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„Experimentelle Untersuchungen zur Wirkung von erhitzten Fetten auf ausgewählte Parameter des Lipidstoffwechsels und der Atherogenese“

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Inhaltsverzeichnis

Abbildungsverzeichnis

Abkürzungsverzeichnis

13-HODE	13-Hydroxy-9,11-octadecadiensäure
13-HPODE	13-Hydroperoxyoctadeca-9,11-diensäure
ABCA1	*adenosine triphosphate binding cassette transporter A1*
ABCG1	*adenosine triphosphate binding cassette transporter G1*
ACO	Acyl-CoA-Oxidase
AMPK	*adenosine monophosphate-activated protein kinase*
AP-1	*activator protein-1*
Apo A-I	Apolipoprotein A-I
CD	*cluster of differentiation*
CDK	*cyclin dependent kinase*
COX-2	*cyclooxygenase-2*
CPT1	Carnitin-Palmitoyl-Transferase 1
CYP4A1	Cytochrom P450 A1
CYP4A10	Cytochrom P450 A10
DNA	*desoxyribonucleic acid*
FAS	*fatty acid synthase*
h	Stunden
HDL	*high-density lipoproteins*
ICAM-1	*intercellular adhesion molecule-1*
IKK	*inhibitor of kappa B kinase*
IL	*interleukin*
IκBα	*inhibitor of kappa B alpha*
LCAD	*long chain acyl CoA dehydrogenase*
LDL	*low-density lipoproteins*
LDLR	*low-density lipoprotein receptor*

LPL	Lipoproteinlipase
LPOP	Lipidperoxidationsprodukte
LXRα	*liver X receptor alpha*
MCAD	*middle chain acyl CoA dehydrogenase*
MCP-1	*monocyte chemoattactant protein-1*
MIP-1α	*macrophage inflammatory protein-1 alpha*
mRNA	*messenger ribonucleic acid*
NF-κB	*nuclear factor kappa B*
Nrf2	*nuclear factor-erythroid 2-related factor 2*
PPARα	*peroxisome proliferator-activated receptor alpha*
PPRE	*peroxisome proliferator response element*
RCT	reverser Cholesteroltransport
RNA	*ribonucleic acid*
ROS	reaktive Sauerstoffspezies
RXR	Retinsäure-X-Rezeptor
SR	*scavenger receptor*
SREBP	*sterol regulatory element-binding protein*
STAT	*signal transducers and activators of transcription*
TGF-β	*transforming growth factor beta*
TNF-α	*tumor necrosis factor-alpha*
VCAM-1	*vascular cell adhesion molecule-1*
VLDL	*very low-density lipoproteins*

1. Einleitung

Die Aufnahme thermisch behandelter Fette über Lebensmittel durch den Menschen steigt infolge der Expansion von Schnellrestaurants, der Beliebtheit von Fertiggerichten sowie durch Lebens- und Ernährungsgewohnheiten. In westlichen Industrienationen sind erhitzte und frittierte Speisen auf Grund ihrer schnellen und kostengünstigen Zubereitung sowie wegen ihrer sensorischen Eigenschaften in Bezug auf Geruch, Geschmack und Textur sehr populär. Lipide durchlaufen während der thermischen Behandlung von Lebensmitteln einen Zerfallsprozess, der als Lipidoxidation bezeichnet wird. Dabei kommt es zu grundlegenden chemischen und physikalischen Umwandlungen der Triglyzeride als Hauptkomponente der Nahrungsfette (Choe und Min, 2007). Die enthaltenen Fettsäuren werden thermisch aktiviert, wobei es zur Abspaltung von Wasserstoffradikalen kommt. Bei Anwesenheit von Sauerstoff entstehen als primäre Oxidationsprodukte im Rahmen einer Kettenreaktion zunächst Lipidperoxide und Lipidhydroperoxide. Zu den Vertretern zählen unter anderem 13-Hydroxy-9,11-octadecadiensäure und 13-Hydroperoxyoctadeca-9,11-diensäure (13-HODE, 13-HPODE), die in erhitzten linolsäurereichen Fetten identifiziert wurden (Toschi *et al.*, 1997). Auf Grund ihrer Instabilität zerfallen diese Produkte bei andauernder Einwirkung von hohen Temperaturen. Als Sekundärprodukte entstehen dabei Dimere und Oligomere wie beispielsweise Epoxyhydroperoxide, Epidioxide und Ketohydroperoxide, die zu Polymeren kondensieren können oder weiter zerfallen zu niedermolekularen Verbindungen, wie Aldehyde, Ketone, Ester oder Furane. Diese sind vor allem für den ranzigen Geruch und Geschmack verdorbener Lebensmittel verantwortlich (Liu und Huang, 1996; Frankel, 1998; Kanner, 2007). Aus Tier- und Humanstudien ist bekannt, dass Lipidperoxidationsprodukte aus erhitzten Nahrungsfetten intestinal absorbiert, zu komplexen Lipiden reverestert und in Chylomikronen und *very low-density lipoproteins* (VLDL) eingebaut werden, bevor sie in die Blutzirkulation gelangen (Naruszewicz *et al.*, 1987; Staprans *et al.*, 1993a; Kanner, 2007).

Fütterungsstudien mit Mäusen, Ratten, Meerschweinchen, Kaninchen und Schweinen zeigen, dass die Aufnahme erhitzter Fette zu vielfältigen Wirkungen, wie der Beeinflussung der Glukosetoleranz (Chao *et al.*, 2007; Liao *et al.*, 2008), der Insulinsensitivität (Tsujinaka *et al.*, 2005), der Schilddrüsenfunktion (Eder und Stangl, 2000; Eder *et al.*, 2002; Skufca *et al.*, 2003) sowie des Fremdstoffmetabolismus (Huang *et al.*, 1988; Liu und Huang, 1995; Liu *et al.*, 2000; Sülzle *et al.*, 2004; Chen *et al.*, 2005; Huang *et al.*, 2009) führen. Daneben beeinträchtigen erhitzte Fette den antioxidativen Status

im Organismus, was sich in verminderten Konzentrationen an Antioxidanzien, wie dem Vitamin E, im Plasma und verschiedenen Geweben äußert (Izaki *et al.*, 1984; Liu und Huang 1995; Quiles *et al.*, 2002; Keller *et al.*, 2004; Tres *et al.*, 2010). Ergebnisse aus tierexperimentellen Untersuchungen zeigen weiterhin, dass erhitzte Fette bzw. isolierte Komponenten, wie oxidierte Fettsäuren, pro-atherogen wirken können. So stellten Kaunitz *et al.* bereits 1965 fest, dass Ratten nach Verabreichung von erhitztem Baumwollsaatöl vermehrt Atherosklerose in ihren Koronargefäßen entwickeln. Aus den Arbeiten von Staprans *et al.* (1993a, 1993b, 1994, 1996a) ist bekannt, dass Komponenten aus erhitzten Nahrungsfetten nach der intestinalen Absorption in Lipoproteine inkorporiert werden. Diese modifizierten Lipoproteine besitzen ein atherogenes Potential, da sie bevorzugt von Makrophagen in der Gefäßwand aufgenommen werden können, wodurch deren Umwandlung zu Schaumzellen gefördert wird (Staprans *et al.*, 1993b). In Fütterungsversuchen mit Kaninchen (Greco und Mingrone, 1990; Staprans *et al.*, 1996b; Zalejska-Fiolka *et al.*, 2007) und Mäusen (Khan-Merchant *et al.*, 2002) konnten nach der Gabe erhitzter Fette bzw. nach der Verabreichung von 13-HODE ebenfalls vermehrt atherosklerotische Gefäßveränderungen festgestellt werden. Obgleich in der wissenschaftlichen Literatur im Wesentlichen von ungünstigen Wirkungen erhitzter Fette ausgegangen wird, zeigen neuere Untersuchungen durchaus auch positive Effekte. So konnte in tierexperimentellen Studien eine Senkung der Konzentration an Blutlipiden nach Gabe erhitzter Fette beobachtet werden (Huang *et al.*, 1988; Eder und Kirchgessner, 1998; Eder und Stangl, 2000; Chao *et al.*, 2001; Ammouche *et al.*, 2002; Eder *et al.*, 2003; Sülzle *et al.*, 2004; Ringseis *et al.*, 2007a; Luci *et al.*, 2007). Mechanistische Studien an Modelltieren und beim Schwein ergaben, dass die Fütterung erhitzter Fette zu einer Aktivierung des *peroxisome proliferator-activated receptor alpha* (PPARα) führt (Chao *et al.*, 2001; Chao *et al.*, 2004; Sülzle *et al.*, 2004; Koch *et al.*, 2007a; Ringseis *et al.*, 2007a). Dieser ligandenaktivierte Transkriptionsfaktor steuert maßgeblich Prozesse, die mit der Verwertung von Fettsäuren im Zusammenhang stehen, wie der Fettsäure-Aufnahme, dem Fettsäure-Transport und der Fettsäure-Oxidation. Eine Genexpressionsanalyse in der Leber von Ratten, die ein erhitztes Fett erhielten, ergab, dass Enzyme, die die Oxidation von Fettsäuren katalysieren, in ihrer Genexpression erhöht waren (Sülzle *et al.*, 2004). Dazu zählten die Acyl-CoA-Oxidase (ACO), die *middle chain acyl CoA dehydrogenase* (MCAD), *long chain acyl CoA dehydrogenase* (LCAD) und Cytochrom P450 A1 (CYP4A1). Weiterhin zeigte sich bei diesen Tieren eine erhöhte Expression der hepatischen Carnitin-Palmitoyl-Transferase 1 (CPT1), die carnitinabhängig Fettsäuren als Substrate für die β-Oxidation vom Cytosol in die

Mitochondrien transportiert. Im Zusammenhang mit einer PPAR*α*-Aktivierung durch erhitzte Fette steht weiterhin eine gesteigerte Aufnahme von Carnitin in die Leber und Synthese von Carnitin in der Leber, wodurch die Fettsäureverwertung gefördert wird (Koch *et al.*, 2007b). Die Aktivierung des PPAR erfolgt durch Ligandenbindung, die eine Konformationsänderung des Rezeptors zur Folge hat. Die sich anschließende Heterodimerisierung mit dem Retinsäure-X-Rezeptor (RXR) und die Degradierung von Co-Repressoren bzw. die Rekrutierung von Co-Aktivatoren ermöglicht eine Bindung des Dimers an definierte DNA-Konsensussequenzen (*peroxisome proliferator response element* (PPRE)) im Promotorbereich von Zielgenen, deren Expression daraufhin gesteigert wird (Abb. 1).

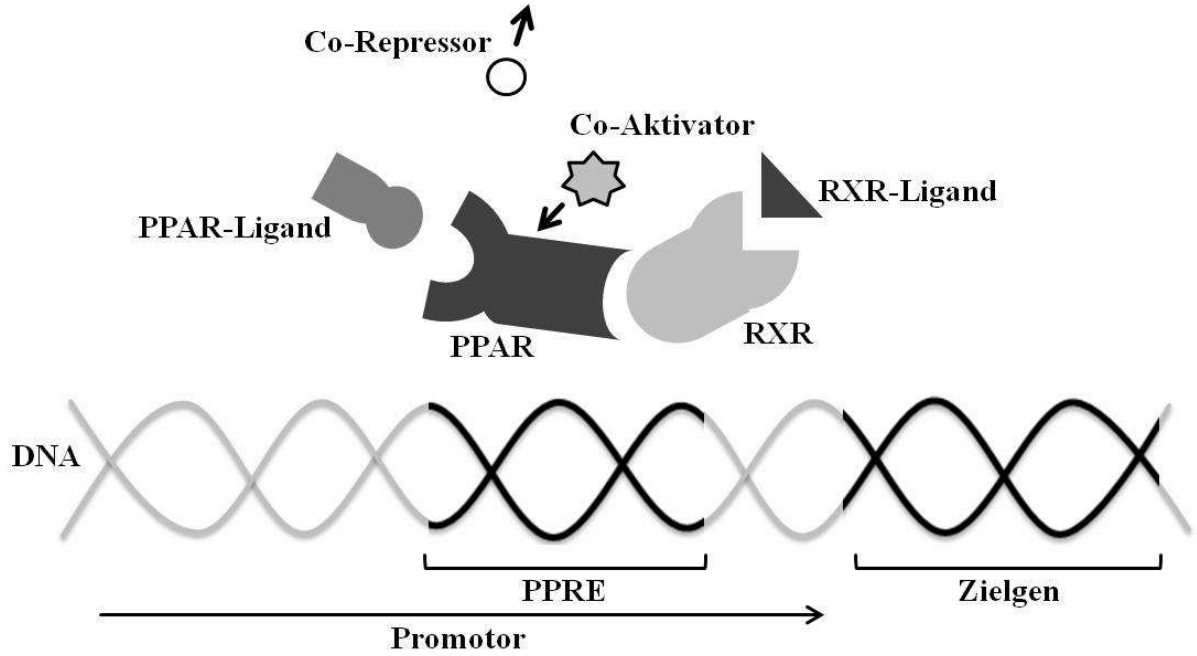

Abb. 1: Die PPAR-Aktivierung nach Ligandenbindung wird durch die Rekrutierung von Co-Aktivatoren sowie durch die Freisetzung von Co-Repressoren ermöglicht. Die transkriptionelle Regulation erfolgt nach Heterodimerisierung mit RXR. Der PPAR-RXR-Komplex interagiert mit spezifischen PPRE im Promotorbereich von Zielgenen, woraufhin deren Expression gefördert wird. Abkürzungen: DNA, *desoxyribonucleic acid*; PPAR, *peroxisome proliferator-activated receptor*; PPRE, *peroxisome proliferator response element*; RXR, Retinsäure-X-Rezeptor

Die Aufnahme von Fibraten, welche als Liganden des PPAR*α* fungieren, führt daher durch eine Steigerung des Fettsäureabbaus zu einer Absenkung der Blutlipide, was deren erfolgreichen Einsatz zur pharmakologischen Behandlung von Hyperlipidämien begründet

(Jialal *et al.*, 2010; Krysiak *et al.*, 2011; Watts und Karpe, 2011). Jüngere Studien ergaben, dass nicht nur Fibrate, sondern auch charakteristische Bestandteile erhitzter Fette, wie die oben erwähnten HODE und HPODE als Agonisten der PPARs wirken (König und Eder, 2006). Somit lässt sich die lipidsenkende Wirkung erhitzter Fette zumindest teilweise durch eine Aktivierung des PPARα in der Leber erklären. In diesem Zusammenhang ist auch zu erwähnen, dass erhitzte Fette zur Hemmung der Ausprägung einer alkoholinduzierten Fettleber in der Lage sind. Auf molekularer Ebene zeigt sich, dass Alkohol zu einer Blockierung der Wirkung des PPARα führt, welche durch die Verabreichung erhitzter Fette wieder aufgehoben wird (Ringseis *et al.*, 2007b).

Neben der zentralen Funktion von PPARα, die Fettsäure-Verwertung zu stimulieren, werden ferner Entzündungsprozesse in der Gefäßwand durch diesen Transkriptionsfaktor reguliert, indem die Aktivität redoxsensitiver Transkriptionsfaktoren wie *nuclear factor kappa B* (NF-κB), *signal transducers and activators of transcription* (STAT) oder *activator protein-1* (AP-1) gehemmt wird (Poynter und Daynes, 1998; Blanquart *et al.*, 2004; Okayasu *et al.*, 2008; Garrido-Urbani *et al.*, 2011). Dieser als Transrepression bezeichnete Vorgang resultiert in einer verminderten Expression inflammatorischer Gene, wie verschiedenen Zytokinen (*tumor necrosis factor-alpha* (TNF-α), *interleukin* (IL)-1β, IL-6), Chemokinen (*monocyte chemoattactant protein-1* (MCP-1), *macrophage inflammatory protein-1 alpha* (MIP-1α)) und Adhäsionsmolekülen (*intercellular adhesion molecule-1* (ICAM-1), *vascular cell adhesion molecule-1* (VCAM-1), E-Selektin), die in der Entstehung der Atherosklerose von Bedeutung sind (Marchesi *et al.*, 2003; Zapolska-Donar und Naruszewicz, 2009; Almanza-Perez *et al.*, 2010). Dadurch konnten in jüngeren Untersuchungen zahlreiche gefäßprotektive Mechanismen auf zellulärer und molekularer Ebene aufgeklärt werden, die mit einer Aktivierung des PPAR in der Gefäßwand einhergehen. Dazu zählt die Hemmung der Expression von Chemokinen und zellulären Adhäsionsmolekülen, die im Rahmen atherosklerotischer Prozesse die Rekrutierung zirkulierender Monozyten sowie deren Adhäsion an Endothelzellen begünstigen (Marx *et al.*, 1999; Okayasu *et al.*, 2008). Die Transmigration von Monozyten in den Subendothelialraum und deren Differenzierung zu gewebsständige Makrophagen stellen Schlüsselfunktionen im Prozess der Atherogenese dar. Die phagozytotische Aktivität dieser Zellen bedingt eine hohe Aufnahme cholesterolhaltiger modifizierter Lipoproteine aus der Blutzirkulation. In dieser Funktion werden sie auch als Schaumzellen bezeichnet. Sie sind maßgeblich an der Bildung von *fatty streaks*, den ersten sichtbaren atherosklerotischen Läsionen, beteiligt (Chinetti *et al.*, 2000). Untersuchungen mit PPAR-Agonisten an

Zellkulturmodellen zeigen, dass dieser Transkriptionsfaktor die Cholesterolhomöostase in Makrophagen günstig beeinflussen und so der Schaumzellbildung entgegenwirken kann (Chinetti *et al.*, 2001; Ogata *et al.*, 2009). Grundlage dafür ist die Tatsache, dass Schlüsselgene für den intrazellulären und transmembranären Transport von Cholesterol durch PPAR reguliert werden (Yuan *et al.*, 2012). Dazu zählen *adenosine triphosphate binding cassette transporter A1* (ABCA1) und *adenosine triphosphate binding cassette transporter G1* (ABCG1) sowie *scavenger receptor* (SR) BI. Sie können unter Energieverbrauch Cholesterol aus dem Zellinneren auf extrazelluläre Akzeptoren, wie Apolipoprotein A-I (Apo A-I), transportieren, wodurch der Transport in *high-density lipoproteins* (HDL) zur Leber ermöglicht wird, wo es weiter verstoffwechselt werden kann. Dieser zelluläre Exportmechanismus stellt den ersten Schritt des reversen Cholesteroltransports (RCT) dar. Dieser gilt als atheroprotektiv, da überschüssiges Cholesterol auf diesem Weg aus peripheren Geweben in die Leber rücktransportiert und dort metabolisiert werden kann (Kreuzer, 2003). Die Bedeutung des PPAR*α* für den Lipidstoffwechsel in Makrophagen wurde in Studien mit synthetischen Agonisten, wie den Fibraten, umfassend beschrieben (Chinetti *et al.*, 2003; Arakawa *et al.*, 2005; Rotllan *et al.*, 2011). Unklar ist jedoch, ob erhitzte Fette durch eine PPAR*α*-Aktivierung ähnliche Wirkungen in diesen Zellen zeigen.

Weitere gefäßprotektive Effekte, die durch eine PPAR-Aktivierung vermittelt werden, sind die Hemmung der Proliferation von glatten Gefäßmuskelzellen sowie deren Migration in den Subendothelialraum (Nigro *et al.*, 2002; Zahradka *et al.*, 2003). Beide Prozesse sind für die fortschreitende Entwicklung der Atherosklerose von enormer Bedeutung, sie fördern den inflammatorischen Zustand im Blutgefäß und tragen wesentlich zur Volumenzunahme atherosklerotischer Plaques bei (Dzau *et al.*, 2002; Hao *et al.*, 2003). Tierexperimentelle Untersuchungen und Zellkulturstudien zeigen, dass, analog zu den Effekten in der Gefäßintima, eine PPAR-Aktivierung die Bildung pro-inflammatorischer Signalmoleküle in glatten Gefäßmuskelzellen hemmt, wodurch deren Proliferation und Migration in den Subendothelialraum behindert wird (Marx *et al.*, 1998; Law *et al.*, 2000; Gizard *et al.*, 2005; Zhang *et al.*, 2011). Neben der erwähnten Transrepression scheint eine Beeinflussung der Zellzykluskontrolle für die Effekte einer PPAR-Aktivierung in diesem Zelltyp mitverantwortlich zu sein (Gizard *et al.*, 2005).

Ergebnisse aus früheren Untersuchungen zeigen somit, dass erhitzte Fette die Konzentration atherogener Blutlipide senken. Weiterhin ist bekannt, dass definierte Bestandteile erhitzter Fette in der Lage sind, PPAR*α* zu aktivieren. Dabei geht eine Aktivierung dieses Transkriptionsfaktors mit der Repression inflammatorischer Signalwege und Mediatoren in

verschiedenen vaskulären Zellen einher, die Atherosklerose-hemmend wirken. Bisher ist jedoch nicht bekannt, ob erhitzte Fette die Entwicklung atherosklerotischer Gefäßveränderungen durch eine Aktivierung von PPARα beeinflussen können.

2. Zielstellung

Die vorliegende Arbeit verfolgt das Ziel, basierend auf den Ergebnissen aus Voruntersuchungen der eigenen Arbeitsgruppe sowie auf den Resultaten vergangener *in vivo*- und *in vitro*-Studien, folgende Fragestellungen zu überprüfen:

Aus tierexperimentellen Studien ist bekannt, dass eine Verabreichung erhitzter Fette zu einer Senkung der Konzentration an Plasmalipiden führen kann (Huang *et al.*, 1988; Sülzle *et al.*, 2004; Luci *et al.*, 2007). Dabei gelten charakteristische Bestandteile dieser Fette als Liganden des Transkriptionsfaktors PPARα (Chao *et al.*, 2004; Sülzle *et al.*, 2004; Koch *et al.*, 2007a; Ringseis *et al.*, 2007a), der maßgeblich an der Regulation des Lipid- und Lipoproteinstoffwechsels in der Leber beteiligt ist. Genexpressionsanalysen der eigenen Arbeitsgruppe zeigen, dass erhitzte Fette auf transkriptioneller Ebene zu einer Steigerung der Fettsäureverwertung in der Leber führen, die für die beobachteten hypolipidämischen Wirkungen nach Aufnahme erhitzter Fette mitverantwortlich ist. Neben einer Begünstigung des Blutlipidprofils geht eine PPAR-Aktivierung in vaskulären Zellen mit der Transrepression inflammatorischer Mediatoren und Signalwege einher. Dadurch können Entzündungsprozesse in der Gefäßwand reguliert werden, die Atherosklerose-hemmend wirken (Marx *et al.*, 1999; Hashizume *et al.*, 2011). Weiterhin zeigen Untersuchungen, dass PPARα an der Aktivierung des RCT in Makrophagen beteiligt ist (Dushkin, 2012). Im Prozess der Atherosklerose akkumulieren diese Zellen vermehrt Cholesterol, was die Ausbildung von Läsionen begünstigt. Eine Aktivierung von PPARα in diesen Zellen führt zu einer gesteigerten Expression von Genen des Cholesterolexports, was den Ausstrom von Cholesterol aus diesen Zellen fördert und damit anti-atherogene Effekte bewirken kann (Chinetti *et al.*, 2001; Nakaya *et al.*, 2011).

Auf den genannten Befunden aufbauend soll daher die Hypothese formuliert werden, dass erhitzte Fette durch eine Aktivierung des PPARα anti-atherogen wirken.

Zur Bestätigung dieser Hypothese wurde zunächst ein Fütterungsversuch mit einem etablierten Tiermodell der Atheroskleroseforschung, den *low-density lipoprotein receptor* (LDLR)-*Knockout*-Mäusen, durchgeführt. Durch die gezielte Inaktivierung des Gens, welches für den LDL-Rezeptor kodiert, akkumulieren bei diesen Tieren vermehrt cholesterolreiche Lipoproteine im Blut. Sie gelten daher auch als Modell für die familiäre Hypercholesterolämie. Darüber hinaus ist bekannt, dass bei diesem Tiermodell auf Grund der erhöhten Cholesterolkonzentration im Blut und nach der Gabe einer fettreichen Diät frühzeitig atherosklerotische Plaques entstehen.

Die Versuchsdiäten der Kontrollgruppe und der Behandlungsgruppen unterschieden sich in der Art der eingesetzten Fette, wobei die Kontrollgruppe frisches hydrogeniertes Palmfett und die Behandlungsgruppen eine Mischung von erhitztem hydrogenierten Palmfett (170°C, 48 h) und frischem Sonnenblumenöl erhielten. Die Verwendung eines Mischfettes war erforderlich, um die erhitzungsbedingten Verluste an mehrfach ungesättigten Fettsäuren im hydrogenierten Palmfett auszugleichen. Somit unterschieden sich die Diäten der Versuchsgruppen nur im Gehalt an Oxidationsprodukten, jedoch nicht im Gehalt an Fettsäuren. Der moderate Erhitzungsprozess des Palmfetts war vergleichbar mit der Behandlung von Fetten zur Zubereitung von Speisen in der Humanernährung (z.B. beim Frittierprozess). Durch dieses Vorgehen entstand ein Diätfett, das unter praxisrelevanten Bedingungen erhitzt wurde und das sich in Bezug auf die Behandlungsintensität an physiologischen Verhältnissen orientierte. Damit unterschied sich diese Diätkomponente vom Oxidationsgrad grundlegend von den Fetten und Ölen, die vorwiegend in früheren tierexperimentellen Untersuchungen zum Einfluss auf die Atherogenese verwendet wurden. So existieren Fütterungsstudien, bei denen pro-atherogene Wirkungen nach Verabreichung stark erhitzter Fette oder Öle mit hohen Anteilen oxidationsempfindlicher ungesättigter Fettsäuren beobachtet wurden (Greco und Mingrone, 1990; Kaunitz *et al.*, 1965; Staprans *et al.*, 1996b; Zalejeska-Fiolka *et al.*, 2004; Zalejeska-Fiolka *et al.*, 2007). Als Folge einer Aufnahme erhitzter Fette ist darüber hinaus bekannt, dass der Verbrauch endogener Antioxidanzien erhöht und der oxidative Status im Organismus beeinträchtigt wird (Liu und Huang, 1996; Keller *et al.*, 2004). Derartige Effekte können die Entwicklung der Atherosklerose fördern (Esterbauer *et al.*, 1993; Eder *et al.*, 2003a) und mögliche atheroprotektive Mechanismen einer PPARα-Aktivierung durch Bestandteile erhitzter Fette beeinträchtigen. Um Sekundäreffekte zu umgehen, erfolgte eine Supplementierung der Diäten mit Vitamin E. Durch Zusatz von synthetischem *all rac*-α-Tocopherylacetat wurde der Vitamin E-Gehalt der Diäten von Kontroll- und Behandlungsgruppe 1 auf jeweils 25 mg α-Tocopheroläquivalente pro kg Diät eingestellt. Diese Konzentration entsprach dem Mindestbedarf an Vitamin E, der sich aus den mit dem Diätfett zugeführten ungesättigten Fettsäuren ergab. Die Diät der Behandlungsgruppe 2 wurde auf 250 mg α-Tocopheroläquivalente pro kg Diät eingestellt, um zu überprüfen, ob die Versuchsergebnisse auf einen veränderten oxidativen Status im Organismus zurückzuführen sind.

Nach Versuchsende wurden die mRNA-Konzentrationen bekannter Zielgene des PPARα in der Leber bestimmt sowie Triglyzeride und Cholesterol in Plasma und Lipoproteinen der Versuchstiere analysiert. Um die Ausprägung der Atherosklerose zu untersuchen, wurden

Gefrierschnitte der Aorta angefertigt und bezüglich der Größe und Zusammensetzung der Läsionen mittels histologischen Standardfärbungen und immunhistochemischen Methoden untersucht. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Studie 1:
Kämmerer I, Ringseis R, Eder K (2011) Feeding a thermally oxidised fat inhibits atherosclerotic plaque formation in the aortic root of LDL receptor-deficient mice. Br J Nutr 105:190-199; reproduced with permission of Cambridge University Press

Als definierte primäre Oxidationsprodukte sind oxidierte Fettsäuren Bestandteil erhitzter Fette und zugleich starke Aktivatoren von PPAR*α*. In Anlehnung an die aufgestellte Hypothese sollte in der zweiten Studie gezeigt werden, dass oxidierte Fettsäuren für die anti-atherogenen Effekte erhitzter Fette mitverantwortlich sind. Ziel der Untersuchung war es nachzuweisen, dass oxidierte Fettsäuren den RCT in Makrophagen stimulieren und diesen als potentiellen Mechanismus der anti-atherogenen Wirkung erhitzter Fette zu identifizieren.
Dafür wurde ein *in vitro*-Modell einer Makrophagen–Zelllinie der Maus genutzt. Die Zellen wurden mit Linolsäure und 13-HODE, dem hydroxylierten Derivat dieser Fettsäure, inkubiert. Anhand eines Transaktivierungsassays sollte die Fähigkeit beider Fettsäuren, PPAR in den Makrophagen zu aktivieren, untersucht werden. Weiterhin wurde die Proteinexpression der transmembranären Cholesteroltransporter ABCA1, ABCG1 und SR-BI sowie des *liver X receptor alpha* (LXR*α*), einem Transkriptionsfaktor, der neben den PPARs die Cholesterolhomöostase reguliert, bestimmt. Um zu überprüfen, ob 13-HODE durch einen Einfluss auf die genannten Transporter den Ausstrom von Cholesterol aus den Makrophagen beeinflusst, wurden Cholesteroleffluxmessungen durchgeführt. Da die Cholesterolhomöostase in Makrophagen durch PPAR*α*, aber auch durch PPAR*γ* beeinflusst werden kann (Rigamonti *et al.*, 2008; Taketa *et al.*, 2008), wurden zusätzlich Inkubationen mit Linolsäure und 13-HODE in Anwesenheit selektiver PPAR-Antagonisten durchgeführt. Auf diese Weise sollte untersucht werden, ob die Effekte der Fettsäuren PPAR-vermittelt sind. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

Studie 2:

Kämmerer I, Ringseis R, Biemann R, Wen G, Eder K (2011) 13-hydroxy linoleic acid increases expression of the cholesterol transporters ABCA1, ABCG1 and SR-BI and stimulates apoA-I-dependent cholesterol efflux in RAW264.7 macrophages. Lipids in Health and Disease 10:222

3. Originalarbeiten

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Feeding a thermally oxidised fat inhibits atherosclerotic plaque formation in the aortic root of LDL receptor-deficient mice

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Abstract

Activators of PPARα have been demonstrated to inhibit atherosclerosis development due to lipid lowering in plasma and direct protective effects on the vasculature. Because dietary oxidised fats (OF) have strong PPARα-activating and lipid-lowering properties, we hypothesised that dietary OF has also an inhibitory influence on atherosclerosis development. To verify our hypothesis, we investigated the effect of feeding diets containing an OF (a 92 : 8 mixture of heated (170°C, 48 h) hydrogenated palm fat and fresh sunflower oil) compared with a fresh fat (fresh hydrogenated palm fat) on the development of atherosclerotic lesions in LDL receptor-deficient ($LDLR^{-/-}$) mice. We observed that a dietary OF caused a strong up-regulation of PPARα-regulated genes in the liver and a marked reduction in plasma concentrations of cholesterol and TAG ($P<0·05$). Cross-sectional lesion area and the lipids and collagen levels in the aortic root were approximately 40–50 % lower in mice fed diets containing OF than in those fed diets containing fresh fat ($P<0·05$). Immunohistochemical analysis of aortic root sections revealed an about 8-fold increased expression of PPARα and a markedly reduced expression of the proinflammatory vascular cell adhesion molecule-1 and smooth muscle cell (SMC)-specific marker α-actin in $LDLR^{-/-}$ mice fed OF ($P<0·05$). We postulate that OF exert anti-atherogenic effects by activation of PPARα both in the liver, which contributes to lipid lowering in plasma, and in the vasculature, which inhibits pro-atherogenic events such as monocyte recruitment and SMC proliferation and migration.

Key words: **Oxidised fat: Atherosclerosis: LDL receptor-deficient mice: PPARα**

In recent years, the contribution of oxidised fats (OF) to total energy intake has markedly increased in industrialised countries[1] due to the rising consumption of deep-fried products. During deep-frying, several chemical reactions occur within the frying oil resulting in the formation of a mixture of chemically distinct lipid peroxidation products. Large quantities of the frying oil are absorbed into the fried foods during deep-frying and are therefore ingested during their consumption.

Although OF are widely considered to have detrimental effects on human health[2–4], feeding experiments in rats have consistently demonstrated an improvement in the blood lipid profile, i.e. a reduction in TAG and cholesterol levels in plasma and VLDL, by OF[5–7]. This effect of OF has been attributed to the ability of OF to activate hepatic PPARα[8–10], a ligand-activated transcription factor that controls a comprehensive set of genes regulating most aspects of lipid catabolism, glucose homoeostasis and inflammation[11,12]. Thus, activation of PPARα results in decreased lipid concentrations in plasma and VLDL, improved glucose tolerance and reduced inflammatory processes. The components of OF supposed to be responsible for PPARα activation are hydroxy and hydroperoxy fatty acids[13] and cyclic fatty acid monomers[14]. Indeed, feeding a diet supplemented with 13-hydroperoxy octadecadienoic acid strongly reduced TAG concentrations in plasma via PPARα-dependent effects[15].

PPARα is also expressed in all the major cells of the vessel wall which are implicated in atherosclerotic lesion development[11]. Activation of PPARα in these cells modulates the expression of several genes implicated in the atherosclerotic process, resulting in decreased monocyte recruitment to endothelial cells[16], enhanced cholesterol removal from macrophages[17] and reduced smooth muscle cell (SMC) proliferation and migration[18]. These direct atheroprotective effects together with the lipid-lowering effects are largely responsible for the observation that pharmacological PPARα activators cause an inhibition of atherosclerosis development[19–22]. Because dietary OF have strong PPARα-activating and lipid-lowering

Abbreviations: CYP4A10, cytochrome P450 isoform 4A10; FF, fresh fat; $LDLR^{-/-}$, LDL receptor deficient; OF, oxidised fat; SMC, smooth muscle cells; VCAM-1, vascular cell adhesion molecule-1.

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properties, it would be expected that dietary OF have also an inhibitory influence on atherosclerosis development. Nevertheless, several earlier reports[2,23–25] demonstrated that feeding OF has pro-atherogenic effects. However, this may be due to the fact that these studies used fats which were strongly oxidised and which contained lipid oxidation products, which are clearly above the limit allowed for 'used frying fats'. Thus, feeding such strongly OF does not reflect the physiological situation in human nutrition. Moreover, feeding such strongly OF causes intense oxidative stress due to the depletion of antioxidants such as tocopherols in serum and tissues[26,27], which is considered to promote the development of atherosclerosis[28]. Hence, a possible atheroprotective effect of OF due to activation of PPARα is probably compromised by the simultaneous induction of intense oxidative stress. It could be demonstrated, however, that oxidative stress and depletion of antioxidants induced by feeding OF is alleviated by supplementation of the diet with a high vitamin E level[27]. The aim of the present study was to investigate the effect of a thermally OF prepared under deep-frying conditions on the development of atherosclerotic lesions. In order to find out whether the effects of the OF in this respect are influenced by oxidative stress, we used diets with moderate or high vitamin E concentrations. As an experimental model of atherosclerosis, we used LDL receptor-deficient ($LDLR^{-/-}$) mice. These mice mimic human lipoprotein disorders that are associated with an increased risk of CHD and develop extensive aortic atherosclerosis which resembles human lesions[29].

Materials and methods

Animals and diets

A total of thirty-six male, adult, 15-week-old $LDLR^{-/-}$ mice (B6.129S7-$Ldlr^{tm1Her}$/J mice; Charles River, Germany) with an initial body weight of 27 (SD 1) g were randomly assigned to three groups of twelve mice each. All mice were kept individually in Macrolon cages in a room maintained at 22 ± 1°C and 50–60 % relative humidity with lighting from 06.00 to 18.00 hours. All the experimental procedures described followed established guidelines for the care and handling of laboratory animals[30] and were approved by the local Animal Care and Use Committee. The mice were fed a semi-purified Western-type diet which consisted of (g/kg diet) maize starch, 285·5; casein, 200; saccharose, 200; experimental fat, 200; vitamin and mineral mixture, 60; cellulose, 50; linseed oil as a source of α-linolenic acid, 3; cholesterol, 1·5. Vitamins and minerals were supplemented according to the recommendations of the American Institute of Nutrition-93M[31].

The experimental fat was varied as follows. The first group (fresh fat group, 'FF25') received 200 g/kg diet of fresh hydrogenated palm fat (Enco, Hamburg, Germany), which is a typical fat used for deep-frying in restaurants. Both the second (OF group 25, 'OF25') and the third groups (OF group 250, 'OF250') received 200 g/kg of a mixture of heated hydrogenated palm fat (Enco) and fresh sunflower oil (92:8, w/w) (AOP, Riesa, Germany). This ratio was chosen to equalise the concentrations of the major fatty acids of the OF diets to that of the FF diet, since the heating process caused a partial loss of PUFA. The OF was prepared by heating the hydrogenated palm fat at a temperature of 170 ± 3°C for 48 h in a domestic fryer (Fryer Model PROFRI 4; Saro Gastro Products, Emmerich, Germany). During the 48 h heating process, a portion of 70 g French fries obtained from a local cafeteria was deep-fried for 6 min every 30 min. The extent of lipid peroxidation in the fats was estimated by assaying the peroxide value[32] and the percentages of polar and unpolar compounds[33] before and after inclusion into the diets.

Because the frying process caused a dramatic loss of tocopherols in the heated hydrogenated palm fat, the native concentrations of tocopherols of all the experimental fats were analysed. Based on the native concentrations of the fats, the vitamin E concentration of the diets was adjusted to 25 mg α-tocopherol equivalents/kg diet in the FF25 diet and the OF25 diet and 250 mg α-tocopherol equivalents/kg diet in the OF250 diet by individually supplementing with all-*rac*-α-tocopheryl acetate (the biopotency of all-*rac*-α-tocopheryl acetate is considered to be 67 % of that of α-tocopherol). Diets were prepared by mixing the dry components with the fat and water and subsequent freeze-drying. The residual water content of the diet was below 5 g/100 g diet. Food was administered daily at 12.00 hours in controlled amounts to standardise the intake.

Experimental diets were fed for 14 weeks. To standardise the food intake, diets were fed in a controlled feeding regimen, whereby each mouse received 2·5 g diet/d during the whole experiment. Energy supplied by this amount of diet was close to the energy requirement of the mice for maintenance[34]. Water was available *ad libitum* for nipple drinkers during the whole experiment.

Sample collection

The mice were killed by decapitation under light anaesthesia with diethyl ether in the non-fasted state. Whole blood was collected into EDTA polyethylene tubes (Sarstedt, Nürnbrecht, Germany). Plasma was separated from the whole blood by centrifugation (1100 *g*; 10 min) at 4°C. Liver, skeletal muscle (*Musculus gastrocnemius*) and visceral adipose tissue were excised immediately and shock frozen with liquid N_2. All the samples were stored at −80°C for pending analysis.

Lipoproteins (VLDL, LDL and HDL) were separated by step-wise ultracentrifugation (900 000 *g*, 1·5 h, 4°C; Mikro-Ultrazentrifuge, Sorvall Products, Bad Homburg, Germany) as described elsewhere[35].

Preparation of aortic tissue and morphometric determination of atherosclerosis

To quantify atherosclerosis, aortic root sections (10 μm thick slices; beginning at the aortic valve area) were prepared and sections were stained with haematoxylin–eosin, oil red O for vascular lipids, Goldner's trichrome for collagen structures and von Kossa for vascular calcification, as described recently in detail[35]. Histomorphological characterisation and computerised morphometric quantification of the atherosclerotic lesions were performed and blinded to the protocol. The cross-sectional surface area of the total vessel, the cross-sectional surface area of the lesion, the calcification area, the collagen area and the lipid area were assessed. The relative lesion area, the relative collagen area, the relative lipid area and the relative calcification area (expressed relative to the total surface area) were used to show individual atherosclerosis development in the aortic root.

Immunohistochemistry

For immunohistochemistry, aortic root sections were immediately fixed in acetone at −20°C for 10 min, and endogenous peroxidase was blocked in 0·3% H_2O_2 in methanol. Three sections were incubated each with 5% blocking serum (either goat, rabbit or sheep depending on the secondary antibody used) in PBS at room temperature for 20 min. Following a washing step, the sections were incubated with primary antibodies against SMC α-actin (Sigma, Taufkirchen, Germany; sections from 190 to 220 μm), vascular cell adhesion molecule (VCAM)-1 (Abcam, Cambridge, UK; sections from 250 to 280 μm), PPARα (Abcam; sections from 280 to 310 μm) and PPARγ (Axxora, Lörrach, Germany; sections from 310 to 340 μm) in a humidifying chamber for various periods (2–14 h depending on the antibody used) at 4°C. After washing in PBS, the sections were incubated with horseradish peroxidase-labelled secondary antibodies (goat anti-rat IgG and sheep anti-rabbit IgG (Serotec, Oxford, UK), and rabbit anti-mouse IgG (Dako, Hamburg, Germany)) at room temperature for 1 h. The immunocomplex was visualised using either diaminobenzidine chromogen (Dako) or Nova Red (Axxora). Subsequently, sections were counterstained with Harris haematoxylin solution. Intensity of staining was measured using LuciaG 3.2 software.

Lipid analysis

TAG and cholesterol concentrations in plasma and lipoproteins were determined using enzymatic reagent kits (DiaSys Diagnostic Systems, Holzheim, Germany, ref. 1.13009990314 and 1.57609990314). The fatty acid composition of the dietary fats was determined by GC. Fats were methylated with trimethylsulphonium hydroxide[36]. Fatty acid methyl esters were separated by GC, using a system (HP 5890; Hewlett-Packard GmbH, Böblingen, Germany) equipped with an automatic on-column injector, a polar capillary column (30 m FFAP, 0·53 mm internal diameter, Macherey and Nagel, Düren, Germany) and a flame ionisation detector. Helium was used as the carrier gas with a flow rate of 5·4 ml/min. Fatty acid methyl esters were identified by comparing their retention times with those of individually purified standards.

Determination of vitamin E concentrations

Concentrations of α-tocopherol in liver, skeletal muscle and epididymal adipose tissue were determined, as described recently, in more detail[37].

RNA isolation and real-time detection PCR

For the determination of hepatic mRNA expression levels of cytochrome P450 isoform 4A10 (CYP4A10), acyl-CoA oxidase and lipoprotein lipase, total RNA was isolated, mRNA reverse transcribed, and target gene mRNA concentrations were determined by real-time detection PCR, as described previously[38]. Sequences of gene-specific primers were as follows (forward, reverse; NCBI GenBank): glyceraldehyde 3-phosphate dehydrogenase (5′-AACGACCCCTTCATTGAC-3′, 5′-TCCACGACATACTCAGCAC-3′; NM_008084), CYP4A10 (5′-TGAGGGAGAGCTGGAAAAGA-3′, 5′-CTGTTGGTGATCAGGGTGTG-3′; NM_010011), acyl-CoA oxidase (5′-CAGGAAGAGCAAGGAAG TGG-3′, 5′-CCTTTCTGGCTGATCCCATA-3′; NM_015729), lipoprotein lipase (5′-GGGCTCTGCCTGAGTTGTAG-3′, 5′-AGAAATTTCGAAGGCCTGGT-3′; BC_158040).

Statistical analysis

Values presented in the text are means and standard deviations. Treatment effects were analysed using one-way ANOVA. For significant F values, means were compared by Fisher's multiple range test. Differences with $P<0·05$ were considered significant.

Results

Characterisation of the dietary fat of the experimental diets

In the OF diets, the dietary fat represented a mixture (92:8, w/w) of heated hydrogenated palm fat and fresh sunflower oil in order to equalise the dietary fat of the experimental diets for their fatty acid composition. This was necessary to avoid the confounding effects resulting from differences in the concentrations of major fatty acids between the experimental diets. As revealed by GC-flame ionoisation detector analysis, the concentrations of the major fatty acids and of the essential fatty acids, linoleic acid (18 : 2 *n*-6) and α-linolenic acid (18 : 3 *n*-3) were similar between

Table 1. Fatty acid composition and concentrations of peroxidation products in the dietary fats after inclusion into the diets

	FF25	OF25	OF250
Major fatty acids (% of total FAME)			
8:0	0·5	0·4	0·5
10:0	0·7	0·8	0·7
12:0	2·5	1·2	1·4
14:0	2·5	1·8	1·9
16:0	42·7	49·0	48·7
18:0	4·3	5·0	4·8
18:1*n*-9	35·5	31·4	32·0
18:2*n*-6	9·9	9·5	9·3
18:3*n*-3	1·1	0·9	0·7
20:0	0·2	0·1	0·1
Peroxidation products			
POV (mEq O_2/kg diet)	5·0	7·5	9·5
Total polar compounds (%)	8·8	26·9	29·4
Total unpolar compounds (%)	91·2	73·1	70·6

FF25, fresh fat group; OF25 and OF250, oxidised fat groups; FAME, fatty acid methyl esters; POV, peroxide value.

all the three experimental diets (Table 1). The concentrations of *trans*-fatty acids such as 18:1 *t*9, 18:2 *c*9*t*11 and 18:2 *t*10*c*12 were below 0·1% of total fatty acids in all the three experimental diets. In contrast, the peroxide value and the percentage of polar compounds in the dietary fat were about 2- and 3-fold, respectively, higher in the OF diets than in the FF25 diet. The percentage of unpolar compounds was lower in the dietary fat of the OF diets than in the FF25 diet (Table 1).

Food intake, body weight changes and relative liver weights

To exclude secondary food intake effects, a controlled feeding system was applied in which each mouse was given an identical amount of diet. Nevertheless, mice of the FF25 group had a slightly higher final body weight at the end of the 14-week feeding period than those of the OF groups (FF25, 35·4 (SD 1·5) g; OF25, 29·5 (SD 1·6) g; OF250, 28·9 (SD 1·9) g; *n* 12, $P<0·05$). No difference in final body weights was observed between the mice of the OF25 group and the mice of the OF250 group. Daily body weight gain during the 14-week feeding period was also slightly higher in the FF group than in the OF groups (FF25, 0·08 (SD 0·02) g; OF25, 0·02 (SD 0·02) g; OF250, 0·02 (SD 0·03) g; *n* 12, $P<0·05$). No difference in daily body weight gain was observed between mice of the two OF groups. Relative liver weights were higher in mice fed the OF diets than in those fed the FF diet (FF25, 4·8 (SD 0·2) g/100 g body weight; OF25, 6·1 (SD 0·3) g/100 g body weight; OF250, 6·1 (SD 0·4) g/100 g body weight; *n* 12, $P<0·05$).

Atherosclerosis in the aortic root

To examine the effect of treatment on atherosclerotic lesion development, serial sections through the aortic root beginning at the level of the aortic valves were taken. Subsequent analysis of the aortic root sections showed that all mice developed severe atherosclerotic lesions covering approximately 20–30% of total vessel area. Atherosclerotic lesion size (cross-sectional lesion area) and the lipids and collagen levels in the aortic root were approximately 40–50% lower in mice of the OF groups than in those of the FF25 group (Figs. 1(A)–(C) and 2(a) and (b); $P<0·05$). The levels of calcifications in the aortic root did not differ between the three groups of mice Figs. 1(D) and 2(c)).

Lipid concentrations in plasma and lipoproteins

To evaluate whether the dietary OF also exerts a lipid-lowering action in $LDLR^{-/-}$ mice, the lipid concentrations in plasma and lipoproteins were determined. Concentrations of TAG in plasma and VLDL + chylomicrons were markedly lower in the OF groups than in the FF25 group (Table 2; $P<0·05$). TAG concentrations in plasma and VLDL + chylomicrons did not differ between both OF groups.

Concentrations of cholesterol in plasma, LDL and HDL were lower in mice fed the OF250 diet than in those fed the FF25 diet (Table 2; $P<0·05$). In mice fed the OF25 diet, only the concentrations of cholesterol in HDL, but not in plasma and LDL, were lower than in those fed the FF25 diet (Table 2). Cholesterol concentrations in VLDL + chylomicrons did not differ among the three groups of mice.

Expression of PPARα and PPARγ in the aortic root

PPAR agonists have been shown to exert antiatherogenic effects through the activation of PPAR in the vasculature. To examine the effect of OF on expression of PPAR, sections of the aortic root were stained for PPARα and PPARγ by immunohistochemistry. Both PPARα and PPARγ were well detectable in the aortic root of $LDLR^{-/-}$ mice, with staining localised largely to the atherosclerotic lesion. Expression of PPARα in the aortic root was about 6- to 8-fold higher in mice fed the OF diets than in those fed the FF25 diet (Fig. 3(A); $P<0·05$). In contrast to PPARα, expression of PPARγ in the aortic root was not different among the three groups of mice (Fig. 3(B)).

Expression of smooth muscle cell α-actin in the aortic root

SMC are the major collagen-producing cell types in the atherosclerotic plaque. To investigate whether changes in SMC content of plaques might be responsible for the reduction of collagen content by OF, sections of the aortic root were stained for the SMC-specific marker α-actin. Immunostaining for SMC α-actin showed a strong expression in the aortic root of mice fed the FF25 diet, with staining localised to atherosclerotic lesions. In the aortic root of mice fed the OF diets, expression of SMC α-actin was strongly reduced (Fig. 4; $P<0·05$).

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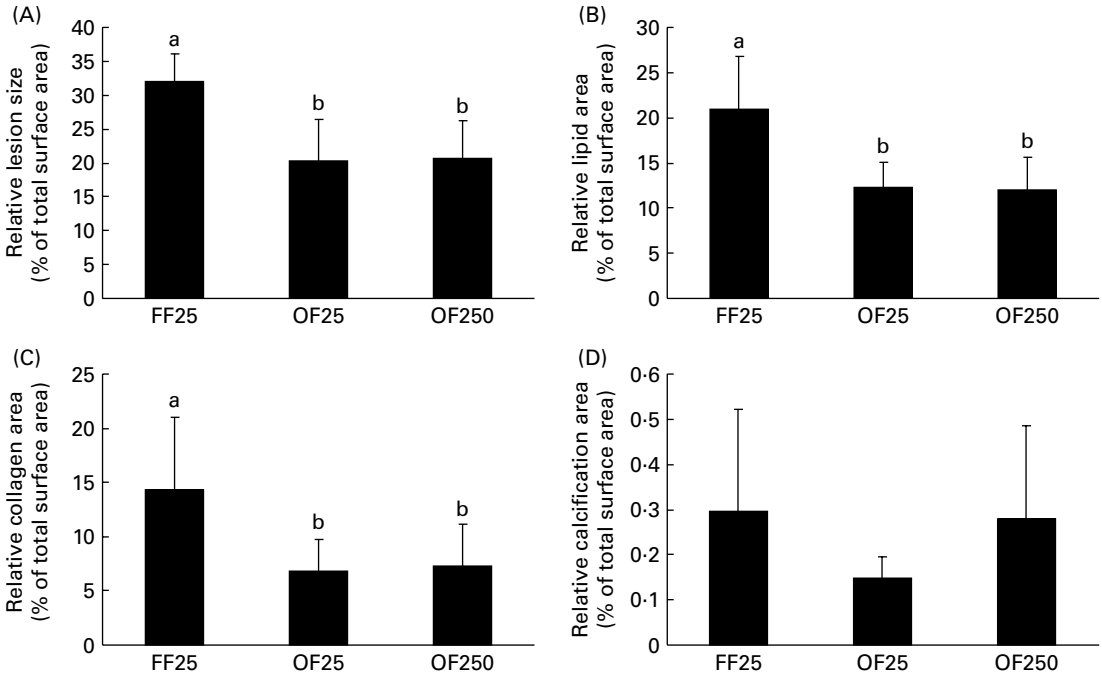

Fig. 1. Effect of treatment on cross-sectional lesion size and lesion composition in the aortic root of LDL receptor-deficient$^{-/-}$ mice fed experimental diets for 14 weeks. (A) Lesion size, (B) lipid area, (C) collagen area and (D) calcified area relative to total surface area. Bars represent means and standard deviations (n 9). ^{a,b} Mean values with unlike letters were significantly different ($P<0{\cdot}05$). FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

Expression of vascular cell adhesion molecule-1 in the aortic root

To evaluate the effect of dietary OF on inflammation, expression of the inflammatory adhesion molecule VCAM-1 in the aortic root sections was determined by immunohistochemistry. Expression of VCAM-1 in the aortic root was approximately 70 % lower in mice fed the OF diets than in those fed the FF25 diet (Fig. 5; $P<0{\cdot}05$). Staining for VCAM-1 was localised mainly to the core region of the atherosclerotic lesions.

Vitamin E status

To evaluate the induction of oxidative stress by the OF, vitamin E concentrations in various tissues were determined in the LDLR$^{-/-}$ mice. Concentrations of total tocopherols in liver, skeletal muscle and epididymal adipose tissue were markedly lower in mice fed the OF25 diet than in those fed the FF25 diet (Table 3; $P<0{\cdot}05$). In mice fed the OF250 diet, concentrations of total tocopherols in liver and epididymal adipose tissue were higher than in mice fed the FF25 diet (Table 3; $P<0{\cdot}05$). Concentrations of total tocopherols in skeletal muscle did not differ between the mice fed the OF250 diet and those fed the FF25 diet (Table 3).

Transcript levels of PPARα target genes in the liver

To investigate whether dietary OF also activates hepatic PPARα in LDLR$^{-/-}$ mice, transcript levels of classical PPARα target genes were determined in the liver. Relative mRNA levels of the PPARα target genes CYP4A10, acyl-CoA oxidase and lipoprotein lipase in the liver were about 4-fold, 2-fold and 1·5-fold higher, respectively, in mice fed the OF diets than in those fed the FF25 diet (Fig. 6; $P<0{\cdot}05$).

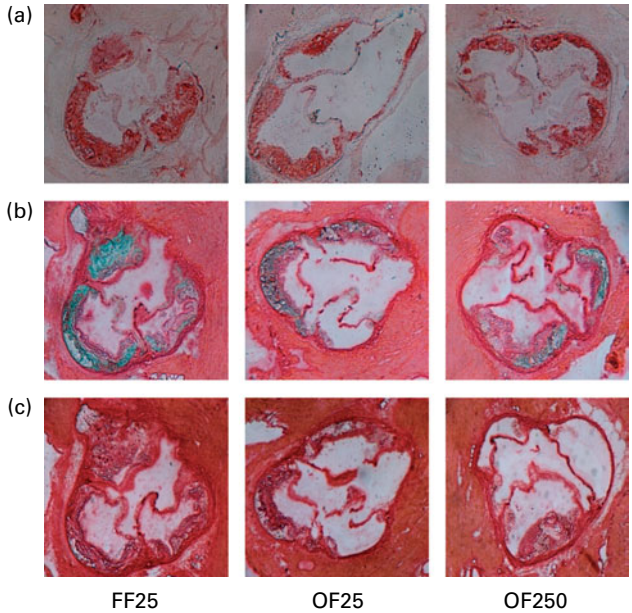

Fig. 2. Stained aortic root sections of LDL receptor-deficient$^{-/-}$ mice fed experimental diets for 14 weeks. (a) Oil red O staining for lipids, (b) Golder's trichrome staining of collagen structures, (c) von Kossa staining of calcifications (3 × magnification). The photographs reflect one representative animal of each experimental group and are taken at an identical distance from the aortic root. FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

British Journal of Nutrition

Table 2. Concentrations of lipids in plasma and lipoproteins of LDL receptor-deficient mice fed the experimental diets for 14 weeks

(Mean values and standard deviations, *n* 12)

	FF25		OF25		OF250	
	Mean	SD	Mean	SD	Mean	SD
TAG (mmol/l)						
Plasma	7·45[a]	1·98	3·54[b]	1·21	3·67[b]	1·11
VLDL + chylomicrons	2·03[a]	0·27	1·03[b]	0·33	1·19[b]	0·43
Cholesterol (mmol/l)						
Plasma	39·4[a]	10·1	33·7[a,b]	6·5	31·6[b]	5·5
VLDL + chylomicrons	20·9	7·5	17·7	4·6	16·7	2·1
LDL	12·6[a]	1·1	11·4[a,b]	1·4	10·6[b]	1·3
HDL	5·9[a]	1·5	4·6[b]	0·6	4·4[b]	0·5

FF25, fresh fat group; OF25 and OF250, oxidised fat groups.
[a,b] Mean values with unlike superscript letters were significantly different ($P<0·05$).

Transcript levels of lipogenic and cholesterogenic genes in the liver

In order to evaluate whether the reduction of lipid concentrations in plasma and lipoproteins by dietary OF is due to decreased lipogenesis and cholesterogenesis in the liver, transcript levels of hepatic lipogenic and cholesterogenic genes were determined. Transcript levels of genes encoding lipogenic enzymes such as fatty acid synthase and acyl-CoA carboxylase and of the rate-limiting enzyme of cholesterol synthesis, hydroxymethylglutaryl-CoA reductase, did not differ among the three groups of mice (data not shown). In addition, transcript levels of the key transcription factors controlling lipogenic and cholesterogenic genes, sterol regulatory element-binding protein-1 and -2, were not different among the three groups (data not shown).

Discussion

In feeding studies dealing with OF, a markedly reduced food intake and growth of the experimental animals has been frequently observed[27,39,40]. This has been attributed to the use of strongly OF containing less PUFA and antioxidants than the equivalent FF and high levels of polymerisation products, thereby causing toxic effects, pronounced oxidative stress and reduction of nutrient digestibility. To avoid these confounding effects, we used a moderately OF (as shown by the comparatively low amount of peroxidation products), which was prepared under deep-frying conditions using hydrogenated palm fat, a typical fat used for such purposes in German restaurants. In addition, dietary fats were equalised for their fatty acid composition by using fat mixtures, and vitamin E concentrations in the diets were adjusted. Moreover, a controlled feeding regimen in which mice of all groups were fed identical amounts of fat was applied. Because we used non-growing mice and the food administered was close to the energy requirement for maintenance, there was only a slight change of body weight during the 14-week feeding period in the three groups of mice. Despite the controlled feeding regimen, weight gain was slightly higher in the FF group than in the OF groups, which might be due to the fact that OF show a slightly lower digestibility than FF[27,39,40]. Nevertheless, the observation that differences in daily weight gains were small between mice fed the FF and those fed the OF indicates that intake of digestible energy did not considerably differ between these groups of mice. We are therefore confident that the metabolic effects of OF reported in this study are not confounded by the slightly reduced weight gain of the OF-fed mice.

The main finding of the present study is that a moderately OF containing levels of lipid oxidation products that are below the limit allowed for 'used frying fats' when fed together with a hyperlipidaemic diet inhibits atherosclerosis development in LDLR$^{-/-}$ mice, as evidenced by

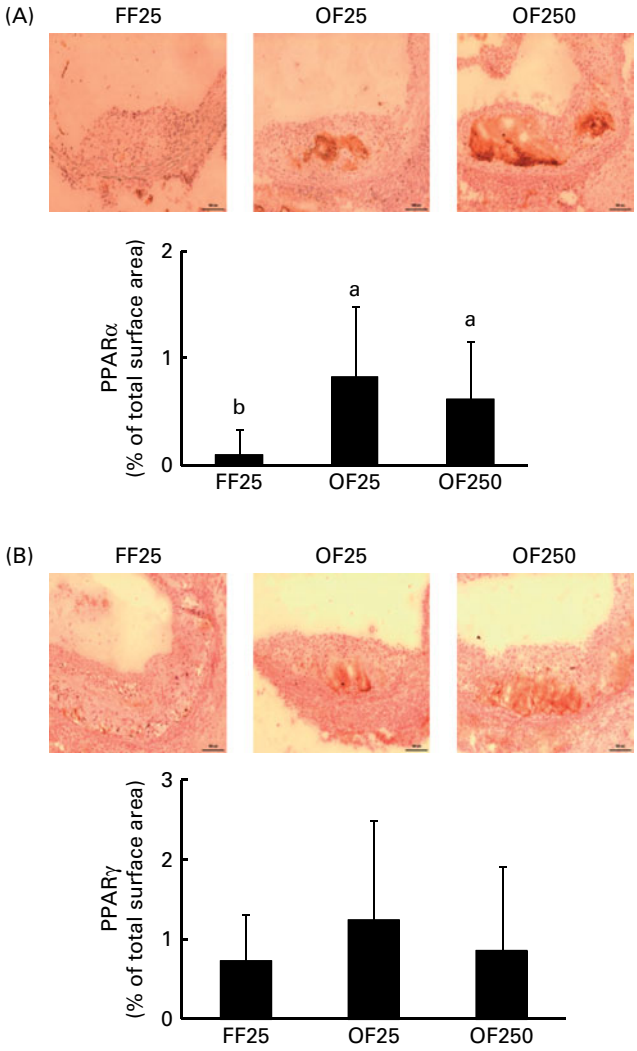

Fig. 3. Quantification of immunohistochemical staining for (A) PPARα and (B) PPARγ in aortic root sections of LDL receptor-deficient$^{-/-}$ mice fed experimental diets for 14 weeks. The photographs reflect one representative animal of each experimental group and are taken at an identical distance from the aortic root (10 × magnification). Bars represent means and standard deviations (*n* 9). [a,b] Mean values with unlike letters were significantly different ($P<0·05$). FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

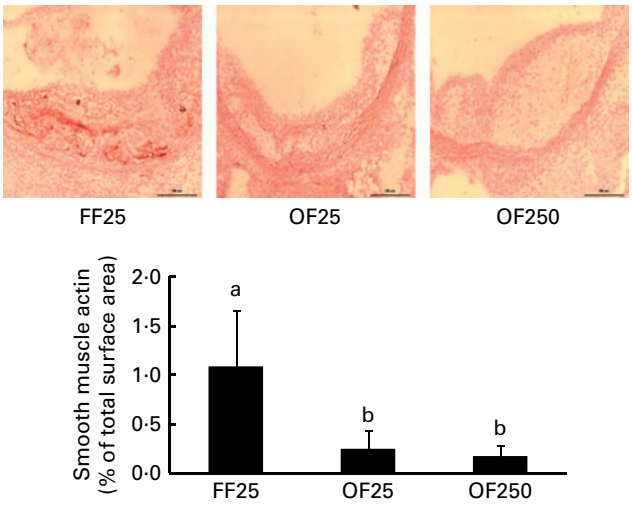

Fig. 4. Quantification of immunohistochemical staining for smooth muscle α-actin in aortic root sections of LDL receptor-deficient$^{-/-}$ mice fed experimental diets for 14 weeks. The photographs reflect one representative animal of each experimental group and are taken at an identical distance from the aortic root (10 × magnification). Bars represent means and standard deviations (n 9). [a,b] Mean values with unlike letters were significantly different ($P<0·05$). FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

a markedly lower lesion size (cross-sectional lesion area) and strongly reduced lipid and collagen contents in the aortic root. Moreover the present study shows that the inhibitory effect of the moderately OF on atherosclerosis development could even be observed when the vitamin E concentration in the diet was moderate. It is likely that this concentration of dietary vitamin E was sufficient to prevent the induction of oxidative stress by OF. In agreement with the recent findings[26,27], we observed that the

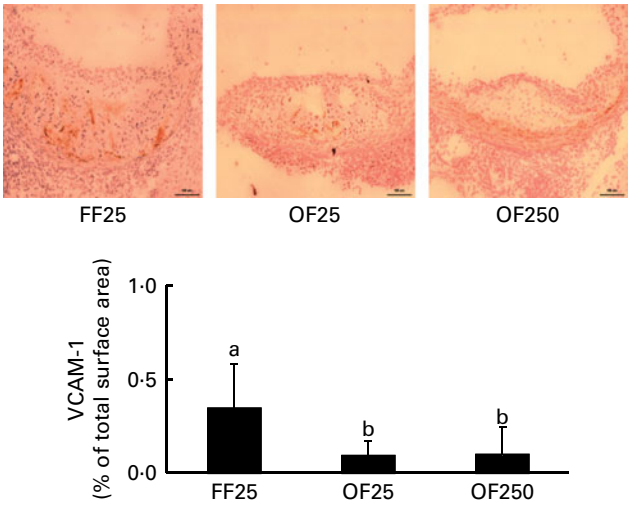

Fig. 5. Quantification of immunohistochemical staining for vascular cell adhesion molecule (VCAM)-1 in aortic root sections of LDL receptor-deficient$^{-/-}$ mice fed experimental diets for 14 weeks. The photographs reflect one representative animal of each experimental group and are taken at an identical distance from the aortic root (10 × magnification). Bars represent means and standard deviations (n 9). [a,b] Mean values with unlike letters were significantly different ($P<0·05$). FF25, fresh fat group; OF25 and OF250, oxidised fat groups.

Table 3. Concentrations of total tocopherols in tissues of LDL receptor-deficient mice fed the experimental diets for 14 weeks

(Mean values and standard deviations, n 12)

	FF25		OF25		OF250	
	Mean	SD	Mean	SD	Mean	SD
α-Tocopherol equivalents (nmol/g)						
Liver	93[b]	10	31[c]	5	221[a]	79
Skeletal muscle	15·1[a]	3·3	8·5[b]	1·4	16·1[a]	4·4
White adipose tissue	47·5[b]	8·5	26·6[c]	5·9	85·3[a]	14·9

[a,b,c] Mean values with unlike superscript letters were significantly different ($P<0·05$).

vitamin E status of the OF-fed mice was compromised by the OF, which suggests induction of oxidative stress.

As a mechanism of action, we suggest that inhibition of atherosclerotic lesion development in LDLR$^{-/-}$ mice fed diets containing OF is, at least partially, due to reduction in plasma cholesterol and TAG concentrations, because elevated blood lipid concentrations are known risk factors for the development of atherosclerosis. It has been shown that the lipid-lowering action of OF is mediated in part by activation of PPARα in the liver, leading to an enhanced fatty acid catabolism and an increased lipolysis of VLDL particles[5–7]. Due to the activation of hepatic PPARα, dietary OF prevent the excessive accumulation of TAG induced by steatosis-inducing agents such as ethanol[41]. Herein, activation of hepatic PPARα could also be observed in LDLR$^{-/-}$ mice as evidenced by the up-regulation of PPARα-dependent genes such as acyl-CoA oxidase, CYP4A10 and lipoprotein lipase in the liver and elevated relative liver weights, which is a typical response to PPARα agonists. A recent study with mice also revealed a strong up-regulation of PPARα and a marked reduction in plasma lipid concentrations in response to feeding a diet supplemented with 13-hydroperoxy octadecadienoic acid, which is derived from peroxidation of linoleic acid[15]. It is therefore likely that, through the activation of hepatic PPARα, components of OF are capable of favourably influencing the blood lipid profile. Thus, we suggest that PPARα activation in the liver contributes to lipid lowering in plasma of LDLR$^{-/-}$ mice, which might in part be responsible for the inhibition of atherosclerotic lesion development. In contrast, transcription of sterol regulatory element-binding protein-regulated lipogenic and cholesterogenic genes, such as fatty acid synthase, LDLR and hydroxymethylglutaryl-CoA reductase, was not influenced by OF in the liver of the mice, suggesting that reduced lipid concentrations in plasma are not due to a decreased synthesis of fatty acids and cholesterol in the liver and/or uptake of cholesterol into the liver[42,43].

We propose that direct activation of PPARα in the vasculature also contributed to the inhibitory effect of OF on atherosclerosis development, because we could observe a markedly increased PPARα expression in the aortic root

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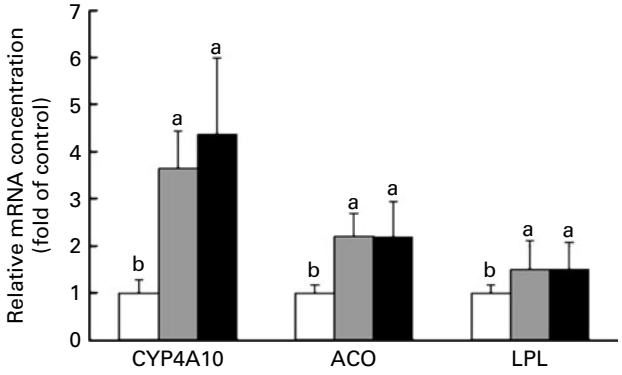

Fig. 6. Effect of treatment on relative mRNA concentrations of PPARα-responsive genes in livers of LDL receptor-deficient$^{-/-}$ mice fed experimental diets for 14 weeks. Bars represent means and standard deviations (n 12). ^{a,b} Mean values with unlike letters were significantly different ($P<0{\cdot}05$). FF25 (□), fresh fat groups; OF25 (▨) and OF250 (■), oxidised fat groups. ACO, acyl-CoA oxidase; LPL, lipoprotein lipase.

lesions of mice that fed the OF. This is probably indicative of an increased expression of PPARα by the plaque cells because lesion size was markedly reduced by the OF. The increased expression of PPARα protein in atherosclerotic lesions by OF has to be considered beneficial because inhibition of atherosclerosis development by anti-atherogenic dietary agents was accompanied by an increased PPARα expression in the atherosclerotic plaque and the aorta, respectively[44]. In line with the increased expression of PPARα by dietary OF is the observation that the expression of the inflammatory protein VCAM-1 and the SMC-specific marker α-actin as well as the lipid and collagen content in the aortic root was also significantly reduced by the OF. VCAM-1 and other adhesion molecules, the expression of which is negatively regulated by PPARα[16], are responsible for monocyte attachment to the luminal surface of the blood vessels and are required for subsequent infiltration of the subendothelial space by monocyte-derived macrophages. Consequently, inhibition of endothelial adhesion molecule expression by PPARα activators inhibits atherosclerotic plaque formation[19,20]. The decreased expression of SMC α-actin suggests that the content of SMC in the aortic root of LDLR$^{-/-}$ mice was reduced by the OF. This might be indicative of an inhibitory effect of OF on the proliferation and/or migration of SMC into the intima, which was shown to be inhibited by PPARα activation[18]. Because SMC are the major collagen-producing cells in the atherosclerotic plaque and collagens substantially contribute to lesion volume[45], it is likely that the decreased aortic SMC content is responsible for the reduced collagen content and lesion size in mice fed OF. In contrast to lipid and collagen content of atherosclerotic lesions, no effect of OF could be observed on the levels of calcification in the aortic root of LDLR$^{-/-}$ mice, suggesting that dietary OF has no major influence on the calcification process and on the complex mechanisms regulating vascular calcification.

Expression of PPARγ, another PPAR isotype with atheroprotective effects that can also be activated by hydroxylated fatty acids present in OF, was not influenced by the OF in the aortic root of LDLR$^{-/-}$ mice. Although this finding does not definitely exclude the possibility that OF caused some of its effects by activation of PPARγ, it is less likely because a recent study revealed only a weak activation of this receptor by OF[37].

Heated oils are a complex mixture of a great number of oxidation products formed during heat treatment. Therefore, it remains unclear which of the components of the OF were responsible for the effects observed in this study. Hydroxy and hydroperoxy fatty acids as well as cyclic fatty acid monomers have been identified as strong PPARα agonists[13–15]. Therefore, these oxidation products are potential candidates which could be responsible for the anti-atherogenic effects induced by the OF. However, Khan-Merchant *et al.*[4] observed that feeding 13-hydroxy octadecadienoic acid, an oxidation product of linoleic acid, did not inhibit but even enhanced the development of atherosclerosis in LDLR$^{-/-}$ mice. Recently, Litvinov *et al.*[46] observed that administration of azelaic acid, an end product of linoleic acid peroxidation, inhibits the development of atherosclerosis in LDLR$^{-/-}$ mice, probably by preventing macrophage accumulation in the arterial wall. Thus, this substance could also account for the anti-atherogenic effect of OF observed in the present study.

In the present study, we used LDLR$^{-/-}$ mice as a well-established experimental model of atherosclerosis. When trying to transfer these findings to human subjects, it must be considered that mice, in contrast to human subjects, have a much higher tissue expression level of PPARα and that the response of many genes to PPARα activation is much stronger[47,48]. As the beneficial effects of the OF observed in the present study might be primarily caused by activation of PPARα, it is expected that the same effects are much weaker in human subjects. Moreover, the results of the present study must not be interpreted in the way that OF could regarded as a health-promoting component of the diet, as components of OF might have several adverse effects in human subjects. The results of the present study rather suggest that OF are a mixture of chemically distinct substances, some of which exhibit a significant biological activity.

In conclusion, the present study demonstrates that feeding an OF prepared under deep-frying conditions containing levels of lipid oxidation products which are below the limit allowed for 'used frying fats' causes anti-atherogenic effects in LDLR$^{-/-}$ mice – effects that are probably due to activation of PPARα in the liver and the vasculature.

Acknowledgements

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analysed data; I. K., R. R. and K. E. wrote the paper. K. E. had primary responsibility for the final content. All the authors read and approved the final manuscript. The authors have no conflicts of interest.

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LIPIDS IN HEALTH AND DISEASE

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13-hydroxy linoleic acid increases expression of the cholesterol transporters ABCA1, ABCG1 and SR-BI and stimulates apoA-I-dependent cholesterol efflux in RAW264.7 macrophages

Ines Kämmerer, Robert Ringseis*, Ronald Biemann, Gaiping Wen and Klaus Eder

Abstract

Background: Synthetic activators of peroxisome proliferator-activated receptors (PPARs) stimulate cholesterol removal from macrophages through PPAR-dependent up-regulation of liver × receptor α (LXRα) and subsequent induction of cholesterol exporters such as ATP-binding cassette transporter A1 (ABCA1) and scavenger receptor class B type 1 (SR-BI). The present study aimed to test the hypothesis that the hydroxylated derivative of linoleic acid (LA), 13-HODE, which is a natural PPAR agonist, has similar effects in RAW264.7 macrophages.

Methods: RAW264.7 macrophages were treated without (control) or with LA or 13-HODE in the presence and absence of PPARα or PPARγ antagonists and determined protein levels of LXRα, ABCA1, ABCG1, SR-BI, PPARα and PPARγ and apolipoprotein A-I mediated lipid efflux.

Results: Treatment of RAW264.7 cells with 13-HODE increased PPAR-transactivation activity and protein concentrations of LXRα, ABCA1, ABCG1 and SR-BI when compared to control treatment ($P < 0.05$). In addition, 13-HODE enhanced cholesterol concentration in the medium but decreased cellular cholesterol concentration during incubation of cells with the extracellular lipid acceptor apolipoprotein A-I ($P < 0.05$). Pre-treatment of cells with a selective PPARα or PPARγ antagonist completely abolished the effects of 13-HODE on cholesterol efflux and protein levels of genes investigated. In contrast to 13-HODE, LA had no effect on either of these parameters compared to control cells.

Conclusion: 13-HODE induces cholesterol efflux from macrophages via the PPAR-LXRα-ABCA1/SR-BI-pathway.

Keywords: Peroxisome proliferator-activated receptors, Cholesterol efflux, Macrophage, Oxidized fatty acids

Background

Although dietary consumption of oxidized fats (OF) is known to cause some unfavourable effects (e.g., oxidative stress, depletion of antioxidants; [1-3]), experiments in laboratory animals and pigs consistently demonstrated that administration of OF reduces lipid concentrations (triacylglycerols and cholesterol) in liver and plasma (reviewed in [4]). Recent evidence suggests that activation of the peroxisome proliferator-activated receptor α (PPARα) pathway in the liver is largely responsible for the lipid lowering action of OF [5-7]. PPARα is a ligand-activated transcription factor which controls a comprehensive set of genes involved in most aspects of lipid catabolism [8,9]. Thus, targeting PPARα by the administration of pharmacological PPARα activators, e.g., fenofibrate, bezafibrate, gemfibrozil, is an effective approach for the treatment of hyperlipidemia [10].

Besides targeting lipid catabolism in the liver and regulating plasma lipid concentrations, synthetic PPARα activators also directly influence vascular function in a beneficial manner through negatively regulating the expression of pro-inflammatory genes in vascular cells such as endothelial cells, smooth muscle cells, and macrophages and inducing genes involved in macrophage cholesterol homeostasis [11-13]. These direct

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atheroprotective together with the lipid lowering effects are largely responsible for the observation that pharmacological PPARα activators cause an inhibition of atherosclerosis development [14-17]. Interestingly, in a recent study it could be demonstrated that dietary administration of an OF also causes activation of PPARα in the vasculature, inhibits expression of pro-inflammatory vascular adhesion molecules, whose expression is negatively regulated by PPARα, and inhibits atherosclerotic plaque development in the low-density lipoprotein receptor deficient mouse model of atherosclerosis [18]. These findings suggest that OF exerts similar effects as pharmacological PPARα agonists.

The components of OF which are supposed to be responsible for PPARα activation are hydroxy and hydroperoxy fatty acids, such as 13-hydroxy octadecadienoic acid (13-HODE) or 13-hydroperoxy octadecadienoic acid (13-HPODE). These substances are formed during oxidation of dietary lipids and absorbed from the intestine following ingestion of these fats [19,20]. Using different experimental approaches, such as ligand binding studies, transactivation assays and cell culture experiments, it was shown that these oxidized fatty acids are potent ligands and activators of PPARα [21-24]. An animal experiment revealed that feeding a diet supplemented with 13-HPODE reduces plasma triacylglycerol concentrations indicating that oxidized fatty acids are indeed the mediators of the lipid lowering effects of OF [25]. Whether oxidized fatty acids are also responsible for the observation that OF modulates the expression of PPAR-dependent genes in the vasculature [18], has not been studied yet. Therefore, the present study aimed to test the hypothesis that the hydroxylated derivative of linoleic acid, 13-HODE, induces genes involved in macrophage cholesterol homeostasis, such as liver × receptor α (LXRα), ATP-binding cassette transporter A1 (ABCA1), ABCG1 and scavenger receptor class B type 1 (SR-BI), and increases cholesterol removal from macrophages in a PPAR-dependent manner. Recent studies showed that synthetic activators of PPARα stimulate cholesterol removal from macrophages, an important step in reverse cholesterol transport, through PPAR-dependent up-regulation of LXRα [26-28], which serves as an intracellular cholesterol sensor and positively regulates expression of cholesterol exporters such as ABCA1, ABCG1 and SR-BI [29].

Materials and methods

Cell culture and treatments

Mouse RAW264.7 cells, obtained from LGC Promochem (Wesel, Germany), were grown in DMEM medium (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum, 4 mmol/L L-glutamine, 4.5 g/L glucose, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate and 0.5% gentamycin. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. RAW264.7 cells were plated in 6-well plates at a density of 1×10^6/well for western blot analysis and at a density of 8×10^5/well for cholesterol analysis. After reaching 80% confluence, cells were treated with LA (≥96% pure) and 13-HODE (≥96% pure; both from Sigma-Aldrich, Taufkirchen, Germany) at the concentrations indicated for 24 h. Cells treated with vehicle alone (ethanol) were used as controls. Incubation media containing fatty acids were prepared by diluting the fatty acid stock solutions (100 mmol/L LA and 2.5 mmol/L 13-HODE in ethanol) with DMEM medium to 100 μmol/L (LA) and 2.5 μmol/L (13-HODE), as also described from others [30]. After addition of the fatty acids to the medium, the medium was gently vortexed at RT to ensure complete solubility of the added fatty acids. No signs of precipitation could be observed. Due to the presence of BSA in the medium, it is expected that most of the added fatty acids was bound to albumin which serves as the natural delivery molecule for free fatty acids in plasma. The concentration of 13-HODE used was based on the knowledge that this fatty acid can be found in human blood in the low μmolar range [31]. Incubation media of control cells contained the same vehicle (ethanol) concentration of 0.1% (v/v). Specific precautions other than appropriate storage conditions (-20°C, in the dark) were not taken to prevent oxidation of LA and 13-HODE. 13-HODE has been reported to be very stable against oxidation as evidenced from air oxidation experiments with 13S-HODE which were carried out by addition of amounts of iron ions greatly surpassing the Fe^{2+} concentration in biological samples [32]. Even under extreme conditions, such as elevated temperature (45-50°C) and enhanced reaction time (2 weeks), 95% of the 13S-HODE was recovered unchanged by GC-MS analysis [32]. For experiments using PPAR inhibitors, cells were pre-treated with either 10 μmol/L of the PPARα selective antagonist GW6471 (Sigma-Aldrich) or 20 μmol/L of the PPARγ selective antagonist GW9662 (Sigma-Aldrich) 4 h before treatment with fatty acids. All experiments were performed between passages 5 and 8.

Western blot analysis

After treatment of cells as indicated above, cells were lysed with RIPA lysis buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Sigma), and protein concentrations of lysates determined by the BCA assay (VWR, Darmstadt, Germany). Equal amounts of protein were electrophoresed by 7.5% SDS-PAGE for ABCA1 and ABCG1 and 10% SDS-PAGE for SR-BI and LXRα and transferred to

a nitrocellulose membrane. The membranes were blocked at 4°C in blocking solution (5% skim milk in Tris buffered saline with Tween-20 [TBS-T]: 50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, 0.2% Tween-20), and then incubated with primary antibodies against ABCA1 (1:1,000, Novus Biologicals), ABCG1 (1:2000, Abcam), β-Actin (1:1,000, Novus Biologicals), SR-B1 (1:1,000, Novus Biologicals), LXRα (1:500, Affinity BioReagents) for 2 h at room temperature or overnight at 4°C depending on the antibody used. The membranes were washed with TBS-T, and incubated with a horseradish peroxidase conjugated secondary anti-mouse IgG antibody (1:10,000, Jackson Immuno Research) or anti-rabbit IgG antibody (1:10,000, Sigma-Aldrich) for 1.5 h at room temperature. Afterwards blots were developed using ECL Advance (GE Healthcare Europe, Freiburg, Germany) for polyclonal antibodies and ECL Plus (GE Healthcare Europe) for monoclonal antibodies. The signal intensities of specific bands were detected with Bio-Imaging system (Syngene, Cambridge, UK) and quantified using Syngene GeneTools software (Nonlinear Dynamics, USA).

Analysis of cholesterol content in medium and cells

After pre-treatment with or without PPAR antagonists and treatment of macrophage cells with or without fatty acids as indicated above, cells were incubated again with the antagonists for 4 h and afterwards with or without the corresponding fatty acids in the presence or absence of apolipoprotein A-I (apoA-I) (30 μg/mL) for 24 h. Afterwards, medium was collected and removed from detached cells by a centrifugation step, and the cell monolayer washed twice with PBS. Cellular lipids were extracted with a mixture of hexane and isopropanol (3:2, v/v) and lipids in the medium were extracted with a mixture of chloroform and methanol (2:1, v/v). Lipid extracts were dried under a stream of nitrogen and total cholesterol concentrations were determined using an enzymatic assay from Biocon (Vöhl-Marienhagen, Germany). Cholesterol concentrations were related to cellular protein content as determined by the BCA protein assay kit.

Transient transfection and dual luciferase assay

RAW264.7 cells were plated in 24-well plates at a density of 5×10^5/well. After reaching 70% confluence, cells were transiently transfected with 500 ng of a 3 × ACO-PPRE reporter vector (containing three copies of consensus PPRE from the ACO promoter in front of a luciferase reporter gene; a generous gift from Dr. Sander Kersten, Nutrigenomics Consortium, Top Institue (TI) Food and Nutrition, Wageningen, Netherlands) using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Cells were also co-transfected with 50 ng of pGL4.74 Renilla luciferase (encoding the renilla luciferase reporter gene; Promega, Mannheim, Germany), which was used as an internal control reporter vector to normalize for differences in transfection efficiency. Following transfection, cells were treated with either WY-14,643 (as positive control), LA, 13-HODE or vehicle only (DMSO and ethanol) at the concentrations indicated for 24 h. Afterwards, cells were washed with PBS and lysed with passive lysis buffer (Promega). Luciferase activities were determined with the Firefly and Renilla Luciferase Assays (PJK, Kleinblittersdorf, Germany) according to the manufacturer's instructions using a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany) as described recently in more detail [33].

Statistical analysis

Data were subjected to either Student's t-test or one-way ANOVA using the Minitab Statistical Software Rel. 13.0 (Minitab, State College, PA, USA). For statistically significant F values, individual means of the treatment groups were compared by Fisher's multiple range test. Means were considered significantly different for $P < 0.05$.

Results

Effects of 13-HODE and LA on PPAR transactivation activity and PPAR protein levels in RAW264.7 macrophages

To study the effect of 13-HODE and LA on the activation of the PPAR signalling pathway in macrophages, RAW264.7 were transiently transfected with a reporter plasmid containing 3 copies of the consensus PPRE in front of a luciferase reporter and studied the stimulation of the reporter activity by 13-HODE and LA as well as by the synthetic PPARα agonist WY-14,643. Treatment with WY-14,643 as a positive control increased PPAR-responsive reporter activity by about 90% compared to treatment with vehicle alone ($P < 0.05$; Figure 1A). Treatment with 13-HODE dose-dependently increased the PPAR-responsive reporter activity compared to treatment with vehicle alone ($P < 0.05$; Figure 1A); incubating RAW264.7 cells with 1.0 and 2.5 μmol/L of 13-HODE increased the PPAR-responsive reporter activity by about 28 and 50%, respectively, compared to vehicle control. Incubation of macrophages with increasing concentrations of LA had no effect on the PPAR-responsive reporter activity when compared to macrophages treated with vehicle alone (Figure 1A); there was only a numerical, but not significant increase in the PPAR-responsive reporter activity at the highest concentration of LA (100 μmol/L) when compared to vehicle control. Protein concentrations of PPARα and PPARγ did not differ between control macrophages and macrophages treated with either 2.5 μmol/L 13-HODE or 100 μmol/L LA (Figure 1B).

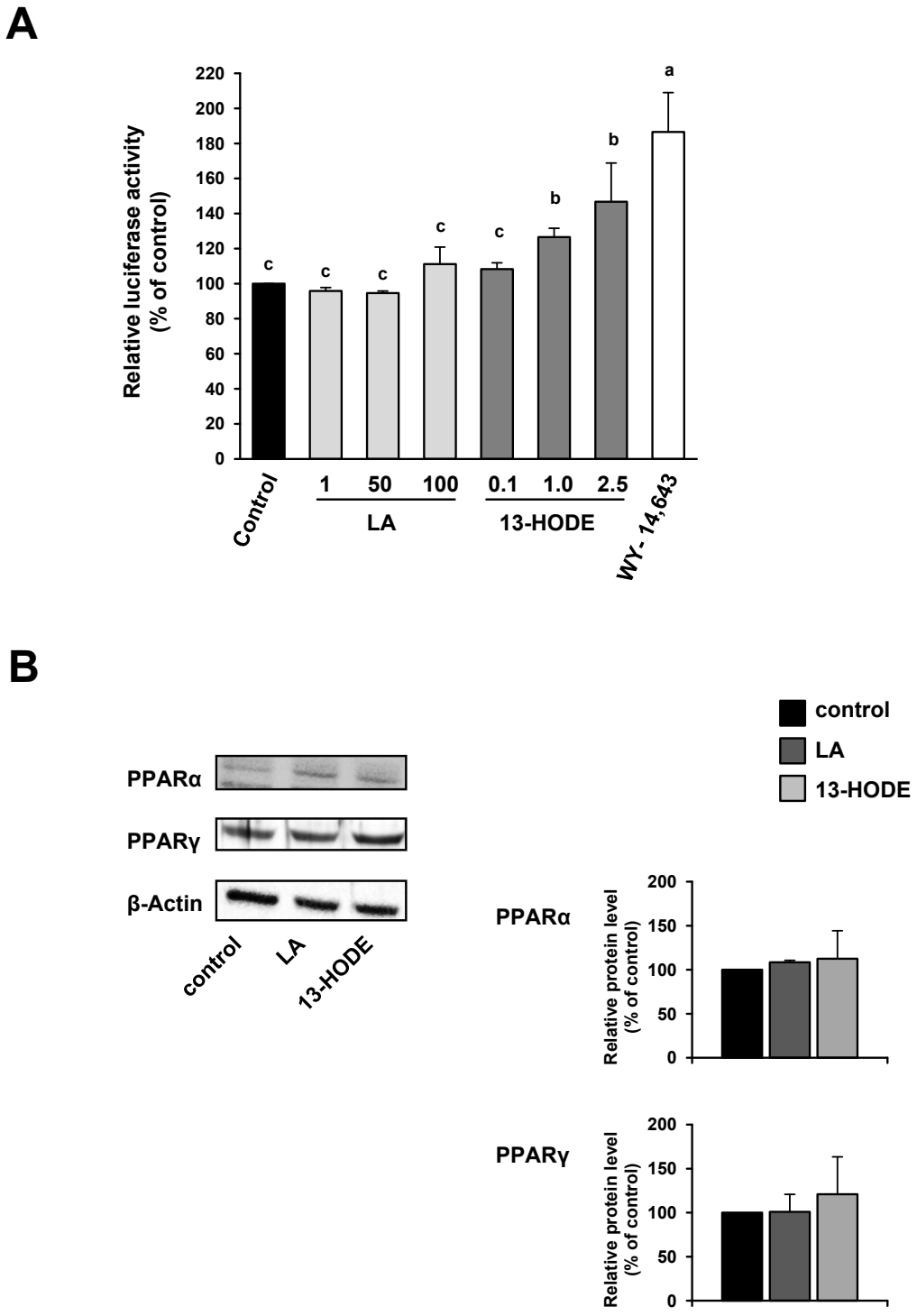

Figure 1 Effects of 13-HODE, LA and WY-14,643 on PPAR/PPRE transactivation activity and PPAR protein levels in RAW264.6 macrophages. A, RAW264.7 cells were transiently transfected with 3 × ACO-PPRE reporter vector. After transfection, cells were treated or not with 0.1-2.5 μmol/L 13-HODE, 1-100 μmol/L LA and 50 μmol/L WY-14,643 for 24 h. Afterwards, cells were lysed, and luciferase activities of the ACO-PPRE firefly luciferase vector and a co-transfected renilla luciferase vector determined by a dual luciferase assay. Bars represent means ± SD from four independent experiments (n = 4). Data are expressed as percentage of relative luciferase activity of vehicle control cells. Results from statistical analysis are indicated: Significant effects are denoted with an asterisk ($P < 0.05$). B, RAW264.7 cells were treated with 2.5 μmol/L 13-HODE, 100 μmol/L LA or vehicle (ethanol) for 24 h. Afterwards, cells were lysed and subsequently processed for western blotting as described in the materials and methods section. Representative immunoblots specific for PPARα, PPARγ, and β-actin which was used for normalization are shown. Bars represent data from densitometric analysis and are means ± SD from three independent experiments (n = 3). Data are expressed as percentage of protein concentration of vehicle control cells.

Effects of 13-HODE and LA in the presence and absence of PPARα and PPARγ selective antagonists on relative protein concentrations of ABCA1, ABCG1, SR-BI and LXRα in RAW264.7 macrophages

To explore the involvement of PPARα and PPARγ in the action of 13-HODE on proteins regulating cholesterol homeostasis, cells were pre-treated without or with selective PPARα and PPARγ antagonists prior to treatment with fatty acids. In the absence of an antagonist, 2.5 μmol/L of 13-HODE increased protein levels of ABCA1, ABCG1, SR-BI and LXRα in RAW264.7 macrophages ($P < 0.05$; Figure 2A and 2B), whereas 100 μmol/L of LA had no effect (Figure 3A and 3B). When cells were pre-treated with either the PPARα antagonist GW6471 or the PPARγ antagonist GW9662 the effect of 13-HODE on the concentrations of these proteins was completely abolished (Figure 2A and 2B). In cells treated with LA, the pre-treatment with GW6471 caused a 15-25% decrease in the protein levels of ABCA1 and SR-BI ($P < 0.05$; Figure 2A and 2B), whereas protein levels of ABCG1 and LXRα remained unaffected. Pre-treatment with GW9662 did not alter the effect of LA on protein levels of ABCA1, ABCG1, SR-BI and LXRα in comparison to treatment without PPARα or PPARγ antagonist (Figure 2A and 2B).

Effects of 13-HODE and LA on cholesterol concentrations in macrophages in the presence and absence of apoA-I and PPARα and PPARγ antagonists

To investigate whether the 13-HODE-induced alterations of the expression of proteins involved in cholesterol homeostasis had an effect on macrophage cholesterol content, we determined the cholesterol concentrations of cells and medium after treatment with 13-HODE and LA, both in the presence and absence of the extracellular lipid acceptor apo-AI. In the absence of apoA-I, cholesterol concentrations in cells and medium did not differ between control macrophages and macrophages treated with either LA or 13-HODE (Figure 3A and 3B). In the presence of apoA-I, treatment with 13-HODE decreased cellular cholesterol concentration by approximately 15% ($P < 0.05$; Figure 3A and 3B) and increased cholesterol concentration in medium by approximately 25% when compared to treatment with vehicle alone ($P < 0.05$; Figure 3A and 3B). In contrast, treatment with LA in the presence of apoA-I had no effect on cholesterol concentrations in cells and medium when compared to control treatment (Figure 3A and 3B). When cells were pre-treated with either the PPARα antagonist GW6471 or the PPARγ antagonist GW9662 the effect of 13-HODE on cellular and medium cholesterol concentration was completely abolished (Figure 3A and 3B).

Discussion

Pharmacological PPAR ligands have been demonstrated to induce cholesterol removal from macrophages and to prevent macrophage foam cell formation through alterations in the expression of genes critically involved in macrophage cholesterol homeostasis [26-28]. Feeding OF was repeatedly shown to cause PPARα activation in tissues of different species [4]. This effect has been attributed to characteristic substances of OF such as hydroxylated fatty acids, e.g. 13-HODE, which are known ligands of PPARs [34,35]. The present study shows that 13-HODE moderately, but significantly lowers the cellular cholesterol content of macrophages while increasing the cholesterol content in the medium when apo-AI, the main apo of high density lipoprotein (HDL) particles, is present in the culture medium as an extracellular cholesterol acceptor. The export of cholesterol to acceptors such as apoA-I or HDL is an important part of the reverse cholesterol transport responsible for redistribution of cholesterol from peripheral tissues to the liver. Recent studies in RAW264.7 macrophages provided evidence that apoA-I is internalized by endocytosis into the macrophage where it acquires free cholesterol from intracellular pools before it is resecreted by exocytosis (novel model of cholesterol efflux called retroendocytosis), and that apoA-I internalization is required for transporter-mediated cholesterol efflux [36]. In the absence of apoA-I, no effect of 13-HODE on macrophage cholesterol content and cholesterol content in the incubation medium was observed. Thus, our findings indicate that 13-HODE stimulates specifically apoA-I-dependent cholesterol efflux in macrophages, an effect that is also known from synthetic PPAR ligands [26-28]. Interestingly, a previous study has shown that dietary oxidized fatty acids enhance intestinal cell apoA-I production via a PPAR-dependent process [37]. Although it has to be considered that plasma HDL levels are also determined by hepatic apoA-I synthesis and nascent HDL particle secretion, these previous findings together with our findings herein may be indicative of the ability of oxidized fatty acids to stimulate reverse cholesterol transport. Interestingly, evidence from feeding studies indeed shows that treatment of rats and guinea pigs with oxidized fat increases HDL cholesterol concentrations in plasma [3,38]. In pigs, however, which are better model objects for humans, no effect of oxidized fat on HDL cholesterol concentrations apoA-I production was found [39]. Epidemiological associations between oxidized fat intake and plasma HDL cholesterol in humans have not been established. This is probably explained by the fact that it is difficult to estimate the intake of oxidized fat.

On the molecular level, reduction of macrophage cholesterol accumulation and stimulation of cholesterol efflux from macrophages to extracellular lipid acceptors by PPAR agonists has been explained by an up-regulation of LXRα and subsequent induction of macrophage

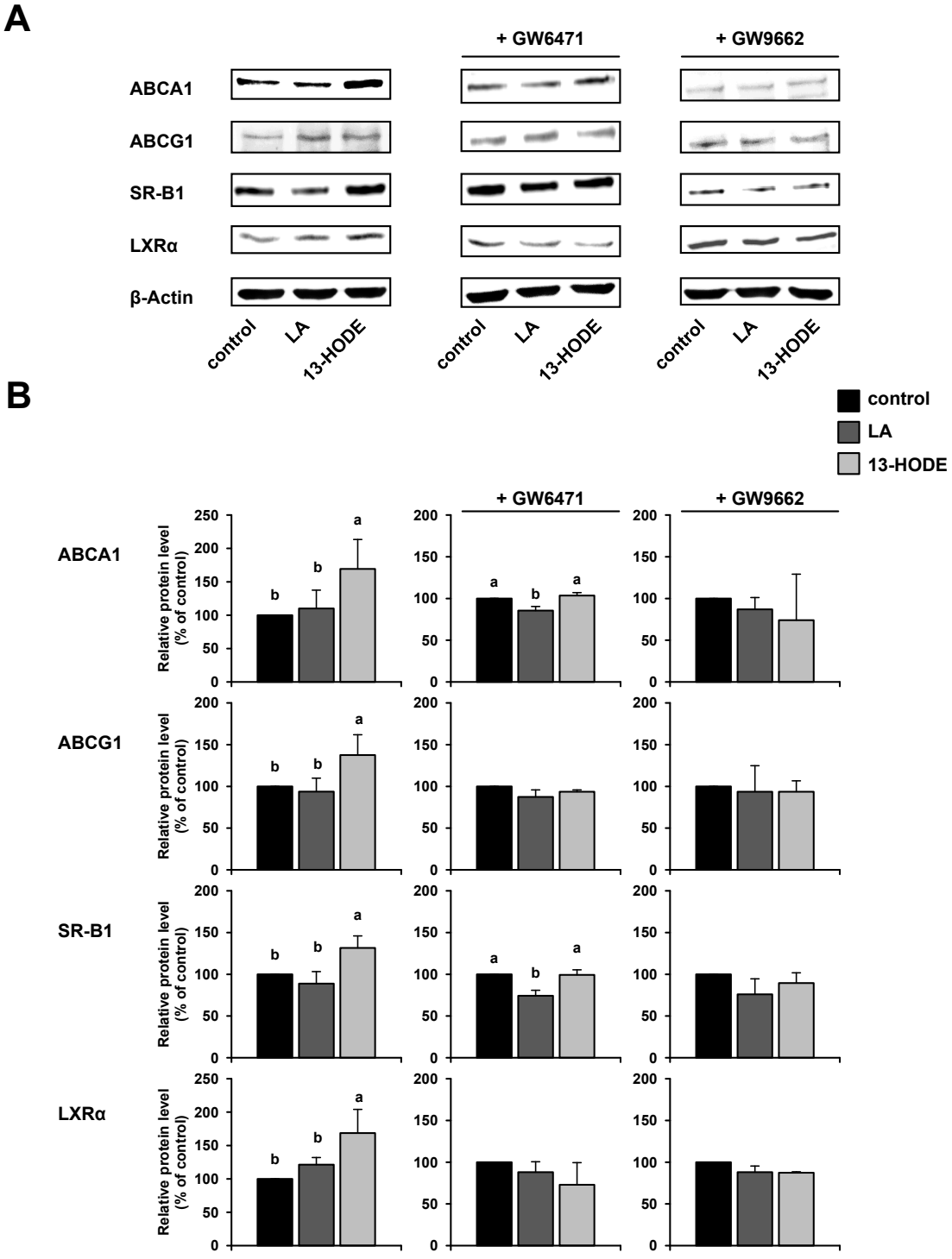

Figure 2 Effects of 13-HODE and LA in the presence and absence of PPARα and PPARγ selective antagonists on molecular markers of cholesterol homeostasis in RAW264.7 macrophages. RAW264.7 cells were pre-treated without or with the PPARα selective antagonist GW6471 or the PPARγ selective antagonist GW9662 and subsequently treated without (vehicle control) or with 2.5 μmol/L 13-HODE or 100 μmol/L LA for 24 h. Afterwards, cells were lysed and subsequently processed for western blotting as described in the materials and methods section. A, Representative immunoblots specific for ABCA1, ABCG1, SR-BI, LXRα, and β-actin which was used for normalization are shown. B, Bars represent data from densitometric analysis and are means ± SD from three independent experiments (n = 3). Data are expressed as percentage of protein concentration of vehicle control cells. Results from statistical analysis are indicated: Significant effects are denoted with superscript letters. Bars marked without a common superscript letter significantly differ ($P < 0.05$).

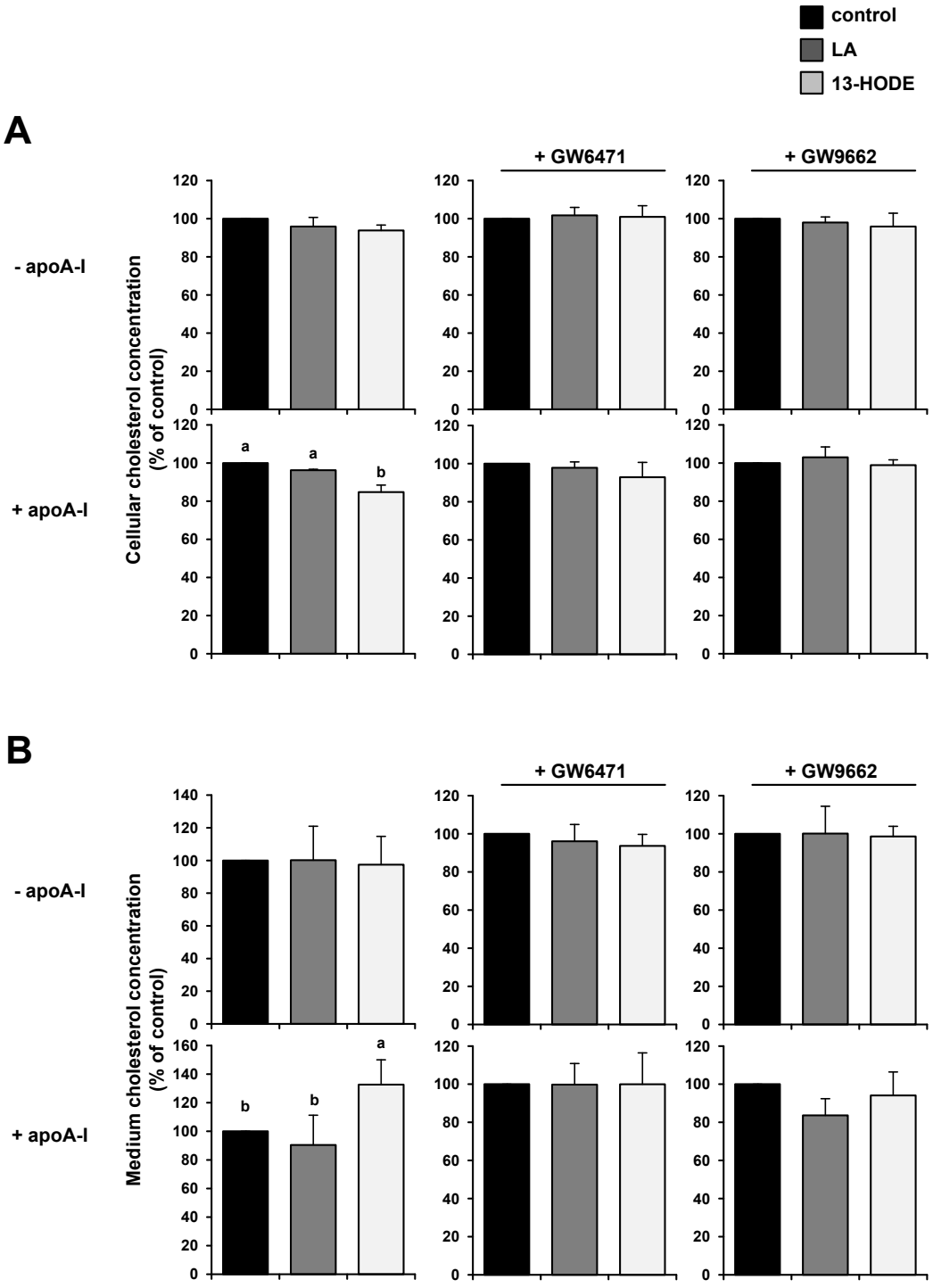

Figure 3 Effects of 13-HODE and LA on cholesterol concentrations in cells and medium of macrophages in macrophages in the presence and absence of apoA-I and PPARα and PPARγ antagonists. After pre-treatment without or with PPAR antagonists for 4 h and treatment of RAW264.7 macrophage cells without (vehicle control) or with 2.5 μmol/L 13-HODE or 100 μmol/L LA for 20 h, cells were incubated again without or with the antagonists for 4 h and afterwards without or with the corresponding fatty acids in the presence or absence of apolipoprotein A-I (apoA-I) (30 μg/mL) for 24 h. Afterwards, medium was collected, and cells were washed with PBS. Total lipids were extracted from medium and cells and concentrations of cholesterol determined as described in the materials and methods section. A, Cellular and B, medium cholesterol concentrations were related to cellular protein content. Bars represent means ± SD from four independent experiments (n = 4). Data are expressed as percentage of cholesterol concentration in cells and medium of control cells. Results from statistical analysis are indicated: Significant effects are denoted with an asterisk ($P < 0.05$).

cholesterol exporters [26-28], like ABCA1 and ABCG1, which are direct LXRα target genes. Induction of SR-BI, which facilitates a bidirectional flux of free cholesterol between cells and lipoproteins, in response to PPAR agonists [40,41] is also considered to contribute to the increased macrophage cholesterol efflux and reverse cholesterol transport. Like ABCA1 and ABCG1, SR-BI promoter activity and protein levels are also positively regulated by LXRα through a functional LXR response element in its gene promoter [42]. Up-regulation of LXRα in response to PPAR agonists is attributed to the fact that LXRα is regulated by PPARs through a functional PPRE in the LXRα gene promoter [28,43]. Given that the blockade of PPARα or PPARγ by the use of selective PPARα or PPARγ antagonists in RAW264.7 cells resulted in a complete loss of the stimulatory effect of 13-HODE on LXRα, ABCA1, ABCG1 and SR-BI and cholesterol efflux, we suggest that 13-HODE exerted its effect on macrophage cholesterol homeostasis in a PPAR ligand-like manner. Conversely, the lack of effect of LA on cellular and medium cholesterol content and expression of LXRα, ABCA1, ABCG1 and SR-BI is probably explained by its failure to cause PPAR activation in RAW264.7 macrophages. The failure of LA to cause PPAR activation is likely due to the lower binding affinity of PPARs for unoxidized fatty acids compared with oxidized fatty acids like 13-HODE [44]. In line with this assumption are observations from several independent groups showing that LA does not induce PPAR target genes in both murine RAW264.7 [45,46] and human THP-1 macrophages [47].

As regards our observations with LA, it has to be mentioned that some studies reported that LA even decreases protein levels of ABCA1 and/or ABCG1 in either J774 macrophages or RAW264.7 macrophages [48-51]. Although it is difficult to provide a definite reason for this discrepancy, it is well known from the literature that cell culture studies dealing with fatty acids, in particular with LA, provided very controversial results [52]. Important reasons that may be responsible for these discrepancies could be differences in the passage number of cells or differences in the treatment regime, such as time of exposure and fatty acid concentration. Regarding the latter point, it is worth mentioning that in two of the abovementioned studies [48,49] the concentration of LA in the medium was higher than in the present study.

Recent studies demonstrated that PPAR activation also stimulates postlysosomal mobilization of cholesterol by induction of Niemann-Pick C (NPC)-1 and NPC-2 [53]. Both proteins control intracellular trafficking of cholesterol from the late endosomal compartment and lysosome, respectively, to the plasma membrane [54]. It has been suggested [53] that up-regulation of NPC-1 and -2 in response to PPAR agonists results in an enhanced availability of cholesterol at the cell membrane, and, thereby, contributes to increases in macrophage cholesterol efflux to extracellular acceptors and reverse cholesterol. For technical reasons we were not capable to determine protein expression of NPC-1 and NPC-2 in RAW264.7 macrophages. However, due to the observed similarities in the action of 13-HODE and synthetic PPAR ligands on macrophage cholesterol homeostasis we postulate that 13-HODE might also stimulate postlysosomal cholesterol mobilization. This has to be clarified in future studies. However, the regulation of cholesterol homeostasis in macrophages is complex and there are several other proteins important for maintenance of cholesterol homeostasis, including low density-lipoprotein (LDL) receptor, acyl-CoA cholesterol:acyltransferase, hydroxymethyl-glutaryl-CoA reductase, sterol regulatory element-binding proteins, steroidogenic acute regulatory (STAR)-related lipid transfer domain proteins, e.g. Star D4, and caveolin-1. Caveolin-1 for instance has been recently reported to be up-regulated by PPARα and PPARγ agonists [55]. It is therefore not unlikely that 13-HODE exerts its effect on macrophage cholesterol homeostasis also by altering the expression of one or more of these proteins. Thus, future studies applying transcriptomics or proteomics may be useful to get a more comprehensive insight into the mode of action of 13-HODE.

Oxidized fatty acids such as 13-HODE were also shown to activate the PPARγ isotype [34,35,56]. Although PPARγ is a less likely candidate for the mediation of the lipid lowering actions of OF, because PPARγ is poorly expressed in tissues with high rates of fatty acid catabolism like liver and skeletal muscle, it may be a putative mediator of the effect of 13-HODE on RAW264.7 macrophage cholesterol homeostasis. PPARγ is abundantly expressed in macrophage cell lines including RAW264.7 cells, as shown herein by western blotting, as well as primary macrophages [35]. In addition, synthetic PPARγ agonists were reported to stimulate macrophage cholesterol efflux by the same mechanisms as PPARα agonists, namely through activating the PPAR-LXR-pathway [12]. From our PPAR/PPRE-transactivation experiments, we cannot distinguish whether the activation of the reporter was due to activation of either PPARα or PPARγ because the PPRE from the mouse ACO promoter contained in the reporter plasmid used is known to be bound by both, PPARα and PPARγ [57]. Collectively, we suggest that the effects observed with 13-HODE on macrophage cholesterol homeostasis may be mediated by activating either PPARα, PPARγ or both of them.

Independent from the stimulatory effect of 13-HODE on proteins involved in macrophage cholesterol efflux, it

is worth mentioning that with respect to 13-HODE also untoward effects have been reported in cell culture experiments, such as up-regulation of scavenger receptor CD36 which mediates the uptake of oxidized LDL [56]. Therefore, future studies using appropriate animal models of atherosclerosis, such as low density-lipoprotein-deficient or apolipoprotein E-deficient mice, have to clarify whether or not diets containing high levels of 13-HODE promote atherosclerosis development. Evidence from epidemiological studies concerning intake of oxidized fatty acids and cardiovascular disease risk is missing due to the lack of appropriate studies correlating the intake of oxidized fats with the incidence of cardiovascular diseases. Correlating the consumption of fried food with cardiovascular disease risk does not contribute to the clarification of this question because the lipid fraction of fried food contains not only oxidized fatty acids, but also large amounts of saturated fatty acids and trans-fatty acids which themselves influence cardiovascular disease risk.

Conclusions

The present study shows that 13-HODE reduces cholesterol content in murine RAW264.7 macrophages and increases cholesterol content in the incubation medium probably by stimulating apoA-I-dependent cholesterol efflux in a PPAR-dependent manner. The 13-HODE-induced increase in cholesterol efflux from macrophages is likely due to PPAR-dependent up-regulation of LXRα and cholesterol transporters (ABCA1, ABCG1, SR-BI) which operate on cholesterol export to extracellular acceptors such as apoA-I/HDL. Because extensive accumulation of cholesterol by macrophages in the arterial wall leads to atherosclerosis, the present findings in macrophages suggest that the recently observed anti-atherogenic effects of OF [18] might be, at least in part, due to the inhibition of macrophage cholesterol accumulation and stimulation of reverse cholesterol transport caused by oxidized fatty acids such as 13-HODE. Future studies in human monocyte/macrophage cell lines, such as THP-1 cells, or human primary macrophages have to show whether the effects observed in murine macrophages also occur in human macrophages.

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Authors' contributions

IK carried out the experiments and participated in the interpretation of the results and the preparation of the manuscript. RR participated in the design of the study and in the interpretation of the results and prepared the manuscript. RB and GW carried out the experiments. KE conceived of the study and its design, coordinated work, participated in the interpretation of the results, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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4. Diskussion

Thermisch erhitzte Fette sind zentraler Bestandteil der Humanernährung, ihre physiologischen und pathophysiologischen Wirkungen im Organismus sind seit Jahrzehnten Gegenstand intensiver Forschung. Daher ist bekannt, dass neben dem Glukose-, dem Schilddrüsen- und dem Fremdstoffwechsel erhitzte Fette vor allem den Lipid- und Lipoproteinmetabolismus in der Leber beeinflussen. Als etablierter Mechanismus gilt eine Aktivierung des Transkriptionsfaktors PPARα in diesem Organ durch Bestandteile erhitzter Fette und der damit verbundenen Steigerung der Fettsäureverwertung. Beide Prozesse sind wesentlich an der hypolipidämischen Wirkung erhitzter Fette beteiligt. Daneben zeigen Studien, dass PPARs den RCT aktivieren und vaskuläre Entzündungsprozesse regulieren, die an der Vermittlung anti-atherogener Effekte beteiligt sind. Somit liegt die Vermutung nahe, dass sich eine PPAR-Aktivierung auf die Entwicklung atherosklerotischer Gefäßwandveränderungen auswirken kann. Bisher fehlen jedoch wissenschaftliche Untersuchungen zur Wirkung erhitzter Fette auf die Expression der PPARs in der Gefäßwand und der damit verbundenen Wirkung auf die Atherosklerose. Im Fokus der vorliegenden Arbeit steht deshalb neben der Untersuchung ausgewählter und bereits etablierter PPARα-abhängiger Regulationsmechanismen in der Leber von Mäusen die Überprüfung der Hypothese, nach der erhitzte Fette durch eine Aktivierung des PPARα Atherosklerose-hemmend wirken.

Um die Hypothese zu überprüfen, wurde ein Versuchstiermodell gewählt, das in einem überschaubaren Zeitraum atherosklerotische Plaques entwickelt. Nager gelten in der wissenschaftlichen Literatur als etabliertes Modell zur Untersuchung der Wirkung von nutritiven Inhaltsstoffen, wie erhitzten Fetten, bezogen auf die Funktion des PPARα (Chao *et al.*, 2004; Sülzle *et al.*, 2004; Ringseis *et al.*, 2007a). Wildtyp-Ratten und -Mäuse weisen allerdings relativ hohe Konzentrationen an anti-atherogenem HDL-Cholesterol im Plasma auf, wodurch die Entwicklung ausgeprägter atherosklerotischer Läsionen verhindert wird. Die Generierung spezifischer *Knockout*-Modelle macht jedoch ihren Einsatz in der Atheroskleroseforschung möglich. Von besonderer Bedeutung sind ApoE- und LDLR-*Knockout*-Mäuse. Ersteren fehlt das Gen für ApoE, einer Proteinkomponente triglyzeridreicher Chylomikronen- und VLDL-*Remnants*, die für die Bindung und Aufnahme dieser Lipoproteine über Rezeptoren, wie dem LDLR, verantwortlich sind. LDLR-*Knockout*-Mäuse gelten seit ihrer Existenz 1992 als etabliertes Tiermodell zur Untersuchung atherosklerotischer Läsionen. Auf Grund des Fehlens funktionsfähiger LDLR in der Leber und anderen extrahepatischen Geweben kommt es bei diesen Tieren zu erhöhten

Plasmakonzentrationen von Apo B100- und Apo E-tragenden cholesterolreichen Lipoproteinen, wie den *low-density lipoproteins* (LDL). Durch den erzielten Gendefekt wird eine Stoffwechselsituation imitiert, die der humanen Hyperlipoproteinämie Typ II ähnelt. LDLR-*Knockout*-Mäuse entwickeln nach Gabe einer fettreichen Diät innerhalb eines kurzen Versuchszeitraums von der proximalen Aorta ausgehend ausgeprägte atherosklerotische Läsionen (Zandelaar *et al.*, 2007).

Im Rahmen der ersten Studie wurde den Mäusen eine *western diet* mit moderat erhitztem Fett verabreicht, wobei die Vitamin E-Versorgung mit der Diät in einer Gruppe bedarfsdeckend und in einer anderen Gruppe stark erhöht war. Es sollte zunächst überprüft werden, ob, entsprechend den Resultaten aus vergangenen Studien, das Blutlipidprofil sowie die Expression ausgewählter PPARα-abhängiger Gene des hepatischen Lipidstoffwechsels auch bei LDLR-*Knockout*-Mäusen durch die Behandlung beeinflusst werden. Im Ergebnis führte die Verabreichung erhitzter Fette erwartungsgemäß zu einer Aktivierung des PPARα in der Leber. Dieser Effekt äußerte sich indirekt in einer Zunahme des durchschnittlichen relativen Lebergewichts der behandelten Tiere sowie direkt in einer erhöhten Expression lipolytischer und fettsäurekataboler Zielgene des PPARα in diesem Organ (ACO, Cytochrom P450 A10 (CYP4A10), Lipoproteinlipase (LPL)). In Übereinstimmung mit Resultaten aus vergangenen Untersuchungen der eigenen Arbeitsgruppe an Ratten (Eder und Kirchgessner, 1998; Eder *et al.*, 2003a; Eder *et al.*, 2003b; Koch *et al.*, 2007a; Ringseis *et al.*, 2007b) konnten in der vorliegenden Arbeit bei LDLR-*Knockout*-Mäusen ebenfalls verminderte Plasmakonzentrationen an Triglyzeriden bzw. Cholesterol nach der Verfütterung erhitzter Fette beobachtet werden. Anhand der Genexpressionsanalyse in der Leber der Versuchstiere konnte gezeigt werden, dass die Senkung der Triglyzeridkonzentration im Plasma zumindest teilweise auf eine PPARα-vermittelte Steigerung der Lipolyse und der Fettsäureoxidation in diesem Organ zurückzuführen ist (Schoonjans *et al.*, 1996; Srivastava *et al.*, 2006; Tenenbaum und Fisman, 2012). Hinweise aus Studien, die zeigen, dass lipidsenkende Eigenschaften des PPARα durch eine Beeinflussung der Transkriptionsfaktoren *sterol regulatory element-binding protein* (SREBP)-1 bzw. SREBP-2 vermittelt werden (Koch *et al.*, 2007a; Hebbachi *et al.*, 2008; König *et al.*, 2009), ließen sich anhand der Ergebnisse dieser Untersuchung nicht bestätigen.

Zusammenfassend lässt sich in Bezug auf den Lipidstoffwechsel in der Leber der LDLR-*Knockout*-Mäuse schlussfolgern, dass die molekularen Mechanismen, die für die hypolipidämischen Wirkungen erhitzter Fette verantwortlich sind, auch in diesem Versuchstiermodell aufgezeigt wurden. Ausgehend von der Senkung der Blutlipide und der

Aktivierung von PPARα in der Leber, sollte darüber hinaus die anti-atherosklerotische Wirkung erhitzter Fette in der Blutgefäßwand geprüft werden. Dazu wurden die Ausprägung sowie die Zusammensetzung atherosklerotischer Läsionen im Bereich des Aortensinus der LDLR-*Knockout*-Mäuse untersucht.

Bedingt durch den Genotyp der Versuchstiere sowie die Verabreichung der *western diet* konnten bei allen Tieren nach 14-wöchiger Fütterung der Versuchsdiäten ausgeprägte atherosklerotische Läsionen in der proximalen Aorta festgestellt werden. Die histologische Untersuchung der atherosklerotischen Läsionen dieser Tiere ergab jedoch, dass die Fütterung erhitzter Fette, unabhängig vom Vitamin E-Gehalt der Diät, zu einer signifikant verminderten Plaquefläche im Aortensinus führt. Dieser protektive Effekt auf die Ausprägung der Atherosklerose steht zunächst im Kontrast zu Resultaten früherer tierexperimenteller Untersuchungen, bei denen nach Verabreichung thermisch erhitzter Fette überwiegend pro-atherogene Wirkungen beobachtet wurden (Staprans *et al.*, 1996b; Staprans *et al.*, 2000; Penumetcha *et al.*, 2002; Zalejska-Fiolka *et al.*, 2007). Als möglicher Erklärungsansatz kann der unterschiedliche Oxidationsgrad der verwendeten Diätfette in Betracht gezogen werden. So konnte eine Atherosklerose-fördernde Wirkung bei Kaninchen nach Verabreichung von erhitztem Maiskeimöl (Staprans *et al.*, 1996b) und von erhitztem Sojaöl (Greco und Mingrone, 1990) festgestellt werden. Im Gegensatz zu Palmfett, welches als Diätfett in der vorliegenden Studie verwendet wurde, enthalten die genannten Pflanzenöle hohe Anteile an ein- und mehrfach ungesättigten Fettsäuren (Misra *et al.*, 2010), die anfällig für oxidative Veränderungen sind (Boler *et al.*, 2012). Neben Intensität und Dauer der Erhitzung kann der Oxidationsgrad eines Diätfetts auch davon abhängen, ob kontinuierlich erhitzt oder zwischenzeitlich die thermische Behandlung unterbrochen wurde (Frankel, 1998). Ng *et al.* (2012) postulieren, dass es bei Ratten durch die Verabreichung von wiederholt erhitztem Fett, nicht aber von einmalig erhitztem Fett, zu einer Einschränkung der Endothelzellfunktion kommt, die in der Pathogenese kardiovaskulärer Erkrankungen, wie Hypertonie oder Arteriosklerose, von Bedeutung ist (Mendizábal *et al.*, 2013; Natali und Ferrannini, 2012). Die Autoren sind der Ansicht, dass die Oxidationsstabilität des Öls durch den diskontinuierlichen Erhitzungsprozess stark verringert wird und die mit der Diät zugeführten Lipidperoxidationsprodukte (LPOP) endogene antioxidative Abwehrmechanismen beeinträchtigen. Diese Umstände begünstigen die Entstehung von oxidativem Stress, der voraussichtlich für die Endothelzellschädigung sowie für die erhöhte Expression von VCAM-1 als Marker einer endothelialen Dysfunktion (Potenza *et al.*, 2009; Rubio-Guerra *et al.*, 2009) nach Aufnahme von wiederholt erhitztem Fett in dem genannten

Rattenmodell mitverantwortlich ist (Ng *et al.*, 2012). Um Sekundäreffekte zu vermeiden, die auf eine übermäßig starke Erhitzung des Diätfettes, eine unzureichende Versorgung der Versuchstiere mit Antioxidanzien oder auf eine mangelnde Futteraufnahme auf Grund der sensorischen Veränderung der Diät zurückzuführen sind, wurden die Diätfette im Vitamin E-Gehalt und im Gehalt an den Majorfettsäuren standardisiert. Um eine Orientierung der Fette an physiologische Verhältnisse zu gewährleisten, wurden die Fettart sowie die Dauer und Intensität der thermischen Behandlung so gewählt, dass die Tiere ein Diätfett erhielten, welches auch in der Humanernährung westlicher Industrienationen verwendet wird. Die Analyse der Fettkennzahlen zeigte dabei die moderate oxidative Veränderung des eingesetzten Diätfettes an, so dass im Rahmen der vorliegenden Untersuchung negative Begleiterscheinungen vergangener tierexperimenteller Untersuchungen, wie Futterverweigerung, Wachstumsdepressionen und Diarrhö durch die Fütterung thermisch stark erhitzter Fette nahezu ausblieben (López-Varela *et al.*, 1995).

Anhand der immunhistologischen Auswertung zeigte sich, dass die verminderte Ausprägung der atherosklerotischen Läsionen in der Aorta durch die Verfütterung erhitzter Fette mit einer signifikant erhöhten Proteinkonzentration von PPARα in den krankhaft veränderten Gefäßwandabschnitten einhergeht. Damit konnte in der vorliegenden Arbeit erstmals gezeigt werden, dass die Verabreichung erhitzter Fette mit einer gesteigerten Expression von PPARα in der Gefäßwand eines murinen Atherosklerosemodells verbunden ist. In Übereinstimmung mit Befunden aus vergangenen tierexperimentellen Untersuchungen mit potenten PPARα-Agonisten kann als wahrscheinlich angenommen werden, dass der anti-atherogene Effekt erhitzter Fette auf die Ausprägung der Atherosklerose im Zusammenhang mit der gesteigerten vaskulären Expression des PPARα steht (Duez *et al.*, 2002; Li *et al.*, 2004; Srivastava *et al.*, 2006; Toomey *et al.*, 2006). Dennoch konnte in der vorliegenden Arbeit mittels immunhistologischer Analysen nicht abschließend geklärt werden, in welchen Gefäßwandzellen es zu einer erhöhten Expression von PPARα durch die Verabreichung erhitzter Fette kam, obgleich bekannt ist, dass vor allem Endothelzellen, glatte Gefäßmuskelzellen und Monozyten PPARs exprimieren und bevorzugt im Frühstadium der Atherogenese beteiligt sind (Marx *et al.*, 2004; Tiwari *et al.*, 2008). Dass die Läsionen der Versuchstiere in dieses Stadium einzuordnen sind, konnte anhand histologischer Standardfärbungen festgestellt werden. Dabei wurde deutlich, dass Lipideinlagerungen und Kollagenstrukturen die dominierenden Komponenten der atherosklerotischen Veränderungen sind, wohingegen kalzifizierte Areale, die typisch für ausgeprägte Läsionen in späten Entwicklungsstadien sind, nur spärlich vorzufinden waren. Anhand der mikroskopischen

Beurteilung konnten keine Anzeichen für komplizierte Läsionen, wie die Bildung von Fissuren, Hämatomen oder auch Thromben, festgestellt werden (Stary, 2000; Müller *et al.*, 2008).

Das Frühstadium der Atherogenese ist gekennzeichnet durch eine eingeschränkte Endothelzellfunktion (endotheliale Dysfunktion) auf Grund andauernder schädigender Einflüsse, wie beispielsweise die Bildung freier Radikale während des Zigarettenkonsums, infektiöse Mikroorganismen (Herpesviren, *Chlamydia pneumoniae*) oder auch durch erhöhte Konzentrationen an atherogenen LDL (Ross, 1999; Andrés, 2004). Die damit verbundene lokale Aktivierung der Endothelzellen fördert die Gefäßpermeabilität und geht mit einer verstärkten Expression von Adhäsionsmolekülen, wie ICAM-1, VCAM-1 oder P-Selektin einher, woraufhin es Monozyten und Leukozyten ermöglicht wird, an die Zelloberfläche der Gefäßintima zu binden, um transendothelial in darunterliegende Wandschichten zu migrieren (Carlos und Harlan, 1994; Nakashima *et al.*, 1998). Im Subendothelialraum angekommen, sezernieren sie als ortsansässige Makrophagen eine Reihe inflammatorischer Chemokine und Zytokine, die die Progression atherosklerotischer Läsionen fördern. Die eigenen Untersuchungen konnten erstmalig zeigen, dass die Verabreichung erhitzter Fette die Expression von VCAM-1 in den Läsionen der Aorta hemmt. Dass dieser Befund im Zusammenhang mit einer Aktivierung von PPARα steht, zeigen Zellkulturstudien an Endothelzellen, bei denen die Zytokin-induzierte Expression von VCAM-1 durch Fibrate inhibiert werden konnte (Marx, 1999; Srivastava *et al.*, 2006). *In vivo* stellten Li *et al.* (2004) fest, dass die Behandlung mit dem PPARα-Agonisten GW6747 nicht nur zu einer verringerten Plaquefläche im Aortenursprung von LDLR-*Knockout*-Mäusen führt, sondern auch mit einer verminderten Expression von VCAM-1 und weiteren pro-atherogenen Chemokinen, wie MCP-1, Interferon-γ, IL-1β und TNFα, verbunden ist, die an der Rekrutierung immunkompetenter Zellen, wie Monozyten und Leukozyten, zu aktivierten Endothelzellen beteiligt sind.

Die Expression der Adhäsionsmoleküle wird voraussichtlich nicht direkt durch PPARα reguliert. Zahlreiche inflammatorische Mediatoren in der Gefäßwand, wie Zytokine, Chemokine und auch die zellulären Adhäsionsmoleküle unterliegen der genregulatorischen Kontrolle durch den Transkriptionsfaktor NF-κB (Marx *et al.*, 1999; Pamukcu *et al.*, 2011). Durch Promotoranalysen ist bekannt, dass die Gene VCAM-1, ICAM-1 und E-Selektin über definierte Bindungsstellen für NF-κB verfügen (Collins, 1995; Marx, 1999). In der Literatur existieren mehrere Hinweise, dass PPARα an der Vermittlung antiinflammatorischer Wirkungen in der Gefäßwand durch eine Hemmung der transkriptionellen Aktivität von

NF-κB beteiligt ist (Staels *et al.*, 1998; Delerive *et al.*, 2002; Ogata *et al.*, 2004; Babaev *et al.*, 2007). Dabei werden verschiedene Mechanismen der PPAR-vermittelten Transrepression diskutiert. In einer humanen Endothelzelllinie wurde gezeigt, dass PPARα nach Aktivierung direkt mit den NF-κB-Untereinheiten p50 und p65 interagieren kann und auf diese Weise ihre Retention im Zytosol fördert, woraufhin diese Zellen mit einer verminderten Expression der inflammatorischen Zielgene MCP-1 und IL-8 reagierten (Mishra *et al.*, 2004). Als weiterer potentieller Mechanismus kann die Hemmung der Aktivität von *inhibitor of kappa B kinase* (IKK) in Betracht gezogen werden. In einer Studie von Okayasu *et al.* (2008) führte die liganden-abhängige Aktivierung von PPARα in humanen Endothelzellen zur Phosphorylierung sowie zur Aktivierung von *adenosine monophosphate-activated protein kinase* (AMPK). Diese Aktivierung war verbunden mit einer Verminderung der Aktivität des IKK-Enzymkomplexes, die die Maskierung der NF-κB-Untereinheiten durch IκBα-Proteine begünstigt und zu einer verminderten Expression von VCAM-1, ICAM-1 sowie E-Selektin in diesen Zellen führte. PPARα scheint weiterhin in der Lage zu sein, durch eine direkte Bindung von NF-κB-Cofaktoren, wie p300, die Bindungsaktivität des Transkriptionsfaktors am VCAM-1-Promotor herabzusetzen (Na *et al.*, 1998; Marx *et al.*, 1999). Das Ziel künftiger mechanistischer Untersuchungen sollte daher sein, umfassend abzuklären, inwiefern der NF-κB-Signalweg in Zellen der Gefäßwand durch die Verabreichung erhitzter Fette beeinflusst werden kann.

Insgesamt kann indessen vermutet werden, dass sich die verminderte Expression von VCAM-1 in diesem Versuch atheroprotektiv auf die Läsionsentwicklung ausgewirkt hat. Dies erklärt sich vermutlich durch eine PPARα-vermittelte Blockierung der NF-κB-Bindungsaktivität, die eine verminderte Expression inflammatorischer Chemokine und Adhäsionsmoleküle zur Folge hat und mit einer beeinträchtigten Interaktion von Monozyten und Endothelzellen einhergeht.

Neben der lokalen Anhäufung von immunkompetenten Zellen sowie deren Transmigration in den Subendothelialraum ist die Progression der Atherosklerose gekennzeichnet durch eine Akkumulation cholesterolüberladener Makrophagen (Ross, 1999). Auf Grund ihrer tropfenförmigen Ansammlung von Lipiden im Zytosol werden sie allgemein auch als Schaumzellen bezeichnet (Ouimet und Marcel, 2012). Als zelluläre Hauptkomponente der *fatty streaks* fördern Makrophagen-Schaumzellen die Synthese chemotaktischer Mediatoren und somit die Migration weiterer Immunzellen aus der Blutzirkulation in die Gefäßwand, was die Weiterentwicklung von Läsionen begünstigt (Stary *et al.*, 1994). Die Cholesterolhomöostase in Makrophagen als Teil des RCT stellt einen bedeutenden

atheroprotektiven Mechanismus dar, der einer Schaumzell- und Läsionenbildung entgegenwirken kann (Bouhlel *et al.*, 2007). Ob die anti-atherogene Wirkung erhitzter Fette auch durch die Beeinflussung des RCT vermittelt wird, sollte im Rahmen einer zweiten Studie an RAW264.7-Makrophagen der Maus untersucht werden. Diese Zelllinie gilt allgemein als etabliertes Modell zur Untersuchung der Wirkung pharmakologischer sowie nutritiver Substanzen auf Atherosklerose-assoziierte Prozesse (Venkateswaran *et al.*, 2000; Lorenzi *et al.*, 2008). Die Resultate der *in vitro*-Studie bestätigten die aufgestellte Hypothese, dass 13-HODE als ein Vertreter oxidierter Fettsäuren, die definierte Bestandteile erhitzter Fette und potentielle Liganden von PPARα darstellen (König und Eder, 2006), den Cholesterolexport aus Makrophagen als bedeutenden Mechanismus des RCT in diesem Zellkulturmodell stimuliert. Anhand der eigenen Untersuchungen konnte deutlich gezeigt werden, dass 13-HODE die Cholesterolkonzentration in Makrophagen vermindert und gleichzeitig den Cholesterolefflux auf den extrazellulären Akzeptor Apo A-I erhöht. Dieser Effekt war mit signifikanten Veränderungen der Proteinexpression verschiedener, in die Cholesterolhomöostase involvierter Gene verbunden. Die Induktion der Cholesterolexportwege (ABCA1, ABCG1, SR-BI) erklärt sich dabei vermutlich durch die gesteigerte PPAR-Transaktivierung durch 13-HODE und die konsekutive Expressionssteigerung des Zielgens LXRα als Modulator der Cholesterolhomöostase in Makrophagen (Tall *et al.*, 2002; Nakaya *et al.*, 2011). ABCA1 und ABCG1 gelten dabei als bedeutende Vermittler des RCT, indem sie den Transfer von Phospholipiden und unverestertem Cholesterol unter ATP-Verbrauch von intrazellulären Kompartimenten sowie von der äußeren Plasmamembran zu extrazellulären Akzeptoren ausführen (Rye und Barter, 2004; Yvan-Charvet *et al.*, 2010; Tarling und Edwards, 2011). Weiterhin ist davon auszugehen, dass sich die 13-HODE-initiierte Expression des SR-BI, der den bidirektionalen Flux von Cholesterol zwischen Zellen und HDL-Partikeln vermittelt (Tall *et al.*, 2002; Pennings *et al.*, 2006), effluxfördernd in diesem Zellmodell ausgewirkt hat. Im Ergebnis zeigen die durchgeführten Effluxmessungen, dass 13-HODE den Cholesterolausstrom aus Makrophagen, unter Verwendung von Apo A-I als extrazellulären Cholesterolakzeptor, signifikant erhöht. Durch das Ausbleiben der Effekte von 13-HODE nach dem Einsatz selektiver Antagonisten der Isotypen PPARα und PPARγ wird die Eigenschaft oxidierter Fettsäuren deutlich, als Liganden von PPARα und PPARγ zu fungieren (Nagy *et al.*, 1998; Bull *et al.*, 2003; Cimen *et al.*, 2011). Daraus lässt sich folgern, dass im Rahmen der vorliegenden Studie die Aktivierung dieser Transkriptionsfaktoren für die

13-HODE-induzierten Effekte auf die Cholesterolhomöostase in Makrophagen verantwortlich ist.

Insgesamt lassen die Ergebnisse der Zellkulturstudie den Schluss zu, dass 13-HODE als Vertreter der oxidierten Fettsäuren initiale Mechanismen des RCT durch eine Aktivierung des PPAR-LXR*α*-Signalwegs stimulieren kann. Damit offenbart sich ein Potential für definierte Bestandteile erhitzte Fette, pro-atherogenen Lipidakkumulierungen in Zellen der Gefäßwand und damit der Schaumzellbildung entgegenzuwirken. Obwohl eine Extrapolation dieser Befunde auf die *in vivo*-Situation nicht möglich ist, kann dennoch vermutet werden, dass erhitzte Fette im Rahmen der vorliegenden tierexperimentellen Untersuchung ihre anti-atherogenen Effekte zum Teil durch die Stimulierung des RCT vermitteln.

Weiterhin sollten künftige Studien klären, inwieweit oxidierte Fettsäuren weitere PPAR-abhängige Gene des intrazellulären Cholesteroltransports beeinflussen, wie beispielsweise *Niemann Pick type C proteins-1* und *-2*. Interessant wäre, ob eine erhöhte Verfügbarkeit von Cholesterol in der Plasmamembran durch oxidierte Fettsäuren zu beobachten ist, was vermutlich zum Nettoefflux auf extrazelluläre Akzeptoren beitragen kann.

Die Ausbildung fibröser Strukturen in vaskulären Läsionen ist charakteristisch für die Progression der Atherosklerose, wobei neben lipidbeladenen Makrophagen vor allem glatte Gefäßmuskelzellen als dominierend in diesem Krankheitsstadium gelten (Lusis, 2000; Doran *et al.*, 2008). Unter physiologischen Bedingungen regulieren sie den Tonus der Blut- und Lymphgefäße und sind für deren mechanische Festigkeit verantwortlich (Jackson, 2000; Wang *et al.*, 2009). Infolge einer Zytokin- und Chemokin-vermittelten Aktivierung wandern Gefäßmuskelzellen aus der *Tunica media* in den Subendothelialraum ein und beginnen dort zu proliferieren. Aus bisher noch unzureichend verstandenen Ursachen kommt es dabei zur Veränderung ihrer kontraktilen Eigenschaften *(phenotype switch)* und zu einer zunehmenden Synthese von inflammatorischen Zytokinen und Wachstumsfaktoren sowie von Komponenten der extrazellulären Matrix, die wesentlich an der Volumenzunahme atherosklerotischer Läsionen beteiligt sind (Hao *et al.*, 2003; Doran *et al.*, 2008). Von Bedeutung ist dabei neben der Bildung von Proteoglykanen und Glukosaminoglykanen vor allem die Synthese von Kollagen, das mit einem Anteil von etwa 60% am Gesamtproteingehalt als dominierendes extrazelluläres Matrixprotein atherosklerotischer Plaques gilt (Katsuda und Kaji, 2003). Kollagenfibrillen bilden zusammen mit retikulären und elastischen Fasen den Faseranteil der extrazellulären Matrix, der zusammen mit der Grundsubstanz unter physiologischen Bedingungen für die Bildung von Zellverbänden, die Gewebeelastizität sowie für die Bildung von Organen oder auch die Wasserspeicherung in Geweben verantwortlich ist

(Warburton *et al.*, 2000; Danen und Sonnenberg, 2003; Mithieux und Weiss, 2005; Thorsteinsdóttir *et al.*, 2011). Im Prozess der Atherogenese führt die Kollagensynthese durch mitogen- oder zytokinaktivierte glatte Muskelzellen zur Fibrosierung der Läsionen, die eine Okklusion des Gefäßlumens fördert und damit zunehmend den Blutfluss in den betroffenen Gefäßabschnitten beeinflusst (Nigro *et al.*, 2002).

Die histologische Untersuchung der atherosklerotischen Läsionen der Versuchstiere in der vorliegenden Arbeit konnte erstmals aufzeigen, dass die Aufnahme erhitzter Diätfette mit einer verminderten Expression von *smooth muscle actin*, einem Marker für glatte Muskelzellen, sowie mit einem verminderten Kollagenanteil in den Plaques der Aorta verbunden ist. Bezüglich der zu Grunde liegenden Mechanismen kann eine Beeinflussung des PPAR-Signalweges durch thermisch erhitztes Fett in Betracht gezogen werden. Dafür sprechen Befunde aus Untersuchungen, die zeigen, dass PPAR-abhängige Prozesse an der Hemmung krankhafter Fibrosierungen in Organen wie Herz, Leber und Niere beteiligt sind (Miyahara *et al.*, 2000; Ogata *et al.*, 2002; Toyama *et al.*, 2004; Park *et al.*, 2006). Dabei kommt der PPAR*α*-vermittelten Regulation der Muskelzellproliferation eine besondere Bedeutung zu. Hinweise dafür liefern Ergebnisse aus Zellkulturstudien, bei denen die PPAR*α*-Agonisten Clofibrat und WY14,643 die mitogen-stimulierte Proliferation und Migration von glatten Muskelzellen, die aus Koronararterien von Schweinen isoliert wurden, hemmen konnte. Auf molekularer Ebene konnte eine PPAR*α*-vermittelte Regulation der Aktivität von Regulatorproteinen des Zellzyklus, wie *cyclin dependent kinase* (CDK)- 2, die Proliferation von glatten Muskelzellen beeinflussen, indem gezielt Gene zur Steuerung der DNA-Synthese transaktiviert wurden (Zahradka *et al.*, 2006). Weitere Hinweise auf einen PPAR-vermittelten Einfluss auf den Zellzyklus liefern Untersuchungen von Gizard *et al.* (2005), bei denen die Inkubation von humanen sowie murinen glatten Muskelzellen mit synthetischen PPAR*α*-Agonisten durch eine ansteigende Promotoraktivität des CDK-Inhibitors p16 den Übergang von der Wachstumsphase G1 in die Synthesephase und damit die Muskelzellproliferation hemmen konnte. Neben der Beeinflussung proliferativer Prozesse auf Kollagen-produzierende Zellen kann auch eine direkte Hemmung der Kollagensynthese als Ursache für den reduzierten Kollagenanteil in den vaskulären Läsionen der LDLR-*Knockout*-Mäuse in Betracht gezogen werden. Möglicherweise ist eine PPAR*α*-induzierte Hemmung der Expression inflammatorischer Mediatoren und NF-*κ*B-Zielgene, wie *transforming growth factor beta* (TGF-*β*), MCP-1, *cyclooxygenase-2* (COX-2) und IL-6, die in Nierenzellen sowie in Rattenherzen mit einer verminderten Kollagendeposition einherging (Diep *et al.*, 2004; Ogata *et al.*, 2004; Gelosa *et al.*, 2010;

Boor *et al.*, 2011), zumindest partiell für die Ergebnisse der immunhistologischen Untersuchung der Läsionen im Rahmen der vorliegenden Arbeit verantwortlich.
Insgesamt lässt sich schlussfolgern, dass die verminderte vaskuläre Kollagendeposition durch die Verfütterung erhitzter Fette zu der verminderte Ausprägung der Läsionen beigetragen hat. Diese begründet sich wahrscheinlich durch eine PPAR-induzierte Hemmung der Proliferation von Gefäßmuskelzellen als Hauptproduzenten extrazellulärer Matrixproteine, die eine entscheidende Rolle in der Pathogenese atherosklerotischer Gefäßveränderungen spielen.

Schlussfolgerung

Zusammenfassend lässt sich anhand der Resultate der vorliegenden Arbeit schlussfolgern, dass ein moderat erhitztes Fett, das unter praxisrelevanten Bedingungen hergestellt wurde, im Mausmodell anti-atherosklerotisch wirkt. Als ein wesentlicher Teilmechanismus gilt die PPARα-Aktivierung in der Leber durch erhitzte Fette mit der konsekutiven Steigerung des Fettsäurekatabolismus und die damit verbundene Senkung der Konzentration atherogener Plasmalipide. Weiterhin steht die verminderte Ausbildung der Gefäßläsionen im Zusammenhang mit der Zunahme der PPARα-Expression in den untersuchten Gefäßbereichen. Neben einer indirekten Hemmung von Entzündungsparametern wurde *in vitro* die PPARα-vermittelte Expressionssteigerung von Genen des Cholesteroltransports in Makrophagen durch 13-HODE nachgewiesen. Die Aktivierung initialer Mechanismen des RCT durch oxidierte Fettsäuren kann voraussichtlich für die anti-atherosklerotische Wirkung erhitzter Fette mitverantwortlich sein.
Die Resultate der vorliegenden Arbeit stehen zunächst in Kontrast zu der verbreiteten Auffassung, dass die Aufnahme erhitzter Fette mit negativen Begleiterscheinungen im Stoffwechsel verbunden ist. Dazu zählt vor allem die Entstehung von übermäßigem oxidativen Stress durch die Anwesenheit von LPOP und reaktiver Sauerstoffspezies (ROS), die als proinflammatorische Stimuli redoxsensitive Transkriptionsfaktoren wie NF-κB aktivieren können (Sen und Packer, 1996; Tsujinaka *et al.*, 2005). Demgegenüber ist bekannt, dass LPOP und ROS in moderaten Dosen an der Vermittlung von Signaltransduktionen (Leonarduzzi *et al.*, 2000) sowie an der Aktivierung adaptiver zellulärer Abwehrreaktionen beteiligt sind (Haendeler *et al.*, 2004; Jarrett und Boulton, 2005; Park *et al.*, 2005). So konnten in Makrophagen und in vaskulären Endothelzellen nach Inkubation mit 4-Hydroxynonenal, einem Abbauprodukt der Lipidoxidation von mehrfach ungesättigten Fettsäuren, erhöhte Expressionen von antioxidativen Enzymen, wie Hämoxigenase-1 oder Peroxiredoxin-1 nachgewiesen werden (Ishii *et al.*, 2004; Ishikado *et al.*, 2010).

Untersuchungen der eigenen Arbeitsgruppe zeigen, dass moderat erhitzte Fette im Tiermodell den Transkriptionsfaktor *nuclear factor-erythroid 2-related factor 2* (Nrf2) aktivieren (Varady *et al.*, 2011a; Varady *et al.*, 2011b). Nrf2 induziert bei oxidativem Stress die Expression von antioxidativen und detoxifizierenden Enzymen und Regulatorproteinen, um den zellulären Redoxstatus aufrecht zu erhalten und einer Schädigung durch ROS entgegenzuwirken (Nguyen *et al.*, 2003; Kang *et al.*, 2005; Mann *et al.*, 2007).

Insgesamt machen diese Beobachtungen deutlich, dass moderat erhitzte Fette genregulatorische Prozesse auf molekularer Ebene gezielt beeinflussen können. Darauf beruht voraussichtlich nicht nur ihre anti-atherosklerotische Wirkung sondern auch ihre Eigenschaft, durch das Auslösen von zellulärem Eustress, endogene Abwehrreaktionen einzuleiten.

5. Zusammenfassung

Thermisch erhitzte Fette sind zentraler Bestandteil der Humanernährung in Industrienationen. Aus Zellkulturstudien sowie tierexperimentellen Untersuchungen ist bekannt, dass diese Nahrungskomponente biologisch hochaktive Bestandteile enthält, die zu vielfältigen Wirkungen im Organismus führen können. Von besonderer Bedeutung ist die Eigenschaft erhitzter Fette bzw. einzelner Bestandteile, als natürliche Aktivatoren des Transkriptionsfaktors *peroxisome proliferator-activated receptor alpha* (PPARα) zu fungieren. PPARα ist dabei maßgeblich an der Regulation der Fettsäureverwertung beteiligt, indem gezielt Gene in der Leber aktiviert werden, die die zelluläre Aufnahme, den Transport sowie die Oxidation von Fettsäuren ermöglichen. Diese Mechanismen stehen dabei im Zusammenhang mit zahlreichen Beobachtungen aus tierexperimentellen Untersuchungen, bei denen die Verfütterung thermisch erhitzter Fette mit verminderten Triglyzeridkonzentrationen im Plasma sowie der Leber einherging. Neben der hypolipidämischen Wirkung ist weiterhin bekannt, dass eine PPAR-Aktivierung in vaskulären Zellen zu einer Entzündungshemmung führt und dadurch die Atherosklerose hemmt. Als bedeutender Mechanismus gilt außerdem die PPAR-abhängige Aktivierung des reversen Cholesteroltransports in Makrophagen, indem Gene des Cholesteroltransports verstärkt exprimiert werden. Die atheroprotektive Wirkung beruht dabei auf dem vermehrten Export von überschüssigem Cholesterol aus den Zellen, was der Entwicklung von Schaumzellen als Charakteristikum atherosklerotischer Läsionen entgegenwirken kann. Ziel der vorliegenden Arbeit war daher die Hypothese zu bestätigen, dass erhitzte Fette durch eine PPARα-Aktivierung anti-atherosklerotisch wirken.

Dafür wurde zunächst ein Fütterungsversuch mit einem etablierten Tiermodell der Atheroskleroseforschung, den *low-density lipoprotein receptor* (LDLR)-*Knockout*-Mäusen, durchgeführt. Die Versuchstiere wurden in drei Gruppen aufgeteilt (n=12, Kontrollgruppe, Behandlungsgruppe 1 und 2) und erhielten über einen Zeitraum von 14 Wochen eine semisynthetische Diät mit einem Fettanteil von 20%. Um Sekundäreffekte auf Grund eines unterschiedlichen Fettsäuremusters sowie einer unterschiedlichen α-Tocopherolkonzentration von frischem im Vergleich zu erhitztem Diätfett zu vermeiden, wurde sowohl die Fettsäurezusammensetzung als auch die α-Tocopherolkonzentration der Diätfette angeglichen. Die Diätfette variierten folgendermaßen: Die Kontrollgruppe erhielt frisches hydrogeniertes Palmfett und die Behandlungsgruppen erhielten eine 92:8-Mischung (w/w) von erhitztem hydrogenierten Palmfett (170°C, 48 h) und frischem Sonnenblumenöl. Da es auf Grund des Erhitzungsprozesses zu einem Verlust von Tocopherolen im erhitzten

Palmfett kam, wurde die Vitamin E-Konzentration der Versuchsdiäten auf 25 mg *α*-Tocopheroläquivalente/kg Diät (Kontroll- und Behandlungsgruppe 1) und auf 250 mg *α*-Tocopheroläquivalente/kg Diät (Behandlungsgruppe 2) eingestellt. Um eine einheitliche Futteraufnahme zu gewährleisten, wurde ein restriktives Fütterungssystem angewendet. Im Ergebnis führte die Verabreichung erhitzter Fette zu verminderten Konzentrationen an Triglyzeriden und Cholesterol in Plasma und Lipoproteinen. Weiterhin wurde anhand erhöhter relativer mRNA-Konzentrationen bekannter Zielgene des PPAR*α* (Acyl-CoA-Oxidase (ACO), Lipoproteinlipase (LPL), Cytochrom P450 A10 (CYP4A10)) dessen Aktivierung in der Leber durch erhitzte Fette nachgewiesen. Die Verabreichung der fettreichen Diäten führte bei allen Versuchstieren zur Ausbildung von ausgeprägten atherosklerotischen Läsionen in der proximalen Aorta. Es zeigte sich erstmals, dass die Aufnahme erhitzter Fette im Tiermodell die Ausbildung atherosklerotischer Läsionen im Aortenursprung reduziert und mit einer erhöhten PPAR*α*-Proteinexpression in den krankhaft veränderten Gefäßwandabschnitten einhergeht. Dabei ist anzunehmen, dass die erhöhte PPAR*α*-Expression indirekt für die verminderte Expression der inflammatorischen Markerproteine *vascular cell adhesion molecule-1* (VCAM-1) und *smooth muscle actin* sowie für die verminderten Lipid- und Kollageneinlagerungen in den Läsionen der Versuchstiere, die erhitztes Fett erhielten, verantwortlich ist. Insgesamt zeigen diese Ergebnisse, dass erhitzte Fette anti-atherosklerotisch wirken. Dieser Effekt beruht dabei voraussichtlich auf einer günstigen Beeinflussung des Blutlipidprofils auf Grund einer ligandenabhängigen Aktivierung des PPAR*α* in der Leber sowie auf der erhöhten Expression des Transkriptionsfaktors in der Gefäßwand, die mit einer Hemmung pro-atherogener Mechanismen wie der Rekrutierung von Monozyten und der Proliferation von glatten Gefäßmuskelzellen einhergeht.

Zusätzlich sollte in einer zweiten Studie geprüft werden, ob oxidierte Fettsäuren als Bestandteile erhitzter Fette und starke PPAR*α*-Aktivatoren für die anti-atherogene Wirkung erhitzter Fette mitverantwortlich sind. Im Fokus standen Untersuchungen zur Wirkung von 13-Hydroxy-9,11-octadecadiensäure (13-HODE) auf die Cholesterolhomöostase in murinen RAW264.7-Makrophagen. Dabei zeigte sich, dass die Behandlung der Zellen mit 13-HODE zu einer gesteigerten PPAR-Transaktivierung führt und die Konzentration von Proteinen erhöht, die an der Regulation des zellulären Cholesteroltransports beteiligt sind (*adenosine triphosphate binding cassette transporter A1* (ABCA1), *adenosine triphosphate binding cassette transporter G1* (ABCG1), *scavenger receptor* (SR) BI, *liver X receptor alpha* (LXR*α*). Darüber hinaus führte die Behandlung mit 13-HODE und dem Cholesterolakzeptor

Apolipoprotein A-I (Apo A-I) zu verminderten zellulären Cholesterolkonzentrationen in Makrophagen sowie zu erhöhten Cholesterolkonzentrationen im Kulturmedium. Beim Einsatz selektiver PPARα- und PPARγ-Antagonisten blieben die Effekte von 13-HODE auf den Cholesterolefflux und die ermittelten Proteinexpressionen aus, was darauf hindeutet, dass beide PPAR-Isoformen für die beobachteten Effekte eine Rolle spielen. Die Befunde aus dieser Studie zeigen, dass 13-HODE den Apo A-I-abhängigen Cholesterolefflux aus Makrophagen durch Aktivierung des PPAR-LXRα-Signalwegs stimuliert. Da eine Cholesterolüberladung in Makrophagen die Entwicklung atherosklerotischer Läsionen fördert, deuten die Ergebnisse dieser Studie darauf hin, dass die beobachteten anti-atherosklerotischen Effekte erhitzter Fette in LDLR-*Knockout*-Mäusen zumindest teilweise durch eine Hemmung der Cholesterolakkumulierung und durch eine Stimulierung des reversen Cholesteroltransports in Makrophagen durch oxidierte Fettsäuren, wie 13-HODE, begründet sind.

6. Summary

Thermally heated fats are quantitatively important components of human nutrition in industrial countries. Numerous *in vitro* studies and animal experiments show, that heated fats contain biologically active compounds with a wide range of metabolic effects. The fact, that they are natural activators of the transcription factor peroxisome proliferator-activated receptor alpha (PPARα), is of particular importance. By activating hepatic genes for cellular intake and transportation of fatty acids and for their oxidation, PPARα is the main regulator of the fatty acid metabolism. These mechanisms are linked with numerous observations from feeding experiments demonstrating triglyceride-lowering properties of heated fats in liver and plasma. Besides the hypolipidemic effects, PPARα regulates anti-atherogenic inflammatory processes in vascular cells. In addition, PPARα also activates the reverse cholesterol transport in macrophages by up regulating the expression of genes encoding for cholesterol transport proteins. These atheroprotective effects are the result of an increased export of excess cellular cholesterol, leading to reduced foam cell development as a hallmark of atherosclerotic lesions. The aim of the present work was to investigate anti-atherogenic properties of heated fats.

Therefore, we performed first of all a feeding experiment with low-density lipoprotein receptor (LDLR) knockout mice as a well-established experimental model of atherosclerosis. The animals were spitted into three groups (n=12, control group, treatment group 1, treatment group 2) and were fed semi synthetic diets with 20% fat per diet over a period of 14 weeks. To avoid secondary effects due to differences in fatty acid composition and in concentration of vitamin E between heated and fresh dietary fat, diets were equalized in fatty acid composition as well as in concentration of vitamin E. The experimental fat was varied as follows. The control group received fresh hydrogenated palm fat and both treatment groups received a mixture of heated hydrogenated palm fat (170°C, 48 hours) and fresh sunflower oil (92:8, w/w). Because the frying process caused a loss of tocopherols in the heated palm fat, the vitamin E concentration of the diets was adjusted to 25 mg α-tocopherol equivalents/kg diet (control group, treatment group 1) and 250 mg α-tocopherol equivalents/kg diet (treatment group 2). To standardize daily food intake, diets were fed in a restrictive feeding system. As a result of feeding dietary heated fat, there was a reduction of triglycerides and cholesterol in plasma and lipoproteins. Furthermore, we determined increased relative mRNA concentrations of PPARα target genes (acyl-CoA oxidase, lipoprotein lipase, cytochrome P450 isoform 4A10) in the liver in animals with dietary heated fats as an indicative effect of its hepatic activation. Due to ingestion of high fat diets, the proximal aorta of all LDLR

knockout mice exhibited distinct atherosclerotic lesions. It became apparent for the first time that feeding diets containing heated fats caused a significant reduction in cross-sectional lesion area and increased PPARα protein expression in the aortic root. It can be suggested that the reduction of the proinflammatory marker vascular cell adhesion molecule-1 (VCAM-1) and smooth muscle actin as well as the decrease in lesion lipid and collagen content in the aortic root of mice fed heated fat is a consequence of an enhanced PPARα expression in vascular cells. In conclusion, the results of the first experiment demonstrate anti-atherogenic effects of heated fats. Therefore, we assume that these effects are due to ligand–dependent activation of PPARα in the liver, which contributes to plasma lipid lowering but also due to increased PPARα expression in vascular cells which inhibits pro-atherogenic events like monocyte recruitment and proliferation of vascular smooth muscle cells.

The aim of a second study was to verify whether oxidized fatty acids as inherent part of heated fats and strong PPARα activators are also responsible for the anti-atherogenic effects of heated fats. The focal point was to investigate the effects of 13-hydroxy-9,11-octadecadienoic acid (13-HODE) on cholesterol homeostasis in murine RAW264.7 macrophages. Treatment of cells with 13-HODE increased PPAR-transactivation activity and concentrations of proteins involved in cellular cholesterol transport (adenosine triphosphate binding cassette transporter A1 (ABCA1), adenosine triphosphate binding cassette transporter G1 (ABCG1), scavenger receptor (SR) BI, liver X receptor alpha (LXRα)). In addition, 13-HODE decreased cellular cholesterol concentration in macrophages during incubation with the extracellular lipid acceptor apolipoprotein A-I as well as 13-HODE increased cholesterol concentration in the culture medium. Pre-treatment of macrophages with a selective PPARα or PPARγ antagonist completely abolished the effects of 13-HODE on cholesterol efflux and protein levels of genes investigated, suggesting an involvement of both PPAR isotypes.

The results indicate a stimulatory effect of 13-HODE on apolipoprotein A-I-dependent cholesterol efflux from macrophages due to PPAR-LXRα-pathway. Because extensive cholesterol accumulation by macrophages in the arterial wall promotes atherosclerotic lesion development, these findings suggest that the observed anti-atherogenic effects of heated fats in LDLR knockout mice might be, at least in part, due to inhibition of macrophage cholesterol accumulation and stimulation of reverse cholesterol transport caused by oxidized fatty acids such as 13-HODE.

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Eigenständigkeitserklärung

„Ich erkläre:
Ich habe die vorgelegte Dissertation

Experimentelle Untersuchungen zur Wirkung von erhitzten Fetten auf ausgewählte Parameter des Lipidstoffwechsels und der Atherogenese

selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der ‚Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis' niedergelegt sind, eingehalten."

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Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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