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Einfluss Lipid-basierter Signaltransduktion auf inflammatorische Prozesse

Habilitationsschrift

zur Erlangung der Venia Legendi

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vorgelegt von Dr. med. Martina Barbara Schaefer

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Teil II

Anlagen in numerischer Reihenfolge

Vorwort und Danksagung

Die vorliegende Habilitationsschrift besteht aus Untersuchungen zur inflammatorischen Lipidmodulation, die in der Zeit von 1998 bis 2017 im Labor von Herrn Prof. Dr. Konstantin Mayer am Zentrum für Innere Medizin, Medizinische Klinik II, (Direktor Prof. Dr. Werner Seeger) entstanden. Die räumlichen und apparativen Voraussetzungen zur Durchführung dieser Untersuchungen sind im Wesentlichen von Herrn Prof. Dr. W. Seeger, Herrn Prof. Dr. Dr. F. Grimminger und Herrn Prof. Dr. K. Mayer geschaffen worden. Zudem wurden diese Untersuchungen zum großen Teil durch Mittel der Deutschen Forschungsgemeinschaft sowie durch eine Anschubfinanzierungs-Förderung des Fachbereichs Medizin der Justus-Liebig-Universität Gießen unterstützt. Ergänzend kam die Absolvierung des internationalen Graduiertenkollegs „Molecular Biology und Medicine of the Lung“ (MBML) hinzu.

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Die Arbeit beruht auch auf medizinischen und veterinärmedizinischen Dissertationen, die unter meiner Betreuung entstanden und abgeschlossen wurden, oder deren Abschluss bevorsteht.

Die vorliegende kumulative Habilitationsschrift besteht aus zwei Teilen. Im ersten Teil wird ein Überblick über den wissenschaftlichen Hintergrund, der der Entwicklung der Fragestellung zugrunde liegt, gegeben. Außerdem werden im ersten Teil alle wesentlichen Ergebnisse der Arbeit zusammengefasst und im Kontext des aktuellen wissenschaftlichen Kenntnisstandes diskutiert. Auch mögliche therapeutische Perspektiven, die sich aus den Untersuchungen ableiten lassen, werden berücksichtigt. Der erste Teil wurde zur besseren Verständlichkeit mit Abbildungen versehen. Die zitierte Literatur ist im Literaturverzeichnis wiedergegeben. Aus Gründen der Übersichtlichkeit wurde auf eine detaillierte Schilderung der verwendeten Methodik sowie weitestgehend auf die Wiedergabe numerischer Messergebnisse verzichtet, diese werden aus den Originalarbeiten im Anhang ersichtlich.

Der zweite Teil beinhaltet die in Anlage beigefügten Originalarbeiten zum Thema. Dabei handelt es sich ausschließlich um bereits veröffentlichte Publikationen.

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1. Einfluss Lipid-basierter Signaltransduktion auf inflammatorische Prozesse – Übersicht und Entwicklung der Fragestellung

Übersicht

Inflammatorische Prozesse am pulmonalen Endothel und am intestinalen Epithel

Die Inflammation ist die Antwort des Immunsystems auf eine Verletzung von Zellen und Gewebe. Diese kann verursacht werden durch Pathogene wie Viren oder Bakterien, oder auch durch chemische oder physikalische Noxen, sowie durch autoimmune Reaktionen. Obwohl die Entzündungsreaktion für den betroffenen Organismus schmerzhaft ist, so ist die Inflammation im Allgemeinen eine gesunde, heilende Antwort. Die inflammatorische Reaktion stellt eine komplexe Interaktion von löslichen Faktoren und Zellen dar [1]. Zu den löslichen Faktoren zählen u. a. pro-inflammatorische Zytokine wie beispielsweise der Tumor Nekrose Faktor (TNF)-α, Interleukin-1 oder Interleukin-8. Leukozyten, darunter insbesondere Lymphozyten, Granulozyten und Monozyten sind an der Infektabwehr beteiligt.

Für die Entstehung einer Inflammation, die den Organismus systemisch schädigen kann, sind folgende Komponenten erforderlich:

1. Ein auslösendes Agens (z.B. infektiös wie Viren oder Bakterien)

2. Eine Barriere, die dysfunktional wird (z.B. die epitheliale Darmschranke oder die Endothelschranke des vaskulären Gefäßbettes)

3. Abwehrzellen des Immunsystems (z.B. Leukozyten wie Polymorphonukleäre Granulozyten (PMN) oder Monozyten) und

4. Entzündungsmediatoren, die sezerniert werden (wie beispielsweise Interleukine oder der Plättchen-aktivernde Faktor (PAF)).

Dies sind folglich die Stellschrauben, über welche eine Inflammation beeinflusst werden kann, um sie zum Abklingen zu bringen:

1. Zum auslösenden Agens gilt der hippokratische Leitsatz „Ubi pus, ibi evacua“. Daher ist es das Primärziel bei der Bekämpfung einer Entzündung, das auslösende Agens zu eliminieren. Abgesehen von einer hierfür gegebenenfalls notwendigen chirurgischen Intervention kann dies z.B. mittels zielgerichteter Antibiotika-Therapie erfolgen.

2. Die Barrieren (epithelial oder endothelial), die bei einer Inflammation relevant sind, stellen ein komplexes sowohl in die immunologische Abwehr der Leukozyten als auch in das Netzwerk der sezernierten Entzündungsmediatoren eingebundenes System dar. Kenntnisse über die Funktionalität dieses Systems stellen potentielle Ansatzpunkte für anti-inflammatorische Interventionen in Aussicht.

3. Leukozyten wie PMN und Monozyten stellen die mobile Eingreiftruppe des Immunsystems dar. Sowohl ihre Quantität als auch ihre Qualität sind entscheidende Faktoren in der Regulation der Entzündung. Sie sind an der Bekämpfung sowohl der akuten als auch der chronischen Entzündung ubiquitär im Organismus beteiligt. Hierfür steht ihnen eine Vielzahl von interagierenden Signaltransduktions-Instrumente zur Verfügung, die ebenfalls Ziel anti-inflammatorischer Agentien sein können.

4. Sezernierte Entzündungsmediatoren wie beispielsweise Interleukine oder PAF sind Teil eines zusammenhängenden Netzwerkes, in welchem sich die verschiedenen Signaltransduktionswege gegenseitig auf unterschiedlichen Ebenen beeinflussen. Auch hier ist eine Einflussnahme auf die Inflammation möglich.

Leukozyten modulieren den Entzündungsprozess durch eine Vielzahl unterschiedlicher Mediatoren. Mittels interzellulärer und parakriner Signaltransduktion, beispielsweise durch den *platelet activating factor* (PAF), durch *nitric oxide* (NO), durch die *Human neutrophil elastase* (HNE) oder durch O_2^--Anionen greifen sie in das

Entzündungsgeschehen ein [2-6]. Der Plättchen-aktivierende Faktor (PAF) ist ein inflammatorisches Biolipid. Es entstammt den membranären Glycerophospholipiden und entfaltet seine Wirkung über einen G-Protein gekoppelten Rezeptor [7]. NO kann sowohl anti- als auch pro-inflammatorisch wirken [8]. *Human neutrophil elastase* (HNE) ist eine von polymorphkernigen neutrophilen Granulozyten (PMN) sezernierte Serinprotease, die bei inflammatorischen Erkrankungen eine zentrale Rolle spielt [5]. HNE reguliert andere pro-inflammatorische und anti-inflammatorische Mediatoren wie Zytokine und Chemokine [9]. Reaktive Oxygene wie O_2^--Anionen verstärken die Inflammation über die Schädigung der Plasmamembran. Sie stören die Organisation der Membran und führen zur Freisetzung verschiedener pro-inflammatorischer Mediatoren [6].

Schädlich für den Organismus wird die inflammatorische Reaktion dann, wenn die gezielte Destruktion und Elimination von Pathogenen oder das Abräumen von geschädigtem Gewebe in eine überschießende, unkontrollierte Reaktion übergeht, Zell- und Gewebsschäden resultieren oder es sogar zum Organversagen kommt. Dieser Zustand wird als *Systemic Inflammatory Response Syndrome* (SIRS) bzw. wenn er auf einer Infektion beruht, als *Sepsis* bezeichnet. Beides sind Krankheitsbilder mit einer hohen Mortalität [10-12]. Gefäße stellten eine entscheidende Interaktionsfläche mit den Mediatoren der Inflammation dar. Daher ist die vaskuläre Inflammation von besonderer Bedeutung für Verlauf und Prognose systemischer inflammatorischer Erkrankungen.

Die vaskuläre Inflammation ist durch die Interaktion von Leukozyten und Gefäßendothel gekennzeichnet. Insbesondere die Adhäsion von Monozyten an Endothelzellen spielt im inflammatorischen Prozess eine entscheidende Rolle. Es kommt zunächst im Rahmen des inflammatorischen Geschehens u.a. zu einer Veränderung der rheologischen Eigenschaften, was sich in einer Verlangsamung des Blutflusses (*Flow*) aufgrund von Gefäßdilatation äußert. Dadurch erhöht sich die Wahrscheinlichkeit des zufälligen Kontaktes des Monozyten am Endothel [13, 14]. Die Monozyten „rollen“ nach dem initialen Kontakt auf dem das Gefäß auskleidenden Endothel. Dieses *Rolling* resultiert aus Scherkräften, die bedingt durch die Interaktion des Monozyten mit dem Gefäßendothel entstehen [13]. Das *Rolling* ist in seiner Persistenz und Intensität vom induzierenden Stimulus abhängig. Nach Gewebeverletzungen beginnt es beispielsweise innerhalb von Minuten und bleibt für ca. zwei Stunden weitgehend konstant [15]. Je nach Art und Stärke des inflammatorischen Reizes bleibt eine unterschiedliche Anzahl von rollenden Monozyten fest am Endothel haften (*Sticking*). Die am Endothel haftenden Monozyten werden flacher und breiten sich über dem Endothel aus. Die Kontaktfläche für interzelluläre Bindungen wird dadurch größer und die Monozyten bieten dem Blutstrom eine geringere Angriffsfläche [16, 17]. Teilweise heften sich auch andere Monozyten an bereits adhärente Zellen und bilden so kleine Aggregate. Die durch die Adhäsion und den inflammatorischen Reiz aktivierten Monozyten migrieren über die Oberfläche des Endothels und es kommt zur Diapedese – der transendothelialen Migration - der Monozyten durch die interzellulären Junktionen des Endothels. Die extravasalen Monozyten migrieren nun Chemotaxis-bedingt durch das subendotheliale Gewebe [13, 17, 18]. Nach Carlos und Harlan [13] werden die adhäsiven Interaktionen bei der Emigration des Monozyten in verschiedene Phasen eingeteilt (Abbildung 1.1):

1. Initiale transiente Adhäsion und *Rolling*
2. Aktivierung des Monozyten und feste Adhäsion (*Sticking*)
3. Transendotheliale Migration (Diapedese)
4. Subendotheliale Migration

Spezielle Komponenten der Signaltransduktion des Monozyten, des Endothels und des subendothelialen Gewebes beteiligen sich in jeder Phase an dieser Adhäsionskaskade (Abbildung 1.1).

Proteine der Zelloberfläche, sogenannte Adhäsionsmoleküle, sind im Rahmen der monozytären-endothelialen Adhäsion von entscheidender Bedeutung. Adhäsionsmoleküle lassen sich in Selektine, Integrine und Immunglobulin-ähnliche Moleküle einteilen [19].

Selektine (E-, P- und L-Selektin) und ihre Liganden sind für das *Rolling* von entscheidender

Bedeutung. Bei der festen Adhäsion von Monozyten an die aktivierten Endothelzellen (*Sticking*) ist die Interaktion der Integrine mit den Immunglobulin-ähnlichen Molekülen (*very late activation antigen* (VLA)-Moleküle – *vascular cell adhesion molecule* (VCAM)-1; CD11/CD18-Moleküle – *intercellular adhesion molecule* (ICAM)-1) relevant. Bei der Diapedese der Monozyten durch die interendothelialen Junktionen in das subendotheliale Gewebe spielen Wechselwirkungen zwischen β2-Integrinen und den Immunglobulin-ähnlichen Molekülen eine tragende Rolle; insbesondere CD31 ist dabei relevant. Durch Chemokine und durch die Interaktion von Integrinen mit Komponenten der extrazellulären Matrix (*very late activation antigen* (VLA)-5 – *vascular cell adhesion molecule* (VCAM)-1) wird die subendotheliale Migration der Monozyten zum Ort der Inflammation gesteuert [13, 19].

Offensichtlich scheinen die Schritte der molekularen Adhäsionskaskade jedoch nicht streng sequentiell, sondern überlappend zu sein [17, 18]. Eine gegenseitige Beeinflussung der an der Kaskade beteiligten Moleküle findet ebenfalls statt [13]. Monozyten beschreiten wahrscheinlich auch noch andere bisher noch nicht vollständig erforschte Wege bei der primären Adhäsion und bei der Transmigration [17, 18, 20-22].

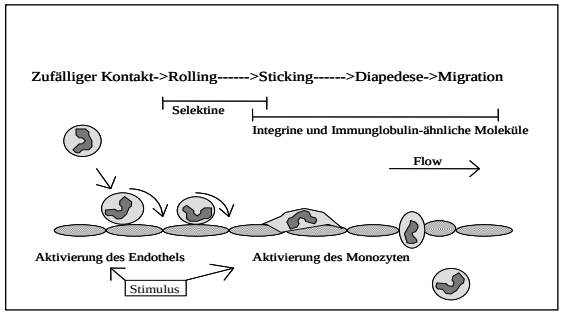

Abb. 1.1: Die monozytäre-endotheliale Adhäsion

Unter rheologischen Bedingungen, die die Charakteristika eines parallelen Flusses aufweisen, wie sie in der post-kapillaren Venole herrschen, rollen die aktivierten Monozyten nach initialem Kontakt zunächst über das inflammatorisch aktivierte Endothel („Rolling"). Bei der festen Adhäsion („Sticking") sind die Interaktionen der Integrine mit den Immunglobulin-ähnlichen Molekülen relevant. Die Diapedese der Monozyten in das subendotheliale Gewebe spielen Wechselwirkungen zwischen β2-Integrinen und den Immunglobulin-ähnlichen Molekülen eine tragende Rolle. Durch Chemokine und durch die Interaktion von Integrinen mit Komponenten der extrazellulären Matrix wird die subendotheliale Migration gesteuert [13, 19].

Die pulmonale Inflammation stellt die relativ uniforme pathophysiologische Antwort der Lunge auf unterschiedliche inflammatorische Trigger dar. Auslöser wie Endotoxin (Lipopolysaccharid (LPS)), Peptidoglykane und Exotoxine aktivieren Effektorzellen wie Leukozyten und Endothelzellen. Sie lösen die systemische und lokale Bildung sekundärer Mediatoren wie Tumor Nekrose Faktor (TNF)-α, Sauerstoffradikale und Stickstoffmonoxid (NO) aus, die zu einer weiteren Schädigung der Lunge führen können [23-26]. Die gesteigerte Permeabilität und die Ausbildung eines eiweißreichen Ödems mit einer Gasaustausch-Störung und einer verminderten *Surfactant*-Funktion durch gesteigerte Inaktivierung steht dabei im Vordergrund [27, 28]. Es kommt zu einer vaskulären Dysfunktion, die mit einer akuten Widerstandserhöhung in der pulmonalen Strombahn einhergeht. Zusätzlich tritt eine Sequestration von inflammatorisch aktivierten Leukozyten in die kapillare Strombahn auf. Eine sekundäre Invasion in das alveoläre Kompartiment ist die Folge. Die leukozytäre inflammatorische Interaktion mit dem Endothel und die konsekutive Beeinträchtigung der endothelialen Barriere ist hier von zentraler Bedeutung [27]. Unter inflammatorischen Bedingungen spielen von der Arachidonsäure abgeleitete Prostanoide eine bedeutende Rolle. Sie sind bei der Anpassung der Ventilation an die Perfusion antagonistisch an der Vasomotorik beteiligt. Ihre Bildung kann unter inflammatorischen Bedingungen gesteigert werden und somit die empfindliche Balance der Anpassung der Ventilation an die Perfusion stören („*Ventilations-Perfusions-Missmatch*") [29, 30]. Die pulmonale Inflammation kann sich klinisch im sogenannten „akuten Lungenversagen" äußern. Ashbaugh und Mitarbeiter prägten 1967 den Begriff des akuten Lungenversagens. Erkrankte beschrieben sie als Patienten mit Lungenversagen, Zyanose, reduzierter pulmonaler Compliance und diffusen Infiltraten im Röntgenbild. Die Patienten zeigten trotz unterschiedlicher Ursachen ein relativ uniformes Bild [31]. Der Begriff der „akuten Lungenschädigung" (*acute lung injury* (ALI)) wurde seit der Berlin-Definition 2011 [32] verlassen. Im experimentellen Bereich ist es jedoch noch sinnvoll, von der akuten Lungenschädigung zu sprechen. Von der Berlin-Definition wird der Begriff des ARDS (*acute respiratory distress syndrome*) differenzierter verwendet. Das ARDS ist definiert durch seinen akuten Beginn (Auftreten

innerhalb von einer Woche), beidseitige Infiltrate in der radiologischen Bildgebung ohne andere sinnvolle Erklärung, nicht durch Herzversagen oder Hypovolämie bedingtes respiratorisches Versagen sowie eine Beeinträchtigung der Oxygenierung bei einem positiven endexpiratorischen Druck von > 5 cmH_2O. Dabei unterscheidet man ein mildes, moderates oder ein schweres ARDS. Bei einem milden ARDS ist das Verhältnis des arteriellen Sauerstoffpartialdruckes zur Fraktion des eingeatmeten inspiratorischen Sauerstoffes (PaO_2/FiO_2) zwischen 200 - 300 mmHg (Horovitz-Quotient). Bei einem moderaten ARDS liegt der Horovitz-Quotient bei < 200 mmHg und bei einem schweren ARDS bei < 100 mmHg [32].

Ein ARDS kann in der Folge einer generalisierten Sepsis auftreten, während der es ebenfalls zu einer leukozytären inflammatorischen Reaktion am intestinalen Epithel kommt. Allerdings kann das intestinale Epithel auch Ausgangspunkt einer Sepsis sein. Das Intestinum ist nicht nur aufgrund seiner koordinierenden Funktion bei der mukosalen Immun-Reaktion sondern auch durch die Barriere-Funktion des Epithels von besonderer Bedeutung im Rahmen der Entzündungsreaktion [33]. Die Zusammenarbeit von Polymorphonukleären Granulozyten (PMN) und intestinalen Epithelzellen ist ein unterschätztes, jedoch wichtiges Konzept [34]. Das Intestinum stellt eine enorme Oberfläche im Körper dar. Seine Integrität und seine Barrierefunktion sind entscheidend für die Abwehr einwandernder Pathogene [33]. Der Darm wird daher auch als Motor der Sepsis bezeichnet. Eine Dysfunktion der epithelialen-leukozytären Interaktion kann in letzter Konsequenz zum Versagen der intestinalen Barriere mit fatalen Konsequenzen wie Septikämie, sekundärem akutem Organversagen und septischem Schock führen. Sepsis ist die häufigste Todesursache auf nicht-kardiologischen Intensivstationen [35, 36].

Lipid-basierte Eicosanoid-abhängige Signaltransduktion vermittelt durch n-3/n-6 Fettsäuren

Lipidmediatoren sind biologisch aktive Moleküle, die lokal als Antwort auf extrazelluläre Stimuli über spezifische biosynthetische Signaltransduktionswege synthetisiert werden. Sie werden extrazellulär transportiert, binden teilweise an spezifische G-Protein gekoppelte Rezeptoren, übermitteln Signale an Zielzellen und werden dann schnell wieder abgebaut. Historisch und strukturell können sie in drei Kategorien eingeteilt werden:

Klasse I: umfasst die Arachidonsäure (AA)-abgeleitete Eicosanoide wie Prostaglandine (PGs), Leukotriene (LTs) und deren Abkömmlinge.

Klasse II: umfasst die Lysophospholipide oder ihre Abkömmlinge wie den Plättchen-aktivierenden Faktor (PAF), die entweder ein Glycerol-Gerüst (wie PAF) oder ein Sphingosin-Gerüst haben.

Klasse II: umfasst die relativ neu identifizierte anti-inflammatorische Lipidmediatoren, die von n-3 PUFA abgeleitet werden wie Resolvine und Protectine [37].

Lipidmediatoren spielen eine zentrale Rolle in der auto- und parakrinen Steuerung der inflammatorischen Aktivierung von Leukozyten, Epithelzellen, Endothelzellen, Thrombozyten, ihren Interaktionen sowie bei der Regulation des Vasotonus. Lipidmediatoren werden auch als *„autacoids“* bezeichnet: Neben der parakrinen Wirkung, bei der das Produkt einer Zelle A Veränderungen in einer nahegelegenen Zelle B verursacht [38], werden sie auch in den gleichen Zellen bzw. Geweben bei Bedarf produziert, in denen sie auch wirken [39]. Dabei agieren sie meist, aber nicht immer lokal. Ein Transport über die Blutbahn und eine systemische Wirkung wurde ebenfalls gezeigt [40]. Als klassische Eicosanoide bezeichnet man die enzymatischen Oxygenierungsprodukte der Arachidonsäure. Eicosanoide sind Produkte der Lipoxygenasen, der Cyclooxygenasen oder der Cytochrom P_{450}-Enzymfamilie. Der Plättchen-aktivierende Faktor (PAF) ist ein weiterer Lipidmediator. Er wird aus acetylierten Derivaten des Membranphospholipids Phosphatidylcholin gebildet. Die enzymatischen Oxygenierungsprodukte der Arachidonsäure beeinflussen die Synthese von Zytokinen sowie die Bildung von Sauerstoffradikalen und Proteasen. Sie können sowohl pro-inflammatorisch als auch anti-inflammatorisch in Entzündungsprozesse eingreifen [41-44].

Arachidonsäure, ein Präkursor für die Synthese der Lipidmediatoren, kann durch Phospholipasen Calcium-abhängig aus der *sn*-2 Position von Membranphospholipiden zur Verfügung gestellt werden [45-47]. Verschiedene Enzymsysteme können freie Arachidonsäure als Substrat nutzen. Die Klassen der Metaboliten unterscheidet man in Lipoxygenase-, Cyclooxygenase- (Abbildung 1.2) oder Cytochrom P_{450}-Metabolite - je nach Enzym, das die erste Oxygenierung einleitet.

Durch die 5-Lipoxygenase (LO) wird Arachidonsäure (AA) zu 5-Hydroperoxyeicosatetraensäure (HPETE) oxidiert. Durch eine Hydroperoxidase kann hieraus 5-Hydroxyeicosatetraensäure (HETE) entstehen. Durch einen weiteren Metabolisierungsschritt der 5-LO wird das instabile Leukotrien (LT) A_4 generiert. LTA_4 zerfällt entweder spontan oder wird zu LTB_4 umgewandelt. LTB_4 wirkt wie 5-HETE chemotaktisch für Leukozyten, ist aber erheblich potenter [48-50]. Des Weiteren steigert LTB_4 die Adhärenz von Leukozyten an Endothelzellen [51, 52]. Alternativ kann LTA_4 zu LTC_4 umgewandelt werden, dem ersten Metaboliten der Cysteinyl-Leukotriene. Cysteinyl-Leukotriene steigern in der pulmonalen Strombahn die endotheliale Permeabilität und führen zu einer post-kapillaren Vasokonstriktion mit Ödembildung [53, 54].

Durch die Cyclooxygenase wird AA zweifach oxidiert, so dass via Prostaglandin (PG) G_2 das zentrale Produkt PGH_2 entsteht. Dies kann zu verschiedenen Prostaglandinen weiter verstoffwechselt werden, je nach Enzymaustattung und Aktivierung der Zellen. Prostaglandin I_2 und Thromboxan (Tx) A_2 sind an der Regulation des pulmonalen Vasotonus beteiligt. Zu den inaktiven Produkten 6-keto-$PGF_{1\alpha}$ und TxB_2 zerfallen sie spontan.

Die Einteilung der Fettsäuren erfolgt in die Klassen der essentiellen und der nicht-essentiellen Fettsäuren. Erstere können vom Menschen nicht selbst synthetisiert werden, sie – oder ihre Vorstufen – müssen mit der Nahrung aufgenommen werden. Zu den essentiellen Fettsäuren gehören neben der Arachidonsäure (AA) [44] die Eicosapentaensäure (EPA) und die Docosahexaensäure (DHA) [55].

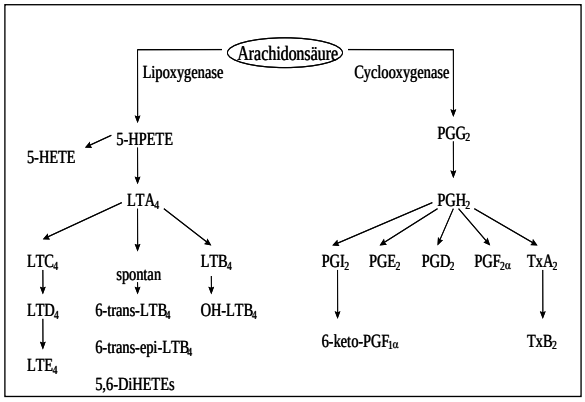

Abb. 1.2: Lipoxygenase- und Cyclooxygenaseweg der Arachidonsäure

Arachidonsäure wird durch die Lipoxygenase über 5-HPETE zu den verschiedenen Leukotrienen metabolisiert. Die Cyclooxygenase metaboliserte Arachidonsäure zu den unterschiedlichen Prostaglandienen und zu Thromboxan. Arachidonsäure (AA); 5-Hydroperoxyeicosatetraensäure (HPETE); 5-Hydroxyeicosatetraensäure (HETE); Leukotrien (LT); Prostaglandin (PG); Thromboxan (Tx).

AA (C20:4 n-6) besteht aus 20 Kohlenstoff(C)-Atomen mit 4 Doppelbindungen. Sie ist eine n-6 (oder ω-6) Fettsäure, d.h. die letzte Doppelbindung befindet sich am 6. C-Atom vom Methyl-Ende aus gesehen. Das entspricht der Doppelbindung zwischen dem 14. und 15. Kohlenstoff-Atom. Bei den n-3 (oder ω-3) Fettsäuren liegt die letzte Doppelbindung am 3. C-Atom vom Methyl-Ende (daher auch „Omega“) aus gesehen. Das entspricht bei EPA (20:5 n-3) der Doppelbindung zwischen dem 17. und 18. C-Atom und bei DHA (22:6 n-3) der Doppelbindung zwischen dem 19. und 20. C-Atom. EPA besitzt 20 Kohlenstoffatome und 5 Doppelbindungen, DHA 22 Kohlenstoffatome und 6 Doppelbindungen. AA, EPA und DHA sind essentiell, d.h. sie können nur aus mit der Nahrung aufgenommenen Vorstufen synthetisiert werden, da dem Menschen die $\Delta 12-$ bzw. die $\Delta 15$-Desaturase fehlt, die in Pflanzen und Algen vorhanden sind. Aus der Ölsäure wird eine Eicosatriensäure (C20:3 n-9, *Mead Acid*), die auch „Hungerfettsäure“ genannt wird, synthetisiert, wenn dem Körper ungenügende Mengen an essentiellen Fettsäuren zugeführt werden (*essential fatty acid deficiency syndrome* (EFAD)). Die *Mead Acid* kann - im Gegensatz zu Arachidonsäure - nicht im Cyclooxygenase- oder Lipoxygenase-Weg metabolisiert werden [56-58].

Die Relevanz der n-3 Fettsäuren wurde zunächst in epidemiologischen Studien demonstriert. Die Gruppe um Bang und Dyerberg fand 1975, dass das

Risiko für koronare Herzkrankheit, Myokardinfarkt und Thrombosen erheblich niedriger bei in Grönland lebenden Inuit war als bei Dänen oder bei in Dänemark lebenden Inuit [59-64]. Andere Gruppen fanden nach diätetischer Applikation von Fischölkapseln eine verminderte Sekretion inflammatorischer Zytokine [65, 66] oder eine reduzierte leukozytäre-endotheliale Adhäsion durch n-3 Fettsäuren [67-69]. Zur Zeit der Frühmenschen und der Entwicklung der Enzymsysteme lag das n-3/n-6 Verhältnis aufgrund der damaligen Ernährung wahrscheinlich bei circa 1/1 bis 1/3. Bei der heutigen westlichen Ernährung und bei den zur parenteralen Ernährung als Standard eingesetzten Lipidemulsionen auf der Basis von Sojabohnen Öl mit hohem Anteil an langkettigen Triglyzeriden (*long chain triglycerids* (LCT)) liegt dieses Verhältnis bei circa 1/10-20 [70]. Durch die industrialisierte Gewinnung von Nahrungsmitteln, insbesondere durch den verstärkten Konsum von Pflanzenölen mit Anreicherung von n-6 Fettsäuren und durch die Abnahme der Ernährung mit n-3- reichen Pflanzen wie beispielsweise Algen und sich von Algen ernährenden Kaltwasserfischen erklärt sich die Verschiebung des n-3/n-6 Verhältnisses. Arachidonsäure-oxydierende Enzymsysteme sind aus evolutionären Aspekten potentiell dazu befähigt, die alternative Fettsäure Eicosapentaensäure zu verstoffwechseln [70].

Eicosapentaensäure kann auch als Substrat von der Cycloxygenase und der Lipoxygenase verwendet werden, da sie eine große strukturelle Ähnlichkeit - eine Doppelbindung mehr bei gleicher C-Atom-Anzahl - zur Arachidonsäure aufweist. Die Stoffwechselprodukte der Eicosapentaensäure unterscheiden sich von denen der Arachidonsäure physiologisch in der Wirkung auf Entzündungszellen und auf die vaskuläre Reagibilität, sowie chemisch durch das Vorhandensein einer zusätzlichen Doppelbindung.

Analog zur Arachidonsäure wird Eicosapentaensäure durch die Cyclooxygenase zu Prostaglandin(PG)G_3 und dem Endoperoxid PGH_3 metabolisiert. Alle Prostanoide der 3-er Serie werden aus diesem Endoperoxid abgeleitet. Thromboxan A_3 und Prostaglandin I_3 können äquivalente Rezeptoren wie Thromboxan A_2 und Prostaglandin I_2 verwenden, allerdings unterscheidet sich ihre biologische Wirksamkeit erheblich. Prostaglandin I_2 und Prostaglandin I_3 besitzen ähnlich starke anti-aggregatorische und vasodilatatorische Effekte. Thromboxan A_3 weist jedoch im Vergleich zu Thromboxan A_2 deutlich schwächere biologische Effekte - eine verminderte Vasokonstriktion und eine reduzierte Thrombozytenaggregation - auf [71, 72]. Eicosapentaensäure führt in Thrombozyten zu einer kompetitiven Inhibition der Thromboxan A_2-Synthese und zu einer protrahierten Verstoffwechslung von Eicosapentaensäure zu Thromboxan A_3. Die Thrombozytenaggregation ist bei einer an n-3 Fettsäuren reichen Diät reduziert. Experimentell wurden bei n-3-reicher Ernährung zur Auslösung der Plättchenaggregation höhere Dosen von Agonisten benötigt [73].

Analog zur Arachidonsäure wird die Eicosapentaensäure durch die Lipoxygenase zu dem instabilen Hydroperoxid *5-hydroperoxy-eicosapentaenoic acid* (HPEPE) oxidiert. 5-HPEPE kann durch eine Hydroperoxidase zu *5-hydroxy-eicosapentaenoic acid* (HEPE) metabolisiert oder alternativ durch eine Dehydratase zu dem sehr instabilem Epoxid Leukotrien (LT) A_5 verstoffwechselt werden. LTA_5 kann wie LTA_4 zu verschiedenen Produkten metabolisiert werden. Die LTA-Hydrolase katalysiert die Bildung von Leukotrien B_5 in Granulozyten oder Makrophagen. Aus LTA_5 geht durch Kooperation von Granulozyten und Endothelzellen LTC_5 hervor, der erste Metabolit der 5-er Serie der Cysteinyl-Leukotriene (LTC_5, LTD_5, LTE_5). Durch eine spontane Hydrolyse Reaktion zerfällt nicht-enzymatisch metabolisiertes LTA_5 in die inaktiven Diastereomeren-Paare 6-trans-LTB_5 und 5,6-DiHEPE [74-76].

Bei simultaner Gabe von freier Arachidonsäure und freier Eicosapentaensäure wird deutlich, dass Eicosapentaensäure effektiver von humanen PMN zu dem Leukotrien B_5 metabolisiert wird als Arachidonsäure zu LTB_4 [77]. Im Modell der isolierten Lunge konnte dies ebenfalls für die kooperative Leukotrien-Synthese und die Cysteinyl-Leukotriene demonstriert werden [74-76]. Im Gegensatz zu der Bevorzugung der Cyclooxygenase für die Arachidonsäure besteht bei der 5-Lipoxygenase eine Präferenz für Eicosapentaensäure.

Lipid-basierte Mediator-abhängige Signaltransduktion vermittelt durch den Plättchen-aktivierenden Faktors (PAF)

Der Plättchen-aktivierende Faktor (PAF) wurde initial als Substanz charakterisiert, die Thromboxan- und ADP-unabhängig eine Thrombozyten-Aggregation auszulösen vermag [78]. Er ist chemisch ein 1-O-Alkyl-2-Acetyl-sn-Glycero-3-Phosphorylcholin. Somit gehört er zur Lipidmediator Klasse II. Der Alkylrest ist in diesem Molekül mit der *sn*-1 Position über eine Etherbindung und nicht durch eine einfach hydrolysierbare Esterbindung gebunden. In Thrombozyten, Leukozyten oder Endothelzellen erfolgt die Synthese des Plättchen-aktivierenden Faktors beispielsweise nach einer Vielzahl von Agonisten, die einen Anstieg des intrazellulären Calciums induzieren [67, 79-83]. Ein 1-O-Alkyl-2-Acyl-Glycero-Phosphorylcholin ist Ausgangspunkt der *Remodeling*-Synthese. Durch eine Phospholipase (PL) A_2 wird dieser PAF-Präkursor zu dem 1-Alkyl-sn-Glycero-Phosphorylcholin, dem Lyso-PAF, hydrolysiert. Bei normaler westlicher Ernährung befindet sich in der *sn-2* Position üblicherweise Arachidonsäure. Daher ist die durch die Phospholipase A_2 hydrolysierte Fettsäure normalerweise Arachidonsäure, so dass gleichzeitig ein pro-inflammatorisches Substrat für die Prostaglandin- oder Leukotrien-Synthese bereitgestellt wird [46].

Wenn allerdings durch vermehrte orale oder intravenöse Zufuhr eine n-3 Fettsäure in der *sn-2* Position ist, wird die PLA_2 inhibiert. Somit entsteht zum einen weniger PAF pro Zeiteinheit, zum anderen wird keine n-6 Fettsäure sondern eine n-3 Fettsäure freigesetzt [84].

Nach diesem Schritt erfolgt die Addition einer Acetylgruppe aus Acetyl-CoA an die *sn*-2 Position durch eine Acetyltransferase und die Generierung des aktiven PAF (Abbildung 1.3). PAF wird von Leukozyten sezerniert, bei Endothelzellen bleibt er membrangebunden [85]. Der Plättchen-aktivierende Faktor ist deutlich pro-inflammatorisch: er führt zur Aggregation von Thrombozyten, aktiviert Granulozyten und Monozyten und steigert die vaskuläre Permeabilität. Endothelial präsentiertes PAF kann durch Bindung an seinen Rezeptor auf Leukozyten zur Erhöhung der Avidität von leukozytären Integrinen und somit zur Adhärenz dieser Zellen an Endothelzellen führen [86]. Die gesteigerte Avidität der Integrine hat eine festere Bindung an den endothelialen Adhäsionsmolekülen zur Folge: Das Rollen der Leukozyten über das Endothel wird durch die Interaktion zwischen β2-Integrinen und dem *intercellular adhesion molecule* (ICAM)-1 in die feste Adhäsion der beiden Zellpopulationen übergeleitet. Insgesamt wird PAF eine bedeutende Rolle als kritischer Mediator in der Sepsis zugesprochen [87].

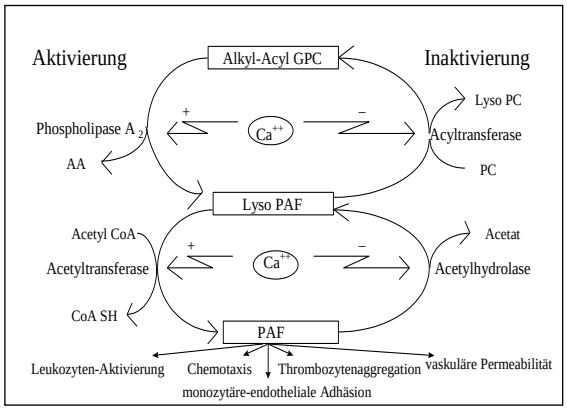

Abb. 1.3: Der metabolische Zyklus des Plättchen-aktivierenden Faktors (PAF).

Aus Alkyl-Acyl Glycero-Phosphorylcholin (GPC) entsteht durch Abspaltung von AA mittels der Phospholipase A_2 Lyso-PAF (1-O-Alkyl-sn-Glycero-3-Phosphorylcholin). Die Acetyltransferase acetyliert mittels Acetyl Coenzym A (Acetyl CoA) Lyso-PAF, wodurch PAF entsteht. Diese beiden enzymatischen Vorgänge werden durch Calcium aktiviert. Die Rückbildung von PAF zu Lyso-PAF über die Acetylhydrolase und über die Acyltransferase zu Alkyl-Acyl GPC wird durch Calcium inhibiert.

Lipid-basierte Kinase-abhängige Signaltransduktion vermittelt durch die PI3-Kinase

Allein die unterschiedliche Metabolisierung durch die genannten Enzymsysteme erklärt nicht ausschließlich die divergente Wirkung von n-3 zu n-6 Fettsäuren. Veränderungen der Phospholipid-Pools der Zellmembran werden für die verschiedenen Effekte von n-3 und n-6 Fettsäuren mit verantwortlich gemacht. *In vitro* kann durch Inkubation mit Eicosapentaensäure bzw. Docosahexaensäure oder durch diätetische Zufuhr

von Fischöl Arachidonsäure durch n-3 Fettsäuren verdrängt werden (*vide supra*). In Endothelzellen konnte dies auch durch Inkubation mit n-3 und n-6 Fettsäuren in weiteren für die intrazelluläre Signaltransduktion wichtigen Phospholipid-Klassen wie dem Phosphatidylinositol (PI) gezeigt werden [56]. Phosphatidylinositol gehört wie PAF zur Lipidmediator Klasse II. Es hat ebenfalls ein Glycerol-Gerüst. In zweierlei Hinsicht ist Phosphatidylinositol für die Signaltransduktion wichtig: Durch die Spaltung einer Phosphatidylinositol-spezifischen Phospholipase (PL) C kann einerseits die Bildung von Inositolphosphaten und Diacylglycerol (DAG) eingeleitet werden. Inositolphosphate und Diacylglycerol sind beides *second messenger* der intrazellulären *Stimulus-Response*-Kopplung. Durch Inositolphosphate kommt es zu einem intrazellulären Calcium-Anstieg, Diacylglycerol aktiviert die Proteinkinase C. Der Anstieg des Calciums aktiviert die *nitric oxide synthase* (NOS), wodurch es zur NO-Produktion kommt. Durch die Fettsäurezusammensetzung des Phosphatidylinositols und des Diacylglycerol kann die intrazelluläre *Stimulus-Response*-Kopplung moduliert werden. Bei regulärer westlicher Diät ist dies meist in der *sn-1* Position des Phosphatidylinositols ein Stearyl-Rest und in der *sn-2* Position ein Arachidonsäure-Rest [88]. Phosphatidylinositol stellt für die Phosphorylierung durch die Phosphatidylinositol-3 Kinase das Substrat dar. Interessante Publikationen diskutieren hier ebenfalls einen Einfluss der Art der Fettsäure-Reste auf die enzymatische Aktivität [89]. Nachgeschaltet erfolgt durch die Phosphatidylinositol-3-Kinase die Aktivierung der Proteinkinase B (Akt). Die Phosphorylierung von Akt aktiviert den Transkriptionsfaktor Bad. So wird über die IkappaB-Kinase I (IKK), ein Regulator des nukleären Faktors kappa B (NFkappaB), die Apoptose inhibiert [90]. PI3-Kinase kann über diverse Signaltransduktionswege die Regulation von Adhäsionsmolelülen und die Adhäsion beeinflussen [91].

Lipid-basierte Membran-abhängige Signaltransduktion vermittelt durch die Lipid Rafts

Lipid Rafts sind Sphingolipid- und Cholesterin-reiche Signaltransduktions-Plattformen in der Plasmamembran. Sie besitzen eine hohe Konzentration an gesättigten Fettsäuren [92]. Diese in der Plasmamembran frei flottierenden *membrane microdomains* zeichnen sich dadurch aus, dass sie *detergent resistent* sind [93, 94]. Mittels dieser Eigenschaft gelingt auch ihre Isolation. Sie können sich sowohl an der inneren als auch an der äußeren Plasmamembran organisieren. Somit stellen sie einen plausiblen Mechanismus dar, um Ereignisse von der äußeren Plasmamembran mit denen an der inneren Plasmamembran und des Zytoplasmas zu koordinieren - und *vice versa* [95]. Die biologische Relevanz der Lipid Rafts ist mittlerweile weithin akzeptiert, da sie Membranfunktionen durch Konzentration der interagierenden Moleküle in spezifischen Regionen der Zelloberfläche organisieren können. Andererseits ist es ihnen möglich, durch Ausschluss der interagierenden Partner eine Aktion zu verhindern [96-98]. Sie sind heterogen in der Lipid- und Protein-Zusammensetzung und können ihre Größe durch die Bildung von *Clustern* variieren. Caveolae sind eine Untergruppe von Lipid Rafts, die sich in der Plasmamembran humaner Endothelzellen befinden. Räumlich gesehen sind Caveolae Invaginationen in der Zellmembran. Als Protein-Marker endothelialer Lipid Rafts gelten Caveolin-1 und die endotheliale NO-Synthase (eNOS); monozytäre Lipid Rafts sind durch die Proteine Lyn und Fyn gekennzeichnet. Die Rekrutierung von Signaltransduktions-Proteinen in Lipid Rafts ist strikt reguliert. Beispielsweise kann durch die Verbindung eines Proteins mit Palmitinsäure (Palmitoylierung) das „Andocken“ an die Rafts der Zellmembran gesteuert werden [99-103]. Eine weitere Möglichkeit ist der aktive Ein- oder Ausschluss von Proteinen mit Hilfe des Zytoskeletts. Bekannt ist, dass die Depletion der Plasmamembran von Cholesterin mittels Methyl-β-Cyclodextrin die Lipid Rafts zerstört und die Signaltransduktion inhibiert. Die Bedeutung der Lipid Rafts unterschiedlicher Zellarten für die inflammatorische Signaltransduktion wurde wiederholt unter Beweis gestellt [99-103].

In neueren Publikationen gibt es Hinweise, dass PUFAs wie DHA die Lipid Rafts auf multiple Art und Weise modulieren können. Dies geschieht, wenn sich DHA in der *sn*-2 Position von Membran-Phosphatidylcholin befindet. Somit findet hier auch eine Lipidmediator-Klasse II-Modulation statt. DHA ist - wie Wassal et al. 2018 allerdings nur an Membran-Modellen publizieren - in dieser Position in der Lage, die Größe der Lipid Rafts positiv zu beeinflussen. Des Weiteren wird die Rekrutierung von für die Signaltransduktion relevanten Proteinen in die Lipid Rafts moduliert und somit auch die Signaltransduktion intrazellulär beeinflusst [104].

Lipid-basierte Transkriptionsfaktor-abhängige Signaltransduktion vermittelt durch PPAR-α

Peroxisom Proliferator-aktivierte Rezeptoren (PPARs) gehören zu den Transkriptionsfaktoren der nuklearen Hormon-Rezeptor Superfamilie, die mit den Retinoid-, den Steroid- und den Thyroid-Rezeptoren verwandt sind [105]. Die Familie der PPARs besteht aus drei Mitgliedern: PPAR-α, PPAR-ß/δ, und PPAR-γ [106]. Der Name PPAR kommt daher, dass die Aktivierung des PPAR bei Nagern zur Proliferation der Peroxisomen in Hepatozyten führt [107]. Im Folgenden soll nur auf PPAR-α näher eingegangen werden. PPAR-α wird in braunem Fettgewebe, in der Leber, in der Niere, im Herz und im Skelettmuskel stark exprimiert [108]. PPAR-α mRNA ist jedoch auch in Lungengewebe von Mäusen detektiert worden [109]. Sie wurde ebenso in humanen Endothelzellen, in glatten Muskelzellen, Monozyten/Makrophagen und in T-Lymphozyten gefunden. Verschiedene Liganden, wie synthetische Fibrate (beispielsweise WY14,643, Clofibrat, Fenofibrat und Bezafibrat) als auch Metaboliten der Arachidonsäure binden an PPAR-α [110, 111]. Nach Aktivierung des PPAR-α dimerisiert dieser mit dem Retinoid X Rezeptor (RXR)-α und bindet an das *Peroxisom Proliferator Response Element* (PPRE). Solche PPRE sind in Promoter-Regionen verschiedener Gene identifiziert worden [112]. Durch die Bindung an das PPRE wird die Protein-Synthese beeinflusst und gesteuert. Obwohl PPAR-α weniger intensiv untersucht wurde als PPAR-γ, wurde für PPAR-α-Liganden auch gezeigt, dass sie regulatorisch in die inflammatorische Antwort eingreifen [113]. Außerdem konnte bei PPAR-α-knockout Mäusen eine abnorm verlängerte Entzündungsreaktion nach Stimulation mit unterschiedlichen pro-inflammatorischen Agenzien nachgewiesen werden [114-116]. Fibrate haben sowohl *in vitro* [117, 118] als auch *in vivo* [113, 119] anti-inflammatorische Eigenschaften. PPAR-α-Liganden können nach Aktivierung durch Zytokine die Expression verschiedener pro-inflammatorischer Gene wie Interleukin (IL)-6, *vascular cell adhesion molecule* (VCAM)-1, den Rezeptor für Plättchen-aktivierenden Faktor und die Cyclooxygenase (COX)-2 – die PGE_2 und TxB_2 generiert - hemmen [119, 120]. Dies liegt möglicherweise zum Teil daran, dass die Aktivierung des nuklearen Faktors [121] NFkappaB inhibiert und die Expression des inhibitorischen Proteins IkappaBα hochreguliert wird [122, 123]. PPAR-α besitzt außerdem protektive Funktionen hinsichtlich der Arteriosklerose. Generell steht seine immunsuppressive Wirkung im Vordergrund [124].

Insgesamt ist PPAR-α in die Regulation von Genen, die in den Lipid-Metabolismus eingreifen, involviert. Da beschrieben ist, dass PPAR-α durch Arachidonsäure und deren pro-inflammatorischen Metabolite wie beispielsweise LTB_4 aktiviert wird, ist hier eine negative Rückkopplungschleife zu vermuten. In bestimmten Zellarten ist auch eine Aktivierung durch n-3 Fettsäuren wie EPA und DHA erwähnt. Daher ist die Untersuchung dieses Transkriptionsfaktors als Stellglied im Lipid-Metabolismus besonders interessant [125, 126].

Fragestellung

Vor diesem Hintergrund war es Ziel dieser Arbeit, die Relevanz der Lipid-basierten Mechanismen auf den unterschiedlichen Ebenen der inflammatorischen Signaltransduktion zu evaluieren.

Hierzu sollten die folgenden Hypothesen überprüft werden:

Die Lipid-basierte Signaltransduktion beeinflusst die Inflammation

1. auf der Ebene der endothelialen-leukozytären Interaktion
2. auf der Ebene der epithelialen-leukozytären Interaktion
3. im Bereich der akuten inflammatorischen Lungenschädigung und der systemischen Inflammation durch n-3 vs. n-6 Fettsäure-basierte Lipidemulsionen differentiell
4. mittels Lipidmediatoren wie PAF
5. mittels Lipid-abhängiger Kinasen wie PI3-kinase
6. über Zellmembran-Komponenten wie Lipid Rafts und
7. durch Lipid-aktivierte Transkriptionsfaktoren wie PPAR-alpha.

Folgende Fragestellungen galt es somit zu bearbeiten:

1. Sind bei der endothelialen-leukozytären inflammatorischen Adhäsion Signaltransduktionswege von Bedeutung, die Einfluss auf die Lipid-basierte Signaltransduktion haben?
2. Sind bei der epithelialen-leukozytären inflammatorischen Interaktion Signaltransduktionswege relevant, die durch die Lipid-basierte Signaltransduktion moduliert werden können?
3. Beeinflussen n-3 basierte Lipidinfusionen im Vergleich mit n-6 basierten Lipidinfusionen die akute inflammatorische Lungenschädigung und die systemische Inflammation differentiell?
4. Ist der Lipidmediator PAF relevant für die Wirkung der n-3/n-6 Fettsäuren bei der inflammatorischen akuten Lungenschädigung und der systemischen Inflammation?
5. Modulieren n-3/n-6 Fettsäuren die endotheliale PI3-Kinase in der Inflammation derart, dass dies Auswirkungen auf Adhäsion und Apoptose hat?
6. Werden monozytäre Lipid Rafts in der Zellmembran signifikant von n-3/n-6 Fettsäuren beeinflusst, so dass die Calcium-Signaltransduktion und die Adhäsion in der Inflammation moduliert werden?
7. Moduliert der Lipid-aktivierte Transkriptionsfaktor PPAR-alpha in der akuten Lungenschädigung die inflammatorische Signaltransduktion?

Der wissenschaftliche Anspruch dieser Arbeit besteht folglich darin, durch die Untersuchung verschiedener Lipid-modulierter Signaltransduktionswege in der Inflammation komplexe Pathomechanismen aufzuzeigen, die kausal an der Entstehung der vaskulären endothelialen bzw. intestinalen epithelialen Inflammation beteiligt sind. Dadurch werden bei klinisch relevanten Krankheitsbildern wie der akuten Lungenschädigung bzw. dem ARDS und der Sepsis pathophysiologische Grundlagen potentieller Therapieansätze evaluiert.

2. Übersicht über die methodischen Schwerpunkte

Auf eine detaillierte Wiedergabe der in der vorliegenden Arbeit eingesetzten Materialen und Methoden wird an dieser Stelle verzichtet, da sie alle aus den entsprechenden Abschnitten in den beigefügten Anlagen hervorgehen. Hier soll zur orientierenden Übersicht lediglich eine kurze Zusammenfassung der wesentlichen Methoden gegeben werden.

Die *in vitro* Untersuchungen wurden mit humanen umbilikal-venösen Endothelzellen (HUVEC) ***(Anlage 1, 4, 5, 6)*** bzw. intestinalen Epithelzellen (CaCo-2 Zellen) ***(Anlage 2)*** durchgeführt. Sie wurden je nach Versuchsansatz mit Lysophosphatidylcholin (LPC), A23187, N-Formyl-methionyl-leucyl-phenylalanin (fMLP), Tumor Nekrose Faktor (TNF)-α, Staurosporin (STS) oder vascular endothelial growth factor (VEGF) stimuliert sowie mit n-3 oder n-6 Fettsäuren vorinkubiert.

PMN wurden von gesunden Spendern aus dem peripheren Blut mittels eines Ficoll-Paque Gradienten und anschließender Erythrozyten-Lyse durch Polyvinylalkohol gewonnen ***(Anlage 2)***.

Für die Versuche zur Bestimmung des *Rolling* und der Adhäsion in der *parallel flow chamber* wurden humane Monozyten aus *Buffy Coats* per Gegenstrom-Zentrifugation-Isolation elutriiert und entsprechend dem Modell nach Lawrence und Springer [120] in der *parallel flow*-Kammer unter Bedingungen des Blutflusses in der post-kapillaren Venole zu den Endothelzellen gegeben. Per Video-Analytik wurde über 10 min. das *Rolling* bewertet und dann in je fünf Vergrößerungsfeldern die Adhärenz ermittelt ***(Anlage 5)***. Die Transmigration wurde in einem statischen *Transwell-Assay* gemessen. Die Monozyten transmigrierten über zwei Stunden durch den HUVEC-Monolayer in das untere Kompartiment und wurden dann mittels Neubauer-Kammer ausgezählt ***(Anlage 4)***.

Die Western Blots der Proteine des Phosphatidyl-Inositol (PI)-3 Signaltransduktionsweges ***(Anlage 4)*** wurden mit VEGF, STS oder TNF-α stimulierten HUVEC durchgeführt. Eine SDS-PAGE (Sodiumdodecylsulfat-Polyacrylalmid Gelelektrophorese) erfolgte im Anschluss. Für die Messung der aktivierten Caspase-3 verwendete man einen kommerziell erhältlichen Assay. Annexin-V wurde mittels FACS (Fluoreszenz-aktivierter Cell Sorter)-Analytik bestimmt. Gleiches galt für die gemessenen Adhäsionsmoleküle und den fMLP-Rezeptor ***(Anlage 1, 2, 4)***.

Lipid Rafts ***(Anlage 5)*** wurden nach Inkubation der mittels Gegenstrom-Zentrifugation-Elutriation gewonnenen humanen Monozyten von gesunden Spendern bzw. von U937-Monozyten mit Triton-X 100 und Durchführung einer Ultrazentrifugation über einen Sucrose-Dichtegradienten isoliert. Anschließend erfolgten Western Blots zur Detektion der Raft-Marker Lyn und Fyn. Anhand dieser Raft-Marker wurden die Raft- und die Non-Raft Fraktionen im Western Blot identifiziert. Diese Raft- und Non-Raft Fraktionen wurden dann einer gaschromatographischen Lipid-Analyse nach Lipid-Extraktion gemäß Bligh und Dyer [127] unterzogen. Die Adhäsion wurde bei den humanen Monozyten in der *parallel flow chamber* wie oben beschrieben und bei den U937-Monozyten mittels eines statischen Assays gemessen.

Die Calcium-Messung wurde Fluoreszenz-basiert mit Hilfe eines Genios-Plate Readers vorgenommen ***(Anlage 2)*** Die Messung des Membranpotentials wurde mittels des Fluorescence-Farbstoffes DiBAC durchgeführt ***(Anlage 1)*.**

Die Sauerstoffradikal-Messung (O_2^-) wurde spektrometrisch mittels der Superoxid-Dismutase inhibierbaren Cytochrom C-Reduktion durchgeführt. Die Messung der humanen neutrophilen Elastase (HNE) erfolgte ebenfalls spektrometrisch ***(*Anlage 2*)***.

Für die Untersuchungen zu PPAR-α wurden je eine Gruppe PPAR-α-/- Mäuse und Wildtyp Mäuse über zwei Wochen entweder mit normalem Futter oder mit einem WY14,643 (WY)-angereichertem Futter versorgt. Im Anschluss erfolgte *ex vivo* nach Stimulation mit Plättchen-aktivierendem Faktor (PAF) die Messung des kapillaren Filtrationskoeffizienten (kf_c) und des pulmonalarteriellen Widerstandes (PAP) im Modell der isoliert perfundierten Mauslunge ***(Anlage 6)***.

Die *in vivo* Experimente zur Relevanz des Plättchen-aktivierenden Faktors (PAF) für die inflammatorische Fettsäure-Modulation wurden an Wildtyp Mäusen, an PAF-Rezeptor -/- Mäusen und an Mäusen, die mit dem PAF-Rezeptor Antagonisten BN5202 behandelt wurden (PAF-RA), vorgenommen. Zunächst erhielten die Mäuse eine NaCl-Infusion. In Narkose erfolgte die offene Präparation der V. jugularis. Ein flexibler Katheter wurde in die V. jugularis nach deren Inzision eingenäht, nach dorsal getunnelt und im Nacken der Maus an eine extern befestigte Mini-Osmotische Pumpe konnektiert ***(Anlage 3)***. Die ersten sieben postoperativen Tage wurde NaCl infundiert, um der Maus eine adäquate Rekonvaleszenzzeit post OP zu gewähren. Danach erfolgte über drei Tage täglich ein Wechsel der osmotischen Pumpe, wozu keine erneute Narkose notwendig war. Während dieser drei Tage wurde entsprechend dem Protokoll n-3 – oder n-6-basierte Lipidemulsion oder NaCl in der Kontrollgruppe i.v. appliziert. Die Blutentnahme erfolgte zeitgleich mit der broncho-alveolären Lavage (BAL) aus der unteren Hohlvene zur Zytokinanalyse ***(Anlage 3).***

Die Lungenfunktion der Mäuse wurde mit einem Body-Plethysmographie-Gerät nach Intubation der anästhesierten Mäuse gemessen. Die BAL der Mauslunge wurde in Narkose bei intubierten Mäusen nach intratrachealer Lipoplysaccharid (LPS)-Gabe vorgenommen. Aus der BAL wurden die Leukozyten mittels Neubauerkammer gezählt und mittels Zytospin analysiert. Eine Proteinbestimmung erfolgte ebenfalls aus der BAL (***Anlage 3, 6***). Die Messung der Eicosanoide Thromboxan B_2 und Prostaglandin E_2 wurde mit kommerziellen Kits aus der Broncho-alveolären Lavage durchgeführt (***Anlage 6***).

Die Zytokinmessungen erfolgten mit kommerziell erhältlichen Antikörper-Paaren (***Anlage 3, 6***).

3. Vorstellung und Diskussion der bearbeiteten Projekte

Die im folgenden Abschnitt dargestellten Resultate der Untersuchungen zum Einfluss Lipid-basierter Signaltransduktion auf inflammatorische Prozesse stellen die Kernbefunde der bearbeiteten Projekte dar. Dabei sind Elemente berücksichtigt, die die Einordnung der Untersuchungsergebnisse in den wissenschaftlichen Gesamtkontext ermöglichen. Weiterführende Einzelheiten zur verwendeten Methodik, zu den Ergebnissen und zur Diskussion können aus den Anlagen entnommen werden.

Inflammatorische Prozesse am Endothel: Modulation der leukozytären Adhäsion durch Ca^{2+}

Die zentrale Fragestellung der vorliegenden Habilitationsschrift geht von der Hypothese aus, dass inflammatorische Prozesse signifikant durch die Lipid-basierte Signaltransduktion beeinflusst werden. Diese Hypothese gilt auch auf der Ebene der endothelialen-leukozytären Interaktion. Die Fragestellung hier war: Sind bei der endothelialen-leukozytären inflammatorischen Adhäsion Signaltransduktionswege von Bedeutung, die Einfluss auf die Lipid-basierte Signaltransduktion haben?

Zunächst sollte deshalb in diesem Abschnitt die Hypothese überprüft werden, dass Calcium die endotheliale-leukozytäre Adhäsion in diesem Setting beeinflusst. Es ist bekannt, dass Calcium beispielsweise die Synthese des Lipid-Mediators PAF stimuliert. Daher ist die Untersuchung der endothelialen-leukozytären Adhäsion, einem zentralen Schritt in der vaskulären Inflammation, hinsichtlich der Modulierbarkeit durch Calcium ein potentielles Prärequisit zur Untersuchung der Lipid-basierten Inflammation. Daraus ergaben sich folgende konkrete operative Fragestellungen für die Durchführung der Studie:

1.1. Führt die Aktivierung des Calcium-abhängigen Kalium-Kanals BK_{Ca}

a. zur Steigerung der monozytären-endothelialen Adhäsion?

b. parallel zur Steigerung der Adhäsion zu einer Hyperpolarisation?

1.2. Wird die BK_{Ca}-bedingte Steigerung der monozytären-endothelialen Adhäsion reduziert durch

a. einen Calcium-Chelator?

b. einen Inhibitor des transmembranären Calcium-Influxes?

c. einen Inhibitor der NAD(P)H-Oxidase?

1.3. Führt die Aktivierung des BK_{ca} zu einer Steigerung der Adhäsionsmoleküle ICAM-1 und VCAM-1?

1.4. Wenn die Aktivierung des BK_{Ca} zu einer Steigerung der Adhäsionsmoleküle ICAM-1 und VCAM-1 führt, ist dies hemmbar durch

a. einen Inhibitor der NAD(P)-Oxidase?

b. einen Inhibitor des transmembranären Calcium-Influxes?

c. einen Calcium-Chelator?

1.0. Ist die inflammatorisch aktivierte monozytäre-endotheliale Adhäsion abhängig von Calcium?

Das Ziel der vorliegenden Studie (***Anlage 1***) [128] war es folglich, herauszufinden, ob eine Lysophosphatidylcholin (LPC)-induzierte Aktivierung des Calcium-aktivierten Kalium-Kanals (BK_{Ca}) zur monozytären-endothelialen Adhäsion beiträgt und somit die darüber erfolgende Veränderung der Calcium-Homöostase für die vaskuläre Inflammation relevant ist.

Hierfür wurde die Adhäsion von U937-Monozyten an HUVEC in einem statischen Assay gemessen. LPC diente dabei als Stimulus der Endothelzellen, was zu einer Steigerung der Adhäsion mit einem Maximum nach 4 h führte (Abb. ***Anlage 1***). Eine Vorbehandlung der U937-Monozyten mit LPC hatte dagegen keinen signifikanten Effekt auf die Adhäsion. Durch LPC erfolgt die Aktivierung des BK_{Ca}. LPC ist der Hauptbestandteil von oxidiertem *low density lipoprotein* (oxLDL), welches in dem chronisch inflammatorischen Prozess der Atherosklerose eine maßgebliche Rolle spielt [129,

130]. Endotheliale Funktionen, wie die Synthese und Freisetzung von NO, als auch die Proliferation und Migration von vaskulären Zellen, werden durch oxLDL beeinflusst. Diese zellulären Funktionen werden durch Veränderungen des intrazellulären Calciums kontrolliert [131]. In Endothelzellen werden Veränderungen im intrazellulären Calcium entweder durch Freisetzung aus intrazellulären Calcium-Speichern oder durch transmembranen Calcium-Influx, der von der Membran-Hyperpolarisation abhängt, bedingt. Das Membranpotential wird von Ionen-Kanälen kontrolliert. Die treibende Kraft für den Calcium-Einstrom ist unter anderem der Calcium-abhängige BK_{Ca}-Kanal [131].

Die durch LPC gesteigerte Adhäsion konnte sowohl durch Vorinkubation mit dem BK_{Ca}-inhibitor IBX (Abb. ***Anlage 1***) als auch mit dem Calcium-Chelator BAPTA und dem Inhibitor des transmembranen Calcium-Flusses 2-APB (Abb. ***Anlage 1***) inhibiert werden. Die Anwendung des NAD(P)H-Oxydase-Inhibitors DPI (Abb. ***Anlage 1***) verhinderte ebenfalls die Wirkung von LPC auf die Adhäsion.

Schließlich konnte gezeigt werden, dass die durch LPC gesteigerte Expression der Adhäsionsmoleküle ICAM-1 und VCAM-1 durch die Applikation des BK_{Ca}-Inhibitors IBX, des NAD(P)H-Oxydase Inhibitors DPI oder des Calcium-Chelators BAPTA signifikant reduziert wurde (Abb. ***Anlage 1***).

Die Tatsache, dass ICAM-1 und VCAM-1 nach Stimulation mit LPC in HUVEC ansteigen, steht in Einklang mit den Ergebnissen anderer Forschergruppen [132, 133]. Wahrscheinlich aufgrund unterschiedlicher Inkubationszeiten und LPC-Dosen fanden einige Gruppen nur ICAM-1 und nicht VCAM-1 hochreguliert [134, 135]. Unsere Ergebnisse zeigen eine verstärkte Adhäsion von U937 an LPC-stimuliert HUVEC. Es wurde auch schon publiziert, dass oxLDL die Adhäsion erhöht, Erl et al. konnten dies jedoch nicht für LPC zeigen, was durch verschiedene U937-Klone bedingt sein könnte [136, 137]. Bei unseren Versuchen hatte eine LPC-Stimulation der U937 keinen steigernden Effekt auf die Adhäsion. Dies steht im Gegensatz zu den Ergebnissen von Frostegard, welcher aber auch längere Inkubations-Zeiten angewendet hat [138]. Wir konnten eine LPC-induzierte Zeit- und Dosis-abhängige Hyperpolarisation der HUVEC ermitteln (Abb. ***Anlage 1***). Dieser LPC-Effekt wurde durch IBX, einem spezifischen BK_{Ca}-Inhibitor, aufgehoben (Abb. ***Anlage 1***). Somit ist klar, dass dieser Ionen-Kanal für die Hyperpolarisation verantwortlich ist. Eine direkte Aktivierung des BK_{Ca} durch LPC konnte schon gezeigt werden [139]. Da die Vorinkubation mit IBX nicht nur die Hyperpolarisation der Endothelzellen, sondern auch die Expression der Adhäsionsmoleküle (Abb. ***Anlage 1***) und die Adhäsion der U937 (Abb. ***Anlage 1***) deutlich verringert hat, zeigen diese Experimente, dass die Aktivierung des BK_{Ca}, und damit die Modulation der Calcium-Homöostase, ein wichtiger Schritt in dieser inflammatorischen Signalkaskade zwischen Endothel und Leukozyten ist.

Als nächstes wurde in dieser Studie gezeigt, dass die Calcium-Chelation sowie die Inhibition des transmembranen Calcium-Influx sowohl die Adhäsion (Abb. ***Anlage 1***) als auch die Expression der Adhäsionsmoleküle (Abb. ***Anlage 1***) signifikant reduziert. In früheren Publikationen konnte demonstriert werden, dass LPC über die Aktivierung der NAD(P)H Oxydase *reactive oxigen species* (ROS) generiert [139, 140]. In den jetzigen Untersuchungen führte eine Blockierung der NAD(P)H Oxydase-abhängigen ROS-Bildung mittels DPI zu einer reduzierten Adhäsion (Abb. ***Anlage 1***) und zu einer verminderten Expression der Adhäsionsmoleküle (Abb. ***Anlage 1***). Diese Ergebnisse sind in Einklang mit denen von Cominacini et al., die pathophysiologisch eine Einbindung des NFkappaB-Signaltransduktionsweges erörtern [133, 141, 142]. Die Einbindung von src-Protein-Tyrosin-Kinasen (PTK) in diesen Signaltransduktionsweg wird ebenfalls diskutiert [143]. Folglich ist zu vermuten, dass LPC durch die Modulation des BK_{Ca}, den daraus resultierende Calcium-Influx und durch die ROS-Bildung die PTK aktiviert, was wiederum zu einer Aktivierung von NFkappaB führt. Darüber können Adhäsionsmoleküle in ihrer Expression und pro-inflammatorische Mediatoren reguliert werden [144, 145].

Neueste Veröffentlichungen weisen auf eine zirkadiane Rhythmik der vaskulären Dysfunktion hin [146]. Dies konnte in der vorliegenden Studie nicht berücksichtigt werden. In der vorliegenden Studie wurde die Modulation der Adhäsionsmoleküle VCAM-1 und ICAM-1 durch Calcium-Chelation untersucht. Tetraspanin CD9 oder *Tetraspanin- enriched microdomains*, welche als Schaltzentralen in der Zellmembran – ähnlich

der Lipid Rafts – als Regulatoren der zellulären Adhäsion auch eine Schlüsselrolle spielen, wurden nicht evaluiert, könnten jedoch über die Modulation von fingerförmigen interzellulären Junktionen („*digitation junctions*“) Einfluss auf die Adhäsion nehmen [147]. Durch CD9 könnte insbesondere die Avidität, d.h. die Stärke der multivalenten Bindung der Adhäsionsmoleküle beeinflusst werden. Die Affinität, d.h. die Kraft der einzelnen Bindung wird nicht beeinflusst durch CD9 [148, 149]. Eine weitere interessante Funktion scheint die durch Scherkraft induzierte mitochondriale ATP Produktion inne zu haben, die wiederum zu einer ATP-Freisetzung in Caveolae führen kann, was purinerge Rezeptoren aktiviert und eine Calcium Freisetzung zur Folge hat [150, 151]. Diese Calcium Freisetzung könnte ebenfalls zur Regulation von VCAM-1 oder ICAM beitragen. Insbesondere die Rolle der Caveolae ist auch für die Lipid-abhängige Signaltransduktion ein lohnender Ansatzpunkt für weitere Untersuchungen.

Insgesamt konnte hier konkret gezeigt werden, dass:

1.1. Die Aktivierung des Calcium-abhängigen Kalium-Kanals BK_{Ca}

a. zur Steigerung der monozytären-endothelialen Adhäsion führt und

b. parallel zur Adhäsionssteigerung eine Hyperpolarisation der EC induziert.

Weiterhin wurde demonstriert, dass

1.2. Die BK_{Ca}-bedingte Steigerung der monozytären-endothelialen Adhäsion durch

a. einen Calcium-Chelator;

b. einen Inhibitor des transmembranären Calcium-Influxes und

c. einen Inhibitor der NAD(P)H-Oxidase reduziert wird;

1.3. Die Aktivierung des BK_{Ca} zu einer Steigerung der Adhäsionsmoleküle ICAM-1 und VCAM-1 führt;

1.4. Dies hemmbar ist durch

a. einen Inhibitor der NAD(P)H-Oxidase;

b. einen Inhibitor des transmembranären Calcium-Influxes und;

c. einen Calcium-Chelator und folglich

1.0: Die inflammatorisch aktivierte monozytäre-endotheliale Adhäsion abhängig ist von Calcium.

Zusammenfassend führte die Aktivierung des endothelialen BK_{Ca} zu einer Hyperpolarisation der Endothelzellen (Abb. ***Anlage 1***). Diese war gefolgt von einem transmembranen Calcium-Influx, welcher wiederum zu einer gesteigerten ROS-Bildung aufgrund der Aktivierung der NAD(P)H Oxydase führte. Durch Blockierung der Hyperpolarisation oder der ROS-Bildung oder des Calcium-Influx kam es zu einer Reduktion der Expression von ICAM-1 und VCAM-1 (Abb. ***Anlage 1***) sowie zu einer entsprechend verminderten LPC-induzierten Adhäsion (Abb. ***Anlage 1***) der U937 an Endothelzellen.

In der vorliegenden Studie konnte folglich demonstriert werden, dass die LPC-induzierte Aktivierung des BK_{Ca} zur monozytären-endothelialen Adhäsion beiträgt und die darüber erfolgende Veränderung der Calcium-Homöostase für die vaskuläre Inflammation relevant ist.

Die Hypothese, dass Calcium die endotheliale-leukozytäre Adhäsion in diesem Setting beeinflusst, wurde bestätigt.

Die Hypothese, dass inflammatorische Prozesse auf der Ebene der endothelialen-leukozytären Interaktion signifikant durch die Lipid-basierte Signaltransduktion beeinflusst werden, konnte mit dieser Studie noch nicht abschließend beantwortet werden.

Aber die Frage, ob bei der endothelialen-leukozytären inflammatorischen Adhäsion Signaltransduktionswege von Bedeutung sind, die Einfluss auf die Lipid-basierte Signaltransduktion haben, konnte ich positiv beantworten, da Calcium u.a. Einfluss auf die PAF-Synthese hat.

Diese Ergebnisse haben das Potential, die pathophysiologischen Grundlagen für mögliche Therapieansätze bei inflammatorischen Erkrankungen zu liefern.

Inflammatorische Prozesse am Epithel: Modulation der leukozytären Inflammation durch NO und durch den Lipidmediator PAF

Die folgende Studie (***Anlage 2***)[152] sollte die Hypothese untersuchen, dass die Lipid-basierte Signaltransduktion die Inflammation auf der Ebene der epithelialen-leukozytären Interaktion beeinflussen kann. In der Sepsis ist die epitheliale-leukozytäre Inflammation bei der Aufrechterhaltung der Darmschranke ein entscheidender Faktor. Daher war es Ziel dieser Studie, zu eruieren, ob die epitheliale-leukozytäre inflammatorische Interaktion durch die Lipid-basierte Signaltransduktion moduliert werden kann.

Daraus ergaben sich folgende konkrete Fragestellungen für die Durchführung der Studie:

2.1. Wie ist die inflammatorische Aktivität gemessen an den O^{2-}-Anionen (respiratorischer Burst) und an der humanen neutrophilen Elastase (HNE), wenn

a. beide Zellarten (Epithelzellen und PMN) durch das Calcium-Ionophor A23187 stimuliert werden?

b. nur PMN durch das Rezeptor-abhängige bakterielle Peptid fMLP aktiviert werden?

2.2. Kann die so gemessene inflammatorische Antwort moduliert werden durch

a. einen Cyclooxygenase (COX)-Inhibitor?

b. einen Inhibitor der NO-Synthase?

c. einen PAF-Rezeptor-Blocker?

2.0 Wird die Inflammation von intestinalen Epithelzellen (CaCo-2) mit Polymorphonukleären Granulozyten (PMN) durch die Lipid-basierte Signaltransduktion moduliert?

Es wurden PMN und CaCo-2 Zellen, separat und in Co-Inkubation mit dem Calcium-Ionophor A23187 oder mit N-Formyl-methionyl-leucyl-phenylalanin (fMLP) stimuliert. A23187 ist von keinem Rezeptor abhängig und induziert einen Calcium-Influx in die Zelle, der verschiedene inflammatorische Effekte, wie beispielsweise die Aktivierung der PAF-Synthese und der PAF-Signaltransduktion zur Folge hat. Das bakterielle Peptid fMLP ist ein G-Protein-Rezeptor-abhängiger Stimulus. Der Rezeptor für fMLP befindet sich auf PMN, jedoch nicht auf CaCo-2 Zellen [153-156]. Um die Modulation der inflammatorischen Signaltransduktion zwischen den verschiedenen Zellen zu evaluieren, wurde die humane neutrophile Elastase (HNE) und die Sauerstoffradikale mittels des respiratorischen Bursts gemessen sowie Inhibitoren der Cyclooxygenase (COX: *acetylsalicylic acid*; ASA), der NO-Synthase (N-Monomethyl-L-Arginin, L-NMMA) und des PAF-Rezeptors (WEB2086) angewandt.

Es zeigte sich, dass eine Co-Inkubation von PMN und CaCo-2 Zellen im Vergleich zu PMN alleine den respiratorischen Burst unter Kontrollbedingungen und nach Stimulation mit A23187 hemmt. CaCo-2 Zellen alleine induzierten generell nur einen schwachen respiratorischen Burst nahe an der Detektionsgrenze (Abb. ***Anlage 2***).

Im Gegensatz dazu modulierte eine Co-Inkubation von PMN und CaCo-2 Zellen den respiratorischen Burst nach einer Stimulation mit fMLP nicht. Der Anstieg des Burst unter den Bedingungen der Co-Inkubation war vergleichbar mit dem von PMN alleine. CaCo-2 Zellen alleine induzierten unverändert einen respiratorischen Burst nahe an der Detektionsgrenze (Abb. ***Anlage 2***). Die Inhibition der Cyclooxygenase durch ASA verhinderte eine Co-Inkubations-bedingte Reduktion des respiratorischen Bursts nach Stimulation mit 0, 0.1 und 10 µM A23187 (Abb. ***Anlage 2***). Die Hemmung der NO-Synthase mittels L-NMMA verhinderte eine Co-Inkubations-bedingte Reduktion des respiratorischen Bursts nur unter Kontrollbedingungen (Abb. ***Anlage 2***). Die Inhibition des PAF-Rezeptors mittels WEB2086 beugte eine Co-Inkubations-bedingte Reduktion des respiratorischen Bursts unter Kontrollbedingungen verglichen mit PMN alleine vor (Abb. ***Anlage 2***). Elastase war nach Stimulation von CaCo-2 Zellen alleine nicht nachweisbar. Eine Co-Inkubation führte zu einer Steigerung der Elastase-Freisetzung im Vergleich zu PMN alleine unter Kontrollbedingungen. Nach Stimulation mit 1 und 10 µM A23187 kam es zu einer signifikanten Reduktion der Elastase unter Co-Inkubationsbedingungen (Abb. ***Anlage 2***).

Nach Stimulation mit fMLP sah man einen Anstieg der Elastase unter Bedingungen der Co-Inkubation. CaCo-2 Zellen alleine induzierten auch nach Stimulation mit fMLP keine Elastase-Freisetzung (Abb. ***Anlage 2***). Die Inhibition der Cyclooxygensase mittels ASA veränderte die Elastase-Produktion weder unter Kontrollbedingungen noch nach Stimulation mit A23187 (Abb. ***Anlage 2***). Die Inhibition der NO-

Synthase mittels L-NMMA führte zu keiner Veränderung unter Kontrollbedingungen. Nach Stimulation mit 1 und 10 µM A23187 kam es jedoch zu einer signifikanten Aufhebung der Co-Inkubations-bedingten Reduktion der Elastase (Abb. ***Anlage 2***). Die Hemmung des PAF-Rezeptors mittels WEB2086 führte zwar unter Kontrollbedingungen zu keiner Änderung, verhinderte aber nach Stimulation mit 1 und 10 µM A23187 eine signifikante Reduktion der Elastase durch die Co-Inkubation der beiden Zelltypen (Abb. ***Anlage 2***).

Unter unseren Versuchsbedingungen konnte ich erstmalig zeigen, dass eine Co-Inkubation von PMN und CaCo-2 Zellen nach inflammatorischer Stimulation mit A23187 zu einer signifikant reduzierten Produktion von Sauerstoffradikalen und Elastase führt.

Es ist davon aus zu gehen, dass wir mit A23187 beide Zelltypen simultan stimulieren [157, 158]. Die Stimulation mit fMLP zeigte in unseren Versuchen keinen modulatorischen Einfluss der Co-Inkubation auf den respiratorischen Burst. In unseren Versuchen führte eine Stimulation mit fMLP zu einer alleinigen Aktivierung der PMN, da wir auf CaCo-2 Zellen keinen fMLP-Rezeptor nachweisen konnten. Vermutlich deshalb ging der kooperative inhibitorische Effekt verloren und war nur vorhanden, wenn beide Zelltypen mit dem Rezeptor-unabhängigem Stimulus A23187 stimuliert wurden. Bezüglich der Co-Inkubations-bedingten Inhibition des Bursts unter Kontrollbedingungen, ist hier eine basale Stimulierung beider Zelltypen, z.B. durch Calcium, ursächlich zu vermuten. A23187 würde diesen Effekt verstärken, wie in unseren Versuchen gezeigt. Allerdings würde fMLP diesen basalen Calcium-induzierten Effekt durch seine stärkere Rezeptor-abhängige Wirkung an PMN überspielen. Das würde erklären, warum fMLP die beobachtete Co-Inkubations-bedingte Inhibition des Bursts unter basalen Kontrollbedingungen verhinderte. Eine Co-Inkubation mit PMN und CaCo-2 Zellen steigerte die Elastase-Freisetzung unter Kontrollbedingungen und nach Stimulation mit fMLP. Da Elastase sich positiv auf die PMN-Transmigration auswirkt [159], könnte eine konkordante Steigerung durch das Zusammentreffen von PMN und CaCo-2 Zellen einer physiologischen Reaktion entsprechen. Es bleibt die Frage, warum eine Stimulation mit A23187 eine Co-Inkubations-bedingte Inhibition der Elastase hervorrief, was unter basalen Bedingungen und nach Stimulation mit fMLP nicht der Fall war. Die gegensätzlichen Ergebnisse von fMLP und A23187 können durch eine singulären versus einer dualen Zellaktivierung erklärt werden, wie oben ausgeführt. Es ist zu vermuten, dass eine Co-Inkubations-abhängige Inhibition des Bursts und der Elastase von Signaltransduktionswegen abhängig ist, die durch das vom A23187 induzierte Calcium aktiviert werden. Hier ist beispielsweise der NO- oder der PAF-Signaltransduktionsweg zu nennen [160-162]. NO- und PAF-Inhibitoren verhinderten eine Co-Inkubations-abhängige Reduktion der Elastase in unseren Versuchen. Die Inhibition der Cyclooxygenase hatte in unserem Versuchsaufbau keinen signifikanten Einfluss auf die Elastase-Freisetzung, antagonisierte aber die Co-Inkubations-bedingte Inhibition der O^{2-}-Produktion leicht. Vermutlich liegt dies an der ASA-induzierten ROS-Produktion in CaCo-2 Zellen [163]. L-NMMA konnte die Co-Inkubations-bedingte Reduktion des Bursts nach Stimulation mit A23187 nicht signifikant modulieren, wohl aber die der Elastase. Folglich ist hier eine Abhängigkeit von der NO-Signaltransduktion zu vermuten. Es ist anzunehmen, dass die NO-Freisetzung ein wichtiger Faktor bei der reduzierten Elastase-Sekretion ist, da NO zu einer Nitrosylierung von (Signal)-Proteinen oder zu einem Anstieg des intrazellulären cGMP führen kann [164, 165]. Weiterhin konnte WEB2086 die Co-Inkubations-bedingte Reduktion des respiratorischen Bursts nach Stimulation mit A23187 nicht signifikant modulieren, wohl aber die der Elastase. Folglich ist hier eine Abhängigkeit von der PAF-Signaltransduktion anzunehmen. Es ist zu erwarten, dass von PMN generierter PAF eine Rolle bei der positiven Rückkopplungsschleife in der Inflammation spielt, da es beispielsweise über NFkappaB IL-8 induzieren kann [166-169]. PAF-PAF-Rezeptor Interaktionen sind in die Adhäsion von PMN an Endothelzellen involviert [14]. Man kann also schlussfolgern, dass der Calcium-abhängige PAF-PAF-Rezeptor-Weg eine wichtige Rolle sowohl für die PMN- als auch für die CaCo-2-abhängige inflammatorische Signaltransduktion, insbesondere für deren Kooperation, spielt [170, 171]. Ein Grund dafür, dass die Co-Inkubations-bedingte Reduktion des Bursts nicht beeinflusst wird, könnte daran liegen, dass *ROS* von beiden Zelltypen produziert werden. Elastase hingegen wird nur von PMN sezerniert [172].

Neueste Veröffentlichungen zeigen, dass Neutrophile auch im *in vivo* Setting zur Homöostase der intestinalen Barriere beitragen. Dies geschieht über eine TGF-beta-abhängige MEK1/2-Aktivierung, welche in intestinalen Epithelzellen zur Bildung von Amphiregulin (AREG) führt. AREG gehört zu den EGFR-Liganden und ist für den Erhalt der Barrierefunktion der intestinalen Epithelzellen und der interstitiellen Homöostase relevant [173]. Somit ist die Kooperation zwischen Neutrophilen und intestinalen Epithelzellen wie in der vorliegenden Studie ebenso dokumentiert.

Andere kommen ebenfalls zu dem Schluss, dass die Interaktion zwischen PMN und Epithelzellen eine wichtige Rolle bei der Kontrolle inflammatorischer Signaltransduktionswege spielen. Es wurde festgestellt, dass intestinale Epithelzellen Pyrophosphatase/Phosphodiesterase-1 exprimieren, welches zur einer Enzymfamilie gehört, die Diadenosinphosphate wie Diadenosintriphosphat (Ap3A), dass von aktivierten Neutrophilen produziert wird, zu Adenosinmonophospat metabolisieren. Das so entstehende Adenosin bindet an epitheliale Adenosin-Rezeptoren, wodurch die Barrierefunktion gestärkt wird [174]. Dies steht in Einklang mit den in der vorliegenden Studie gefundenen Ergebnissen.

Zusammenfassend ist festzuhalten, dass eine Co-Inkubation von PMN und CaCo-2 Zellen den respiratorischen Burst ohne Applikation eines Stimulus reduziert. Dies könnte an einer basalen Stimulation beider Zelltypen liegen, da dies nach Stimulation mit fMLP, das nur PMN aktiviert, nicht mehr nachweisbar ist. Nach Stimulation mit dem Calcium-Ionophor A23187, das beide Zelltypen aktiviert, führte eine Co-Inkubation von PMN und CaCo-2 Zellen zu einer Reduktion von Elastase und Burst. Die Cyclooxygenase könnte zum Teil für die Co-Inkubations-bedingte Reduktion des Bursts verantwortlich sein, da ASA den anti-inflammatorischen Effekt teilweise verhinderte. Die Co-Inkubation steigerte die Elastase-Freisetzung unter Kontrollbedingungen und nach Stimulation mit fMLP. A23187 führte zu einer Co-Inkubations-bedingten Reduktion der Elastase. Sowohl die NO- als auch die PAF-Signaltransduktion scheinen eine relevante Rolle bei der Co-Inkubations-bedingten Reduktion der Elastase zu spielen, da deren Inhibitoren L-NMMA bzw. WEB2086 den anti-inflammatorischen Effekt vereitelten.

Es konnte somit konkret beantwortet werden, dass:

2.1. Die inflammatorische Aktivität gemessen an den O^{2-}-Anionen (respiratorischer Burst) und an der humanen neutrophilen Elastase (HNE),

a. wenn beide Zellarten durch das Calciumionophor A23187 stimuliert werden, vermindert ist;

b. wenn nur PMN durch das Rezeptor-abhängige bakterielle Peptid fMLP aktiviert werden, nicht vermindert ist;

Des Weiteren konnte eruiert werden, dass

2.2. Die so gemessene inflammatorische Antwort moduliert werden kann durch

a. einen Inhibitor der NO-Synthase und

b. einen PAF-Rezeptor-Blocker und somit

2.0 Die Inflammation von intestinalen Epithelzellen (CaCo-2) mit Polymorphonukleären Granulozyten (PMN) durch die Lipid-basierte Signaltransduktion moduliert wird.

Es konnte in dieser Studie folglich herausgearbeitet werden, dass die gleichzeitige Aktivierung von PMN und CaCo-2 Zellen für eine koordinierte Immunantwort notwendig und dabei der Lipidmediator PAF relevant ist.

Somit konnte ich zeigen, dass bei der epithelialen-leukozytären inflammatorischen Interaktion Signaltransduktionswege relevant sind, die durch die Lipid-basierte Signaltransduktion moduliert werden können.

Die Hypothese, dass die Lipid-basierte Signaltransduktion die Inflammation auf der Ebene der epithelialen-leukozytären Interaktion beeinflussen kann, wurde bestätigt.

Diese Ergebnisse könnten für die Entschlüsselung der interaktiven Signaltransduktion zwischen Neutrophilen und intestinalen Epithelzellen bei inflammatorischen Erkrankungen von Bedeutung sein.

Beeinflussung der Inflammation in vivo *durch n-3/n-6 Fettsäuren in Abhängigkeit vom PAF-Rezeptor*

Nun sollte die Hypothese untersucht werden, dass die Lipid-basierte Signaltransduktion durch n-3 vs. n-6 Fettsäure-basierte Lipidemulsionen die Inflammation im Bereich der akuten Lungenschädigung und der systemischen Inflammation differentiell beeinflussen kann.

Des Weiteren sollte die Hypothese evaluiert werden, dass die Lipid-basierte Signaltransduktion die Inflammation mittels eines Lipidmediators wie PAF modulieren kann.

Da zum einen sowohl die monozytäre-endotheliale Adhäsion als auch der Plättchen-aktivierende Faktor in der Inflammation eine Schlüsselrolle besitzen, zum anderen Hinweise für das anti- bzw. pro-inflammatorische Potential der n-3/n-6 Fettsäuren vorlagen, wurde in einer früheren *in vitro* Studie die Relevanz des Plättchen-aktivierenden Faktors (PAF) für den Einfluss der n-3 und n-6 Fettsäuren auf die monozytäre-endotheliale Adhäsion untersucht (***Anlage 7***)[67]. Die *in vitro* gewonnenen Ergebnisse der Relevanz des Plättchen-aktivierenden Faktors für die Wirkungsweise der n-3/n-6 Fettsäuren auf die monozytäre-endotheliale Adhäsion sollte nun *in vivo* unter Verwendung von PAF-Rezeptor-defizienten Mäusen und Langzeit-Lipidinfusionen – analog der auf Intensivstationen verwendeten Lipidinfusionen zur Ernährung septischer Patienten - evaluiert werden (***Anlage 3***)[175].

Es wurde der Frage nachgegangen, ob n-3 basierte Lipidinfusionen im Vergleich mit n-6 basierten Lipidinfusionen die akute inflammatorisch bedingte Lungenschädigung und die systemische Inflammation unterschiedlich beeinflussen.

Des Weiteren wurde die Frage eruiert, ob der Lipidmediator PAF relevant ist für die Wirkung der n-3 Fettsäuren auf die inflammatorische akute Lungenschädigung und die systemische Inflammation.

Daraus ergaben sich diese Fragestellungen für die Durchführung dieser Studie:

3.1. Führt die inflammatorische Stimulation von Wildtyp (WT) Mäusen und PAF-Rezeptor-knockout (PAF-KO) Mäusen mit LPS intratracheal (i.t.) bzw. intraperitoneal (i.p.) zu einer vergleichbaren inflammatorischen Antwort gemessen an der

a. alveolärer Leukozyteninvasion

b. Myeloperoxidase (MPO)-Aktivität im Lungengewebe

c. alveoläre Protein-Leckage

d. TNF-α Konzentration im Alveolarraum

e. MIP-2 Konzentration im Alveolarraum

f. TNF-α Konzentration im Plasma

g. MIP-2 Konzentration im Plasma?

3.2. Moduliert eine intravenöse Langzeit-Infusion mit n-3 bzw. n-6 basierten Lipidemulsionen in diesem Setting diese inflammatorische Antwort unterschiedlich bei WT Mäusen?

3.3. Moduliert eine intravenöse Langzeit-Infusion mit n-3 bzw. n-6 basierten Lipidemulsionen in diesem Setting diese inflammatorische Antwort unterschiedlich bei PAF-KO Mäusen?

3.4. Moduliert eine intravenöse Langzeit-Infusion mit n-3 bzw. n-6 basierten Lipidemulsionen in diesem Setting diese inflammatorische Antwort unterschiedlich bei WT Mäusen nach Applikation eines PAF-Rezeptor-Antagonisten?

3.0 Ist im Modell der murinen akuten pulmonalen bzw. systemischen Inflammation die anti-inflammatorische Wirkung einer Langzeit-Infusion mit n-3 basierten Lipidemulsionen abhängig von der Präsenz eines PAF-Rezeptors?

Ziel war es, die Signifikanz insbesondere für die pulmonale Inflammation - aber auch für die systemische Inflammation - und die Wirksamkeit der intravenösen Applikation im Ganztiermodell unter inflammatorischen Bedingungen unter Beweis zu stellen. Anhand von zwei verschiedenen Modellen konnte gezeigt werden, dass die durch LPS hervorgerufene inflammatorische Antwort durch eine dreitägige Infusion von Lipidemulsionen moduliert werden kann. Im Gegensatz zu n-6(Sojabohnenöl (SO))-basierten Lipidemulsionen reduzierten n-3(Fischöl(FO))-basierte Lipidemulsionen die pulmonale Leukozyteninvasion (Abb. ***Anlage 3***), die Protein-Durchlässigkeit durch die endo-/epitheliale Barriere und die alveoläre und vaskuläre Zytokinsynthese (Abb. ***Anlage 3***). Ebenso wurde demonstriert, dass die divergierenden Effekte der Lipidemulsionen abhängig sind von der Signaltransduktion des PAF-Rezeptors. Sowohl bei Mäusen, denen das PAF-Rezeptor Gen fehlte (PAF-R-/-), als auch bei Mäusen, die mit einem PAF-Rezeptor-Antagonisten behandelt wurden, war die inflammatorische Antwort auf LPS erhalten, der

divergierende Effekt der n-6 im Vergleich zu den n-3 Lipiden war jedoch nicht mehr nachweisbar.

In unserem Modell der akuten Lungenschädigung war die inflammatorische Antwort auf LPS bei PAF-R-/- Mäusen unverändert. Dieser Befund steht in Einklang mit Berichten, die eine intakte Antwort von PAF-R-/- Mäusen in einem *Endotoxic-Shock*-Modell schildern [176].

Ein herausragendes Ergebnis der vorliegenden Studie war die differentielle Beeinflussung der LPS-induzierten Zytokin-Synthese durch die n-3 im Vergleich zu den n-6 Lipiden bei Wildtyp Mäusen (Abb. ***Anlage 3***). Bei Mäusen, die eine konventionelle n-6 Lipidinfusion erhielten, wurde bei beiden verwendeten Modellen ein Anstieg von TNF-α und von *macrophage inflammatory protein* (MIP)-2 (dem murinen IL-8-Äquivalent) beobachtet. Im Gegensatz dazu resultierte eine Infusion mit n-3 Lipiden unter allen untersuchten experimentellen Bedingungen bei Wildtyp Mäusen in einer Reduktion der pro-inflammatorischen Zytokinproduktion. Obwohl dieses Modell einer kontinuierliche Lipidinfusion in der systemischen Inflammation ein Novum ist, sind diese Ergebnisse mit bereits veröffentlichten Daten dahingehend konsistent, dass bereits gezeigt wurde, dass die TNF-α– und IL-1-ß Freisetzung in isolierten mononukleären Zellen durch orale Gabe von n-3 Fettsäuren bei freiwilligen Probanden [65], in isolierten murinen Splenozyten [177], und in isolierten Monozyten von septischen Patienten mit n-3 Lipidinfusion [178] supprimiert wurde. Normalerweise ist eine orale Fischöl-Substitution von mehreren Wochen notwendig, um eine Änderung des Immunstatus zu erreichen. Hingegen war eine intravenöse Applikation über drei Tage ausreichend, um die Zytokinsynthese bei Mäusen in der vorliegenden Studie und bei Patienten, die intravenöse Lipide erhielten, zu verändern [178].

Die pulmonale Leukozytenrekrutierung war bei den Wildtyp Mäusen durch die Fischöl-basierte Lipidinfusion vermindert (Abb. ***Anlage 3***). Im Gegensatz dazu verstärkte die Sojabohnen-basierte n-6 Lipidinfusion die Leukozyteninvasion und die akute Lungenschädigung. Eine größere Schädigung der Lungen unter inflammatorischem Stress durch n-6 Lipide und *vice versa* eine geringere Schädigung durch Applikation von n-3 Lipiden wurde bereits beschrieben [44, 74]. Die diesem protektivem Effekt zugrundeliegenden Mechanismen beruhen, zumindest zum Teil, auf dem oben beschriebenen Einfluss von Fischöl auf die Zytokin-Antwort, die Synthese von weniger potenten Lipid-Mediatoren wie Leukotrien B_5 anstelle von Leukotrien B_4, die Bildung des weniger aktiven Vasokonstriktors Thromboxan A_3 anstelle von Thromboxan A_2, einer verminderten Synthese des Plättchen-aktivierenden Faktors und einer geringeren leukozytären-endothelialen Adhäsion [67, 75, 179].

Die leukozytäre Transmigration durch die endotheliale-epitheliale Barriere ist ein komplexer und strikt regulierter Prozess. Die n-3 Lipide interferieren mit diesem Prozess auf mehreren Ebenen, einschließlich einer verminderten Präsentation endothelialer Adhäsionsmoleküle und einer reduzierten Bildung des Plättchen-aktivierenden Faktors durch die Endothelzellen, was in der Gesamtheit zu einer weniger starken Integrin-Aktivierung auf rollenden Leukozyten führen könnte [67, 180].

Durch die Infusion der Lipidemulsionen wird die physiologische Aufnahme und Verarbeitung der Triglyzeride durch den Gastrointestinaltrakt umgangen. Die Infusion synthetischer Lipidaggregate aktiviert endotheliale Lipoproteinlipasen und induziert eine Translokation des Enzyms von seiner zellulären Bindungsstelle ins vaskuläre Kompartiment. Aktivierung und Translokation des Enzyms resultiert in einem Anstieg der freien Fettsäuren im Plasma [181, 182]. Kinetik und Dauer der erhöhten Plasmaspiegel durch n-3 Lipidinfusion übersteigt das Potential konventioneller orale Fischöl-Zufuhr um ein Vielfaches [183, 184]. Die unterschiedliche Verfügbarkeit von Präkursor-Fettsäuren hat nicht nur Einfluss auf die daraus resultierende Generierung von Lipidmediatoren (z.B. Substitution von Leukotrien B_4 durch B_5), sondern moduliert auch die PAF-Bildung durch die Inkorporation in den Phospholipid-Präkursor-Pool. Diesbezüglich sind mindestens zwei Lipid-abhängige Mechanismen relevant. Erstens führt eine Anreicherung von n-3 Fettsäuren im PAF-Präkursor-Pool zu einer sterischen Inhibition der Phospholipase A_2 [85, 185]. Umgekehrt verstärkt eine vermehrte Verfügbarkeit von Arachidonsäure die Bildung des Plättchen-aktivierenden Faktors [67]. Zweitens könnte eine Anreicherung von n-3 Fettsäuren im Phosphatidyl-Inositol-Pool die leukozytäre Aktivierung durch eine Reduktion der intrazellulären *second messenger* Synthese und durch eine verminderte Aktivierung der Proteinkinase C hemmen [186, 187].

Experimentelle und klinische Untersuchungen sehen einen deutlichen Zusammenhang zwischen der Verfügbarkeit von freier Arachidonsäure, Plättchen-aktivierendem Faktor und Lungenschädigung. Plättchen-aktivierender Faktor und Lipopolysaccharid (LPS) führen zur Lungenschädigung und zur Ödembildung durch eine Sphingomyelinase-abhängige Ceramidsynthese sowie durch die Aktivierung des Cyclooxygenase-Weges, was zur Generierung von Arachidonsäure-abgeleiteten Prostanoiden führt [188]. Eine schnelle Infusion konventioneller n-6-basierter Lipidemulsionen bei beatmeten Patienten mit *acute respiratory distress syndrome* (ARDS) führte zu einem größeren pulmonalen *Shunt*-Fluss bedingt durch eine vermehrte Prostanoid-Bildung. Dies führte zu einer Verschlechterung des Horovitz-Quotienten [121]. Eine weitere Studie mit ARDS-Patienten, die eine Lipidinfusion erhielten, berichtet über einen Anstieg der broncho-alveolären PAF-Konzentration sowie der neutrophilen Granulozyten als auch über eine Verschlechterung der Lungenfunktion mit einem erniedrigten Horovitz-Quotienten [189].

Die Bedeutung von PAF bei der Lipidinfusions-bedingten Verschlechterung der Lungenfunktion wird durch diese Versuche mit PAF-Rezeptor-knockout Mäusen noch untermauert. Durch die Verwendung dieses PAF-Rezeptor-defizienten Stammes konnte gezeigt werden, dass eine intratracheale LPS-Instillation eine vergleichbare pulmonale Leukozytenrekrutierung sowie eine ähnliche TNF-α– und MIP-2- Synthese wie in Wildtyp Mäusen hervorruft (***Anlage 3***). Dieses Phänomen wurde auch schon beschrieben [176]. Die Verschlechterung der pulmonalen Inflammation durch die n-6 Lipide und die Verbesserung durch die Fischöl-basierten Emulsionen war jedoch bei den PAF-Rezeptor-knockout Mäusen nicht nachweisbar. Diese Ergebnisse wurden durch die Behandlung von Wildtyp Mäusen mit einem PAF-Rezeptor Antagonisten bestätigt. Durch die Verwendung von BN52021 bei Wildtyp Mäusen konnte der differentielle Effekt der n-3 vs. n-6 Lipide auf die pulmonale Leukozytenrekrutierung und die Zytokin-Synthese wie bei den PAF-R -/- Mäusen aufgehoben werden. Daher kann man schlussfolgern, dass der negative Einfluss der konventionellen Lipidemulsionen und die positive Wirkung der Fischöl-basierten Lipidemulsionen auf die Inflammation und die Lungenschädigung zum Großteil von der Integrität der PAF - PAF-Rezeptor Signaltransduktion in Mäusen abhängen.

Neueste Veröffentlichungen zeigen, dass auch eine orale auf n-3 Fettsäuren basierte Diät protektiv im Sepsis-Modell der Maus sein kann. Es wurde insbesondere auf die Reduktion pro-inflammatorischer Zytokine und des oxidativen Stresses sowie auf die Hemmung der Autophagie, eines Mechanismus mithilfe dessen die Zelle geschädigte Organellen recycelt, hingewiesen. Allerdings war der Diät auch Molkeprotein zugesetzt [190]. Andererseits wurde bei asthmatischen Ratten kein Effekt von oralen n-3 Lipiden auf die Bioaktivität von PAF im Lungengewebe gefunden [191]. Das steht im Gegensatz zu den in der vorliegenden Studie gefundenen Daten, aber in der hier vorgestellten Studie wurden auch die Lipide intravenös appliziert.

Die Gruppe der SPMs (*specialized pro-resoliving mediators*) gehört ebenfalls zu den Lipidmediatoren, die von PUFA (*polyunsaturated fatty acids*) abgeleitet werden, wie von Dalli und Serhan in der Übersichtsarbeit dargestellt [192]. Es ist mittlerweile ein anerkanntes Faktum, dass die Auflösung der Entzündung ein aktiver zellulärer und biochemischer Prozess ist, der durch SPMs orchestriert wird. Prostaglandine, die in die Initialphase der Entzündung involviert sind (z.B. PGE2 und PGD2) führen zu einer Umschaltung der Lipidmediator-Klasse, indem sie die Translation von mRNAs aktivieren, die Enzyme (z.B. 15-LOX Typ I) für die Produktion von SPMs kodieren, die während der Auflösungs-Phase der Entzündung aktiv sind. SPMs inhibieren pro-inflammatorische Mediatoren wie PGs, LTs und bestimmte Zytokine. Anti-inflammatorisch im Sinne von „immunsuppressiv“ wirken die SPMs jedoch nicht. SPMs lösen die Entzündung auf. Zwei Hauptkennzeichen der Entzündungs-Auflösung (*Resolution*) ist die Hemmung des PMN-Influx und die Stimulation der Makrophagen, apoptotische PMN zu phagozytieren [193]. Dass ein Teil dieser an der Auflösung der Inflammation beteiligten SPMs in der vorliegenden Studie eine Rolle spielen, ist nicht auszuschließen.

Lipoxine (LX) (*lipoxygenase interaction products*) der 4er Serie sind von n-6 Fettsäuren abgeleitete SPM, welche zunächst pro-inflammatorisch (Steigerung der Sauerstoffradikalbildung, Förderung der Degranulation), beschrieben wurden, sich dann aber doch als anti-inflammatorische Mediatoren darstellten. Lipoxine gibt es auch in einer durch Aspirin getriggerten Form (AT-LX) [194, 195]. Da in der vorliegenden Studie die auf n-6 Fettsäuren-basierende Lipidinfusion keine dominant anti-

inflammatorische Auswirkung hatte, ist die Relevanz der Lipoxine der 4er Serie hier wahrscheinlich zu vernachlässigen. Allerdings sind die Lipoxine der 5er Serie von EPA abgeleitet [196] und könnten in der vorliegenden Studie als anti-inflammatorische Komponente eine Rolle spielen. Da Lipoxin A5 und B5 jedoch als Mediator für Erkrankungen unwahrscheinliche Kandidaten sind [197], sind die EPA-abgeleiteten Lipoxine wohl in der realen Inflammation eher zu vernachlässigen. Die Gruppe der Resolvine (*resolution phase interaction products*), Protektine und Maresine stellen eine weitere interessante Fraktion der SPM dar. Resolvine der D-Serie gibt es auch in der Aspirin-getriggerten Form (AT-RvD), ebenso wie Protectin D1 (AT-PD1)[198, 199]. Protectine, Maresine (MaR) (*macrophage mediators in resoving inflammation*) und Resolvine der D-Serie (RvD) sind Polyhydroxyl-Derivate von DHA, Resolvine der E-Serie (RvE) von EPA [200-202]. Die von der Docosapentaensäure (DPA) abgeleiteten Resolvine (RvTs und $RvDs_{n\text{-}3DPA}$) [39, 203] dürften in der vorliegenden Studie keine Rolle spielen, da DPA nicht verwendet wird. Es ist durchaus möglich, dass in der durchgeführten Studie ein Teil der anti-inflammatorischen Potenz der auf n-3 Fettsäuren-basierenden Lipidinfusionen durch Maresine oder Resolvine bedingt ist. Dies ist in weiteren Untersuchungen zu evaluieren. Protectine sind in neuronalem Gewebe und Eosinophilen wirksam, wie in dieser Übersichtsarbeit von Dalli und Serhan dargestellt [192]. Sie sind für die hier vorgestellte Arbeit daher wahrscheinlich nicht von Bedeutung. Da Mar1 und RvE1 primär die Geweberegeneration fördern, RvD1, RvD2, RvD5 und RvE1 hauptsächlich an der Auflösung viraler Infektionen und der Apoptose-Induktion bei Neutrophilen beteiligt sind, fallen diese Mediatoren bei der vorliegenden Studie eher nicht ins Gewicht. Maresine regulieren die Produktion von LTB4 über die direkte Inaktivierung der LTA4 Hydrolase. Außerdem kommt es zu einer Veränderung des Phänotypen bei Makrophagen [204]. Daher können 13S,14S-eMaR oder Mar2 in der vorliegenden Studie schon eine Rolle spielen. Dass DHA in Studien häufig eine potentere anti-inflammatorische Wirkung hat, als EPA [67] ***(Anlage 7)*** [205, 206], könnte unter anderem an der größeren Anzahl von DHA-abgeleiteten SPMs liegen.

Die MCTR (*maresin conjugates in tissue regeneration)*, [207] Sulfid-konjugierte Mediatoren, die bei der Auflösung von E.Coli Infektionen entstehen und die Reparatur und den Verschluss von Wunden fördern, können hinsichtlich der hier erhobenen Ergebnisse ebenfalls wahrscheinlich nicht ausschlaggebend sein. Desgleichen gilt für die PCTR (*protectin conjugates in tissue regeneration*) und die RCTR (*resolvin conjugates in tissue regeneration).* [202, 208].

Unter septischen Bedingungen findet eine verstärkte Lipolyse statt, so dass mehr freie Fettsäuren aus den jeweils vorhanden Fetten entstehen. Freie Fettsäuren können die Inflammation noch über *Toll-like Receptors* (TLRs) und über die *fatty acid translocase* (FAT/CD36) beeinflussen[209-212]. Hier ist interessant, dass die Umschaltung von der frühen akuten Initialphase der Sepsis zur späten Antwort des adaptiven Immunsystems nach TLR4 Stimulation von der Deacetylase Sirtuin(Sir) T1 abhängt. Zwischen der initialen Sepsis-Phase und der späten Phase kommt es zu einem Wechsel von der Glukose- zur Fettsäure-Oxidation, die von SirT6 und SirT1 abhängt. Somit ist der Energiestoffwechsel eng mit der akuten Inflammation verbunden [211]. Das könnte ebenfalls für die Entwicklung neuer Therapiekonzepte in der Sepsis – auch hinsichtlich der hier gefundenen Effekte der Lipidemulsionen - relevant sein.

Bei Patienten, die eine Sepsis nicht überlebt haben, wurde in einer Studie sowohl höhere Konzentrationen der Inflammations-initiierenden Mediatoren wie PGF2α und LTB4 als auch der Inflammations-auflösenden Mediatoren wie RvE1 und RvD5 gefunden, als bei Patienten, die die Sepsis überlebt haben. Höhere Konzentrationen der Inflammation-initiierenden Mediatoren (einschließlich PGF2α) und einzelne Inflammations-auflösende Mediatoren waren mit der Entwicklung eines ARDS assoziiert [40]. Auch bei der bakteriellen Pneumonie konnten SPMs als regulierende Faktoren gefunden werden. Metabololipidomische Profile könnten bei diagnostischen und therapeutischen Überlegungen zu Rate gezogen werden [213, 214]. In der Folge könnten so im peripheren Blut neue Lipid-Biomarker bei der Diagnostik und Therapie der Sepsis entwickelt werden. Dies ist bei zukünftigen Studien dieser Art zu berücksichtigen.

Neueste Untersuchungen zeigen einen interessanten Zusammenhang zwischen dem genetischen Hintergrund (z.B. dem APOE-Genotyp) und der Konzentration bestimmter Fettsäuren im Blut. Auch der BMI (*Body Mass Index*) und das Alter spielen dabei eine Rolle [215, 216]. Die in der vorliegenden Studie verwendeten Mäuse waren bezüglich Alter und Gewicht homogen. Allerdings

ist beispielsweise der APOE-Genotyp-Polymorphismus nicht bekannt und kann somit nicht evaluiert werden.

Zusammenfassend konnte durch diese Studie somit erstmalig im Tiermodell mit PAF-Rezeptor -/- Mäusen gezeigt werden, dass (a) Langzeit-Infusionen mit n-3 basierten Lipidemulsionen zu einer Reduktion der LPS-induzierten pro-inflammatorischen Zytokine, der alveolären Leukozytentransmigration und des *protein leakage* führen, und dass (b) die anti-inflammatorische Potenz der n-3 Lipide in der LPS-induzierten murinen Inflammation abhängig ist von der intakten PAF - PAF-Rezeptor Signaltransduktion.

Daher konnten in dieser Studie die Fragestellungen wie folgt beantwortet werden:

3.1. Die inflammatorische Stimulation von Wildtyp (WT) Mäusen und PAF-Rezeptor-knockout (PAF-KO) Mäusen mit LPS intratracheal (i.t.) bzw. intraperitoneal (i.p.) führt zu einer vergleichbaren inflammatorischen Antwort gemessen an der

a. alveolärer Leukozyteninvasion

b. MPO-Aktivität im Lungengewebe

c. alveoläre Protein-Leckage

d. TNF-α Konzentration im Alveolarraum

e. MIP-2 Konzentration im Alveolarraum

f. TNF-α Konzentration im Plasma

g. MIP-2 Konzentration im Plasma.

3.2. Eine intravenöse Langzeit-Infusion mit n-3 bzw. n-6 basierten Lipidemulsionen modelliert in diesem Setting diese inflammatorische Antwort unterschiedlich mit einem deutlichen anti-inflammatorischem Potential der n-3 Lipidemulsionen bei WT Mäusen.

3.3. Eine intravenöse Langzeit-Infusion mit n-3 bzw. n-6 basierten Lipidemulsionen modelliert in diesem Setting diese inflammatorische Antwort nicht signifikant bei PAF-KO Mäusen.

3.4. Eine intravenöse Langzeit-Infusion mit n-3 bzw. n-6 basierten Lipidemulsionen in diesem Setting modelliert diese inflammatorische Antwort nicht signifikant bei WT Mäusen nach Applikation eines PAF-Rezeptor-Antagonisten.

3.0 Im Modell der murinen akuten pulmonalen bzw. systemischen Inflammation ist die anti-inflammatorische Wirkung einer Langzeit-Infusion mit n-3 basierten Lipidemulsionen abhängig vom der Präsenz eines funktionalen PAF-Rezeptors.

Folglich wurde die Frage positiv beantwortet, ob n-3 basierte Lipidinfusionen im Vergleich mit n-6 basierten Lipidinfusionen die akute inflammatorisch bedingte Lungenschädigung und die systemische Inflammation unterschiedlich beeinflussen.

Des Weiteren konnte ich die Frage positiv beantworten, dass der Lipidmediator PAF für die Wirkung der n-3 Lipide auf die inflammatorische akute Lungenschädigung und die systemische Inflammation relevant ist.

Die Hypothese, dass die Lipid-basierte Signaltransduktion die Inflammation im Bereich der akuten Lungenschädigung und der systemischen Inflammation beeinflussen können, wurde bestätigt.

Ebenso konnte ich die Hypothese verifizieren, dass die Lipid-basierte Signaltransduktion die Inflammation mittels eines Lipidmediators wie PAF modulieren kann.

Die Nutzung von n-3 basierten Lipidinfusionen bei Intensiv-pflichtigen Patienten mit einer akuten hyper-inflammatorischen Erkrankung wie Sepsis oder akute Lungenschädigung bzw. ARDS könnte somit eine adjuvante anti-inflammatorische Therapie darstellen.

Beeinflussung der Inflammation in vitro *durch n-3/n-6 Fettsäuren in Abhängigkeit von der PI3-Kinase: Bedeutung für Adhäsion und Apoptose*

Eine weitere Hypothese war, dass die Lipid-basierte Signaltransduktion die Inflammation mittels Lipid-abhängiger Kinasen wie der PI3-Kinase beeinflussen kann.

Folglich wollte ich in dieser Studie der Frage nachgehen, ob n-3 vs. n-6 Fettsäuren die endotheliale PI3-Kinase in der Inflammation derart modulieren, dass dies Auswirkungen auf Adhäsion und Apoptose hat.

Daraus ergaben sich folgende operativen Fragestellungen für die Durchführung dieser Studie:

4.1. Beeinflussen eine Präinkubation mit AA vs. DHA und TNF-alpha-Stimulation von HUVEC

a. das Rolling

b. die Adhäsion bzw.

c. die Transmigration

von Monozyten unterschiedlich?

4.2. Beeinflussen eine Präinkubation mit AA vs. DHA und TNF-alpha-Stimulation von HUVEC die Expression von

a. VCAM-1

b. ICAM-1 bzw.

c. E-Selektin

auf HUVEC differentiell ?

4.3. Beeinflussen eine Präinkubation mit AA vs. DHA und eine TNF-alpha- bzw. VEGF- und STS-Stimulation von HUVEC

a. die Phosphorylierung von Akt

b. die Caspase-3-Aktivität bzw.

c. die Annexin-V-Expression

bei HUVEC verschieden?

4.4. Beeinflussen eine Präinkubation mit AA vs. DHA und TNF-alpha-Stimulation von HUVEC nach Applikation der PI3-Kinase-Inhiboren LY294002 bzw. Wortmannin

a. Rolling

b. Adhäsion bzw.

c. Transmigration

von Monozyten divergent?

4.0 Ist die Wirkung der n-3 vs. n-6 Fettsäuren auf die inflammatorisch aktivierte monozytäre-endotheliale Adhäsion und auf die Apoptose abhängig von der PI3-Kinase?

Die Fähigkeit der n-3/n-6 Fettsäuren, über den Einbau in spezielle Membranphospholipide die nachgeschaltete Signaltransduktion zu beeinflussen, wurde aus vorherigen Arbeiten ersichtlich. Daher stellte sich nun die Frage, inwieweit die Fettsäuren durch die PI3-Kinase-abhängige Signaltransduktion in der Lage sind, sowohl die Induktion der Inflammation als auch die Resolution der Inflammation zu modulieren (***Anlage 4***)[217].

Dazu standen hier die Endothelzelle und deren adhäsiven Interaktionen mit humanen Monozyten sowie die endotheliale Apoptose im Zentrum der Untersuchungen. In dieser Studie wurde folglich der Einfluss von DHA versus AA auf *Rolling* und Adhäsion mittels einer *Parallel-Flow-Chamber* und auf die Transmigrationen mittels eines statischen *Transwell Assays* untersucht. DHA, im Gegensatz zu AA, reduzierte monozytäres Rolling, Adhäsion und Transmigration durch einen TNF-α-aktivierten endothelialen Monolayer (Abb. ***Anlage 4***). Diese divergierenden Effekte der beiden Fettsäuren waren von der Expression endothelialer Adhäsionsmoleküle unabhängig, konnten aber nach Applikation eines PI3-Kinase Inhibitors nicht mehr nachgewiesen werden (Abb. ***Anlage 4***). Weitere Untersuchungen zur Fettsäure-Abhängigkeit der Signaltransduktion der PI3-Kinase zeigten, dass eine Vorinkubation der HUVEC mit DHA verglichen mit AA die Phosphorylierung von Akt nach Stimulation mit VEGF-, TNF-α-, und Staurosporin (STS) deutlich reduzierte (Abb. ***Anlage 4***). Als nächstes konnte nachgewiesen werden, dass DHA die STS-induzierte endotheliale Apoptose – gemessen mit zwei unabhängigen Methoden – steigerte (***Anlage 4***).

Dass Fettsäuren die leukozytären-endothelialen adhäsiven Interaktionen modulieren, war bekannt [67, 68, 180]. Diese Studie demonstriert, dass eine Vorinkubation der Endothelzellen mit DHA, im Vergleich zu einer Vorinkubation mit AA, eine hemmende Wirkung auf das monozytäre *Rolling*, die monozytäre-endotheliale Adhäsion und die monozytäre Transmigration durch TNF-α-aktivierte HUVECs hat. Das steht in Einklang mit vorhergehenden Publikationen, die sich mit der Rolle der n-3 Fettsäuren bei der leukozytären-

endothelialen Interaktion befassen [67, 68, 180], obwohl die Reduktion der Transmigration durch DHA in diesem Ansatz ein Novum darstellt. Bei Verwendung von physiologischen Konzentrationen von n-3 Fettsäuren konnte kein Unterschied in der Expression der endothelialen Adhäsionsmoleküle (ICAM-1, VCAM-1, E-Selektin) gefunden werden, was auch schon vorher gezeigt werden konnte (***Anlage 7***) [67]. Im Gegensatz dazu fanden andere Untersucher eine reduzierte Expression endothelialer Adhäsionsmoleküle nach Exposition endothelialer Zellen mit n-3 Fettsäuren [68, 180]. Jedoch führten diese Untersucher ihre Studien unter anderen Bedingungen durch. So wurden beispielsweise - verglichen mit den von uns eingesetzten physiologischeren Konzentrationen (10 µmol/l) - höhere Fettsäure-Konzentrationen (bis zu 300 µmol/l) verwendet und längere Inkubationszeiten vorgenommen. Die fehlende differentielle Regulation der Adhäsionsmoleküle durch DHA oder AA passt jedoch auch zu der fehlenden Modulation der monozytären Adhäsionsmoleküle in einer Studie mit freiwilligen Probanden, die entweder eine Fischöl-basierte Lipidinfusion oder eine Sojabohnenöl-basierte Lipidinfusion erhielten. Trotz deutlicher Änderungen der adhäsiven Eigenschaften wurde bei den Monozyten dieser Probanden keine differentielle Regulation der Adhäsionsmoleküle festgestellt [218].

Die Mechanismen, die dem protektivem Effekt der n-3 Fettsäuren auf das Rolling, die Adhäsion, und die Transmigration zugrunde liegen, können – wie schon erwähnt - vielfältig sein [56, 67, 69, 219]. Um die Auswirkung der Zusammensetzung der Zellmembran auf die PI3-Kinase-abhängigen Effekte zu untersuchen, und um die Effekte der klassischen Eicosanoide vernachlässigen zu können, wurde DHA (und nicht EPA) als n-3 Fettsäure verwendet. In publizierten Studien konnte auch gezeigt werden, dass die Zugabe von DHA in Endothelzellen unter inflammatorischen Bedingungen den EPA-Gehalt der Zellmembran nicht verändert [56].

In vorhergehenden Studien konnte dargelegt werden, dass Fettsäuren Einfluss auf die Signaltransduktion der Membranphospholipide haben, die für die inflammatorisch bedingte Adhäsion relevant ist [56, 67]. Da die PI3-Kinase für ihre Fähigkeit bekannt ist, das Zytoskelett zu modulieren, die Integrine zu beeinflussen sowie Auswirkungen auf die leukozytäre Adhäsion und Transmigration zu haben [220], wurde der Einfluss der PI3-Kinase auf die Fettsäure-induzierte Modulation der Adhäsionskaskade in der Inflammation untersucht. Durch den PI3-Kinase/Akt/NO - Signaltransduktionsweg kann die ICAM-1-Expression hochreguliert werden. Das führt zur Endothelzellmigration [91]. Fettsäuren können über die Inkorporation in die Membran-Phospholipid-Pools die Signaltransduktion der PI3-Kinase durch ihr Substrat Phosphatidyl-Inositol beeinflussen [56, 69, 186]. Um den Effekt der PI3-Kinase und der Fettsäuren auf die Adhäsionskaskade zu untersuchen, wurden die Endothelzellen mit zwei verschiedenen PI3-Kinase-Inhibitoren vorbehandelt: Wortmannin und LY294002. Nach Verwendung der PI3-Kinase-Inhibitoren war der divergente Effekt der Vorinkubation mit AA und DHA auf das Rolling, die Adhäsion und die Transmigration der Monozyten nicht mehr vorhanden (Abb. ***Anlage 4***).

Die klassische PI3-Kinase-Signaltransduktion, d.h. die Phosphorylierung von Akt und die Apoptose, wurde in der Folge auf eine Fettsäure-Abhängigkeit untersucht. Nach Stimulation der HUVEC mit VEGF wurde eine signifikante Steigerung der Akt-Phosphorylierung festgestellt. Nach DHA-Vorinkubation der HUVEC fand jedoch eine Reduktion der Akt-Phosphorylierung statt, wenn man VEGF als hochspezifischen Stimulus für die Aktivierung des PI3-Kinase-abhängigen Signaltransduktionsweges verwendete. Die Reduktion der Akt-Phosphorylierung nach Vorinkubation der HUVEC mit DHA war im Vergleich zu AA signifikant. Setzte man TNF-α als generellen inflammatorischen Stimulus ein, wurde ebenfalls eine Steigerung der Akt-Phosphorylierung beobachtet (Abb. ***Anlage 4***). Nach Vorinkubation mit DHA zeigte sich jedoch wieder eine Hemmung der Akt-Phosphorylierung im Gegensatz zu AA. Staurosporin als klassischer Apoptose-Stimulus führte zu einer Hochregulation der Akt-Phosphorylierung. Unter diesen Bedingungen führte AA zu einem leichten Anstieg während DHA eine signifikante Reduktion von phospho-Akt induzierte (Abb. ***Anlage 4***). Die Tatsache, dass DHA und n-3 Fettsäuren im Allgemeinen die Phosphorylierung von Akt hemmen und dadurch einen Einfluss auf die Inflammation und Apoptose haben, wurde auch von anderen berichtet [221-223]. Die in dieser Studie gezeigte Steigerung der Apoptose in Endothelzellen durch Hemmung der Akt-Phosphorylierung durch DHA im Vergleich zu

AA sowie die Tatsache, dass DHA in Abhängigkeit von der PI3-Kinase anti-inflammatorisch und pro-apoptotisch in HUVEC wirkt, ist in diesem Versuchsansatz neu und erstmalig in dieser Studie gezeigt. Die Ergebnisse stehen jedoch in Einklang mit den publizierten Daten über den pro-apoptotischen DHA-Effekt in proliferierenden Endothelzellen [224]. Das Ergebnis dieser Studie steht jedoch im Gegensatz zu der berichteten anti-apoptotischen Rolle von DHA in der Stress-induzierten Apoptose durch die gleiche Gruppe [225]. Allerdings wurde die Signaltransduktion der PI3-Kinase und ihr Einfluss auf die Apoptose von dieser Gruppe nicht bestimmt [224, 225]. Des Weiteren betonen einige Veröffentlichungen die Rolle der Apoptose bei der Auflösung, der Resolution, der Inflammation [226, 227]. Möglicherweise können Fettsäuren nicht nur die Induktion der Inflammation, sondern auch die Resolution der Inflammation durch die Beeinflussung der Apoptose von Entzündungszellen modulieren. Das könnte zur Prävention einer chronischer Entzündung beitragen [228] und steht möglicherweise in Zusammenhang mit der Bildung der Resolvine, die von n-3 Fettsäuren abgeleitet werden, aber in dieser Studie nicht untersucht wurden [229]. Natürlich kann die Wirkung der n-3 Fettsäuren auf die Apoptose auch abhängig vom verwendeten Stimulus und dem Versuchsaufbau variieren, wie unterschiedliche Berichte aus der gleichen Gruppe verdeutlichen [224, 225]. Andere Gruppen zeigten, dass freie Fettsäuren, die bei akuten inflammatorischen Zuständen durch gesteigerte Lipolyse aus den jeweils vorhanden Fettsäuren vermehrt freigesetzt werden, über *toll-like receptors* (TLRs) die Phosphorylierung von AKT und nachgeschaltete NFkappaB-Aktivierung und COX-2-Expression beeinflussen [209, 210, 230]. Bezüglich der therapeutischen Relevanz könnte die Bildung von Kollateralen bei Koronarer Herzkrankheit durch endotheliale Proliferation einen wünschenswerten Effekt darstellen. Im Übrigen könnte die apoptotische Wirkung von DHA im zentralen Nervensystem, insbesondere im Gehirn und in der Retina, die reich an DHA sind, im Status der Inflammation sogar gefährlich sein. Weitere Untersuchungen sind notwendig, um die unterschiedlichen Effekte und Aspekte hinsichtlich einer chronischen bzw. einer akuten Inflammation zu evaluieren.

Neueste Untersuchungen zeigen, dass n-3 Fettsäure-abgeleitete SPM Inflammasome beeinflussen können [231]. Das Inflammasom ist eine intrazelluläre molekulare Maschinerie, die die inflammatorische Antwort initiiert. Besonders das Inflammasom *nucleotide-binding domain leucine-rich repeat-containing protein 3* (NLRP3), das aus einem proteolytischen Komplex (NLRP3), dem Adaptor-Protein ASC (*apoptosis-associated speck-like protein*) - dass eine *caspase activation and recruitment domain* (CARD) enthält - und aus der Caspase-1 besteht, ist gut charakterisiert [232]. Inflammasome sind an der *innate immune surveillance* beteiligt, indem sie mikrobielle Produkte detektieren. Sie aktivieren die Caspase-1 (CASP1), die wiederum die Zytokine IL-1ß und IL18 aktiviert und einen lytischen Zelltod initiiert, der *„Pyroptosis“* genannt wird. Von Moltke et al. zeigten, dass eine Inflammasom-Aktivierung in einem “Eicosanoid-Sturm” (bestehend aus PGE2 und LTB4) mündet, einer von der Calcium-abhängigen cytosolischen Phospholipase C (PLA2)-abhängigen pathologischen Freisetzung von Lipidmediatoren, die zu einer schnellen Initiierung der Inflammation führt [233-235]. Andererseits wurde auch publiziert, dass SPMs zu einer Hemmung von Inflammasomen in Makrophagen führen [236]. Die der PI3-Kinase vorgeschalteten Phosphatidylinositole modulieren ebenfalls die Inflammasom-Signaltransduktion [237]. Somit ist in der Zusammenschau davon auszugehen, dass in dieser Studie von n-3 Fettsäuren abgeleitete SPMs auch potentiell hier zu einer Hemmung der Inflammasom-Signaltransduktion führen können und dieser Signaltransduktionsweg mit zu der festgestellten Reduktion der Inflammation durch n-3 Fettsäuren beitragen kann. Damit wären die PI3-Kinase-abhängige Signaltransduktion und die Inflammasom-abhängige Signaltransduktion möglicherweise in die Rückkopplungsschleife der SPMs eingebunden.

Zusammenfassend lässt sich feststellen, dass eine DHA-Vorinkubation im Vergleich zu AA – unabhängig vom verwendeten Stimulus (Wachstumsfaktor, inflammatorischer Stimulus, oder klassischer Apoptose-Stimulus) immer zu einer deutlich reduzierten PI3-Kinase-abhängigen Akt-Phosphorylierung führte. Die der PI3-Kinase und Akt nachgeschalteten Apoptose – gemessen mittels Annexin-V- und Caspase-3-Analyse – zeigte

eine pro-apoptotische Wirkung von DHA auf die Endothelzellen verglichen mit AA.

Folglich konnte in der vorliegenden Studie in diesem Setting gezeigt werden, dass die n-3 Fettsäure DHA durch die PI3-Kinase-abhängige Signaltransduktion in der Lage ist, sowohl die Induktion der Inflammation (Rolling, Adhäsion, Transmigration) als auch die Resolution der Inflammation durch Hemmung der Phosphorylierung von Akt und Steigerung der Apoptose, zu modulieren. Somit konnte das Wissen über die Mechanismen, durch die DHA die Kinase-abhängige Signaltransduktion in der Induktion und der Resolution der Entzündung beeinflusst, vertieft werden.

In dieser Studie fand ich somit folgende konkrete Antworten auf meine Fragen:

4.1. Eine Präinkubation mit AA vs. DHA und TNF-alpha-Stimulation von HUVEC beeinflusst

a. das Rolling

b. die Adhäsion bzw.

c. die Transmigration

von Monozyten unterschiedlich, mit einer Reduktion durch DHA.

4.2. Eine Präinkubation mit AA vs. DHA und TNF-alpha-Stimulation von HUVEC beeinflusst die Expression von

a. VCAM-1

b. ICAM-1 bzw.

c. E-Selektin

auf HUVEC nicht differentiell.

4.3. Eine Präinkubation mit AA vs. DHA und TNF-alpha- bzw. VEGF- und STS-Stimulation von HUVEC beeinflusst

a. die Phosphorylierung von Akt

b. die Caspase-3-Aktivität bzw.

c. die Annexin-V-Expression

bei HUVEC verschieden, mit einer Hemmung der Phosphorylierung von Akt und einer Steigerung bei Caspase-3 und Annexin-V durch DHA.

4.4. Eine Präinkubation mit AA vs. DHA und TNF-alpha-Stimulation von HUVEC nach Applikation der PI3-Kinase-Inhiboren LY294002 bzw. Wortmannin beeinflusst

a. Rolling

b. Adhäsion bzw.

c. Transmigration

von Monozyten nicht mehr divergent.

4.0 Die Wirkung der n-3/n-6 Fettsäuren auf die inflammatorisch aktivierte monozytäre-endotheliale Adhäsion und auf die Apoptose ist abhängig von der PI3-Kinase.

Die Hypothese, dass die Lipid-basierte Signaltransduktion die Inflammation mittels Lipid-abhängiger Kinasen wie der PI3-Kinase beeinflussen können, konnte somit verifiziert werden.

Folglich wurde in dieser Studie die Frage positiv beantwortet, dass n-3/n-6 Lipide in Form von Fettsäuren die endotheliale PI3-Kinase in der Inflammation derart modulieren, dass dies Auswirkungen auf Adhäsion und Apoptose hat.

Die Hypothese, dass die Lipid-basierte Signaltransduktion die Inflammation auf der Ebene der endothelialen-leukozytären Interaktion beeinflusst, konnte ich in dieser Studie ebenfalls bestätigen.

Auch war ich in der Lage, die Frage, ob bei der endothelialen-leukozytären inflammatorischen Adhäsion Signaltransduktionswege von Bedeutung sind, die durch die Lipid-basierte Signaltransduktion moduliert werden können, positiv zu beantworten.

Die Signaltransduktion der PI3-Kinase scheint für einen Teil der anti-inflammatorischen Wirkung der n-3 Fettsäure DHA von Bedeutung zu sein. Dies könnte bei der Erforschung der Pathophysiologie von Therapieansätzen in Bezug auf inflammatorischen Erkrankungen wie Sepsis oder akute Lungenschädigung bzw. ARDS hilfreich sein.

Beeinflussung der Inflammation in vitro *durch n-3/n-6 Fettsäuren in Abhängigkeit von den Lipid Rafts*

Angesichts des Potentials der Fettsäuren, die Membran-Phospholipid-Pools zu modulieren, wurde nun der Hypothese nachgegangen, dass sie auch Zellmembran-Komponenten wie Lipid Rafts und deren Signaltransduktion in der Inflammation beeinflussen können. Diese Hypothese beruhte zum einen darauf, dass die mehrfach ungesättigten Fettsäuren potentiell mit den an gesättigten Fettsäuren reichen Lipid Rafts interferieren könnten und zum anderen auf deren Fähigkeit, die Palmitoylierung von Proteinen zu beeinflussen und somit die Integration von Proteinen in Lipid Rafts im inflammatorischen Prozess zu modulieren.

Folglich wollte ich in dieser Studie die Frage beantworten, ob monozytäre Lipid Rafts in der Zellmembran signifikant von n-3/n-6 Lipiden beeinflusst werden, so dass die Calcium-Signaltransduktion und die Adhäsion in der Inflammation moduliert werden.

Dazu ergaben sich als konkrete Fragestellungen für die Durchführung dieser Studie:

5.1. Beeinflusst eine Präinkubation mit AA vs. DHA und TNF-Stimulation die Zusammensetzung der

a. Lipid Rafts Fraktion in U937 Monozyten

b. Non-Raft Fraktion in U937 Monozyten

c. Lipid Raft Fraktion in humanen Monozyten

d. Non-Raft Fraktion in humanen Monozyten?

5.2. Beeinflusst eine Präinkubation mit AA vs. DHA und TNF-Stimulation

a. die statische Adhäsion von U937 Monozyten

b. die dynamische Adhäsion von humanen Monozyten

c. das Rolling von humanen Monozyten?

5.3. Beeinflusst eine Präinkubation mit AA vs. DHA und TNF-Stimulation

a. die Expression von Adhäsionsmolekülen (CD11b, CD18, CD49d) auf U937 Monozyten?

b. die Expression von Adhäsionsmolekülen (CD11b, CD18, CD49d) auf humanen Monozyten?

5.4. Wie wirkt sich die Applikation eines Calcium-Chelators nach Präinkubation mit AA vs. DHA und TNF-Stimulation aus auf

a. die statische Adhäsion von U937 Monozyten

b. die dynamische Adhäsion von humanen Monozyten?

5.5. Wie ist das intrazelluläre Calcium nach Präinkubation mit AA vs. DHA und Stimulation mit TNF

a. in U937 Monozyten

b. in humanen Monozyten?

5.6. Wie wirkt sich eine Zerstörung der Lipid Rafts mittels MCD aus auf

a. die Adhäsion von U397 Monozyten

b. die Adhäsion von humanen Monozyten

c. das Rolling von humanen Monozyten

d. Calcium in U937 Monozyten

e. Calcium in humanen Monozyten

5.0 Sind monozytäre Lipid Rafts in der Inflammation relevant für die Fettsäure-bedingte Modulation des Calciums und der Adhäsion?

Im Zentrum des Interesses standen hier die Monozyten, ihr Calcium-*Signalling* und deren adhäsive Interaktionen mit Endothelzellen (***Anlage 5***)[238]. Es konnte gezeigt werden, dass die Vorinkubation mit AA und DHA die Zusammensetzung monozytärer Lipid Rafts effektiv moduliert. Der Einbau von DHA und AA erfolgte, wie die Analyse der Raft- und der Non-Raft-Membranfraktion zeigte, verstärkt in der Raft- und weniger in der Non-Raft-Fraktion. Dies war sowohl bei der monozytären Tumorzelllinie U937 als auch bei frisch isolierten humanen Monozyten aus Spenderblut der Fall. Dabei kam es insbesondere zu einem imposanten Anstieg von DHA nach entsprechender Vorinkubation (Abb. ***Anlage 5***). Die Adhäsion der U937-Monozyten an HUVEC sowie die Adhäsion und das Rolling von humanen Monozyten an HUVEC wurde durch die n-3 und die n-6 Fettsäuren differentiell beeinflusst (Abb. ***Anlage 5***). Nach einer Vorinkubation der Monozyten mit AA und Stimulation mit 1 ng/ml TNF-α zeigte sich ein signifikanter Unterschied in der Ausprägung der Adhäsion verglichen mit DHA. Bei Einsatz von 10 ng/ml TNF-α steigerte AA die Adhäsion nicht weiter und DHA führte nur zu einer

geringeren Reduktion der Adhäsion. Da der Schwerpunkt darauf lag, Fettsäure-induzierte Modulationen zu untersuchen, wurden die Experimente mit 1 ng/ml TNF-α fortgeführt. Die Analyse der monozytären Adhäsionsmoleküle CD11b, CD18, und CD49d ergab in der quantitativen Auswertung sowohl der U937 als auch der humanen Monozyten keine signifikante Änderung durch die Fettsäure-Vorinkubation (***Anlage 5***). Daher konnte ein Einfluss von AA oder DHA auf die Expression der Adhäsionsmoleküle unter diesen Versuchsbedingungen ausgeschlossen werden.

Es stellte sich heraus, dass Calcium für die Fettsäure-induzierten Veränderung der Adhäsion maßgeblich verantwortlich war. Sowohl bei U937 als auch bei humanen Monozyten wurde nach Stimulation mit TNF-α das intrazelluläre Calcium durch eine Vorinkubation mit DHA reduziert (Abb. **Anlage 5**). Nach Vorinkubation mit dem Calcium-Chelator BAPTA war die differentielle Modulation durch die Fettsäuren nicht mehr nachweisbar (***Anlage 5***). Dazu kam, dass gezeigt werden konnte, dass eine Zerstörung der monozytären Lipid Rafts mit Methyl-β-Cyclodextrin (MCD) sowohl die Fettsäure-induzierte Modulation des intrazellulären Calciums als auch die Fettsäure-bedingte differentielle Beeinflussung der monozytären-endothelialen Adhäsion und des Rolling aufhob (Abb. ***Anlage 5***).

Daher kann man schlussfolgern, dass die Modulation der monozytären-endothelialen Adhäsion und des intrazellulären Calciums durch die n-3 und n-6 Fettsäuren zumindest zum Teil von Lipid Rafts und deren Zusammensetzung abhängig sind.

Es konnte nachgewiesen werden, dass mehrfach ungesättigte Fettsäuren wie AA und DHA ohne weiteres in Lipid Rafts inkorporiert werden; ein Phänomen, das für Monozyten bisher noch nicht beschrieben wurde. Da Lipid Rafts dadurch charakterisiert sind, dass sie reich an gesättigten Fettsäuren sind, widerspricht dieses Phänomen gängigen Vorstellungen, die allerdings vornehmlich auf der Analyse von Lymphozyten beruhen [93, 94]. Eine Erklärung dafür könnte sein, dass sich die Lipid Rafts der T-Zellen in ihrer Fähigkeit, mehrfach ungesättigte Fettsäuren zu inkorporieren von denen der monozytären Lipid Rafts unterscheiden. Eine andere Erklärung stellt die These dar, dass die erhöhte Verfügbarkeit von freien Fettsäuren – wie in dieser Studie – im Gegensatz zu den veresterten Fettsäuren, die in den meisten anderen Studien verwendet wurden, den Einbau der mehrfach ungesättigten Fettsäuren in die von Natur aus an einem Mangel an mehrfach ungesättigten Fettsäuren leidenden Lipid Rafts forciert.

Die Tatsache, dass die monozytäre Adhäsion an Endothelzellen durch n-3 und n-6 Fettsäuren moduliert wird, steht in Einklang mit bisherigen Veröffentlichungen [67, 180, 223]. Jedoch wurden in dieser Studie nicht die Endothelzellen [67], sondern die Monozyten mit Fettsäuren vorinkubiert. Im Gegensatz zu anderen Wissenschaftlern, die mit Monozyten [239, 240] oder Endothelzellen [180, 223] arbeiteten, konnte hier kein Einfluss von AA und DHA auf die Expression der Adhäsionsmoleküle in U937 und in humanen Monozyten gefunden werden. Allerdings erklären möglicherweise die unterschiedlichen Versuchsbedingungen diese Varianz der Ergebnisse, da die Monozyten *in vitro* inkubiert wurden - im Gegensatz zur oralen Aufnahme von Fischöl durch Probanden [240]. Auch wurden hier im Vergleich zu supra-physiologischen Konzentrationen oder Triglyzeriden niedrige physiologische Fettsäure-Konzentrationen verwendet. Nichtsdestotrotz steht dieses Versuchsergebnis in Einklang mit früheren Berichten, die weder zeigen konnten, dass Fettsäuren einen Einfluss auf die Expression von endothelialen Adhäsionsmolekülen noch auf die Expression von Adhäsionsmolekülen frisch isolierter Monozyten von Probanden hatten, die eine Fischöl-Infusion erhielten [67, 218]. Obwohl keine Veränderungen der Adhäsionsmoleküle der Zellmembran festgestellt wurde, könnte es trotzdem zu einer Modulation der Integrin-Rekrutierung in die Lipid Rafts durch die Fettsäuren gekommen sein, da diese die Zusammensetzung der Lipid Rafts verändert haben. Dies wurde jedoch in dieser Studie nicht untersucht.

Der Einfluss der Fettsäuren auf Monozyten wurde bereits anhand der Antigen-Präsentation oder der Adhäsion sowohl *in vivo* als auch *in vitro* demonstriert [218, 241, 242]. Ebenso ist bekannt, dass Fettsäuren inflammatorisch aktivierte Zellen differentiell beeinflussen und Auswirkungen auf verschiedene Lipid-abhängige Signaltransduktions-wege haben [41, 243].

Die Bedeutung von Calcium für die Adhäsion ist weithin bekannt [244, 245] und einige Veröffentlichungen berichten auch über die Wirkung von Fettsäuren und ihrer Metaboliten auf die zelluläre Calcium-abhängige Signaltransduktion [246, 247]. Verschiedene Arachidonsäure-abgeleitete Metaboliten wie die 5-Lipoxygenase abgeleitete 5-Hydroperoxyeicosatetraensäure und

das Cytochrome-P450-Hydroxylase-Produkt 20-Hydroxyeicosatetraensäure wurden als Trigger für den Calciumeinstrom in B-Zellen identifiziert [246, 247]. Bisher wurde allerdings noch nicht über eine Calcium-Abhängigkeit der Fettsäure-bedingten Modulation der monozytären-endothelialen Adhäsion in diesem Setting berichtet. In dieser Studie liegt der Fokus auf den Monozyten und der Fettsäure-bedingten Modulation der Adhäsion. Es konnte gezeigt werden, dass der divergierende Effekt von AA und DHA Calcium-abhängig ist, da er nach Vorbehandlung der Monozyten mit dem Calcium-Chelator BAPTA nicht mehr nachweisbar war (***Anlage 5***). Die differentielle Calcium-Modulation in Monozyten durch AA und DHA in einem inflammatorischen Modell ist ein Novum. Eine Co-Inkubation von Fettsäuren und BAPTA führte zu einem verminderten Calcium-Anstieg und machte den Fettsäure-bedingen Unterschied des intrazellulären Calciums zunichte. Daher kann man schlussfolgern, dass der Fettsäure-Einfluss auf das intrazelluläre Calcium zur Modulation der monozytären-endothelialen Adhäsion durch die Fettsäuren beiträgt.

Eine Calcium-Abhängigkeit der Lipid-Raft-Signaltransduktion wurde schon von einigen Gruppen gezeigt [248, 249]. In dieser Studie konnte demonstriert werden, dass durch eine Zerstörung der Lipid Rafts mittels MCD die Fettsäure-induzierte Calcium-Modulation und die Fettsäure-bedingte Modulation der Adhäsion aufgehoben werden (Abb. ***Anlage 5***). Bereits veröffentlichte Daten deuten auf einen Einfluss der Fettsäuren auf die Lipid Raft-abhängige Signaltransduktion hin; jedoch wurden die meisten Untersuchungen an T-Zellen vorgenommen [250-256]. Protein-Modifikationen durch Acylierung wie N-Myristylierung und Palmitoylierung dienen der Adressierung von Proteinen in die Lipid Rafts [257]. Ein möglicher Mechanismus, wie die Fettsäuren die Signaltransduktion der Lipid Rafts beeinflussen können - abgesehen von der Modulation der Zusammensetzung – stellt die Interaktion mit der Acylierung von Proteinen der Signaltransduktion dar [250].

Eine Lipid Raft-abhängige Modulation des intrazellulären Calciums durch Fettsäuren in Monozyten konnte in dieser Studie so erstmalig gezeigt werden. Zu klären bleibt, wie die monozytären Lipid Rafts die Calcium-Signaltransduktion beeinflussen und über welche Wege genau das intrazelluläre Calcium die monozytäre-endotheliale Adhäsion moduliert. Eine Möglichkeit, die den Lipid Rafts nachgeschaltete Signaltransduktion zu beeinflussen, scheint die Modulation der Toll-like Rezeptoren, über die die Fettsäuren auch wirken können, zu sein [258].

Neueste Veröffentlichungen zeigen zudem, dass Analysemethoden aus dem Bereich der Systembiologie wie *targeted liquid chromatography-tandem mass spectrometry-based metabololipidomics* ein großes Potential für die Lipidmediator-Analyse haben. So konnten *specialized pro-resolving metabolites* (SPMs) der n-3 Fettsäure Docosapentaensäure (DPA) in Colon-Biopsien von Patienten mit entzündlichen Darmerkrankungen detektiert werden. Diese SPMs trugen als Lipidmediatoren zur Hemmung der neutrophilen-endothelialen Adhäsion bei und wirkten anti-inflammatorisch [259]. In dieser Studie von Gobbetti et al. waren nicht wie in der vorliegenden Studie die Modulation von Lipid Rafts durch n-3 Fettsäuren und die dadurch bedingte Hemmung der leukozytären-endothelialen Adhäsion von primärer Bedeutung, sondern die von der n-3 Fettsäure DPA abgeleiteten SPMs, die auch zu den Lipidmediatoren gehören. Allerdings gibt es Hinweise, dass n-3-abgeleitete SPMs Lipid Rafts beeinflussen können. Nicht wie in der vorliegenden Studie die Fettsäure DHA, sondern die davon abgeleiteten SPMs RvD1 und 17S-HDHA unterdrückten die Hypercysteinämie-induzierte Bildung von Lipid Raft Signaltransduktions-Plattformen und der nachgeschalteten Sauerstoffradikalproduktion in Podozyten in einem Modell der Podozytenschädigung und Glomerulosklerose [231]. Es ist also durchaus möglich, dass EPA- und DHA-abgeleitete SPMs auch in der vorliegenden Studie bei der Modulation der Lipid Rafts eine Rolle spielen.

Metabololipidomics setzt sich aus *metabolomics* und *lipidomics* zusammen. *Metabololipidomics* gehört zur Wissenschaft der Systembiologie. *Metabolomics* ist das wissenschaftliche Studium chemischer Prozesse, die Metabolite beinhalten, bzw. das systematische Studium eines einzigartigen chemischen Fingerabdrucks, den spezifische zelluläre Prozesse hinterlassen. Es wird folglich ein Profil der vorhandenen Metabolite erstellt. Das Metabolom umfasst alle Metabolite in einer Zelle, in einem Gewebe, in einem Organ oder in einem Organismus. *Lipidomics* umschreibt ein Teilgebiet von *Metabolomics*, das die Lipidmediatoren (und nicht die Nukleotid-, Zucker- oder Protein/Aminosäure-abgeleiteten Mediatoren) untersucht. Forschungsgruppen, die sich mit *Metabololipidomics* beschäftigen, erstellen Profile bioaktiver Lipidmediatoren aus seiner

großen Anzahl biologischer Proben und validieren ihre Interaktionen untereinander und mit anderen Mediatoren in biologischen Systemen [260-262].

Im Bereich *Lipidomics* werden Analysemethoden wie LC-MS/MS (Flüssigchromatographie mit Massenspektrometrie-Kopplung) genutzt. Das wäre auch in der vorliegenden Studie ein Mittel gewesen, um Lipid Rafts zu analysieren [263]. Auch die Differenzierung von M1 und M2 Makrophagen und die Analyse der entsprechenden Lipid Rafts könnte ein interessantes Projekt für zukünftige Studien sein, da die lipidomische Analysen einen deutlichen Unterschied im SPM-Profil zwischen M1 und M2 Makrophagen gezeigt haben, wobei im M2 Makrophagen die *pro-resolving* Lipidmediatoren dominierten [261]. Die Untersuchen der Lipid Rafts des Endoplasmatischen Retikulums sind ebenfalls ein spannendes Feld für zukünftige Studien [264].

Im Übrigen sind auch Lipid Rafts in Mikropartikeln detektiert worden [265, 266]. Mikropartikel sind kleine Membran-Vesikel, die nach zellulärer Aktivierung von verschiedenen Zelltypen durch exozytotische Knospung aus der Plasmamembran freigesetzt werden und sowohl bei physiologischen Prozessen auch bei klinischen Therapie-Konzepten eine Rolle spielen können [267]. Vorläufer der SPMs hat man ebenfalls in Mikropartikeln gefunden [268]. Daher wäre die Analyse des lipidomischen Profils von Lipid Rafts in Mikropartikeln von großem Interesse für anstehende Projekte.

Zusammenfassend konnte in der vorliegenden Studie sowohl in U937 als auch in humanen Monozyten erstmalig gezeigt werden, dass (a) n-6 und n-3 Fettsäuren die Fettsäurezusammensetzung der monozytären Lipid Rafts deutlicher verändern als die der Non-Raft Membranfraktion, und dass (b) die differentielle Beeinflussung der TNF-α-induzierten Adhäsion und des intrazellulären Calciums durch die Fettsäuren mit Zerstörung der Lipid Rafts aufgehoben wird.

Somit konnte ich In dieser Studie konkret folgende Antworten auf meine Fragen finden:

5.1. Eine Präinkubation mit AA vs. DHA und TNF-Stimulation beeinflusst die Zusammensetzung der

a. Lipid Raft Fraktion in U937 Monozyten stärker als

b. die Non-Raft Fraktion in U937 Monozyten und

c. die Lipid Raft Fraktion in humanen Monozyten stärker als

d. die Non-Raft Fraktion in humanen Monozyten.

5.2. Eine Präinkubation mit AA vs. DHA und TNF-Stimulation beeinflusst

a. die statische Adhäsion von U937 Monozyten und

b. die dynamische Adhäsion von humanen Monozyten sowie

c. das Rolling von humanen Monozyten differentiell mit einer allgemein hemmenden Wirkung von DHA.

5.3. Eine Präinkubation mit AA vs. DHA und TNF-Stimulation beeinflusst

a. die Expression von Adhäsionsmolekülen (CD11b, CD18, CD49d) auf U937 Monozyten sowie

b. die Expression von Adhäsionsmolekülen (CD11b, CD18, CD49d) auf humanen Monozyten nicht.

5.4. Die Applikation eine Calcium-Chelators nach Präinkubation mit AA vs. DHA und TNF-Stimulation hebt die Wirkung der Fettsäure-Präinkubation auf

a. die statische Adhäsion von U937 Monozyten und

b. die dynamische Adhäsion von humanen Monozyten auf.

5.5. Das intrazelluläre Calcium wird nach einer Präinkubation mit AA vs. DHA und Stimulation mit TNF

a. in U937 Monozyten und

b. in humanen Monozyten differentiell beeinflusst mit einer Hemmung durch DHA.

5.6. Eine Zerstörung der Lipid Rafts mittels MCD hebt den differentiellen Effekt einer Präinkubation mit AA vs. DHA und TNF-Stimulation auf

a. die Adhäsion von U397 Monozyten,

b. die Adhäsion von humanen Monozyten,

c. das Rolling von humanen Monozyten,

d. Calcium in U937 Monozyten und

e. Calcium in humanen Monozyten auf.

Daher komme ich zu dem Schluss, dass

5.0 Monozytäre Lipid Rafts in der Inflammation relevant für die Fettsäure-bedingte Modulation des Calciums und der Adhäsion sind.

Die Hypothese, dass die Lipid-basierte Signaltransduktion auch Zellmembran-Komponenten wie Lipid Rafts und deren Signaltransduktion in der Inflammation beeinflussen können, konnte ich verifizieren.

Ebenso konnte ich die Frage positiv beantworten, dass monozytäre Lipid Rafts in der Zellmembran signifikant von n-3/n-6 Lipiden in Form von Fettsäuren beeinflusst werden, so dass die Calcium-Signaltransduktion und die Adhäsion in der Inflammation moduliert werden.

Diese Studie konnte die Hypothese, dass Lipide die inflammatorische Signaltransduktion auf der Ebene der endothelialen-Leukozytären Interaktion beeinflussen, auch bestätigen. Die Frage, ob bei der endothelialen-leukozytären inflammatorischen Adhäsion Signaltransduktionswege von Bedeutung sind, die durch die Lipid-basierte Signaltransduktion moduliert werden können, konnte genauso positiv beantwortet werden. Es wurde deutlich, dass n-3/n-6 Fettsäuren die leukozytäre-endotheliale Adhäsion auch nach alleiniger Vorinkubation von Monozyten (und nicht von Endothelzellen) modulieren können.

Diese Ergebnisse stellen einen Ansatzpunkt für die Entwicklung neuer medikamentöser Optionen in der Therapie von inflammatorischen Erkrankungen dar.

Beeinflussung der Inflammation in vivo *in Abhängigkeit von dem Lipid-aktivierten Transkriptionsfaktor PPAR-α*

Bisher konnte in dieser Arbeit auf den unterschiedlichsten Ebenen der inflammatorischen Signaltransduktion ein Einfluss Lipid-basierter Mechanismen demonstriert werden. Nun sollte die Hypothese evaluierte werden, dass die Lipid-basierte Signaltransduktion die Inflammation durch Lipid-aktivierte Transkriptionsfaktoren wie PPAR-α beeinflussen können. Die Frage war also, ob der Lipid-aktivierte Transkriptionsfaktor PPAR-α in der akuten Lungenschädigung die inflammatorische Signaltransduktion moduliert.

Daraus ergaben sich folgende Fragestellungen für die Durchführung dieser Studie:

6.1. Hat die Fütterung mit dem PPAR-α Aktivator WY14,643 bei Wildtyp vs. PPAR-α -/- Mäusen einen differentiellen Einfluss auf

a. die Leukozytenzahl in der BAL

b. TNF-alpha in der BAL

c. MIP-2 in der BAL

d. den Proteingehalt in der BAL

e. TxB_2 in der BAL

f. PGE_2 in der BAL

g. die Lungencompliance

gemessen je 8 und 24 Stunden nach intratrachealer Stimulation mit 1 bzw. 10 µg LPS?

6.2. Hat die Fütterung mit dem PPAR-α Aktivator WY14,643 bei Wildtyp vs. PPAR-α -/- Mäusen einen differentiellen Einfluss auf den kapillaren Filtrationskoeffizienten nach PAF-Stimulation?

6.0 Hat die Aktivierung des Lipid-aktivierten Transkriptionsfaktor PPAR-α in einem murinen Modell der LPS-induzierten akuten Lungenschädigung Einfluss auf die inflammatorische Signaltransduktion und das Ausmaß der akuten Lungenschädigung?

In dieser Studie (***Anlage 6***)[269] konnte gezeigt werden, dass die Aktivierung des Transkriptionsfaktors PPAR-α durch das Fibrat WY14,643 (WY) in einem murinen Modell der akuten Lungenschädigung *in vivo* und im PAF-induzierten Anstieg des „*vascular leakage*“ in isoliert perfundierten Mäuselungen protektiv wirkt. Die Aktivierung von PPAR-α reduzierte die LPS-induzierte Leukozyteninvasion in das alveoläre Kompartiment und hemmte die Synthese inflammatorischer Mediatoren TNF-α, MIP-2, TxB_2, und PGE_2 (Abb. ***Anlage 6***). Außerdem war die Integrität der endothelialen-alveolären Barriere durch die Fibrat-Behandlung besser erhalten, was man aus dem erniedrigten Proteingehalt der broncho-alveolären Lavage und dem geringeren kapillaren Filtrationskoeffizient schließen konnte. Als weiterer funktioneller Parameter wurde die Lungenfunktion bei Mäusen untersucht, die LPS intratracheal erhalten hatten. Die PPAR-α Aktivierung durch das Fibrat WY verbesserte die durch die intratracheale LPS-Behandlung hervorgerufene akute Verschlechterung der pulmonalen Compliance (Abb. ***Anlage 6***). Bei PPAR-α-defizienten Mäusen führte die Fibrat-Behandlung weder im *in vivo* Modell der akuten Lungenschädigung noch im Modell der isoliert perfundierten Mäuselunge zu diesen positiven Effekten. Man muss jedoch berücksichtigen, dass das LPS-Modell ein „*single-hit*“ Modell ist, bei dem mit nur einer Endotoxin-Gabe eine inflammatorische Antwort hervorgerufen wird. Solch ein Modell unterscheidet sich natürlich von einem durch eine prolongierte bakterielle Infektion hervorgerufenem *acute respiratory distress syndrome* (ARDS).

In Leber, Nieren, Muskeln und im Herz besteht eine hohe PPAR-α-Expression [124]; PPAR-α wird aber auch in glatten Muskelzellen, Monozyten/Makrophagen und T-Lymphozyten exprimiert [270]. PPAR-α-abhängige Gene sind an der Regulation des Fettsäure- und des Lipoprotein-Metabolismus beteiligt. Jedoch mehren sich die Hinweise, dass Fibrate, die PPAR-α aktivieren, nicht nur Lipid-senkende Eigenschaften haben, sondern sich auch positiv auf pulmonale Erkrankungen wirken [270]. Die meisten Autoren beschreiben einen Benefit der PPAR-α-Aktivierung in Langzeit-Modellen pulmonaler Erkrankungen oder auf Atemwegs- oder Pleura-Erkrankungen [271-273].

In einer Bleomycin-induzierten Langzeit-Schädigung der Lunge (über 15 Tage) bei Mäusen konnte die protektive Wirkung von PPAR-α-Liganden auf die inflammatorische Antwort nachgewiesen werden [271]. In einem Langzeit-Modell mit chronischer intranasaler LPS-Applikation zur Untersuchung der Inflammation der Atemwege stiegen Leukozyteninvasion, TNF-α, Monozyten-chemotaktisches Protein (MCP-1), Keratinozyten-Chemokin (KC) und Matrix-Metallo-Proteinasen nach fünf Tagen bei Mäusen ohne

funktionellem PPAR-α-Rezeptor an. Eine Behandlung mit Fenofibrat bei Wildtyp-Mäusen hingegen reduzierte alle untersuchten Parameter [272]. In Einklang mit diesen Ergebnissen stehen Daten, die bei einer allergischen Atemwegsinflammation eine Reduktion durch Fenofibrat zeigen [273]. In einigen Publikationen wurde nachgewiesen, dass die PPAR-α-Aktivierung mit der Expression pro-inflammatorischer Gene wie *vascular cell adhesion molecule* (VCAM)-1, Plättchen-aktivierender Faktor (PAF) Rezeptor und Cyclooxygenase (COX)-2 [119, 120] einhergeht. Dies könnte zumindest zum Teil an der verminderten Aktivierung von NFkappaB und der vermehrten Expression seines Inhibitors IkappaBα liegen [122, 123]. Beide Effekte könnten nach inflammatorischer Stimulation zu einer reduzierten nuklearen Translokation von p50/p65 NFkappaB führen und dadurch die nukleare Transkription von abhängigen Genen wie TNF-α und IL-1ß hemmen.

Da die der LPS-Stimulation nachgeschalteten Effekte zumindest teilweise durch die Aktivierung von NFkappaB bedingt sind, ist die Inhibition dieses Signaltransduktionsweges möglicherweise auch verantwortlich für die Reduktion der akuten Lungenschädigung. Damit konsistent sind die von uns erhobenen Daten einer verminderten Bildung von TNF-α, MIP-2, TxB_2 und PGE_2 nach LPS-Stimulation bei Mäusen (Abb. ***Anlage 6***).

Für die positiven Effekte von WY auf die pulmonale Permeabilität jedoch reicht diese Erklärung nicht ganz aus. Es ist bekannt, dass PAF in Mäuselungen einen akuten Anstieg der Vasokonstriktion und der Permeabilität induziert [274]. Die akute Vasokonstriktion wird durch die Cysteinyl-Leukotriene und durch Thromboxan (Tx) A_2 vermittelt [274]. Die akute Bildung von PGE_2 durch die COX-1 und die Aktivierung der *Acid Sphingomyelinase* (ASM) mit konsekutiver Ceramid-Synthese sind die wichtigsten Mediatoren der Permeabilität [188]. In isoliert perfundierten Mäuselungen war die Vasokonstriktion – gemessen am Perfusionsdruck – trotz PPAR-α-Aktivierung durch WY bei unseren Versuchen konstant. Im Gegensatz dazu war der als Permeabilitätsmarker analysierte PAF-induzierte Anstieg des kapillaren Filtrationskoeffizienten nach PPAR-α-Aktivierung fast nicht mehr nachweisbar (***Anlage 6***). Obwohl PPAR-α–Agonisten über sekundäre Mediatoren sowohl die Vasokonstriktion als auch die Permeabilität und die PAF-Rezeptor-Expression vermindern [275], scheint dieser Mechanismus die Modulation bei dem verwendeten Modell nicht adäquat zu erklären. Es ist anzunehmen, dass WY durch eine Inhibition des PGE_2 (COX-1)- oder des Ceramid(ASM)-Signaltransduktionsweges die Lungen vor der PAF-induzierten Permeabilität schützt [188]. Die Hinweise mehren sich, dass die PPAR-α-Aktivierung einen Ceramid-Anstieg induziert – diese Daten wurden jedoch in Herzen und nicht in Lungen gewonnen [276, 277]. Da auch in der BAL nach LPS-Stimulation eine Reduktion des PGE_2 *in vivo* gefunden wurde (Abb. ***Anlage 6***), ist zu vermuten, dass die PPAR-α–Aktivierung die Permeabilität in diesem Ansatz hauptsächlich über den COX-1- und den COX-2-Signaltransduktionsweg beeinflusst.

Wie bereits erwähnt, wurde gezeigt, dass eine PPAR-α–Aktivierung die pulmonale Inflammation reduziert (***Anlage 6***). *Vice versa* führte eine genetische Deletion des PPAR-α zu einer gesteigerten und prolongierten Entzündungsreaktion, wie man an der vermehrten Synthese pro-inflammatorischer Zytokine, der gesteigerten Leukozyteninfiltration und der höheren Mortalität sieht [271, 272, 278, 279]. Die Daten von dem hier verwendeten Modell der akuten Lungenschädigung sind mit dem ersten Teil dieser Beobachtungen konsistent. Allerdings konnte hier nur ein kleiner, nicht signifikanter Anstieg der LPS-induzierten inflammatorischen Antwort bei PPAR-α-knockout Mäusen gezeigt werden. Diese voneinander abweichenden Resultate mögen an den unterschiedlichen Zeitpunkten und Modellen liegen.

Es bestätigte sich, dass der Benefit der Fibrat-Therapie in der pulmonalen Inflammation spezifisch von einem funktionellen PPAR-α abhängt. Bei PPAR-α -/- Mäusen war der protektive Effekt des WY im Modell des akuten Lungenversagens auf Zytokine, Eicosanoide, Leukozyteninfiltration und *vascular leakage* nicht mehr nachweisbar.

Diese Beobachtungen stehen in Einklang mit früheren Berichten anderer Gruppen, die WY oder Fenofibrat verwendet haben [271, 272, 278, 279]. Trifilieff et al. konnten nach Verwendung des PPAR-α Agonisten GW 9578 bei Mäusen nicht den gleichen protektiven Effekt feststellen [280]. Allerdings applizierten sie GW 9578 intranasal eine Stunde vor LPS-Instillation und fanden weder eine reduzierte TNF-α-Synthese in der BAL noch eine Veränderung des Neutrophilen-Influx. Ein Grund für diese divergenten Ergebnisse könnte die unterschiedliche Applikationsart der Fibrate darstellen. Die Verabreichung von GW 9578 lokal intranasal nur eine Stunde vor dem

inflammatorischen Stimulus war möglicherweise zu kurz und wurde evtl. nicht ausreichend resorbiert, um die positiven Effekte der PPAR-α-Aktivierung zum Tragen zu bringen. Die Änderungen in der Proteinexpression der NFkappaB-abhängigen Gene benötigen mehr Zeit. Obwohl die Expression von PPAR-α in murinen Lungen [109] und ein direkter Einfluss der Fibrate auf die glatten Muskelzellen der Atemwege schon beschrieben wurde [281], ist möglicherweise die Aktivierung von lokalen als auch von ortsfernen Leukozyten und Endothelzellen durch das Fibrat notwendig, um den besten positiven Effekt zu erzielen.

Die leukozytäre Transmigration in den alveolären Raum stellt ein Charakteristikum der pulmonalen Inflammation und der akuten Lungenschädigung dar [282]. Es konnte gezeigt werden, dass die PPAR-α-Aktivierung zu einer reduzierten akuten Invasion der Neutrophilen in dem verwendeten Modell des akuten Lungenversagens führt. Da die neutrophile Transmigration in das alveoläre Kompartiment die konzertierte Interaktion von Neutrophilen, Endothelzellen und Epithelzellen voraussetzt, ist ein Einfluss des Fibrates auf alle erwähnten Zellpopulationen möglich. Von einer verminderten VCAM-1-Expression in Endothelzellen und einer reduzierten PAF-Rezeptor-Expression in Leukozyten wurde bereits berichtet [119, 120] und beide Systeme sind in die leukozytäre Adhäsion und Transmigration involviert [283]. Abgesehen davon stellen die reduzierte Synthese von MIP-2 (dem murinen Äquivalent des Chemotaxins Interleukin-8 bei Menschen) und eine verminderte TxA_2-Bildung zwei weitere Gründe für eine geringere leukozytäre Infiltration in den alveolären Raum dar.

Schließlich konnte gezeigt werden, dass die intratracheale LPS-Instillation die pulmonale Compliance signifikant beeinträchtigt (Abb. ***Anlage 6***). Diese Beeinträchtigung wird durch die WY-induzierte PPAR-α-Aktivierung deutlich reduziert. Die Bestimmung der Compliance ist ein anerkanntes Verfahren zur Evaluation der Lungenschädigung [284] und eine Beeinträchtigung der Compliance findet man bei Patienten mit akuter Lungenschädigung [285]. Die Tatsache, dass eine PPAR-α-Aktivierung die Compliance verbessert, untermauert die positiven Effekte der Fibrat-bedingten PPAR-α-Aktivierung auf die akute Lungenschädigung.

Nicht untersucht wurde in dieser Studie der Einfluss anderer Aktivatoren des PPAR-α, wie beispielsweise oxidierter Fettsäuren oder Metabolite der Arachidonsäure (z.B. LTB4, 20-HETE oder 8(S)-HETE). Die Aktivierung durch Metabolite der Arachidonsäure könnte auch das manchmal gezeigte Paradox der pro-inflammatorischen Wirkung der PPAR-α Aktivierung mit erklären [286]. PPAR-α gilt auch als intrazellulärer Lipid-Sensor, da viele der Fettsäuren eine sehr hohe Affinität zu PPAR-α haben, die im nanomolar-Bereich liegt [287]. Die Untersuchung der Lipid-Aktivatoren von PPAR-α, insbesondere der Unterschied zwischen n-3 und n-6 abgeleiteter Aktivatoren, ist Gegenstand weiterer Studienplanungen.

Neueste Untersuchungen zeigen, dass PPAR-α durch die microRNA miR-21 reguliert wird [288]. MicroRNA (miRNA) spielen bei der Regulation der Transkription eine wichtige Rolle [289]. In einem Rattenmodel des chronischen Nierenversagens war miR-21-5p erhöht. MiR-21-5p ist ein PPAR-α Ziel und die miR-21-5p Suppression veränderte die PPAR-α nachgeschaltete Genexpression im linken Ventrikel. Außerdem schützte die Gabe des PPAR-α Agonisten vor einem linksventrikulären Versagen und verbesserte die Herzfunktion auch unter septischen Bedingungen [288, 290].

In der vorliegenden Studie wurde nicht die Herzfunktion, wohl aber die pulmonale Funktion nach inflammatorischer Aktivierung, gemessen an der Compliance und der *vascular leakage,* PPAR-α-vermittelt verbessert. Dies steht in Einklang mit Ergebnissen anderer experimenteller Untersuchungen im septischen Tiermodell [291].

Gugliandolo et al. konnten im Modell der pulmonalen Infektion mit Pseudomonas Aeruginosa ebenfalls eine PPAR-α-vermittelte anti-inflammatorische Wirkung zeigen [292].

Obwohl einiges für die Vernetzung von PPAR-α mit der inflammatorischen Signaltransduktion spricht, einschließlich der Beeinflussung von NFkappaB, haben Fibrate noch nicht den Weg in die Therapie inflammatorischer Erkrankungen gefunden. Die Fähigkeit von Simvastatin, PKC zu beeinflussen, stimuliert indirekt die PPAR-α Transrepressions-Aktivität hinsichtlich NFkappaB. Das könnte die Statin-mediierten anti-inflammatorischen Effekte zum Teil erklären [293]. Andererseits wirken NFkappaB-gesteuerte Zytokine wie TNF-α, IL1ß und IL6 auf die Expression von PPAR-α ein; und die PPAR-α Expression moduliert wiederum die PPAR-α Aktivierung, wie von Bourgarne et al in der Übersichtsarbeit dargestellt [294]. Dies steht in Einklang mit den hier erhobenen Daten der PPAR-α-abhängigen Reduktion der pro-

inflammatorischen Zytokine nach inflammatorischer Aktivierung.

Auch in Patienten-Studien wurde die anti-inflammatorische Fähigkeit der PPAR-α Signaltransduktion gezeigt [295]. Um das therapeutische Potential von PPAR-α als Ziel für die medikamentöse Therapie zu maximieren, wurde selektive PPAR-α-Modulatoren (SPPARMα) wie beispielsweise Pemafibrat, das sich in klinischen Studien in Phase III befindet, entwickelt [296]. Im Tierversuch konnte die hemmende Wirkung von Pemafibrat auf den Entzündungsprozess schon demonstriert werden [297]. Somit ist zu hoffen, dass so das hier gezeigte anti-inflammatorische Potential der PPAR-α Aktivierung bald therapeutisch genutzt werden kann.

In Zusammenhang mit der Therapie der Sepsis ist die Relevanz des Zeitpunktes der Therapie offensichtlich [298]. Die zirkadiane Uhr scheint hier ebenfalls eine wichtige Rolle zu spielen. Ein PPAR-α-Zielgen, Rev-erbα [299], war bei einer Studie, die Abhängigkeit der zirkadianen Rhythmik bei perioperativen Myokardinfarkten untersuchte, protektiv [300]. Folglich ist bei Untersuchungen, die auf der vorliegenden aufbauen werden, die zirkadiane Rhythmik zu berücksichtigen.

Zusammenfassend konnte in dieser Studie sowohl im Tiermodell der akuten Lungenschädigung mit Wildtyp und PPAR-α -/- Mäusen als auch im Organmodel der isoliert perfundierten Mauslunge gezeigt werden, dass durch eine Vorbehandlung mit dem Fibrat WY die Synthese pro-inflammatorischer Zytokine und Eicosanoide reduziert, die alveoläre Leukozyteninvasion vermindert und die Compliance wie auch die *vascular leakage* verbessert wurde.

In dieser Studie konnte ich somit folgende konkrete Antworten auf meine Fragen finden:

6.1. Die Fütterung mit dem PPAR-α Aktivator WY14,643 hat bei Wildtyp vs. PPAR-α -/- Mäusen einen differentiellen Einfluss auf

a. die Leukozytenzahl in der BAL

b. TNF-alpha in der BAL

c. MIP-2 in der BAL

d. den Proteingehalt in der BAL

e. TxB_2 in der BAL

f. PGE_2 in der BAL und

g. die Lungencompliance

gemessen je 8 und 24 Stunden nach intratrachealer Stimulation mit 1 bzw. 10 µg LPS mit einem generell hemmenden Einfluss auf die Inflammation bzw. Verbesserung der Lungencompliance bei Präsenz des PPAR-α Rezeptors.

6.2. Die Fütterung mit dem PPAR-α Aktivator WY14,643 bei Wildtyp vs. PPAR-α -/- Mäusen hat einen differentiellen Einfluss auf den kapillaren Filtrationskoeffizienten nach PAF-Stimulation mit einem protektivem Effekt bei Präsenz des PPAR-α Rezeptors, der einen Anstieg des kapillaren Filtrationskoeffizienten verhinderte.

6.0 Die Aktivierung des Lipid-aktivierten Transkriptionsfaktor PPAR-α in einem murinen Modell der LPS-induzierten akuten Lungenschädigung hat einen protektiv-hemmenden Einfluss auf die inflammatorische Signaltransduktion und das Ausmaß der akuten Lungenschädigung.

Die Hypothese, dass Lipide die inflammatorische Signaltransduktion durch Lipid-aktivierte Transkriptionsfaktoren wie PPAR-α beeinflussen können, wurde somit verifiziert. Die Frage, ob der Lipid-aktivierte Transkriptionsfaktor PPAR-α in der akuten Lungenschädigung die inflammatorische Signaltransduktion moduliert, konnte ich positiv beantworten.

Diese Beeinflussung der Inflammation durch den Lipid-aktivierten Transkriptionsfaktor PPAR-α kann für zukünftige Therapieoptionen der pulmonalen Inflammation von erheblichem Interesse sein.

4. Therapeutische Perspektiven

Die vorgestellten Untersuchungen belegen die Relevanz der Lipid-basierten Signaltransduktion für inflammatorische Prozesse. Sie zeigen, dass die Lipid-basierte Signaltransduktion die Inflammation auf verschiedenen Ebenen effektiv modulieren kann.

In dieser Arbeit konnte ich zeigen, dass die inflammatorische Signaltransduktion auf der Ebene der endothelialen- und epithelialen-leukozytären Interaktion, im Bereich der akuten inflammatorischen Lungenschädigung und der systemischen Inflammation durch n-3 vs. n-6 Fettsäuren, Fettsäure-basierte Lipidinfusionen, mittels PAF, der PI3-Kinase, den Lipid Rafts und des PPAR-α beeinflusst werden kann.

Die Wirkung der Lipid-basierten Signaltransduktion erfolgt dabei über multiple Effektoren. In den durchgeführten Untersuchungen waren das die Calcium-Signaltransduktion sowie direkte Effekte des Lipidmediators PAF. Der Einfluss der Fettsäure-basierten Lipidinfusionen auf inflammatorische Zytokine wie TNF-α, Chemokine wie MIP-2 und Enzyme wie die Myeloperoxidase wurde mit den daraus folgenden Auswirkungen wie Leukozytenrekrutierung in die Lunge und Proteinleckage demonstriert. Ebenso konnte ein Einfluss der Fibrat-Aktivierung des PPAR-α auf die Cyclooxygenase mit entsprechender Modulation ihrer Metaboliten, der Thromboxane und Prostaglandine gezeigt werden. Dies hatte entsprechende Folgen für die Organfunktion, wie an einer veränderten Lungencompliance oder dem modulierten kapillaren Filtrationskoeffizienten dargestellt. Des Weiteren wurde analysiert, wie die Fettsäuren über den Einbau in die Zellmembran die Inflammation beeinflussen können. Hier sind die Lipid Rafts zu nennen. Auch das hatte Auswirkungen auf pathophysiologische Ereignisse wie die Adhäsion und die weitere Signaltransduktion des Calciums. Es konnte sogar evaluiert werden, dass Fettsäuren die Signaltransduktion der PI3-Kinase und darüber nicht nur die Adhäsion sondern auch die Apoptose steuern können. Somit wurden mehrere Mechanismen aufgezeigt, wie Fettsäuren in der Lage sind, den für die Inflammation zentralen Prozess der Adhäsion zu modulieren.

Durch diese große Bandbreite der Wirkungsweisen und der verschiedenen Interventionsmöglichkeiten beispielsweise über Lipidinfusionen oder über orale Zufuhr von Fibraten wird das therapeutische Potential einer Einflussnahme auf die Lipid-basierte Signaltransduktion ersichtlich.

Die Tatsache, dass die Lipid-basierte Signaltransduktion multiple Signalkaskaden simultan beeinflussen kann, macht sie für die Therapie der Sepsis besonders interessant.

Hinzu kommt, dass die Lipid-basierte Signaltransduktion durch intravenöse Ernährungstherapie – wie ebenfalls hier *in vivo* demonstriert - aktiviert wird. Dadurch besteht die Möglichkeit, über die Zusammensetzung der applizierten Lipidemulsionen die Signaltransduktion modulieren zu können.

Bei hospitalisierten Patienten, die intensivpflichtig sind, weil sie an einer akuten inflammatorischen Erkrankung, wie beispielsweise der akuten inflammatorischen Lungenschädigung bzw. ARDS oder einer systemischen Inflammation wie der Sepsis leiden, kann eine intravenöse Ernährung notwendig sein. Die klinisch einsetzbaren n-3 basierten Lipidemulsionen wurden auch in den von mir durchgeführten Studien verwendet. Da in dieser Arbeit demonstriert wurde, dass sich intravenöse n-3 basierte Lipidemulsionen positiv auf den Verlauf der Inflammation auswirken, ist hier ein Ansatzpunkt für therapeutische Möglichkeiten dargelegt worden. Dabei ist zu berücksichtigen, dass intravenös applizierte Lipidemulsionen deutlich potenter sind als die orale Applikation von Fischöl, bei der es mehrere Wochen dauert, bis entsprechende Plasmaspiegel erreicht sind [301, 302]. Ambulant sind es meist Patienten, die an einer malignen Erkrankung leiden, die einer intravenösen Ernährung bedürfen. Auch bei Krebserkrankungen gibt es Hinweise, dass Fischöl sich positiv auf den Krankheitsverlauf auswirken kann [303]. Durch die in dieser Arbeit aufgezeigten Pathomechanismen, die durch Lipide beeinflusst werden, könnten hieraus weitere Ansätze für medikamentöse Optionen zur therapeutischen Intervention entstehen. Wenn man zum Beispiel die demonstrierte pro-apoptotische Wirkung von DHA gezielt auf Krebszellen richten könnte, und so eine gezielte anti-carcinogene Therapie entwickeln würde, wäre

das eine therapeutische Option, die man von dieser Arbeit ableiten kann.

Die Anwendung der n-3 Lipide bei septischen Patienten beruht auf der Vorstellung, dadurch die Phase der Hyper-Inflammation zu mildern und in der Folge die immunsuppressive Phase abzuschwächen oder gar zu verhindern. Insbesondere durch die Verbesserung des Verhältnisses der n-6 zu den n-3 Fettsäuren soll dieser Effekt zustande kommen [178, 304]. In dieser Arbeit konnte dies mittels der durchgeführten *in vivo* Modelle bestätigt und somit diese therapeutische Idee untermauert werden.

Die deutschen Leitlinien zur Sepsis-Therapie und zur klinischen Ernährung in der Chirurgie werden zurzeit überarbeitet. Die aktuellen internationalen *Sepsis Guidelines* der *Surviving Sepsis Campain* von 2016 sprechen sich gegen die Anwendung von Omega-3 Fettsäuren in der Sepsis oder im septischen Schock aus (starke Empfehlung, niedriges Evidenzniveau) [305]. Studien zeigen jedoch einen positiven Einfluss unter anderem auf Mortalität, Liegezeit und Immunreaktionen durch die Supplementierung der parenteralen Ernährung mit n-3 Lipiden bei septischen Patienten in der Chirurgie [306-309]. Besonders im Vergleich mit konventionell verwendeten Lipidinfusionen scheint die Addition intravenöser n-3 Lipide sich günstig auf die Liegezeiten auszuwirken [310]. Ebenfalls konnte eine multizentrische Beobachtungsstudie bei Patienten mit *Systemic Inflammatory Response Syndrom* (SIRS), Sepsis, Pankreatitis und Peritonitis eine deutliche Reduktion des Antibiotikaverbrauchs, der Infektionen und der Komplikationen durch die Verwendung von n-3 Lipidinfusionen zeigen [311]. In dieser Arbeit wurde durch die Analyse der zugrundeliegenden Pathomechanismen dieser therapeutische Ansatz fundiert.

Abgesehen von akuten inflammatorischen Erkrankungen wie Sepsis, können n-3 Lipide auch bei subakuten, chronischen Erkrankungen in der adjuvanten Therapie von Nutzen sein. Die *American Heart Association* rät bei Patienten nach einem Myokardinfarkt zur Einnahme von 1 g EPA + DHA pro Tag in einer Klasse II Empfehlung [312] [313]. In der Primärprävention von kardiovaskulären Ereignissen zeigen neueste Studien (VITAL und ASCEND) keinen therapeutischen Nutzen einer oralen Supplementierung mit n-3 Fettsäuren [314, 315]. Hierbei ist allerdings zu beachten, dass die Zufuhr von n-3 Fettsäuren nicht über die Nahrung z.B. mittels Seefisch erfolgte. Da der Triglyzerid-senkende Effekt von n-3 Fettsäuren bekannt war, wurde die REDUCE-IT Studie durchgeführt. Dies ist eine randomisierte, doppel-blinde Placebo kontrollierte Phase 3 Studie. Hier erhielten Patienten mit manifester kardiovaskulärer Erkrankung oder Diabetes mit einem zusätzlichen Risikofaktor und Hypertriglyceridämie unter Statin-Therapie 4 g Icosapent-Ethyl, eines aufgereinigten Ethyl-Esters von EPA. Dieses Präparat reduzierte signifikant die kardiovaskulären Ereignisse [316, 317]. Bei weiteren chronisch inflammatorischen Erkrankungen wie Psoriasis und rheumatoider Arthritis, wird der Einsatz der n-3 Lipide als adjuvante Therapie in klinischen Studien evaluiert [318]. Insbesondere die hier durchgeführten Untersuchungen zur monozytären-endothelialen Interaktion sowie auch die Studie zu den monozytären Lipid Rafts können hier weitere Mechanismen hinsichtlich der Interventionsoptionen in der Therapie aufzeigen. Die Relevanz der Calcium-Signaltransduktion sollte - wie in dieser Arbeit gezeigt – in Bezug auf potentielle medikamentöse Therapieoptionen nicht unterschätzt werden.

Abgesehen davon, dass die Ernährung die n-3/n-6 -Balance bestimmt, gibt es Interaktionen zwischen dem genetischen Hintergrund und der Ernährung, der hormonellen und epigenetischen Regulation, wie auch Erkrankungs-bedingte Faktoren, die die individuelle Kapazität, aus den C18 Vorläuferstufen n-3 und n-6 PUFAs zu synthetisieren, beeinflussen [296][297]. Folglich tragen alle diese Faktoren dazu bei, dass Patienten, die eine EPA/DHA-Supplementation erhalten, sich im *Baseline*-Niveau der n-3 vs. n-6 PUFAs unterscheiden. Ebenso werden diese Faktoren Einfluss haben auf das Modulationspotential der EPA/DHA-Supplementation bezüglich des endogenen Fettsäureprofils [193, 298]. Allerdings hatte in der *Multi-Ethnic Study of Atherosclerosis* (MESA) der ethnische Hintergrund zwar einen Einfluss auf die PUFA-Spiegel, aber keinen Einfluss auf die Korrelation zwischen PUFA-Spiegeln und inflammatorischen Biomarkern im Blut [319].

Die hier durchgeführte Studie, die frisch isolierte humane Monozyten mit der Ziellinie der U937

Monozyten verglich, zeigte ebenfalls Differenzen, die jedoch marginal waren, zwischen diesen beiden Zellenarten. Die epigenetische Varianz der frisch isolierten humanen Monozyten könnte dem zugrunde liegen. Somit liefern die hier durchgeführten Untersuchungen ebenfalls Anhaltspunkte für die Relevanz der Epigenetik bei der Therapie, die auch andere gezeigt haben [320].

N-3 basierte Lipidemulsionen können ebenfalls Auswirkungen auf die SPMs haben. Das wurde in den hier durchgeführten Studien nicht untersucht. Allerdings ist es durchaus möglich, dass SPMs bei der Wirkung der n-3 Lipide in den durchgeführten Untersuchungen eine Rolle spielen.

Aktuell wird mit Hilfe von Knockout-Mäusen für den BLT-1 Rezeptor bzw. für den ChemR-Rezeptor die Relevanz von LTB4 bzw. RvE1 in der pulmonalen Inflammation evaluiert. Diese Projekte sind noch nicht abgeschlossen.

Abgesehen von den hier durchgeführten *in vivo* Studien, die ein Wirksamkeit der n-3 Lipidemulsionen in der Inflammation zeigen, sind weitere klinische Studien notwendig, um die n-3 Lipide bei inflammatorischen Erkrankungen adäquat in die bisher vorhandenen Therapiekonzepte zu integrieren.

Ein weiterer Lipid-basierter Wirkmechanismus mit therapeutischen Perspektiven, der in dieser Studie *in vivo* untersucht wurde, ist die Aktivierung des Transkriptionsfaktors PPAR-α mittels eine Fibrates. Fibrate werden bislang zur Therapie der Hypertriglyzeridämie eingesetzt.

Die Lipid-senkenden Eigenschaften der n-3 Fettsäuren können ebenfalls bei der Hypertriglyzeridämie therapeutisch empfehlenswert sein. Hier kommt wahrscheinlich auch die Wirkung auf die PPARs zum Tragen [321, 322]. Ein PPAR-alpha und –delta-Agonist hat eine Phase III Studie zur Therapie der *nonalcoholic steatohepatitis* (NASH) erfolgreich durchlaufen [323]. Hier konnte also analog zur von mir durchgeführten Studie auch ein Effekt auf eine Inflammation gezeigt werden. Bei pulmonalen Erkrankungen kommen Fibrate bisher noch nicht zum Einsatz, obwohl *in vitro* der Einfluss auf die pulmonale Inflammation durch Aktivierung des Transkriptionsfaktors PPAR-α gezeigt wurde [324]. Weitere Studien zur anti-inflammatorischen Wirkung im pulmonalen System sind abzuwarten.

In dieser Arbeit wurde die Hypothese *in vitro* bestätigt, dass Lipide über Zellmembran-Komponenten wie Lipid Rafts die inflammatorische Signaltransduktion beeinflussen. Die Modulation der Lipid Raft-abhängigen Signaltransduktion ist klinisch bei inflammatorischen Erkrankungen noch ungenügend untersucht. Es ist jedoch zu vermuten, dass Lipid Rafts nicht nur *in vitro* sondern auch *in vivo* bei Patienten mit inflammatorischen Erkrankungen durch n-3 Lipide beeinflusst werden. Weitere Agenzien, die Auswirkungen auf die Lipid Rafts haben, sind Cholesterin-Synthese-Enzym-(CSE)-Inhibitoren (Statine) [325, 326]. Insbesondere für Statine konnte eine anti-inflammatorische Wirkung [327] sowie ein positiver Effekt in der Sepsis gezeigt werden [328]. Hier sind weitere experimentelle und klinische Untersuchungen abzuwarten. Da Statine unter anderem mit Lipid Rafts und deren Signaltransduktion interferieren [329], erklären die hier durchgeführten Untersuchungen auch partiell die anti-inflammatorischen Effekte von Statinen.

Ein interessantes Konzept ist das der therapeutisch nutzbaren künstlich hergestellten Mikropartikeln, die auch Lipid Rafts enthalten können und durch das Lipid-*Signalling*, insbesondere auch durch SPMs beeinflusst werden können [265, 266, 268].

Insgesamt sind aufgrund der hier erarbeiteten pathophysiologischen Mechanismen weitere therapeutische Ansätze in der Therapie der Inflammation abzusehen.

Basierend auf den bisherigen Ergebnissen meiner Forschung plane ich perspektivisch meine wissenschaftliche Tätigkeit wie folgt zu strukturieren:

1. Fokussierung auf die Reversibilität / Auflösung der Inflammation.

2. Richtung des Augenmerks auf die Rolle des Zytoskelettes, der epigenetischen Veränderungen, des Calciums und der Lipid-basierten Signaltransduktion sowie auf das lymphatische System.

3. Durchführung der Untersuchungen jeweils im Modell der Zellkultur, im Tiermodell und in Probanden.

4. Die Entwicklung von therapeutischen Konzepten beruhend auf den oben genannten Faktoren.

Hierbei steht somit insbesondere die Entwicklung von medikamentösen therapeutischen Optionen als finales Ziel im Fokus.

5. Zusammenfassung

Ziel dieser Arbeit war es, die Relevanz der Lipid-basierten Mechanismen auf den unterschiedlichen Ebenen der inflammatorischen Signaltransduktion zu evaluieren.

Die folgenden Hypothesen konnten verifiziert werden:

Die Lipid-basierte Signaltransduktion beeinflusst die Inflammation

1. auf der Ebene der endothelialen-leukozytären Interaktion

2. auf der Ebene der epithelialen-leukozytären Interaktion

3. im Bereich der akuten inflammatorischen Lungenschädigung und der systemischen Inflammation durch n-3 vs. n-6 Fettsäure-basierte Lipidemulsionen differentiell

4. mittels Lipidmediatoren wie PAF

5. mittels Lipid-abhängiger Kinasen wie der PI3-Kinase

6. über Zellmembran-Komponenten wie Lipid Rafts und

7. durch Lipid-aktivierte Transkriptionsfaktoren wie PPAR-α

Die in der Fragestellung dargelegten Fragen konnten somit wie folgt beantwortet werden:

1. Bei der endothelialen-leukozytären inflammatorischen Adhäsion sind Signaltransduktionswege von Bedeutung, die Einfluss auf die Lipid-basierte Signaltransduktion haben. Hier wurde die Calcium-Signaltransduktion identifiziert, die beispielsweise die Synthese des Lipidmediators PAF beeinflussen kann. Außerdem konnte ich zeigen, dass die Fettsäuren AA vs. DHA die leukozytäre-endotheliale Adhäsion nach alleiniger Vorinkubation der Endothelzellen und nach alleiniger Vorinkubation der Leukozyten modulieren können. Letztere hat ebenfalls Auswirkungen auf die Calcium Signaltransduktion. Somit scheint Calcium in die Lipid-basierte Signaltransduktion insgesamt eingebunden zu sein. Es sind folglich Signaltransduktionswege untersucht worden, die Einfluss auf die Lipid-basierte Signaltransduktion haben und solche, die von der Lipid-basierten Signaltransduktion beeinflusst werden.

2. Bei der epithelialen-leukozytären inflammatorischen Interaktion sind Signaltransduktionswege relevant, die durch die Lipid-basierte Signaltransduktion moduliert werden können. Hier wurde der Lipidmediator PAF identifiziert.

3. N-3 basierte Lipidinfusionen im Vergleich mit n-6 basierten Lipidinfusionen modulieren die akute inflammatorische Lungenschädigung und die systemische Inflammation differentiell mit einer hemmenden Wirkung der n-3 Lipide.

4. Der Lipidmediator PAF ist für die Wirkung der n-3/n-6 Fettsäuren bei der inflammatorischen akuten Lungenschädigung und der systemischen Inflammation relevant.

5. N-3 Fettsäuren modulieren die endotheliale PI3-Kinase im Vergleich mit n-6 Fettsäuren in der Inflammation derart, dass dies inhibierend auf die Adhäsion und pro-apoptotisch wirkt.

6. Monozytäre Lipid Rafts in der Zellmembran werden signifikant von n-3 Fettsäuren sowohl in ihrer Zusammensetzung als auch in ihrer Signaltransduktion beeinflusst, so dass die Calcium-Signaltransduktion und die Adhäsion in der Inflammation im Vergleich mit n-6 Fettsäuren negativ moduliert werden.

7. Die Aktivierung des Lipid-aktivierten Transkriptionsfaktors PPAR-α in der akuten Lungenschädigung wirkt sich hemmend auf die inflammatorische Signaltransduktion aus.

Somit konnte demonstriert werden, dass auf multiplen Ebenen der inflammatorischen Signaltransduktion Lipid-basierte Mechanismen von entscheidender Bedeutung sind. Es wurden komplexe Pathomechanismen aufgezeigt, die kausal an der Entstehung der vaskulären endothelialen bzw. intestinalen epithelialen Inflammation beteiligt sind und somit konnten pathophysiologische Grundlagen potentieller Therapieansätze evaluiert werden.

Die Lipid-basierte Signaltransduktion ist für den Verlauf der Inflammation von entscheidender Bedeutung. Die leukozytäre Inflammation wird sowohl an der endothelialen als auch an der

epithelialen Barriere durch Lipid-Mediatoren wie beispielsweise der Plättchen-aktivierende Faktor, die PI3-Kinase, Eicosanoide und Lipid-modulierte Transkriptionsfaktoren wie PPAR-α als auch durch Membranbestandteile wie Lipid Rafts beeinflusst. Insbesondere die adhäsiven Interaktionen der Leukozyten und Endothelzellen, die eine Schlüsselrolle bei inflammatorischen Prozessen besitzen, werden durch die Lipid-basierte Signaltransduktion signifikant moduliert.

Die therapeutische Nutzung dieser Lipid-basierten Modulation steht bislang erst am Anfang. Bei inflammatorischen Erkrankungen, wie der akuten Lungenschädigung bzw. dem ARDS und der Sepsis könnte eine notwendige Ernährungs-Therapie mit einer potentiellen therapeutischen Intervention kombiniert werden. Insbesondere die hier durchgeführten *in vivo* Studien belegen dafür das anti-inflammatorische Potential in der n-3 basierten Lipidemulsionen. Die Ergebnisse dieser Arbeit demonstrieren insgesamt die zugrundeliegenden Pathomechanismen möglicher zukünftiger Lipid-basierter Therapieoptionen.

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7. Abkürzungsverzeichnis

AA	Arachidonsäure (*arachidonic acid*)
ASA	Acetylsalizylsäure (acetylsalicylic acid)
ARDS	Akutes Lungenversagen (*acute respiratory distress syndrome*)
COX	Cyclooxygenase
DHA	Docosahexaensäure (*docosahexaenoic acid*)
EPA	Eicosapentaensäure (*eicosapentaenoic acid*)
HNE	humane neutrophile Elastase
ICAM-1	*Intercellular adhesion molecule-1*
IL	Interleukin
L-NMMA	L-Monomethyl-L-Arginin
LPS	Lipopolysaccharid (Endotoxin)
LT	Leukotrien
NO	Stickoxid (*nitric oxide*)
PAF	Plättchen-aktivierender Faktor
PG	Prostaglandin
PMN	Polymorphkernige neutorphile Granulozyten (*polymorphonuclear neutrophils*)
PPAR	Peroxysom Proliferator-aktivierter Rezeptor
SIRS	*Systemic inflammatory response syndrome*
STS	Staurosporin
TNF-α	Tumor-Nekrose Faktor-α
VCAM-1	*Vascular cell adhesion molecule-1*
VEGF	*Vascular endothelial growth factor*

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9. Liste der Anlagen

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Anlage 1

Erdogan A#, Schaefer MB#, Kuhlmann CR, Most A, Hartmann M, Mayer K, Renner FC, Schaefer C, Abdallah Y, Hoelschermann H, Schaefer CA

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Activation of Ca^{2+}-activated potassium channels is involved in lysophosphatidylcholine-induced monocyte adhesion to endothelial cells

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Abstract

Objective: Ca^{2+}-activated K^+-channels (BK_{Ca}) play an important role in lysophosphatidylcholine (LPC)-induced endothelial dysfunction. Aim of our study was to investigate whether LPC-induced activation of BK_{Ca} is also involved in monocyte adhesion to endothelial cells (EC).
Methods and results: Measurement of membrane potential (MP) was performed using the fluorescence dye DiBAC. Adhesion of the monocytotic cell line U937 to EC was analysed by ^{3}[H]-thymidine-adhesion-assay. Expression of ICAM-1 and VCAM-1 were analyzed by FACS. LPC induced a hyperpolarization of EC in a dose-dependent manner with the maximum seen with 2 μM. This was prevented by the BK_{Ca}-inhibitor iberiotoxin (IBX, 100 nM). Adhesion of U937 cells to EC was increased after stimulation of EC with LPC. This effect was time-dependent with the maximum seen after 4 h. LPC-induced adhesion was significantly reduced when EC were co-incubated with IBX, or NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI, 5 μM) and also blocked by addition of 2-aminoethoxydiphenylborate (2-APB, 100 μM) or the calcium-chelator BAPTA (10 μM). Stimulation of U937 cells with LPC did not result in an increased adhesion to unstimulated EC.
Conclusion: Activation of the endothelial BK_{Ca} plays an important role in monocyte adhesion to endothelial cells.

Keywords: LPC; Potassium channel; Membrane potential; Endothelial cells; Adhesion

1. Introduction

Atherosclerosis has been shown to be a chronic inflammatory process that is characterized by enhanced expression of adhesion molecules including intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) in the endothelium overlaying atherosclerotic lesions [1,2]. Within an atherosclerotic lesion an accumulation of mononuclear cells in the vascular wall [3] as well as an increased cellular turnover [4] and apoptotic and necrotic cell death [5] occur. Oxidation of low-density lipoproteins (LDL) seem to play a major role in the pathogenesis of atherosclerosis [6–8]. The mechanism by which oxidized LDL (oxLDL) increases atherogenicity is not yet completely understood, but its major component, lysophosphatidylcholine (LPC), appears to be responsible for most of its effects [9,10]. Endothelial functions, such as the synthesis and release of

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nitric oxide, as well as the proliferation and migration of vascular cells, are influenced by oxLDL [9,11]. These cell functions are controlled by changes in the intracellular Ca^{2+}-homeostasis [12]. In endothelial cells, which do not express voltage-gated Ca^{2+}-channels [13] changes in intracellular calcium concentrations are mainly due to Ca^{2+}-release from internal stores or through transmembrane Ca^{2+}-influx, which depends on membrane hyperpolarization [12]. The membrane potential in turn is controlled by activity of ion channels. The driving force for Ca^{2+}-entry is mainly controlled by large conductance Ca^{2+}-dependent BK_{Ca}-channels, inwardly rectifying K^+ channels and at least two types of Cl^--channels [12].

As we have published recently [14], LPC-induced activation of Ca^{2+}-activated K^+-channels (BK_{Ca}) increases generation of reactive oxygen species (ROS) in endothelial cells (EC) to 190% compared to control, as measured by means of DCF-fluorescence intensity. We were also able to demonstrate that this effect was due to activation of NAD(P)H oxidase. ROS-generation in turn promoted proliferation of EC [14]. The aim of the present study was to investigate whether LPC-induced activation of BK_{Ca} also contributes to monocyte adhesion to EC as an approach to vascular inflammation and atherosclerotic plaque-formation.

2. Materials and methods

2.1. Cell culture

Human endothelial cells derived from umbilical cord veins (HUVEC) were isolated by a collagenase digestion procedure and cultured as described by Jaffe et al. [15]. Cells of the passages 1–3 were used in all experiments.

The monocytotic cell line U937 was cultured in RPMI culture medium with the supplementation of 5% fetal calf serum (FCS), and 1% penicillin/streptomycin (all from PAA, Linz, Austria). Cells were splitted 1–4 every 48 h.

2.2. Adhesion-assay

As a model for monocyte adhesion to endothelial cells, the interaction of HUVEC and U937 cells was examined as described before [16]. U937 cells were labeled with [^{3}H]-thymidine (10 μCi/ml; Amersham, Freiburg, Germany) overnight. HUVECs grown to confluency on 24-well plates were stimulated over a period of 15 min to 8 h with combinations of the following substances: LPC (20 μM), iberiotoxin (100 nM, both from Sigma, Deisenhofen, Germany), BAPTA (10 μM), 2-aminoethoxydiphenylborate (2-APB, 100 μM), diphenyleneiodonium (DPI, 5 μM, all from Calbiochem, Bad Soden, Germany). After stimulation, [^{3}H]-labeled U937 cells were added to HUVEC at a density of 1×10^6 cells/ml. Adhesion was allowed for 1 h (37 °C, 5% CO_2). Then, HUVEC were washed three times with PBS (PAA, Linz, Austria) to remove non-adherent cells. HUVEC and adherent [^{3}H]-labeled U937 cells were lysed with NaOH (1 mol/l), and the cell lysate was analyzed by liquid scintillation using a β-counter (Canberra Packard, Dreieich, Germany). Counts were set in relation to the untreated control group, and data is demonstrated as adherence in percentage of control.

2.3. Expression of adhesion molecules

The expression of ICAM-1 and VCAM-1 was detected using a fluorescence-activated cell sorter (FACScalibur, Becton Dickinson) as described before [17]. HUVEC were incubated with LPC (20 μM), DPI (5 μM), IBX (100 nM), or BAPTA (10 μM) for 4 h. Afterwards, cells were trypsinized and incubated with antibodies directed against human ICAM-1 and VCAM-1 (Santa Cruz Biotechnologies, Santa Cruz, USA).

2.4. Measurement of membrane potential

Membrane potential (MP) was assessed as described before [18] using the fluorescence dye bis-1,3-dibutylbarbituric acid-trimethine oxonol (DiBAC). Cells were incubated in a HEPES-buffered bath solution containing 0.5 μM DiBAC for 15–30 min. Coverslips were mounted into a temperature-controlled incubation chamber adapted to a fluorescence microscope. After 1 min, LPC was added. Fluorescence intensities were acquired during intervals of 6 s and averaged over 1 min. Fluorescence was excited at 490 nm and emitted light was detected at 535 nm. Changes in endothelial cell MP were analyzed with the TILL Photonics imaging system. Data were expressed as changes of DiBAC-fluorescence intensity in percent. In some experiments HUVEC were additionally treated with the BK_{Ca}-inhibitor iberiotoxin (100 nM). Calibration of the signal was performed according to the method described by Daut and Langheinrich [19]. The result of these calibration measurements was that changes of 1.026 % of DiBAC-fluorescence intensity equal a change of membrane potential of 1 mV.

2.5. Statstical analysis

The results of all experiments are shown as mean values ± S.E.M. Statistical significant differences were determined by ANOVA followed by a post-hoc Tukey test or a post-hoc holm-sidak test (SPSS for windows, version 11.0).

3. Results

3.1. Increased adhesion of U937 cells to LPC-stimulated HUVEC

The adherence of the monocytotic U937 cells to LPC-stimulated HUVEC was examined. After 4 and 8 h LPC

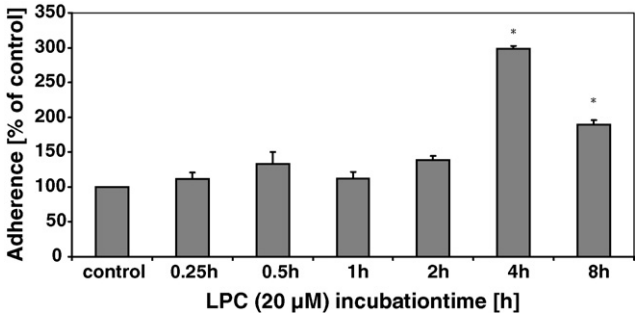

Fig. 1. Adhesion of monocytes to HUVEC is induced by LPC in a time-dependent manner. The adhesion of [^{3}H]-labeled U937 cells to HUVEC after 0.25–4 h of incubation with LPC (20 μM) was measured. Results are expressed as mean values ± S.E.M. of adherent U937 cells in percentage of untreated control ($^*p < 0.01$ vs. control; $n = 6$).

caused a significant increase of U937 adhesion with a maximum seen at 4 h ($n = 6$, $^*p < 0.01$ versus untreated control; Fig. 1). In detail, the adhesion was 111.3 ± 9.7% (15 min), 133.6 ± 16.3% (30 min), 112.0 ± 9.7% (1 h), 138.8 ± 5.3% (2 h), 298.2 ± 4.3% (4 h) and 189.7 ± 5.8% (8 h).

3.2. Adhesion of LPC-stimulated U937 cells to unstimulated HUVEC is not increased

On the other hand, pretreatment of U937 cells with LPC for up to 8 h did not induce a significant increase of adhesion to unstimulated HUVEC ($n = 6$, n.s. versus untreated control, data not shown).

3.3. LPC-induced hyperpolarization of HUVEC

As already described above in Section 2 the membrane potential was assessed using the fluorescence dye DiBAC. Between 5 and 20 μM LPC induced a dose-dependent hyperpolarization of HUVEC with a maximum at 20 μM. Higher doses of LPC did not further enhance hyperpolarization. The effect of LPC was abolished by the BK_{Ca}-inhibitor IBX (100 nM) while IBX alone did not affect the membrane potential ($n = 20$, $^*p < 0.05$ versus control, $^{**}p < 0.01$ versus control; Fig. 2).

3.4. LPC-induced adhesion of U937 cells to HUVEC is modulated by BK_{Ca}

LPC caused a significant increase of U937 adhesion that was significantly reduced by pre-incubation of HUVEC with the BK_{Ca}-inhibitor IBX (100 nM) ($^\#p < 0.01$ versus LPC; Fig. 3). IBX alone did not influence the adhesion of U937 cells to HUVEC ($n = 6$, $^*p < 0.01$ versus untreated control). In detail, adhesion was 323.2 ± 33.2% (LPC), 140.6 ± 11.8% (LPC + IBX), 103.7 ± 5.37% (IBX).

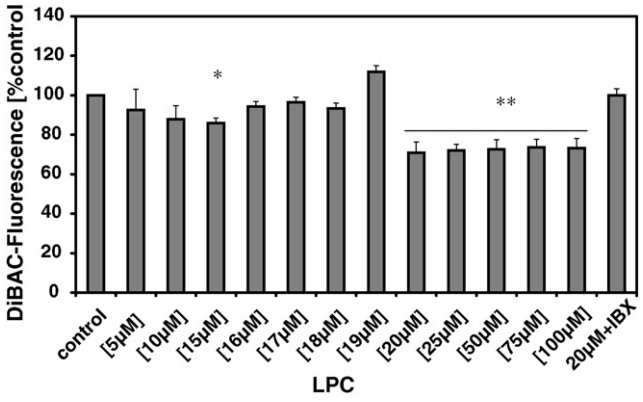

Fig. 2. LPC-induced hyperpolarization of HUVEC. Membrane potential was measured using DiBAC-imaging. Data demonstrate changes of fluorescence-intensity 5 min after application of LPC (5–100 μM) and the combination of LPC (20 μM) + IBX (100 nM) ($n = 20$, $^*p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control).

3.5. LPC-induced adhesion of U937 cells to HUVEC depends on calcium-influx

LPC-induced adhesion of U937 cells to HUVEC was also significantly reduced pre-incubating HUVEC with the calcium-chelator BAPTA (10 μM) or an inhibitor of transmembrane calcium-influx (2-APB, 100 μM; $n = 6$, $^\#p < 0.01$ versus LPC; Fig. 4). In detail, adhesion was 323.2 ± 33.2% (LPC), 77.9 ± 7.5% (LPC + BAPTA), 52.0 ± 8.4% (BAPTA), 57.8 ± 10.2% (LPC + 2-APB), 65.2 ± 7.6% (2-APB).

3.6. LPC-induced adhesion of U937 cells to HUVEC depends on ROS-formation

We were able to demonstrate that the effect of LPC was also significantly reduced by pre-incubation of HUVEC with the NAD(P)H oxidase inhibitor DPI (5 μM; $n = 6$, $^\#p < 0.01$

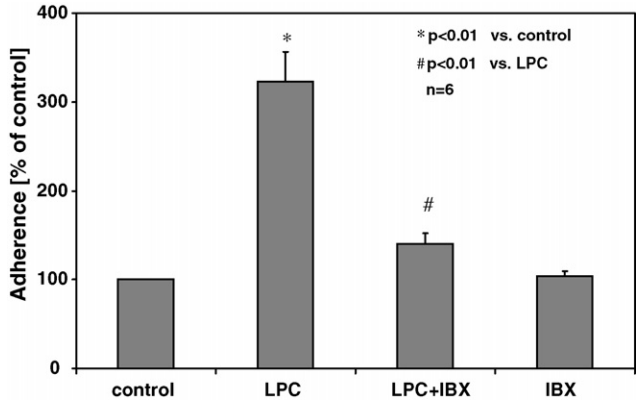

Fig. 3. LPC-induced adhesion of U937 cells to HUVEC is modulated bei BK_{Ca} The adhesion of [^{3}H]-labeled U937 cells to HUVEC after 4 h of incubation with either LPC (20 μM), IBX (100 nM) or LPC + IBX was measured. Results are expressed as mean values ± S.E.M. of adherent U937 cells in percentage of untreated control ($^*p < 0.01$ vs. control, $^\#p < 0.01$ vs. LPC; $n = 6$).

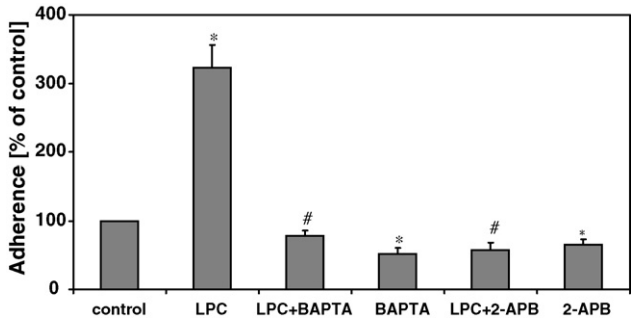

Fig. 4. LPC-induced adhesion of U937 cells to HUVEC depends on Ca^{2+}-influx The adhesion of U937 cells to HUVEC after incubation with combinations of LPC (20 μM), BAPTA (10 μM) or 2-APB (100 μM) was measured. Results are given as mean values ± S.E.M. of adherent U937 cells in percentage of untreated control ($^{*}p < 0.01$ vs. control, $^{\#}p < 0.01$ vs. LPC; $n = 6$).

versus LPC; Fig. 5). In detail, adhesion was 323.2 ± 33.2% (LPC), 68.2 ± 7.6% (LPC + DPI), 68.4 ± 8.5% (DPI).

3.7. Ca^{2+} and ROS are essential for LPC-induced expression of adhesion molecules

The expression of ICAM-1, and VCAM-1 was examined using FACS-analysis. After 4 h LPC (20 μM) significantly increased the expression of both adhesion molecules by more than 50% ($n = 4$; $^{*}p < 0.05$ versus control). This effect of LPC was significantly reduced in the presence of the BK_{Ca}-inhibitor IBX (100 nM), the NAD(P)H-oxidase inhibitor DPI (5 μM), or the Ca^{2+}-chelator BAPTA (10 μM; $n = 4$, $^{\#}p < 0$–05 versus LPC; Fig. 6). In detail, expression of ICAM-1 was 150.2 ± 5.8% (LPC), 115.9 ± 4.1% (LPC + IBX), 112.4 ± 35.8% (LPC + DPI), 100.0 ± 9.8% (LPC + BAPTA) and expression of VCAM-1 was 164.5 ± 33.2% (LPC), 100.4 ± 17.7% (LPC + IBX), 111.9 ± 9.2% (LPC + DPI), 57.1 ± 11.4% (LPC + BAPTA).

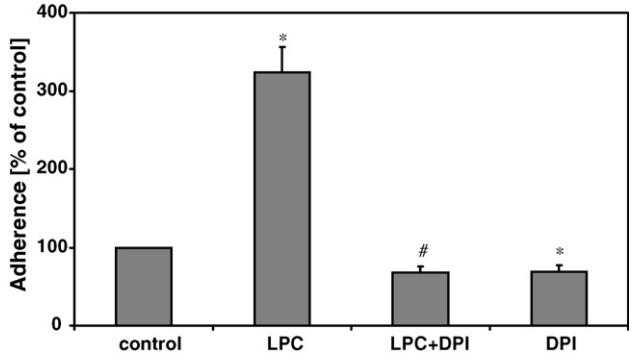

Fig. 5. LPC-induced adhesion of U937 cells to HUVEC depends on ROS-formation. Adhesion was measured after incubation with either LPC (20 μM), LPC + DPI (5 μM) or DPI alone. Results are expressed as mean values ± S.E.M. of adherent U937 cells in percentage of untreated control ($^{*}p < 0.01$ vs. control, $^{\#}p < 0.01$ vs. LPC; $n = 6$).

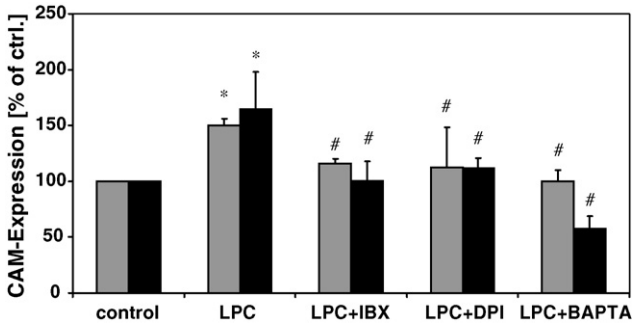

Fig. 6. Ca^{2+} and ROS are essential for LPC-induced expression of adhesion molecules. The expression of ICAM-1 (grey bars) and VCAM-1 (black bars) was detected by FACS-analysis. LPC (20 μM) induced a significant increase of expression of both adhesion molecules after 4 h of incubation time. This effect was significantly reduced in the presence of the BK_{Ca}-inhibitor iberiotoxin (IBX, 100 nM), the NAD(P)H-oxidase inhibitor DPI (5 μM), or the Ca^{2+}-chelator BAPTA (10 μM). Results are expressed as mean values ± S.E.M. of four independent experiments in percentage of untreated control ($^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. LPC).

4. Discussion

The aim of our study was to investigate whether LPC-induced activation of BK_{Ca} contributes to vascular inflammation and atherosclerotic plaque-formation by enhancing monocyte adhesion to EC. As an approach to vascular inflammation adhesion of U937 cells to HUVEC, and expression of endothelial ICAM-1 and VCAM-1 was examined. The expression of both adhesion molecules was significantly increased after stimulation with LPC. Our results are in line with the findings of Chen et al. [20] and Cominacini et al. [21,22]. They observed increased levels of ICAM-1 and VCAM-1 expression in HUVEC after pretreatment with oxLDL or LPC. Contrary to our results, other groups only found ICAM-1, but not VCAM-1 to be up-regulated in HUVEC [23–25]. This discrepancy could be due to differences in incubation times or doses of LPC used.

We also demonstrated an increase of U937 adhesion to LPC-stimulated EC. On the other side, stimulation of U937 cells with LPC did not cause enhancement of adhesion to unstimulated EC. Again, our findings are consistent with previously published data showing an increase of adhesion of monocytes or U937 cells after stimulation of EC with oxLDL [24,26,27]. In contrast to our results, Erl et al. only found oxLDL but not LPC to induce monocyte adhesion to EC [28]. Furthermore, in their setting only the adhesion of MonoMac-6 but not of U937 cells was increased. The discrepancy could be due to dose and incubation-time used, as far as LPC is concerned. As Erl et al pointed out in their article, the different behaviour of U937 cells could be due to different clones of U937 cells and a different expression of the ICAM-1-counter receptor Mac-1.

Frostegard et al. were able to demonstrate that pre-incubation of U937 cells with oxLDL is followed by an increase of adhesion to HUVEC [29]. We were not able to demonstrate that LPC-stimulation of U937 induced enhance-

ment of adhesion. This difference in reactivity could be due to the much longer incubation-time used in their settings. Of course, it would also be possible, that not LPC but other compounds of oxLDL are responsible for the effect described by Frostegard et al. As our experiments demonstrate, LPC induced a time- and dose-dependent hyperpolarization of HUVEC with a maximum seen with 20 μM. Higher doses of LPC did not further strengthen this effect, therefore this dose was used in all other experiments. The effect of LPC was abolished by pre-incubation with IBX, a specific inhibitor of the BK_{Ca}, indicating that this ion channel is responsible for the hyperpolarization. Recently, by measuring single-channel membrane currents using the patch clamp technique, we could directly demonstrate activation of BK_{Ca} by LPC [14]. Since pre-incubation with IBX not only abolished hyperpolarization of EC, but also inhibited expression of the adhesion molecules and adhesion of U937 cells, our experiments demonstrate that activation of BK_{Ca} is an important step in the signalling pathway leading to an increase of ICAM-1 and VCAM-1. To our knowledge, this is the first study indicating involvement of the endothelial BK_{Ca} in LPC-induced expression of adhesion molecules.

The next parameter investigated in this study was the role of cytosolic Ca^{2+}. As we have recently shown, LPC-dependent changes in intracellular calcium homeostasis are due to activation of BK_{Ca}. We were also able to show that generation of ROS via NAD(P)H oxidase depends on transmembrane calcium-influx and is also significantly reduced by inhibition of BK_{Ca} [14]. In the present study chelation of Ca^{2+} and also inhibition of transmembrane calcium-influx resulted in a significant decrease of monocyte adhesion to EC, as well as in a reduced expression of ICAM-1 and VCAM-1. Previously published data demonstrate that LPC induces generation of ROS due to activation of NAD(P)H oxidase [9,14]. In the present study, the impact of LPC-induced ROS-generation on monocyte adhesion to HUVEC was analyzed.

Interestingly, we demonstrated that blocking NAD(P)H oxidase dependent ROS-formation by pretreatment of cells with DPI prevents LPC-induced monocyte adhesion to HUVEC and reduces expression of ICAM-1 and VCAM-1. These results are in line with data published by Cominacini et al. They demonstrated that after binding to the LOX-1 receptor oxLDL activates the redox-sensitive transcription factor nuclear factor kappa B (NF-kappaB) [30], which is involved in the signal-transduction pathway of ICAM-1 and VCAM-1. They were also able to show a reduced expression of these adhesion molecules inhibiting ROS-generation [21,22].

There are several other studies demonstrating that LPC activates NF-kappaB and thereby modulates expression of adhesion molecules and other pro-inflammatory mediators like monocyte chemotractant protein-1 [31,32]. There is also data suggesting involvement of src-protein tyrosine kinases (PTK) in this process [31]. As Nakashima et al. and den Hertog et al. pointed out, activation of PTK also depends on ROS-formation [33,34]. Therefore we suggest that by modulating BK_{Ca} and inducing calcium-influx and ROS-formation LPC activates PTK, and in turn NF-kappaB. Further studies will be needed to identify the specific PTK(s) involved in the LPC-induced signalling pathway.

In conclusion, the present study outlines part of the intracellular signalling mechanism which is involved in the inflammatory response of human endothelial cells induced by LPC. Activation of the endothelial BK_{Ca} results in a hyperpolarization of EC. This is followed by transmembrane calcium-influx, which in turn leads to an increase of ROS-formation due to activation of the NAD(P)H oxidase. Blocking either hyperpolarization, generation of ROS or calcium-influx results in a reduced expression of ICAM-1, VCAM-1 and prevents LPC-induced adhesion of U937 cells to EC.

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Anlage 2

Schaefer MB, Schaefer CA, Hecker M, Morty RE, Witzenrath M, Seeger W, Mayer K.

Co-incubation of PMN and CaCo-2 cells modulates inflammatory potential.

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Co-incubation of PMN and CaCo-2 cells modulates inflammatory potential

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Abstract: Polymorphonuclear granulocytes (PMN) are activated in inflammatory reactions. Intestinal epithelial cells are relevant for maintaining the intestinal barrier. We examined interactions of PMN and intestinal epithelial cell-like CaCo-2 cells to elucidate their regulation of inflammatory signalling and the impact of cyclooxygenase (COX), nitric oxide (NO) and platelet-activating factor (PAF). Human PMN and CaCo-2 cells, separately and in co-incubation, were stimulated with the calcium ionophore A23187 or with N-Formyl-methionyl-leucyl-phenylalanin (fMLP) that activates PMN only. Human neutrophil elastase (HNE) and respiratory Burst were measured. To evaluate the modulation of inflammatory crosstalk we applied inhibitors of COX (acetyl salicylic acid; ASA), NO-synthase (N-monomethyl-L-arginin; L-NMMA), and the PAF-receptor (WEB2086). Unstimulated, co-incubation of CaCo-2 cells and PMN led to significantly reduced Burst and elevated HNE as compared to PMN. After stimulation with A23187, co-incubation resulted in an inhibition of Burst and HNE. Using fMLP co-incubation failed to modulate Burst but increased HNE. Without stimulation, all three inhibitors abolished the effect of co-incubation on Burst but did not change HNE. ASA partly prevented modulation of Burst. L-NMMA and WEB2086 did not change Burst but abolished mitigation of HNE. Without stimulation, co-incubation reduced Burst and elevated HNE. Activation of PMN and CaCo-2 cells by fMLP as compared to A23187 resulted in a completely different pattern of Burst and HNE, possibly due to single vs. dual cell activation. Anti-inflammatory effect of co-incubation might in part be due to due to COX-signalling governing Burst whereas NO- and PAF-dependent signalling seemed to control HNE release.

Key word: Polymorphonuclear granulocytes; CaCo-2 cells; Inflammation; Nitric oxide; Platelet-activating factor.

Introduction

The intestine plays a major role in inflammation not only orchestrating the mucosal immune response but also due to its epithelial barrier function (1). The co-operation of polymorphonuclear neutrophils (PMN) and intestinal epithelial cells in inflammatory signalling is an underestimated but important concept (2). Furthermore, PMN are the first line of defence against infection maintaining epithelial integrity.

The intestine makes up an enormous surface within the body being one of the most relevant barriers for invading pathogens; and its integrity is of tremendous importance for survival of the host (1). In inflammation, dysfunction between intestinal epithelial cells and PMN plays a major role and may lead to failure of the intestine barrier with the fatal consequences of septicaemia, secondary acute organ failure, and septic shock. In fact, the gut is recognized as the motor of sepsis with sepsis being the leading cause of death in non-coronary intensive care units (3, 4).

PMN modulate inflammation using a multitude of different mediators. Intercellular and paracrine signal transduction is governing their actions, e.g. via platelet activating factor (PAF) or nitric oxide (NO) (5-7). PAF is an inflammatory biolipid produced from membrane glycerophospholipids acting through the activity of its G-protein coupled receptor (8). NO can exert both protective and pro-inflammatory actions in the gastrointestinal tract (9). Human neutrophil elastase (HNE) a serine protease released by PMN plays a key role in inflammatory diseases (10). It has been used as a marker of inflammation in CaCo-2 cells, a well-established intestinal epithelial cell line (2, 11). In PMN, HNE regulates other inflammatory and anti-inflammatory players like cytokines and chemokines (12). Reactive oxygen species like O^{2-}-anions promote inflammation by acting on the plasma membrane, damaging its organization and releasing various pro-inflammatory agents (13). Measuring O^{2-}-anions (respiratory Burst) is a recognized means to evaluate inflammation in PMN (14, 15) and in CaCo-2 cells (16, 17).

The aim of this study was to investigate PMN and CaCo-2 interaction in inflammation. Therefore, human PMN and CaCo-2 cells, separately and in co-incubation, were stimulated with the calcium ionophore A23187 or with N-Formyl-methionyl-leucyl-phenylalanin (fMLP). A23187 is a non-receptor operating stimulus that induces an influx of calcium into the cell which leads to various inflammatory cellular effects e.g. promotion of PAF signalling. The bacterial chemotactic peptide fMLP is a receptor operating inflammatory stimulus with its receptor found on PMN but not on CaCo-2 cells (18-21). To evaluate the modulation of inflammatory crosstalk

between the different cells we measured HNE and respiratory Burst and applied inhibitors of cyclooxygenase (COX: acetylsalicylic acid; ASA), NO-synthase (N-monomethyl-L-arginine; L-NMMA), and the PAF-receptor (WEB2086).

Materials and Methods

Materials

Chemicals of highest purity were obtained from Merck (Darmstadt, Gernany). fMLP, superoxide dismutase (SOD), cytochrome c and bovine serum albumin (BSA) were bought from Sigma Chemical (Deisenhofen, Germany). Acetylsalicylic acid (ASA) was ordered from Bayer (Leverkusen, Germany), WEB2086 from Boehringer Ingelheim (Ingelheim, Germany) and L-NMMA as well as A23187 by Calbiochem (Bad Soden, Germany). Tissue culture plastic supplies were purchased from Becton-Dickinson (Heidelberg, Germany). Cell culture reagents, buffer, and media were from PAN (Aidenbach, Germany).

Isolation of PMN

Peripheral venous blood samples from healthy volunteers that were matched for gender and age were drawn. EDTA-anticoagulated blood was centrifuged in a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient and erythrocytes were sedimented using polyvinyl alcohol (Merck-Schuchardt, Hohenbrunn, Germany). PMN were isolated as described (22). PMN purity was > 98% (Pappenheim staining) and viability was 92-100 % (trypan blue exclusion) throughout.

Oxidative Burst

Superoxide anion generation (O_2^-) generation was assessed as superoxide dismutase-inhibitable reduction of cytochrome C according to Cohen (23) as described (24).

PMN, CaCo-2 cells, or co-incubations of PMN with CaCo-2 cells were activated according to the experimental protocol for 15 min in Hank´s balanced salt solution (HHBSS++).

Duplicate reaction mixtures containing cytochrome in the presence or absence of superoxide dismutase were performed at 37°C. Incubations were terminated by incubation on ice for 5 min followed by centrifugation at 4 °C at 1.200×g. O_2^- release was quantified as relative extinction at 546 nm in an Uvicon Spectrophotometer and expressed as nmol/ml of O_2^-.

Elastase

Elastase release was taken as marker for PMN degranulation and measured according to standard procedures (25, 26).

For induction of elastase release, PMN, CaCo-2 cells or co-incubations of PMN with CaCo-2 cells were activated according to the experimental protocol for 15 min in HHBSS++. Incubations were terminated by incubation on ice for 5 min followed by centrifugation at 4 °C at 1.200×g. The cell-free supernatant was harvested and analyzed as described above for elastase activity expressed in U/l in an Uvicon Spectrophotometer.

Cell culture

CaCo-2 cells were generously donated from Prof. H. Daniel, who had obtained them from ATCC (HTB 37, passage 31). Cells were cultured and passaged in Dulbecco's Modified Eagle Medium as previously described (27). CaCo-2 cells were used for experiments a) after being confluent and b) when measurements of brush border enzyme alkaline phosphatase (AP) reached a plateau phase occurring usually at day 14 of culture.

Detection of fMLP-receptor

Using FACScan from Becton Dickinson (Mountain View, CA, USA), PMN and CaCo-2 were examined for fMLP receptors applying fMLP-FITC/F1314 from Molecular Probes (Eugene, OR, USA) as described (28).

Experimental protocol

Culture media was removed and CaCo-2 cells were washed twice followed by a two hour incubation time with HHBSS++ at 37°C with 5% CO2 in an incubator in 12-well plates. In experiments with inhibitors, 300µM ASA, 10µM L-NMMA, or 10µM WEB 2086 were applied during that time. Then, HHBSS++ was removed. For measuring respiratory Burst, cytochrome or cytochrome/SOD solution was added. For measuring elastase, no cytochrome was added. In experiments with inhibitors, these were again applied. 5 million PMN per well were given to those sets of experiments running with PMN. A 15 min incubation time at 37°C in a water bath either with 0, 0.1, 1, or 10µM fMLP or 0, 0.1, 1, or 10 µM A23187 ensued.

Statistics

Data are given as mean ± SEM. Two-way analysis of variance and Student-Newman-Keuls post-hoc test was performed to test for differences between different experimental groups. Probability (p) values < 0.05 were considered to indicate statistical significance. Analysis was carried out using SigmaStat® version 3.5.

Results

Co-incubation of PMN and CaCo-2 inhibited respiratory Burst under control conditions and after stimulation with A23187.

CaCo-2 cells, PMN, as well as both cells in co-incubation were stimulated with 0, 0.1, 1, and 10 µM A23187. At any concentration, incubation CaCo-2 cells induced a respiratory Burst close to the detection limit. Under control conditions, co-incubation of PMN and CaCo-2 cells reduced respiratory Burst significantly compared to PMN ($p<0.05$; n=5-6; fig. 1a). Stimulation of PMN resulted in an increase to 0.133 ± 0.026 nmol/ml, 0.184 ± 0.024 nmol/ml, 0.254 ± 0.009 nmol/ml and 0.199 ± 0.021 nmol/ml, respectively. However, co-incubation of PMN and CaCo-2 cells led to a significant reduced respiratory Burst compared to PMN, with 0.052 ± 0.014 nmol/ml, 0.067 ± 0.015 nmol/ml, 0.090 ± 0.017 nmol/ml and 0.121 ± 0.020 nmol/ml, respectively ($p<0.05$; n=5-6; fig. 1a).

Co-incubation of PMN and CaCo-2 did not modify respiratory Burst after stimulation with fMLP.

CaCo-2 cells, PMN, as well as both cells in co-incu-

bation were stimulated with 0, 0.1, 1, and 10 µM fMLP. Under control conditions, co-incubation of PMN and CaCo-2 cells reduced respiratory Burst significantly, compared to PMN ($p<0.05$; n=7; fig. 1b). Stimulation of PMN led to a respiratory Burst of 0.122 ± 0.035 nmol/l, 0.188 ± 0.033 nmol/l, 0.221 ± 0.025 nmol/l and 0.205 ± 0.034 nmol/l, respectively; but stimulation of CaCo-2 cells induced a respiratory Burst close to the detection limit at any concentration. Co-incubation of PMN and CaCo-2 cells did not modify respiratory Burst significantly compared to PMN (n=7; fig. 1b).

ASA partly abolished reduction of respiratory Burst by co-incubation of PMN and CaCo-2 under control conditions and after stimulation with A23187.

Stimulation of PMN with 0, 0.1, 1, and 10 µM A23187 in the presence of ASA resulted in a respiratory Burst of 0.053 ± 0.013 nmol/ml, 0.138 ± 0.039 nmol/ml, 0.227 ± 0.053 nmol/ml and 0.196 ± 0.043 nmol/ml, respectively. Stimulation of CaCo-2 cells led to a minor respiratory Burst. When PMN and CaCo-2 cells were stimulated in co-incubation with 0.1 and 10 µM A23187 and under control conditions, ASA prevented a significant reduction of respiratory Burst (n=4-5; fig. 1c).

L-NMMA prevented a reduction of respiratory Burst by co-incubation of PMN and CaCo-2 under control conditions but did not influence the modulation after stimulation with A23187.

CaCo-2 cells, PMN, as well as both cells in co-incubation were stimulated in the presence of L-NMMA with A23187 as detailed. Stimulation of CaCo-2 cells led to a respiratory Burst close to the detection limit. Under control conditions, L-NMMA prevented the co-incubation induced reduction of Burst. L-NMMA did not change the pattern of reduced release of superoxide anions in the setting of co-incubation ($p<0.05$; n=4-5; fig. 1d).

WEB2086 prevented a reduction of respiratory Burst by co-incubation of PMN and CaCo-2 under control conditions but did not influence the modulation after stimulation with A23187.

CaCo-2 cells, PMN, as well as both cells in co-incubation were stimulated with A23187 in the presence of WEB2086 as detailed. Incubation of CaCo-2 cells resulted in a Burst close to the detection limit. Under control conditions, WEB2086 prevented a co-incubation induced reduction of Burst. The co-incubation dependent reduction of Burst was not modified by WEB2086

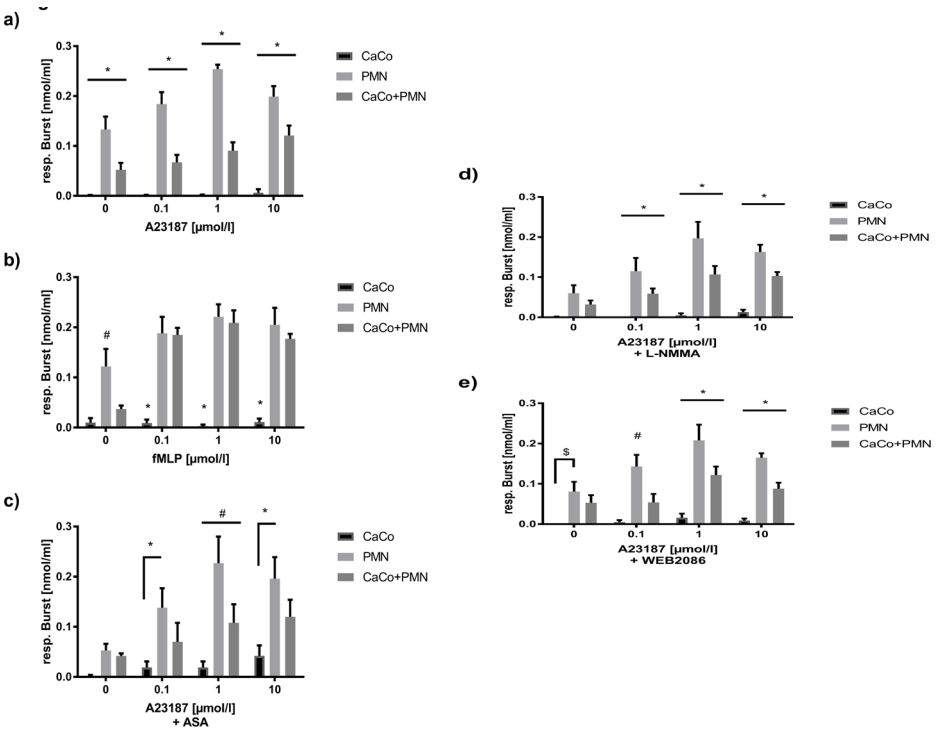

Figure 1. For analysis of respiratory Burst, CaCo-2 cells and PMN separately as well as in co-incubation were stimulated with 0, 0.1, 1, and 10 µM A23187 (a, c, d, e) or with 0, 0.1, 1, and 10 µM fMLP (b). **a)** CaCo-2 cells differed significantly from both other groups with PMN present (*, $p<0.05$; n=5-6). Co-incubation of PMN and CaCo-2 cells led to a significant reduced respiratory Burst compared to PMN stimulated separately (*, $p<0.05$; n=5-6). **b)** After stimulation with fMLP, CaCo-2 cells differed significantly from both other groups with PMN present (*, $p<0.05$; n=7). In control experiments, CaCo-2 cells were significantly different to the PMN group only (#, $p<0.05$ vs. PMN; n=7) but co-incubation with CaCo-2 cells led to a decrease compared to the PMN group. After stimulation with fMLP, co-incubation of PMN and CaCo-2 did not modify respiratory Burst significantly compared to PMN though in control experiments. **c)** In experiments applying the COX-inhibitor ASA, CaCo-2 cells differed significantly from the PMN group (*, $p<0.05$; n=4-5) but in the control experiments. Using 1 µM A23187, all groups differed significantly from each other (#, $p<0.05$, n=4-5). Stimulating with 0.1 and 10 µM A23187, ASA prevented a significant co-incubation-dependent reduction (n=4-5). **d)** In experiments applying the NO-inhibitor L-NMMA, all groups differed significantly from each other (*, $p<0.05$; n=4-5) after stimulation, while in the control group no significant differences were found. **e)** In experiments applying the PAF-inhibitor WEB2086, the PMN group differed significantly from the CaCo-2 group in all settings ($, #, *, $p<0.05$, n=4). Using 0.1 µM A23187, PMN cells were also significantly different from the co-incubation group (#, $p<0.05$, n=4). Applying 1 and 10 µM A23187, all groups differed significantly from each other (*, $p<0.05$, n=4). Data are given as mean ± SEM.

stimulating with 1 or 10 µM A23187 ($p<0.05$, n=4; fig. 1e).

Co-incubation of PMN and CaCo-2 increased elastase under control conditions but led to a reduction after stimulation with A23187.

Stimulation of CaCo-2 cells did not lead to a release of elastase at neither concentration of A23187. Measurement of elastase after stimulation of PMN with 0, 0.1, 1 and 10 µM A23187 revealed 1.7 ± 0.4 U/l, 3.7 ± 1.8 U/l, 9.5 ± 1.8 U/l and 12.7 ± 1.7 U/l, respectively. Under control conditions, co-incubation led to a significant increase of elastase ($p<0.05$; n=6; fig 2a). Interestingly, co-incubation of PMN and CaCo-2 cells resulted in a significant reduced elastase release when stimulated with 1 and 10 µM A23187 ($p<0.05$; n=6; fig 2a).

Co-incubation of PMN and CaCo-2 increased elastase release under control conditions and after stimulation with fMLP.

CaCo-2 cells, PMN, as well as both cells in co-incubation were stimulated with 0, 0.1, 1, and 10 µM fMLP. Stimulation of CaCo-2 cells did not induce an elastase release at neither concentration of fMLP. Stimulation of PMN led to an elastase release of 1.7 ± 0.4 U/l, 2.0 ± 0.3 U/l, 1.9 ± 0.3 U/l and 2.1 ± 0.3 U/l, respectively. However, without and with stimulation co-incubation of PMN and CaCo-2 cells resulted in a significantly increased elastase release compared to PMN with 3.3 ± 0.6 U/l, 7.4 ± 1.5, 7.4 ± 1.2 and 7.6 ± 1.3, respectively ($p<0.05$; n=6; fig. 2b).

ASA failed to modulate elastase release by co-incubation of PMN and CaCo-2 under control conditions and after stimulation with A23187.

The cells were stimulated with A23187 in the presence of ASA as described. While stimulation of CaCo-2 cells resulted in an elastase release close to the detection limit, stimulation of PMN induced an elastase release of 1.4 ± 0.2 U/l, 3.8 ± 1.3 U/l, 11.8 ± 2.7 U/l and 18.2 ± 1.2 U/l, after stimulation with 0, 0.1, 1 and 10 µM A23187, respectively. Co-incubation of PMN and CaCo-2 cells with or without ASA resulted in a similar pattern of reduced elastase release ($p<0.05$; n=5; fig. 2c).

L-NMMA did not change pattern under control conditions but modulated the reduced elastase release by co-incubation of PMN and CaCo-2 after stimulation with A23187.

CaCo-2 cells, PMN as well as both cells in co-incubation were stimulated with A23187 in the presence of L-NMMA as detailed. Stimulation of CaCo-2 cells resulted in an elastase release close to the detection limit. L-NMMA did not modify the pattern of elastase release under control conditions. In contrast, when PMN and CaCo-2 cells were stimulated with 1 or 10 µM A23187,

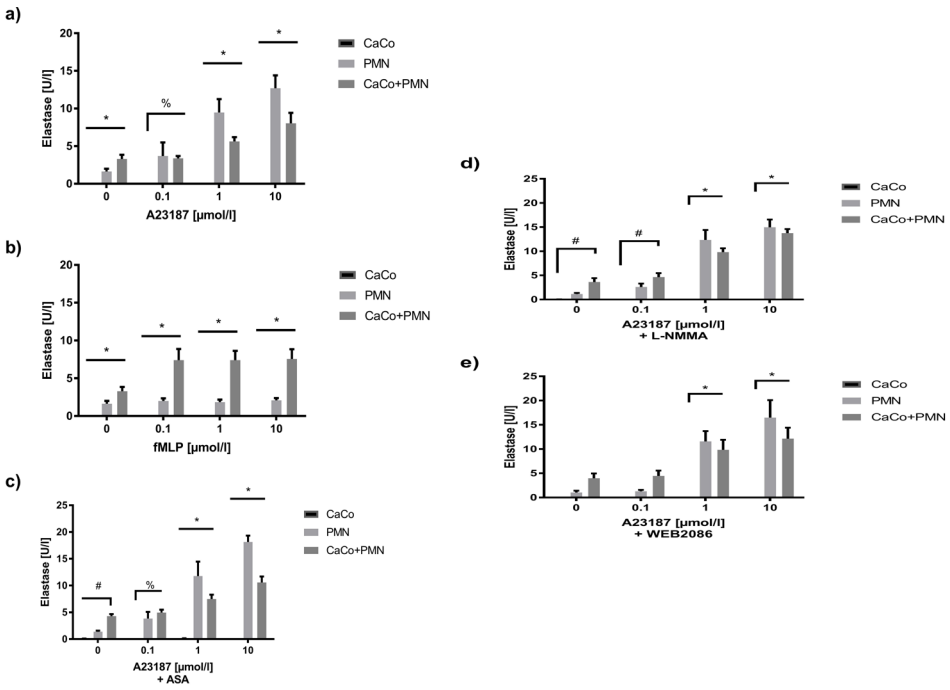

Figure 2. For analysis of elastase release, CaCo-2 cells and PMN separately as well as in co-incubation were stimulated with 0, 0.1, 1, and 10 µM A23187 (a, c, d, e) or with 0, 0.1, 1, and 10 µM fMLP (b). In all experiments CaCo-2 did yield elastase close to detection limits. **a)** CaCo-2 cells differed significantly from both other groups in all experimental settings (*, %, $p<0.05$; n=6). Under control conditions, co-incubation increased elastase significantly compared to PMN (*, $p<0.05$). 0.1 µM A23187 induced a significant difference only between CaCo-2 cells and both other groups (%, $p<0.05$). Co-incubation of PMN and CaCo-2 cells resulted in a significant reduced elastase release when stimulating with 1 and 10 µM A23187 (*, $p<0.05$). **b)** All groups differed significantly from each other with the co-incubation group inducing the highest elastase release (*, $p<0.05$; n=6-7). **c)** In all experiments applying the COX inhibitor ASA, CaCo-2 cells differed significantly from the co-incubation group (#, %, *, $p<0.05$; n=5); after stimulation with A23187 CaCo-2 differed also from the PMN group (%, *, $p<0.05$). Co-incubation reduced elastase compared to PMN significantly using 1 and 10 µM A23187 (*, $p<0.05$). **d)** In experiments applying the NO-inhibitor L-NMMA using 0 and 0.1 µM A23187, CaCo-2 cells differed significantly from CaCo-2 co-incubation group (#, $p<0.05$; n=5). At concentrations of 1 and 10 µM A23187, CaCo-2 cells differed significantly from both other groups (*, $p<0.05$; n=5). **e)** In experiments applying the PAF-inhibitor WEB2086, stimulating with 1 and 10 µM A23187, the CaCo-2 group differed significantly from the PMN and from co-incubation group (*, $p<0.05$; n=4). Data are given as mean ± SEM.

L-NMMA prevented the significant co-incubation-dependent inhibition of elastase production ($p<0.05$; n=5; fig. 2d).

WEB2086 did not change pattern of elastase release under control conditions but abolished the co-incubation-dependent reduction of elastase.

CaCo-2 cells, PMN, or both cells in co-incubation were stimulated with A23187 in the presence of WEB2086 as detailed. Incubating CaCo-2 cells resulted in a non-detectable elastase release. WEB2086 did not modify the pattern of elastase release under control conditions. Interestingly, WEB2086 prevented a co-incubation-dependent reduction of elastase release when stimulated with 1 or 10 μM A23187 ($p<0.05$; n=4; fig. 2e).

Discussion

The aim of this study was to investigate interaction of PMN and CaCo-2 under inflammatory conditions. Therefore, human PMN and CaCo-2 cells, separately and in co-incubation, were stimulated with the calcium ionophore A23187 or with fMLP. Under control conditions, co-incubation of PMN and CaCo-2 cells reduced Burst and elevated HNE as compared to PMN. When stimulated with A23187, co-incubation of CaCo-2 cells and PMN as compared to PMN alone reduced release of HNE and Burst significantly. This reduction was not found using fMLP. The co-incubation induced reduction of Burst seen under control conditions was not present after stimulation with fMLP. Instead, fMLP induced a similar Burst of PMN alone and in co-incubation.

Under control conditions and after stimulation with fMLP, HNE was increased in co-incubation experiments compared to PMN. FMLP did not induce a dose-dependent increase in HNE of PMN alone. Though, co-incubation resulted in an enhanced release of HNE displaying a ceiling-effect.

In control setting, all three inhibitors (ASA, L-NMMA and WEB2086) abolished the inhibitory effect of co-incubation on Burst but did not change pattern of HNE. Interestingly, only after stimulation with A23187 all three inhibitors provoked a slightly stronger CaCo-2 response in Burst compared to experiments without inhibitors with ASA being the most effective one. This was not the case in HNE experiments. ASA partly influenced the co-incubation dependent reduction in respiratory Burst significantly but failed to modulate HNE. However, using inhibitors of the NO-synthase and PAF, Burst was unchanged but the mitigation of HNE release was abolished after stimulation with A23187.

The model of PMN and CaCo-2 co-incubation to evaluate inflammation is well recognized in literature (29). Using this model, we could demonstrate that under control conditions and after stimulation with A23187, co-incubation of PMN and CaCo-2 inhibited respiratory Burst. In contrast, fMLP abolished co-incubation induced suppression of Burst seen under control conditions, but did not provoke a difference between PMN and PMN and CaCo-2 after stimulation. It has been already observed that intestinal epithelial cells like CaCo-2 cells may exert bactericidal activity (30). Though, in this inflammatory setting, we could demonstrate for the first time that a co-incubation of PMN and CaCo-2 cells led to a significant diminished 0^{2-} and elastase production after stimulation with A23187. In contrast, a pro-inflammatory effect of supernatant from hypoxia-re-oxygenation challenged CaCo-2 cells on PMN was described (31). However, this model deprived both cells from their direct interaction. Our experimental setting does not allow to determine if the effects observed are due to direct cell-cell contact or due to indirect – e.g. paracrine – mediators. Further experimental settings, e.g. using transwells, would be necessary to address this question.

Wang et al. showed that calcium ionophore A23187 in similar concentrations as used in our study increased intracellular calcium in CaCo-2 cell and initiated apoptosis in a dose dependent manner (32). Since the effect of A23187 on PMN is well recognized (33) we conclude that using A23187, we definitively stimulated both cell types. In contrast to the A23187-induced effect, our experiments revealed that co-incubation of PMN and CaCo-2 did not modify the respiratory Burst after stimulation with fMLP. We speculate that this effect is due to CaCo-2 cells lacking a fMLP-receptor and therefore an inflammatory signalling after challenge with fMLP. Supporting this notion, we did not find an expression of fMLP-receptor on the surface of our CaCo-2 cells using FACS. In contrast to our findings, Carlson et al. found that the polarized human intestinal epithelial cell line Caco2bbe could be stimulated with fMLP and this effect could be prevented using a fMLP-receptor antagonist (21). The difference in the two cells lines polarized versus non-polarized may be fully responsible inducing the contrasting results. In consequence, stimulating with fMLP actually led to a stimulation of PMN only. We assume this to be the reason why the co-operative inhibitory effect was lost when both cell types were stimulated using the receptor-independent stimulus A23187. In addition, Foster et al. pointed out that fMLP is absorbed by the intestinal oligopeptide transporter, hPEPT1, and is transported across CaCo-2 cells (34). This might also lead to a clearance of fMLP, but also to activation of alternative signalling pathways in CaCo-2 cells.

Considering the effect of co-incubation dependent inhibition of Burst we observed under control condition without any stimulation, we speculate that it must be due to a basal stimulation of both cell types e.g. by calcium. As a calcium ionophore, A23187 would increase this effect as demonstrated in our experiments. Though, fMLP would abolish the basal calcium-induced effect by its strong receptor-dependent activation of PMN only. This would explain, why we saw a basal co-incubation dependent inhibition of Burst that is abolished by fMLP.

We observed that co-incubation of PMN and CaCo-2 increased elastase release under control conditions and after stimulation with fMLP. Since elastase facilitates PMN transmigration (35), a concordant elevation when CaCo-2 meet PMN and with the chemoattractant fMLP appears to be a physiological reaction. In contrast, a previous study from another group showed a reduced elastase release after PMN and CaCo-2 co-incubation after hypoxia/re-oxygenation and bacterial challenge (11). The question remains why stimulation with A23187 induced a co-incubation dependent inhibition of elastase in contrast to basal conditions and to stimulation with fMLP. The contrasting effects of fMLP

and A23187 could be explained by the single vs. dual cell activation, respectively as explained above. The difference under basal conditions of elastase and Burst remain a subject for further investigations. One hypothesis could be that basal dual cell activation by calcium is not that effective in elastase experiments.

We speculate that the co-incubation dependent inhibition of Burst and HNE might depend on signalling pathways activated by A23187- induced calcium like those of cyclooxygenase, NO or PAF (36-38). We observed that inhibitors against cyclooxygenase (ASA), NO (L-NMMA) or PAF (WEB2086) all abolished the co-incubation induced inhibitory effect under basal conditions in Burst experiments. This seemed to be basically due to a slightly diminished PMN response when applying the inhibitors.

Analysing elastase, inhibitors did not change the pattern of increased elastase after co-incubation.

Our data depicted that ASA, an inhibitor of the cyclooxygenase, partly abolished the reduction of respiratory Burst but failed to modulate reduction of elastase release. We conclude that co-incubation dependent inhibition of Burst is in part related to cyclooxygenase signal transduction. Fukui et al. found that ASA-induced ROS (reactive oxygen species) production specifically modified the expression of ZO-1 protein and induced increased cell permeability, which may ultimately cause small intestinal mucosal injury (39). However, the concentrations ASA used (10mmol/l (39) vs. 300µmol/l) are not comparable. Nevertheless, a small effect of ASA on the ROS production may counteract the inhibitory effect due to the co-incubation. In line with our results, Egger et al. did not observe a significant alteration in PMN elastase detected in the blood of atherosclerosis patients after ASA treatment (40). We conclude that inhibiting cyclooxygenase in our setting has no significant effect on elastase release but slightly antagonized the reduction of O^{2-} production by PMN and CaCo-2 co-incubation. We speculate this might be due to ASA- induced ROS production in CaCo-2 cells (39).

NO is an important final effector of mucosal injury in inflammatory bowel disease (41). Therefore, we examined the effect of L-NMMA, an inhibitor of the nitric oxide synthase, on PMN and CaCo-2 co-incubation in inflammation. L-NMMA failed to significantly modulate reduction of respiratory Burst by co-incubation of PMN and CaCo-2 after stimulation with A23187. Though, our data indicated that L-NMMA did significantly modulate reduced elastase release under these conditions. We conclude that co-incubation-dependent inhibition of elastase after stimulation with A23187 is in part related to NO-signal transduction.

Brasse-Lagnel et al. demonstrated that the inhibitory effect of IL1-1β on argininosuccinate synthase activity is linked to the production of NO since it was totally blocked in the presence of L-NMMA. They resumed that such an inhibitory effect of NO may be related, at least in part, to S-nitrosylation of the proteins in CaCo-2 cells (42). Banan et al. proved that ethanol-induced microtubule skeleton damage and intestinal barrier dysfunction require iNOS activation followed by NO overproduction and $ONOO^-$ formation (43). We do not think that iNOS does play a role in our setting as its induction required a longer term challenge with IFN-γ-containing combinations of cytokines in CaCo-2 cells (44). However, NO attenuated elastase release from PAF-primed PMN through an intracellular cGMP-dependent signal transduction pathway (45). We think that NO-release is an important contributor to the reduced elastase release possibly by nitrosylation of (signalling)-proteins or by increasing intracellular cGMP.

We furthermore showed that the PAF-receptor inhibitor WEB2086 failed to significantly modulate the reduction of respiratory Burst by co-incubation of PMN and CaCo-2 after stimulation with A23187 but abolished the reduction of elastase release under these conditions. We conclude that co-incubation dependent inhibition of elastase after stimulation with A23187 is in part dependent on PAF-signal transduction. PAF is an important paracrine and autocrine mediator in PMN and CaCo-2 cells and its receptor is constitutively expressed and regulated (46). PAF-induced NF-κB activation may lead to IL-8 production in intestinal epithelial cells (47, 48). PAF and LTB4 may prime human PMN for the concordant release of elastase, generation of O^{2-}, and CD11b up-regulation. Therefore, PAF produced by PMN could play a role as positive feedback loop for further inflammatory activation (49). PAF-PAF-receptor interactions are involved in the adhesion of PMN to endothelial cells; inhibition of the PAF-receptor may reduce PMN adhesion to endothelial cells possibly by interfering with the outside-in signalling leading to firm adhesion (50). In differentiated CaCo-2 cells, infection by *S. enteritidis* increased PAF levels and activated the enzymes of the remodelling pathway cytosolic phospholipase A_2, which catalyses the formation of the PAF precursor lyso-PAF, and lyso-PAF acetyltransferase in a Ca^{2+}-dependent pathway (51). We conclude that the PAF-PAF-receptor signal transduction seemed to play an important role in both, PMN (52) and CaCo-2 inflammatory signalling, in particular in their cooperation.

It is interesting that different inhibitors of NO-synthase and PAF influenced the inhibition of elastase after PMN and CaCo-2 co-incubation but not the reduction in O^{2-} production. Released elastase might be responsible at least in part for PMN transmigration towards an inflammation focus through the intestinal epithelial barrier. Therefore, a tightly regulated release linked to multiple signalling pathways may ensure their way through the epithelium. Serine proteases like elastase activate specifically pro-inflammatory cytokines, lead to the activation of different receptors and act as key regulators of cell signalling during inflammation (53).

On the other hand, an overproduction of O^{2-} might damage both cell types. Therefore a reduction in O^{2-} generation due to co-operative inhibition if both cells are in close contact may be a useful mechanism to avoid mutual damage.

Another reason why elastase release but not respiratory Burst was modulated by NO- and PAF-inhibitors might be related to the fact that ROS may be produced by both, PMN and CaCo-2 cells, whereas elastase is only generated by PMN. Nevertheless, an interaction between both cell types may influence the release of the enzyme (54). This finding requires further investigation.

Other investigators also considered the migration of PMN across the intestinal epithelium as a histopathological hallmark of many mucosal inflammatory dis-

eases (55). Therefore, it is of relevance to further investigate the interaction of PMN and intestinal epithelial cells in inflammation. This might help understand regulatory mechanisms in inflammatory signalling. In consequence, a better and more specific treatment for patients suffering from intestinal inflammatory disorders should be available in future.

Without stimulation, PMN and CaCo-2 co-incubation reduced respiratory Burst. This might be due to cell-cell based interaction or a basal stimulation of both cell types since it was abolished by fMLP which only stimulates PMN. When stimulated in co-incubation by the calcium ionophore A23187, which affects both cell types, Burst and elastase release was inhibited. Cyclooxygenase might be in part relevant for co-incubation dependent inhibition of Burst since ASA partly abolished this anti-inflammatory effect.

Co-incubation increased elastase release under basal conditions and after stimulation with fMLP. When PMN and CaCo-2 were co-incubated, A23187 provoked an inhibition of elastase. NO- and PAF-signalling seemed to play an important role in the inhibitory signalling measuring elastase under conditions of co-incubation since L-NMMA and WEB2086 abolished the anti-inflammatory effect.

These findings might be relevant for the interactive signalling between neutrophils and intestinal epithelial cells in inflammatory diseases.

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This manuscript includes portions of the doctoral thesis of Christian Schaefer.

Authors´ contribution

MBS wrote the manuscript. CAS carried out the experiments. MH and REM helped analysing and interpreting the results. MW and WS supervised the study. KM conceived the study and developed the design. All authors participated in analysing and interpreting the results, read and approved the final manuscript.

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Anlage 3

Schaefer MB, Ott J, Mohr A, Bi MH, Grosz A, Weissmann N, Ishii S, Grimminger F, Seeger W, Mayer K.

Immunomodulation by n-3- versus n-6-rich lipid emulsions in murine acute lung injury - role of platelet-activating factor receptor.

Critical Care Med 2007; 35(2):544-554.

Immunomodulation by n-3- versus n-6-rich lipid emulsions in murine acute lung injury—Role of platelet-activating factor receptor

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Objective: **Cytokines, platelet-activating factor (PAF), and eicosanoids control local and systemic inflammation. Conventional soybean oil-based lipid emulsions used for parenteral nutrition may aggravate the leukocyte inflammatory response or adhesion to the vessel wall. Fish oil-based lipid emulsions, in contrast, may exert an anti-inflammatory effect.**

Design: **We investigated the impact of lipid emulsions on leukocyte invasion, protein leakage, and cytokines in two murine models of acute inflammation.**

Setting: **Research laboratory of a university hospital.**

Subjects: **Mice that were 8–12 wks old (18–21 g weight).**

Interventions: **●●●.**

Measurements and Main Results: **Preinfusion with soybean oil resulted in increased leukocyte invasion, myeloperoxidase activity, and protein leakage and exaggerated release of tumor necrosis factor (TNF)-α as well as macrophage inflammatory protein (MIP)-2 into the alveolar space after intratracheal lipopolysaccharide challenge. In contrast, preinfusion with fish oil reduced leukocyte invasion, myeloperoxidase activity, protein leakage, and TNF-α as well as MIP-2 generation. Corresponding profiles were found in plasma following intraperitoneal lipopolysaccharide application: Soybean oil increased but fish oil decreased the TNF-α and MIP-2 formation. When PAF-receptor-deficient mice were challenged with lipopolysaccharide, leukocyte invasion, lung tissue myeloperoxidase, cytokine generation, and alveolar protein leakage corresponded to those observed in wild-type animals. Fish oil and soybean oil lost their diverging effects on leukocyte transmigration, myeloperoxidase activity, leakage response, and cytokine generation in these knockout mice. Similarly, the differential impact of both lipid emulsions on these lipopolysaccharide-provoked changes was suppressed after pretreating animals with a PAF-receptor antagonist.**

Conclusions: **Fish oil- vs. soybean oil-based lipid infusions exert anti- vs. proinflammatory effects in murine models of acute inflammation. The PAF/PAF-receptor-linked signaling appears to be a prerequisite for this differential profile. (Crit Care Med 2007; 35:●●●–●●●)**

KEY WORDS: platelet-activating factor; fish oil; lipid emulsions; inflammation; sepsis; acute lung injury

Acute lung injury, the systemic inflammatory response syndrome, and sepsis are common in intensive care patients (1, 2). At early time points, all three entities are associated with an excessive inflammatory response (3), but in later stages of systemic inflammatory response syndrome or sepsis, a reduced immune response may be detected, termed compensatory anti-inflammatory response syndrome (4). Lipids, lipid mediators, and inflammation are closely interrelated (3, 5). The generation of pro- and anti-inflammatory as well as vasoactive eicosanoids (such as prostaglandin E_2, prostaglandin I_2, and thromboxane A_2) is coupled to the generation of free arachidonic acid from phospholipids. In the context of lipids and inflammation, eicosanoids represent a major focus of interest due to their strong proinflammatory and anti-inflammatory potencies (3). Among the n-6 fatty acids in the Western diet and current nutritional regimes applied in intensive care units, linoleic acid is the most prominent fatty acid, giving rise to its elongation and desaturation product and eicosanoid precursor arachidonic acid. The n-3 fatty acids, including eicosapentaenoic acid and docosahexaenoic acid, make up an appreciable part of the fat in cold-water fish and seal meat. Eicosapentaenoic acid-derived 5-series leukotrienes generated by the 5-lipoxygenase and the cyclooxygenase product thromboxane A_3 possess markedly reduced inflammatory and vasomotor potencies compared with the arachidonic acid-derived lipid mediators and may even exert antagonistic functions (6).

In addition to acting as a precursor for eicosanoid formation, n-3 vis-à-vis n-6 fatty acid incorporation into membrane (phospho)-lipid pools was suggested to influence lipid-related intracellular signaling events (7). Subclasses of phosphatidyl-

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choline such as the platelet-activating factor (PAF)-precursor pool, as well as phosphatidylinositol and sphingomyelin pools, may be particularly relevant in this respect. Next, gene transcription is modulated as nuclear translocation of nuclear factor-κB and is inhibited by n-3 fatty acids involving signaling by plasma membrane translocation and activation of protein kinase C (8). Furthermore, research work has linked a specific genetic background to the reduction in proinflammatory cytokine generation in volunteers (9).

PAF is a major lipid mediator derived in two steps from phosphatidylcholine yielding free arachidonic acid and the active mediator (10). Furthermore, PAF-like molecules may be generated by oxidative attack on unsaturated fatty acids in phosphatidylcholine, which may aggravate sepsis and tissue injury (11). PAF promotes adhesion of leukocytes to endothelial cells, and application of PAF-receptor antagonists (PAF-RA) ameliorates features of sepsis and shock in experimental models (12), but effects on mortality in septic patients were not reported in clinical phase III studies (13, 14). Mice carrying a targeted disruption of the PAF-receptor (PAF-R) gene exhibit an ameliorated response to an acid-aspiration lung injury model; however, they remain sensitive to lipopolysaccharide (LPS) and endotoxin-induced shock (15).

Different groups reported a major influence of nutrition including n-3 fatty acids on morbidity of intensive care patients. In the adult respiratory distress syndrome, a tailored nutrition with eicosapentaenoic acid, γ-linoleic acid, and antioxidants was reported to improve oxygenation, reduce length of mechanical ventilation, decrease incidence of new organ failures, and shorten length of stay in the intensive care unit (16). Using fish oil supplements, however, several days to weeks are required to effectively influence the fatty acid composition of membrane (phospho)-lipids and thereby the lipid mediator profile in humans (17). In contrast, when administering a fish oil-based lipid emulsion via the intravenous route in volunteers or septic patients, we recently demonstrated rapid changes in cell membrane fatty acid composition and leukocyte functions (18, 19).

In the present study, we developed a murine model suitable for continuous long-term intravenous lipid infusions and subsequently submitted mice to intratracheal or intraperitoneal LPS injection. In addition to analyzing systemic cytokine generation, we focused on compartmentalized inflammatory events such as cytokine appearance in the bronchoalveolar space and recruitment of leukocytes to the alveolar compartment.

MATERIALS AND METHODS

Reagents. Lipoven 10% (soybean oil, or SO) and Omegaven 10% (fish oil, or FO) were purchased from Fresenius-Kabi (Bad Homburg, Germany). Analysis of fatty acid composition of the lipid emulsions is given in Table 1. Chemicals of highest purity were obtained from Merck (Darmstadt, Germany). LPS (O111:B4) from *Escherichia coli* was from Sigma-Aldrich (Dreisenhofen, Germany). The PAF antagonist BN52021 originated from Biomol (Hamburg, Germany).

Animals. Local government authorities and university officials responsible for animal protection approved the study. Parent and offspring PAF-R −/− mice on the BALB/c background and wild-type animals (WT) were kept under standard conditions with a 12-hr day/night cycle under specific pathogen-free conditions. Animals 8–12 wks old (18–21 g weight) were used for experiments. For implantation of a jugular vein catheter, mice were anesthetized by an intraperitoneal injection of a 1:1 mixture of xylazine at 80–100 mg per kilogram of body weight (Bayer, Leverkusen, Germany)/ketamine (Pharmacia & Upjohn, Erlangen, Germany). When animals were anesthetized and spontaneously breathing, points of incisions were shaved and disinfected. A silicon catheter (Braun, Melsungen, Germany) was inserted into the left jugular vein and tied. The catheter was tunneled to the neck of the animal and connected to an osmotic minipump (Alzet, Cupertino, CA) filled with NaCl 0.9% situated in an external device tied to the back allowing easy access and exchange of pumps without anesthesia.

Table 1. Fatty acid composition of the soybean oil (SO)-based and fish oil (FO)-based lipid-emulsion (g/L)

Fatty Acid	SO	FO
C14:0	—	4.9
C16:0	12.4	10.7
C16:1n-7	—	8.2
C18:0	5.0	2.4
C18:1n-9	24.1	12.3
C18:2n-6	52.2	3.7
C18:3n-3	8.2	1.3
C20:4n-6	—	2.6
C20:5n-3	—	18.8
C22:5n-3	—	2.8
C22:6n-3	—	16.5
Others	—	16.1

The SO-based emulsion (Lipoven) and the FO-based lipid emulsion (Omegaven) were manufactured with identical techniques and additives. Repetitive gas chromatographic controls of both lipid emulsions revealed <0.3% free eicosapentaenoic acid or arachidonic acid as related to the esterified amounts of these fatty acids.

Murine Model of Acute Lung Injury. Mice were anesthetized with xylazine/ketamine, a small catheter was inserted in the trachea, and LPS (0, 1, or 10 μg in 50 μL of normal saline/mouse) was instilled. Twenty-four hours after LPS application, mice were killed by an overdose of isoflurane (Abbot, Wiesbaden, Germany), and bronchoalveolar lavage (BAL) was performed as described (20). An additional BAL was performed after 4 hrs in mice receiving 10 μg of LPS as a precaution since the escalation in LPS dose could have increased mortality in the model. However, even after receiving the higher dose of LPS, all animals survived the observation period. Alveolar-recruited leukocytes recovered from lungs of LPS-challenged and control mice were counted in a counting chamber. Differentiation of leukocytes in blinded fashion was done on differential cell counts of Pappenheim-stained cytocentrifuge preparations, using overall morphologic criteria, including differences in cell size and shape of nuclei. Protein in bronchoalveolar lavage was determined according to Lowry et al (21).

Model of Intraperitoneal Inflammation. Mice were anesthetized with xylazine/ketamine, and LPS (2 μg/mouse) or vehicle was injected intraperitoneally. For cytokine measurements from plasma and white blood cell count in peripheral blood, mice were exsanguinated 2 hrs after LPS treatment in deep anesthesia.

Enzyme-Linked Immunosorbent Assay. Cytokine enzyme-linked immunosorbent assays for tumor necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-2 were performed according to the manufacturer's (R&D, Wiesbaden, Germany) instructions.

Myeloperoxidase Assay. Lung myeloperoxidase (MPO) was determined as an index of tissue neutrophil accumulation 24 hrs after LPS challenge as described (22). After weighing of lung stored at −80°C, the frozen lung was homogenized, sonicated, and centrifuged at 25,000 × *g*. MPO activity was calculated from change in absorbance (460 nm) resulting from decomposition of H_2O_2 in the presence of o-dianisidine.

Wet-to-Dry Ratio. To assess pulmonary edema, determination of lung wet weight was performed after removal of extraneous bronchial and cardiac structures as described (23). To measure dry weight, lungs were incubated in a drying oven a week at 80°C and then reweighed.

Peripheral White Blood Cell Counts. White blood count of peripheral blood was measured as previously described (24).

Experimental Protocol. Seven days after central venous catheter implantation in mice, exchange of pumps was performed. Then, 200 μL per day of either SO, FO, or 0.9% NaCl was infused over 3 days with the mice being allowed access to water and chow *ad libitum*. The amount of lipids infused is equivalent to

1.0 g/kg/day. However, the energy expenditure of mice is nearly three times higher that that of humans. Therefore, the infused lipids were considered to be close to lower limits of recommended amount of lipids in parenteral nutrition. While receiving infusions, mice were subjected to low-dose unfractionated heparin injected subcutaneously. In experiments with WT mice treated with PAF-RA, 10 mg/kg of body weight BN52021 (Biomol, Hamburg, Germany) was injected into the tail vein 30 mins before intratracheal LPS application.

Statistics. Data are given as the mean ± SEM. Two-way analysis of variance was performed to test for differences between different infusion groups and mice strains (WT, PAF-R −/−, PAF-RA). *Post hoc* analysis was carried out using Student-Newman-Keuls' test. As data of protein in lavage and leukocytes in BAL (1 μg, 24 hrs) were not normally distributed, log-transformation was performed. Wet-to-dry ratios between unstimulated and stimulated groups were compared using Student's *t*-test. Probability (p) values $<.05$ were considered to indicate statistical significance. Analysis was carried out using SigmaStat.

RESULTS

Effect of Lipid Emulsions on Alveolar Leukocyte Recruitment and Wet-to-Dry Dry Ratio in LPS-Induced Acute Lung Injury. Without LPS challenge, we found $0.10 \pm 0.01 \times 10^6$ leukocytes in the BAL fluid without significant variation between NaCl and lipid infusion groups. After stimulation of WT mice with 1 μg of LPS, leukocytes migrated into the alveolar space, with their numbers in BAL fluid rising to $1.09 \pm 0.08 \times 10^6$ cells after 24 hrs (Fig. 1*A*). Preinfusion of SO massively increased leukocyte recruitment by nearly 100% ($p < .01$ vs. NaCl). In contrast, in mice receiving FO, leukocytes were significantly reduced to <60% ($p < .01$ vs. SO and NaCl). When using 10 μg of LPS in the normal saline group, we

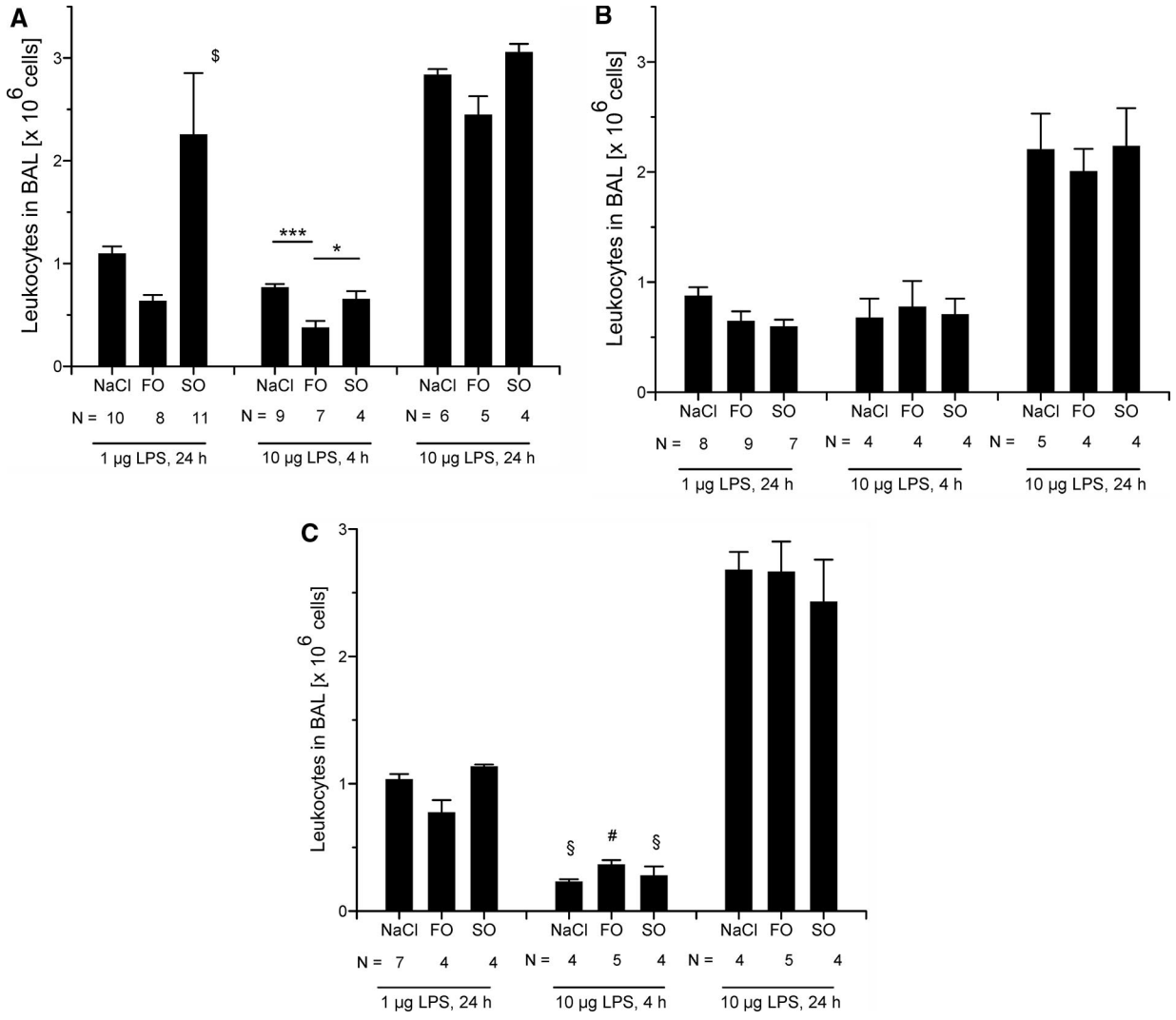

Figure 1. Impact of fish oil (*FO*)- vs. soybean oil (*SO*)-based lipid infusions on leukocytes migrated into the alveolar space in wild-type (WT) mice, mice lacking the platelet-activating factor-receptor (PAF-R −/−), and WT mice treated with platelet-activating factor receptor antagonist (PAF-RA) in a model of acute lung injury. WT mice (*A*), PAF-R −/− mice (*B*), and WT mice treated with a PAF-RA (*C*) were infused with saline (*NaCl*) or FO- or SO-based lipid emulsions, followed by application of 1 or 10 μg of endotoxin (lipopolysaccharide, LPS) intratracheally 4 or 24 hrs before performing a bronchoalveolar Lavage (*BAL*). Total lavage leukocyte counts are given. Only in WT mice, a significant difference of SO- vs. FO-based lipid emulsions, and of saline (control) vs. FO-based lipid emulsion, respectively, was detectable ($\$p < .01$ all groups differed significantly from each other; $*p < .05$ FO vs. SO; $***p < .001$ FO vs. control). Animals exposed to 10 μg of LPS for 4 hrs in the PAF-RA groups exhibited a reduction of leukocyte numbers ($§p < .05$ vs. WT and PAF-R −/−; $\#p < .05$ vs. PAF-R −/−). Data are given as mean ± SEM. Numbers of animals per group are detailed below columns. Error bars are missing when falling into symbol.

found $0.76 \pm 0.04 \times 10^6$ leukocytes after 4 hrs rising to $2.83 \pm 0.06 \times 10^6$ leukocytes after 24 hrs in the BAL. After 4 hrs, infusion of FO-based lipid emulsions induced a significant reduction of transmigrated leukocytes by nearly 50% ($p < .001$ vs. NaCl and $p < .05$ vs. SO), whereas SO had no impact on leukocyte invasion compared with NaCl. After 24 hrs in mice receiving SO, leukocyte invasion was comparable to mice receiving NaCl, whereas infusions of FO-based lipids led to a small reduction.

Without LPS challenge, differential count of leukocytes in BAL was 2.5 ± 0.8% granulocytes, 97.0 ± 0.9% monocytes/macrophages, and 0.5 ± 0.3% lymphocytes without effect of lipid emulsions on this feature. At 24 hrs after challenge with 1 μg of LPS, 78.1 ± 3.2% granulocytes, 2.0 ± 0.4% lymphocytes, and 20.2 ± 3.1% monocytes/macrophages were detected in the BAL of WT mice receiving NaCl. This profile of predominant neutrophil invasion was not changed by infusion of lipids or LPS dose.

Wet-to-dry ratio was determined to evaluate degree of lung injury. Without LPS, wet-to-dry ratio was 4.62 ± 0.07 in control animals with both lipid infusions exhibiting no further impact. After instillation of 10 μg of LPS in mice receiving normal saline, wet-to-dry ratio increased to 5.12 ± 0.09 after 24 hrs ($p < .01$ vs. control) whereas both lipid emulsions did not modulate this feature and did not differ significantly from the NaCl group.

Significance of PAF-R for Recruitment of Leukocytes Into the Alveolar Space and Its Modulation by Lipid Emulsions. Bronchoalveolar lavage performed 4 or 24 hrs after intratracheal LPS challenge with 1 or 10 μg of LPS in PAF-R −/− mice submitted to infusion of normal saline (control) indicated a similar response as encountered in corresponding WT animals. Quantity (Fig. 1*B*) and cell differentiation of recruited leukocytes were not different in both strains. The diverging effect of preinfused lipid emulsions on alveolar recruitment of leukocytes was, however, fully lost in PAF-R −/− mice. In both lipid infusion groups, numbers of recruited leukocytes were slightly but not statistically significantly different compared with normal saline control mice. This finding was irrespective of LPS dose or time of lavage.

To confirm our results in PAF-R −/− mice, we treated WT mice with the PAF-RA BN52021. WT mice were infused for 3 days with normal saline (NaCl control) or with FO- or SO-based lipid emulsions followed by application of 1 or 10 μg/mouse LPS intratracheally 4 or 24 hrs before performing a BAL (Fig. 1*C*). A dose of 10 mg/kg BN52021 was injected into the tail vein 30 mins before LPS application. In mice treated with normal saline, the absolute leukocyte numbers and differential count in BAL did not differ from WT or PAF-R −/− mice 24 hrs after LPS challenge. However, 4 hrs after instillation of 10 μg of LPS, we found a strong reduction of leukocytes in all PAF-RA groups. The reduction was significant in animals receiving NaCl or SO compared with respective WT and PAF-R −/− groups ($p < .05$ for each comparison). In animals receiving FO, leukocytes were significantly lower compared with the FO-PAF-R −/− group ($p < .05$) but comparable to WT mice infused with FO-based emulsions.

As in PAF-R −/− mice, FO or SO no longer modulated the amount of alveolar recruited leukocytes after treatment with a PAF-RA. This was consistent irrespective of time of lavage and dose of LPS. In particular, in contrast to WT mice, SO-based lipid emulsions lost their capacity to double alveolar leukocyte transmigration.

LPS-Induced Accumulation of Neutrophils in Lung Tissue. MPO activity was measured before and 24 hrs after LPS challenge to assess neutrophil accumulation in lung tissue. In lungs of WT mice without LPS exposure, MPO activity was 0.9 ± 0.3 units/g without significant effect of lipid emulsions (Table 2). After instillation of 10 μg of LPS, MPO increased to 6.6 ± 0.7 units/g in control animals. After infusion of SO-based lipid emulsions, MPO was nearly two-fold higher compared with the FO and normal saline groups ($p < .05$ for each comparison). After application of 1 μg of LPS intratracheally, we found the same trend for MPO determination in WT animals, but results failed to reach the level of significance. The diverging effect of preinfused lipid emulsions on neutrophil accumulation in lung tissue was, however, lost in PAF-R −/− mice and after application of the PAF-RA in mice receiving 10 μg of LPS.

LPS-Induced Alveolar Protein Leakage. To examine impact of lipids on lung injury, we determined protein in the BAL as marker for vascular leakage. In WT animals without LPS challenge, protein concentration was determined as 32 ± 3 μg/mL without significant modulation by lipid emulsions (Table 3). After intratracheal challenge with 1 μg or 10 μg of LPS, protein in BAL increased to 273 ± 16 μg/mL or 551 ± 34 μg/mL, respectively, after 24 hrs in WT animals. Infusion of FO significantly decreased alveolar protein leakage after 1 μg ($p < .01$ vs. NaCl and SO) and 10 μg of LPS, whereas after SO infusion protein concentrations slightly increased. Due to the marked reduction of protein in BAL after FO after 1 μg of LPS, FO-WT animals differed significantly from FO-PAF-R −/− mice ($p < .05$).

Generation of MIP-2 After LPS Instillation. We then examined the impact of lipid emulsions on intra-alveolar cytokine generation in our model. Without LPS stimulation, MIP-2 concentration in BAL was below detection limit regardless of

Table 2. Myeloperoxidase activity in lung homogenates (units/g of tissue)

	Without LPS	1 μg of LPS (24 Hrs)	10 μg of LPS (24 Hrs)
Wild-type			
NaCl	0.9 ± 0.3 (n = 4)	3.3 ± 0.7 (n = 4)	6.6 ± 0.7 (n = 8)
FO	1.0 ± 0.4 (n = 4)	3.4 ± 0.4 (n = 4)	6.8 ± 1.4 (n = 4)
SO	0.9 ± 0.4 (n = 4)	5.1 ± 0.9 (n = 4)	11.3 ± 0.9 (n = 4)[a]
PAF-R −/−			
NaCl	0.8 ± 0.4 (n = 4)	ND	9.1 ± 1.0 (n = 4)
FO	0.9 ± 0.4 (n = 4)	ND	9.4 ± 1.4 (n = 4)
SO	0.9 ± 0.3 (n = 4)	ND	7.7 ± 0.5 (n = 4)
PAF-RA			
NaCl	0.9 ± 0.4 (n = 4)	ND	9.5 ± 1.5 (n = 4)
FO	0.8 ± 0.4 (n = 4)	ND	8.3 ± 0.4 (n = 4)
SO	0.9 ± 0.5 (n = 4)	ND	9.6 ± 1.6 (n = 4)

LPS, lipopolysaccharide; NaCl, saline; FO, fish oil; SO, soybean oil; PAF-R −/−, mice lacking the platelet-activating factor receptor; ND, not done; PAF-RA, platelet-activating factor receptor antagonist.

[a] $p < .05$ for comparison with NaCl and FO. Wild-type mice, PAF-R −/− mice, and wild-type mice treated with a PAF-RA were infused for 3 days with NaCl (control) or with FO- or SO-based lipid emulsions, followed by stimulation with 1 or 10 μg of endotoxin (LPS) intratracheally for 24 hrs.

Table 3. Protein concentration in bronchoalveolar lavage (μg/mL)

	Without LPS	1 μg of LPS (24 Hrs)	10 μg of LPS (24 Hrs)
Wild-type			
NaCl	32 ± 3 (n = 4)	273 ± 16 (n = 21)	551 ± 34 (n = 4)
FO	33 ± 4 (n = 4)	148 ± 21 (n = 6)[a,b]	416 ± 21 (n = 5)[c]
SO	31 ± 5 (n = 5)	313 ± 40 (n = 11)	641 ± 11 (n = 4)
PAF-R −/−			
NaCl	31 ± 4 (n = 4)	347 ± 36 (n = 11)	382 ± 89 (n = 6)
FO	33 ± 5 (n = 4)	338 ± 69 (n = 9)	462 ± 43 (n = 4)
SO	32 ± 5 (n = 4)	304 ± 67 (n = 7)	654 ± 115 (n = 5)
PAF-RA			
NaCl	33 ± 4 (n = 4)	325 ± 40 (n = 4)	552 ± 86 (n = 4)
FO	33 ± 3 (n = 4)	253 ± 50 (n = 4)	424 ± 102 (n = 6)
SO	32 ± 4 (n = 4)	331 ± 70 (n = 4)	527 ± 119 (n = 6)

LPS, lipopolysaccharide; NaCl, saline; FO, fish oil; SO, soybean oil; PAF-R −/−, mice lacking the platelet-activating factor receptor; PAF-RA, platelet-activating factor receptor antagonist.

[a]Within WT, $p < .01$ FO vs. NaCl and SO; [b]within FO infusion groups, $p < .05$ WT vs. PAF-R−/−; [c]within WT, $p < .05$ FO vs. SO. Wild-type mice (WT), PAF-R −/− mice, and WT mice treated with a PAF-RA were infused for 3 days with NaCl (control) or with FO-, or SO-based lipid emulsions, followed by stimulation with 1 or 10 μg of endotoxin (LPS) intratracheally for 24 hrs.

infusion used or mouse line examined. At 24 hrs after challenge with 1 μg of LPS, we found an increase in lavage MIP-2 concentration to 112 ± 6 pg/mL in mice infused with normal saline (Fig. 2*A*). In mice receiving SO, MIP-2 was similar compared with the NaCl group. However, in mice receiving FO, MIP-2 concentration was significantly reduced ($p < .001$ vs. NaCl and $p < .05$ vs. SO). When using 10 μg of LPS in the normal saline group, we found a tremendous increase in MIP-2 after 4 hrs to 1225 ± 96 pg/mL and a decline to 165 ± 18 pg/mL after 24 hrs. After 4 hrs, infusion of FO-based lipid emulsions induced a small reduction of MIP-2, but preinfusion with SO provoked a significant increase of nearly 25% compared with the NaCl group ($p < .05$ vs. FO or NaCl). After 24 hrs in mice receiving SO, MIP-2 concentration remained massively elevated (741 ± 73 pg/mL, $p < .001$ vs. NaCL or FO) and was reduced to 140 ± 18 pg/mL in the FO group.

In PAF-R −/− mice receiving normal saline, MIP-2 levels 24 hrs after challenge with 1 or 10 μg of LPS were comparable to WT animals. The MIP-2 concentrations in both lipid infusion groups did not differ significantly from each other and from saline control irrespective of time of lavage or dose of LPS (Fig. 2*B*). After 4 hrs in mice receiving 10 μg of LPS, MIP-2 concentrations were significantly higher compared with corresponding WT animals in the NaCl and FO groups ($p < .05$) but did not differ from the animals receiving SO.

To confirm our results in PAF-R −/− mice, we treated WT mice with the PAF-RA BN52021. At 24 hrs after challenge with 1 μg of LPS, PAF-RA pretreatment exhibited a reduction of MIP-2 down to about one third compared with WT and PAF-R −/− mice receiving normal saline (Fig. 2*C*, $p < .05$). This reduction by PAF-RA pretreatment was also found in mice receiving either lipid emulsion compared with WT and PAF-R −/− animals ($p < .05$).

Using 10 μg of LPS in the PAF-RA group, MIP-2 concentrations after 4 and 24 hrs were similar to the concentrations detected in WT and PAF-R −/− mice receiving NaCl. In contrast to WT animals, but similar to PAF-R −/−, application of PAF-RA abolished differential impact of FO vs. SO lipid infusions on intra-alveolar MIP-2 concentrations irrespective of dose of LPS or time of lavage. Due to the massive increase after infusion of SO-based lipid emulsions, WT animals differed significantly from PAF-R −/− and PAF-RA groups ($p < .01$).

TNF-α Concentration in BAL After LPS Challenge. Next, we examined impact of lipid emulsions on TNF-α concentration in BAL. Without LPS stimulation, TNF-α was undetectable in BAL in all groups irrespective of mouse line and infusion used. At 24 hrs after challenge with 1 μg of LPS, we found an increase in TNF-α concentration to 162 ± 12 pg/mL in mice infused with normal saline (Fig. 3*A*). In mice receiving SO, a similar TNF-α concentration was detected. In contrast, infusion of FO resulted in a significant reduction of TNF-α ($p < .05$ vs. NaCl and SO). After challenge with 10 μg of LPS in mice receiving normal saline, TNF-α concentration massively rose to 1496 ± 92 pg/mL after 4 hrs and to 564 ± 126 pg/mL after 24 hrs. After 4 hrs, infusion of SO led to a further small increase. In contrast, in the FO group, TNF-α was significantly reduced by nearly 25% compared with the NaCL group ($p < .05$ vs. NaCl and SO). However, in the BAL performed after 24 hrs, TNF-α concentration was not significantly modulated by lipid emulsions.

In PAF-R −/− mice receiving NaCl infusions, TNF-α was slightly lower after LPS challenge as compared with WT animals (Fig. 3*B*). TNF-α concentrations in the FO or SO group did not differ significantly from each other and from saline control irrespective of time of lavage or dose of LPS.

To confirm our results in PAF-R −/− mice, we treated WT mice with the PAF-RA BN52021. At 24 hrs after challenge with 1 μg of LPS, TNF-α concentration in PAF-RA-pretreated mice receiving NaCl was 100 ± 12 pg/mL, exhibiting a significant reduction by nearly 40% compared with saline-infused WT mice ($p < .05$, Fig. 3*C*). When instilling 10 μg of LPS, TNF-α concentrations after 4 and 24 hrs were similar to concentrations determined in WT and PAF-R −/− mice receiving normal saline. In contrast to WT animals, but similar to PAF-R −/−, injection of PAF-RA inhibited the differential impact of FO and SO lipid infusions on intra-alveolar TNF-α concentrations irrespective of dose of LPS or time of lavage.

After 4 hrs in mice stimulated with 10 μg of LPS, TNF-α concentrations were increased in all infusion groups receiving PAF-RA. In mice infused with FO, the PAF-RA mice differed significantly from the WT and PAF-R −/− animals ($p < .05$). As TNF-α was also increased in WT mice after SO infusion, the PAF-RA SO group and the WT SO group differed significantly from the PAF-R −/− mice ($p < .05$).

Cytokine Concentrations in Plasma After Intraperitoneal LPS Instillation. Next, we asked if lipid emulsions would have similar effects in a model of abdominal inflammation. In pilot experiments, we determined that TNF-α concentration in plasma peaked at 2 hrs after intra-abdominal LPS instillation in our model. Using the previous infusion setting, we challenged mice with 2 μg LPS intraperitoneally 2 hrs before kill and bleeding.

Before LPS challenge, leukocyte counts in peripheral blood were 6.6 ± 0.4 g/L (n = 5) without significant impact of the lipid emulsions. Two hours after intraperi-

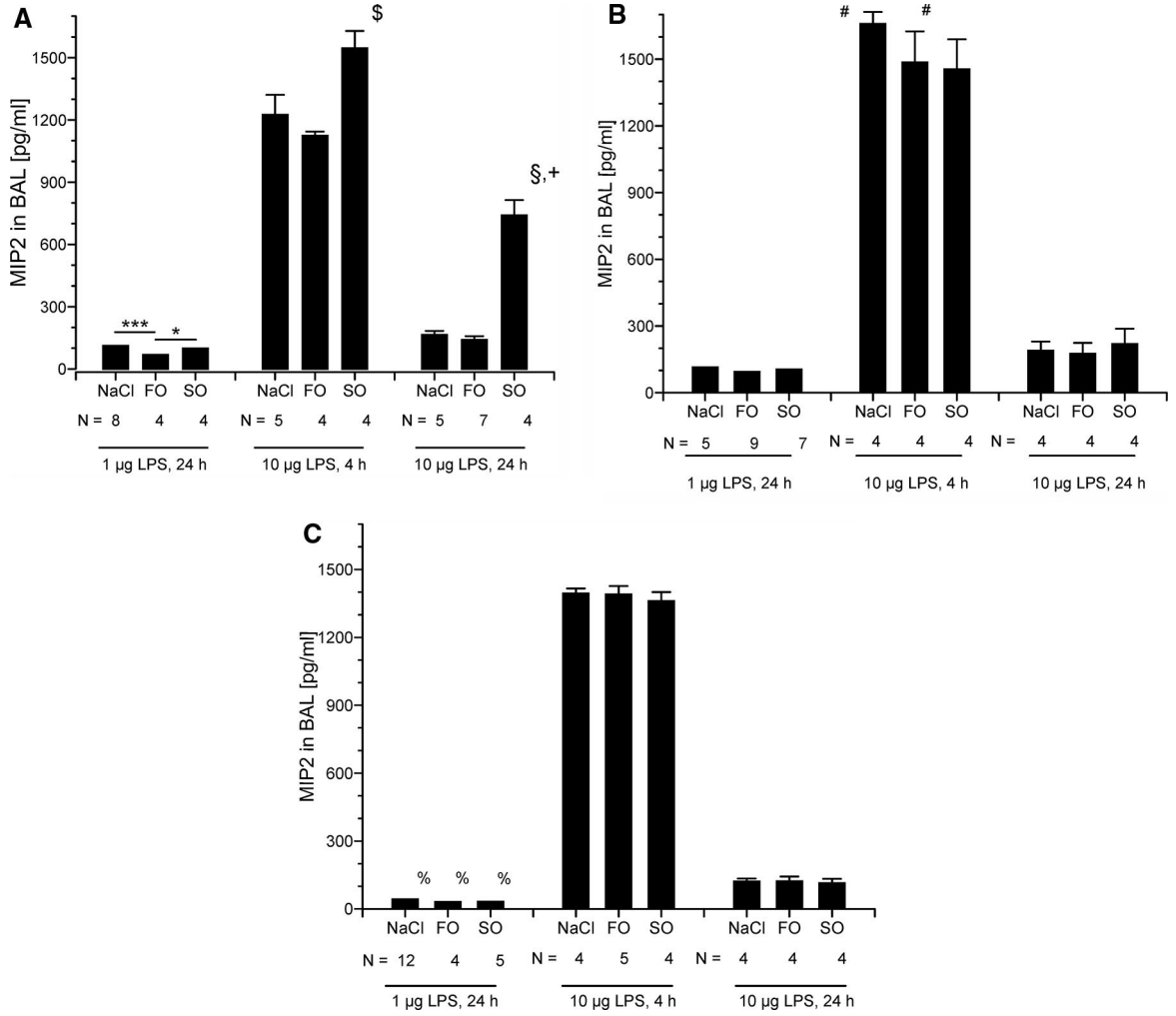

Figure 2. Impact of fish oil (*FO*)- vs. soybean oil (*SO*)-based lipid infusions on alveolar macrophage inflammatory protein (*MIP*)-2 generation in wild-type (WT) mice, mice lacking the platelet-activating factor-receptor (PAF-R −/−), and WT mice treated with platelet-activating factor receptor antagonist (PAF-RA) in a model of acute lung injury. WT mice (*A*), PAF-R −/− mice (*B*), and WT mice treated with a PAF-RA (*C*) were infused for 3 days with normal saline (control) or FO- or SO-based lipid emulsions, followed by stimulation with 1 or 10 μg of endotoxin (lipopolysaccharide, LPS) intratracheally 4 or 24 hrs before lavage. MIP-2 concentrations in bronchoalveolar lavage (*BAL*) were determined by enzyme-linked immunosorbent assay. Infusion of FO lipid emulsions resulted in decreased MIP-2 formation in WT mice (*$p < .05$ vs. SO; ***$p < .001$ vs. control). Infusion of SO resulted in increased MIP-2 generation compared with control and FO in both 10-μg groups ($\$p < .05$; $\S p < .001$). In PAF-R −/− or in WT mice treated with PAF-RA, no significant impact of lipid emulsions could be detected. MIP-2 concentrations in PAF-R −/− mice receiving 10 μg of LPS were higher after 4 hrs compared with corresponding WT controls ($\#p < .05$). After 24 hrs in WT-SO mice challenged with 10 μg of LPS, MIP-2 was significantly higher compared with the corresponding infusion groups of PAF-R −/− and PAF-RA mice ($+p < .01$). All infusion groups of PAF-RA mice receiving 1 μg of LPS were significantly lower than corresponding PAF-R −/− and WT groups ($\%p < .05$). Data are given as mean ± SEM. Numbers of animals per group are detailed below columns. Error bars are missing when falling into symbol.

toneal LPS challenge, 7.5 ± 0.8 g/L leukocytes were found in peripheral blood in mice receiving normal saline (n = 4). After FO infusion, leukocytes were slightly lower and SO slightly increased peripheral leukocytes; however, no significant difference was found.

Before LPS instillation in PAF-R −/− mice, leukocyte counts in peripheral blood were slightly higher compared with WT mice (7.2 ± 0.5 g/L, n = 4). Again, no significant impact of the lipid emulsions was found. After LPS injection into the peritoneal cavity, we detected 7.9 ± 1.6 g/L leukocytes in mice receiving NaCl. After FO or SO treatment, 8.5 ± 1.8 g/L and 7.1 ± 1.9 g/L leukocytes were measured (n = 4 each). However, no significant difference was found between infusion regimes.

Before LPS stimulation, no MIP-2 was detectable in plasma in either group. In WT controls, LPS challenge induced an increase to 9.0 ± 1.9 ng/mL. Infusion of FO-derived lipid emulsions resulted in a diminished MIP-2 serum concentration, whereas application of SO-based lipids increased MIP-2 levels significantly compared with the FO group ($p < .05$, Fig. 4*A*). In PAF-R −/−, LPS stimulation induced a three-fold increase in MIP-2 concentrations compared with WT in mice receiving NaCl ($p < .01$). This increase was also detected in both lipid infusion groups; however, differences between the infusion groups were abolished (Fig. 4*B*). The increase in MIP-2 was significant comparing PAF-R −/− mice with WT animals receiving FO ($p < .01$) but not in mice infused with SO.

Before LPS challenge, we did not detect TNF-α in plasma in all groups. Following intraperitoneal LPS application,

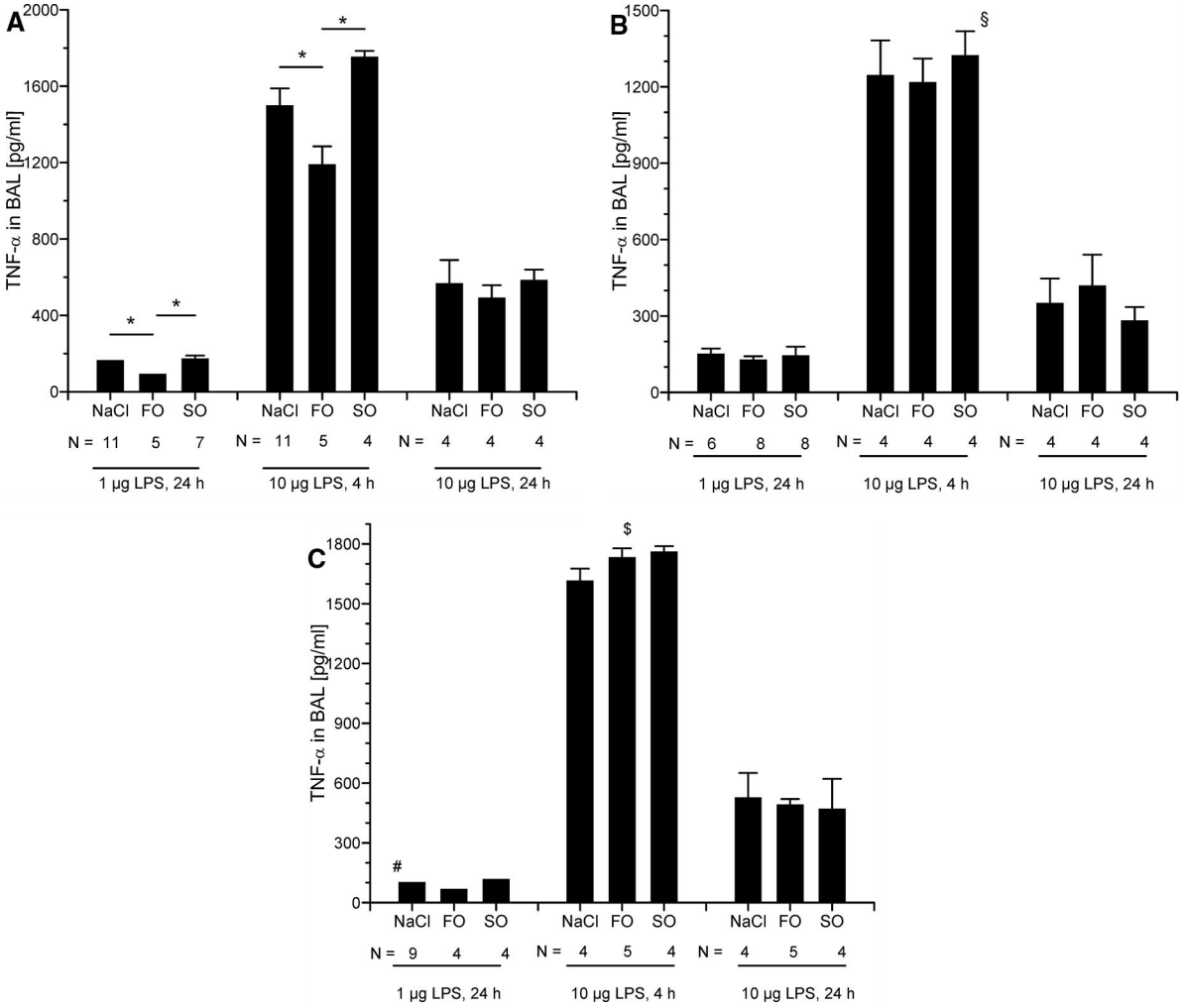

Figure 3. Impact of fish oil (*FO*)- vs. soybean oil (*SO*)-based lipid infusions on alveolar tumor necrosis factor (*TNF*)-α generation in wild-type (WT) mice, mice lacking the platelet-activating factor-receptor (PAF-R −/−), and WT mice treated with platelet-activating factor receptor antagonist (PAF-RA) in a model of acute lung injury. WT mice (*A*), PAF-R−/− mice (*B*), and WT mice treated with a PAF-RA (*C*) were infused for 3 days with saline (control) or FO- or SO-based lipid emulsions, followed by stimulation with 1 or 10 μg of endotoxin (lipopolysaccharide, LPS) intratracheally 4 or 24 hrs before lavage. TNF-α concentration in bronchoalveolar lavage was determined by enzyme-linked immunosorbent assay. Infusion of FO resulted in decreased TNF-α formation in WT mice (*$p < .05$ vs. SO and control). In PAF-R−/− mice or WT mice treated with PAF-RA, no significant impact of lipid emulsions could be detected. In PAF-RA mice challenged with 1 μg of LPS, TNF-α concentration of the NaCl group was lower compared with corresponding WT animals (#$p < .05$). After infusion of FO in PAF-RA mice, TNF-α concentration was higher compared with the WT-FO and PAF-R−/−-FO animals receiving 10 μg of LPS after 4 hrs ($$p < .05$). TNF-α concentration was lower in the PAF-R−/−-SO group after 10 μg of LPS and 4 hrs compared with the WT-SO and PAF-RA-SO animals (§$p < .05$). Data are given as mean ± SEM. Numbers of animals per group are detailed below columns. Error bars are missing when falling into symbol.

plasma TNF-α concentration rose to 303 ± 20 pg/mL in control animals. Infusion of SO-based lipids resulted in a significant increase of nearly 50% in TNF-α plasma concentration (Fig. 4*C*, $p < .01$ vs. control). After infusion of FO-derived lipids, TNF-α decreased significantly to 181 ± 12 pg/mL ($p < .05$ vs. control and $p < .001$ vs. SO). LPS challenge in PAF-R −/− mice infused with NaCl evoked a similar TNF-α concentration compared with WT controls (Fig. 4*D*). Again, no significant difference between the different infusion groups became detectable.

DISCUSSION

Using two different models of acute inflammation in mice, we have demonstrated that a 3-day course of lipid emulsion infusions is sufficient to modulate inflammatory responses induced by LPS. In contrast to SO-based lipid emulsions, FO-based lipid emulsions reduced pulmonary leukocyte invasion, protein leakage, and cytokine generation as well as cytokine appearance in the intravascular compartment. We present evidence that diverging effects of lipids emulsions are linked to PAF-R signaling. In mice carrying the disrupted PAF-R gene (PAF-R −/−), as well as in mice treated with a PAF-RA, the general response to endotoxin remained intact, but the differential response to FO vs. SO lipids was lost. A drawback of both models applied is the use of LPS in a single-hit model to induce inflammatory responses. Such a model is clearly different from clinical and experimental sepsis induced by bacterial infection.

Responses to endotoxin in PAF-R −/− mice were intact in our acute lung injury model. These data are well in line with a

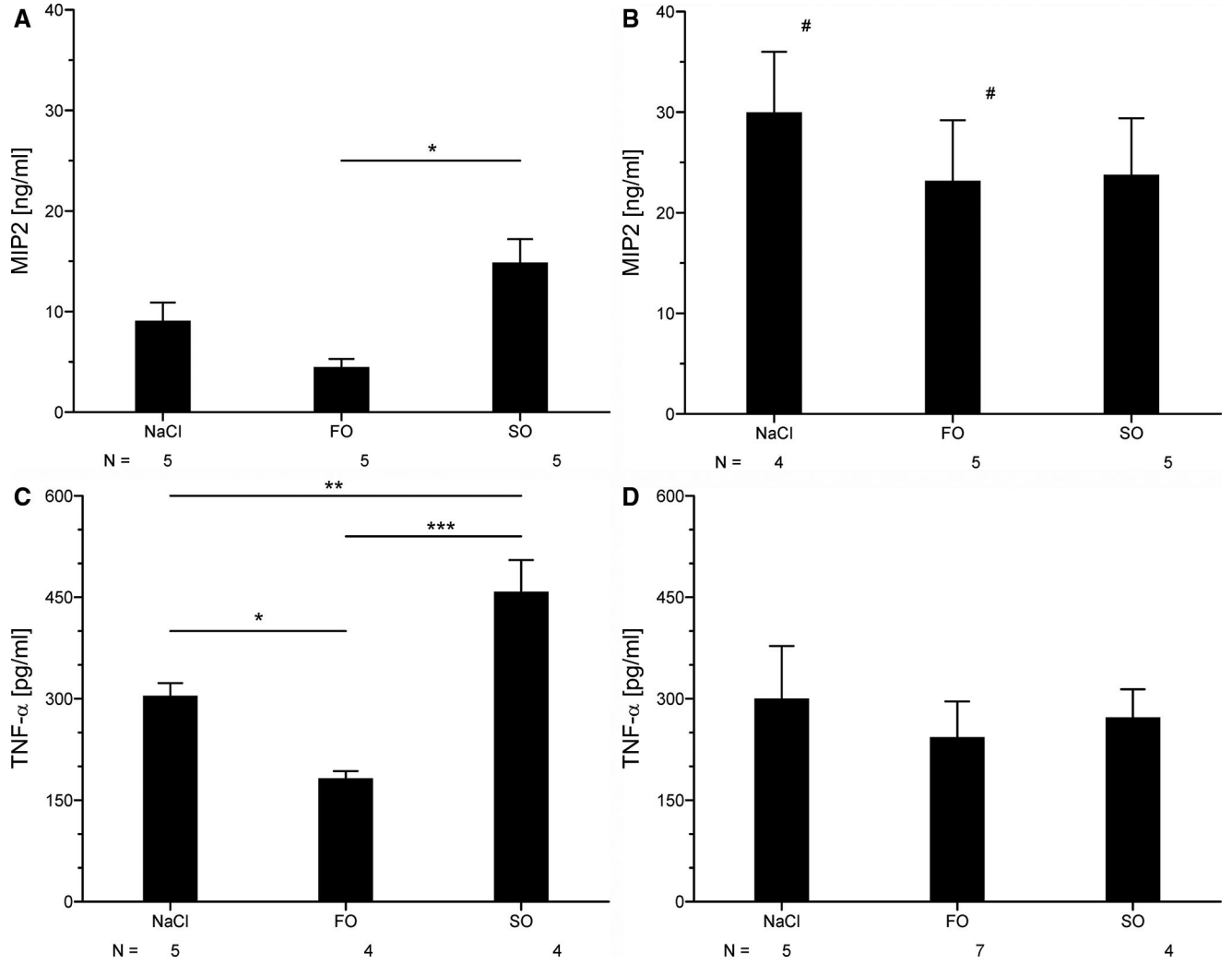

Figure 4. Impact of fish oil (*FO*) vs. soybean oil (*SO*) lipid infusions on plasma macrophage inflammatory protein (*MIP*)-2 and tumor necrosis factor (*TNF*)-α in wild-type (WT) mice and mice lacking the platelet-activating factor-receptor (PAF-R −/−) in a model of intraperitoneal inflammation. WT mice (*A*, *C*) or PAF-R−/− mice (*B*, *D*) were infused for 3 days with saline (control) or FO- or SO-based lipid emulsions, followed by stimulation with endotoxin (lipopolysaccharide, LPS) intraperitoneally 2 hrs before kill. MIP-2 (*A*, *B*) and TNF-α (*C*, *D*) in plasma were determined by enzyme-linked immunosorbent assay. Infusion of FO resulted in decreased MIP-2 formation in WT mice (*$p < .05$ vs. SO) but not in PAF-R−/− mice. TNF-α was increased after SO but decreased after FO compared with NaCl in WT mice receiving LPS (*$p < .05$; **$p < .01$; ***$p < .001$). MIP-2 was higher in PAF-R−/− mice after infusion with NaCl or FO compared with corresponding WT groups ($p < .01$). Data are given as mean ± SEM. Numbers of animals per group are detailed below columns.

previous report demonstrating regular responses of PAF-R −/− mice in an endotoxic shock model (15). Our data documenting elevated plasma levels of MIP-2 in PAF-R −/− mice compared with WT mice remain enigmatic. Elevated MIP-2 levels were observed both after lipid infusions and in saline controls. We speculate that this increase may be part of a long-term compensatory mechanism due to PAF-R deficiency, since PAF and MIP-2 may both act as chemoattractants. Further investigations are required to explain this phenomenon. In the PAF-RA group, the overall response to endotoxin remained intact. However, we found a delay in transmigration of leukocytes compared with WT mice: In mice receiving 10 μg of LPS, after 4 hrs leukocyte numbers were reduced but rose to an equal number of leukocytes after 24 hrs. Furthermore, we found a reduction of MIP2 and TNF-α in BAL in mice receiving 1 μg of LPS but not after 10 μg of endotoxin.

A striking finding of the present study was the differential impact of the FO vs. SO on cytokine generation provoked by endotoxin challenge in WT mice. In mice undergoing conventional SO-based lipid infusion, an augmentation of TNF-α and MIP-2 (the murine equivalent of interleukin-8) concentration in response to LPS challenge was observed irrespective of the model employed. In contrast, infusion of FO in WT mice resulted in reduced proinflammatory cytokine generation under all experimental conditions investigated. Although this is the first report of continuous lipid infusion in murine acute lung injury, our observations are consistent with previous findings demonstrating that TNF-α and interleukin-1 release was suppressed by dietary n-3 fatty acids in isolated murine splenocytes (25) and in isolated monocytes obtained from septic patients undergoing FO-based lipid infusions (18). Several days to weeks of oral FO supplementation are usually necessary to achieve such a change, whereas a 3-day infusion course sufficed to cause

changes in cytokine synthesis in the present study and in patients receiving intravenous lipids (18). However using continuous enteral feeding, Gadek et al. (16) found a rapid increase in n-3 fatty acids in plasma phospholipids. We speculate that a key issue may be the continuous delivery of higher doses of FO to achieve fast changes. Preliminary data in our model suggest a rapid increase in n-3 fatty acids in plasma after infusion of FO and increase in n-6 polyunsaturated fatty acids in the SO group.

Pulmonary leukocyte recruitment was reduced in WT mice receiving FO-derived lipid emulsions. In contrast, SO-derived n-6 lipids increased leukocyte invasion and lung injury. We and others have described increased injury to lungs undergoing inflammatory stress due to n-6 lipids and, vice versa, an amelioration of damage by administration of n-3 lipids (26, 27). Mechanisms underlying this protective effect include, at least in part, the effect of FO on cytokine response described previously, generation of less potent lipid mediators as leukotriene B_5 instead of leukotriene B_4, formation of the less active vasoconstrictor thromboxane A_3 instead of thromboxane A_2, a reduction in platelet-activating factor synthesis, and reduced adhesion of leukocytes to endothelial cells (28–30). Transmigration of leukocytes through the endothelial-epithelial structures is a complex and tightly regulated process. The n-3 lipids interfere with this process at multiple stages, involving reduced presentation of endothelial adhesion molecules and reduced formation of platelet-activating factor by endothelial cells, which may then result in diminished activation of integrins on rolling leukocytes (28, 31). Furthermore, addition of arachidonic acid to endothelial cells increased thrombin-induced formation of PAF; in contrast, supplementation with n-3 fatty acids reduced its formation (28). As PAF generated by endothelial cells is not secreted to the supernatant but remains bound to the cell membrane, it may activate rolling leukocytes by binding to their PAF-R and initiate adhesion and transmigration (32). This mechanism may be at least in part responsible for the reduced rolling and adhesion of monocytes to endothelial cells after exposure to n-3 fatty acids and may translate in reduced transmigration of leukocytes (28).

Infusion of lipid emulsions bypasses physiologic uptake and processing of triglycerides by the gastrointestinal tract. Instead, infusion of synthetic lipid aggregates activates endothelial lipoprotein lipases, with translocation of the enzyme from its cellular binding sites into vascular compartment. Activation and translocation of this enzyme result in an increase in plasma free fatty acids due to avoidance of local cellular uptake mechanisms (33). The kinetics and duration of elevated plasma n-3 lipid levels thus exceed the corresponding alterations in response to conventional dietary FO uptake by orders of magnitude (34). Different availability of precursor fatty acids not only has an impact on subsequent generation of lipid mediators (e.g., substitution of leukotriene B_5 for B_4) but also reduces the generation of PAF due to incorporation into the phospholipid-precursor pool. In this respect, at least to two lipid-dependent mechanisms may be relevant. First, enrichment of n-3 fatty acids in the PAF precursor pool may result in steric inhibition of phospholipase A_2 (32). Conversely, the increasing availability of arachidonic acid enhances generation of platelet-activating factor (28). Second, increasing n-3 fatty acids in phosphatidyl-inositol may impair the activation of leukocytes through reduction in intracellular second-messenger generation and activation of protein kinase C (7).

Recent experimental and clinical investigations suggest a strong link between the availability of free arachidonic acid, PAF, and lung injury. PAF and LPS promote lung injury and edema through sphingomyelinase-dependent formation of ceramide and activation of the cyclooxygenase pathway, which leads to the generation of arachidonic acid-derived prostanoids (35). Rapid infusion of conventional lipid emulsions in mechanically ventilated patients suffering from acute respiratory distress syndrome increased pulmonary shunt, notably linked to enhanced prostanoid generation resulting in a deterioration of the $Pa_{O_2}/F_{I_{O_2}}$ ratio (36). A second study in acute respiratory distress syndrome patients undergoing lipid infusions reported an increase in BAL PAF concentration and neutrophil counts paralleled by a deterioration in lung function, as measured by decreased $Pa_{O_2}/F_{I_{O_2}}$ (37). Despite these results and experimental use of PAF-RAs exhibiting beneficial properties in models of sepsis and acute lung injury (12, 38), phase III clinical studies using PAF-RAs or PAF-acetylhydrolase in septic patients have failed to show a difference in survival (13, 14). There may be a gap between experimental studies, when sepsis is initiated by a single LPS challenge with simultaneously started treatment, and clinical reality. Furthermore, as the response to LPS was intact in our model using PAF-R −/− mice, it remains to be determined if other inflammatory response systems may compensate for the inhibited PAF pathway.

However, the role of PAF in lipid infusion-related deterioration of lung function is underscored by our experiments using mice with a targeted disruption of the PAF receptor gene. Employing this strain, we were able to demonstrate that intratracheal LPS instillation provoked recruitment of leukocytes, as well as TNF-α and MIP-2 generation to the same extent as in WT mice, a phenomenon already described (15). Nevertheless, aggravation of lung inflammation by SO and its amelioration by FO-derived emulsions were essentially abolished in mice lacking this receptor. These results were confirmed in WT mice treated with a PAF-RA. Using BN52021, the differential impact of FO vs. SO on leukocyte recruitment and cytokine generation was also blocked. We conclude that major proinflammatory effects of conventional lipid emulsions and the ameliorating impact of FO-derived lipid emulsions on inflammation and pulmonary injury are linked to the integrity of PAF and PAF-R-related signaling in mice.

The correct timing and dosing of any pro- or anti-inflammatory drug in inflammation and sepsis are currently unsettled. N-3/n-6 ratios of 7.6:1 (FO) or 1:370 (safflower oil) have immunesuppressive features in a heart transplantation model (39), and application of SO (ratio 1:6.4) in septic patients increased the cytokine response (19). However, a ratio of 1:2 was shown to have only a minor impact on immunity in the heart transplantation model (39). Applying an n-3/n-6 ratio of 1:2 or 1:3 may therefore be a means to evade immune-modulating effects. Whereas FO in a hyperinflammatory state may be judged as an adjunct therapy, it may prove not to be beneficial in patients with already reduced immune response. Nevertheless, recent data from an observational study in 661 patients including 276 septic patients suggest that supplementation of parenteral nutrition with 0.1–0.2 g/kg/day FO had favorable effects on survival rate, infection rates, and length of stay in this subgroup (40). In addition, current studies using an enteral immune-modulating diet including n-3 fatty acids in patients with acute respiratory

distress syndrome or sepsis show improvement in Pa_{O_2}/F_{IO_2} ratio, reduction in ventilation time, and even improvement in survival (16, 41, 42).

CONCLUSIONS

We demonstrated that infusion of FO-based lipid emulsions reduces LPS-induced proinflammatory cytokines, alveolar leukocyte transmigration, and protein leakage. In contrast, SO-based lipids lead to a further increase in the inflammatory response. However, the effect of lipid emulsions in murine inflammation is dependent on an intact PAF/PAF-R signaling. Administration of lipid emulsions not only may be regarded as a simple supply of calories but may also modulate the inflammatory response.

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Anlage 4

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Fatty acids differentially influence phosphatidyl-inositol 3-kinase signal transduction in endothelial cells: Impact on adhesion and apoptosis.

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Fatty acids differentially influence phosphatidylinositol 3-kinase signal transduction in endothelial cells: Impact on adhesion and apoptosis☆

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Abstract

In contrast to *n*-6 fatty acids like arachidonic acid (AA), the anti-inflammatory potential of *n*-3 fatty acids such as docosahexaenoic acid (DHA) has been demonstrated. We examined the phosphatidylinositol (PI)3-kinase dependent effects of AA versus DHA on monocyte rolling, adhesion and transmigration through inflammatory activated human umbilical venous endothelial cells (HUVEC) as well as on apoptosis, to investigate the impact on vascular inflammation. HUVEC were pre-incubated with AA, DHA or sham, and stimulated with VEGF, TNF-α or staurosporine. Rolling and adhesion were investigated by means of a parallel flow chamber; transmigration was performed in a static assay. Activation of PI3-kinase was measured as phosphorylation of protein kinase B (Akt). Apoptosis was determined by caspase-3 activity and annexin-V analysis. Pre-incubation of HUVEC with DHA markedly decreased TNF-α-induced monocyte rolling, adhesion, and transmigration, although expression of endothelial adhesion molecules was unchanged. In contrast, AA increased TNF-α-induced rolling. Both fatty acids did not alter TNF-α-mediated upregulation of the adhesion molecules ICAM-1, VCAM-1, and E-selectin. The divergent effects of AA and DHA were abrogated with PI3-kinase inhibitors. After pre-incubation with DHA, VEGF-, TNF-α- and staurosporine-induced phosphorylation of Akt was decreased when compared to AA. DHA pre-incubation significantly increased staurosporin-induced apoptosis. In addition, DHA in comparison to AA augmented staurosporin-mediated increase in caspase-3 activity. In conclusion, DHA-induced a reduction in rolling, adhesion and transmigration of monocytes through inflammatory activated HUVEC that is in part PI3-kinase dependent. PI3-kinase driven phosphorylation of Akt and apoptosis of HUVEC as contribution to the resolution of inflammation is differentially modulated by DHA versus AA.

Keywords: Apoptosis; Endothelial function; Lipid signaling; Inflammation; Signal transduction

1. Introduction

Chronic and acute inflammatory diseases like atherosclerosis and coronary artery disease (CAD) as well as pneumonia and adult respiratory distress syndrome (ARDS) represent major causes of patient mortality and morbidity worldwide [1,2]. Lipids, lipid mediators, and inflammation are closely connected [3,4]. In the context of lipid mediators and inflammatory sequelae, eicosanoids represent a major focus of interest due to their strong regulatory, pro- and anti-inflammatory potencies [3,4]. It has been also demonstrated that *n*-3 fatty acids reduce chronic and acute inflammation in patients. The *n*-6 fatty acids, including arachidonic acid (AA), are the predominant polyunsaturated fatty acids in common western diets. The *n*-3 fatty acids like docosahexaenoic acid (DHA) constitute an appreciable part of the fat in cold-water fish and seal meat [5–9].

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n-3 *vis-a-vis* *n*-6 fatty acid incorporation into membrane (phospho)-lipid pools was suggested to influence lipid-related intracellular signaling events. De Caterina et al. demonstrated an inhibitory effect of *n*-3 fatty acids on cytokine-induced generation of adhesion proteins and inflammatory signaling although an impact on apoptosis by influencing PI3-kinase signaling was not elucidated in detail [10,11]. The plasma membrane phospholipid phosphatidylinositol is used as substrate by phosphatidylinositol 3-kinase (PI3-kinase). Therefore, *n*-3 and *n*-6 fatty acid incorporation into this phospholipid could potentially influence phosphorylation differentially in addition to modulating their binding capacities to pleckstrin homology (PH) domains. Vascular endothelial growth factor (VEGF) and tumor-necrosis factor (TNF)-α lead to recruitment of PI3-kinase generating phosphatidylinositol-3,4 bisphosphate (PIP_2) and phosphatidyl inositol-3,4,5 trisphosphate (PIP_3) [12] and subsequently the PI3-kinase pathway to start [13]. The PH domain of Akt binds to the newly formed PIP_2 or PIP_3 resulting in translocation of the kinase to the plasma membrane. Phosphorylation of Akt substrates Bad, the family of forkhead transcription factors, and the nuclear factor kappa B (NF-κB) regulator IκB kinase I (IKK) leads to inhibition of apoptosis or directly promotes cell survival [14]. The *n*-6 or *n*-3 fatty acids may therefore differentially influence PI3-kinase-dependent signaling, activation, and cell survival, and evidence exists, that *n*-3 fatty acids possess anti-proliferative properties [15,16]. PI3-kinase signaling also coordinates integrin activation, cytoskeletal rearrangements, the process of adhesion and transmigration, and nuclear responses in inflammation [17,18].

Recently, resolution of inflammation has been a major focus of investigations [19,20]. In this context, in particular in vascular inflammation apoptosis of inflamed cells represents a key player [21,22]. The aim of our study was to evaluate the significance of PI3-kinase signaling for fatty acid-dependent modulation of inflammation and its resolution. In the present study performed in human endothelial cells undergoing growth factor and inflammatory stimulation *in vitro*, the impact of AA versus DHA on PI3-kinase-dependent inflammatory monocyte–endothelial interaction, Akt phosphorylation, and EC apoptosis were investigated.

2. Materials and methods

2.1. Materials

Chemicals of highest purity were obtained from Merck (Darmstadt, Germany). AA and DHA were obtained from Sigma (Deisenhofen, Germany). Tissue culture plastic dishes, anti-total Akt antibody to measure total Akt and flow cytometry analysis supplies were from Becton-Dickinson (Heidelberg, Germany). Cell culture reagents and media were from PAN (Aidenbach, Germany). VEGF (A65), staurosporine, MCP-1, and TNF-α were bought from R&D Systems (Wiesbaden, Germany). Annexin-V was obtained from Roche Diagnostics (Penzberg, Germany) and propidium iodine was from Serva (Heidelberg, Germany). LY294002 and wortmannin were obtained from Calbiochem (Schwalbach, Germany). Secondary antibodies were from Santa Cruz (Heidelberg, Germany). Anti-phospho Akt antibody was from Cell Signaling (Frankfurt, Germany) and ECL was from Amersham (Freiburg, Germany).

2.2. Preparation of endothelial cells

EC were obtained from human umbilical veins (HUVEC) according to the method described by Jaffe et al. [23].

2.3. Monocyte isolation

Human monocytes were isolated from platelet pheresis residues by Ficoll-Hypaque density gradient centrifugation, followed by counterflow centrifugation elutriation using a Beckman JE-5.0 rotor as described [24]. Monocyte purity (94–98%) was confirmed by light scatter [fluorescence-activated cell sorter (FACS) scan; Becton Dickinson]. Cell viability was above 96% throughout the study.

2.4. Immunofluorescence staining of endothelial cells

Immunofluorescent labeling of HUVEC was performed as described [25]. Antibodies directed against ICAM-1 [clone R1/1 (CD54); Bender MedSystems; Vienna, Austria], VCAM-1 [clone 1G11 (CD106), Coulter-Immunotech; Marseille, France], E-selectin [clone BBIG-E1 (CD62E), R&D Systems; Wiesbaden, Germany], major histocompatibility complex-I (MHC-I, positive control, W6/32.HL, generously provided by A. Ziegler, Berlin, Germany), and isotype controls (negative control; Dianova, Hamburg, Germany) were used.

2.5. Leukocyte rolling and adhesion assay

Leukocyte rolling and adhesion was determined as described using a parallel plate flow chamber according to Lawrence and Springer [24]. Confluent endothelial monolayers were pre-incubated with fatty acids and TNF-α according to the *experimental protocol*.

2.6. Monocyte transmigration across HUVEC (modified Boyden Chamber assay)

HUVEC were grown to near-confluence on transwell-inserts, washed, and incubated according to the *experimental protocol*. 1×10^6 monocytes/well were allowed to transmigrate the endothelium barrier for 120 min at 37 °C and 5% CO_2 with 100 ng MCP-1 and 10 ng/ml TNF-α in the lower

compartment. Monocytes in the lower compartment were counted to determine the number of transmigrated cells.

2.7. Western blots

Confluent HUVEC were harvested after treatment according to *experimental protocol*. Cell pellets were lysed with RIPA buffer containing a protease inhibitor cocktail, centrifuged, and assayed for total protein concentration. Protein was determined according to Lowry [26]. Total protein (30 μg) was separated in an 8% SDS-PAGE and transferred to a PVDF membrane. Membranes were probed with primary antibody and then were probed with horseradish peroxidases-conjugated secondary antibody after washing with PBS. The exposed film (Hyperfilm TM; Amersham Biosciences, Buckinghamshire, UK) was developed in an AGFA CURIX HAT 530-U (Mortsel, Belgium) developer. Specific bands of total Akt were unchanged throughout the experiments. Blot analysis was performed with BioDoc II Biometra (Göttingen, Germany) with Scan Pack 3.0 software.

2.8. Annexin-V analysis

HUVEC were treated according to *experimental protocol*. Adherent cells were released by trypsinization, washed, the supernatant was aspirated, and the pellet was resuspended in incubation buffer ($CaCl_2$ 5 mM, NaCl 140 mM, HEPES 10 mM, pH 7.4). Annexin-V was diluted 1:50 prior to an incubation time of 15 min at room temperature in a light protected area. Cells were washed twice and flow cytometry analysis was performed after adding 20 μg/ml propidium iodide. The samples were analyzed by a Becton Dickinson fluorescence cytometer using CellQuest software. The procedure allowed to distinguish intact cells (annexin −/propidium iodide −) from apoptotic cells (annexin +/propidium iodide −) and necrotic cells (annexin +/propidium iodide +) after treatment with an apoptotic stimulus.

2.9. Caspase-3 activity assay

Caspase-3 activity assay was purchased from R&D (Wiesbaden, Germany) and performed according to manufacturer's instructions.

2.10. Experimental protocol

HUVEC were grown to confluence, the culture medium was exchanged, and free fatty acids (AA and DHA) dissolved in ethanol (final volume ≤1%, v/v) were added to the culture medium at a final concentration of 10 μmol/l and incubated for 6 h. Controls were sham incubated with solvent only. In case of transmigration experiments, HUVEC were grown on inserts. $N = 8–12$ independent experiments for rolling, adhesion, and transmigration were performed. Without change of incubation medium, TNF-α (10 ng/ml), staurosporine (30 ng/ml), or VEGF (30 ng/ml) were added, and HUVEC were incubated for another 24 h respectively for another 30 min in the case of Akt. Western blots of phospho Akt and total Akt were carried out in 35 mm culture dishes as detailed above ($n = 6$ independent experiments). For monocyte adhesion experiments, HUVEC were grown on slides, incubation medium was discarded, and cells were gently washed directly before use in the flow chamber. The PI3-kinase inhibitors (LY294002, 200 nM; wortmannin, 10 nM) were added to HUVEC 1 h prior to the start of superfusion experiments in $n = 6–8$ independent experiments each.

2.11. Statistics

Data are given as mean ± S.E.M. One-way analysis of variance was performed to test for differences between different groups. *Post hoc*, Student–Newman–Keuls test was performed. Probability (p) values <0.05 were considered to indicate statistical significance. Analysis was carried out using SPSS for Windows® (Release 11.0.0, SPSS; Chicago, IL).

3. Results

3.1. Influence of TNF-α and fatty acids on monocyte rolling, adhesion, and transmigration

Superfusing monocytes over a HUVEC monolayer incubated under control conditions resulted in a low number of rolling and adherent monocytes (Fig. 1a and b). Monocyte rolling and adhesion both were markedly enhanced by nearly a factor of ten by a preceding 24 h exposure of HUVEC to TNF-α (10 ng/ml; $p < 0.01$). Pre-incubation of the HUVEC for 6 h with DHA with subsequent stimulation with TNF-α reduced this increase in rolling monocytes by nearly 40%. In contrast, AA increased number of rolling monocytes significantly compared to TNF and highly significantly compared to DHA ($p < 0.05$ vs. TNF-α; $p < 0.01$ vs. DHA, Fig. 1a). Incubation with TNF-α and AA reduced monocyte adhesion to HUVEC by 23% compared to sole TNF-α application ($p < 0.05$, Fig. 1b). Combined treatment with TNF-α + DHA reduced adhesion by nearly 67% ($p < 0.01$ vs. TNF-α; $p < 0.05$ vs. TNF + AA).

For static transmigration experiments, HUVEC grown in transwells were used. Transmigration of monocytes was measured after TNF-α pre-incubation of endothelial cells and MCP-1 and TNF-α as chemoattractant in the lower compartment (Fig. 1c). After 2 h of transmigration, TNF-α increased number of transmigrated monocytes by nearly 150% ($p < 0.01$ vs. control). Combined TNF-α and DHA pre-incubation resulted in a highly significant reduction of transmigrated monocytes compared to sole TNF-α application ($p < 0.01$). In contrast, simultaneous TNF-α and AA pre-incubation lead to a further small increase in number of transmigrated monocytes as compared to single TNF-α-application but differed significantly from TNF-α + DHA ($p < 0.01$).

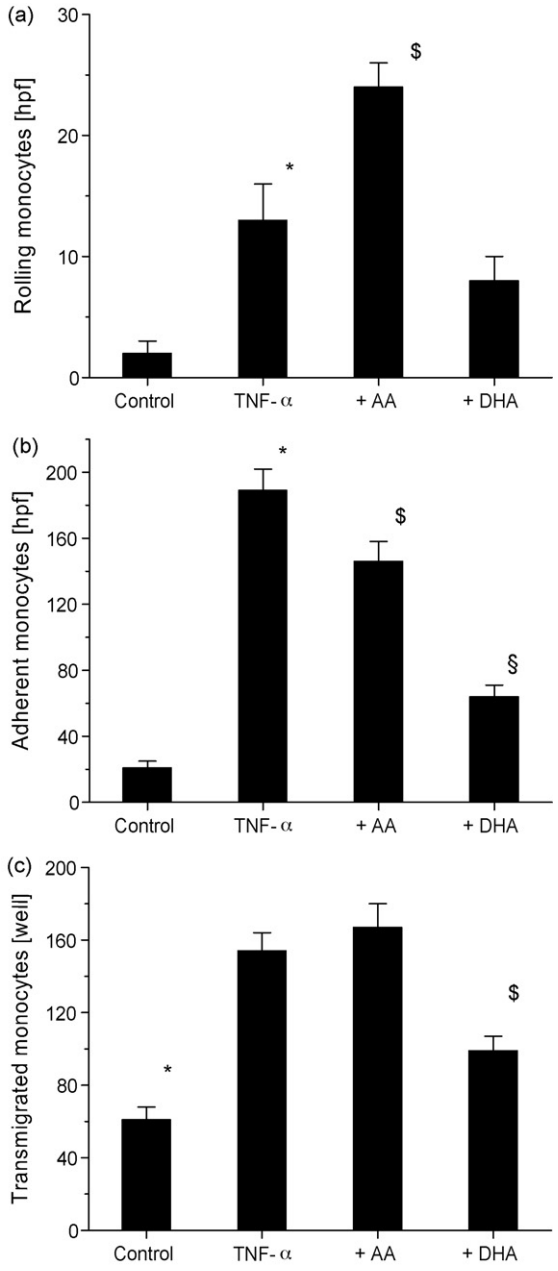

Fig. 1. Effects of DHA versus AA on monocyte rolling, adhesion and transmigration through tumor necrosis factor (TNF)-α-activated endothelial cells. (a) Monocyte rolling was significantly increased as compared to control by TNF-α ($^{*}p < 0.05$). TNF-α and arachidonic acid (AA) co-application further increased rolling ($^{\$}p < 0.05$ vs. TNF-α and $p < 0.01$ vs. control). After addition of docosahexaenoic acid (DHA), rolling was significantly reduced compared to AA-application ($^{\$}p < 0.01$). (b) Adhesion was increased by addition of TNF-α under all conditions as compared to controls (TNF: $^{*}p < 0.01$; +AA: $^{\$}p < 0.01$; +EPA: $^{\S}p < 0.05$). The increase in adhesion mediated by single TNF-addition differed from combined TNF-α and AA incubation ($^{\$}p < 0.05$) as well as combined TNF-α and DHA application ($^{\S}p < 0.01$). After pre-incubation with DHA, adhesion was lower as compared to AA application ($^{\S}p < 0.01$). (c) Transmigration was increased by addition of TNF-α under all conditions as compared to controls ($^{*}p < 0.01$ vs. TNF and TNF + AA, $p < 0.05$ vs. TNF + DHA). Transmigration was reduced after application of DHA ($^{\$}p < 0.01$ vs. TNF and vs. +AA). Data are given as mean ± S.E.M., $n = 8$–12 independent experiments each.

3.2. Influence of AA and DHA on TNF-α-induced endothelial adhesion molecule expression

To address the hypothesis that fatty acids might suppress monocyte adhesion by reducing the expression of endothelial adhesion molecules, FACS analysis of HUVEC was performed. E-selectin, VCAM-1, and ICAM-1 were significantly upregulated by TNF-α. However, neither AA nor DHA exerted a significant effect on this increase ($n = 5$–6 independent experiments, data not shown) as previously reported by us [27].

3.3. Effect of pre-incubation of HUVEC with PI3-kinase inhibitors on rolling, adhesion, and transmigration of monocytes

Confluent HUVEC were pre-incubated with fatty acids and TNF-α as detailed. The PI3-kinase inhibitors wortmannin (10 nM) or LY 294002 (200 nM) were added to HUVEC 60 min prior to superfusion or transmigration experiments. Cell viability as judged by trypan blue method was not impaired. Compared to TNF-treated controls, PI3-kinase inhibitors reduced rolling (TNF-α + wortmannin to 71 ± 10%; TNF-α + LY to 92 ± 7%), adhesion (TNF-α + wortmannin to 65 ± 19%; TNF-α + LY to 85 ± 10%), and transmigration (TNF-α + Wortmannin to 89 ± 3%; TNF-α + LY to 94 ± 1%). Pre-incubation of HUVEC with the PI3-kinase inhibitor wortmannin (data not shown) or LY294002 (Fig. 2) abolished the diverging effect of AA or DHA on rolling, adhesion, and transmigration: both PI3-kinase inhibitors prevented the DHA-induced reduction of rolling, adhesion, and transmigration. Consistent with this finding, AA was unable to increase rolling and transmigration of monocytes when HUVEC were pre-treated with either Wortmannin or LY294002.

3.4. Impact of fatty acids on phosphorylation of Akt in HUVEC after stimulation with VEGF, TNF-α, or staurosporine (STS)

EC were pre-incubated with or without fatty acids and stimulated with either VEGF, TNF-α, or staurosporine (STS) as detailed. After protein-extraction, Western blots were performed and probed for phospho Akt and total Akt. Total Akt was unchanged under all conditions. AA or DHA pre-incubation of HUVEC without stimulation did not result in significant changes as compared to control cells. Phosphorylation of Akt without addition of VEGF was 15 ± 5% when VEGF application was normalized to 100%. Both fatty acids decreased VEGF-induced phosphorylation: AA reduced Akt phosphorylation to 84 ± 3%, compared with VEGF-control, while DHA reduced Akt phosphorylation to 70 ± 4% ($p < 0.05$ vs. AA; Fig. 3a). Slightly different results were found using TNF-α. Phosphorylation of Akt without cytokine application was only 7 ± 2% when TNF-α-induced stimulation was set as 100%. Compared to TNF-α alone,

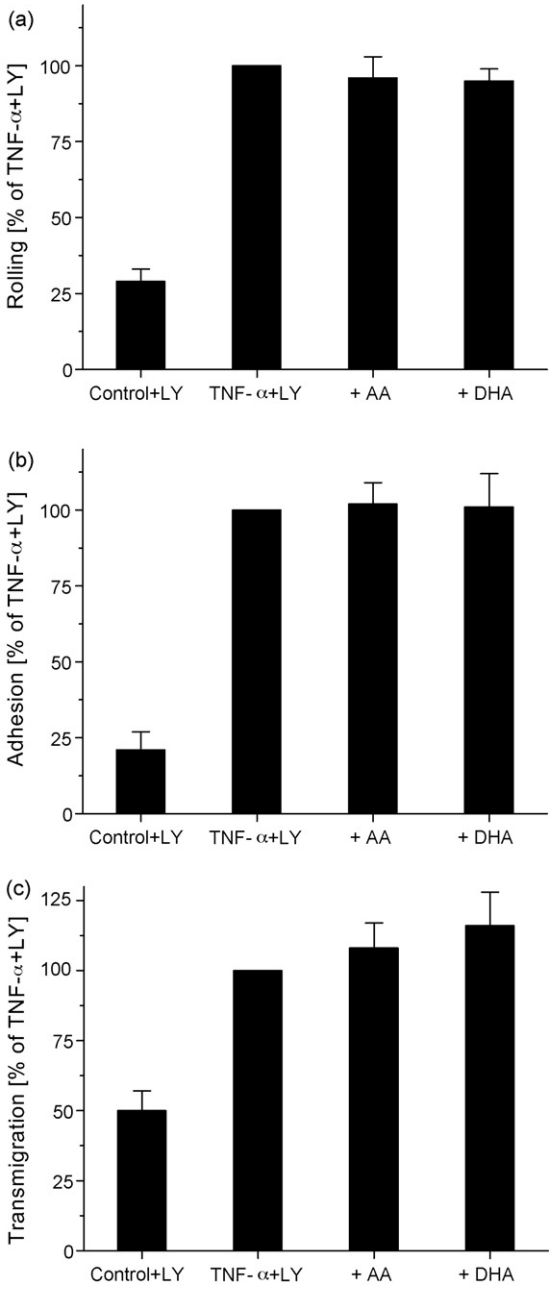

Fig. 2. Endothelial pre-incubation with PI3-kinase inhibitors prevents the effect of DHA or AA on rolling, adhesion, and transmigration of monocytes. Confluent HUVEC were pre-incubated with fatty acids, TNF-α, and the PI3-kinase inhibitor LY294002 (LY). Pre-incubation of HUVEC with LY lead to a slight reduction of rolling, adhesion and transmigration compared to experiments without PI3-kinase inhibitor. For comparison, TNF + LY was set as 100%. LY294002 abolished diverging effects of DHA and AA on rolling (a), adhesion (b), and transmigration (c). Data are given as mean ± S.E.M., $n = 6$–8 independent experiments.

co-application of TNF-α + AA increased phosphorylation of Akt slightly, but co-administration of TNF-α + DHA caused a significant reduction in Akt phosphorylation to 68 ± 5% ($p < 0.05$ vs. AA; Fig. 3b). A representative Western blot of phospho Akt and total Akt after TNF-stimulation is shown in Fig. 3c.

Staurosporin (STS) was used as alternative stimulus for Akt phosphorylation. Akt phosphorylation after staurosporin was set as 100% and phospho-Akt was determined as 62 ± 4% under control conditions (Fig. 3d). AA pre-incubation followed by STS application induced a rise to 126 ± 9% that differed significantly from pre-incubation with DHA causing a reduction to 82 ± 10% ($p < 0.05$, $n = 6$ independent experiments).

3.5. Effect of fatty acids on staurosporine (STS)-induced apoptosis

To investigate PI-3 kinase-dependent downstream events, we tested the impact of fatty acids on STS-induced apoptosis. Under control conditions, pre-incubation with fatty acids did not influence endothelial apoptosis as defined as annexin-V positive cells. Apoptosis of HUVEC increased from 8 ± 2% to 26 ± 1% after addition of STS. Pre-incubation of HUVEC with AA followed by stimulation with STS increased annexin-V-positive cells insignificantly. Pre-incubation with DHA, however, nearly doubled STS-induced apoptosis from 26 ± 1% to 51 ± 7% ($p < 0.05$ vs. STS, Fig. 4a; $n = 6$ independent experiments).

To confirm the effects of fatty acids, we measured caspase-3 activity in HUVEC after STS-administration. Incubation of fatty acids without STS-application did not significantly change baseline caspase-3 activity. Baseline caspase-3 activity was 21 ± 5% when STS-induced activity was set as 100% (Fig. 4b). Pre-incubation of AA reduced STS-induced caspase-3 activity to 86 ± 7%. In contrast, DHA increased STS-induced caspase-3 activity to 109 ± 7% ($p < 0.05$ vs. AA; $n = 6$ independent experiments).

4. Discussion

Investigating the impact of DHA versus AA on TNF-induced adhesive interactions, we found that in contrast to AA, DHA attenuated monocyte rolling, adhesion to, and transmigration through a TNF-α-activated endothelial layer. These diverging effects of both fatty acids were independent of simultaneous changes in expression of endothelial adhesion molecules but could be abolished using PI3-kinase inhibitors. Exploring further PI3-kinase signaling dependency on fatty acids, we found that pre-incubation of HUVEC with DHA significantly reduced phosphorylation of Akt after VEGF-, TNF-α-, and STS-stimulation when compared to pre-incubation with AA. Next, we showed that DHA increased STS-induced endothelial apoptosis as judged by two independent methods.

It has been previously demonstrated that fatty acids modulate leukocyte-endothelial adhesive interactions [27–29]. Our study demonstrates that pre-incubation of EC with DHA reduces rolling, inhibits adhesion, and ameliorates transmigration of monocytes through TNF-α activated HUVEC as compared to AA. This is well in line with previous work from other researchers and also from us concerning the role of *n*-

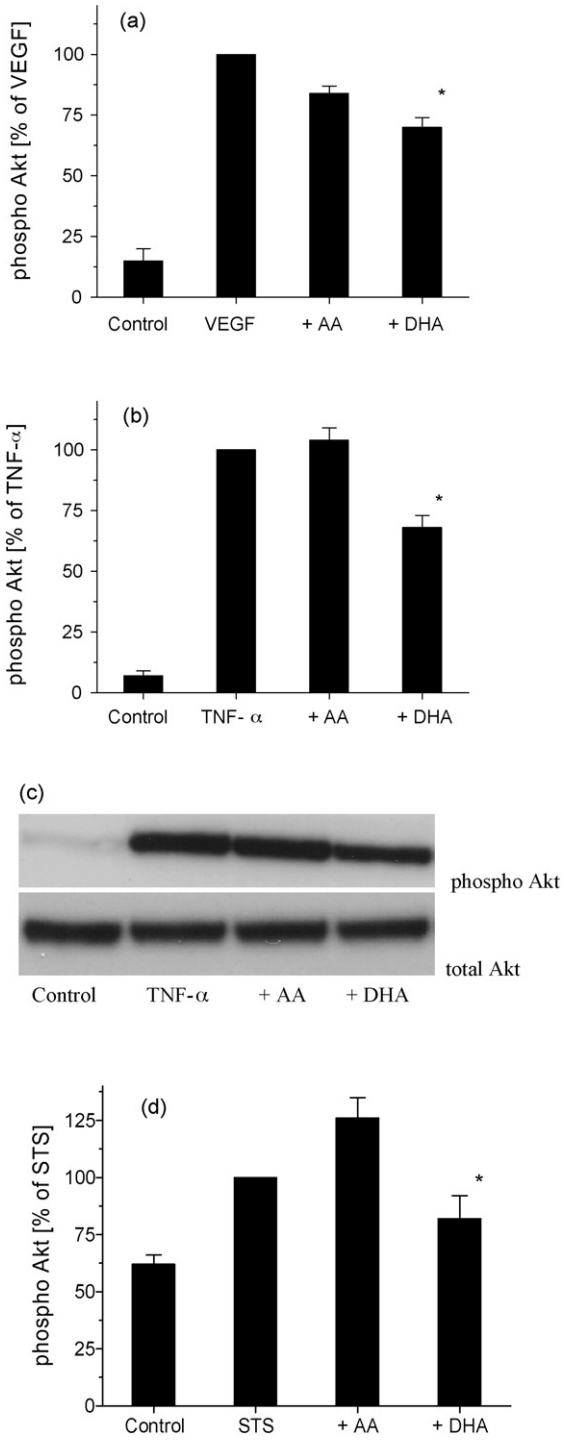

Fig. 3. DHA in contrast to AA reduces phosphorylation of Akt after VEGF-, TNF-α-, or staurosporin-stimulation. HUVEC were pre-incubated with fatty acids and stimulated with VEGF (a), TNF-α (b, c), or staurosporin (STS) (d). Western blots were probed for phospho Akt and total Akt. Total Akt was unchanged in all groups. (a) After VEGF stimulation, DHA reduced phosphorylation of Akt significantly compared to AA (*$p < 0.05$). (b) After TNF-α application, DHA induced a significantly diminished phosphorylation of Akt compared to AA (*$p < 0.05$). A representative blot after TNF-α stimulation is shown in (c). After STS challenge, DHA lead to a significant lower phosphorylation of Akt compared to AA (*$p < 0.05$). Data are given as mean ± S.E.M., $n = 6$ independent experiments each.

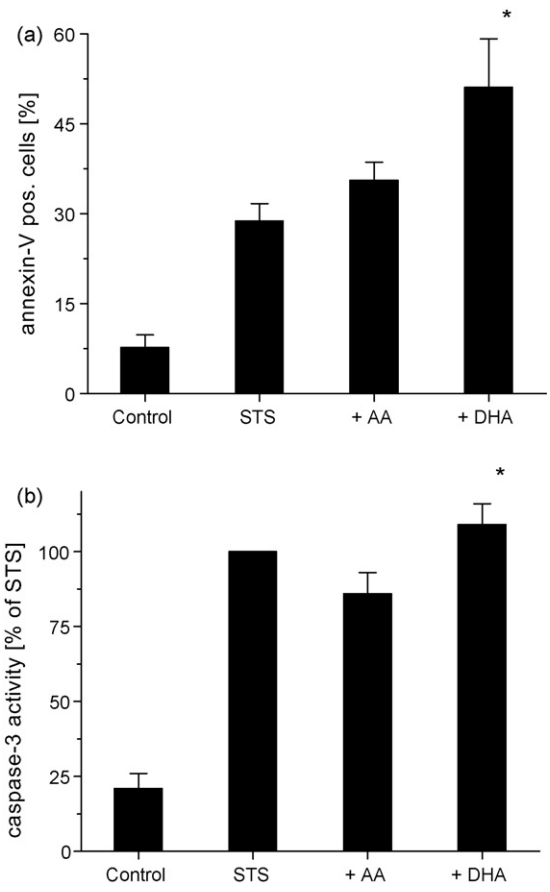

Fig. 4. DHA in contrast to AA increases apoptosis determined by annexin-V and caspase 3-activity. (a) DHA pre-incubation with subsequent STS-application resulted in a significant rise in apoptotic cells compared to sole staurosporine administration as judged by annexin-V positive cells ($p < 0.05$). (b) DHA pre-incubation significantly augmented caspase-3 activity in HUVEC as compared to AA ($p < 0.05$). Data are given as mean ± S.E.M., $n = 6$ independent experiments each.

3 fatty acids in endothelial-leukocyte interactions [27–29] although reduction of monocyte transmigration by DHA in this setting is a new result. Using physiological concentrations of *n*-3 fatty acids, we did not find a difference in the expression of endothelial adhesion molecules (ICAM-1, VCAM-1, E-selectin) which has been previously demonstrated by us [27], contrasting the results of other investigators who found a reduced presentation of adhesion molecules after exposure of endothelial cells to *n*-3 fatty acids [28,29]. However, their results were obtained under different conditions e.g. using higher concentrations of fatty acids (up to 300 μmol/l) and longer incubation times as compared to the more physiologic concentration (10 μmol/l) employed by us. However, the lack of a diverging regulation in adhesion molecules by DHA or AA is in line with a lack of regulation of adhesion molecules in monocytes isolated from volunteers undergoing infusion of fish oil-based or soybean oil-based lipid emulsions. Despite a major change in adhesive properties no difference in expression of adhesion molecules was detected [24].

Mechanisms underlying the protective effect of *n*-3 fatty acids on rolling, adhesion, and transmigration may also include a reduction in platelet-activating factor synthesis and other lipid mediators as prostaglandins, reduced expression and activation of leukocyte adhesion molecules, altered avidity and affinity of endothelial adhesion molecules as well as cytoskeletal rearrangement and membrane modulation [11,27,30]. Generation of 5-series leukotrienes, 3-series prostaglandins, and thromboxane A_3 is dependent on EPA. In order to investigate effects of the cell membrane on PI3-kinase-dependent effects and to be able to neglect the effect of eicosanoids, DHA but not EPA was used as *n*-3 fatty acids. In previous investigations, we could show that addition of DHA to endothelial cells under inflammatory conditions does not change EPA content in the cell membrane [30]. Due to these considerations, DHA was chosen as *n*-3 fatty acid.

In previous studies, we demonstrated an impact of fatty acids on membrane phospholipid signaling that was relevant to inflammatory induced adhesion [27,30]. Since PI3-kinase is also known to effect cytoskeletal rearrangements, integrin modulation, and leukocyte adhesion and transmigration [18] we evaluated the impact of PI3-kinase on fatty acid-induced modulation of the adhesion cascade in inflammation. Through the PI3-kinase/Akt/nitric oxide pathway, ICAM-1 expression can be up-regulated which leads to microvascular endothelial cell migration [17]. On a molecular basis, fatty acids might influence PI3-kinase signaling via its substrate phosphatidyl-inositol by incorporation into membrane (phospho)-lipid pools [10,11,30]. To define the effect of PI3-kinase and fatty acids on the adhesion cascade, we pre-treated EC with two different PI3-kinase inhibitors: wortmannin and LY294002. When PI3-kinase inhibitors were employed, the diverging effects pre-incubation with AA and DHA on rolling, adhesion and transmigration of monocytes were no longer detectable.

Now, the classical PI3-kinase signaling pathway, encompassing phosphorylation of Akt and apoptosis, remained to be evaluated for its dependency on fatty acids. After VEGF-application in HUVEC, a significant upregulation of phosphorylated Akt could be detected. We found a down-regulation of Akt phosphorylation by DHA when VEGF was employed as a highly specific stimulus to activate the PI3-kinase-dependent signaling cascade. The down-regulation after pre-incubation of HUVEC with DHA was significant compared to AA. When TNF-α was employed as a general stimulus of inflammation, we observed a significant elevation of phospho Akt. However, TNF-α stimulation after DHA pre-incubation resulted in a marked inhibition of Akt phosphorylation, which contrasted the effect of AA. Staurosporine as a classical stimulus of apoptosis also lead to an upregulation of phospho Akt. In this setting, AA resulted in a slight increase whereas DHA induced a significant decrease of phospho Akt. The notion that DHA and more general *n*-3 fatty acids decrease phosphorylation of Akt and thereby have an impact on inflammation, cell survival signaling, and apoptosis is also reported by others [31–33].

Our study demonstrating that DHA enhances endothelial apoptosis by decreasing phosphorylation of Akt compared to AA and demonstrating that DHA exerts its anti-inflammatory and pro-apoptotic effects in a PI3-kinase dependent manner in HUVEC is well in line with a pro-apoptotic effect of DHA in proliferating endothelial cells [34]. It contrasts the finding of an anti-apoptotic role of DHA in stress-induced apoptosis by the same group [35]. However, PI3-kinase signaling and its impact on apoptosis were not determined in these investigations [34,35]. Furthermore, recent reports emphasize the role of apoptosis in resolution of inflammation [19,36]. Fatty acids might not only modulate the induction but also the resolution of inflammation by inducing apoptosis of inflamed cells. This could contribute to prevent chronic inflammation [37] and may speculatively be linked to the generation of recently detected resolvins derived from *n*-3 fatty acids which were not investigated here [38]. It has to be taken into account that the effect of *n*-3 fatty acids on apoptosis may differ depending on the environment and stimulus as differing reports from the same group suggest [34,35]. Next, proliferation of endothelial cells in case of formation of collateral vessels in coronary artery disease may represent a very desirable effect. In addition, apoptotic effects of DHA in the central nervous system including brain and retina being rich in DHA may be regarded even dangerous in an inflammatory setting. There may be different consequences in chronic versus acute inflammation which warrants further investigation.

To summarize, independent of the applied stimulus (growth factor, inflammatory stimulus, or classical inducer of apoptosis) DHA pre-incubation always resulted in a marked diminished PI3-kinase-dependent phosphorylation of Akt compared to AA. Investigating downstream pathways of PI3-kinase and Akt using annexin-V analysis and caspase-3 measurement, we found that DHA compared to AA promotes EC apoptosis. PI3-kinase signaling seems to be important for parts of the anti-inflammatory action of the *n*-3 fatty acid DHA.

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Anlage 5

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N-3 vs. n-6 fatty acids differentially influence calcium signalling and adhesion of inflammatory activated monocytes: impact of lipid rafts

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Abstract

Background Anti-inflammatory n-3 fatty acids (FA) like docosahexaenoic acid (DHA) opposed to the pro-inflammatory n-6 FA arachidonic acid (AA) might modulate lipid rafts within the cell membrane by differential incorporation. In inflammation, monocyte adhesion to endothelial cells is a crucial step mediated by intracellular calcium changes. We investigated whether lipid rafts mediate FA-induced modulation of adhesion and intracellular calcium.

Methods In isolated human monocytes and monocytic U937 cells we measured adhesion to human umbilical vein endothelial cells (HUVEC) using a parallel flow chamber and a static assay, adhesion molecules by FACScan, and intracellular calcium by fluorescence. Monocyte lipid rafts were isolated by ultracentrifugation and submitted to gas chromatography for FA analysis.

Results Pre-incubation with AA or DHA resulted in a predominant incorporation of the respective FA into raft compared to non-raft fraction. DHA as compared to AA significantly reduced monocyte adhesion and calcium release after stimulation with TNF-α while expression of

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adhesion molecules remained unchanged. Pre-treatment with a calcium chelator abolished the effect of FA on calcium and adhesion. Disruption of lipid rafts prevented FA-induced modulations.
Conclusion Incorporation of FA into lipid rafts seem to be crucial for modulation of adhesion under inflammatory conditions.

Keywords Lipid rafts · Calcium · Inflammation · Adhesion · Fatty acids

Background

Enhanced transmigration of circulating blood monocytes across the vascular endothelium is considered to be an important contributor to the pathogenesis of chronic and acute inflammatory diseases. Here, atherosclerosis [1] as well as sepsis and multi-organ failure [2] are the most prominent examples [3]. This process of vascular inflammation requires adhesion of leukocytes to and migration through endothelium. Interaction between several adhesion molecules were shown to be involved in the transendothelial migration of monocytes, as well as enhanced calcium signalling, and other pro-inflammatory mediators like tumor-necrosis factor (TNF)-α [4–6]. It has also been demonstrated that TNF-α mediated nuclear factor-κB activation is dependent on lipid rafts [7].

Lipid rafts are sphingolipid- and cholesterol-rich signalling platforms within the plasma membrane. These membrane microdomains are characterized by being detergent resistant and liquid ordered [8]. They can organize domains on the inner leaflet, coupling events between the outer leaflet to others in the inner leaflet and the cell cytoplasm. Membrane microdomains influence membrane functions by concentrating signalling molecules in these particular regions of the surface for facilitated interaction. Otherwise, by excluding molecules from the rafts their interaction and subsequent signalling may be prevented [9, 10]. It has also been demonstrated in U937-monocytes [11] and in human monocytes [12] that rafts are important for inflammatory signal transduction [13, 14].

In the context of lipids and inflammation, eicosanoids represent a focus of interest due to their strong pro-inflammatory and anti-inflammatory properties [15, 16]. Different groups reported a major influence of nutrition including n-3 fatty acids (FA) on morbidity and mortality of intensive care patients as well as patients suffering from coronary artery disease [17, 18]. The family of n-6 FA, including arachidonic acid (AA), are the predominant polyunsaturated fatty acids (PUFA) in common western diet. The n-3 FA, including docosahexaenoic acid (DHA), are the predominant fat in cold-water fish and seal meat. N-3 vis-à-vis n-6 FA incorporation into membrane (phospho)-lipid pools was suggested to influence lipid-related intracellular signaling events [19].

It has been demonstrated that FA are capable of modulating lipid raft composition and raft related signalling [20]. Due to the ability of PUFA to inhibit palmitoylation, n-3 vs. n-6 FA might possess different abilities to target signalling proteins into rafts [21, 22]. Modulation of membrane composition due to incorporation of different FA represents a means by which PUFA could differentially influence rafts and subsequently raft-dependent signal transduction. Reports demonstrate that in particular anti-inflammatory capacity of n-3 FA might be in part due to raft modulation, namely due to their potential to alter both the composition of signalling molecules and the lipid composition within the rafts [22, 23].

The aim of this study was firstly, to investigate whether n-3 vs. n-6 FA differentially influence lipid raft fatty acid composition in U937-monocytes as well as in freshly isolated human blood monocytes. Secondly, we investigated whether a differential fatty acid composition of lipid rafts influenced intracellular calcium and monocyte adhesion to human umbilical venous endothelial cells (HUVEC) after TNF-α stimulation.

Methods

Materials

Chemicals of highest purity were obtained from Merck (Darmstadt, Gernany). AA and DHA were obtained from Sigma Chemical (Deisenhofen, Germany). Tissue culture plastic supplies were purchased from Becton–Dickinson (Heidelberg, Germany). Cell culture reagents, buffer, and media were from PAN (Aidenbach, Germany). Lyn and Fyn antibody for Western Blots were obtained from BD Biosciences (Pharmingen, Germany). Secondary antibodies were purchased from Santa Cruz (Heidelberg, Germany). Blot analysis was performed with BioDoc II Biometra (Göttingen, Germany) with Scan Pack 3.0 software.

Experimental protocol

U937-monocytes were cultured, human blood monocytes were isolated and HUVEC were grown to confluence. Then, the culture medium was exchanged, and free fatty

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acids (AA and DHA) dissolved in ethanol (final volume ≤1 %, vol/vol), or vehicle were admixed to the culture medium at a final concentration of 10 µmol/l in all experiments with U937 and fresh monocytes. The concentration was derived from our previous experiments and due to the fact that this concentration of free AA and DHA is similar to that detected in human plasma [24–26]. Controls were sham incubated with solvent only.

TNF-α was added after FA pre-incubation to U937 and to fresh monocytes in all experiments at a concentration of 1 ng/ml. In static adhesion experiments with U937, additionally a second concentration of 10 ng/ml was applied. In dynamic flow adhesion investigations using freshly isolated monocytes, HUVEC were incubated with 10 ng/ml TNF-α for 20 h prior to the beginning of the experiments.

FA pre-incubation times were 24 h in all experiments with U937, and 2 h in all experiments with freshly isolated monocytes. Due to the nature of the tumor cell line, pre-incubation and stimulation time as well as the kind of adhesion assay varied between U937 and freshly isolated human blood monocytes.

After FA pre-incubation, TNF-α stimulation followed for 4 h in experiments with U937 and for 2 h in experiments with freshly isolated monocytes. Only in experiments for calcium measurements in U937, TNF-α stimulation time was 1 h.

In experiments with calcium-chelator [10 µmol/l 1,2-*bis*(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra acetoxymethyl-ester(BAPTA); (Calbiochem; Bad Soden, Germany)] a co-incubation with BAPTA and FA was performed. In experiments with disruption of lipid rafts, methyl-ß-cyclodextrin (MCD, Sigma, Dreisenhofen, Germany) was used at a concentration of 10 mmol/l for 10 min at 37 °C after FA incubation and prior to TNF-α stimulation.

Cell culture

Endothelial cells were obtained from human umbilical veins according to the method described by Jaffe et al. [27]. Cells of the passages 1–4 were used in all experiments.

The monocyte tumor cell line U937 was cultured in RPMI culture medium with supplementation of 5 % fetal calf serum (FCS), and 1 % penicillin/streptomycin (both PAA, Linz, Austria). Cells were splitted 1–4 every 48 h.

Fluorescence imaging: calcium measurement using a microplate reader

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were determined using the fluorescent Ca^{2+} indicator Fluo-3-AM (Molecular Probes, Leiden, Netherlands). Monocytes were labelled with 5 µmol/l Fluo-3-AM. After an incubation period of 90 min, extracellular Fluo-3-AM was removed and the medium was exchanged using PBS buffer. Changes of $[Ca^{2+}]_i$ in monocytes were analysed by a "GENios Plus multi-detection microplate reader with enhanced fluorescence" (Tecan Inc., Research Triangle Park, NC) after 1 h stimulation with 1 ng/ml TNF-α. The excitation wavelength was set at 485 nm and emitted light was detected at 535 nm. Fluorescence background determination and calibration were performed according to methods described by Fowler [28] and Baskin [29].

Static adhesion assay

The static interaction assay between monocytes and endothelial cells has been described previously [30]. Briefly, after FA pre-incubation, U937-monocytes were labelled with 3 µg/ml 2,-7-bis2carboxyethyl5and6carbox fluorescein, acetoxymethylester (BCECF-AM) (Molecular Probes, Leiden, Netherlands) at 37 °C for 30 min, washed, and resuspended in medium. 250 000 BCECF-AM-loaded U937-monocytes per well were co-incubated with confluent HUVEC for 4 h in a 24-well plate after stimulation with 1 or 10 ng/ml TNF-α. After aspiration of the medium, three washing steps with PBS followed to remove non-adherent cells. Adherent cells were lysed using 1 mol/l NaOH for 45 min. Fluorescence was measured by multi-detection microplate reader including fluorescence background determination and calibration as detailed above. All measurements were corrected by subtraction of the fluorescence of non-treated cells as background.

Isolation of human blood monocytes

Human monocytes were isolated from platelet pheresis residues by Ficoll-Hypaque density gradient centrifugation, followed by counterflow centrifugation elutriation using a Beckman JE-5.0 rotor as described [31]. Monocyte purity (88–98 %) was confirmed by light scatter [fluorescence-activated cell sorter (FACS) scan; Becton–Dickinson]. Cell viability was above 96 % throughout the study measured by trypan blue test.

Flow-chamber adhesion assay

Adhesion of freshly isolated human blood monocytes to HUVEC was determined as described previously [26] using a parallel plate flow chamber according to Lawrence and Springer [32]. Confluent endothelial monolayers were pre-incubated with fatty acids and TNF-α according to the experimental protocol. HUVEC were pre-incubated with 10 ng/ml TNF-α for 20 h [26]. A suspension of 2×10^6 monocytes was perfused through the chamber at a constant wall shear stress of 1.0 dyn/cm^2 (syringe pump sp100i, WPI; Sarasota, FL). Interactions were visualized using a phase contrast video microscope (IMT-2, Olympus Optical,

Hamburg, Germany, with a KP-C551 CCD camera, Hitachi, Rodgau, Germany) and videotaped (JVC HR-S7000, JVC; Friedberg, Germany) over the entire time period of monocyte perfusion. Rolling in the parallel plate flow chamber was measured in one high-power field for each experiment. "Rolling" was expressed as the number of rolling cells per high power field (20× objective) during a 10-min observation period. Monocytes were considered to be adherent after 30 s of stable contact with the monolayer. Adhesion was determined after 10 min of perfusion by analysis of five random high magnification fields (20×) from videotape. Results are expressed as adherent cells per high magnification field.

FACScan immunofluorescence analysis of adhesion molecules

U937-monocytes and freshly isolated human monocytes were subjected to immunofluorescence staining by incubation of samples with appropriately diluted PE-labeled anti-CD11b (Mac-2)mAb or anti-CD18 mAb or anti-CD49d mAb (very late antigen (VLA)-4) mAb (R&D Systems, Wiesbaden, Germany) for 30 min at ambient temperature followed by flow cytometric analysis using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was performed using the CellQuest software package (BD Biosciences).

Detergent-resistant membrane (lipid raft) partitioning

Lipid raft isolation was performed by a modified method according to Waheed and Jones [33]. Briefly, cell pellets from U937-monocytes and from freshly isolated human monocytes were resuspended and incubated for 20 min at 4 °C in ice cold buffer containing 0.5 % Triton X-100 to give a detergent to protein ratio of 5:1. The samples were adjusted to 35 % (v/v) OptiPrep (Sigma; Dreisenhofen, Germany) and the lipid raft fraction separated by centrifugation on a 5/30/35 % OptiPrep sucrose-gradient. After ultra-centrifugation, nine fractions were collected. Determination of two raft-marker (Scr-family protein tyrosine kinases Lyn and Fyn) by Western Blot was performed demonstrating detection of Lyn and Fyn within fraction one and two. Thereby we defined fractions one and two as raft fractions and fractions three to nine as non-raft fractions.

Membrane fatty acid analysis

Membrane fatty acid analysis of U937-monocytes and freshly isolated human monocytes of pooled raft fractions one and two and the pooled non-raft fractions three to nine was done by gas chromatography as described [34] after lipid extraction according to Bligh and Dyer [35].

Fig. 1 Impact of AA and DHA-incubation on U937-monocyte and freshly isolated human blood monocyte membrane FA composition: analysis of raft and non-raft fraction. Membrane fatty acid analysis of U937- and freshly isolated human blood monocytes raft fraction and non-raft fraction was performed after lipid raft separation. A western blot depicting monocyte raft marker Lyn (**a**) and Fyn (**b**) in the first two of nine fractions is shown. Fractions one and two were analysed as raft fraction; fractions three to nine as non-raft fraction. In U937-monocytes, arachidonic acid (AA) pre-incubation and TNF-α stimulation resulted in a non-significant rise of AA in non-raft fraction (**c**) whereas a highly significant rise was noted in raft fraction (**d**, $p < 0.001$). In U937-monocytes, docosahexaenoic acid (DHA) pre-incubation and TNF-α stimulation induced a highly significant rise in both non-raft (**e**) and raft (**f**) fraction ($p < 0.001$) though with a marked enhanced incorporation into raft fraction. In freshly isolated human blood monocytes, AA pre-incubation and TNF-α stimulation resulted in a significant rise of AA in non-raft fraction (**g**) though in raft fraction a significant rise of AA compared to TNF-α and compared to DHA was detected which was more pronounced (**h**, $p < 0.05$). In freshly isolated human blood monocytes, DHA pre-incubation and TNF-α stimulation induced a highly significant rise in both non-raft (**i**) and raft (**j**) fraction ($p < 0.001$) though with a marked enhanced incorporation into raft fraction. Two-way analysis of variance revealed no significant impact of TNF-α on the incorporation of AA into raft or non-raft fractions as compared to control conditions. While DHA incorporation into the non-raft fraction was not influenced by TNF-α, DHA was significantly increased under inflammatory conditions in the raft fraction (*j*; §, $p < 0.05$). Data are given as mean ± SEM

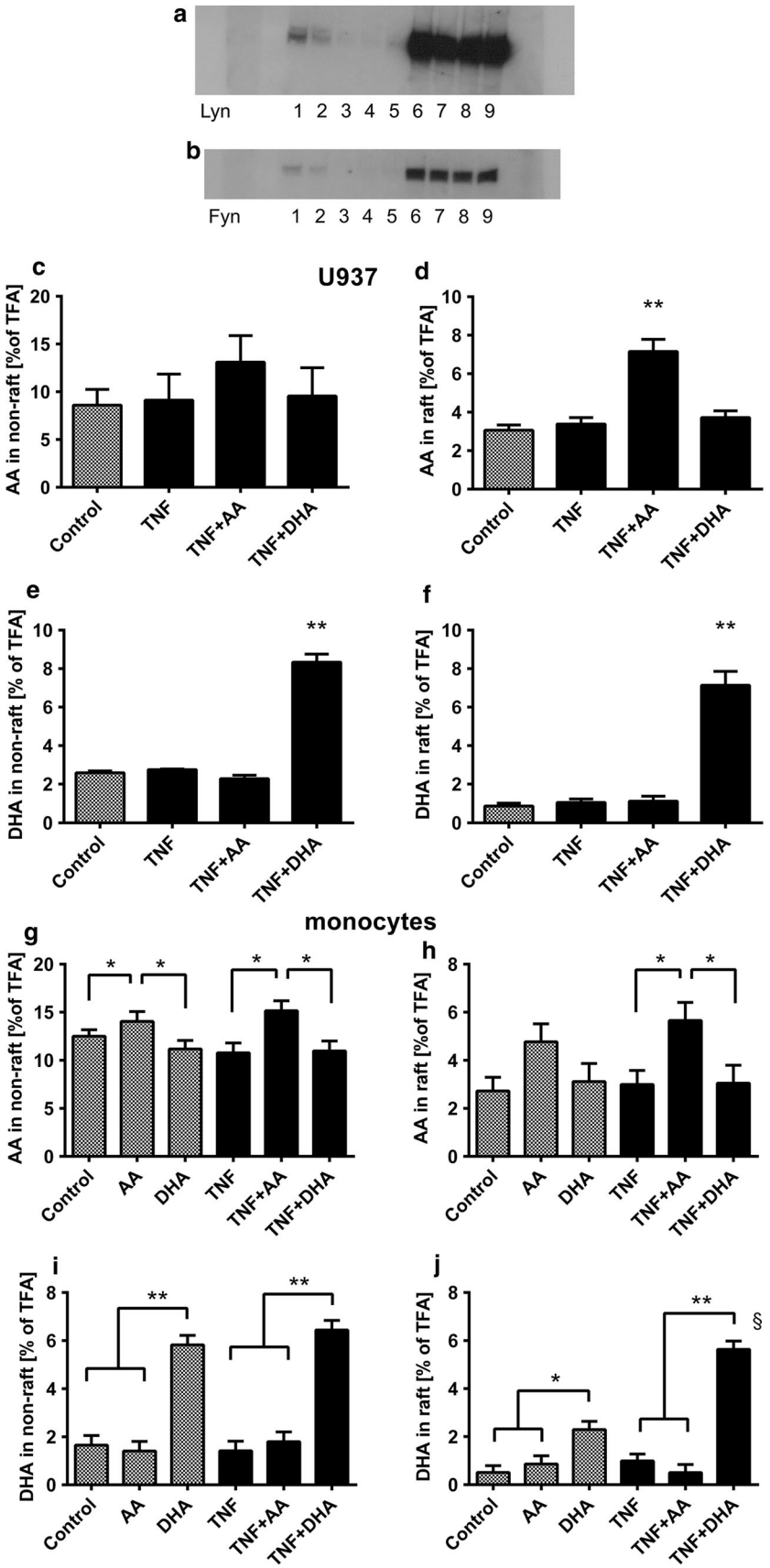
Statistics

Data are given as mean ± SEM. One-way analysis of variance and Student–Newman–Keuls post hoc test was performed to test for differences between different experimental groups in experiments with U937-monocytes. Since freshly isolated human monocytes also underwent experiments where fatty acids were examined without TNF-α, two-way analysis of variance and Student–Newman–Keuls post hoc test was performed to test for differences between different experimental groups. Data generated from rolling experiments after treatment with MCD were not distributed normally. Therefore, log-transformation was applied. Probability (p) values <0.05 were considered to indicate statistical significance. Analysis was carried out using SigmaStat® version 3.5.

Results

Fatty acid composition of raft and non-raft membranes of U937-monocytes

U937-monocytes were pre-incubated with FA and stimulated with 1 ng/ml TNF-α followed by separation of raft and non-raft membrane fractions as described in the method section. A western blot of the monocyte raft


a
Lyn
1 2 3 4 5 6 7 8 9
b
Fyn
1 2 3 4 5 6 7 8 9
U937
c
AA in non-raft [%of TFA]
d
AA in raft [%of TFA]
e
DHA in non-raft [% of TFA]
f
DHA in raft [% of TFA]
Control
TNF
TNF+AA
TNF+DHA
monocytes
g
AA in non-raft [%of TFA]
h
AA in raft [%of TFA]
i
DHA in non-raft [% of TFA]
j
DHA in raft [% of TFA]
AA
DHA
§

marker Lyn and Fyn is depicted. Lyn and Fyn are situated in the first and second lane (raft fraction). Both proteins cannot be detected in the non-raft fractions and but in the "pellet" fractions to demonstrate a proper separation of raft and non-raft parts of the membrane (Fig. 1a, b). Subsequently, raft and non-raft fractions were submitted to FA analysis as described.

Incubation with AA and TNF-α resulted in a 1.4-fold but not significant increase of AA in total fatty acids (TFA) of the non-raft membrane fraction to 13.11 ± 2.78 % compared to sole TNF-α stimulation with 9.12 ± 2.74 % ($n = 3$–5; Fig. 1c). In contrast, incubation with AA and TNF-α induced a highly significant 2.1-fold rise in AA in raft TFA to 7.15 ± 0.64 % compared to stimulation with TNF-α (3.39 ± 0.33 %, $p < 0.001$; $n = 4$–7; Fig. 1d). In line with these findings, stimulation with DHA and TNF-α lead to a 3.0-fold rise in DHA to 8.34 ± 0.41 % compared to TNF-α stimulated cells with 2.75 ± 0.04 % of TFA in the non-raft membrane fraction ($p < 0.001$; $n = 3$–7; Fig. 1e). Though in raft membrane fraction, incubation with DHA and TNF-α enhanced DHA of TFA even stronger (6.8-fold) to 7.14 ± 0.72 % compared to TNF-α with 1.05 ± 0.18 % ($p < 0.001$; $n = 3$–7; Fig. 1f).

Fatty acid composition of raft and non-raft membranes of freshly isolated human blood monocytes

Freshly isolated human blood monocytes were pre-incubated with FA and stimulated with 1 ng/ml TNF-α followed by separation of rafts and non-raft membrane and subsequently submitted to FA analysis as described.

Incubation of AA without TNF-α lead to a significant rise of AA in total fatty acids (TFA) to 15.51 ± 1.023 % compared to control and DHA in the non-raft fraction ($p < 0.05$, Fig. 1g; $n = 3$–7). Incubation with AA and TNF-α resulted also in a 1.4-fold increase of AA in TFA of the non-raft membrane fraction to 15.16 ± 1.03 % compared to sole TNF-α stimulation with 10.78 ± 1.03 % ($p < 0.05$; Fig. 1g; $n = 3$–7).

In raft-fraction, incubation of AA without TNF-α lead to a not significant rise of AA in TFA to 4.77 ± 0.75 % compared to control with 2.72 ± 0.58 %. Notably, incubation with AA and TNF-α induced a significant 1.9-fold rise in AA in raft TFA to 5.66 ± 0.75 % compared to control cells stimulated with TNF-α (3.00 ± 0.58 %, $p < 0.05$; Fig. 1h; $n = 3$–6).

In line with these findings, in non-raft fraction, incubation of DHA without TNF-α lead to a highly significant rise of DHA in TFA to 5.82 ± 0.40 % compared to control with 1.66 ± 0.40 % ($p < 0.001$).

Pre-incubation with DHA and subsequent stimulation by TNF-α lead to a 4.5-fold rise in DHA to 6.44 ± 0.40 % compared to TNF-α stimulated cells with 1.42 ± 0.40 % of TFA in the non-raft membrane fraction ($p < 0.001$; Fig. 1i; $n = 3$).

Though in raft membrane fraction, incubation of DHA without TNF-α lead to a significant rise of DHA in TFA to 2.30 ± 0.34 % compared to control with 0.51 ± 0.29 % ($p < 0.05$).

Incubation with DHA + TNF-α enhanced DHA of TFA 5.7-fold to 5.64 ± 0.34 % compared to TNF-α with 0.99 ± 0.29 % ($p < 0.001$; Fig. 1j; $n = 3$–4).

Two-way analysis of variance revealed no significant impact of TNF-α on the incorporation of AA into raft or non-raft fractions as compared to control conditions. While DHA incorporation into the non-raft fraction was not influenced by TNF-α, DHA was significantly increased under inflammatory conditions in the raft fraction ($p < 0.05$; Fig. 1j).

Impact of fatty acids on static adhesion of U937-monocytes after TNF-α stimulation

Adhesion of unstimulated U937-monocytes to HUVEC was determined as 492.73 ± 40.46 fluorescence units (FU) which was highly significantly different from all TNF-α treated groups ($p < 0.001$, Fig. 2a). Stimulation of U937-monocytes with 10 ng/ml TNF-α for 4 h increased adhesion to HUVEC (3335.77 ± 111.84 FU). Pre-incubation with AA + TNF-α resulted in a comparable adhesion (3212.02 ± 288.73 FU). However, DHA + TNF-α pre-treatment induced a significant reduction of fluorescence (2900.48 ± 142.65 FU) compared to AA + TNF-α and compared to TNF-α ($p < 0.05$, $n = 7$–11; Fig. 2a).

Stimulation of U937-cells with 1 ng/ml TNF-α for 4 h increased subsequent adhesion to endothelial cells. Fluorescence increased from 168.30 ± 4.94 FU (control) to 183.564 ± 2.14 FU. Pre-incubation of monocytes with DHA + TNF-α resulted in a slightly reduced adhesion (181.24 ± 3.00 FU) compared to TNF-α. In contrast, incubation of U937-monocytes with AA + TNF-α induced a highly significant difference in adhesion compared to DHA and compared to TNF-α [(274.16 ± 6.61 FU); ($p < 0.001$, $n = 4$–5; Fig. 2b)]. Since we aimed at investigating the FA-induced modulation, we continued our experiments using the lower TNF-α dose (1 ng/ml) that allowed a highly significant difference in adhesion between FA groups and did not provoke a ceiling effect as observed with 10 ng/ml TNF-α.

Impact of fatty acids on dynamic flow-chamber adhesion of freshly isolated human monocytes after TNF-α stimulation

Adhesion of unstimulated freshly isolated human monocytes under flow conditions to HUVEC was determined as

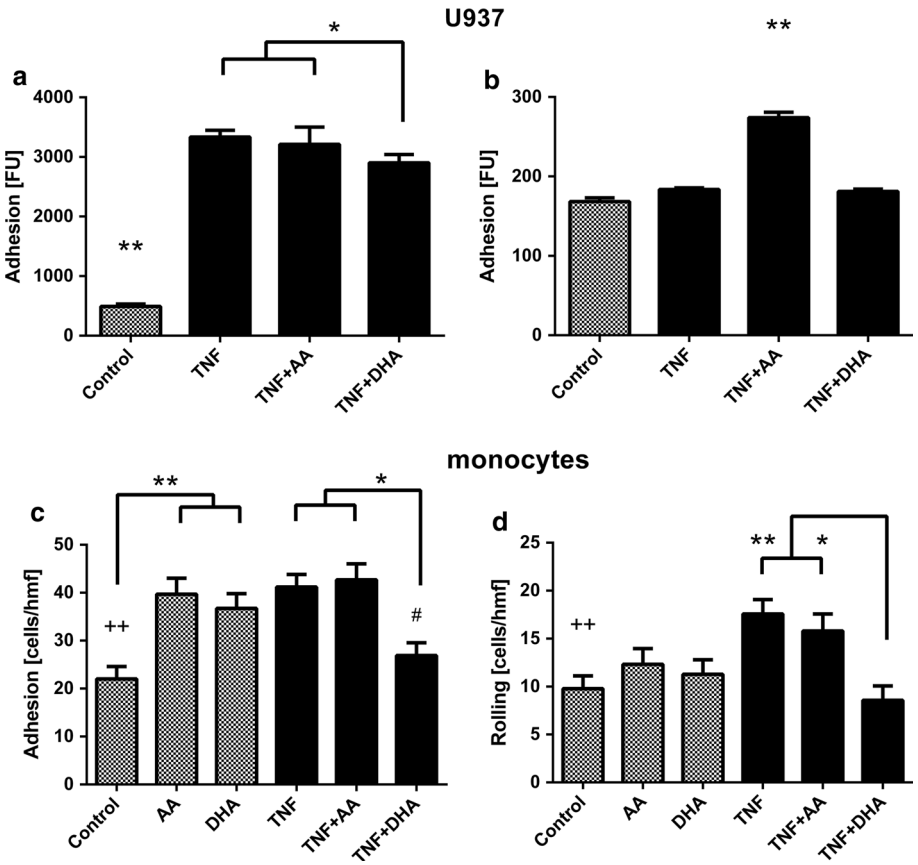


Fig. 2 Effect of FA pre-incubation on U937- and on human monocytes adhesion and rolling. U937-monocytes were pre-incubated with arachidonic acid (AA), docosahexaenoic acid (DHA), or vehicle followed by stimulation with TNF-α. Adhesion of U937 to HUVEC was measured using a fluorescence-based static assay. After stimulation with 10 ng/ml TNF-α adhesion was significantly different between the FA groups (**a**, $p < 0.05$). After stimulation with 1 ng/ml TNF-α FA induced a highly significant modulation of adhesion (**b**, $p < 0.001$). Freshly isolated human monocytes were pre-incubated with arachidonic acid (AA), docosahexaenoic acid (DHA), or vehicle followed by stimulation with 1 ng/ml TNF-α. Rolling and adhesion to HUVEC under laminar flow conditions was measured using a parallel plate flow chamber. Pre-incubation with DHA induced a highly significant reduction compared to TNF-α and a significant down-regulation compared to AA-pre-incubation and TNF-α stimulation (**c**, $p < 0.001$ and $p < 0.05$, respectively). DHA induced significantly less rolling after stimulation with 1 ng/ml TNF-α than AA and highly significantly less rolling than sole TNF-α stimulation (**d**, $p < 0.05$ and $p < 0.001$, respectively). Data are given as mean ± SEM

22 ± 3 adherent cells per high magnification field, and increased upon pre-incubation of freshly isolated human monocytes with 10 μM of AA to 40 ± 3 adherent cells and with DHA to 37 ± 3 adherent cells per high magnification field. AA and DHA differed highly significantly from unstimulated control. Stimulation of freshly isolated human monocytes with TNF-α increased adhesion to HUVEC highly significantly to 41 ± 3 adherent cells per high magnification field compared to unstimulated control. Pre-incubation with AA + TNF-α augmented adhesion to 43 ± 3 adherent cells per high magnification field. DHA + TNF-α pre-treatment induced a highly significant reduction to 27 ± 3 adherent cells per high magnification field compared to TNF-α and a significant reduction compared to TNF-α + AA as well as to DHA without TNF-α ($p < 0.001$ and $p < 0.05$; $n = 6$–10; Fig. 2c).

Impact of fatty acids on dynamic flow-chamber rolling of freshly isolated human monocytes after TNF-α stimulation

Rolling of unstimulated freshly isolated human monocytes under flow conditions to HUVEC was determined as 10 ± 1 rolling cells in 10 min, and increased upon pre-incubation of freshly isolated human monocytes with 10 μM of AA to 12 ± 2 rolling cells per high magnification field and with DHA to 11 ± 2 rolling cells per high magnification field. Stimulation of freshly isolated human monocytes with TNF-α increased rolling to HUVEC highly significantly to 18 ± 2 rolling cells per high magnification field compared to unstimulated control. Pre-incubation with AA + TNF-α resulted in rolling of 16 ± 2 cells per high magnification field. DHA + TNF-α pre-treatment

induced a highly significant reduction to 9 ± 2 rolling cells per high magnification field compared to TNF-α and a significant reduction to TNF-α + AA ($p < 0.001$ and $p < 0.05$, $n = 5$–9; Fig. 2d).

Analysis of surface expression of adhesion molecules on U937-monocytes

Immunofluorescence analysis of CD11b (Mac-2), CD18, or CD49d (very late antigen (VLA)-4) on U937-monocytes by FACScan under the above mentioned conditions did not show a significant modulation between any groups (data not shown).

Analysis of surface expression of adhesion molecules on freshly isolated human monocytes

Immunofluorescence analysis of CD11b (Mac-2), CD18, or CD49d (very late antigen (VLA)-4) on freshly isolated human monocytes by FACScan under the above mentioned conditions did not show a significant modulation between any groups (data not shown).

Effect of the calcium-chelator BAPTA on FA-modulation of U937- and freshly isolated human monocyte adhesion to HUVEC after TNF-α exposure

Co-incubation of U937- and human monocytes with 10 μmol/l BAPTA and AA or DHA subsequent stimulation with 1 ng/ml TNF-α induced a similar increase in adhesion as TNF-α when compared to unstimulated cells (negative control) and were no longer able to differentially modulate adhesion. AA or DHA incubation of HUVEC without TNF-α-stimulation did not differ from negative control (data not shown).

Differential impact of FA on intracellular calcium in U937-monocytes after TNF-α stimulation

Stimulation of U937-monocytes with 1 ng/ml TNF-α induced an increase to 191.80 ± 8.25 % of intracellular calcium measured after 1 h with Fluo-3-AM-fluorescence with unstimulated U937-cells (negative control) set as 100 %. Pre-incubation of U937 with 10 μM DHA for 24 h and subsequent stimulation with TNF-α induced a significant reduction in calcium release to 153.97 ± 10.16 % compared to TNF-α and TNF-α + AA with 191.80 ± 8.25 % and 190.33 ± 7.34 %, respectively ($p < 0.05$; $n = 7$–8; Fig. 3a). Co-stimulation with FA and calcium-chelator BAPTA did not result in any significant modulation of intracellular calcium after TNF-α stimulation (data not shown).

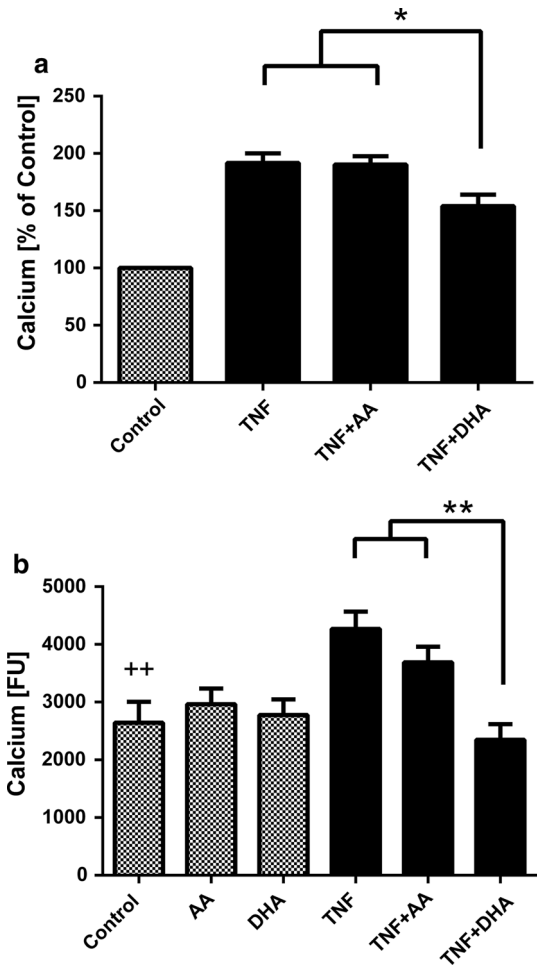


Fig. 3 Intracellular calcium is modulated by U937- and freshly isolated human monocyte pre-incubation with FA. U937-monocytes were pre-incubated arachidonic acid (AA), docosahexaenoic acid (DHA), or vehicle followed by stimulation with 1 ng/ml TNF-α. Intracellular calcium was measured using a fluorescence-based assay. DHA induced a significant reduction of intracellular calcium compared to AA and compared to TNF-α alone (**a**, $p < 0.05$). Using the same assay to measure intracellular calcium but examining freshly isolated human monocytes, pre-incubation with DHA and stimulation with 1 ng/ml TNF-α resulted in a highly significant reduced value of intracellular calcium compared to AA-pre-incubation with TNF-α stimulation and sole TNF-α stimulation (**b**, $p < 0.001$). Data are given as mean ± SEM

Differential impact of FA on intracellular calcium in freshly isolated human monocytes after TNF-α stimulation

Stimulation of freshly isolated human monocytes with 1 ng TNF-α induced a highly significant increase to 4270 ± 297 Fluorescence Units(FU) of intracellular calcium measured with Fluo-3-AM-fluorescence compared to unstimulated human monocytes with 2643 ± 364 FU. FA incubation without TNF-α resulted in equal levels of intracellular calcium as negative control with AA inducing 2966 ± 269

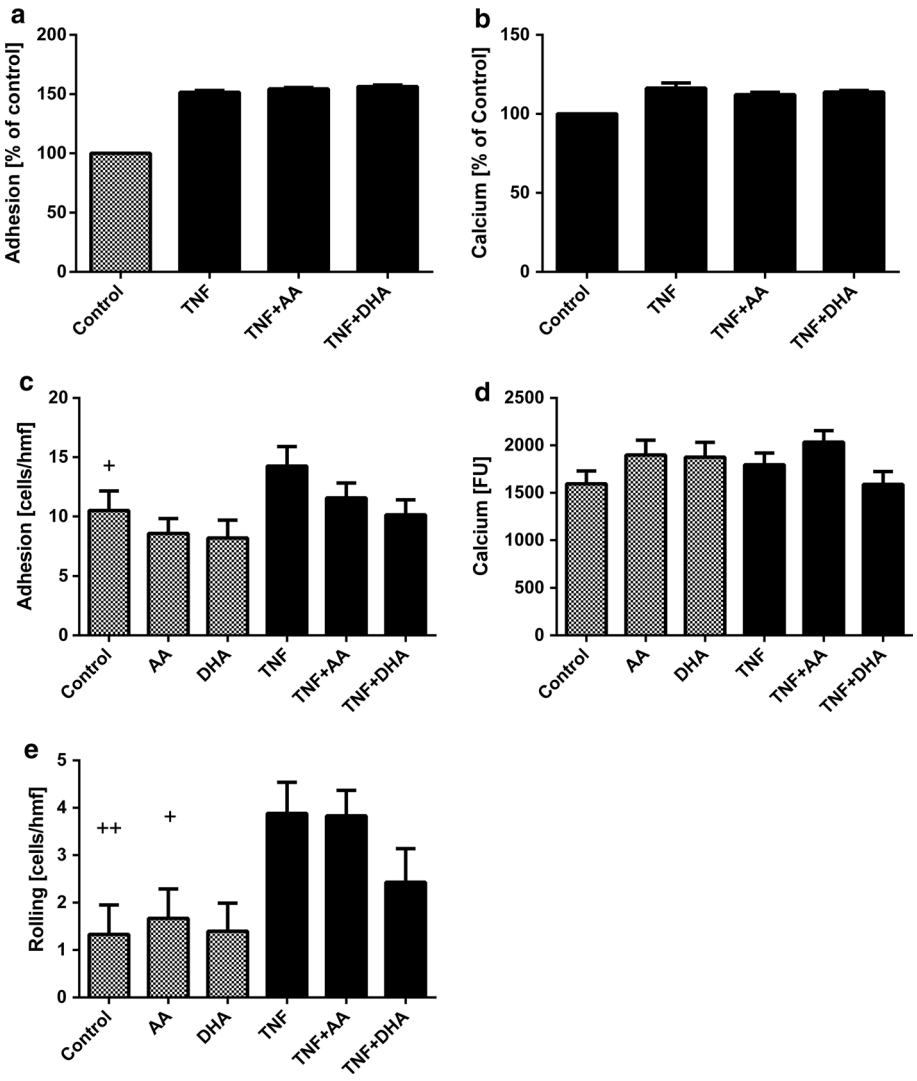


Fig. 4 Disruption of U937- and freshly isolated human monocyte lipid rafts by MCD abolishes FA-induced modulation of adhesion, intracellular calcium and human monocyte rolling. Treatment of U937-monocytes with MCD prior to TNF-α stimulation abolished FA-induced effect on adhesion (**a**) and intracellular calcium (**b**). Treatment of freshly isolated human monocytes with MCD prior to TNF-α stimulation prevented a significant modulation of adhesion under flow conditions (**c**), intracellular calcium (**d**) and of rolling under flow conditions (**e**) by fatty acids. Data are given as mean ± SEM

FU and DHA inducing 2777 ± 269 FU. Pre-incubation of human monocytes with 10 μM DHA and subsequent stimulation with TNF-α induced a highly significant reduction in calcium release to 2349 ± 269 FU compared to TNF-α and TNF-α + AA with 4270 ± 297 FU and 3690 ± 269 FU, respectively ($p < 0.001$; $n = 6$–11; Fig. 3b). Co-stimulation with FA and calcium-chelator BAPTA did not result in any significant modulation of intracellular calcium after TNF-α stimulation (data not shown).

Effect of raft disruption by MCD on FA-induced modulation of U937-monocyte adhesion and intracellular calcium after TNF-α stimulation

Adding 10 mmol/l MCD for 30 min prior to stimulation with 1 ng/ml TNF-α of U937-monocytes for cholesterol depletion and thus lipid raft disruption resulted in a reduced adhesion of U937-cells to HUVEC. With adhesion of MCD-treated but unstimulated monocytes set as 100 %, TNF-α increased adhesion to 151.51 ± 1.57 %. Under these conditions, AA (154.41 ± 1.16 %) and DHA (156.43 ± 1.31 %) failed to modulate adhesion ($n = 5$–7; Fig. 4a).

After MCD-treatment, addition of TNF-α induced an increase to 116.27 ± 3.33 % of intracellular calcium measured with Fluo-3-AM-fluorescence with unstimulated monocytes set as 100 %. Under conditions of raft-disruption, pre-incubation with DHA (113.87 ± 1.00 %) or AA (112.21 ± 1.44 %) did not modulate TNF-α-induced calcium release ($n = 4$–7; Fig. 4b).

Effect of raft disruption by MCD on FA-induced modulation of freshly isolated human monocyte adhesion under flow conditions and intracellular calcium after TNF-α stimulation

Adding MCD prior to stimulation with TNF-α of freshly isolated human monocytes for cholesterol depletion and thus

lipid raft disruption resulted in a reduced adhesion under flow conditions of human monocytes to HUVEC with 14.25 ± 1.66 cells compared to 22.90 ± 1.39 cells without MCD. Applying MCD, control without TNF-α treatment was at 10.50 ± 1.66 adherent cells with MCD. After MCD-treatment without TNF-α, AA and DHA induced 8.57 ± 1.26 cells and 8.2 ± 1.49 cells to adhere, respectively. After MCD treatment, TNF-α stimulation induced a significant difference to control ($p < 0.05$, $n = 4$–7; Fig. 4c).

After MCD-treatment, intracellular calcium control was elevated to 1595 ± 136 FU(fluorescence units). MCD-treatment increased TNF-α stimulated calcium to 1797 ± 122 FU. Without addition of TNF-α, MCD induced an increase of intracellular calcium measured with Fluo-3-AM-fluorescence after AA- and DHA-treatment to 1899 ± 157 FU and to 1876 ± 157 FU, respectively. Notably, MCD-treatment plus TNF-α stimulation resulted in no significant differences in calcium between AA pretreated cells with 2034 ± 122 FU, DHA pretreated cells with 1588 ± 136 FU and TNF-α with 1797 ± 122 FU. Under conditions of raft-disruption, pre-incubation with DHA or AA did not modulate TNF-α-induced calcium release ($n = 3$–5; Fig. 4d).

Effect of raft disruption by MCD on fatty acids induced modulation of dynamic flow-chamber rolling of freshly isolated human monocytes after TNF-α stimulation

Rolling of unstimulated freshly isolated human monocytes after MCD-treatment under flow conditions to HUVEC was determined as 1.33 ± 0.62 rolling cells in 10 min, and increased upon pre-incubation of freshly isolated human monocytes with 10 μM of AA to 1.67 ± 0.62 rolling cells per high magnification field and with DHA to 1.40 ± 0.59 rolling cells per high magnification field. Control cells without MCD-treatment showed a rolling of 2.86 ± 0.71 cells which increased after TNF-α-treatment to 8.78 ± 0.62 rolling cells. Stimulation of freshly isolated MCD-treated human monocytes with TNF-α increased rolling to HUVEC to 3.88 ± 0.66 rolling cells per high magnification field compared to unstimulated control. Pre-incubation with MCD + DHA + TNF-α pre-treatment induced 2.43 ± 0.71 rolling cells per high magnification field. MCD + AA + TNF-α resulted in a rolling of 3.83 ± 0.54 cells per high magnification field. Within the MCD-treated cells, rolling did not differ significantly ($n = 7$–11; Fig. 4e).

Discussion

The present study elucidates pathways influenced by n-3 (DHA) and n-6 (AA) FA in vascular inflammation. We could demonstrate that AA and DHA effectively modulate U937- and human monocyte lipid raft composition. As judged by FA analysis of raft and non-raft membrane fraction incorporation of DHA and AA was more pronounced in lipid rafts than in non-raft membrane fraction. Adhesion of U937- and freshly isolated human monocytes to HUVEC as well as human monocyte rolling was modulated differentially by n-3 and n-6 FA: AA pre-incubation of U937-monocytes induced a highly significant difference in adhesion compared to DHA after stimulation with 1 ng/ml TNF-α. Using 10 ng/ml TNF-α, AA did not further increase adhesion and DHA lead only to a small reduction. To prevent this "ceiling"-effect and since we aimed at investigating FA-induced modulation, we continued our experiments using 1 ng/ml TNF-α where a greater difference between FA could be observed. Analysis of adhesion molecules CD11b, CD18, and CD49d of U937- and human monocytes did not reveal any modulation in quantitative expression thus ruling out an impact of AA or DHA on expression of adhesion molecules under our conditions.

Next, our study proved calcium to play a role in FA-induced change in adhesion since differential modulation was abolished by pre-treatment with the calcium-chelator BAPTA. In addition, we found that disruption of U937- and freshly isolated human monocyte-lipid rafts by MCD also abolished the modulation of intracellular calcium and monocyte-endothelial adhesion as well as differential influence on human monocyte rolling by FA. We conclude that modulation of monocyte-endothelial adhesion and intracellular calcium by n-3 and n-6 FA is at least in part dependent on lipid rafts and their modulation of FA composition.

We could show that the approved method of lipid raft isolation using Triton X-100 and a sucrose-gradient was efficient using U937- and human monocytes [33]. After ultra-centrifugation, nine fractions were collected and raft-marker detection (Lyn and Fyn) by Western Blot was performed demonstrating fraction one and two as raft-fraction and fraction three to nine as non-raft fraction.

Polyunsaturated FA as AA and DHA are readily incorporated into lipid rafts, a feature not described for monocytes up to now. Incorporation of AA and DHA in U937- and human monocytes was higher in raft fraction than in non-raft fraction compared to TNF-α control with DHA being even more readily incorporated. This contrasts current ideas, because rafts are characterised by containing mostly saturated FA [8]. An explanation might be that T-cells lipid rafts are different in their ability to incorporate polyunsaturated FA as compared to monocyte lipid rafts. Another explanation would be that increased availability of free FA as used in our study—contrasting the esterified FA used in most other studies—targets polyunsaturated FA preferentially to naturally PUFA-deprived lipid rafts. Particular attention should be paid to the inflammatory setting

in which incorporation of FA into membranes is different than under resting conditions [36]. The fact that TNF-α significantly increases the content of DHA in the raft fractions of the membrane is an interesting finding. One might speculate that this preferred allocation into the signalling platform might be a part of the altered inflammatory behaviour of monocytes after exposure to n-3 fatty acids. Furthermore, studying the effect of n-3 FA on lipid rafts in a biophysical model might also lead to different results than studying living cells. Though, reports also demonstrate a modulation of lipid rafts in different cells by n-3 FA some of them owing attention to the fact that n-3 FA incorporation could inhibit lipid raft signalling by altering its size or by simply disturbing its order [37–41].

We found that there is a differential influence of FA on adhesion and rolling with DHA exerting a reduction also compared to TNF-α except for the static U937 adhesion assay which might be due to the lack of flow conditions and the nature of the tumor cell line. The finding that monocyte adhesion and rolling to endothelial cells is modulated by n-3 and n-6 FA is well in line with previous reports from our group and others [25, 42, 43]. However, in this study we did not pre-incubate endothelial cells [25] but monocytes with FA. The finding that AA and DHA had no impact on expression of adhesion molecules in U937- and human monocytes contrasts the finding of other investigators in isolated monocytes [44] and endothelial cells [42]. However, different experimental conditions may fully compensate for the failure as we incubated monocytes in vitro (contrasting ingestion of fish oil by volunteers), and used low physiologic concentrations of free fatty acids as opposed to e.g., supraphysiologic concentrations of triglycerides. However, the finding is in line with previous reports of our group and others that did not show an impact of fatty acids on expression of adhesion molecules in endothelial cells or isolated monocytes derived after infusion of fish oil in volunteers [25, 31, 45]. Despite failure to detect an overall change in adhesion molecules recruitment of integrins into lipid rafts may be differentially modulated by fatty acids due to their effect on lipid composition of rafts. FA might alter adhesion via lipid rafts by exclusion or inclusion of signalling proteins into lipid rafts or changes of avidity or affinity of receptors which we did not investigate [46–48]. In inflammatory activated cells, also the high turnover of the membranes might play a significant role in modulation of signal transduction by FA [49]. There is evidence that the PAF–PAF-receptor system as well as the PI3-kinase system is affected by fatty acids in the process of adhesion [25, 43]. These receptor systems might also be affected in their affinity to lipid rafts by FA. Monocytes were already shown to be influenced by FA as judged by presentation of antigens in addition to adhesion in vitro as well as in vivo [31, 50]. Modulation of the inflammatory activated cell correlates with the situation of the intensive care patient suffering from an acute inflammatory disease requiring parenteral nutrition. Since parenteral nutrition consists of lipid emulsions based on triglycerides, fatty acids need to be liberated from triglycerides in order to become active. In septic conditions with catecholamines and heparin in the bloodstream, free fatty acids are augmented by order of magnitude compared to non-inflammatory conditions [31, 51]. It has been demonstrated that FA differently affect inflammatory activated cells and modulate various lipid-dependent signalling pathways [15, 19].

Control of adhesion by calcium signalling is generally recognized [52] and the fact that FA or their metabolites might have an impact on cellular calcium signalling has been investigated [53]. Several arachidonic acid-derived derivatives were identified that trigger Ca^{2+} entry into B cells, including the 5-lipoxygenase derived 5-hydroperoxyeicosatetranenoic acid and the cytochrome P450 hydroxylase product 20-hydroxyeicosatetraenoic. Influence of FA on U937-monocyte calcium has been observed [54] However, investigations demonstrating a calcium dependency of FA modulation of monocyte-endothelial adhesion like in our setting were missing until now. Our experiments demonstrated that DHA induces a decline of intracellular calcium in U937- and human monocytes in an inflammatory setting. This effect was more prominent using human monocytes. Here, we focus on modulation of adhesion by FA and were able to show a calcium-dependency of diverging effects of AA and DHA on adhesion since it was abolished after pre-treatment of monocytes with calcium-chelator BAPTA. Co-incubation of FA and BAPTA lead to a diminished calcium peak and abolished differences in intracellular calcium content between FA. We conclude that FA-influence on intracellular calcium contributes to modulation of monocyte-endothelial adhesion seen with FA. In U937-monocytes, we observed an enhanced intracellular calcium after TNF-α stimulation but in the static adhesion assay did not translate into an enhanced adhesion of U937-monocytes. This may be due to the non-physiological situation of the tumor cell line U937 combined with the static adhesion assay. Using freshly isolated human monocytes and a more physiological adhesion assay under flow conditions we could demonstrate a concordant significant difference between control and TNF-α stimulation in adhesion, rolling and intracellular calcium.

Reports demonstrating calcium dependency of lipid raft signalling have been published [55]. It could be depicted in endothelial cells that DHA reduces calcium in a raft-dependent manner [56]. In this study, we could demonstrate that disrupting monocyte lipid rafts with MCD abolished FA modulation of calcium signalling and FA influence on

monocyte-endothelial adhesion and rolling. The significant difference between control and TNF-α stimulated monocytes seen in adhesion, rolling and calcium experiments could not always be detected after MCD treatment. An explanation would be that by destroying lipid rafts with MCD part of the raft-dependent-inflammatory signalling is lost. A toxic effect of MCD is to be neglected since dose and duration of MCD treatment was used as published previously and monocytes were tested for viability [57]. This would also be reasonable with respect to the decreased TNF-α-effect. Here, a hint is given at the recognized role of lipid rafts for inflammation in general [58]. But a more detailed investigation would be beyond the scope of this manuscript. Interesting work revealed an impact of FA on lipid raft dependent signal transduction. Though, most studies were performed in T-cells [20–23]. Modification of proteins by acylation as *N*-myristoylation and palmitoylation serves to target proteins into lipid rafts [59]. A possible mechanism of interference by FA with lipid raft signalling—besides altering raft FA composition—is the interaction with acylation of signalling or target proteins [21]. Signal transduction components like PAF(platelet-activated-factor)-PAF-receptor pathway or lipoprotein lipase A_2, interacting with FA could be dependent on lipid rafts and be less influence able by FA when lipid rafts are destroyed. This may also explain why calcium modulation by FA is lost after lipid raft disruption [60].

To our knowledge, this study is the first report demonstrating in U937- and human monocytes that modulation of intracellular calcium and adhesion by FA is dependent on lipid rafts. It remains to be clarified how monocyte lipid rafts influence calcium signalling and via which route exactly intracellular calcium affects monocyte-endothelial adhesion.

Conclusions

We could demonstrate that n-6 and n-3 FA modulate FA content of monocyte lipid rafts to a greater extent than non-raft membrane fraction. Modulation of TNF-α-induced adhesion, rolling and intracellular calcium signalling by FA is abolished by disruption of lipid rafts. Therefore, lipid rafts seem to be crucial for inflammatory modulation by n-6 and n-3 FA in monocytes. This could help elucidating the role of FA in inflammatory diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Anlage 6

Schaefer MB, Pose A, Ott J, Hecker M, Behnk A, Schulz R, Weissmann N, Günther A, Seeger W, Mayer K.

Peroxisome proliferator-activated receptor (PPAR)-α reduces inflammation and vascular leakage in a murine model of acute lung injury.

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Peroxisome proliferator-activated receptor-α reduces inflammation and vascular leakage in a murine model of acute lung injury

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ABSTRACT: Acute lung injury (ALI) still represents a major cause of morbidity and mortality in intensive care units. Beneficial effects have been described after activation of the peroxisome proliferator-activated receptor (PPAR)-α by fibrates such as WY 14,643 (WY) in inflammatory models. In the present study, the impact of WY was investigated in a model of endotoxin (lipopolysaccharide; LPS)-induced ALI in mice.

Intratracheal LPS challenge dose-dependently resulted in leukocyte invasion, protein leakage and release of tumour necrosis factor-α as well as macrophage inflammatory protein-2, prostaglandin E_2 and thromboxane B_2 into the alveolar space after 8 and 24 h. Lung ventilator compliance was reduced at both time-points. In isolated perfused mouse lungs, platelet-activating factor (PAF) induced an acute increase in pulmonary artery pressure (P_{pa}) and in capillary filtration coefficient (K_{fc}). WY significantly improved all features of ALI *in vivo* and blunted the increase in K_{fc} in isolated perfused mice lungs. In mice with genetic deletion of PPAR-α, all characteristics of ALI, P_{pa}, and K_{fc} were not significantly different from wild-type mice but WY failed to improve ALI and PAF-induced increase in K_{fc}.

Activation of peroxisome proliferator-activated receptor-α by WY 14,643 reduced acute lung injury and vascular leakage. Fibrates may possess beneficial effects in acute pulmonary diseases beyond their lipid-lowering capability.

KEYWORDS: Acute lung injury, inflammation, peroxisome proliferator-activated receptor-α, vascular leakage

Acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) are common clinical disorders characterised by alveolar epithelial and endothelial injury leading to the development of a protein-rich pulmonary oedema, elevation of pulmonary artery pressure (P_{pa}) and finally acute respiratory failure. According to recent data, the incidence of ALI or ARDS was 4.5–7.1% of all patients admitted to an intensive care unit (ICU), increasing to 12.5% when considering only patients treated for >24 h in the ICU [1, 2]. The high mortality rate associated with ARDS and ALI has declined to 30–40% in recent randomised trials [3] but still there is no proven pharmacological treatment, despite a multitude of strategies being successful in animal models [4]. Pathophysiological features of ALI include a compromised endothelial–alveolar barrier, leading to increased vascular permeability mirrored by the capillary filtration coefficient (K_{fc}), neutrophil migration into the lung tissue, and formation of pro-inflammatory mediators such as cytokines and eicosanoids (*e.g.* thromboxane (Tx)B_2 and prostaglandin (PG)E_2) [5].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors, which are related to retinoid, steroid and thyroid hormone receptors [6]. The PPAR subfamily comprises three members: PPAR-α, PPAR-β/δ and PPAR-γ [7]. The name PPAR is derived from the fact that activation of PPAR-α by xenobiotics results in peroxisome proliferation in rodent hepatocytes. PPAR-α is highly expressed in brown adipose tissue, and to a lesser extent by liver, kidney, heart and skeletal muscle [8]. PPAR-α mRNA has been detected in murine lung tissue [9]. It has also been found in human endothelial cells,

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STATEMENT OF INTEREST
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as well as in smooth muscle cells, monocytes/macrophages and T-lymphocytes [10–12]. A diverse set of ligands binds to PPAR-α, such as arachidonic acid metabolites and synthetic fibrate drugs, including WY 14,643 (WY), clofibrate, fenofibrate and bezafibrate [13, 14]. Although PPAR-α has been less studied than PPAR-γ, PPAR-α ligands have also been shown to regulate inflammatory responses [15]. In addition, it has been demonstrated that PPAR-α-deficient mice have abnormally prolonged responses to different inflammatory stimuli [16–18]. Fibrates have exhibited anti-inflammatory properties *in vitro* [19, 20] as well as *in vivo* [15, 21]. In particular, it has been reported that PPAR-α ligands can inhibit the expression of various pro-inflammatory genes, such as interleukin (IL)-6, vascular cell adhesion molecule-1, platelet-activating factor (PAF) receptor and cyclooxygenase (COX)-2 (generating PGE_2 and TxB_2), in response to cytokine activation [21, 22]. This may, in part, be dependent on the inhibition of functional nuclear factor (NF)-κB activation and on the increase of expression of the inhibitory protein IκBα [23, 24]. The present study was carried out in order to gain a better understanding of the possible influence of PPAR-α in a mouse model of ALI.

MATERIALS AND METHODS

Reagents

Chemicals of the highest purity were obtained from Merck (Darmstadt, Germany). Lipopolysaccharide (LPS) from *E. coli* strain O111:B4, and WY, were from Sigma-Aldrich (Dreisenhofen, Germany).

Animals

The present study was approved by local government authorities (Giessen, Germany) and university officials responsible for animal protection (Justus-Liebig-University Giessen, Giessen). Parent and offspring PPAR-$\alpha^{-/-}$ and wild-type (WT) mice on the Sv129 background were kept under standard conditions with a 12-h day/night cycle under specific pathogen-free conditions. Animals 8–12 weeks old (18–21 g weight) were used for experiments. For intratracheal LPS instillation and measurement of compliance, mice were anaesthetised as described previously [25].

Determination of lung compliance by ventilator

When properly anaesthetised, mice were tracheotomised and ventilated in a volume-driven mode at a positive end-expiratory pressure of 0 kPa as described previously [26]. The respiration rate was set at 20 breaths·min^{-1} and ventilation pressure was recorded while inflating the lung at a tidal volume of 200 μL. The ventilator compliance is given and was corrected for animal weight.

Murine model of ALI

Mice were anaesthetised, a small catheter was inserted in the trachea, and LPS (1 or 10 μg in 50 μL normal saline per mouse) was instilled into the lung as described previously [25]. The mice were sacrificed 8 or 24 h after LPS application, and bronchoalveolar lavage (BAL) was performed [25]. Alveolar recruited leukocytes recovered from the lungs of LPS-challenged and control mice were counted using a counting chamber. Differentiation of leukocytes was performed in a blinded fashion using differential cell counts of Pappenheim-stained cytocentrifuge preparations, by overall morphological criteria, including differences in cell size and shape of nuclei. Protein in BAL was determined according to the method of LOWRY *et al.* [27].

Isolated perfused and ventilated lung model

For determination of K_{fc} and haemodynamic measurements, a ventilated and perfused mouse lung preparation was used as previously described [28]. Briefly, mice were deeply anaesthetised and anticoagulated. After intubation *via* a tracheostoma, the mice were ventilated with a 250 μL tidal volume, 90 breaths·min^{-1} and 2 cmH_2O (0.2 kPa) positive end-expiratory pressure. Following midsternal thoracotomy, catheters were inserted into the pulmonary artery and left atrium. Sterilised perfusion circuit tubing was used throughout. Perfusion was performed using a peristaltic pump and Krebs-Henseleit buffer, containing 120 mM NaCl, 4.3 mM KCl, 1.1 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$ and 13.32 mM glucose, as well as 5% (weight/volume) hydroxyethylamylopectin (molecular weight 200 kDa). The pH was adjusted to 7.37–7.40 with $NaHCO_3$. After rinsing the lungs, the perfusion circuit was closed for recirculation and the left atrial pressure was set at 2.0 mmHg. Under steady-state conditions, perfusion flow was 2 mL·min^{-1}. P_{pa} and left atrial pressure were registered continuously *via* small-diameter catheters. The lungs were removed from the thorax and were placed in a temperature-equilibrated, humidified chamber at 37.0°C, freely suspended from a force transducer for monitoring of organ weight. The K_{fc} and the total vascular compliance were determined gravimetrically from the slope of the lung weight gain curve induced by a 7.5 mmHg-step elevation of the venous pressure for 8 min, as previously described [29].

Enzyme-linked immunosorbent assay

Tumour necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-2, PGE_2 and TxB_2 from BAL were determined by ELISA according to the manufacturer's instructions.

Experimental protocol

WY was given orally to WT and PPAR-$\alpha^{-/-}$ mice for 14 days at a dose of 1 mg·day^{-1}. This dose was chosen after performing pilot experiments with 0.5, 1 and 2 mg WY and determination of an upregulation of PPAR-α-dependent genes (*e.g.* fatty acid binding protein and lipoprotein lipase) in lung and liver homogenates by PCR, liver weight and bromodeoxyuridine staining in liver histology.

BAL for cytokine measurements (4–8 samples), protein determination (4–8 samples) and cell counts (4–8 samples), and lung compliance measurements (5–6 samples) were performed 8 or 24 h after intratracheal LPS instillation (0, 1 or 10 μg). In the isolated lung, after a steady-state period of 30 min, K_{fc} was determined (5–6 samples). PAF was injected into the pulmonary artery at 100 nmol·L^{-1} final concentration. Determination of K_{fc} was repeated 30 and 60 min after injection.

Statistics

Data are presented as mean±SEM. Two-way ANOVA was performed to test for differences between treatment groups (WT±WY, PPAR-$\alpha^{-/-}$±WY) and LPS dose (0, 1 and 10 μg) at each time-point. Repeated measures two-way ANOVA was used in the case of K_{fc} to detect differences between treatment groups (WT±WY, PPAR-$\alpha^{-/-}$±WY) and different time-points. *Post hoc* analysis was carried out using the Student-Newman-Keuls test. As the values for leukocytes, TNF-α, TxB_2 and PGE_2

were not normally distributed, log transformation was performed. Values of $p<0.05$ were considered statistically significant.

RESULTS

PPAR-α and alveolar transmigration of leukocytes in LPS-induced ALI

Without LPS, $0.24\pm0.05\times10^6$ leukocytes were recovered from BAL in WT mice. Comparable numbers were found after WY feeding and in PPAR-$\alpha^{-/-}$ mice, irrespective of WY treatment (fig. 1). The number of leukocytes in BAL increased to $0.81\pm0.06\times10^6$ and $0.96\pm0.13\times10^6$ 8 h after intratracheal instillation of 1 and 10 μg LPS, respectively. This increase in LPS-challenged mice was virtually the same in all groups examined (with or without WY, in WT and PPAR-$\alpha^{-/-}$ mice; $p<0.05$ *versus* respective baseline groups).

Leukocyte numbers in BAL increased further after 24 h to $1.49\pm0.18\times10^6$ and $2.12\pm0.13\times10^6$ in WT mice stimulated with 1 and 10 μg LPS, respectively. The rise in leukocyte numbers was significant in all groups receiving LPS, compared with the respective baseline groups ($p<0.05$). Similar numbers were detected in PPAR-$\alpha^{-/-}$ mice irrespective of WY treatment. In WT mice treated with WY, transmigration of leukocytes in the alveolar space was significantly reduced. After WY application and stimulation with 1 μg LPS, leukocytes decreased to 50% compared with WT mice not treated with WY, differing significantly from all other groups receiving this dose at this time-point ($p<0.05$; fig. 1). After challenge with 10 μg LPS, the WT+WY group exhibited a 33% reduction in leukocyte transmigration compared with WT mice, differing significantly from all other groups ($p<0.05$; fig. 1).

Without LPS challenge, the differential count of leukocytes in BAL in WT mice was $2.3\pm0.6\%$ granulocytes, $96.7\pm0.8\%$ monocytes/macrophages and $0.9\pm0.3\%$ lymphocytes. The distribution did not significantly differ from PPAR-$\alpha^{-/-}$ mice or from both groups receiving WY. In the BAL of WT mice, 24 h after challenge with 1 μg LPS, $75.3\pm2.9\%$ granulocytes, $22.7\pm3.0\%$ monocytes/macrophages and $1.9\pm0.3\%$ lymphocytes were detected. This profile of predominantly neutrophil invasion was not significantly changed in both PPAR-$\alpha^{-/-}$ groups or in WT mice with WY application. A similar distribution of leukocytes was detected in all groups receiving 10 μg LPS.

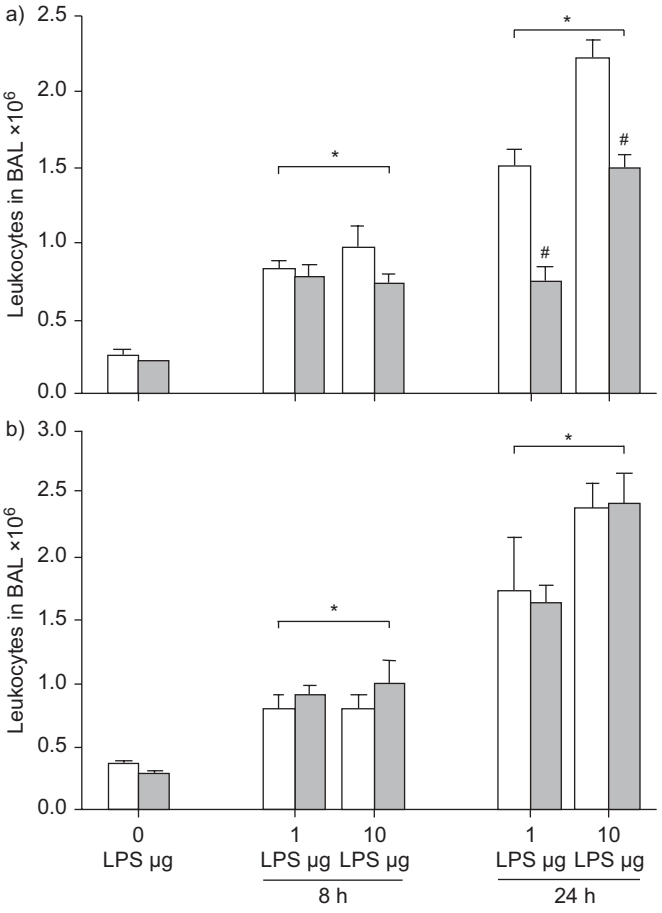

FIGURE 1. Peroxisome proliferator-activated receptor (PPAR)-α activation and leukocytes in bronchoalveolar lavage (BAL) in a model of acute lung injury. Wild-type (WT; a) and PPAR-$\alpha^{-/-}$ (b) mice were fed a diet enriched in WY 14,643 (WY; ■) or regular chow (□). Leukocytes were recovered from BAL at baseline (0 h), 8 h and 24 h after 1 or 10 μg intratracheal lipopolysaccharide (LPS) application. Leukocyte numbers increased after LPS stimulation, with all groups differing from their respective baseline. The rise in leukocyte numbers was reduced after WY pretreatment in WT mice compared with all other groups receiving the same dose. Data are presented as mean±SEM, 4–8 independent experiments each. Error lines are missing when contained within bar. #: $p<0.05$ for WT+WY after LPS applications compared with other groups receiving the same LPS dose. *: $p<0.05$ for groups exposed to LPS *versus* respective baseline.

PPAR-α and TNF-α in LPS-induced ALI

The baseline TNF-α concentration in BAL of WT mice without LPS application was 49 ± 14 pg·mL^{-1} in control animals, and comparable concentrations were found in WT+WY as well as in both PPAR-$\alpha^{-/-}$ groups (fig. 2a and b). TNF-α in BAL rose to $1{,}262\pm126$ pg·mL^{-1} and $1{,}587\pm33$ pg·mL^{-1} 8 h after intratracheal instillation of 1 and 10 μg LPS in WT mice, respectively. This increase in LPS-challenged mice was nearly the same in all groups examined ($p<0.01$ *versus* baseline).

The concentration of TNF-α in BAL dropped after 24 h to 416 ± 45 pg·mL^{-1} and 534 ± 87 pg·mL^{-1} in WT mice stimulated with 1 and 10 μg LPS, respectively. Similar concentrations were measured in PPAR-$\alpha^{-/-}$ mice irrespective of WY treatment after application of 1 μg LPS. In both PPAR-$\alpha^{-/-}$ groups challenged with 10 μg LPS, TNF-α was slightly but not significantly higher compared with WT mice without WY treatment. All groups receiving LPS differed significantly from their baseline groups ($p<0.01$). WT mice treated with WY exhibited a reduction in TNF-α concentration to 33% and 59% after 1 and 10 μg LPS application, respectively. Both WT+WY groups differed significantly from all other groups receiving the same dose of LPS (1 μg: $p<0.01$; 10 μg: $p<0.05$) and from their respective baseline groups (1 μg: $p<0.05$; 10 μg: $p<0.01$).

PPAR-α and MIP-2 in LPS-induced ALI

The baseline MIP-2 concentration in BAL of WT mice without LPS application was 150 ± 53 pg·mL^{-1} in control animals and 115 ± 16 pg·mL^{-1} in WY-treated mice (fig. 2c). In PPAR-$\alpha^{-/-}$ mice (fig. 2d), MIP-2 was slightly but not significantly higher

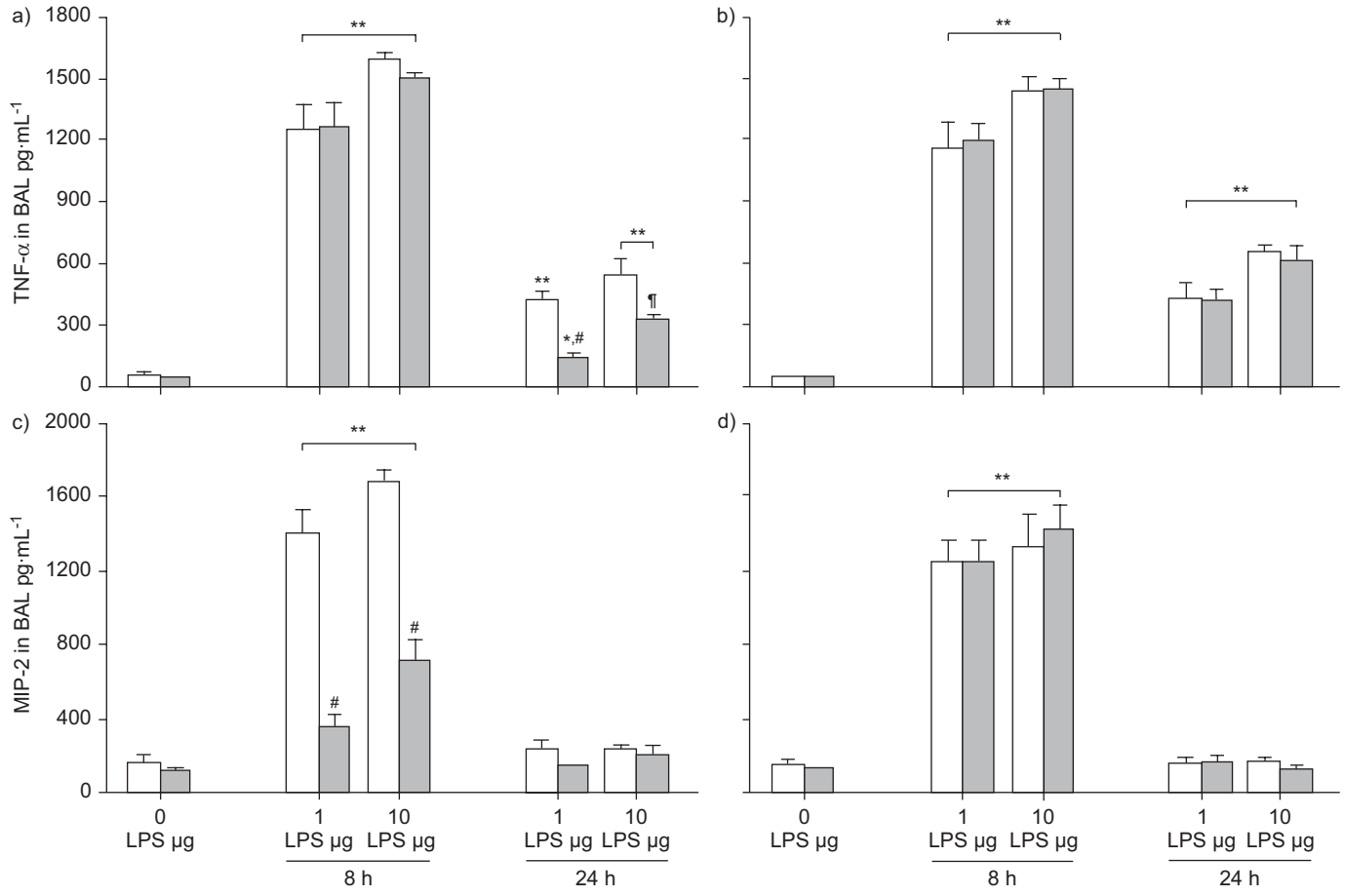

FIGURE 2. Peroxisome proliferator-activated receptor (PPAR)-α activation and cytokine generation in bronchoalveolar lavage (BAL) in a model of acute lung injury. Wild-type (WT; a and c) and PPAR-$\alpha^{-/-}$ (b and d) mice were fed a diet enriched in WY 14,643 (WY; ■) or regular chow (□). Tumour necrosis factor (TNF)-α (a and b) and macrophage inflammatory protein (MIP)-2 (c and d) concentrations were determined in BAL at baseline (0 h), 8 h and 24 h after 1 or 10 µg intratracheal lipopolysaccharide (LPS) application. TNF-α was significantly different for all groups exposed to LPS *versus* respective baseline. MIP-2 rose significantly after LPS exposure, with all LPS-treated groups differing from their baseline. Concentrations of TNF-α and MIP-2 in WT+WY after LPS applications were significantly lower compared with all other groups receiving the same dose. Data are presented as mean±SEM, 4–8 independent experiments each. Error lines are missing when contained within bar. #: $p<0.01$; ¶: $p<0.05$ for WT+WY after LPS applications compared with other groups receiving the same LPS dose. *: $p<0.05$; **: $p<0.01$ for groups exposed to LPS *versus* respective baseline.

compared with WT animals. MIP-2 in BAL rose to 1,400±120 pg·mL^{-1} and 1,681±57 pg·mL^{-1} 8 h after intratracheal instillation of 1 and 10 µg LPS in WT mice, respectively. All groups receiving LPS differed significantly from baseline ($p<0.01$). MIP-2 was slightly but not significantly higher in PPAR-$\alpha^{-/-}$ mice challenged with 1 µg LPS and comparable to WT mice after application of 10 µg LPS. Treatment with WY reduced the rise in MIP-2 to 26% or 42% in WT mice after challenge with 1 or 10 µg LPS, respectively. WT mice receiving WY differed significantly from all other groups with the same LPS dose ($p<0.01$ for both doses; fig. 2c).

After 24 h, the concentration of MIP-2 in BAL dropped to 232±44 pg·mL^{-1} and 231±18 pg·mL^{-1} in WT mice receiving 1 and 10 µg LPS, respectively. All other groups (WT+WY, PPAR-$\alpha^{-/-}$±WY) also returned to baseline and no significant difference was observed.

PPAR-α and lung compliance in LPS-induced ALI

Baseline lung compliance in WT mice without LPS application was 5.27±0.29 L·kPa^{-1}·kg^{-1} in control animals and there was no significant variation in all other groups at baseline (fig. 3). Compliance was markedly reduced in WT mice 8 h after intratracheal challenge with 1 and 10 µg LPS (4.02±0.12 and 3.43±0.17 L·kPa^{-1}·kg^{-1}, respectively), with PPAR$^{-/-}$±WY exhibiting similar values ($p<0.05$ *versus* baseline). In WT mice receiving WY, compliance remained higher and animals receiving 10 µg LPS differed from all other groups with that dose of LPS ($p<0.05$).

After 24 h, compliance was further reduced in WT animals and both PPAR-$\alpha^{-/-}$ groups ($p<0.05$ *versus* baseline). In contrast, an improvement in lung compliance was found in WT animals receiving WY ($p<0.05$ *versus* all other groups receiving 1 µg LPS at 24 h).

PPAR-α and protein concentration in LPS-induced ALI

Before LPS challenge, protein concentration in BAL was 0.21±0.03 µg·mL^{-1}, with no significant differences due to strain or WY treatment (fig. 4a and b). Protein concentration increased to nearly 400% of baseline 8 h after 1 µg LPS instillation in WT mice as well as in both PPAR-$\alpha^{-/-}$ groups ($p<0.05$ *versus* baseline).

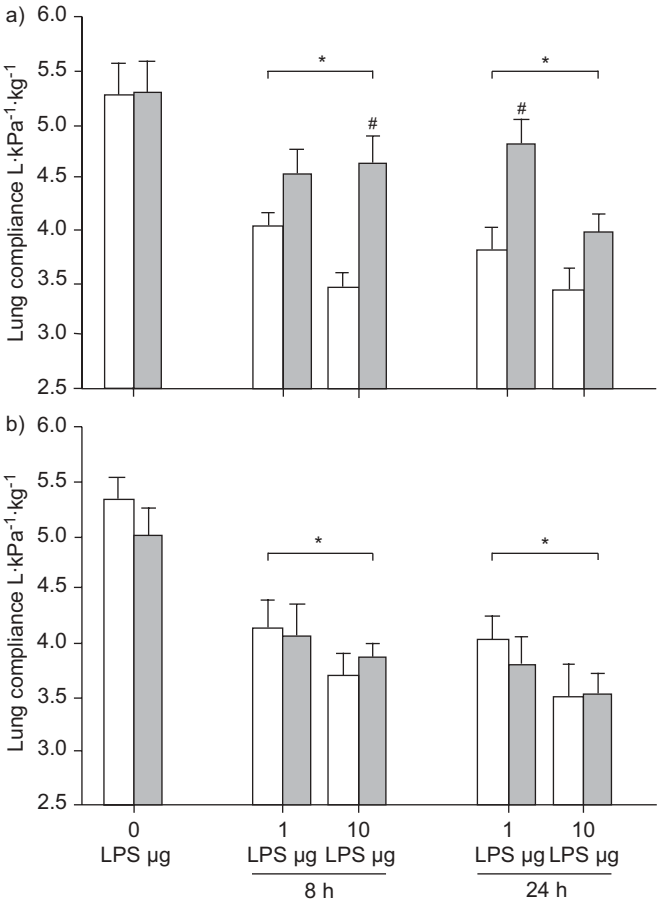

FIGURE 3. Peroxisome proliferator-activated receptor (PPAR)-α activation and lung compliance in a model of acute lung injury. Wild-type (WT; a) and PPAR-$\alpha^{-/-}$ (b) mice were fed a diet enriched in WY 14,643 (WY; ■) or regular chow (□). Lung compliance was determined at baseline (0 h), 8 h and 24 h after 1 or 10 µg intratracheal lipopolysaccharide (LPS) application. Lung compliance was decreased after LPS instillation in WT mice and both PPAR-$\alpha^{-/-}$ groups compared with baseline. WT mice receiving WY exhibited protection against loss of compliance after 10 µg LPS at 8 h and after 1 µg LPS at 24 h compared with all other groups at the respective time-points. Data are presented as mean±SEM, 5–6 independent experiments each. #: $p<0.05$ for WT+WY after LPS applications compared with other groups receiving the same LPS dose. *: $p<0.05$ for groups exposed to LPS *versus* respective baseline.

In WT mice receiving WY, the increase was markedly blunted and the protein concentration rose only to 0.45 ± 0.06 µg·mL^{-1} ($p<0.05$ *versus* all other groups receiving 1 µg LPS and *versus* baseline). Protein concentration was further increased in WT animals after instillation of 10 µg LPS after 8 h, with similar values determined in both PPAR-$\alpha^{-/-}$ groups ($p<0.05$ *versus* baseline). Again, a marked reduction by nearly 50% was found in WT mice after WY treatment ($p<0.05$ *versus* all other groups receiving 10 µg LPS and *versus* baseline). After 24 h, protein concentrations in animals receiving 1 or 10 µg LPS were slightly lower compared with corresponding groups at 8 h. However, at this time-point, the differences were not statistically significant.

PPAR-α and Kfc in isolated perfused lungs

To examine whether the increased BAL protein concentration would be mirrored by a reduction in endothelial barrier function, *K*fc was measured in a murine isolated perfused lung model. Lungs were isolated from WT and PPAR-$\alpha^{-/-}$ mice without LPS challenge and 8 or 24 h after LPS instillation. However, the current authors were not able to obtain stable baseline conditions at any time after LPS challenge. Therefore, only the data from mice prior to LPS challenge, with PAF as acute stimulus, are reported in the isolated perfused lung model. Baseline *K*fc was similar in all groups (fig. 4c). Challenge with PAF resulted in an acute increase in *P*pa by 5.1 mmHg with no significant difference between the groups (data not shown). After 30 min, *K*fc was nearly tripled in WT mice and in PPAR-$\alpha^{-/-}$ mice irrespective of WY treatment. WT animals pretreated with WY exhibited a blunted increase in *K*fc ($p<0.01$ *versus* all other groups at 30 min). *K*fc returned to baseline values 60 min after PAF challenge. The increase in *K*fc directly after PAF challenge was significant compared with baseline as well as with the determination after 60 min ($p<0.01$ for all groups apart from WT+WY).

PPAR-α and TxB_2 in LPS-induced ALI

Before LPS challenge, the concentration of TxB_2 in BAL was 214 ± 27 pg·mL^{-1}, with no significant differences due to strain or WY treatment (fig. 5a and b). After 8 h, the concentrations of TxB_2 increased to 471 ± 36 pg·mL^{-1} and 535 ± 54 pg·mL^{-1} in WT mice challenged with 1 or 10 µg LPS, respectively. The rise in TxB_2, irrespective of LPS dose, was significant for these three groups (WT, PPAR-$\alpha^{-/-}$ with WY and PPAR-$\alpha^{-/-}$ without WY) compared with baseline ($p<0.05$). WT mice receiving WY showed a blunted increase in TxB_2 concentrations after 1 and 10 µg LPS of nearly 20% or nearly 30%, respectively, which reached a significant level only after challenge with 10 µg LPS ($p<0.05$ *versus* all other groups receiving the same dose). The increase in TxB_2 after LPS in these mice was not significant compared with baseline.

After 24 h, 466 ± 54 pg·mL^{-1} and 599 ± 78 pg·mL^{-1} TxB_2 were measured in WT mice receiving 1 and 10 µg LPS, respectively (fig. 5a). Similar concentrations were detected in corresponding PPAR-$\alpha^{-/-}$ groups, irrespective of WY application (fig. 5b). All groups differed from their respective baseline ($p<0.05$). WT mice with WY pretreatment exhibited a marked reduction in TxB_2 concentration at this time-point, reaching a significant level in mice receiving 10 µg LPS ($p<0.05$ *versus* all other groups receiving this dose; fig. 5a and b). The increase in TxB_2 after LPS application was not significant compared with baseline in both groups.

PPAR-α and PGE_2 in LPS-induced ALI

Before LPS instillation, the concentration of PGE_2 in BAL was 296 ± 28 pg·mL^{-1}, with similar values measured in both PPAR-$\alpha^{-/-}$ groups (fig. 5c and d). PGE_2 was lower in WT mice receiving WY, but the difference was not statistically significant. In WT mice, 8 h after LPS challenge, PGE_2 rose to 873 ± 127 pg·mL^{-1} and $2{,}048\pm185$ pg·mL^{-1} in mice challenged with 1 and 10 µg LPS, respectively. The PGE_2 concentration was significantly different from baseline in all groups after both doses of LPS ($p<0.05$). Concentrations found in all PPAR-$\alpha^{-/-}$ groups at this time-point did not differ significantly from WT mice. However, in WT mice receiving WY, the rise in PGE_2 was blunted to 601 ± 31 pg·mL^{-1} and 723 ± 35 pg·mL^{-1} ($p<0.05$ *versus* all other groups) after 1 and 10 µg LPS,

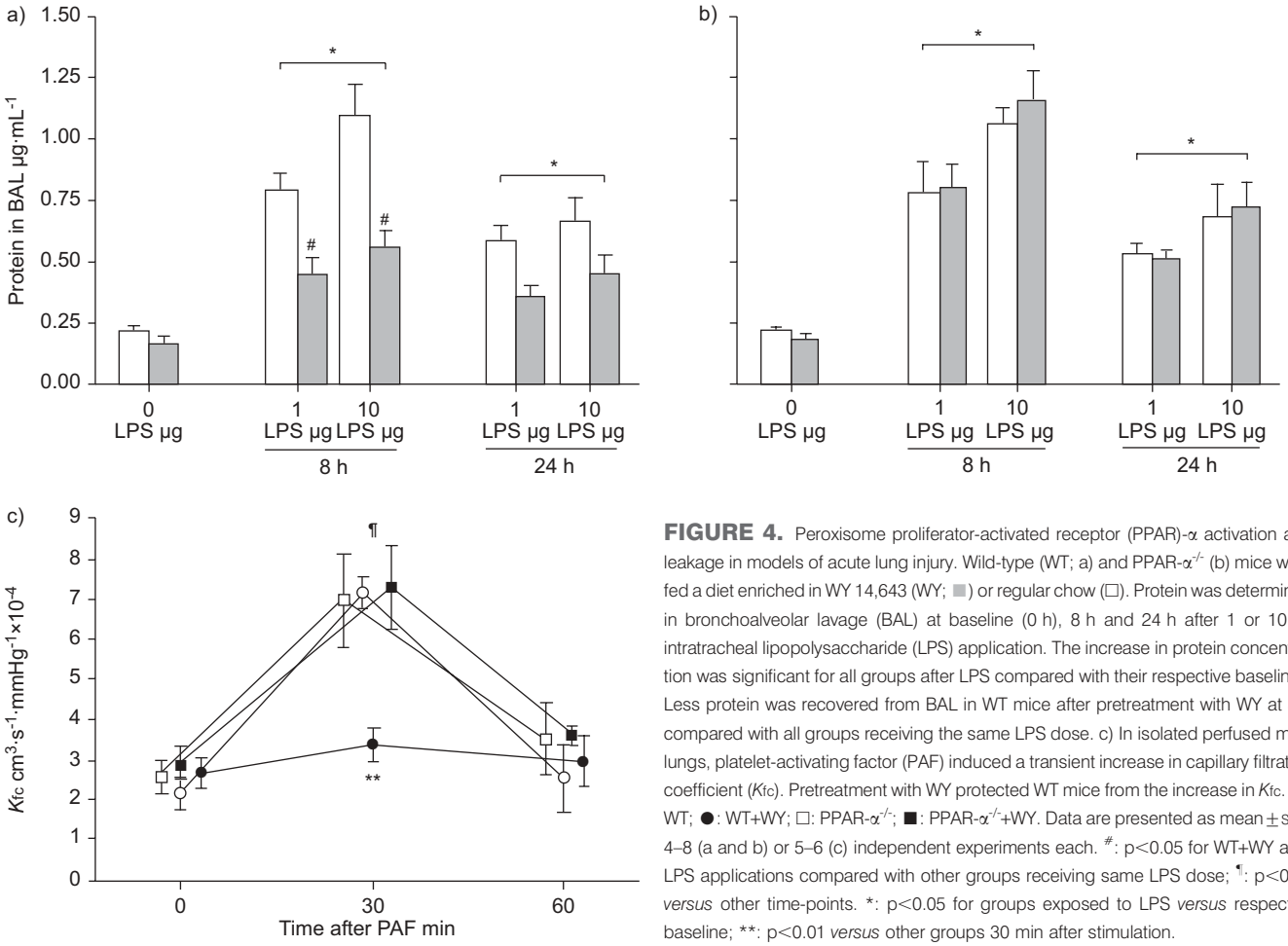

FIGURE 4. Peroxisome proliferator-activated receptor (PPAR)-α activation and leakage in models of acute lung injury. Wild-type (WT; a) and PPAR-$\alpha^{-/-}$ (b) mice were fed a diet enriched in WY 14,643 (WY; ■) or regular chow (□). Protein was determined in bronchoalveolar lavage (BAL) at baseline (0 h), 8 h and 24 h after 1 or 10 μg intratracheal lipopolysaccharide (LPS) application. The increase in protein concentration was significant for all groups after LPS compared with their respective baselines. Less protein was recovered from BAL in WT mice after pretreatment with WY at 8 h compared with all groups receiving the same LPS dose. c) In isolated perfused mice lungs, platelet-activating factor (PAF) induced a transient increase in capillary filtration coefficient (K_{fc}). Pretreatment with WY protected WT mice from the increase in K_{fc}. ○: WT; ●: WT+WY; □: PPAR-$\alpha^{-/-}$; ■: PPAR-$\alpha^{-/-}$+WY. Data are presented as mean±SEM, 4–8 (a and b) or 5–6 (c) independent experiments each. #: $p<0.05$ for WT+WY after LPS applications compared with other groups receiving same LPS dose; ¶: $p<0.01$ *versus* other time-points. *: $p<0.05$ for groups exposed to LPS *versus* respective baseline; **: $p<0.01$ *versus* other groups 30 min after stimulation.

respectively. The difference in PGE_2 after 1 and 10 μg LPS was significant ($p<0.05$) in all groups except in WT mice after treatment with WY.

PGE_2 rose further after 24 h to 1,172±107 pg·mL^{-1} and 2,460±158 pg·mL^{-1} in WT mice receiving 1 μg and 10 μg LPS, respectively (fig. 5c). No significant difference from the respective PPAR-$\alpha^{-/-}$ groups was found (fig. 5d). In contrast, PGE_2 was reduced to ~60% irrespective of LPS challenge in WT mice after feeding with WY. The difference was significant compared with all corresponding groups receiving the same LPS dose ($p<0.05$). PGE_2 concentrations differed significantly from baseline after both LPS challenges in all groups ($p<0.05$). Again, a dose-dependent increase in PGE_2 was found, with concentrations after challenge with 1 μg LPS being significantly lower compared with those after 10 μg LPS in all groups ($p<0.05$).

DISCUSSION

In the present study, it was demonstrated that activation of the transcription factor PPAR-α by the fibrate WY is protective in a murine model of ALI *in vivo* and in PAF-induced increase in vascular leakage in isolated perfused mice lungs. WY was able to reduce LPS-provoked invasion of neutrophils into the alveolar space and generation of inflammatory mediators such as TNF-α, MIP-2, TxB_2 and PGE_2. Furthermore, integrity of the endothelial–alveolar barrier was preserved by treatment with WY, as judged by decreased protein concentration in the BAL and a reduced K_{fc}. As an additional functional parameter, WY reduced acute deterioration of lung compliance in LPS-challenged mice. In PPAR-α-deficient mice, treatment with WY did not evoke these beneficial changes in ALI either *in vivo* or in isolated perfused mice lungs. However, it should be kept in mind that a drawback of the LPS model is the use of endotoxin in a single-hit model to induce inflammatory responses. Such a model is clearly different from ARDS or ALI due to prolonged bacterial infection.

Expression of PPAR-α is high in liver, kidney, muscle and heart [30], but has also been detected in smooth muscle cells, monocytes/macrophages and T-lymphocytes [10]. PPAR-α-dependent genes regulate metabolism of fatty acids and lipoproteins but evidence is accumulating that fibrates activating PPAR-α not only possess lipid-lowering properties but are also exerting beneficial actions in pulmonary diseases [10]. Most authors describe positive effects of PPAR-α activation in prolonged models of pulmonary diseases but an impact in per-acute lung injury (not focusing on airway or pleural inflammation) was lacking. Protection from the inflammatory response by a PPAR-α ligand was found in a prolonged (15 days) murine lung injury model induced by bleomycin [31]. Activation of PPAR-α by WY induced a reduction of the bleomycin-induced increases in TNF-α, IL-1, poly-ADP-ribose and mortality. In contrast, loss of the

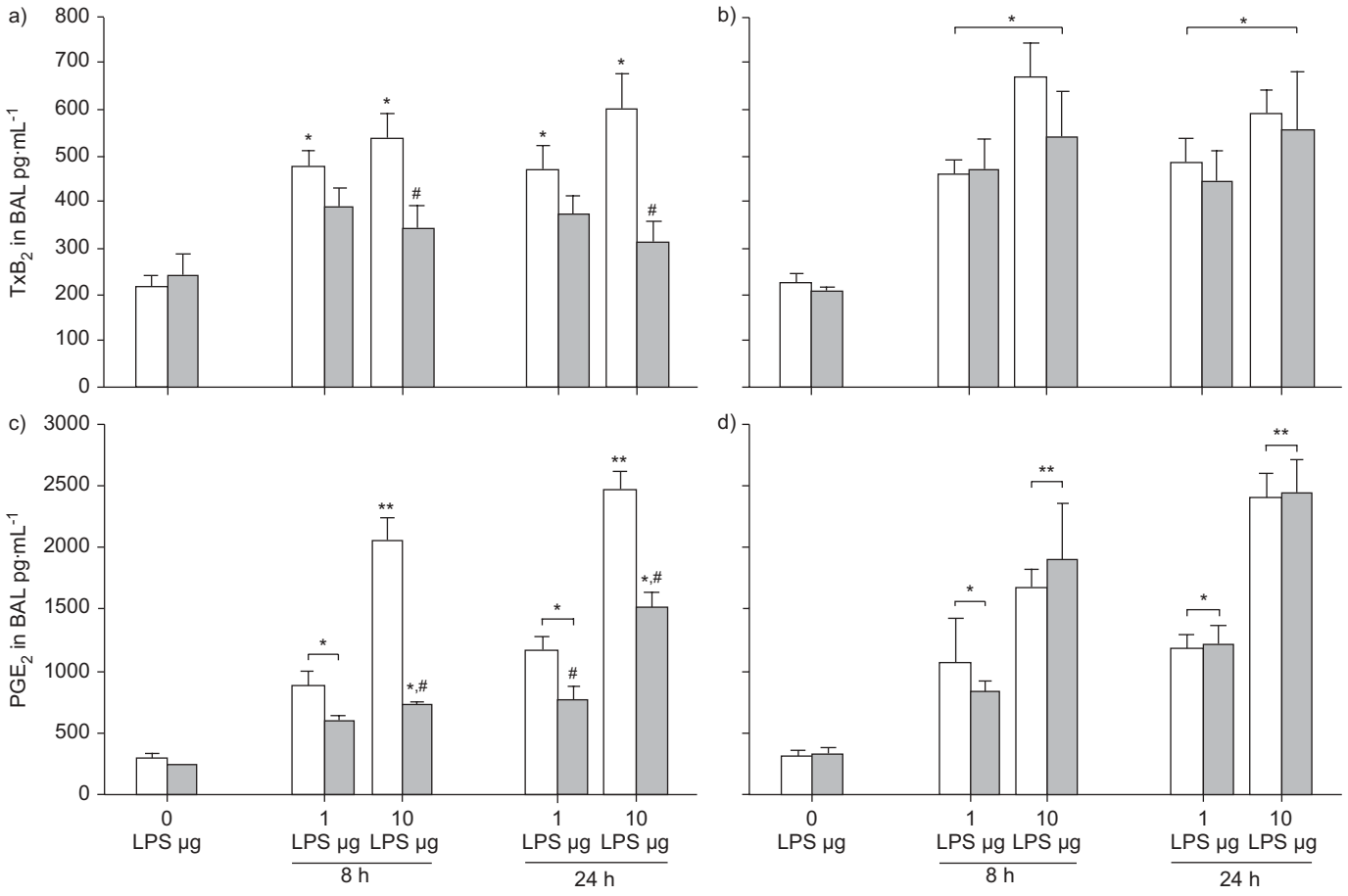

FIGURE 5. Peroxisome proliferator-activated receptor (PPAR)-α activation and prostanoid generation in bronchoalveolar lavage (BAL) in a model of acute lung injury. Wild-type (WT; a and c) and PPAR-$\alpha^{-/-}$ (b and d) mice were fed a diet enriched in WY 14,643 (WY; ■) or regular chow (□). Thromboxane (Tx)B_2 (a and b) or prostaglandin (PG)E_2 (c and d) were determined in BAL at baseline (0 h), 8 h and 24 h after 1 or 10 µg intratracheal lipopolysaccharide (LPS) application. After challenge with 1 and 10 µg LPS, TxB_2 increased in all groups except WT mice receiving WY. The WT+WY group differed from all other groups after 10 µg LPS. PGE_2 also rose after LPS challenge, with all groups differing from their respective baseline. Compared with all other groups receiving the same LPS dose, PGE_2 concentrations were significantly lower in WT+WY mice after 10 µg LPS (at 8 h and 24 h) and after 1 µg LPS (at 24 h). Data are presented as mean±SEM, 4–8 independent experiments each. Error lines are missing when contained within bar. #: $p<0.05$ for WT+WY after LPS applications compared with other groups receiving the same LPS dose. *: $p<0.05$; **: $p<0.01$ for groups exposed to LPS *versus* respective baseline.

functional PPAR-α pathway in PPAR-$\alpha^{-/-}$ mice increased all reported parameters [31]. In a model of carrageen-induced pleurisy, PPAR-$\alpha^{-/-}$ mice showed increased generation of TNF-α, IL-1 and FAS-ligand, and increased leukocyte infiltration [32]. In a model of prolonged airway inflammation induced by chronic intranasal LPS challenge, leukocyte infiltration, TNF-α, monocyte chemoattractant protein-1, keratinocyte-derived chemokine and matrix metalloproteinases were increased after 5 days in mice lacking functional PPAR-α receptor. In contrast, treatment with fenofibrate reduced all examined parameters [33]. In line with these findings, allergic airway inflammation was reduced by fenofibrate in ovalbumin-sensitised mice [34]. It has been demonstrated that activation of PPAR-α interferes with the expression of pro-inflammatory genes such as vascular cell adhesion molecule (VCAM)-1, PAF receptor and COX-2 [21, 22]. This may be mediated at least partly by reduced activation of NF-κB and increased expression of its inhibitor IκBα [23, 24]. Both effects may result in a decreased nuclear translocation of p50/p65 NF-κB after inflammatory stimulation, thereby affecting nuclear transcription of dependent genes such as TNF-α and IL-1. As downstream effects of LPS are mediated at least in part by activation of NF-κB, inhibition of this pathway may also be responsible for the reduction of ALI. The present data, showing reduced generation of TNF-α, MIP-2, TxB_2 and PGE_2 after LPS challenge in mice, are consistent with this analysis.

However, this reasoning may not fully explain the beneficial effects of WY on pulmonary permeability. In murine lungs, PAF induced an acute increase in vasoconstriction and permeability [35]. The acute vasoconstriction is mediated by generation of cysteinyl-leukotrienes and TxA_2 [35]. Immediate formation of PGE_2 by COX-1 and activation of the acid sphingomyelinase (ASM) with subsequent synthesis of ceramides are the key events inducing permeability [36]. In isolated lungs, the current authors found an unchanged vasoconstriction, mirrored by the perfusion pressure, despite activation of PPAR-α by WY. In contrast, the PAF-induced

increase in K_{fc} (a marker of permeability) was nearly abolished. As both vasoconstriction and permeability involve receptor-dependent activation of secondary mediators, a downregulation of PAF receptors by PPAR-α agonists, as found in human macrophages [22], seems unlikely in the mice used in the present study. The current authors speculate that WY protected the lungs from PAF-induced permeability due inhibition of the PGE_2 (COX-1) or ceramide (ASM) pathway [36]. Evidence is accumulating that activation of PPAR-α induces an increase in ceramides, although these data were generated in hearts and not in lungs [37, 38]. Taking this together with the reduction in PGE_2 as determined in the BAL after LPS challenge *in vivo*, the COX-1 and COX-2 pathways can be considered primary targets of PPAR-α in effects on permeability in the present model.

As already discussed, activation of PPAR-α was reported to reduce inflammation in the lungs. Conversely, genetic deletion of PPAR-α induced prolonged and increased inflammation, as judged, for example, by increased generation of pro-inflammatory cytokines, leukocyte infiltration and mortality [31–34]. Whereas the data from the present ALI model are consistent with the first part of these findings, only a small (not significant) increase in the LPS-induced response was found in mice lacking functional PPAR-α and thus lacking responses from exogenous or endogenous activators. These diverging results may be due to the different time-points and models. While the present study used per-acute models of ALI, taking minutes in the isolated lung and 24 h *in vivo* as time period, the diverging results are derived from models that are more chronic and focus mainly on airway or pleural inflammation involving time spans of 5–14 days. In contrast, the present study confirmed that the beneficial effects of WY in pulmonary pathologies were specifically dependent on functional PPAR-α. Using PPAR-$\alpha^{-/-}$ mice, the protective effects of WY on cytokines, eicosanoids, leukocyte infiltration and vascular leakage in models of ALI were abolished. These findings are in line with previous studies using WY or fenofibrate [31–34]. TRIFILIEFF *et al.* [39] were unable to detect the same protective effects of the PPAR-α agonist GW 9578 in mice. However, they applied GW 9578 intranasally 1 h before LPS instillation and did not find reduced TNF-α generation in BAL or a change in neutrophil influx. A clue to the understanding of these diverging results may be the difference in the application of fibrates. The application of GW 9578 just 1 h before the inflammatory challenge may have been too short for the beneficial actions of PPAR-α activation, *e.g.* for subsequent changes in protein expression of NF-κB-dependent genes to take place. Next, TRIFILIEFF *et al.* [39] used a local (intranasal) application whereas other studies and the present study used a systemic approach: feeding the mice or using intraperitoneal injection. Despite expression of PPAR-α in murine lungs [9] and a direct impact of fibrates on airway smooth muscle cells [40], it is possible that local as well as remote leukocytes and endothelial cells must also be targeted for the full effect of fibrates to take place.

Leukocyte transmigration into the alveolar space represents a characteristic of pulmonary inflammation and ALI [41]. The present study showed that activation of PPAR-α reduced acute neutrophil invasion in the model of ALI. As neutrophil transmigration into the alveolar space needs a concerted action of neutrophils, endothelial cells and epithelial cells, an impact of WY on all cell populations may be possible. A reduced expression of VCAM-1 in endothelial cells and PAF receptor in leukocytes has already been shown [21, 22], and both systems are involved in adhesion and transmigration of leukocytes [42]. Furthermore, reduced generation of MIP-2 (the murine equivalent of the chemotaxin IL-8 in humans) as found in the present study and decreased TxA_2 synthesis represent two further causes of the reduced leukocyte infiltration in the lungs.

Finally, the present study provides evidence that LPS instillation into the lungs significantly impairs lung compliance. This impairment is ameliorated by WY-induced PPAR-α activation. Determination of compliance represents a recognised means to evaluate lung injury [43] and impairment is found in patients with ALI [44]. The fact that PPAR-α activation improved compliance underscores the beneficial effects of PPAR-α activation on ALI.

The beneficial impact of fibrates in the present ALI model is best described through the blunting and shortening of the ability of LPS to induce an inflammatory response. The invasion of leukocytes into the alveolar space was decreased 24 h after the initiation of the lung injury. This may be attributed to the reduced generation of MIP-2 at 8 h after LPS instillation, which, in addition to the decreased release of TxB_2 at both time-points, may be responsible for the lower influx of neutrophils. The impact of fibrates on TNF-α was only visible at the second time-point, as the generation of TNF-α was unchanged at 8 h but was decreased at 24 h after the injury. This is in contrast to the effect of fibrates on COX-derived PGE_2 and TxB_2, on protein in BAL and on compliance. All these variables were generally improved at both time-points after LPS instillation by application of fibrates. This feature adds to the idea that inhibition of COX may be a key player in translating the effects of fibrates in ALI.

In conclusion, the present study demonstrated that pretreatment with WY 14,643 reduced generation of pro-inflammatory cytokines and eicosanoids, blunted alveolar leukocyte invasion and improved compliance as well as vascular leakage in mice. The protective potency of WY 14,643 is dependent on functional peroxisome proliferator-activated receptor-α. Further investigations are warranted to explore the effect of fibrates in patients with inflammatory pulmonary pathologies.

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Anlage 7

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ω-3 fatty acids suppress monocyte adhesion to human endothelial cells - role of endothelial PAF generation.

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ω-3 Fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation

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[1]*Medizinische Klinik II, Justus Liebig University, D-35392 Giessen; and* [2]*Medizinische Klinik mit Schwerpunkt Infektiologie, Charité, Humboldt University, D-13353 Berlin, Germany*

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Mayer, Konstantin, Martina Merfels, Marion Muhly-Reinholz, Stephanie Gokorsch, Simone Rosseau, Jürgen Lohmeyer, Nicole Schwarzer, Matthias Krüll, Norbert Suttorp, Friedrich Grimminger, and Werner Seeger. ω-3 Fatty acids suppress monocyte adhesion to human endothelial cells: role of endothelial PAF generation. *Am J Physiol Heart Circ Physiol* 283: H811–H818, 2002; 10.1152/ajpheart.00235.2002.—Monocyte-endothelium interaction is a fundamental process in many acute and chronic inflammatory diseases. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are fish oil-derived alternative (ω-3) precursor fatty acids implicated in the suppression of inflammatory events. We investigated their influence on rolling and adhesion of monocytes to human umbilical vein endothelial cells (HUVEC) under laminar flow conditions in vitro. Exposure of HUVEC to tumor necrosis factor (TNF-α) strongly increased *1*) surface expression of intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1), and E-selectin, *2*) platelet-activating factor (PAF) synthesis as assessed by thrombin challenge, and *3*) rate of rolling and adhesion of monocytes. Preincubation of HUVEC with EPA or DHA markedly suppressed PAF synthesis, monocyte rolling, and adherence, whereas expression of endothelial adhesion molecules was unchanged. Also, PAF receptor antagonists markedly suppressed the adhesion rate of monocytes, and EPA or DHA revealed no additional inhibitory capacity. In contrast, arachidonic acid partially reversed the effect of the antagonist. We conclude that ω-3 fatty acids suppress rolling and adherence of monocytes on activated endothelial cells in vitro by affecting endothelial PAF generation.

eicosapentaenoic acid; arachidonic acid; adhesion molecules; leukocytes; platelet-activating factor

THE EMIGRATION OF LEUKOCYTES, e.g., monocytes from the intravascular compartment into the tissue, is a fundamental process in many acute and chronic inflammatory diseases. This process requires adhesion of the leukocytes to and migration through vascular endothelium. Several adhesion molecules were shown to be involved in the transendothelial migration of monocytes, including β_2-integrins (CD11/CD18 complex), the β_1-integrin VLA-4 (very late antigen-4), selectins, intercellular adhesion molecule (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), and vascular cell adhesion molecule (VCAM-1) (1, 23, 27, 28, 33, 36, 38, 43). For firm monocyte-endothelium adherence, CD11/CD18-ICAM-1 and VLA-4-VCAM-1 interactions were noted to be particularly relevant, with directional motility through the interendothelial gaps into the subendothelial tissue apparantly demanding reversible integrin-endothelial and subsequent monocyte-matrix interactions as, e.g., communicated via VLA-5 (43). Under conditions of inflammation, mimicked by endothelial cytokine pretreatment in in vitro studies, endothelial adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 are upregulated, and endothelial monocyte adhesion is markedly increased, with the role of VLA-4-VCAM-1 interaction being particularly prominent under these conditions (6–8, 33, 36, 43). This is consistent with in vivo studies addressing the role of both β_1- and β_2-integrins in monocyte migration into inflammatory sites or cytokine-induced lesions in vivo (1, 13, 45). In addition to the "classical" adhesion molecules, platelet-activating factor (PAF) expression in the endothelial membrane, interacting with monocyte PAF receptors, was suggested to contribute to the adhesive interaction of the mononuclear cells with the endothelium (47, 48).

Enhanced transmigration of circulating blood monocytes across the vascular endothelium is considered as an important contributor to the pathogenesis of acute and chronic systemic inflammatory diseases with sepsis and multiorgan failure (29) as well as atherosclerosis (24, 34) representing prototypic entities. In both diseases, lipid mediators have additionally been implicated in the pathogenesis of vascular abnormalities, and, of interest, supplementation with ω-3 instead of ω-6 fatty acid-rich diets is considered to be a therapeutic approach (10, 35). The family of ω-6 fatty acids, including arachidonic acid (AA), represents the predominant polyunsaturated fatty acids in common diets

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of the Western world. In contrast, ω-3 fatty acids make up an appreciable part of the fat in cold-water fish and seal meat. In this family of fatty acids, the last double bond is located between the third and fourth carbon atom from the methyl end, with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) being important representatives. AA is metabolized via multiple metabolic pathways, including cyclooxygenases and various lipoxygenases to prostanoids, leukotrienes, and other lipoxygenase products with well-described vascular effects (12). The anti-inflammatory potency of the ω-3 fatty acids is largely ascribed to the fact that they serve as alternative lipid precursors for all metabolic pathways hitherto recognized for AA. Arising metabolites are trienoic prostanoids, thromboxane A_3, 5-series cysteinyl-leukotrienes, and leukotriene B_5, which possess markedly reduced inflammatory and vasomotor potencies compared with the AA-derived lipid mediators and exert even antagonistic functions. In addition to being precursors for different eicosanoid formation, ω-3 versus ω-6 fatty acid incorporation into membrane (phospho) lipid pools was suggested to have impact on lipid-related intracellular signaling events (9, 39, 44). Phosphatidylinositol and sphingomyelin pools, but also subclasses of phosphatidylcholine such as the PAF precursor pool, may be particularly relevant in this respect.

In the present study performed on human endothelial cells undergoing cytokine stimulation in vitro, the impact of AA versus EPA and DHA on monocyte-endothelial interaction was investigated under laminar flow conditions. Incubation of the endothelial cells with ω-3 fatty acids turned out to suppress monocyte rolling and adhesion significantly, with DHA being even more potent than EPA. Evidence is forwarded that this ω-3 fatty acid effect is related to suppression of endothelial PAF generation. Such an impact on monocyte-endothelium interaction may contribute to the dampening of inflammatory events observed under ω-3-rich enteral or parenteral diets in acute and chronic inflammatory diseases.

MATERIALS AND METHODS

Materials. AA, EPA, and DHA were obtained from Sigma Chemical (Deisenhofen, Germany). Chromatographic supplies included HPLC-grade solvents, glass-distilled (Fluka; Heidelberg, Germany) octadecylsilyl (5 μm, Hypersil) and silica gel 5-μm column packing (Machery-Nagel; Duren, Germany), and C-18 Sep-Pac cartridges (Waters; Milford, MA). RPMI-1640 medium and fetal calf serum were from Boehringer Mannheim (Mannheim, Germany). Collagenase (type CLS type II) was purchased from Worthington Biochemical (Freehold, NJ). Medium 199, fetal calf serum, HEPES, Hanks' balanced salt solution, phosphate-buffered saline, trypsin-EDTA solution, and antibiotics were obtained from GIBCO (Karlsruhe, Germany). [^{3}H]PAF, lyso-[^{3}H]PAF, [^{3}H]acetate, and [^{3}H]serotonin were obtained from Amersham (Dreieich, Germany). The PAF receptor antagonist WEB-2086 was generously supplied by Boehringer Ingelheim (Ingelheim, Germany). Tissue culture plastic was purchased from Becton-Dickinson (Heidelberg, Germany). 1-*O*-hexadecyl-2-ace-tyl-*sn*-glycero-3-phosphocholine (PAF) and thrombin were obtained from Sigma Chemical. The PAF receptor antagonist BN-50730 (46) was generously supplied by Dr. P. Braquet (Institute Henri Beaufour, Le Plessis-Robinson, France), and the PAF receptor antagonists CV-3988 and CV-6209 were bought from Biomol (Hamburg, Germany). All other biochemicals were obtained from Merck (Munich, Germany).

Preparation of endothelial cells. Endothelial cells were obtained from human umbilical veins (HUVEC) according to the method described by Jaffe et al. (15).

Monocyte isolation. Human monocytes were isolated from platelet pheresis residues by centrifugation on Ficoll-Hypaque density gradient centrifugation, followed by counterflow centrifugation elutriation using a Beckman JE-5.0 rotor. Monocyte purity (88–90%) was confirmed by light scatter [fluorescence-activated cell sorter (FACS) scan; Becton Dickinson]. Cell viability ranged above 96% throughout the study.

Leukocyte adhesion assay. Leukocyte adhesion was determined as described previously (16) using a parallel plate flow chamber according to Lawrence and Springer (19). Confluent endothelial monolayers were preincubated with fatty acids and tumor necrosis factor-α (TNF-α) according to the experimental protocol. A suspension of 4×10^6 monocytes per milliliter was perfused through the chamber at a constant wall shear stress of 1.0 dyn/cm^2 (syringe pump sp100i, WPI; Sarasota, FL). Interactions were visualized using a phase-contrast video microscope (IMT-2, Olympus Optical, Hamburg, Germany, with a KP-C551 CCD camera, Hitachi, Rodgau, Germany) and videotaped (JVC HR-S7000, JVC; Friedberg, Germany) over the entire time period of leukocyte perfusion. Rolling in the parallel plate flow chamber was measured in a high-power field for each experiment. "Rolling" was expressed as the number of rolling cells per high power field (×20 objective) during a 10-min observation period. Leukocytes were considered to be adherent after 30 s of stable contact with the monolayer. Adhesion was determined after 10 min of perfusion by analysis of five random high magnification fields (×20) from videotape (16, 19). Results are expressed as adherent cells per high magnification field.

Measurement of PAF by bioincorporation of radiolabel. Endothelial cell PAF production was quantified by post-HPLC liquid scintillation counting using the radiochromatogram imaging system (5LS Raytest). Endothelial cells were stimulated in the presence of 50 μCi [^{3}H]acetate (7.75 Ci/mmol) with 0.1 U/ml thrombin according to Tessner et al. (42) as adapted by Suttorp et al. (41). Reactions were stopped by addition of three volumes of chloroform:methanol (2:1 vol/vol), and extraction was performed according to Bligh and Dyer (31).

Post-HPLC PAF Bioassay. In addition, PAF production in HUVEC was quantified by induction of [^{3}H]serotonin release from prelabeled rabbit platelets. After HUVEC incubation, the total cellular and extracellular PAF content was lipid-extracted and subjected to straight phase HPLC separation as described above. Eluate fractions were collected at the appropriate PAF retention time, again lipid extracted for removal of phosphoric acid present in the mobile phase, evaporated to dryness, and redissolved in 50 μl of assay buffer for induction of platelet serotonin release. Preparation of platelets and the protocol of the bioassay were essentially as published by Pinkard et al. (30) and Suttorp et al. (41). Aliquots of each sample were used to ascertain the specificity of platelet secretion by the inhibitory effect of the PAF-receptor antagonist BN-50730.

Immunofluorescence staining of endothelial cells. Immunofluorescence labeling of HUVEC was performed as previously

described (21). Antibodies directed against ICAM-1 [clone R1/1 (CD54); Bender MedSystems; Vienna, Austria], VCAM-1 [clone 1G11 (CD106), Coulter-Immunotech; Marseille, France], E-selectin [clone BBIG-E1 (CD62E), R&D Systems], major histocompatibility complex-I (MHC-I, positive control, W6/32.HL, generously provided by A. Ziegler, Berlin, Germany), and isotype controls (negative control; Dianova) were used.

Cell surface ELISA for P-selectin. Expression of P-selectin was determined by cell surface ELISA as previously described (17). The primary monoclonal antibody against P-selectin [clone 9E1 (CD62P), R&D] was used.

Experimental protocol. HUVEC were grown to confluence, the culture medium was exchanged, and free fatty acids (AA, EPA, and DHA) dissolved in ethanol (final volume <1%, vol/vol) were admixed to the culture medium at a final concentration of 10 μmol/l and incubated for 6 h. Controls were sham incubated with solvent only. Without exchange of incubation medium, admixture of TNF-α (0, 1, or 10 ng/ml, as detailed) was then performed, and HUVEC were incubated for another 20 h. Expression of the endothelial adhesion molecules was then carried out after treatment with trypsin and transfer of the HUVEC to a FACS. For the monocyte adhesion experiments, HUVEC were grown on slides under the detailed experimental conditions, the incubation medium was discarded, and cells were gently washed directly before use in the flow chamber.

Statistics. For statistical comparison, one-way analysis of variance was performed. A level of $P < 0.05$ was considered to be significant. Analysis was carried out with SPSS for Windows (Release 8.0.0, SPSS; Chicago, IL).

RESULTS

Influence of TNF-α and fatty acids on monocyte-endothelium rolling and adhesion. Superfusing monocytes over HUVEC monolayer incubated under control conditions resulted in a low number of rolling and adherent monocytes (~5–9 and 10–12 monocytes/high magnification field, respectively). Monocyte rolling and adhesion were enhanced by nearly one order of magnitude by a preceding 20-h exposure of the HUVEC to TNF-α (10 ng/ml; Fig. 1). Preincubation of the HUVEC for 6 h with free EPA or DHA (10 μmol/l each), followed by the stimulation with TNF-α (20 h), significantly reduced this increase in monocyte rolling compared with TNF-α alone to 52% and 38%, respectively. In contrast, free AA (10 μmol/l) increased monocyte rolling to 149% (Fig. 1). Adhesion of monocytes to endothelial cells was significantly reduced by both ω-3 fatty acids. Compared with TNF-α alone set at 100%, EPA reduced this adhesion to ≈61%, and DHA to ≈55%, respectively. AA led to some reduction of monocyte adhesion but did not reach level of significance. The higher potency of the ω-3 fatty acids compared with AA was statistically highly significant ($P < 0.005$, Fig. 1).

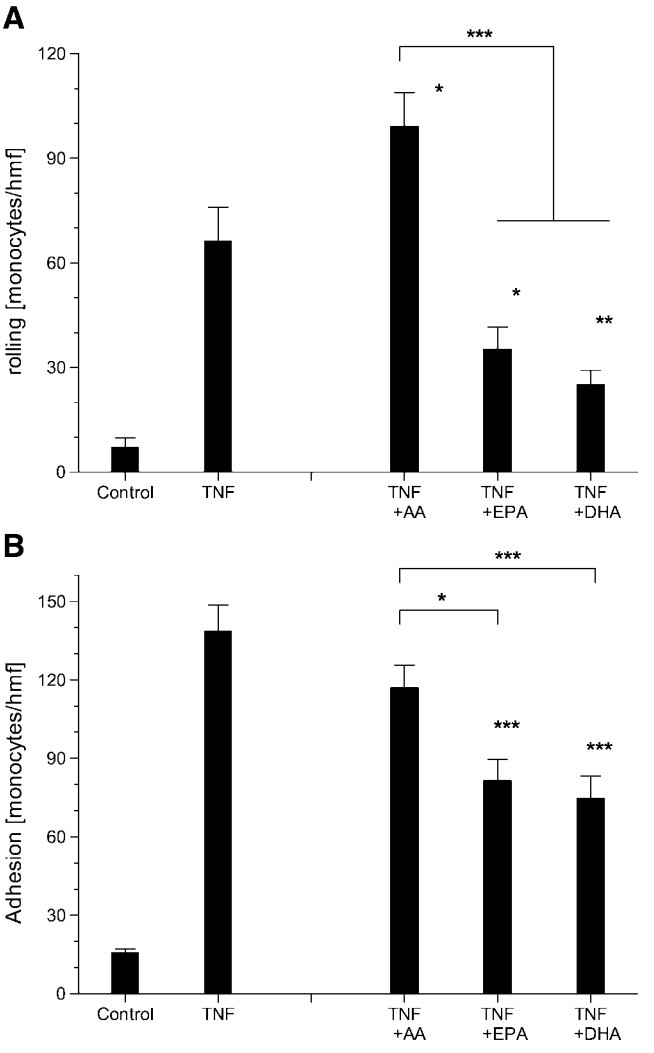

Fig. 1. Effects of free fatty acids on monocyte rolling and adhesion to tumor necrosis tactor (TNF)-α-activated endothelial cells. A total of 4×10^6 monocytes/ml was injected into the flow system and perfused over human umbilical vein endothelial cell (HUVEC) monolayer for 10 min. Cell rolling (*A*) was measured by playback of videotape as described. Monocyte adhesion (*B*) was determined at the end by analysis of 5 random high magnification fields (×20) from videotape. Preincubation with various fatty acids was performed as detailed under *Experimental protocol.* AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Values are means ± SE of 10 independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ for comparison with the TNF-α group; $^{***}P < 0.005$ for direct comparison between groups with different fatty acid pretreatment.

Influence of TNF-α and fatty acids on endothelial adhesion molecule expression. To address the hypothesis that fatty acids might suppress monocyte adhesion by reducing the expression of endothelial adhesion molecules, FACS analysis of EC was performed. E-selectin, VCAM-1, and ICAM-1 were upregulated by TNF-α (Fig. 2); however, none of the fatty acids exerted an significant effect on this increase. In addition, preincubation of HUVEC with AA, EPA, or DHA in the absence of TNF-α also did not affect the expression of E-selectin, VCAM-1, or ICAM-1. Analysis of endothelial P-selectin showed no signal on these cultured cells even after TNF-α challenge, which did not change in response to fatty acid preincubation.

Influence of PAF-receptor antagonists on monocyte adhesion to TNF-α-activated endothelial cells. To assess a putative role of PAF in the TNF-α- and fatty acid-induced alterations of monocyte-endothelium interaction, the effect of the selective PAF-receptor an-

tagonists BN-50730, CV-3988, and CV-6209 were investigated. BN-50730 dose dependently inhibited the adhesion of monocytes to TNF-α-activated HUVEC (Fig. 3). Used in concentrations of 0.01, 0.1, and 1 μmol/l, BN-50730 reduced monocyte adhesion to 86%, 70%, and 39%, respectively. The solvent control was without influence (data not shown). CV-3988 (10 nmol/l-10 μmol/l) and CV-6209 (10 nmol/l-10 μmol/l) dose dependently reduced TNF-α-enhanced adhesion of monocytes, with a maximal reduction to 49% and 52%, respectively. In the presence of 1 μmol/l BN-50730, neither EPA nor DHA further suppressed the amount of adherent monocytes to TNF-α-activated endothelial cells (Fig. 4). Under these conditions, AA even slightly increased the monocyte-endothelium adhesion (TNF+AA+BN compared with the TNF+BN in Fig. 4).

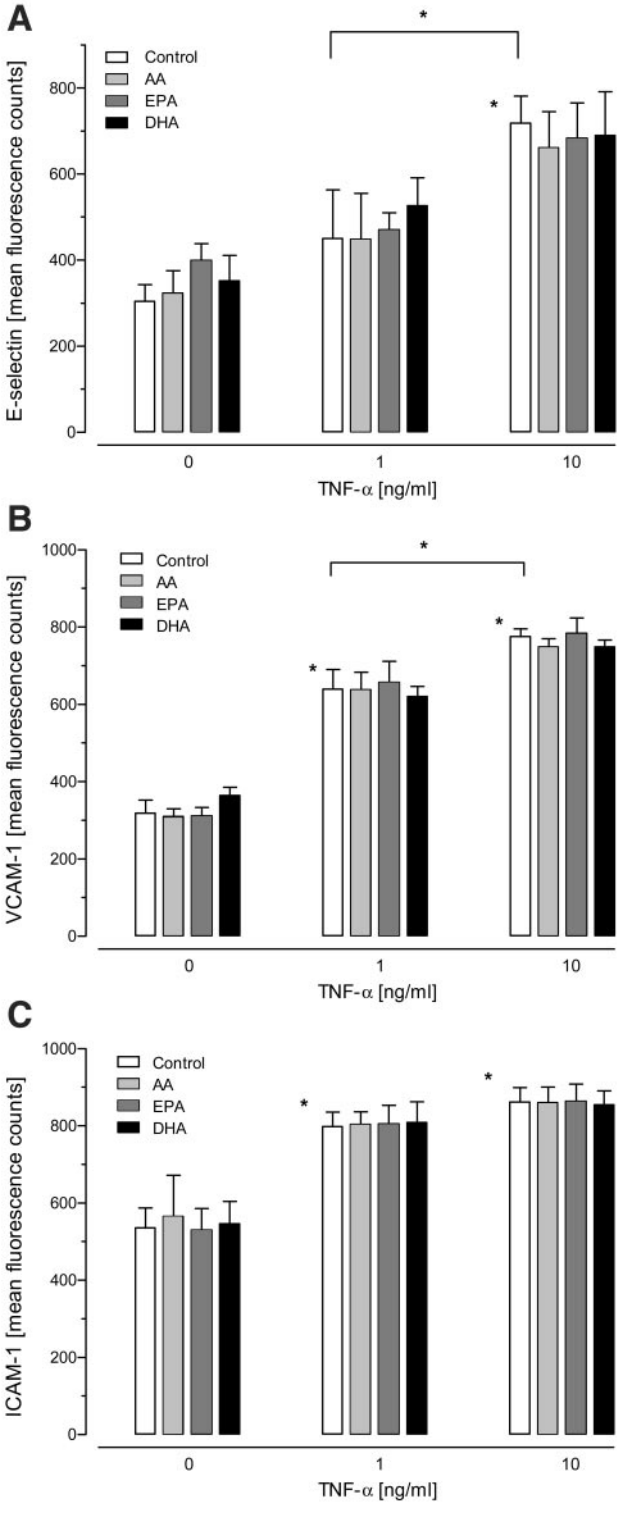

Fig. 2. TNF-α-induced increase of endothelial adhesion molecules. Surface expression of adhesion molecules on HUVEC was measured by fluorescence-activated cell sorter (FACS) analysis. Monoclonal mouse anti-human antibodies directed against E-selectin (*A*), vascular adhesion molecule (VCAM-1) (*B*), or intercellular adhesion molecule (ICAM-1) (*C*) were employed. Preincubation with various fatty acids and TNF-α was performed as detailed under *Experimental protocol*. Values are means ± SE of 4 independent experiments each. $^*P < 0.05$ for comparison of the TNF-α-treated HUVEC (absence of fatty acids) with controls in the absence of TNF-α or for direct comparison between different TNF-α concentrations. No statistical significance to the corresponding control group was detected in any of the fatty acid-incubated cells.

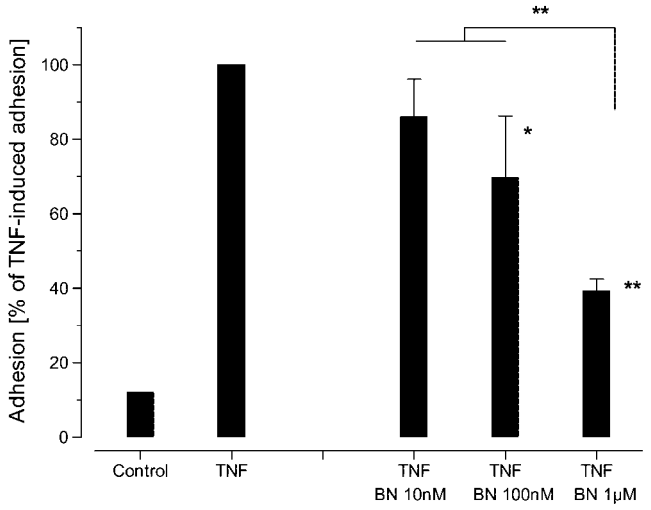

Fig. 3. Effects of the platelet-activating factor (PAF)-receptor antagonist BN-50730 on monocyte adhesion to TNF-α-activated endothelial cells. A total of 4×10^6 monocytes/ml was injected into the flow system and perfused over the HUVEC monolayer for 10 min. Cell adhesion was then determined by analysis of 5 random high magnification fields (×20) from videotape. Adhesion of monocytes to HUVEC stimulated with TNF-α was set at 100%. PAF-antagonist BN-50730 was admixed to the monocyte suspension 15 min before the flow experiments. Values are means ± SE of 6 independent experiments each. $^*P < 0.05$; $^{**}P < 0.01$ for comparison with the TNF-α group or for direct comparison between groups with different BN-50730 concentrations.

Influence of ICAM-1 or VCAM-1 blockade on monocyte adhesion to TNF-α-activated endothelial cells. In separate experiments, saturating amounts of adhesion-blocking antibodies against ICAM-1 [clone R1/1 (CD54)] and VCAM-1 [clone 1G11 (CD106)] were admixed to the endothelial incubation medium of TNF-α-pretreated HUVEC 30 min before the adhesion assay. Monocyte adhesion was reduced to 57.0 ± 4.2 (anti-ICAM-1) and 46.3 ± 3.8% (anti-VCAM-1) compared

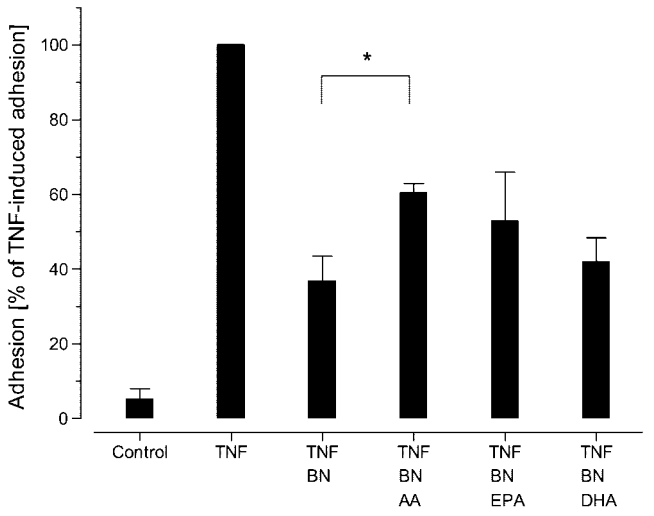

Fig. 4. Effects of coapplication of the PAF-receptor antagonist BN-50730 and free fatty acids on monocyte adhesion to TNF-α-activated endothelial cells. A total of 4×10^6 monocytes/ml was injected into the flow system and perfused over the HUVEC monolayer for 10 min. Cell adhesion was then determined by analysis of 5 random high magnification fields (×20) from videotape. Adhesion of monocytes to HUVEC preexposed to TNF-α was set at 100%. Preincubation with various fatty acids was performed as detailed under *Experimental protocol*. PAF-antagonist BN-50730 was admixed to the monocyte suspension 15 min before the flow experiments. Values are means ± SE of 5 independent experiments. Only the TNF+BN+AA group differed significantly from the TNF+BN group ($*P < 0.05$).

with TNF-α controls in the absence of antibodies monoclonal antibodies.

Fatty acids and endothelial cell PAF generation. To address the impact of the fatty acids on endothelial PAF generation in a more direct fashion, short-term provocation of PAF synthesis by challenge of HUVEC with thrombin was performed, because examination of HUVEC after superfusion of monocytes yielded PAF levels below the detection limit of our assays. Pretreatment of the endothelial cells with TNF-α and fatty acids was undertaken as described above. Thrombin provoked a dose-dependent generation of PAF in control HUVEC, as demonstrated by both PAF bioassay (Fig. 5) and release of bioincorporated [^{3}H]acetate (data not shown). Thrombin-induced PAF-synthesis was increased by approximately equal to one order of magnitude when endothelial cells were pretreated with TNF-α. A further increase in PAF quantities in lipid-extracted HUVEC was found after preincubation with AA (Fig. 5). In contrast, incubation with EPA and even more with DHA resulted in a suppression of thrombin-induced PAF synthesis.

DISCUSSION

Monocytes spontaneously adhere to endothelial cell monolayers under static conditions; however, substantial monocyte-endothelial adhesion under flow demands preceding cytokine stimulation of the endothelial cells: E-selectin-L-selectin, ICAM-1-β_2 integrin, and in particular VCAM-1-VLA-4- interactions were shown to represent predominant adhesive forces under these conditions (6–8, 14, 33, 36, 43). It is well in line with these preceding observations that monocyte rolling and firm adhesion as currently addressed under laminar flow conditions was increased by approximately one order of magnitude after TNF-α pretreatment of the HUVEC, in companion with markedly enhanced endothelial ICAM-1 and VCAM-1 expression. Moreover, the monocyte adhesion to the cytokine-stimulated endothelial cells was reduced to 46% by blocking antibodies against VCAM-1 and to 57% by antibodies against ICAM-1.

In addition to these adhesion molecule interactions, the present data suggest endothelial PAF synthesis as an important contributor to monocyte-HUVEC adhesion. First, endothelial PAF synthesis as probed by thrombin challenge was increased by one order of magnitude upon prolonged TNF-α incubation. Second, monocyte adhesion to cytokine-stimulated endothelial cells was dose dependently inhibited by PAF-receptor antagonists. PAF synthesis in endothelial cells was first described in HUVEC (5, 31) but is in fact a function of endothelial cells from all vascular beds activated by receptor-operated stimuli or undergoing injurious attack (3, 18). Endothelial PAF remains associated with the cell surface, even in the presence of albumin for binding of this hydrophobic molecule, and studies from Zimmerman and co-workers (20, 48, 49) suggested that binding of endothelial PAF and the leukocyte PAF receptor contribute to adhesive interactions between endothelial cells and monocytes, followed by a juxtacrine activation of the adherent mononuclear cells. This concept is fully supported by the

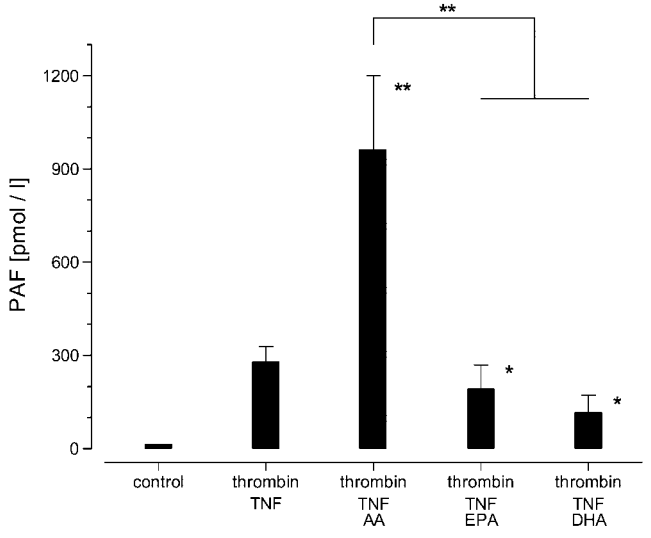

Fig. 5. Impact of free fatty acids on thrombin-induced PAF-generation in endothelial cells. PAF generation was measured after short-term incubation of HUVEC with thrombin (0.1 U/ml, 10 min) by PAF bioassay using [^{3}H]serotonin-labeled platelets. Preincubation with TNF-α in the absence and presence of the different fatty acids was performed as detailed under *Experimental protocol*. Values are means ± SE of 5 independent experiments. $*P < 0.05$; $**P < 0.01$; for comparison with the thrombin/TNF-α group or for direct comparison between groups with different fatty acid preincubation.

presently noted efficacy of the PAF receptor antagonist. A role of P-selectin coexpression with PAF for the endothelial attraction of monocytes under condition of laminar flow (49) may be excluded for the present investigation. Cultured HUVEC demonstrate hardly any P-selectin expression after stimulation with TNF-α. This study did not address the question whether adhesive forces between PAF and its receptor may directly contribute to the firm monocyte attachment on the endothelial cells or whether the PAF system is largely operative via monocyte activation and enhanced mononuclear integrin expression and/or avidity as suggested as juxtacrine PAF-induced mechanisms (22, 49). Receptor-operated regulation of integrin affinity has, indeed, been disclosed as an important mechanism inducing rapid leukocyte adhesion to endothelial cell surfaces (26, 40, 43).

The most important finding of the present study is the fact that ω-3 fatty acid preincubation of the HUVEC markedly reduced monocyte rolling and adhesion to endothelial cells, with DHA being even more potent than EPA; monocyte adherence was reduced to 55% and 61%, respectively, compared with control cells incubated with TNF-α in the absence of these fatty acids. As assessed by random videotape analysis, firmly adhering monocytes all subsequently transmigrate the endothelial barrier, and this feature was not changed by endothelial fatty acid incubation (data not shown). Thus the ω-3 fatty acid-effected decrease in the amount of rolling and adhering monocytes directly translates into a reduction (in absolute numbers) of transmigrating monocytes.

The suppression of monocyte adherence to the EPA- or DHA-preexposed endothelial cells might be exerted by a reduction of adhesion molecule expression on the endothelial surface. Previous investigations indeed supplied evidence that ω-3 fatty acid incubation of cytokine-stimulated endothelial cells reduced VCAM-1 expression (9, 44). In the present study in HUVEC, however, quantification of endothelial surface expression of VCAM-1, ICAM-1, E-selectin, and P-selectin by FACS analysis did not detect any change in response to AA, EPA, or DHA pretreatment. This discrepancy may be explained by the fact that the fatty acid concentrations in these previous studies ranged between 100 and 300 μmol/l, whereas 10 μmol/l was employed in the present study to meet the physiological plasmatic fatty acid concentrations. The current finding of unchanged adhesion molecule expression on the EPA- or DHA-exposed HUVEC does, however, not exclude the possibility that the ω-3 fatty acids might have impact on the affinity of these adhesion molecules. Moreover, we did not address the endothelial surface expression of a L-selectin ligand suggested to contribute to monocyte-endothelial adherence under flow conditions (23).

The present study did, however, forward evidence that the prominent impact of the ω-3 fatty acids on the monocyte-HUVEC interaction is related to endothelial PAF generation. First, coapplication of the PAF-receptor antagonist BN-50730 and EPA or DHA did not exert additive effects on the rate of monocyte adhesion, but the levels of suppression achieved by either BN-50730 or EPA/DHA or a combination of ω-3 fatty acid and BN-50730 were not statistically different from each other. Second, when probing with thrombin, an established stimulus for rapid PAF synthesis in endothelial cells (48), the appearance of this lipid mediator in the cytokine preexposed HUVEC was found to be markedly suppressed by EPA and DHA. These findings are well compatible with the concept that preincubation of the HUVEC with EPA or DHA inhibits endothelial PAF generation and subsequently reduces monocyte adhesion. Interestingly, recent studies of the lipid composition of the endothelial membrane pools under cytokine challenge demonstrated marked loss of long-chain polyunsaturated fatty acids within 22 h, mimicking an "essential fatty acid deficiency" syndrome, with exogenous ω-3 fatty acids being rapidly incorporated into the *sn*-2 position of the phosphatidylcholine, the phosphatidylethanolamine, and the phosphatidylinositol pool, including their PAF precursor subclasses under these conditions (K. Mayer and W. Seeger, personal communication). This is well in line with data in eosinophilic leukocytes, where preincubation with DHA resulted in incorporation into the phosphatidylcholine pool, a reduction of PAF generation, and free AA release (37). Moreover, the activity of the phospholipase A_2, hydrolyzing the *sn*-2 acyl residue from the PAF precursor as an initial step in endothelial PAF synthesis, is known to be dependent on the type of fatty acid located in the *sn*-2 position (32, 37), and DHA and EPA might well exert their suppressive effect on PAF synthesis via this route. This suggestion for the mode of action of EPA and DHA does, of course, not exclude that these ω-3 fatty acids may have major impact on additional lipid-related signaling events finally contributing to the rate of monocyte adherence to endothelial cells.

In contrast to EPA and DHA, the endothelial PAF liberation as probed by thrombin challenge was increased upon preincubation of HUVEC with AA, which is well compatible with the finding that similar to the ω-3 fatty acids, AA is rapidly incorporated into PAF-precursor pools of cytokine-stimulated HUVEC, and that PAF precursors with AA in the *sn*-2 position are preferred substrates for the phospholipase A_2 attack, the rate-limiting step in PAF synthesis (32). In parallel, an increase of rolling monocytes on HUVEC was found. Accordingly, AA preincubation of the endothelial cells significantly antagonized the BN-50730-effected decrease in monocyte-HUVEC adherence. When given as sole agent, AA significantly differed from EPA and DHA with respect to the suppressive effect on endothelial monocyte adhesion; however, still some not significant reduction of adherence was noted. Thus further effects of AA, not related to its impact on PAF synthesis, must be assumed to underlie the influence of this fatty acid on the monocyte-HUVEC interaction.

In conclusion, firm adhesion of monocytes on cytokine-stimulated HUVEC was found to be largely depressed by preincubation of the endothelial cells with EPA and DHA. This effect of the ω-3 fatty acids oc-

curred independent of the endothelial expression of the adhesion molecules ICAM-1, VCAM-1, E-selectin, and P-selectin but is probably related to the suppression of endothelial PAF synthesis by EPA and DHA. The applied concentrations of ω-3 fatty acids (10 μmol/l) are in the range of free plasmatic EPA/DHA levels appearing under long-term oral supplementation with fish oil (4) and under short-term infusion of fish oil-based ω-3-based lipid emulsions; the latter approach even provoked plasmatic-free EPA/DHA concentrations of >100 μmol/l in septic patients (11, 25). The impressive effect of ω-3 fatty acids on monocyte-endothelium interaction may be of interest for dampening inflammatory processes in acute and chronic diseases, in which activation and transmigration of mononuclear cells largely contribute to the pathogenic sequels.

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