34]. This hypothesis is confirmed by our present data, showing that GRA supplied Se-deficient rats (G15) had a higher colonic mRNA abundance of a number of AE and PIIE compared to those with isolated Se deficiency (C15). The missing systemic effect of GRA in the liver has been shown in two other rat studies [64, 65].

Because nearly all currently available dietary GRA supplements lack myrosinase activity, the addition of myrosinase to these supplements should be considered in the future.

Views and News on the Indicator Function of Nrf2 and of Keap1 on the Regulation of ARE Regulated Genes

Finally, a very important topic should be addressed with regard to the effects of Se and GRA on the ARE-containing Nrf2 target genes. Besides studying ARE-regulated genes, we have additionally investigated the influence of Se and GRA on the expression of the transcription factor Nrf2 and on its cytosolic adapter protein Keap1. The results of these analyses are summarised in Fig. 2 and Table 7. Figure 2 shows the connection between the average net response of all target genes and changes in Nrf2 and Keap1 expression due to the different dietary conditions in the jejunum (Fig. 2a, b), the colon (Fig. 2c, d) and the liver (Fig. 2e, f). In Table 7, the correlations between Nrf2 and Keap1 expression and ARE-regulated genes are displayed. Our data revealed (Fig. 2 and Table 7) that on the basis of gene expression and on correlation analyses Keap1 seems to be a better indicator of ARE-driven gene expression than Nrf2. In the colon, both Nrf2 and Keap1 were similar good predictors for target gene expression (Fig. 2c, d). However, as an example, demonstrating the deviation of our current results from the opinion, that Nrf2 is a good indicator of ARE-driven gene expression, our expression data for the liver can be consulted. According to the general opinion, every would have been expected a strong increase in Nrf2 expression due to oxidative stress in Se deficiency (cf. Table 4, Fig. 2e, f). But quite in contrast Nrf2 expression was low in Se-deficient rats (C15). On the other hand Keap1 expression sensitively indicated the prooxidative conditions and consequently the up-regulation of ARE-containing target genes. One explanation for this phenomenon may consist in the different fates of Nrf2 and Keap1 in cells (Fig. 3).

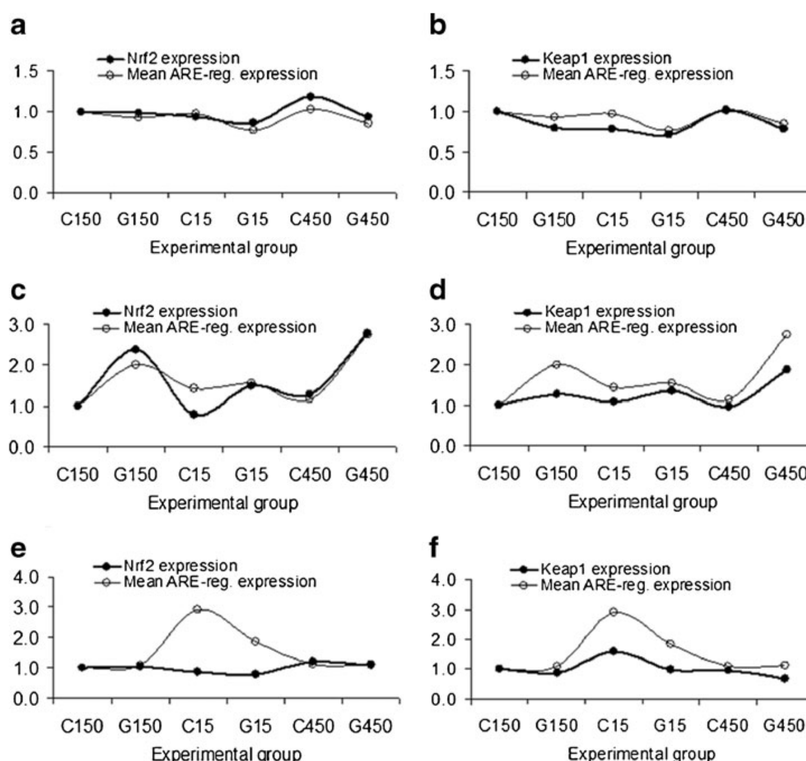

Fig. 2 Coherence between Nrf2 and Keap1 mRNA expression and the average mRNA expression of ARE-regulated antioxidant and phase II genes in the jejunum (**a**, **b**), the colon (**c**, **d**), and the liver (**e**, **f**) of rats

Table 7 Pearson correlations between Nrf2 and Keap1 mRNA expression and the average mRNA expression of ARE-regulated antioxidant and phase II genes in the jejunum (A, B), the colon (C, D), and the liver (E, F) of rats

Correlation investigated	Jejunum	Colon	Liver
Nrf2:ARE reg. genes	Correlation: 0.792	Correlation: 0.908	Correlation: 0.678
	p value: 0.061	*p* value: 0.012	*p* value: 0.139
	Fig. 1a	Fig. 1c	Fig. 1e
Keap1:ARE reg. genes	Correlation: 0.833	Correlation: 0.941	Correlation: 0.882
	p value: 0.040	*p* value: 0.005	*p* value: 0.020
	Fig. 1b	Fig. 1d	Fig. 1f

Nrf2 in fact is the transcription factor initiating the gene expression of ARE-containing genes. However, as discussed above, a number of interactions exist, and further transcription factors are involved into the regulation of PIIE (e.g., the AhR the CAR, the PXR and the PPARα) [53, 55]. These interactions may cause changes in Nrf2 gene expression that do not reflect oxidative stress adequately. On the other hand, Keap1 per se is a target gene of Nrf2 that undergoes oxidative or covalent modifications at sensitive cysteine SH groups due to the reaction with reactive oxygen species or isothiocyanates [66]. In turn, these modifications, coupled to reduced Nrf2 degradation and increased Nrf2 translation, may force Keap1 gene expression. Therefore, the newly synthesised Keap1 seems to turn off the Nrf2 signal. Finally a most recent study has clearly shown that the three sensitive cysteine residues of Keap1 have an individual response to different prooxidants and electrophiles [67].

Due to our current observations, we conclude that, on the basis of mRNA expression, Keap1 indicates changes in ARE-driven gene expression even better than Nrf2. This implicates that in future investigations regarding, this topic Keap1 mRNA and protein expression should be considered to a higher extent.

Influence of GRA and Se on Genes Involved in Inflammation

Inflammation is a well recognised risk factor in carcinogenesis [68]. Recent in vitro studies revealed that chemopreventive

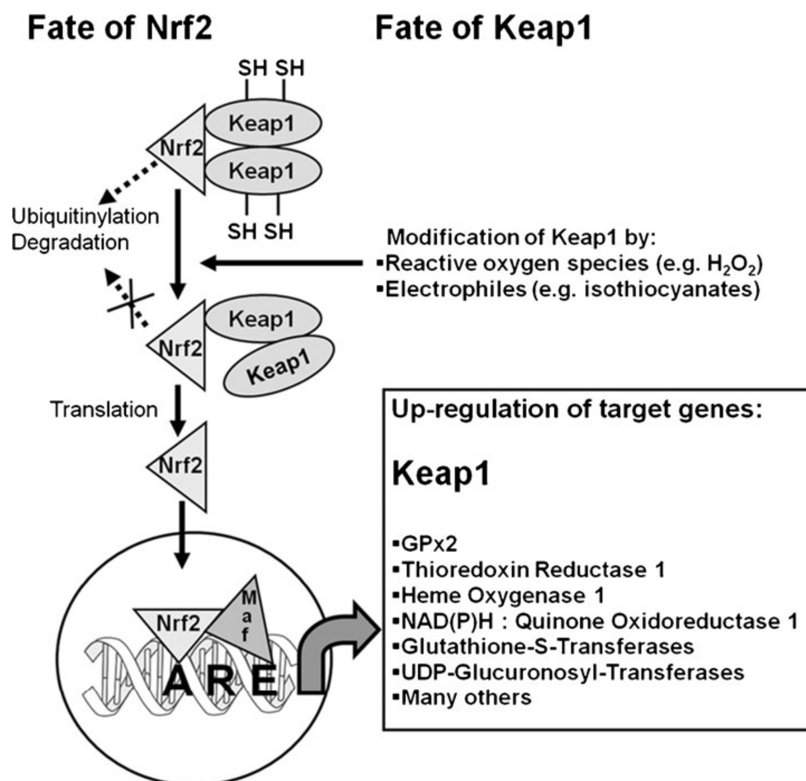

Fig. 3 The fates of Nrf2 and Keap1 in cells (modified from Li and Kong [9]). Under balanced pro- and antioxidant conditions, Nrf2 is bound to a dimer of its cytosolic adapter protein Keap1 through 2 binding motifs. Nrf2 can be regulated by different mechanisms: (1) Keap1 acts as a linker for a Cullin-dependent E3 ubiquitin ligase complex and leads to the proteasomal degradation of Nrf2. (2) Keap1 acts as a redox sensor. Electrophiles and reactive oxygen species initiate the modification of different cysteine-SH groups of Keap1. Subsequently one bond between Keap1 and Nrf2 is disrupted and Nrf2 degradation is inhibited. Furthermore, Nrf2 translation is increased and the free Nrf2 pool expands. Free Nrf2 can translocate into the nucleus and bind to ARE sequences in the promoter region of genes of antioxidant and phase II enzymes [9]

effects of SFN do not only base on the induction of detoxifying and antioxidant enzymes (AE), but that SFN also develops anti-inflammatory effects. One trial showed that the treatment of endothelial cells with SFN inhibited TNFα induced VCAM1 and MCP1 expression [69]. SFN also down-regulated LPS mediated induction of iNOS, COX2 and TNFα in RAW 264.7 macrophages [70]. Lin et al. [28] demonstrated that SFN decreased LPS induced TNFα, COX2 and interleukin-1 in peritoneal macrophages. Under conditions without an inflammatory stimulus our current data for the colon showed, that GRA under Se-deficient conditions counteracted COX2 expression (C15 vs. G15). Moreover colonic COX2 expression was lower under all other dietary regimes tested than in C15 rats. These data indicate the anti-inflammatory potential of Se and GRA. Another remarkable result in our trial was reflected by the down-regulation of COX1 expression due to supranutritive Se and/or GRA supplementation. Because COX1 is constitutively expressed, our results suggest, that this constitutive expression can be reduced by supranutritive Se and/or GRA. Rats with Se deficiency (C15) also showed the highest colonic MCP1 expression which could be reduced most efficiently by combined supplementation of supranutritive Se plus GRA.

In the context of the anti-inflammatory potential of Se and GRA, the following aspects are discussed in the literature.

The results of a study with endothelial cells showed that the SFN-dependent inhibition of VCAM1 and MCP1 expression rather depended on the inhibition of p38 MAP kinase than on an influence on the Nrf2/ARE pathway. The authors concluded that SFN treatment develops anti-inflammatory actions by two pathways: (1) the more acute action of SFN on p38 MAP kinase inhibition and (2) a long-term effect depending on Nrf2 [69]. Besides SFN Se also is an important anti-inflammatory agent. In a murine macrophage cell line the effect of cellular Se status on the expression of COX2 was investigated. The results indicated that Se supplementation in macrophages decreases the activity and expression of COX2 but not of COX1 [71, 72]. An increased potential to scavenge ROS via the increased GPx activity has been suggested as the underlying mechanism for this effect [71]. Several GPx are able to inhibit the activity of COX by removal of hydroperoxides [73]. COX, in turn, needs hydroperoxides for catalysing eicosanoide synthesis [74]. Therefore, an increased GPx activity may result in an inhibition of COX2 expression and activity [73, 75, 76]. In our study, we could confirm a distinct increase in COX2 mRNA levels in Se deficiency in the colon. This was in accordance with the decreased expression and activity of colonic GPx2. GRA supplementation counteracted the effect of Se deficiency and decreased the mRNA expression of colonic COX2 nearly to the levels of the control group. In a study with mice it could be demonstrated that SFN exhibited proinflammatory effects under marginal Se deficiency by increasing apoptosis in colonic crypt bases and by triggering colitis. Moreover the mice in this study underwent inflammation-induced carcinogenesis with azoxymethane (AOM) and dextrane sulphate sodium (DSS) [29], whereas our rats were healthy and the Se deficiency was not marginal but distinct.

In summary, we conclude that:

- GRA supplementation affects the expression and activity of ARE regulated AE and PIIE in an organ specific manner.
- In contrast to in vitro studies, GRA had no mentionable local effects on ARE regulated genes in the small intestine.
- As indicated by a very strong induction of colonic ARE regulated AE and PIIE, the effective cleavage of GRA to SFN and glucose takes place in the large intestine.
- GRA and Se synergistically affect the induction of ARE regulated AE and PIIE in the large intestine.
- GRA and slightly supranutritive Se may represent an optimum formula to protect against colon cancer and inflammation in the large intestine.
- On the basis of mRNA expression Keap1 seems to be a much more sensitive indicator of ARE-driven gene expression than Nrf2. In future investigations on ARE-driven gene expression, besides Nrf2, the role of Keap1 should be considered to a higher extent.
- Future studies should investigate if higher GRA concentrations or GRA in combination with microencapsulated myrosinase influence also the expression of ARE regulated genes in the small intestine and in the liver.

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3.5 Studie 5

Bei ***Studie 5*** handelt es sich um einen Fütterungsversuch mit 28 wachsenden männlichen Wistar Ratten (Charles River, Sulzfeld, Deutschland) mit einem mittleren Anfangsgewicht von 63,1 ± 1,25 g. Die Ratten wurden in vier Gruppen (siehe **Tabelle** 2) zu jeweils 7 Tieren eingeteilt.

Tabelle 2: Versuchsdesign des Fütterungsversuches mit 28 wachsenden Wistar-Ratten

	K	Se^-	SFN50	SFN100
Selen	150 µg	<15 µg	150 µg	150 µg
Sulforaphan	0	0	50 µmol	100 µmol

Anzahl der Tiere pro Gruppe: n = 7

Drei Gruppen (K, SFN50, SFN100) erhielten für 6 Wochen eine methioninreiche Hochfettdiät (1,5 % Methionin, 20 % Schweineschmalz und 0,15 % Cholesterin), die Natriumselenat in Höhe der Empfehlung enthielt (150 µg Selen pro kg Diät). Die Gruppe Se^- wurde mit der gleichen methioninreichen Hochfettdiät gefüttert, allerdings ohne Selenzusatz (<15 µg Selen pro kg Diät). Zwei Tage vor Versuchsende wurde den Ratten der Gruppen SFN50 und SFN100 oral 50 µmol beziehungsweise 100 µmol Sulforaphan (gelöst in Sonnenblumenöl) oral verabreicht. Die Ratten der Gruppen K und Se^- erhielten reines Sonnenblumenöl. Zwölf Stunden nach der letzten Sulforaphanapplikation wurden die Ratten mit CO_2 narkotisiert und dekapitiert. Für die folgenden Analysen wurde das Plasma gewonnen sowie das Jejunum, das Kolon und die Leber entnommen.

Die Ergebnisse der ***Studie 5*** wurden bislang nicht veröffentlicht und sind in den folgenden **Abbildungen 2** und **3** sowie in den **Tabellen 3-5** zusammengefasst.

Tabelle 3: Relative mRNA-Konzentrationen von Enzymen des Homocystein-, Glutathion- und Fettstoffwechsels sowie von antioxidativen Enzymen und Phase-II-Enzymen in der Leber

	K	Se^{-}	SFN50	SFN100
Enzyme des Homocystein- und Glutathionstoffwechsels				
BHMT	1.00 ± 0.08	1.00 ± 0.12	0.84 ± 0.14	0.90 ± 0.13
CBS	1.00 ± 0.09	0.95 ± 0.13	1.09 ± 0.08	0.80 ± 0.11
GCLC	1.00 ± 0.07	1.20 ± 0.11	1.20 ± 0.09	1.27 ± 0.08
GCLM	1.00 ± 0.05^{a}	1.45 ± 0.0^{b}	1.02 ± 0.12^{ac}	1.26 ± 0.07^{bc}
GNMT	1.00 ± 0.10	0.81 ± 0.11	0.77 ± 0.11	0.81 ± 0.09
GS	1.00 ± 0.19^{ab}	0.78 ± 0.13^{a}	1.49 ± 0.12^{b}	0.73 ± 0.20^{a}
Mrp4	1.00 ± 0.08	0.86 ± 0.09	0.94 ± 0.06	0.97 ± 0.11
Mtr	1.00 ± 0.10^{ab}	1.01 ± 0.10^{ab}	1.19 ± 0.07^{a}	0.76 ± 0.07^{b}
SAMDC	1.00 ± 0.06	1.00 ± 0.08	1.01 ± 0.20	1.21 ± 0.14
Antioxidative Enzyme und Phase- II-Enzyme				
GPx1	1.00 ± 0.06^{ac}	0.09 ± 0.01^{b}	1.12 ± 0.07^{a}	0.89 ± 0.09^{c}
GPx2	1.00 ± 0.15^{a}	0.75 ± 0.08^{a}	1.41 ± 0.12^{b}	1.41 ± 0.11^{b}
GSTT1	1.00 ± 0.08^{ab}	1.20 ± 0.07^{a}	0.86 ± 0.05^{b}	1.11 ± 0.07^{a}
NQO1	1.00 ± 0.09^{a}	1.93 ± 0.13^{bc}	1.59 ± 0.07^{b}	2.56 ± 0.44^{c}
UGT1a6	1.00 ± 0.06^{a}	2.85 ± 0.23^{b}	1.09 ± 0.08^{a}	1.23 ± 0.17^{a}
Enzyme des Fettstoffwechsels				
ABCG8	1.00 ± 0.26^{a}	3.02 ± 0.64^{bc}	1.84 ± 0.30^{ab}	3.87 ± 0.86^{c}
Cyp7a1	1.00 ± 0.18^{a}	0.85 ± 0.14^{a}	0.38 ± 0.08^{ab}	0.32 ± 0.05^{b}
FAS	1.00 ± 0.15	0.71 ± 0.10	0.92 ± 0.12	0.65 ± 0.08
HMGCoAR	1.00 ± 0.071^{a}	1.09 ± 0.08^{ab}	0.83 ± 0.08^{ab}	0.80 ± 0.11^{b}
LDLR	1.00 ± 0.05^{a}	1.01 ± 0.05^{a}	1.52 ± 0.17^{ab}	1.69 ± 0.31^{b}
LXRα	1.00 ± 0.07^{a}	0.86 ± 0.03^{ab}	0.90 ± 0.02^{ab}	0.81 ± 0.07^{b}
SREBP1c	1.00 ± 0.11	0.77 ± 0.08	1.07 ± 0.08	0.85 ± 0.12
SREBP2	1.00 ± 0.06^{a}	0.90 ± 0.04^{a}	1.16 ± 0.12^{ab}	1.47 ± 0.17^{b}

Die relativen mRNA-Konzentrationen sind als Mittelwerte ± SEM als Vielfaches der Kontrollgruppe (K = 1) dargestellt; zur Normalisierung dienten β-Aktin und das Ribosomale Protein L13a (RPL13a); unterschiedliche Buchstaben kennzeichnen signifikante Unterschiede ($P \leq 0,05$); es wurde je nach Varianzhomogenität der LSD- oder der Games-Howell-Test verwendet.

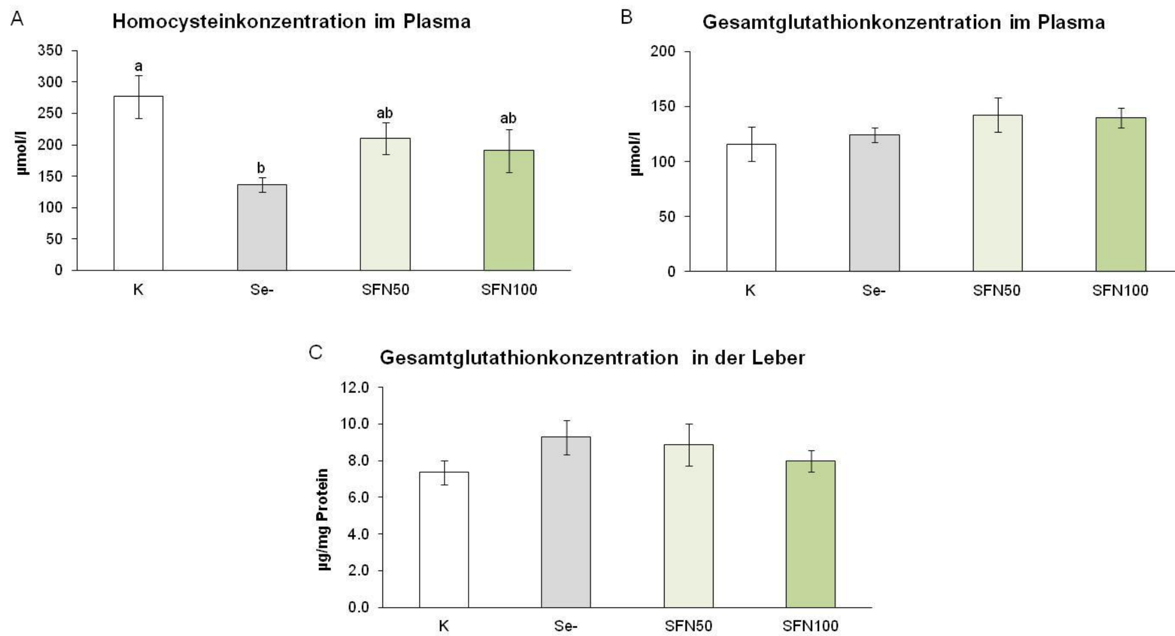

Abbildung 2: Einfluss der Versuchsdiäten auf die Homocysteinkonzentration im Plasma sowie die Gesamtglutathionkonzentration in Plasma und Leber

Die Werte sind als Mittelwerte ± SEM dargestellt; unterschiedliche Buchstaben kennzeichnen signifikante Unterschiede ($P \leq 0{,}05$); es wurde je nach Varianzhomogenität der LSD- oder der Games-Howell-Test verwendet.

Tabelle 4: Relative mRNA-Konzentrationen von ABCG8 sowie von antioxidativen Enzymen und Phase-II-Enzymen in Jejunum und Kolon

	K	Se⁻	SFN50	SFN100
Jejunum				
ABCG8	1.00 ± 0.20[a]	2.78 ± 0.58[b]	2.21 ± 0.39[ab]	2.54 ± 0.70[b]
GPx2	1.00 ± 0.21	0.60 ± 0.06	1.10 ± 0.23	1.00 ± 0.18
GSTT1	1.00 ± 0.14[a]	1.06 ± 0.10[a]	1.40 ± 0.18[a]	2.20 ± 0.23[b]
NQO1	1.00 ± 0.10[a]	1.06 ± 0.14[a]	4.70 ± 0.94[b]	16.05 ± 4.51[c]
UGT1a6	1.00 ± 0.13[a]	0.87 ± 0.16[a]	2.20 ± 0.67[ab]	8.67 ± 2.42[b]
Kolon				
ABCG8	1.00 ± 0.14[a]	0.58 ± 0.17[b]	0.60 ± 0.04[b]	0.81 ± 0.11[ab]
GPx2	1.00 ± 0.10[a]	0.91 ± 0.06[a]	1.45 ± 0.15[ab]	1.89 ± 0.39[b]
GSTT1	1.00 ± 0.06[a]	0.98 ± 0.06[a]	1.24 ± 0.08[b]	1.27 ± 0.10[b]
NQO1	1.00 ± 0.10[a]	0.93 ± 0.08[a]	2.65 ± 0.27[b]	3.26 ± 0.48[b]
UGT1a6	1.00 ± 0.21[ab]	0.81 ± 0.08[a]	1.29 ± 0.16[b]	0.83 ± 0.17[ab]

Die relativen mRNA-Konzentrationen sind als Mittelwerte ± SEM als Vielfaches der Kontrollgruppe (K = 1) dargestellt; zur Normalisierung dienten β-Aktin und das Ribosomale Protein L13a (RPL13a); unterschiedliche Buchstaben kennzeichnen signifikante Unterschiede ($P \leq 0{,}05$); es wurde je nach Varianzhomogenität der LSD- oder der Games-Howell-Test verwendet.

Tabelle 5: Enzymaktivitäten der GPx und der NQO1 in Leber, Jejunum und Kolon

	K	Se-	SFN50	SFN100
GPx Leber	201 ± 13.1^{a}	4.01 ± 0.58^{b}	231 ± 19.7^{a}	213 ± 22.1^{a}
GPx Jejunum	29.3 ± 2.72^{a}	4.99 ± 0.44^{b}	26.3 ± 1.75^{a}	26.2 ± 2.65^{a}
GPx Kolon	53.7 ± 10.9^{a}	4.11 ± 0.69^{b}	67.2 ± 6.63^{ac}	81.9 ± 7.85^{c}
NQO1 Leber	56.7 ± 5.35^{a}	119 ± 10.3^{b}	61.5 ± 4.83^{a}	70.5 ± 5.48^{a}
NQO1 Jejunum	133 ± 8.29^{a}	133 ± 9.67^{a}	347 ± 35.4^{b}	423 ± 49^{b}
NQO1 Kolon	127 ± 9.34^{ab}	99.2 ± 6.66^{a}	150 ± 17.7^{ab}	173 ± 21.7^{b}

Die Enzymaktivitäten sind in mU/mg Protein angegeben und als Mittelwerte ± SEM als Vielfaches der Kontrollgruppe (K = 1) dargestellt; unterschiedliche Buchstaben kennzeichnen signifikante Unterschiede (P ≤ 0,05); es wurde je nach Varianzhomogenität der LSD- oder der Games-Howell-Test verwendet.

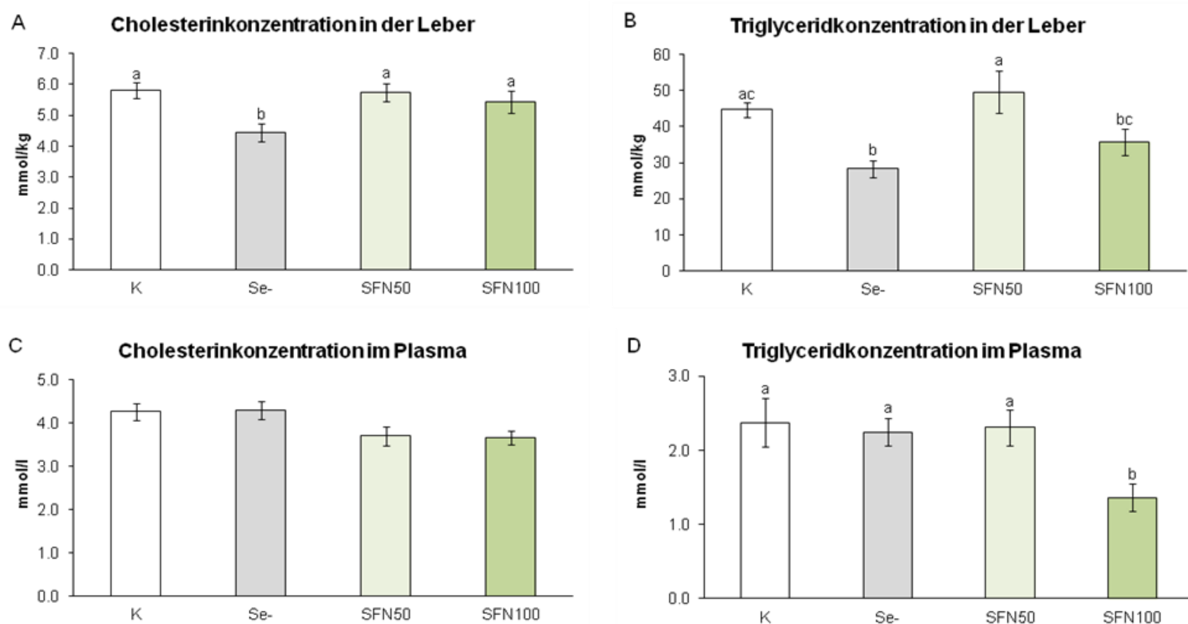

Abbildung 3: Einfluss der Versuchsdiäten auf die Cholesterin- und Triglyceridkonzentrationen in Plasma und Leber

Die Werte sind als Mittelwerte ± SEM dargestellt; unterschiedliche Buchstaben kennzeichnen signifikante Unterschiede (P ≤ 0,05); es wurde je nach Varianzhomogenität der LSD- oder der Games-Howell-Test verwendet.

4. Diskussion

4.1 Einfluss von Selen auf Funktionen des Intermediärstoffwechsels sowie auf Phase-II-Enzyme

4.1.1 Einfluss von Selen auf den Cholesterin- und Triglyceridstoffwechsel

Auf Grund der protektiven Eigenschaften von Selenoproteinen gegenüber oxidativem Stress wird Selen eine Rolle in der Prävention von Krebs und kardiovaskulären Erkrankungen zugeschrieben (Stranges et al. 2010b). Im Gegensatz zu dieser Annahme ist nach aktueller Datenlage die Effektivität von Selen bei der Prävention von Insulinresistenz, Typ II Diabetes und Hyperlipidämien umstritten (s. 1.1.2).

Auch die zu Grunde liegenden Mechanismen für den Einfluss von Selen auf den Lipidstoffwechsel sind noch unklar. Ein Enzym, das in diesem Zusammenhang eine Rolle zu spielen scheint, ist die PTP1B. Diese steuert die Dephosphorylierung der β-Untereinheit des Insulinrezeptors und des Insulinrezeptorsubstrates 1 und wirkt somit dem Insulinsignal entgegen (Koren und Fantus 2007, Tonks 2003). Darüber hinaus ist die PTP1B auch an der Regulation des Fettsäurestoffwechsels beteiligt (Shi et al. 2007, Shimizu et al. 2003). Die PTP1B kann physiologisch durch Wasserstoffperoxid gehemmt werden. Dies erfolgt mittels der reversiblen Oxidation der –SH Gruppe des Cysteinrests 215 im katalytisch aktiven Zentrum des Enzyms zur Sulfensäure (Denu und Tanner 1998). Die weitere Oxidation kann durch die Bildung eines zyklischen Sulphenylamids verhindert werden (Salmeen et al. 2003). Dieses Sulphenylamid kann mit reduziertem und oxidiertem Glutathion durch Glutathionylierung zu einem gemischten Disulfid reagieren (Barrett et al. 1999). Die reversibel oxidierte sowie die glutathionylierte Form der PTP1B können teilweise durch Zusatz von Dithiotreitol (DTT) oder Thioltransferasen wieder in die katalytisch aktive Form umgewandelt werden (Salmeen et al. 2003, Barrett et al. 1999). Da Selen über die Selenoenzyme GPx und TrxR an der Modulierung des Peroxid-Tonus und somit an der Regulation des antioxidativen Systems beteiligt ist, sollte in ***Studie 1*** der Einfluss der Selenkonzentration auf die Regulation der PTP1B an wachsenden Ratten untersucht werden. Es konnte gezeigt werden, dass eine niedrige Selenaufnahme, die mit niedrigen Enzymaktivitäten antioxidativer Selenoenzyme (GPx1, GPx3, GPx4 und TrxR1) und erhöhten Lipidhydroperoxidkonzentrationen einhergeht, zu einer vermehrten Inaktivierung der PTP1B durch Glutathionylierung führt (siehe ***S1***: Tabelle 2 und Abbildung 1). Die Regulation erfolgte nicht nur auf posttranskriptionaler Ebene. Dies zeigte sich durch die unterschiedliche PTP1B-Aktivität, die trotz der Behandlung mit DTT existierte. Außerdem war auch die durch Immunfluoreszenz ermittelte Proteinexpression bei den selensupplementierten Ratten höher

als in der selenarm ernährten Gruppe. Dass eine erhöhte PTP1B-Proteinexpression mit einer gesteigerten Enzymaktivität antioxidativer Enzyme einhergeht, konnte auch in einer Untersuchung mit Katalase-Überexpression gezeigt werden (Dong et al. 2006). Der Zusammenhang zwischen der PTP1B und der Selensupplementation beziehungsweise der Aktivität verschiedener Selenoenzyme (***S1***) wurde in einer weiteren Untersuchung mit Ratten für supranutritive Selenkonzentrationen (1,0 mg und 2,0 mg Selen/kg Diät) bestätigt. Es konnte außerdem gezeigt werden, dass Natriumselenat eine geringere PTP1B-Glutathionylierung als Natriumselenit bewirkte (Mueller et al. 2009).

In Untersuchungen an Ratten, die fruktosereiche Diäten erhielten, ging eine Steigerung der PTP1B-Aktivität mit der Aktivierung von SREBP1c und dessen Zielgen FAS einher. Diese Wirkung konnte in ***Studie 1*** ebenfalls für die Supplementation mit Selen (75 µg und 150 µg Selen/kg Diät) bestätigt werden. Darüber hinaus wurden erhöhte Lebertriglyceridspiegel nachgewiesen. Elchebly et al. (1999) konnten in ihrer Arbeit zeigen, dass sowohl PTP1B$^{-/-}$- als auch PTP1B$^{+/-}$-Mäuse im Vergleich zu Wildtypmäusen bei Fütterung einer Hochfettdiät resistent gegenüber einer Gewichtszunahme waren und insulinsensitiv blieben. Die Autoren schreiben der PTP1B daher eine wichtige Rolle in der Behandlung von Typ II Diabetes und Übergewicht zu. In den eigenen Untersuchungen konnte gezeigt werden, dass die verringerte PTP1B-Aktivität bei selenarm ernährten Ratten im Vergleich zu selensupplementierten Ratten mit niedrigeren Endgewichten einher gingen (***S1, S2*** und ***S3***). Demzufolge könnte die Regulation der PTP1B einen möglichen Mechanismus darstellen, über den Selen die Erhöhung der Triglyceride bedingt und schließlich zu Adipositas führt.

Zudem wurde in den bereits erwähnten Humanstudien (Stranges et al. 2010a, Laclaustra et al. 2010, Bleys et al. 2008) sowie in Tierexperimenten (Dhingra und Bansal 2006a, b) ein Einfluss von Selen auf den Cholesterinstoffwechsel belegt. Dhingra et al. (2006a) konnten zeigen, dass die Supplementation von 1 mg Selen pro kg Diät bei Ratten mit einer cholesterinhaltigen Diät (2 % Cholesterin), im Vergleich zu adäquat mit Selen gefütterten Ratten (200 µg/kg Diät), zu einer Reduktion der Plasmacholesterinspiegel sowie einer Erhöhung der LDLR-Aktivität und -mRNA-Expression führt. In einer weiteren Untersuchung beschreiben die Autoren eine Senkung der HMGCoAR-mRNA-Konzentration sowie niedrigere Konzentrationen an Gesamt-Cholesterin, LDL-Cholesterin und Apolipoprotein B (apoB) (Dhingra et al. 2006b). Auch in ***Studie 2*** führte die Selensupplementation im Vergleich zur selenarmen Fütterung zu einer Erhöhung der hepatischen LDLR-mRNA-Expression, zu einer Senkung des Plasmacholesterins sowie zu einer Erhöhung der Cholesterinkonzentration in der Leber. Allerdings konnte kein Einfluss auf die relative

mRNA-Konzentration der HMGCoAR festgestellt werden. Jedoch unterscheiden sich die beiden Untersuchungen in den verwendeten Selenkonzentrationen. Während Dhingra et al. (2006a) eine Selensupplementation in Höhe der Empfehlung (200 µg/kg Diät) mit einer supranutritiven Selenkonzentration von 1,0 mg pro kg Diät verglichen, wurde in ***Studie 2*** die Verabreichung einer selenarmen Diät mit einer Selensupplementation in Höhe der Empfehlung (150 µg/kg Diät) beziehungsweise in leicht supranutritiver Dosierung (450 µg/kg Diät) geprüft. Die unterschiedliche Regulation der HMGCoAR ist möglicherweise auch auf die verschiedenen Cholesterinkonzentrationen der Diäten zurückzuführen, da Dhingra et al. (2006a) eine Hochcholesterindiät (2 % Cholesterin) fütterten. Die HMGCoAR wird über den Transkriptionsfaktor SREBP2 reguliert. SREBP werden durch Bindung an das SREBP *Cleavage-Activating Protein* (SCAP) zum Golgi-Apparat transportiert, wo die aktive Form des SREBP entsteht. Hohe intrazelluläre Cholesterinkonzentrationen führen zu einer Konformationsänderung von SCAP. Dadurch bleibt SCAP im ER, unterbindet die SREBP-Reifung und vermindert die Lipidsynthese (Yabe et al. 2002). Möglicherweise war also in der Untersuchung von Dhingra et al. (2006a) die Lebercholesterinkonzentration so hoch, dass SREBP2 und somit die HMGCoAR herunterreguliert wurde. In ***Studie 2*** wurde weder die HMGCoAR-, noch die SREBP2-mRNA-Konzentration durch Selensupplementation beeinflusst. Darüber hinaus konnte in ***Studie 2*** als Ursache für die höheren Lebercholesterinkonzentrationen bei den selenversorgten Ratten, im Vergleich zu den selenarm ernährten Ratten, eine Herunterregulation des hepatobiliären Cholesterinexporters ABCG8 festgestellt werden.

Zusammenfassend konnte gezeigt werden, dass eine selenarme Diät bei Ratten sowohl eine Reduktion der Triglyceridkonzentration als auch der Cholesterinkonzentration in der Leber bedingt. Die Regulation der Triglyceride erfolgte vermutlich über die PTP1B, während die gesenkten hepatischen Cholesterinspiegel auf eine vermehrte Expression von ABCG8 und eine verminderte Expression des LDLR zurückzuführen sind. Da sich das Lipidprofil von Ratten und Menschen stark unterscheidet, könnten zukünftige Untersuchungen am Modelltier Schwein durchgeführt werden, da dessen Lipidmetabolismus eher dem humanen Stoffwechsel entspricht.

4.1.2 Einfluss von Selen auf den Homocystein- und Glutathionstoffwechsel

Erhöhte Konzentrationen an Plasmahomocystein werden mit der Entstehung einer Vielzahl von Erkrankungen, wie beispielsweise von kardiovaskulären Erkrankungen, neurodegenerativen Krankheiten, Osteoporose sowie Krebserkrankungen assoziiert (Bekaert

et al. 2008). Der Homocysteinstoffwechsel kann durch verschiedene Faktoren beeinflusst werden. Dazu zählen außer genetischen und physiologischen Einflussfaktoren auch unterschiedliche Nährstoffe wie Folsäure, Vitamin B6 sowie B12 (Clarke et al. 2001, Sumner et al. 1996, Franken et al. 1994). Dabei spielen die Folsäure und das Vitamin B12 vor allem als Cofaktoren bei der Remethylierung eine Rolle, während das Vitamin B6 für die an der Transsulfurierung beteiligten Enzyme CBS und Cystathionase von Bedeutung ist (Bekaert et al. 2008). Auch das Spurenelement Selen wird als potentieller Einflussfaktor auf den Homocysteinstoffwechsel diskutiert. Allerdings sind die aktuellen Forschungsergebnisse diesbezüglich nicht einheitlich. In humanen Querschnittsuntersuchungen wurde eine inverse Korrelation zwischen der Plasmahomocysteinkonzentration und dem Selenstatus festgestellt (Kłapcińska et al. 2005, González et al. 2004, Bates et al. 2002). Zwei Humanstudien, die den Effekt von einer Selensupplementation (200 µg Selenomethionin/Tag für 20 Wochen beziehungsweise 100-300 µg selenreiche Hefe pro Tag für 6 Monate) untersuchten, konnten keine Selen-bedingte Veränderung des Plasmahomocysteinspiegels nachweisen (Bekaert et al. 2008, Venn et al. 2003). Im Gegensatz zu Humanstudien zeigten Tierexperimente an Hühnern, Mäusen und Ratten, dass eine selenarme Ernährung zu einer Senkung der Plasmahomocysteinkonzentration führt (Uthus und Ross 2009, 2007, Uthus et al. 2002, Halpin und Baker 1984). Die Untersuchungen dieser Arbeit (***S2*** und ***S3***) ergaben ebenfalls, dass eine selenarme Ernährung bei Ratten eine starke Reduktion des Plasmahomocysteinspiegels bewirkt. In beiden Studien hatte die Erhöhung der Selenzufuhr von 15 µg pro kg Diät auf 50 µg pro kg Diät eine Verdopplung der Plasmahomocysteinkonzentration zur Folge. Allerdings bewirkte eine Erhöhung der Selensupplementation auf 150 µg beziehungsweise 450 µg pro kg Diät keine weitere Veränderung des Plasmahomocysteins. Der fehlende Effekt der Selensupplementation auf das Plasmahomocystein in Humanstudien ist daher vermutlich auf diesen Schwelleneffekt zurückzuführen. Die niedrigsten Plasmaselenkonzentrationen in den Humanstudien reflektieren keinen starken Selenmangel wie in den Tierstudien, sondern eher einen leichten Selenmangel. Venn et al. (2003) vermuten einen solchen Schwellenwert bei Menschen ab einer mittleren Plasmaselenkonzentration von weniger als 1,2 µmol/l. Demzufolge wäre eine Beeinflussung der Homocysteinwerte durch den Selenstatus möglicherweise bei Populationen mit einer sehr niedrigen Selenaufnahme, wie in einigen Regionen Chinas, möglich (Venn et al. 2003).

Die erniedrigten Plasmahomocysteinwerte unter selenarmer Fütterung gingen mit einer Erhöhung der Gesamtglutathionkonzentration im Plasma und in der Leber einher (***S 2*** und

S3). In Übereinstimmung mit anderen Untersuchungen (Uthus und Ross 2007, Uthus et al. 2002, Hill und Burk 1982) konnte als Ursache hierfür eine vermehrte Aktivierung des Transsulfurierungsweges nachgewiesen werden. Dabei war vor allem die Regulation der GCL von Bedeutung. Die GCL ist das geschwindigkeitsbestimmende Enzym bei der Glutathionbiosynthese und ist für die Bildung des Dipeptids γ-Glutamylcystein aus Glutaminsäure und Cystein verantwortlich. Es handelt sich um ein heterodimeres Enzym, das aus einer katalytischen Untereinheit (GCLC) und einer modulatorischen Untereinheit (GCLM) besteht. In den Untersuchungen dieser Arbeit führte Selenmangel im Vergleich zur Selensupplementation zu einer Erhöhung der relativen mRNA-Konzentration der GCLC (***S2***) sowie der GCLM (***S2*** und ***S3***). Des Weiteren war auch die mRNA-Expression der GS unter Selenmangel deutlich höher als unter Selenversorgung. Sowohl die GCL als auch die GS werden über den Transkriptionsfaktor Nrf2 reguliert (Copple et al. 2010), welcher durch oxidativen Stress, wie er unter Selenmangel vorliegt, induziert wird.

Im Vergleich zu anderen Untersuchungen aus der Literatur wurden in den ***Studien 2*** und ***3*** nicht nur die Homocysteinkonzentrationen im Plasma, sondern auch in der Leber analysiert. Dabei wurden in ***Studie 2*** die höchsten hepatischen Homocysteinwerte in selenarm ernährten Ratten nachgewiesen, obwohl die mRNA-Expression der CBS in dieser Gruppe am höchsten war. Allerdings war die relative mRNA-Konzentration der BHMT und somit die Remethylierung des Homocysteins in der selenarm gefütterten Gruppe niedriger als bei den selenversorgten Ratten. Im Gegensatz hierzu konnte in ***Studie 3*** kein Effekt von Selenmangel auf die Leberhomocysteinkonzentration festgestellt werden. Außerdem war die relative mRNA-Konzentration der BHMT in der selenarm ernährten Gruppe im Vergleich zu den selenversorgten Tieren erhöht. Daher kann auf eine erhöhte Remethylierung von Homocystein geschlossen werden, welche möglicherweise die niedrigeren hepatischen Homocysteinkonzentrationen erklären könnte. Die Ursache für die unterschiedlichen Ergebnisse der beiden Studien ist unklar und bedarf weiterer Untersuchungen. In anderen Rattenstudien wurde, übereinstimmend mit ***Studie 2***, eine erniedrigte mRNA-Konzentration der BHMT sowie der BHMT-Aktivität festgestellt. Allerdings wurde das Leberhomocystein nicht ermittelt. Das SAH in der Leber war jedoch durch Fütterung unterschiedlicher Selenkonzentrationen nicht beeinflusst (Uthus und Ross 2007). Es konnte erstmals gezeigt werden, dass die niedrigeren Plasmahomocysteinkonzentrationen sowie höhere beziehungsweise gleichbleibende Leberhomocysteinkonzentrationen unter Selenmangel mit einem reduzierten Export von Homocystein mittels Slco1a4 einhergehen. Die erhöhten Glutathionkonzentrationen im Plasma sind auf einen gesteigerten Glutathionexport via Mrp4

zurückzuführen. Mrp4 ist für den ATP-abhängigen Cotransport von Glutathion und Gallensalzen über die basolaterale Hepatozytenmembran in das Blutsystem von Bedeutung (Rius et al. 2003).

4.1.3 Wirkungen von Selen auf Nrf2-regulierte antioxidative Enzyme sowie Phase-II-Enzyme

In ***Studie 4*** wurde der Einfluss verschiedener Selenkonzentrationen (<15 µg/kg Diät, 150 µg/kg Diät und 450 µg/kg Diät) auf die mRNA-Konzentration und die Aktivität verschiedener Nrf2-regulierter Gene ermittelt. Im Gegensatz zu anderen Untersuchungen wurden in ***Studie 4*** nicht nur einzelne GST-Isoformen, sondern ein breites Spektrum an GSTs analysiert.

Die Ergebnisse zeigten, dass vor allem Selenmangel die verschiedenen Nrf2-Zielgene beeinflusst. Diese Wirkung war abhängig vom untersuchten Gewebe. In der Leber bewirkte Selenmangel, neben einer Erhöhung der relativen mRNA-Konzentrationen der ARE-regulierten Glutathionbiosyntheseenzyme GCLM und GS (***S3***), auch einen Anstieg der relativen mRNA-Konzentrationen der anderen untersuchten Gene (GPx2, EPHX1, HO1, NQO1, UGT1a6, GSTA3, K1, M5, O1, P1, T1, T2). Dieser Effekt konnte auf Aktivitätsebene für die GSTA, die GSTP und die NQO1 bestätigt werden (***S4***). Die durch Selenmangel-bedingte Induktion verschiedener Nrf2-regulierter Gene in der Leber ist bereits in der Literatur beschrieben (Raines und Sunde 2011, Burk et al. 2008, Olsson et al. 1993, Lawrence et al. 1978, Correia und Burk 1976). In den 70er Jahren konnte bei Ratten gezeigt werden, dass die Aktivität der HO1 während eines Selenmangels erhöht ist (Correia und Burk 1976). In weiteren Untersuchungen führte Selenmangel auch zu Aktivitätssteigerungen der GST und der NQO (Olsson et al. 1993, Lawrence et al. 1978). In einem Mäuseexperiment bewirkte die Fütterung mit einer selenarmen Diät ebenfalls eine Aktivitätssteigerung von NQO, HO1 und GST in der Leber. Außerdem hatte Selenmangel einen Anstieg der hepatischen ARE-Rezeptor Enzymaktivität zur Folge. Die Autoren dieser Arbeit konnten allerdings auch zeigen, dass die Erhöhung der NQO- sowie der HO1-Aktivität nur teilweise über Nrf2 reguliert wird, da eine Deletion von Nrf2 während des Selenmangels immer noch mit einer gesteigerten Enzymaktivität einherging (Burk et al. 2008). In ***Studie 4*** wurde keine Deletion von Nrf2 untersucht. Allerdings ist auch hier davon auszugehen, dass neben dem Nrf2-ARE-System weitere Regulationsmechanismen an der Selenmangel-bedingten Induktion von antioxidativen Enzymen und Phase-II-Enzymen beteiligt sind, da auch GSTs die kein ARE in ihrer Promotorregion aufweisen durch Selenmangel vermehrt exprimiert wurden. Zu den an

der Regulation der GST zusätzlich beteiligten Transkriptionsfaktoren zählen der *Constitutive Androstane Receptor* (CAR), der *Pregnane* X R*eceptor* (PXR), der *Aryl Hydrocarbon Receptor* (AhR) sowie der *Peroxisome Proliferator-Activated Receptor* α (PPARα) (Knight et al. 2008). Bei selenarmer Ernährung war die hepatische mRNA-Konzentration der GSTT1 im Vergleich zu den anderen GSTs am höchsten (siehe ***S4***: Tabelle 4). Auch McLeod et al. (1997) zeigten, dass die GSTT1 während eines Selenmangels überexprimiert wird. Aus der Literatur ist bekannt, dass ein Fehlen der GSTT1 mit einem erhöhten Risiko für verschiedene Krebsarten einhergeht (Landi 2000). Zu den Substraten der GSTT1 zählen unter anderem Dichlormethan, Dibrommethan und 1,2,3,4-diepoxybutan (Sherratt et al. 1997; Thier et al. 1996). Bei Ratten kann auf Grund der erhöhten GSTT1-mRNA-Konzentration während eines Selenmangels vermutet werden, dass diese sensitiver auf die toxischen Effekte dieser Verbindungen reagieren, als selenadäquat gefütterte Ratten (McLeod et al. 1997). Aus der Literatur geht hervor, dass auch hohe Selenkonzentrationen die hepatischen Nrf2-Zielgene induzieren (Zhang et al. 2008, Xiao und Parkin 2006, 't Hoen et al. 2002). Eine Erhöhung der diätetischen Selenkonzentration von 150 µg auf 450 µg pro kg Diät veränderte die relative mRNA-Konzentration der untersuchten Nrf2-Zielgene in der Leber nicht (***S4***). Da die Induktion durch hohe Selenkonzentrationen vermutlich auf vermehrten oxidativen Stress zurückzuführen ist, wären wahrscheinlich noch höhere Selenkonzentrationen notwendig gewesen, um die nukleäre Translokation von Nrf2 zu bewirken. So wurde beispielsweise in einem Versuch mit Mäusen die Induktion der Nrf2-Zielgene durch die intraperitoneale Injektion von 2 mg Natriumselenit erzielt (Zhang et al. 2008).

Im Jejunum konnte kein Einfluss des Selenmangels auf die relative mRNA-Konzentration der untersuchten Nrf2-Zielgene festgestellt werden. Lediglich die relative mRNA-Konzentration der GPx2 war trotz des Selenmangels signifikant erhöht. Die GPx2 unterliegt neben einer Selen-abhängigen Regulation auch einer ARE-Regulation (Banning et al. 2005). Die Dominanz von ARE über Selen bei selenarmer Ernährung lässt auf die protektive Funktion des Enzyms schließen. Außerdem ist die GPx2 auf Grund ihres hohen Ranges in der Hierarchie der Selenoproteine am längsten resistent gegenüber Selenmangel (Wingler et al.1999).

Im Kolon bewirkte Selenmangel nur bei wenigen der untersuchten Gene eine leichte, jedoch nicht signifikante Steigerung der mRNA-Konzentration (***S4***). Im Gegensatz zu den eigenen Ergebnissen führte in einer Untersuchung mit Mäusen ein marginaler Selenmangel (86 µg Selen pro kg Diät) sowohl im Duodenum als auch im Kolon zu erhöhten mRNA-Konzentrationen verschiedener Nrf2-regulierter Gene, während in der Leber kein Effekt auf

die untersuchten Nrf2-Zielgene festgestellt wurde (Müller et al. 2010). Die gegensätzlichen Ergebnisse der eigenen Beobachtungen und der Untersuchung von Müller et al. (2010) könnten zum einen auf die verschiedenen Tierarten (Maus vs. Ratte) und zum anderen auf die unterschiedlichen diätetischen Selenkonzentrationen zurückzuführen sein. Während im Mäuseexperiment durch Fütterung von 86 µg Selen pro kg Diät nur ein marginaler Selenmangel bestand, erhielten die Ratten weniger als 15 µg Selen pro kg Diät. Da bereits eine Selenkonzentration von 50 µg zu einer deutlichen Aktivierung der GPx1 (***S3***) und vermutlich auch der anderen Selenoproteine führt, könnte ein unterschiedlicher oxidativer Status für die verschiedenen Ergebnisse der beiden Untersuchungen verantwortlich sein.

4.2 Einfluss von Methionin auf den Glutathion- und Homocysteinstoffwechsel sowie auf den Cholesterinstoffwechsel

4.2.1 Einfluss von Methionin auf den Homocystein- und Glutathionstoffwechsel

Der Homocysteinstoffwechsel kann, wie bereits erwähnt, durch zahlreiche Nahrungsinhaltsstoffe, wie zum Beispiel Methionin, Vitamin B6, Vitamin B12, Cholin und Folsäure beeinflusst werden (Davis und Uthus 2003). Untersuchungen konnten zeigen, dass beim Menschen zwei Drittel der Homocysteinämien mit einem hohen Konsum an tierischen Proteinen aus Milch und Fleisch, welche sehr methioninreich sind, und einem gleichzeitigen Mangel an den Vitaminen B12, B6 und Folsäure einhergehen (Bieżanowska-Kopeć und Leszczyńska 2012). Auch bei Ratten und Mäusen führte die Fütterung von methioninreichen Diäten zu einer erhöhten Plasmahomocysteinkonzentration (Velez-Carrasco et al. 2008, Hirche et al. 2006a, b). In ***Studie 2*** bewirkte eine fünffache Erhöhung des Methioningehaltes in der Diät in allen Gruppen, unabhängig von der Selenkonzentration, eine Verdoppelung der Plasmahomocysteinkonzentration (***S2***). Dies ist vor allem auf eine verminderte Glutathionbiosynthese zurückzuführen. Die Ergebnisse von Humanstudien, die den Einfluss von Methionin auf den Plasmahomocysteinspiegel untersuchten, sind kontrovers hinsichtlich der kritischen Methioninkonzentration. Ward et al. (2001) zeigten in einer Untersuchung mit gesunden Männern, dass eine erhöhte Methioninaufnahme erst ab der fünffachen Dosis im Vergleich zur normalen Aufnahme zu erhöhten Plasmahomocysteinspiegeln führt. Im Gegensatz dazu konnte bei finnischen Männern sogar ein inverser Zusammenhang zwischen der Methioninaufnahme und der Plasmahomocysteinkonzentration festgestellt werden. Allerdings war hier, trotz der fehlenden Erhöhung des Homocysteins, das Risiko für koronare Erkrankungen durch eine hohe Methioninaufnahme gesteigert (Virtanen et al. 2006). Weitere

Humanstudien konnten keine Erhöhung des Plasmahomocysteins auf Grund einer hohen Methioninaufnahme feststellen (Haulrik et al. 2002, Ward et al. 2000, Shimakawa et al. 1997). Eine mögliche Erklärung für den fehlenden Einfluss der Methioninaufnahme auf den Plasmahomocysteinspiegel beim Menschen ist, dass sich die Enzyme des Methioninstoffwechsels an die hohe Methioninaufnahme anpassen und dadurch eine normale Homocysteinkonzentration in der Zirkulation aufrecht erhalten (Virtanen et al. 2006). Daraus kann geschlussfolgert werden, dass sich der Homocysteinstoffwechsel von Ratten von dem des Menschen unterscheidet.

4.2.2 Einfluss von Methionin auf den Cholesterinstoffwechsel

Es ist bekannt, das pflanzliche Proteine im Vergleich zu Proteinen tierischen Ursprungs in Tier- sowie Humanstudien zu niedrigeren Plasmacholesterinkonzentrationen führen (Carroll und Kurowska 1995, Kritchevsky 1993, Sirtori et al. 1993, Sugano und Koba 1993). Als Ursache wird die unterschiedliche Aminosäurezusammensetzung diskutiert. Vor allem Schwefel-haltige Aminosäuren, wie Methionin, das vor allem in Proteinen tierischen Ursprungs enthalten ist, gelten als bedeutende Einflussfaktoren auf den Lipidmetabolismus (Sugiyama et al. 1997). Die Forschungsergebnisse zum Einfluss von Methionin auf Parameter des Lipidstoffwechsels sind jedoch nicht einheitlich. Die Untersuchungen wurden mit stark unterschiedlichen Methioninkonzentrationen durchgeführt und erzielten teilweise widersprüchliche Ergebnisse. Bei älteren Probanden führte ein Methioninbelastungstest zu gesteigerten Plasmahomocystein- und Triglyceridkonzentrationen sowie zu einer Erhöhung des Gesamtcholesterin-HDL-Verhältnisses (Hart et al. 2006). Auch bei Ratten konnte ein Einfluss von methioninhaltigen Diäten auf die Cholesterin- und Lipoproteinkonzentration nachgewiesen werden (Bieżanowska-Kopeć und Leszczyńska 2012, Hirche et al. 2006 a, b, Sugiyama et al. 1996, 1997). In ***Studie 2*** führte die fünffache Erhöhung der Methioninzufuhr in Kombination mit Selen (150 µg sowie 450 µg Selen/kg Diät) zu einer signifikanten Steigerung der Lebercholesterinkonzentration. Dies stimmt mit den Ergebnissen anderer Rattenversuche überein (Hirche et al. 2006a, Woo et al. 2005). Jedoch konnte in der aktuellen Arbeit durch die Erhöhung der Methioninkonzentration im Vergleich zu anderen Untersuchungen (Hart et al. 2006, Hirche et al. 2006a, Woo et al. 2005) kein Anstieg des Plasmacholesterins festgestellt werden. Allerdings enthielt die Kontrolldiät, entsprechend den Empfehlungen der AIN-93G Diät, bereits 3,0 g Methionin pro kg Diät. Im Gegensatz hierzu konnten Hirche et al. (2006a) bereits mit Methioninkonzentrationen von 3,55 g pro kg Diät im Vergleich zu 2,6 g pro kg Diät einen signifikanten Anstieg des Plasmacholesterins feststellen.

Höhere Methioninkonzentrationen (4,5 g und 6,0 g pro kg Diät) konnten im Vergleich zu der Diät mit 3,5 g Methionin pro kg Diät keine weitere Steigerung des Plasmacholesterins bewirken. Demzufolge könnte die Methioninkonzentration der Kontrolldiäten in ***Studie 2*** (3 g pro kg Diät) schon über der Schwelle für den Plasmacholesterinanstieg liegen. Bieżanowska-Kopeć und Leszczyńska (2012), die Effekte der Methioninsupplementation der AIN-93G Diät auf den Homocystein- und Lipidstoffwechsel untersuchten, konnten bei einer Supplementation mit niedrigen Methioninkonzentrationen (2-8 g/kg Diät) ebenfalls keine Beeinflussung des Plasmacholesterins zeigen. Die Anreicherung der Diät mit 16 g Methionin pro kg Diät führte nur zu einem leichten, nicht signifikanten Anstieg des Plasmacholesterins. Erst die Supplementation von 32 g Methionin pro kg Diät bewirkte eine signifikante Erhöhung der Plasmacholesterinkonzentration. Neben den unterschiedlichen Methioninkonzentrationen der Diäten variierten auch das Alter sowie das Anfangsgewicht der Ratten, obwohl es sich in den drei Studien jeweils um wachsende Ratten handelte. Bei ausgewachsenen Ratten ist der Einfluss des Nahrungsmethionins auf die Plasmacholesterinkonzentration nicht mehr so ausgeprägt wie bei wachsenden Ratten (Hirche et al. 2006b). Auch die Fütterungsdauer variierte zwischen 2 und 6 beziehungsweise 8 Wochen. Demzufolge untersuchten Hirche et al. (2006a) über einem Zeitraum von zwei Wochen nur den kurzfristigen Einfluss von Methionin, während in ***Studie 2*** die längerfristigen Wirkungen von Methionin auf dem Lipidstoffwechsel ermittelt wurden.

Die molekularen Mechanismen, die dem Einfluss von hohen diätetischen Methioninkonzentrationen auf den Lipidstoffwechsel zu Grunde liegen, sind noch nicht exakt geklärt. Es ist auch weitestgehend unbekannt, ob der Einfluss des hohen Nahrungsmethionins auf das Methionin selbst oder auf die dadurch bedingte Erhöhung des Plasmahomocysteins zurückzuführen ist. Woo et al. (2005) gehen davon aus, dass das hohe Plasmahomocystein für die Veränderungen im Cholesterinstoffwechsel verantwortlich ist. Hirche et al. (2006a) dagegen vermuten, dass das Plasmahomocystein nicht selbst die Ursache für den Cholesterinanstieg war, da nur eine schwach positive Korrelation zwischen der Plasmahomocysteinkonzentration und dem Plasmacholesterinwert vorlag. In ***Studie 2*** konnte zwar ein Anstieg des Plasmahomocysteins durch die Erhöhung der Methioninkonzentration erzielt werden, allerdings bestand keine Korrelation mit der Plasmacholesterinkonzentration. Die Ergebnisse einer Rattenstudie, welche die Effekte von 17 g Methionin pro kg Diät untersuchte, lassen darauf schließen, dass die durch Hyperhomocysteinämie bedingte Aktivierung von SREBP2, *cAMP Response Element-Binding Protein* (CREB) und *Nuclear Factor Y* (NF-Y) für die erhöhte Cholesterinbiosynthese verantwortlich ist. Dies geht mit der

transkriptionellen Regulation der hepatischen HMGCoAR-mRNA-Konzentration einher (Woo et al. 2005). Auch in einer weiteren Rattenstudie führte die Erhöhung der Methioninzufuhr auf 6 g pro kg Diät zu einer Erhöhung der HMGCoAR-mRNA-Konzentration (Hirche et al. 2006a). In der eigenen ***Studie 2*** bewirkte die Supplementation von 15 g Methionin pro kg Diät im Vergleich zu Diäten mit 3 g pro kg Diät keine Veränderung der relativen mRNA-Konzentration von SREBP2 und der HMGCoAR. Allerdings führte die hohe Methioninkonzentration zu einem signifikanten Anstieg der mRNA-Konzentration des LDLR. Die vermehrte mRNA-Konzentration des LDLR bei unveränderter Expression des hepatischen Cholesterinexporters ABCG8 liefert eine mögliche Erklärung für die erhöhte Lebercholesterinkonzentration und den fehlenden Anstieg des Plasmacholesterins. In einer anderen Untersuchung wurde jedoch kein Einfluss des Nahrungsmethionins auf die LDLR-mRNA-Konzentration festgestellt (Hirche et al. 2006a). In ***Studie 2*** führte lediglich die Supplementation von 15 g Methionin in Kombination mit einer selenarmen Diät zu einer signifikanten Erhöhung der relativen ABCG8-mRNA-Konzentration. Dies lässt darauf schließen, dass eine selenarme Ernährung in Kombination mit hohen Methioninkonzentrationen die hepatobiliäre Ausscheidung des Cholesterins über den Transporter ABCG8 fördert. In dieser Gruppe konnte allerdings im Vergleich zu den mit Selen supplementierten Gruppen keine Änderung der Lebercholesterinkonzentration ermittelt werden.

4.3 Einfluss von Glucoraphanin- und Sulforaphansupplementation auf Funktionen des Intermediärstoffwechsels und Phase-II-Enzyme

4.3.1 Einfluss von Glucoraphanin- und Sulforaphansupplementation auf den Homocystein- und Glutathionstoffwechsel

Das Genom von Säugetieren enthält zahlreiche ARE-regulierte Gene. Dazu zählen auch die an der Glutathionbiosynthese beteiligten Enzyme GCL und GS (Copple et al. 2010). Das Ziel der ***Studie 3*** war es daher zu untersuchen, ob Sulforaphan über die Aktivierung der ARE-regulierten Gene die Glutathionbiosynthese sowie die Homocysteinkonzentration beeinflussen kann. Die Ergebnisse ergaben, dass die Fütterung mit Glucoraphanin-angereicherten Diäten (700 µmol/kg Diät) bei wachsenden Ratten keinen Einfluss auf die Homocysteinkonzentrationen in Leber und Plasma hat. Lediglich die relative mRNA-Konzentration der CBS wurde durch Glucoraphaninsupplementation reduziert. Dies müsste mit erhöhten Homocysteinwerten und reduzierten Cystathioninkonzentrationen einhergehen. Allerdings war keine signifikante Veränderung der Homocysteinkonzentration in der Leber zu

beobachten. Da jedoch nur die Regulation auf transkriptioneller Ebene analysiert wurde, sollten zukünftige Untersuchungen auch die Regulation auf Ebene der Proteinexpression und der Aktivität berücksichtigen. Die relative mRNA-Konzentration der SAMDC der Ratten mit Selenmangel konnte durch Glucoraphanin signifikant reduziert werden. Die SAMDC ist an der Synthese der Polyamine Spermin und Spermidin beteiligt, welche als krebsfördernd diskutiert werden. SAMDC-Inhibitoren gelten als krebsprotektiv (Milovica et al. 2001). Da Selenmangel mit einem erhöhten Krebsrisiko assoziiert wird (Combs 2005), könnte die Supplementation mit Glucoraphanin dieses Risiko durch die Hemmung der SAMDC senken.
Auch in Bezug auf die Gesamtglutathionkonzentrationen in Leber und Plasma hatte die Fütterung von Glucoraphanin keinen nennenswerten Einfluss. Ebenso konnte keine Wirkung auf die mRNA-Konzentrationen der Glutathionbiosyntheseenzyme GCL und GS festgestellt werden. Die GCL besteht aus zwei Untereinheiten. Die katalytische Untereinheit GCLC ist für die Konjugation von Cystein mit L-Glutamat verantwortlich, während die modulatorische Untereinheit GCLM den K_m-Wert der GCLC für Glutamat erniedrigt und den K_i-Wert für Glutathion erhöht (Chen et al. 2005). Sowohl die katalytische als auch die modulatorische Untereinheit der GCL besitzt in der Promotorregion AREs und beide werden über den Transkriptionsfaktor Nrf2 reguliert (Copple et al. 2010, Shenvi et al. 2009). An Striatumzellen der Ratte konnte gezeigt werden, dass Sulforaphan sowohl die Proteinkonzentration der GCL, als auch die Gesamtglutathionkonzentration erhöht (Mizuno et al. 2011). Bei humanen Prostatazellen führte die Inkubation mit Sulforaphan ebenfalls zu einer Erhöhung des reduzierten Glutathions sowie zu einem Expressionsanstieg der GCL. Dieser wurde allerdings nur für die modulatorische Untereinheit (GCLM) und nicht für die katalytische Untereinheit (GCLC) nachgewiesen (Brooks et al. 2001). Aus der Arbeit von Mizuno et al. (2011) geht nicht hervor, welche GCL-Untereinheit analysiert wurde. Es könnte sich um einen speziesabhängigen Effekt handeln. Die fehlende Wirkung von Glucoraphanin auf die Glutathionbiosynthese in der eigenen Untersuchung könnte möglicherweise darauf zurückzuführen sein, dass nicht ausreichend Sulforaphan in die Leber gelangte um Nrf2 zu aktivieren.
Da von einer Sulforaphansupplementation auf Grund der besseren Bioverfügbarkeit stärkere systemische Effekte als durch die Verabreichung von Glucoraphanin erwartet werden, sollte in ***Studie 5*** die Wirkung von reinem Sulforaphan auf den Glutathion- und Homocysteinstoffwechsel untersucht werden. Die Homocysteinkonzentration im Plasma der Ratten war durch Supplementation von 100 µmol Sulforaphan verglichen mit der Kontrollgruppe erniedrigt ($p = 0{,}060$; siehe 3.5, **Abbildung** 2). Während Glucoraphanin keine

Wirkung auf die relativen mRNA-Konzentrationen der GCL-Untereinheiten aufwies, bewirkte Sulforaphan eine signifikante Erhöhung der GCLM-mRNA-Konzentration (siehe 3.5, **Tabelle** 3). Damit wurde das Ergebnis von Brooks et al. (2001) bestätigt. Die Plasmaglutathionkonzentration war jedoch nur leicht erhöht.

Zusammenfassend konnte gezeigt werden, dass die Supplementation mit Glucoraphanin keinen Einfluss auf die Homocystein- und Glutathionkonzentration besitzt, während Sulforaphansupplementation eine leichte Reduktion des Plasmahomocysteins bewirkt. Da dieser Effekt konzentrationsabhängig war, sollten zukünftige Untersuchungen überprüfen, ob höhere Sulforaphankonzentrationen noch deutlichere Wirkungen erzielen.

4.3.2 Wirkungen von Glucoraphanin- und Sulforaphansupplementation auf Nrf2-regulierte antioxidative Enzyme und Phase-II-Enzyme

Aus der Literatur ist bekannt, dass das Isothiocyanat Sulforaphan vor allem bei der Induktion von Phase-II-Enzymen eine bedeutende Rolle spielt (Fahey and Talalay 1999). Auf diesem Weg, sowie durch die Inhibierung von Phase-I-Enzymen, hemmt Sulforaphan die Karzinogenese auf der Stufe der Initiation (Clarke et al. 2008). In ***Studie 4*** sollte die Wirkung der Sulforaphan-Vorstufe Glucoraphanin auf die Induktion von Phase-II-Enzymen in verschiedenen Organen bestimmt werden. Die Ergebnisse weisen daraufhin, dass die Wirkung des Glucoraphanins stark vom analysierten Organ abhängig ist. Die Untersuchung des Jejunums sollte Aufschluss darüber geben, ob Glucoraphanin selbst eine induzierende Wirkung auf die Phase-II-Enzyme hat, da das untersuchte Brokkoliextrakt keine Myrosinase enthielt. Außerdem konnte *in vitro* gezeigt werden, dass die Verdauungsenzyme des oberen Gastrointestinaltraktes Glucoraphanin strukturell nicht verändern und die bakterielle Hydrolyse zu Sulforaphan erst im Zäkum stattfindet (Lai et al. 2010). Im Jejunum konnte durch Fütterung der Ratten mit dem glucoraphaninhaltigen Brokkoliextrakt auf mRNA-Ebene keine Induktion der Phase-II-Enzyme sowie des Transkriptionsfaktors Nrf2 erzielt werden. Ausnahmen stellten lediglich die mRNA-Konzentrationen der GSTP1 und der GPx2 dar. Die GSTP1 war im Jejunum die einzige durch Glucoraphanin induzierbare GST (***S4***). Sie ist im Vergleich zu den anderen GSTs effektiver bei der Detoxifizierung von elektrophilen α,β-ungesättigten Carbonylverbindungen, die bei Radikalreaktionen von Lipiden entstehen (Berhane et al. 1994). Die GSTs der P-Klasse spielen vor allem bei der Zelltransformation und der Krebsprävention eine Rolle (Nakae et al. 1998, Hu et al. 1997). Die Ergebnisse bezüglich der relativen mRNA-Konzentrationen der Nrf2-regulierten Phase-II-Enzyme weisen darauf hin, dass Glucoraphanin in einer Konzentration von 13 µmol pro Ratte und Tag

nicht ausreicht, um im Jejunum das mRNA-Konzentrations- und Aktivitätsprofil verschiedener antioxidativer Enzyme und von Phase-II-Enzymen bedeutend zu verändern. Darüber hinaus ist Glucoraphanin im Vergleich zu Sulforaphan auf Grund seiner Struktur nicht in der Lage Nrf2 sowie dessen Zielgene zu beeinflussen. Im Gegensatz zur Fütterung der Ratten mit Glucoraphanin führte die orale Applikation von Sulforaphan im Jejunum zur Erhöhung der relativen mRNA-Konzentrationen von NQO1, GSTT1 und UGT1a6 (siehe 3.5, **Tabelle** 4). Dies unterstützt die Hypothese, dass Glucoraphanin im Jejunum noch nicht zu Sulforaphan hydrolysiert wurde, sondern als nicht bioaktives Glucosinolat vorliegt. Außerdem konnte eine dosisabhängige Wirkung des Sulforaphans gezeigt werden. Während 50 µmol Sulforaphan nur zu einer leichten, teilweise nicht signifikanten, Erhöhung der genannten Gene führten, bewirkten 100 µmol Sulforaphan 1,6- bis 3,9-fach gesteigerte relative mRNA-Konzentrationen der GSTT1, der NQO1 und der UGT1a6 im Vergleich zu 50 µmol Sulforaphan. Auch die Aktivität der NQO1 war im Jejunum durch Sulforaphan signifikant erhöht (siehe 3.5, **Tabelle** 5). In einer Untersuchung von Veeranki et al. (2013) führte die orale Gabe von täglich 150 µmol Sulforaphan pro kg Körpergewicht über sieben Tage zu einer 2,2- beziehungsweise 2,4-fachen Steigerung der GST- respektive der NQO1-Aktivität. Die eingesetzte Konzentration von 150 µmol Sulforaphan pro kg Körpergewicht entspricht ungefähr den in ***Studie 5*** verabreichten 50 µmol Sulforaphan pro Ratte. In der eigenen Arbeit führte die Verdopplung der Sulforaphankonzentration auf Ebene der jejunalen NQO1-Aktivität nur zu einer leichten Steigerung, während bezüglich der mRNA-Konzentration eine stärkere Erhöhung erzielt werden konnte. Veeranki et al. (2013) untersuchten zwar nicht die Dosisabhängigkeit, konnten aber zeigen, dass auch die Versuchsdauer eine Rolle spielt. So ergab die Analyse der Sulforaphanäquivalente im Jejunum nach siebenmaliger Applikation signifikant höhere NQO1-Konzentrationen als nach einmaliger Applikation. In den eigenen Untersuchungen konnten die oben genannten Nrf2-regulierten Gene im Jejunum durch Sulforaphan hochreguliert werden, während die relative mRNA-Konzentration der jejunalen GPx2 unverändert blieb. Interessanterweise war die jejunale GPx2 neben der GSTP1 in der Untersuchung mit Glucoraphaninsupplementation das einzige Gen dessen mRNA-Konzentration erhöht wurde, während sich bei den anderen Genen keine Veränderungen ergaben. Eine mögliche Ursache für den fehlenden Anstieg der GPx2-mRNA-Konzentration durch Sulforaphansupplementation könnte darin bestehen, dass auf Grund der Erhöhung der anderen Nrf2-regulierten Gene auf Transkriptions- und Aktivitätsebene der oxidative Stress bereits sehr gering war.

Während Glucoraphanin durch die Verdauungsenzyme des oberen Gastrointestinaltraktes nicht verändert wird, können die Mikroorganismen des Zäkums und des Kolons die Hydrolyse zu Sulforaphan bewirken (Lai et al. 2010). Daher bestand ein weiteres Ziel der ***Studie 4*** darin, den Einfluss von oral verabreichtem Glucoraphanin ohne Myrosinasezusatz auf die Induktion verschiedener Nrf2-regulierter Gene sowie des Transkriptionsfaktors Nrf2 im Kolon zu untersuchen. Für Ratten ist bekannt, dass die Verabreichung von aufgereinigtem Glucoraphanin (550 µmol/kg Körpergewicht), sowohl über die Diät als auch über eine Schlundsonde einen signifikanten Anstieg der NQO1-Aktivität im Kolon bedingt (Zhu et al. 2010, Lai et al. 2008). Die eigenen Ergebnisse bestätigten dies für das Glucoraphanin-haltige Brokkoliextrakt sowohl auf Aktivitäts- als auch auf mRNA-Konzentrationsebene der NQO1. Somit konnte gezeigt werden, dass im Vergleich zu den oben genannten Studien auch schon deutlich geringere Glucoraphaninkonzentrationen (37 µmol/kg Körpergewicht vs. 550 µmol/kg Körpergewicht) ausreichen, um die NQO1 zu induzieren. Dies könnte für die Übertragbarkeit auf den Menschen relevant sein, da kleinere Glucoraphaninkonzentrationen leichter durch die Nahrungsaufnahme (z.B. durch gekochten Brokkoli) erreicht werden können. Darüber hinaus konnte das Glucoraphanin-haltige Brokkoliextrakt auch die relative mRNA-Expression der weiteren untersuchten Nrf2-regulierten Gene erhöhen (mit Ausnahme der GSTA3 und der GSTT1). Des Weiteren waren auch die relative mRNA-Konzentration sowie die Proteinexpression des Transkriptionsfaktors Nrf2 in der mit Glucoraphanin gefütterten Gruppe höher als in der Kontrollgruppe. Lai et al. (2010) zeigten sowohl *ex vivo* als auch *in situ*, dass Mikroorganismen, die natürlicherweise im Zäkum von Ratten vorkommen, dazu fähig sind Glucoraphanin zu hydrolysieren. Das dabei entstandene Sulforaphan kann über die Enterozyten des Zäkums absorbiert werden. Da die Glucoraphaninsupplementation im Jejunum keine Wirkung auf die Nrf2-regulierten Gene hatte, ist die Induktion des Nrf2 und der Nrf2-regulierten Gene im Kolon vermutlich nicht auf Glucoraphanin selbst, sondern auf dessen bioaktives Hydrolyseprodukt Sulforaphan und/oder andere Abbauprodukte zurückzuführen. Die Verabreichung von reinem Sulforaphan führte in ***Studie 5*** zu einer leichten (aber nicht signifikanten) Erhöhung der NQO1-Aktivität im Kolon (siehe 3.5, **Tabelle** 5). Auch die relative mRNA-Konzentration der NQO1 sowie der GSTT1 und der GPx2 konnte durch Sulforaphan gesteigert werden (siehe 3.5, **Tabelle** 4). Lediglich die relative mRNA-Konzentration der UGT1a6 blieb trotz Sulforaphansupplementation unverändert. Dieser Effekt war für die mRNA-Konzentration und die Aktivität von GPx2 und NQO1 abhängig von der eingesetzten Sulforaphankonzentration. Matusheski und Jeffery (2001) konnten an Fischer 344 Ratten zeigen, dass die Schlündelung von Sulforaphan die

NQO- und GST-Aktivität im Kolon im Vergleich zur Kontrollgruppe steigert. Dieser Effekt war jedoch stark konzentrationsabhängig. Sowohl bei der NQO- als auch bei der GST-Aktivität konnte durch 200 µmol Sulforaphan pro kg Körpergewicht keine signifikante Erhöhung erzielt werden. Erst 500 µmol beziehungsweise 1000 µmol Sulforaphan pro kg Körpergewicht bewirkten eine signifikante Aktivitätssteigerung. Dementsprechend könnte der geringe Aktivitätsanstieg der NQO1 sowie die fehlende Induktion der UGT1a6-mRNA-Konzentration auf zu geringe Sulforaphankonzentrationen (150 und 300 µmol/kg Körpergewicht) zurückgeführt werden. Denn auch in dieser Untersuchung konnte gezeigt werden, dass die Induktion der analysierten Gene konzentrationsabhängig ist. In einer weiteren Untersuchung an Fischer 344 Ratten bewirkte die orale Gabe von Sulforaphan (150 µmol/kg Körpergewicht) eine leichte Steigerung der NQO- und GST-Aktivität. Die Autoren konnten zudem zeigen, dass die Aktivität der NQO und der GST im Gastrointestinaltrakt, vom Duodenum bis hin zum Rektum, abnimmt. Die Enzymaktivität korrelierte mit den analysierten Sulforaphan-Gewebekonzentrationen (Veeranki et al. 2013).

Um die systemischen Effekte von Glucoraphanin- und Sulforaphansupplementen zu vergleichen, wurde in den ***Studien 4*** und ***5*** die Leber analysiert. Da die Leber auf Grund des „*First-Pass*"-Effekts hohen Konzentrationen an Xenobiotika und anderen chemischen Stoffen ausgesetzt ist, ist hier die Aktivierung von Abwehrmechanismen besonders wichtig. Dementsprechend könnte die Induktion von antioxidativen und zytoprotektiven Mechanismen durch den Transkriptionsfaktor Nrf2 beim Schutz vor verschiedenen Lebererkrankungen von Bedeutung sein. Die Anreicherung der Diät mit einem glucoraphaninhaltigen Brokkoliextrakt führte auf mRNA-Ebene weder zu einer Erhöhung des Nrf2 noch zu einer Steigerung der untersuchten Nrf2-regulierten Gene. Auch auf Aktivitätsebene konnten keine Veränderungen durch Glucoraphaninsupplementation erzielt werden (***S4***). Zhu et al. (2010) und Lai et al. (2008) stellten fest, dass hitzebehandelte Brokkoliblüten und -sprossen sowie aufgereinigtes Glucoraphanin die hepatische NQO1-Aktivität nicht beeinflussen. Die Autoren vermuten, dass auf Grund einer begrenzten Absorption aus den unteren Darmabschnitten die Plasmakonzentration den notwendigen Schwellenwert nicht erreicht. Folglich könnten höhere Dosierungen eventuell auch zu hepatischen Effekten führen. Darüber hinaus sind bereits kleine Mengen an Myrosinase ausreichend, um die hepatische NQO1-Aktivität zu steigern (Zhu et al. 2010). Demzufolge müsste die Supplementation mit reinem Sulforaphan ebenfalls zu einer Erhöhung der NQO1-Aktivität führen. In ***Studie 5*** bewirkte Sulforaphan eine konzentrationsabhängige Erhöhung der relativen NQO1-mRNA-Konzentration in der Leber. Auf Aktivitätsebene konnte jedoch nur eine leichte, nicht signifikante Steigerung erzielt

werden (siehe 3.5, **Tabelle** 5). Sulforaphan steigerte auch die GPx2-mRNA-Konzentration, während die GSTT1- und die UGT1a6-mRNA-Konzentrationen unverändert blieben. Übereinstimmend bewirkten 150 µmol Sulforaphan pro kg Körpergewicht bei Veeranki et al. (2013) ebenfalls keine beziehungsweise nur eine geringe Steigerung der NQO- sowie der GST-Aktivität. Auch in anderen Organen, wie Herz, Pankreas, Lunge und Prostata, war der Einfluss auf die Enzymaktivitäten eher gering (<Faktor 1,4). Dies ging mit niedrigen Sulforaphankonzentrationen in diesen Organen einher (Veeranki et al. 2013). Möglicherweise könnten auch in der Leber durch Verwendung höherer Sulforaphankonzentrationen Effekte erzielt werden.

Insgesamt konnte mit den ***Studien 4*** und ***5*** gezeigt werden, dass Glucoraphanin- und Sulforaphansupplemente in unterschiedlichen Darmabschnitten eine Induktion von Nrf2 und eines breiten Spektrums an Nrf2-Zielgenen bewirken. Dabei erfolgt eine Glucoraphanin-abhängige Induktion ausschließlich im Kolon, während Sulforaphan vor allem im Jejunum, aber auch im Kolon wirkt. Hieraus kann geschlossen werden, dass sich Glucoraphanin und Sulforaphan auch in ihrer chemopräventiven Wirkung unterscheiden. Während Glucoraphanin vor allem vor Kolonkrebs schützt, könnte Sulforaphan bereits im Dünndarm eine chemopräventive Wirkung haben.

4.3.3 Einfluss von Sulforaphansupplementation auf den Fettstoffwechsel

Bei Mäusen konnte ein Zusammenhang zwischen dem Transkriptionsfaktor Nrf2 und dem Lipidstoffwechsel festgestellt werden (Huang et al. 2010, Kitteringham et al. 2010, Tanaka et al. 2008). Auch für Brokkolisprossen sowie Brokkoliextrakt konnte in Human- und Tierstudien ein Wirkung auf den Lipidmetabolismus nachgewiesen werden (Bahadoran et al. 2012, Rodríguez-Cantú et al. 2011). Daher war das Ziel der ***Studie 5*** den Zusammenhang zwischen Sulforaphansupplementation und dem Fettstoffwechsel zu untersuchen. Da sich Selenmangel, wie bereits beschrieben (s. 4.1.1), positiv auf den Lipidmetabolismus auswirkt, sollte ermittelt werden, ob Sulforaphan ähnlich effektiv ist. Während die selenarme Fütterung die hepatische Cholesterin- und Triglyceridkonzentration senkte, hatte die Supplementation von Sulforaphan keinen Einfluss auf diese Parameter (siehe 3.5, **Abbildung** 3). Jedoch bewirkte Sulforaphan in beiden verwendeten Konzentrationen eine tendenzielle Abnahme der Plasmacholesterinwerte. Die Verabreichung von 100 µmol Sulforaphan führte außerdem zu einer signifikanten Reduktion der Plasmatriglyceride. Während hier die Wirkung von reinem Sulforaphan ermittelt werden sollte, prüfte eine Untersuchung mit männlichen und weiblichen Hamstern die Wirkung von Brokkolisprossen beziehungsweise –extrakten die reich an

Glucoraphanin- oder Sulforaphan waren. Im genannten Experiment führte sowohl die Fütterung mit Brokkolisprossen als auch mit einem sulforaphanhaltigen Brokkolisprossenextrakt, das eine Äquivalenzdosis von 20 µmol Sulforaphan pro Tier und Tag enthielt, bei männlichen und bei weiblichen Hamstern zu einer signifikanten Reduktion der hepatischen Cholesterinkonzentration. Im Plasma dagegen konnten keine Veränderungen hinsichtlich der Cholesterinwerte nachgewiesen werden. Die Autoren vermuten als Grund für die fehlende Korrelation zwischen der Cholesterinkonzentration in der Leber und im Plasma, Unterschiede in der biliären Cholesterinausscheidung, untersuchten diese jedoch nicht (Rodríguez-Cantú et al. 2011). Die Unterschiede zwischen ***Studie 5*** und der Hamsterstudie könnten auf die unterschiedliche Tierart zurückzuführen sein. Die eigenen Ergebnisse zeigten, dass die relative mRNA-Konzentration des Transkriptionsfaktors SREBP2 durch Sulforaphansupplementation gesteigert werden kann (siehe 3.5, **Tabelle** 3). SREBP2 reguliert unter anderem die HMGCoAR sowie den LDLR (Horton et al. 2002). In ***Studie 5*** war allerdings trotz der durch Sulforaphan erhöhten SREBP2-mRNA-Konzentration die relative mRNA-Konzentration der hepatischen HMGCoAR reduziert. Die relative mRNA-Konzentration des LDLR war jedoch signifikant gesteigert. Dies könnte die niedrigere Plasmacholesterinkonzentration infolge einer Sulforaphansupplementation erklären, die aus einer vermehrten Aufnahme in die Leber resultiert. Dass die Lebercholesterinkonzentration im Vergleich zur Kontrollgruppe trotz vermehrter Aufnahme nicht anstieg, ist möglicherweise auf eine erhöhte Cholesterinausscheidung über die Galle durch ABCG8 zurückzuführen. Der Transporter ABCG8 war sowohl auf mRNA-Ebene als auch auf Proteinebene erhöht. Die relative mRNA-Konzentration der Cholesterol-7-alpha-Hydroxylase (Cyp7a1), das Schlüsselenzym der Gallensäuresynthese aus Cholesterin, war jedoch durch Sulforaphansupplementation stark erniedrigt. Diese Reduktion korrelierte positiv mit der relativen mRNA-Konzentration des nukleären Rezeptors Leber-X-Rezeptor α (LXRα), der die Cyp7a1 reguliert (Zhao und Dahlman-Wright 2010). Ein weiteres interessantes Ergebnis der eigenen Arbeit war, dass die Verabreichung von 100 µmol Sulforaphan zur Reduktion der plasmatischen Triglyceridkonzentration um circa 50 % führte. Die hepatische Triglyceridkonzentration wurde jedoch nicht beeinflusst. In der Hamsterstudie wurde nur die Triglyceridkonzentration im Plasma untersucht. Diese konnte bei männlichen Hamstern durch die Verabreichung des sulforaphanreichen Brokkolisprossenextraktes ebenfalls gesenkt werden, während bei weiblichen Hamstern kein Unterschied zwischen der Kontrollgruppe und den behandelten Gruppen festgestellt werden konnte. Untersuchungen der mRNA-Konzentrationen ergaben kaum Korrelationen zwischen den untersuchten Genen (SREBP1, -

2, FAS, ApoB100) und dem Cholesterin senkenden Effekt in der Leber (Rodríguez-Cantú et al. 2011). In ***Studie 5*** dagegen bewirkte die Verabreichung von 100 µmol Sulforaphan eine leichte Abnahme der SREBP1c- sowie der FAS-mRNA-Konzentration. Auch in Humanstudien bewirkte der Verzehr von Brokkolisprossen eine Senkung der Triglyceridkonzentration im Serum sowie des Verhältnisses von oxidiertem LDL zu LDL. Außerdem konnte die Konzentration des HDLs erhöht werden (Bahadoran et al. 2012).

Während in anderen Untersuchungen Brokkolisprossen sowie deren Extrakte eingesetzt wurden, sollte in der eigenen Arbeit die direkte Wirkung des Isothiocyanates Sulforaphan ermittelt werden. Es konnte erstmals gezeigt werden, dass die Supplementation von 100 µmol Sulforaphan bei männlichen Ratten eine Reduktion der Cholesterin- und Triglyceridkonzentrationen im Plasma bewirkt. Des Weiteren konnte als mögliche Ursache für diese Effekte eine veränderte mRNA-Konzentration verschiedener am Lipidmetabolismus beteiligter Gene demonstriert werden. Da diese Gene nicht nur auf transkriptionaler Ebene reguliert werden, sondern auch posttranskriptional und posttranslational, sollte dies bei zukünftigen Untersuchungen berücksichtigt werden.

4.3.4 Wechselwirkungen von Selen- und Glucoraphaninsupplementation

Wie bereits erwähnt wurde, sind sowohl Selen als auch Sulforaphan an der Regulation des Transkriptionsfaktors Nrf2 beteiligt. Beiden wird außerdem eine chemopräventive Wirkung zugesprochen. In den vergangenen Jahren wurde die Hypothese etabliert, dass Selen und Sulforaphan vor allem in Bezug auf ihre chemopräventive Wirkung synergistisch wirken (Brigelius-Flohé und Banning 2006). In ***Studie 4*** konnte erstmals *in vivo* an Ratten gezeigt werden, dass Glucoraphanin in Kombination mit einer leicht supranutritiven Selenkonzentration (450 µg Selen pro kg Diät) verschiedene Nrf2-regulierte Gene stärker induziert als Glucoraphanin in Kombination mit der empfohlenen Selenkonzentration (150 µg Selen pro kg Diät). Allerdings konnte dieser synergistische Effekt auf Ebene der Enzymaktivität für die GPx, die GST der Klassen A und P sowie die NQO1 nicht nachgewiesen werden. Daher sollte in zukünftigen Untersuchungen auch die Proteinebene mittels Immunoblot oder ELISA berücksichtigt werden. Außerdem war auch eine synergistische Wirkung in Bezug auf die relative mRNA-Konzentration von Keap1 zu beobachten, während die relative mRNA-Konzentration des Transkriptionsfaktors Nrf2 im Vergleich zu der Gruppe mit adäquater Selenversorgung durch die Kombination aus Glucoraphanin und 450 µg Selen pro kg Diät nur leicht höher war. Demzufolge könnte im weiteren Zeitverlauf auch auf Ebene der mRNA-Expression eine Gegenregulation folgen.

Hierzu sind weitere Studien notwendig um die Langzeitwirkung von Glucoraphaninsupplementen in Kombination mit leicht supranutritiven Selenkonzentrationen zu untersuchen. Bisher konnte *in vitro* an HepG2 Zellen demonstriert werden, dass es synergistische Effekte zwischen Sulforaphan und Selen bezüglich der mRNA-Expression und Aktivität der TrxR1 gibt. Dadurch waren die Zellen vor oxidativem Stress geschützt. Diese Wirkung ist vor allem auf die Steigerung der TrxR1-mRNA-Konzentration durch Nrf2 sowie auf die anschließende Erhöhung der Proteinlevel und die spezifische Enzymaktivität durch Selen zurückzuführen (Zhang et al. 2003). Auch an der humanen Endothelzelllinie Eahy926 konnte bestätigt werden, dass Selenit und Sulforaphan in Bezug auf die TrxR1-Expression synergistisch wirken. Dieser synergistische Effekt konnte auch bei Induktion von oxidativem Stress via *tert*-Butylalkohol erzielt werden (Campbell et al. 2007). Neben der TrxR1 ist die GPx2 ein Selenoenzym, welches ein ARE in der Promotorregion aufweist. Beiden Selenoproteinen werden zytoprotektive Effekte sowie mögliche chemopräventive Eigenschaften zugeschrieben (Barrera et al. 2012). In ***Studie 5*** konnte gezeigt werden, dass auch die mRNA-Konzentration der GPx2 durch Glucoraphaninsupplementation erhöht wird. Barrera et al. (2012) untersuchten an Kolonkrebszellen den Einfluss von Selenit und Selenomethylselenocystein sowie von Sulforaphan auf die Induktion von TrxR1 und GPx2. Die Autoren konnten zeigen, dass eine Kombination aus Selenit und Sulforaphan die Proteinexpressionen von GPx2 und TrxR1 nach 24-stündiger Inkubation stärker erhöht als die Einzelsubstanzen. Außerdem bewirkte die Kombination von Selen und Sulforaphan einen stärkeren Schutz der Zellen nach Inkubation mit Wasserstoffperoxid als die Einzelkomponenten. Der Knockdown von Nrf2 führte zu einer geringeren Zellviabilität als ein GPx2- und TrxR1-Knockdown. Dies lässt darauf schließen, dass neben der GPx2 und der TrxR1 auch weitere Nrf2-Zielgene am Selen- und Sulforaphan-induzierten Schutz der Zellen vor oxidativem Stress beteiligt sind. Allerdings muss dieser Effekt kritisch betrachtet werden, da es sich um eine Krebszelllinie handelt. Die Kombination aus Selen und Sulforaphan könnte die Kanzerogenese aufrechterhalten, indem die Krebszellen vor Zelltod durch oxidativen Stress bewahrt werden. Die Autoren vermuten jedoch, dass dieser protektive Effekt auch auf gesunde Zellen übertragbar ist (Barrera et al. 2012). Daher ist es notwendig zu unterscheiden, ob es sich um gesunde oder um maligne Zellen handelt (Barrera et al 2012, Brigelius-Flohé et al. 2012).

Die Ergebnisse aus ***Studie 4*** bestätigen, dass Selen und Glucoraphanin synergistisch wirken. Im Gegensatz zu anderen Studien handelt es sich nicht um *in vitro* Untersuchungen, sondern um Ergebnisse aus einem *in vivo* Versuch an Ratten. Der synergistische Effekt konnte nicht

nur für die GPx2, sondern auch für weitere Nrf2-regulierte Gene gezeigt werden. Allerdings wurde die synergistische Wirkung nur im Kolon und nicht in der Leber oder im Jejunum erzielt. Daher könnte eine Kombination aus Selen und Glucoraphanin einen optimalen Schutz vor Kolonkrebs bieten. Da die Aufnahme von Fremdstoffen aus dem Dickdarm vermindert ist, sinkt eventuell auch das Risiko für andere Krebsarten. Um weitere Aussagen zur chemopräventiven Wirkung von leicht supranutritiven Selenkonzentrationen in Kombination mit Glucoraphanin beziehungsweise Sulforaphan zu machen, sind weitere Untersuchungen nötig.

5. Zusammenfassung

Das essentielle Spurenelement Selen ist Bestandteil verschiedener Selenoproteine. Diese schützen vor oxidativem Stress und ihnen werden protektive Wirkungen gegenüber verschiedenen Erkrankungen wie Krebs und der koronaren Herzkrankheit zugeschrieben. Allerdings ist die Studienlage zum Einfluss von Selen in Bezug auf verschiedene Erkrankungen uneinheitlich. In dieser Arbeit sollte daher die Wirkung von verschiedenen Selenkonzentrationen auf den Lipid-, Homocystein- und Fremdstoffmetabolismus geprüft werden sowie mögliche zu Grunde liegende Mechanismen analysiert werden.

In der Literatur wird beschrieben, dass ein hoher Selenstatus respektive eine Selensupplementierung, das Risiko für Hyperlipidämien erhöht. Im Rahmen dieser Arbeit wurde die Wirkung von drei verschiedenen Selenkonzentrationen (<20 µg, 75 µg und 150 µg Selen/kg Diät) auf den Fettstoffwechsel ermittelt. Überdies wurden die Aktivität und die Regulation der PTP1B als mögliche Trigger für Leberverfettung und Hyperlipidämie analysiert. Die PTP1B ist ein zytosolisches Enzym, das an der Balancierung des Insulinsignals beteiligt ist und über die Aktivierung des SREBP1c und der FAS die Lipogenese fördert. Es konnte gezeigt werden, dass eine selenarme Fütterung der Ratten zu einer Reduktion der hepatischen Triglyceridkonzentration sowie zu einer Abnahme der relativen mRNA-Konzentrationen von SREBP1c und dessen Zielgen der FAS führt. Dies ging mit einer Inhibierung der PTP1B durch eine posttranslationale Modifizierung über die Anlagerung von Glutathion (Glutathionylierung) einher. Dieser Mechanismus wird möglicherweise durch höhere Peroxidspiegel bei niedriger Selenzufuhr verstärkt. Des Weiteren war auch der hepatische Cholesterinspiegel im Selenmangel erniedrigt. Als mögliche Ursachen hierfür wurden eine vermehrte Expression des hepatischen Cholesterinexporters ABCG8 und eine verminderte mRNA-Konzentration des hepatischen LDLR ermittelt.

In der Literatur wird auch der Einfluss von Selen auf das Homocystein, als Risikofaktor für kardiovaskuläre Erkrankungen, kontrovers diskutiert. Eine hohe Methioninzufuhr wird als weiterer möglicher Risikofaktor für Hyperhomocysteinämien in Betracht gezogen. Daher sollte in dieser Arbeit die Wirkung verschiedener Selenkonzentrationen in Kombination mit zwei verschiedenen Methioninkonzentrationen (3 g und 15 g pro kg Diät) auf den Homocystein- und Glutathionstoffwechsel untersucht werden. Es stellte sich heraus, dass eine selenarme Ernährung bei Ratten zu einer reduzierten Plasmahomocysteinkonzentration und zu einer erhöhten Gesamtglutathionkonzentration im Plasma und in der Leber führt. Als Ursache hierfür konnte eine vermehrte Aktivierung des Transsulfurierungsweges durch eine Erhöhung

der relativen mRNA-Konzentrationen von GCL und GS nachgewiesen werden. Außerdem wurde erstmals gezeigt, dass Änderungen der Gesamtglutathion- und Homocysteinkonzentration im Plasma und in der Leber möglicherweise auf einem verminderten Homocysteinexport durch Reduktion der Slco1a4-mRNA-Konzentration und auf einem vermehrten Glutathionexport in das Plasma durch eine erhöhte mRNA-Konzentration des Transporters Mrp4 basieren. Darüber hinaus wurde festgestellt, dass eine Verfünffachung des diätetischen Methioningehaltes zu einer Erhöhung der Plasmahomocysteinkonzentration sowie zu einem Anstieg des hepatischen Cholesterinwertes führt. Da diese Versuchsergebnisse am Modell Ratte nicht mit den Daten aus Humanstudien übereinstimmen, kann geschlussfolgert werden, dass sich die Regulation des Homocysteinstoffwechsels bei Ratten und beim Menschen unterscheidet. Ferner wurde bestätigt, dass eine selenarme Fütterung die Induktion von Nrf2-regulierten Genen bewirkt. Dieser Effekt ist allerdings vom untersuchten Organ abhängig und konnte nur in der Leber gezeigt werden.

Neben erhöhtem oxidativem Stress durch Selenmangel gilt auch das Isothiocyanat Sulforaphan als potenter Induktor für Nrf2-regulierte Gene. Sulforaphan ist ein sekundärer Pflanzenstoff, der aus seiner Vorstufe Glucoraphanin mittels Hydrolyse entsteht und vor allem in Kreuzblütengewächsen enthalten ist. Ein weiteres Ziel dieser Arbeit war es daher, die Wirkung von Glucoraphanin und Sulforaphan auf ein breites Spektrum an Nrf2-regulierten Genen in verschiedenen Organen zu ermitteln. Da sowohl die GCL als auch die GS ein ARE besitzen, sollte zunächst die Wirkung von Glucoraphanin (700 µmol/kg Diät) und Sulforaphan (50 µmol oder 100 µmol pro Ratte und Tag) auf den Glutathion- und Homocysteinstoffwechsel geprüft werden. Während Glucoraphanin keinen nennenswerten Einfluss auf die Homocystein- und Gesamtglutathionkonzentration sowie auf die Glutathionbiosyntheseenzyme hatte, führten 100 µmol Sulforaphan zu einer Erhöhung der relativen mRNA-Konzentration der GCLM sowie zu einer leichten Reduktion des Plasmahomocysteins. Des Weiteren wurden die Effekte von Glucoraphanin und Sulforaphan in Bezug auf antioxidative Enzyme und Phase-II-Enzyme im Jejunum, im Kolon und in der Leber der Ratten untersucht. Dabei wurde eine organabhängige Induktion der untersuchten Enzyme festgestellt. Während Glucoraphanin hauptsächlich im Kolon eine Aktivierung der Nrf2-Zielgene bewirkte, führte Sulforaphan vor allem im Jejunum, aber auch im Kolon zu einer Induktion dieser Gene. Systemische Effekte in der Leber konnten durch Glucoraphanin nicht und durch Sulforaphan nur in geringem Maße erzielt werden.

In der aktuellen Literatur wird ein Zusammenhang zwischen der Sulforaphanaufnahme und dem Lipidmetabolismus vermutet. Ein weiterer Aspekt dieser Arbeit bestand daher darin, mögliche Effekte von Sulforaphan auf den Fettstoffwechsel männlicher Ratten zu ermitteln. Dabei wurde erstmals gezeigt, dass die Verabreichung von 100 µmol Sulforaphan eine Senkung der Cholesterin- und Triglyceridkonzentrationen im Plasma bewirkt. Als mögliche Ursache wurden veränderte mRNA-Konzentrationen des nukleären Rezeptors LXRα, sowie verschiedener am Lipidmetabolismus beteiligter Transkriptionsfaktoren und deren Zielgene ermittelt.

Außerdem wurden die Wechselwirkungen von Selen und Glucoraphanin untersucht, da beide an der Regulation des Transkriptionsfaktors Nf2 beteiligt sind sowie chemopräventive Wirkungen aufweisen. Demzufolge könnten supranutritive Selenkonzentrationen in Kombination mit Glucoraphanin besser vor Kolonkrebs schützen, als Selen oder Glucoraphanin alleine. Es wurde festgestellt, dass Glucoraphanin in Kombination mit 450 µg Selen pro kg Diät im Kolon höhere relative mRNA-Konzentrationen der untersuchten Nrf2-Zielgene bewirkte, als in Kombination mit 150 µg Selen pro kg Diät.

Zusammenfassend wurde festgestellt, dass der Zusammenhang zwischen der Selenzufuhr und der Entstehung verschiedener Erkrankungen nach wie vor kritisch betrachtet werden sollte. Das Glucosinolat Glucoraphanin kann dabei sowohl synergistisch als auch antagonistisch wirken. Außerdem wurde gezeigt, dass Glucoraphanin und Sulforaphan möglicherweise vielversprechend in Bezug auf die Prävention und Therapie von Kolonkrebs und Hyperlipidämien wirken. Zukünftige Studien sollten daher weitere Aspekte der Dosis-Wirkungs-Beziehungen untersuchen. Darüber hinaus sollten die zu Grunde liegenden Mechanismen noch detaillierter geprüft werden. Dabei sollte besondere die Proteinregulation im Mittelpunkt stehen.

6. Summary

The essential trace element selenium is an integral part of different selenoproteins. These so-called functional selenoproteins protect from oxidative stress and are believed to mediate protection against various diseases like cancer and coronary heart disease. However, the results of studies investigating the role of selenium with regard to different diseases are inconsistent. The present study should thus contribute to understanding the effects and the mechanisms by which different selenium concentrations influence the metabolism of lipids, homocysteine and xenobiotics.

Current research postulates that a high selenium status or selenium supplementation increases the risk of hyperlipidemia. The present study therefore investigated the effects of three different selenium concentrations (<20 µg, 75 µg and 150 µg selenium/kg diet) on lipid metabolism. In addition, the activity and regulation of PTP1B, a possible trigger of non-alcoholic fatty liver disease (NAFLD) and hyperlipidemia were analysed. PTP1B is a cytosolic enzyme that is involved in the balance of insulin signalling and that promotes lipogenesis by activating SREBP1c and FAS. It could be shown that feeding rats a selenium-deficient diet reduced the animals' hepatic triglyceride concentration and decreased the relative mRNA levels of SREBP1c and its target gene FAS. These changes were accompanied by a reduced PTP1B activity due to the posttranslational modification of the enzyme via the addition of glutathione to the active site cysteine residue (glutathionylation). This particular inhibition mechanism is presumably enhanced through the existence of increased peroxide levels under low dietary selenium availability. Moreover, hepatic cholesterol concentration was reduced in selenium deficiency. It is possible that an increased expression of the hepatic cholesterol exporter ABCG8 and the reduced mRNA concentration of the hepatic LDLR caused this effect.

In the literature also controversial reports on the role of selenium on homocysteine, a risk factor for cardiovascular diseases, exist. A high dietary methionine intake is considered another possible risk factor for hyperhomocysteinemia. The present study thus investigated the influence of different dietary selenium concentrations in combination with two different methionine concentrations (3 g and 15 g per kg diet) on homocysteine and glutathione metabolism. It could be shown that rats that were fed a selenium-deficient diet had significantly reduced plasma homocysteine concentrations and increased total glutathione levels in their plasma and liver. An increased pull on the transsulphuration pathway through the augmented expression of the glutathione biosynthesis enzymes GCL and GS may cause these effects. Furthermore, it could be demonstrated for the first time that the change of total

glutathione and homocysteine levels in plasma and liver possibly derives from a decreased homocysteine export and an increased glutathione export into the plasma. It could also be shown that a fivefold increase in dietary methionine drastically augmented plasma homocysteine and hepatic cholesterol levels. Since these results were obtained from a rat trial and are not completely consistent with data from human studies, it can be concluded that the regulation of homocysteine metabolism differs between humans and rats. In the present study, selenium deficiency turned out to be a trigger for the induction of Nrf2-regulated genes. This observation is in accordance with results of other trials. However, the specifity and strength of this effect depends on the organ analysed and could be identified only in the liver.

In addition to oxidative stress during selenium deficiency, the isothiocyanate sulforaphane is also a potent inducer of Nrf2-regulated genes. Sulforaphane is a secondary plant compound of crucifers that is liberated from its precursor glucoraphanin via hydrolysis. In this context, another main objective of the present study was to examine the influence of glucoraphanin and sulforaphane on the induction of a broad spectrum of Nrf2-regulated genes in various organs. Since both GCL and GS are genes regulated via an ARE, the influence of glucoraphanin (700 µmol/kg diet) and sulforaphane (50 µmol or 100 µmol per rat and day) on glutathione and homocysteine metabolism was investigated. Glucoraphanin had no influence on either the enzymes of glutathione biosynthesis or on total glutathione and homocysteine concentration. In contrast, 100 µmol sulforaphane increased the relative mRNA concentration of GCLM and slightly reduced plasma homocysteine. Moreover, the effects of glucoraphanin and sulforaphane regarding antioxidant enzymes and phase II enzymes in jejunum, colon and liver of rats were investigated. An organ-dependent induction of the enzymes analysed was detected. While glucoraphanin was effective mainly in the colon, sulforaphane induced Nrf2 target genes mainly in the jejunum but also in the colon. Whereas glucoraphanin had no systemic effects in the liver, sulforaphane had small effects on liver ARE-regulated genes.

Current research assumes a link between sulforaphane intake and lipid metabolism. The present study thus sought to determine possible effects of sulforaphane on lipid metabolism in male rats. It could be shown for the first time that the administration of 100 µmol sulforaphane reduced cholesterol and triglyceride levels in the plasma. Changes in the mRNA concentrations of the nuclear receptor LXRα and of other transcription factors related to lipid metabolism and of their target genes could be identified as possible reasons for this effect.

Another aim of this study was to examine the interaction of selenium and glucoraphanin, because both substances have chemopreventive effects due to their involvement in the regulation of the transcription factor Nrf2. It could be shown that glucoraphanin in

combination with 450 µg selenium per kg diet increased the relative mRNA concentrations of Nrf2 target genes to a higher extent than glucoraphanin combined with 150 µg selenium per kg diet. Therefore, slightly supranutritive selenium concentrations in combination with glucoraphanin may provide better protection against colon cancer than selenium or glucoraphanin alone.

In sum, the present study contributes to a more detailed understanding of the connection between selenium intake and the development of different diseases. It can be concluded that taking selenium supplements should still be viewed critically. The glucosinolate glucoraphanin could be demonstrated to have both selenium-synergistic and selenium-antagonistic effects. Furthermore, it could be demonstrated that glucoraphanin and sulforaphane are promising substances for the prevention and therapy of colon cancer and hyperlipidemia. Future studies should investigate the dose-response effects of glucoraphanin and sulforaphane in more detail. These studies should focus on the examination of differential protein regulation by glucoraphanin and sulforaphane using two-dimensional difference gel electrophoresis.

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Lebenslauf

Name:	Blum
Geburtsname:	Wolf
Vorname:	Nicole Michaela
Geburtsdatum:	05.10.1981
Geburtsort:	Heidelberg
Familienstand:	verheiratet

Schulausbildung

1988 – 2001	Besuch allgemeinbildender Schulen, Grundschule Mainaschaff und Friedrich-Dessauer-Gymnasium Aschaffenburg **Abschluss: Allgemeine Hochschulreife**

Studium

10/2001 – 09/2004	Studium der Ökotrophologie an der Justus-Liebig-Universität in Gießen **Abschluss: Bachelor of Science**
10/2004 – 11/2006	Studium der Ernährungswissenschaften an der Justus-Liebig-Universität in Gießen **Abschluss: Master of Science**

Berufliche Tätigkeiten

10/2007 – 03/2009	Wissenschaftliche Mitarbeiterin am Institut für Tierernährung und Ernährungsphysiologie Justus-Liebig-Universität, Gießen
04/2009 – 07/2013	Wissenschaftliche Mitarbeiterin am Institut für Agrar- und Ernährungswissenschaften Abteilung „Präventive Ernährung“ Martin-Luther-Universität, Halle-Wittenberg

Walldürn, den

Nicole Blum

Weitere Publikationen

Mueller K, **Blum NM**, Mueller AS. (2013) Examination of the Anti-Inflammatory, Antioxidant, and Xenobiotic-Inducing Potential of Broccoli Extract and Various Essential Oils during a Mild DSS-Induced Colitis in Rats. ISRN Gastroenterol. 2013:710856. doi: 10.1155/2013/710856.

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Mueller K, **Blum NM**, Mueller AS. (2013): Influence of dextran sulfate sodium (DSS) on real-time polymerase chain reaction amplification in the colon of rats exposed to DSS. Proceedings of the Society of Nutrition Physiology Vol. 22, 72, ISBN: 978-3-7690-4106-4, Seite 102

Blum NM, Mueller K, Mueller AS. (2012) Influence of selenium deficiency and sulforaphane on lipid metabolism in growing rats. 28th GMS Conference at Karlsruhe University. 11th to 13th October

Mueller K, **Blum NM**, Kluge H, Wendler K, Mueller AS. (2012) Effects of broccoli extract and essential oils on xenobiotic enzymes in the intestine of piglets. Proceedings of the Society of Nutrition Physiology, Vol. 21, ISBN: 978-3-7690-4105-7

Blum NM, Müller K, Müller AS. (2012) Influence of selenium (Se) and glucoraphanin (GRA) on Nrf2-regulated genes in intestine and liver of growing rats. Deutsche Gesellschft für Ernährung (DGE): Proc. Germ. Nutr. Soc., Vol 17, ISBN: 978-3-88749-230-4

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Blum NM, Mueller K, Mueller AS. (2011) Influence of selenium and sulforaphane on homocysteine metabolism in growing rats. International Oskar Kellner Symposium Warnemünde. 9th to 11th September, ISSN: 0946-1981.

Mueller K., **Blum NM**, Kluge H, Mueller AS. (2010) Einfluss verschiedener phytogener Futterzusätze auf die Expression von Phase II Enzymen und weiterer Parameter des antioxidativen Schutzsystems in unter-schiedlichen Darmabschnitten sowie in der Leber wachsender Ross 308 Broiler. Tagungsband 11. Tagung Schweine- und Geflügelernährung, Universitätsdruckerei der Martin-Luther-Universität Halle-Wittenberg, ISBN: 978-3-8304-1082-9.

Wolf NM, Mueller K., Pallauf J., Mueller AS. (2009) Selenium and methionine affect homocysteine and cholesterol metabolism in growing rats. 25th.GMS Conference at Vienna University. 1st to 3rd October, ISBN: 978-3-8316-4090-4.

Mueller AS, **Wolf NM**, Pallauf J. (2008) PTP1B seems to be one molecular link between selenium and liver triglyceride metabolism. 24th.GMS Conference at Berlin University. 13th to 15th November.

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit mit dem Titel: **„Einfluss von Selen auf ausgewählte Funktionen des Intermediärstoffwechsels und die differenzielle Regulation von Phase-II-Enzymen sowie die antagonistische und synergistische Wirkung von Methionin und Glucoraphanin auf diese Prozesse“** selbstständig und ohne fremde Hilfe verfasst habe. Es wurden keine anderen als die in der Arbeit angegebenen Quellen und Hilfsmittel benutzt. Die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Hiermit erkläre ich, dass ich noch keine vergeblichen Promotionsversuche unternommen habe und die vorliegende Dissertation nicht in der gegenwärtigen bzw. in einer anderen Fassung bereits einer anderen Fakultät / anderen wissenschaftlichen Einrichtung vorgelegt habe.

Walldürn, den

Nicole Blum