Medizinische Universität Graz

Dissertation

Impact of Lysophosphatidylcholines and Endothelial Lipase-modified HDL on vascular reactivity

submitted by

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, ----------

Summary

CHAPTER I

Previously we identified palmitoyl-, oleoyl-, linoleoyl-, and arachidonoyl-lysophosphatidylcholine (LPC 16:0, 18:1, 18:2 and 20:4) as the most prominent LPC species generated by endothelial lipase (EL). In the present study, we examined the impact of those LPC on acetylcholine (ACh)- induced vascular relaxation. All tested LPC attenuated ACh-induced relaxation, measured ex vivo, using mouse aortic rings and wire myography. The rank order of potency was as follows: 18:2.20:4.16:0.18:1. The attenuating effect of LPC 16:0 on relaxation was augmented by indomethacin-mediated cyclooxygenase (COX)-inhibition and CAY10441, a prostacyclin (PGI_2)-receptor (IP) antagonist. Relaxation attenuated by LPC 20:4 and 18:2 was improved by indomethacin and SQ29548, a thromboxane A_2 (TXA_2)- receptor antagonist. The effect of LPC 20:4 could also be improved by TXA_2- and PGI_2-synthase inhibitors. As determined by EIA assays, the tested LPC promoted secretion of PGI_2, TXA_2, PGF_{2a}, and PGE_2, however, with markedly different potencies. LPC 16:0 was the most potent inducer of superoxide anion production by mouse aortic rings, followed by LPC 18:2, 20:4 and 18:1, respectively. The strong antioxidant tempol recovered relaxation impairment caused by LPC 18:2, 18:1 and 20:4, but not by LPC 16:0. The tested LPC attenuate ACh-induced relaxation through induction of proconstricting prostanoids and superoxide anions. The potency of attenuating relaxation and the relative contribution of underlying mechanisms are strongly related to LPC acyl-chain length and degree of saturation.

CHAPTER II

In the second part we studied the impact of EL on HDL composition and function. Inflammation causes dramatic changes in HDL composition and function, which can turn HDL profile from anti-atherogenic to pro-atherogenic in nature. Considering capacity of EL to provoke structural and compositional alterations in HDL, we hypothesize that EL induced alterations of the lipid and protein composition of HDL might yield dysfunctional HDL, with dramatically impaired endothelial function. We observed that aortas of EL overexpressing mice were unable to relax as efficiently to ACh as compared to their LacZ controls. HDL isolated from EL-overexpressing mice (EL-HDL) upon adenoviral injection exhibited, compared with LacZ-HDL, markedly decreased relative PL-, moderately increased triglyceride (TG)-, slightly decreased total cholesterol (TC)- and slightly increased protein content. As revealed by mass spectrometry various phosphatidylcholine and lysophosphatidylcholine species were markedly decreased in EL- compared with LacZ-HDL. The LC-MS/MS analysis/spectral counting, Western blotting and arylesterase activity measurements revealed decreased apoM and PON1 in EL-HDL. As found by non-denaturing gradient gel electrophoresis (GGE) EL-HDL was slightly smaller in size than LacZ-HDL. Most importantly, wire myography measurements using mouse aortic rings precontracted with norepinephrine (NE) revealed markedly diminished vasorelaxing capacity of EL- compared with LacZ-HDL. Incubation of human HDL with EL-overexpressing HepG2 cells for 16 h resulted in similar alterations in lipid and protein composition in human EL-HDL as found for mouse EL-HDL. However, in contrast to mouse EL-HDL, human EL-HDL was profoundly smaller in size compared with LacZ-HDL, as found by non-denaturing GGE. Most importantly, vasorelaxing capacity of human EL-HDL was markedly decreased when compared with human LacZ-HDL.

Based on these results we concluded that EL-modification of human and mouse HDL markedly diminishes vasorelaxing capacity of HDL, most likely due to EL-mediated alterations in lipid and protein composition of HDL.

Zusammenfassung

Kapitel I

Palmitoyl-, Oleoyl, Linoleoyl und Arachidonoyl-Lysophosphatidylcholine (LPC 16:0, 18:1, 18:2 und 20:4) wurden bereits als die am häufigsten von der endothelialen Lipase (EL) gebildeten LPC-Spezies identifiziert. In dieser Studie wurde der Einfluss dieser LPC auf die Acetylcholin (Ach)- induzierte vaskuläre Relaxation untersucht. Alle Ex Vivo - unter Verwendung von Aortenringen der Maus und einem Myographen - getesteten LPC verringerten die ACh-induzierte Relaxation. Die Reihenfolge der Wirksamkeit der LPC auf die Relaxation ist dabei wie folgt: 18:2>20:4>16:0>18:1. Der verringernde Effekt von LPC 16:0 auf die Relaxation wurde sowohl durch die Indomethacin-vermittelte Inhibition der Cyclooxygenase (COX), als auch durch CAY10441, einem Prostacyclin (PGI_2)-receptor (IP) Antagonisten, verstärkt. Die durch LPC 20:4 und 18:2 verringerte Relaxation konnte durch Indomethacin und SQ29548, einem Thromboxan A_2 (TXA_2)-rezeptorantagonisten verbessert werden. Zusätzlich konnte der Effekt von LPC 20:4 durch TXA_2- und PGI_2- Synthaseinhibitoren verbessert werden.

Die getesteten LPC förderten die Sekretion von den über EIA Assays gemessenen PGI_2, TXA_2, PGF_{2a} und PGE_2 deutlich unterschiedlich stark. LPC 16:0, gefolgt von 18:2, 20:4 und 18:1, zeigte dabei die stärkste Induktion der Produktion von Superoxidanionen in Aortenringen der Maus.

Das starke Antioxidanz Tempol stellte die durch die LPC 18:2, 18:1 und 20:4, aber nicht durch LPC 16:0, verringerte Relaxation wieder her. Die getesteten LPC verringerten die ACh-induzierte Relaxation durch Induktion von prokonstriktierenden Prostanoiden und Superoxidanionen. Das Ausmaß der Verringerung der Relaxation und der relative Beitrag der zugrundeliegenden Mechanismen stehen stark in Beziehung mit der Acylkettenlänge der LPC und deren Grad der Sättigung.

Kapitel II

Im zweiten Teil wurde der Einfluss von EL auf die Zusammensetzung und Funktion von HDL untersucht. Entzündungen haben deutliche Auswirkungen auf die Zusammensetzung und Funktion von HDL, wodurch HDL seine antiatherogenen Eigenschaften verlieren und sogar proatherogene Eigenschaften haben kann. Aufgrund der Fähigkeit von EL, Veränderungen in der Struktur und Zusammensetzung von HDL herbeizuführen, wurde die Hypothese aufgestellt, dass EL-induzierte Veränderungen in der Lipid- und Proteinzusammensetzung von HDL zu dysfunktionalem HDL führen, welche mit einer deutlich verringerten endothelialen Funktion einhergehen.

Dabei wurde beobachtet, dass die Aorten von EL-überexprimierenden Mäusen keine effiziente Relaxation nach Behandlung mit ACh im Vergleich zu lacZ-Kontrollen zeigten. Das aus adenoviral EL-überexprimierenden Mäusen isolierte HDL (EL-HDL) zeigte im Vergleich zu den lacZ-Kontrollen einen deutlich verringerten PL-, leicht erhöhten Triglycerid (TG)-, leicht verringerten Total Cholersterol (TC)- und einen leicht erhöhten Proteingehalt. Mit Hilfe der Massenspektrometrie konnte gezeigt werden, dass verschiedene Phosphatidylcholin- und Lysophosphatidylcholinspezies in EL-HDL im Vergleich zu lacZ-HDL deutlich verringert waren.

Die Analysen mittels LC-MS/MS, Western Blotting und Arylesterase-Aktivitätsmessungen, zeigten eine Verringerung von apoM und PON1 in EL-HDL.

Mit Hilfe der nicht denaturierenden Gradientengelelektrophorese (GGE) konnte gezeigt werden, dass die Größe von EL-HDL im Vergleich zu lacZ-HDL etwas abnahm. Messungen am Myographen mit Aortenringen der Maus, welche mit Norepinephrin (NE) präkontraktiert wurden, zeigten eine deutlich verminderte Vasorelaxationskapazität von EL- im Vergleich zu lacZ-HDL. Die Inkubation von humanem HDL auf EL-überexprimierenden HepG2-Zellen für 16 h führte zu ähnlichen Veränderungen in der Lipid und Proteinkomposition. Über die GGE konnte gezeigt werden, dass im Gegensatz zum murinen EL-HDL, das humane EL-HDL im Vergleich zum humanen lacZ-HDL deutlich kleiner war. Auch die vasorelaxierenden Eigenschaften von humanem EL-HDL waren im Vergleich zu humanem lacZ-HDL deutlich erniedrigt.

Basierend auf diesen Resultaten kamen wir zu dem Schluss, dass die EL-Modifikation die vasorelaxierenden Eigenschaften des humanen und murinen HDLs, vermutlich aufgrund der Veränderungen in der Lipid- und Proteinzusammensetzungen, deutlich verringert.

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LIST OF ABBREVIATIONS

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AA	arachidonic acid
AC	adenylate cyclase
ADMA	Asymmetric dimethyl amine
ACh	acetylcholine
Ag-II	angiotensin II
cAMP	cyclic adenylate monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
cPLA2	cytosolic PLA2
DAG	diacylglycerol
EC	endothelial cell
EDCF	endothelium-derived constricting factor
EDHF	endothelium-derived hyperpolarizing factor
EDR	endothelium-dependent relaxation
EDRF	endothelium-derived relaxing factor
EETs	epoxyeicosatrienoic acids
ET-1	endothelin-1
GC	guanylyl cyclase
GPCR	G-protein coupled receptor
H_2O_2	hydrogen peroxide
HDL	high density lipoprotein
HETEs	hydroxyeicosatetraenoic acids
ICAM-1	intercellular adhesion molecule-1
IL-8	interleukin-8
IP3	inositol triphosphate
iPLA2	Ca2+-independent intracellular PLA_2
KCa	Ca2+-sensitive K+ channels
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
L-NMMA	**N**G-monomethyl-L-arginine methyl ester

LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
LPLD	lysophospholipase D
MCP-1	monocyte chemoattractant protein
NO	nitric oxide
eNOS	endothelial nitric-oxide synthase
O2 -	superoxide
OH·	hydroxyl radicals
ONOO -	peroxynitrite
oxLDL	oxidized low density lipoprotein
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLA_2	phosphalipase A_2
PLC	phospholipase C
PLD	phospholipase D
ROS	reactive oxygen species
SMC	smooth muscle cell
SNP	sodium nitroprusside
SOD	superoxide dismutases
SQ-29548	[1S-[1a,2a(Z),3a,4a]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino] methyl]-7-oxabicyclo [2.2.1]hept-2-yl]-5-heptanoic acid
TP	thromboxane A_2 receptor
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
VCAM-1	vascular cell adhesion molecule-1

List of Abbreviations

CHAPTER II

ABCA1	ATP-binding cassette, sub-family A, member 1
ABCG1	ATP-binding cassette, sub-family G, member 1
ABCG5	ATP-binding cassette, sub-family G, member 5
Apo	Apolipoprotein
ApoA1	Apolipoprotein A1
ApoE	Apolipoprotein E
ApoM	Apolipoprotein M
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CE	cholesteryl-ester
CETP	cholesteryl-ester transfer protein
CM	chylomicrons
CVD	cardiovascular disease
EC	endothelial cells
EL	endothelial lipase
EL-HDL	endothelial lipase- modified HDL
eNOS	endothelial nitric oxide synthase
FA	fatty acid
FC	free cholesterol
FFA	free fatty acid
HDL	high-density lipoprotein
HDL-C	HDL-cholesterol

HL	hepatic lipase
HPLC	high performance liquid chromatography
ICAM-1	intercellular adhesion molecule 1
IDL	intermediate-density lipoprotein
IL-1β	Interlukin1β
KBr	Potassium bromide
kDa	kilodalton
LacZ	Beta galactosidase
LacZ-HDL	LacZ-modified HDL
LCAT	lecithin-cholesterol acyl transferase
LDL	Low-density lipoprotein
LPC	Lysophosphatidylcholine
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoctractant protein-1
NO	Nitric oxide
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
PL	Phospholipid
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLTP	Phospholipid transfer protein
PON	Paroxonase
RCT	Reverse cholesterol transport
rHDL	Reconstituted high- density lipoproteins

S1P	Sphingosine-1-phosphate
SAA	Serum amoyloid A
SMC	Smooth muscle cells
SR-BI	Scavenger receptor class B type 1
TG	Triglycerides
TNF-α	Tumor necrosis factor α
VLDL	Very low- density lipoproteins

Chapter I

1. Introduction

1.1. Lysophosphatidylcholine (LPC)

LPC also known, as lysolecithin is a bioactive phospholipid derived from phosphatidylcholine (PC), found in mammalian tissues. LPC is known as a proinflammatory molecule, produced due to pathological processes. The physiological concentration of LPC in blood is quiet high and ranges between 100 to 170μM (1). LPC are usually bound to albumin or other carrier proteins that serve as a store for LPC and control its bioavailability (2, 3). Free LPC may be produced by excessive lipolysis that produces fatty acid (FA) and LPC that exceeds the binding capacity of albumin. Due to this, LPC tends to associate with lipoproteins, from where it is delivered to cells. Here it can either get reacylated to produce PC or deacylated to produce FA and choline (4). Lecithin-cholesterol acyl transferase (LCAT) is mainly involved in generating circulating LPC, by transfering a fatty acid from phosphatidylcholine (PC) to cholesterol (5). Phospholipase A2 (PLA2) produces LPC and arachidonic acid (AA) by hydrolysis of PC (6). LPC can also be produced by the action of endothelial lipase (EL) on high-density lipoproteins (HDL). By their sn-1 phospholipase activity both hepatic lipase (HL) and EL generate saturated as well as different species of unsaturated LPC by cleaving HDL-PC. Native LDL contains only about 1-5% LPC, but due to oxidation about 40% of PC contained within LDL gets converted to LPC (7).

1.1.1 Role of LPC in atherosclerosis

Atherosclerosis is an inflammatory disease associated with the development of fibrofatty plaques within the arterial intima. Oxidized LDL was found in high percentage in atherosclerotic plaques. Oxidized LDL consists of about 40% LPC, this lead to various studies focusing on the pathophysiological properties of LPC (8). LPC is said to be involved in the progression of atherosclerosis and other inflammatory diseases. It does so by altering the functions of a multitude of cell-types including endothelial cells (EC), smooth muscle cells (SMC), monocytes, macrophages, neutrophils and T-cells (9). On the cell surface LPC induces membrane

PLA2 to break down phospholipids, producing Arachidonic acid (AA) further required for prostanoid synthesis. LPC is also produced as a by-product of this process. Prostanoid biosynthesis involves oxidation and isomerization of unesterified AA, whereby AA is converted to prostaglandin H_2 (PGH_2) by cyclooxygenase (COX-1) or (COX-2). PGH_2 is further converted to various prostanoids depending on the prostanoid synthase acting on it and can have pro or anti-inflammatory effects on cells (10).

1.2. Structure and activity of LPC

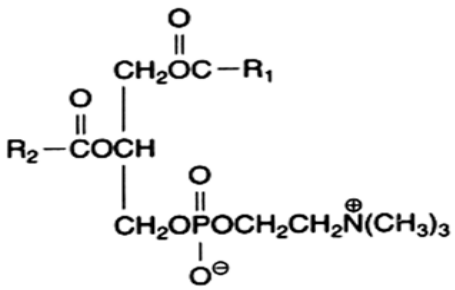


Figure.1. Structure of LPC

LPC is composed of a mixture of various molecular groups with different acyl groups (11). It has been suggested from a number of studies that the LPC acyl-chain length and degree of saturation may play an important role in deciding its biological activity. Only LPC containing greater than 14 carbon acyl groups could induce pathologic effects (12). Higher proportion of long-chain acyl group (>= C16:0) LPC was detected in hyperlipidemic subjects. The long chain LPC was able to produce impaired endothelium-dependent relaxation in rat aortic rings (7). An acyl chain-dependent difference was observed in the effect of LPC on neutrophils in vitro. In this study along with acyl-chain length of LPC the degree of saturation was also found to play a role. Enhanced and sustained reactive oxygen species (ROS) were produced by unsaturated LPC. In contrast, saturated LPC species induced significantly less ROS, but were found to be potent inducers of cytoplasmic calcium and plasma membrane permeability (13). Palmitoyl (16:0) LPC was found as the most abundant LPC in vivo in humans and rats (14, 15). Upon oxidation, LDL also consists of higher proportion of LPC 16:0, which is also found in atherosclerotic aortas (16). LPC are phospholipids that exist as free monomers in aqueous buffer at a concentration below, and as micelles at concentrations above, the critical micelle concentration (17). The

critical micelle concentration of LPC in Krebs-Henseleit buffer at pH 7.4 was found to be 40-50μM/L. The effects of LPC on cells depends on the crictical micelle concentration, such that alteration in cellular function occurs at lesser concentrations and cytoxicity occurs at concentration higher than critical micelle concentration (18).
LPC is shown to have diverse effects on intracellular signaling processes, including inhibition of inositol 1,4,5-triphosphate production and calcium elevation (18), inhibition of G protein-mediated signal transduction (19), inhibition of sodium fluoride stimulation of adenylate cyclase, stimulation of nitric oxide synthase (20) and either stimulation or inhibition of protein kinase C (21). Rather than showing direct actions on specific proteins, LPC mainly act by altering the membrane environment with which these proteins are associated and thereby alter their affinity for substrate or cofactors (22). Previously we found that the capacity and underlying mechanisms of palmitoyl-LPC (16:0 LPC), oleoyl-LPC (18:1 LPC), linoleoyl-LPC (18:2 LPC) and arachidonoyl-LPC (20:4 LPC) to modulate endothelial prostanoid production were remarkably different and related to the acyl-chain length as well as degree of saturation (4).

Table 1: Accumulation of LPC molecular species in human atherosclerotic aorta

	Normal Aorta		Atherosclerotic Aorta	
LPC	Average ± SEM	No. of patients	Average ± SEM	No. of patients
16:0	0.83 ±0.45	3	30.1 ±9.6	10*
18:0	0.62 ±0.31	3	22.0 ±7.6	10*
18:2	0.23 ±0.14	4	8.0 ±2.2	10
20:4	0.22 ±0.15	4	4.4 ±1.1	10
22:6	ND	4	0.9 ±0.2	10

Lipids from normal and atherosclerotic human aorta samples (50 mg) were extracted into chloroform in the presence of 14:0 lysophosphatidylcholine and quantitatively analyzed by ESI-MS for lysophosphatidylcholine molecular species. Values are expressed as picomol of each lysophosphatidylcholine molecular species per nanomole of inorganic phosphate. ND indicates not detectable. ***P**<0.01 and **P**<0.005, respectively, for comparisons between normal and atherosclerotic aorta for each molecular species using the Student **t** test. Adapted from (16).

1.3. LPC metabolism

1.3.1. LPC-generating enzyme: PLA2

PLA_2 breaks down membrane phospholipids to produce arachidonic acid. PLA_2 controls the rate-limiting step of the biosynthetic pathway starting from membrane phospholipids breakdown, to lysolipids, arachidonic acid and finally eicosanoid production. The eicosanoids thus formed have a number of biological effects. LPC is produced as a byproduct of this pathway and it can have differential effects on eicosanoid production and finally alter the biological effects produced by cells and tissues. PLA_2 is considered as an independent risk factor for cardiovascular disease (23). Among the different isoforms of PLA_2, cytosolic PLA_2 ($cPLA_2$) is mainly involved in agonist mediated AA release (24). Among isoforms, $cPLA_2$ plays a central role in the agonist stimulated AA liberation, and the following generation of lipid mediators (23). On the other hand, $sPLA_2$ and $Lp\text{-}PLA_2$ are especially important in mediating the proinflammatory properties of LPC.

1.3.2 LPC degrading enzyme: Lysophospholipase D (LPLD)

LPLD is an enzyme located in the plasma membrane and is involved in the conversion of LPC to LPA. The importance of LPLD is associated more with its LPA generating property than its LPC degrading role. Similar to LPC, LPA belongs to a family of lipid metabolites and induces a number of cellular responses (25). Some effects of LPC may be associated with the formation of LPA. It was suggested that LPC causes neuropathic pain by forming LPA and activating LPA receptors in mice lacking LPA receptor gene (18). In rat coronary endothelial cells, LPC was found to augment lipoprotein lipase enzyme by formation of LPA (26).

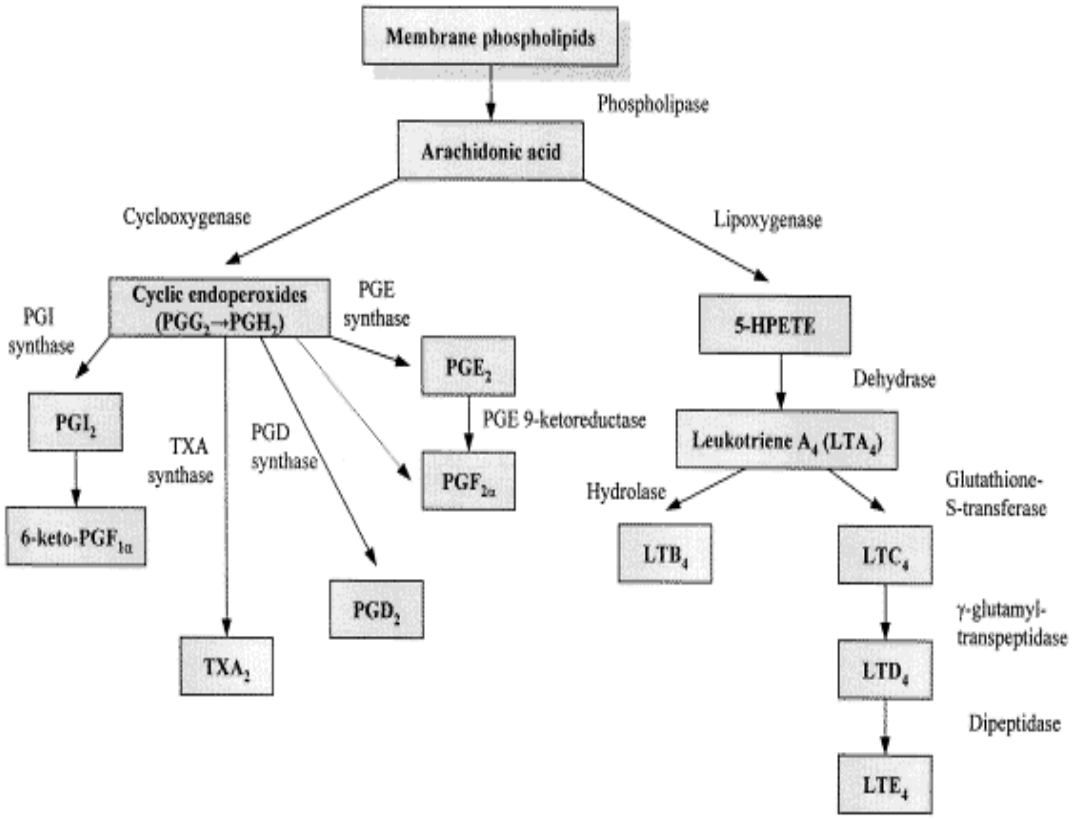


Figure .2. Pathways for the metabolism of arachidonic acid (AA)

Arachidonic acid is present mainly at the sn-2 position of cell membrane phospholipids, from where it is released primarily by cPLA2 (27), following an increase in intracellular calcium. The released AA can then be metabolized via several different pathways. In the cyclic pathway, initially AA is irreversibly converted to prostaglandin endoperoxide H_2 (PGH_2) by the enzymatic action of cyclooxygenase (COX-1 and COX-2). This is mostly the rate-limiting step in the process. The final step is the isomerization of PGH_2 to various end products, including TxA_2, PGI_2 and prostaglandins (PGE_2, $PGF_{2\alpha}$, and PGD_2). The lipoxygenase pathway synthesizes leukotrienes. AA is also metabolized by cytochrome P450 (CYP) to epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acid (HETE's) (28).

1.4. LPC receptors

Several studies suggested that LPC may act via some G-protein coupled receptors (GPCR), including GPR4, G2A, OGRI (ovarian cancer-G-protein coupled receptor 1). GPR4 has highest tissue distribution as compared to the other putative receptors. It is also well expressed in endothelial cells and its expression is enhanced by

inflammation (29). G2A and GPR4 were initially cloned in immune and tumour cells (30, 31). It was later found that G2A is expressed in human and murine macrophages within atherosclerotic lesions and may be involved in LPC mediated development of atherosclerotic lesions (32). It was also observed that G2A deficient LDL receptor knock out mice had suppressed atherosclerotic lesion formation (33). In contrast, it was shown that GPR4 and not G2A was expressed in human microvascular endothelial cells (29). Another group showed that GPR4 over-expression in rat endothelial cell- line enhanced LPC induced expression of adhesion molecules(34). Although initial papers were retracted as they failed to reproduce the binding of LPC to GPR4 and G2A respectively, there are several studies showing LPC mediated cellular activities via GPR4 (34). However, there are also some studies that failed to demonstrate the LPC mediated effects in GPR4 expressing cells such as intracellular signaling and cAMP accumulation (35). The use of specific antibodies or antagonists of GPR4 and G2A may help in elucidating their function as LPC receptors.

1.5. Endothelium mediated control of vascular tone

The term “endothelium-dependent responses” was coined after the seminal work of Robert Furchgott in 1980 (36). This elucidated the role of the endothelial cells in regulating the tone of the underlying vascular smooth muscle cells. Indeed, endothelial cells control the tone of the underlying vascular smooth muscle cells by releasing various relaxing and contracting factors and also by interacting directly with the vascular smooth muscle cells (37).

An artery consists of innermost single layer of cells called as endothelial cells. These cells line the luminal surface of blood vessels and come in direct contact with vasoactive substances. Hence, playing a primary role in the regulation of cardiovascular homeostasis that includes vascular tone control, immune and inflammatory responses and vascular remodeling (38). Endothelial dysfunction is the hallmark of cardiovascular diseases like hypertension, atherosclerosis, coronary artery disease, chronic heart failure, diabetes and chronic renal failure and has been identified as a common link for all cardiovascular disease risk factors (39). Endothelial dysfunction may be triggered by a number of factors like smoking, aging, diabetes, shear stress and oxidative stress, resulting in vascular remodeling, loss of

antithrombotic factors, increase in vasoconstrictor and prothrombotic products, along with impaired vasoreactivity (40). The maintenance of normal vascular tone is largely dependent on the capability of vascular endothelium to maintain the fine balance between endothelium-derived relaxing factors (EDRF) and endothelium-derived contracting factors (EDCF). Upon stimulation with various agonists EDRF and EDCF released from vascular endothelial cells diffuse to underlying smooth muscle cells, where they act on specific receptors and cause relaxation or contraction (41). While nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) are principal EDRF, prostanoids, the products of cyclooxygenase (COX-1) and -2, may act as both EDRF and EDCF. Endothelium-derived PGI_2 promotes relaxation of underlying vascular smooth muscle cells through activation of PGI_2- (IP) receptors (42).

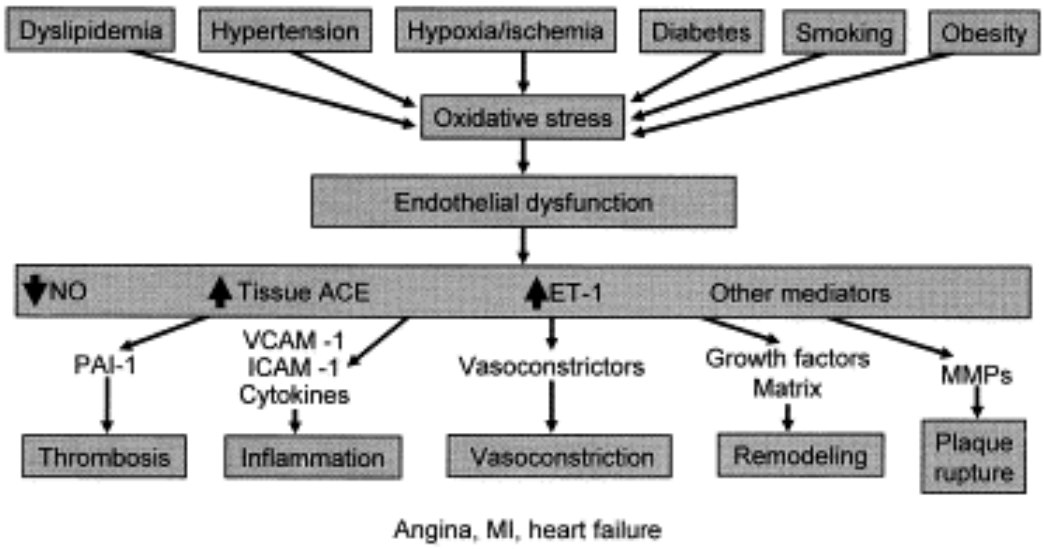


Figure .3. Risk factors leading to endothelial dysfunction

Associated risk factors of the hypertension syndrome contribute to oxidative stress, which causes endothelial dysfunction. Because the endothelium is a dynamic mediator of a wide spectrum of vasoactive substances, endothelial dysfunction can result in vascular abnormalities, leading to cardiovascular morbidity and mortality. NO = nitric oxide; ACE = angiotensin converting enzyme; ET-1 = endothelin-1; PAI-1 = plasminogen activator inhibitor; VCAM-1 = vascular cell adhesion molecule–1; ICAM-1 = intercellular adhesion molecule–1; MMP = matrix metalloproteinase; MI = myocardial infarction (43).

1.5.1. Endothelium –derived relaxing factors (EDRF)

Endothelial cells release vasoactive substances that cause arterial relaxation, which are termed as endothelium derived relaxing factors (EDRF). The most important EDRF in aortic tissues was found to be nitric oxide (NO). In the mesenteric arteries prostacyclin (PGI_2) is the more predominant EDRF. Endothelium derived hyperpolarizing factor (EDHF) is also found to be a significant EDRF in certain vascular beds (44).

1.5.1.1. Nitric oxide (NO)

NO is the most important EDRF in large arteries, responsible for about 80% of tissue relaxation. Under physiological conditions, two constitutive forms of NOS, endothelial NOS (eNOS) and neuronal NOS (nNOS) play a role in NO production. The activation of another isoform of NOS, inducible NOS (iNOS), results in excess NO production and is considered to contribute to many pathological conditions. Endothelial nitric oxide synthase (eNOS) converts L-Arginine to NO in endothelial cells. The activation of eNOS requires a number of co-factors, namely, calcium, calmodulin, nicotinamide adenine dinucleotide phosphate (NADPH) and 5,6,7,8-tetra-hydrobiopterine (BH4). Association of eNOS with caveolin-1 or glycosylation of the enzyme decreases activity. Assymetric dimethyl arginine (ADMA), a metabolite of L-arginine, competitively binds to eNOS, thus decreasing NO production. Therefore, NO is considered as a risk factor for the development of cardiovascular disease (not correct NO is protective and not risk factor) (45). After release from the endothelial cells, NO diffuses into the underlying smooth muscle cells and activates soluble guanylyl cyclase (GC), forming cyclic guanosine monophosphate (cGMP), and the subsequent activationof cGMP-dependent protein kinase G (PKG). PKG either inhibits Ca2+ influx, suppresses the sensitivity of contractile proteins to Ca2+, or phosphorylates and inactivates myosine light chain kinase thus causing the relaxation of smooth muscle (46). Basal NO-release is known to occur in both conduit and resistance vessels. NO not only promotes vascular relaxation, it also inhibits aggregation of platelets, expression of adhesion molecules on endothelial cell surface, endothelin production and smooth muscle cell proliferation, thus protecting the overall functioning of the artery wall.

1.5.1.2. PGI_2

PGI_2 is produced as one of the products of AA metabolism. Cyclo-oxygenase (COX), exists as COX-1 and COX-2 isoforms. COX metabolizes AA producing vasoactive prostanoids. Briefly, by the action of COX AA is first converted to prostaglandin G2 (PGG_2) and then prostaglandin H2 (PGH_2). These are further metabolized by individual synthases producing a number of eicosanoids of which PGI_2 is produced abundantly in mouse aorta (47). PGI_2 elicits biological functions by activating its cell-surface receptors (IP) that are G-protein-coupled to adenylate cyclase (AC) and thereby elevate cyclic adenylate monophosphate (cAMP) levels causing smooth muscle relaxation. Prostacyclin synthase is also expressed in vascular smooth muscle cells, heart, lung, kidneys, gastric mucosa and intestinal epithelial cells, brain, macrophages, oviducts and embryonic cells (48). In addition to being a vasodialator, PGI_2 is also a potent platelet aggregation inhibitor. PGI_2 has numerous other physiological functions. It is involved in overall renal function by regulating renal blood flow, glomerular filtration rate and renin release. PGI_2 is also involved in the development, transport and implantation of the embryo, in pain tolerance, in gastric acid secretion and regulating gene transcription (49, 50). PGI_2 analogues are currently being used to treat pulmonary hypertension (51), and are likely to be effective in the management of atherothrombosis. In recent studies in hypertensive rats, COX-1 derived PGI_2 has been implicated to cause endothelial dysfunction (52). In the aorta of spontaneously hypertensive rats (SHR) PGI_2 causes contraction and not relaxation. This impaired response to PGI_2 starts at a very early age, approximately 12 weeks. It is hypothesized that this impaired relaxation to PGI_2 may be due to decreased aortic expression of IP receptors or impairment in adenylyl cyclase (53). Another hypothesis that needs validation could be the oxidative damage of IP receptors (54). Endothelium-dependent contraction and PGI_2- dependent contraction, both involve the activation of TP receptors (53). PGI_2 has also been found to produce vasoconstriction in normal arteries from pigs and mice, by activating TP receptors (55). Which factors trigger the proconstricting activity of PGI_2 needs to be studied more in detail to get a better understanding of this prostanoid.

1.5.1.3. EDHF

Apart from NO and PGI_2 which are the known and well characterized vasodialators, there exist other pathways by which smooth muscle cells undergo hyperpolarization to produce tissue relaxation. These additional pathways were all grouped into a single term EDHF. In general, EDHF-mediated responses consist of increase in intracellular calcium concentration, followed by opening of calcium-activated potassium channels of small and intermediate conductance and hyperpolarization of endothelial cells leading to hyperpolarization of underlying smooth muscle cells. This could be evoked by direct electrical coupling by myo-endothelial junctions or also by accumulation of potassium ions in the intercellular space. AA metabolites can also produce hyperpolarization of SMC. In some blood vessels, including coronary arteries, the endothelial cells produce AA-metabolites derived from cytochrome P450 monooxygenases. The epoxyeicosatrienoic acids (EET) can hyperpolarize SMC by activating calcium –activated potassium channels (56). EETs might act as both intracellular and extracellular messengers. EETs affect Ca2+ signalling, the Ca2+ sensitivity of KCa channels and the generation of cAMP by AC, as well as gap junctional coupling in the endothelial cells. EETs and/or their metabolites can also diffuse to SMCs and activate large-conductance KCa channels (BKCa channels) (57). The endothelium also produces lipoxygenases derivatives or hydrogen peroxide to produce SMC hyperpolarization. These different mechanisms may not be mutually exclusive and can occur simultaneously.

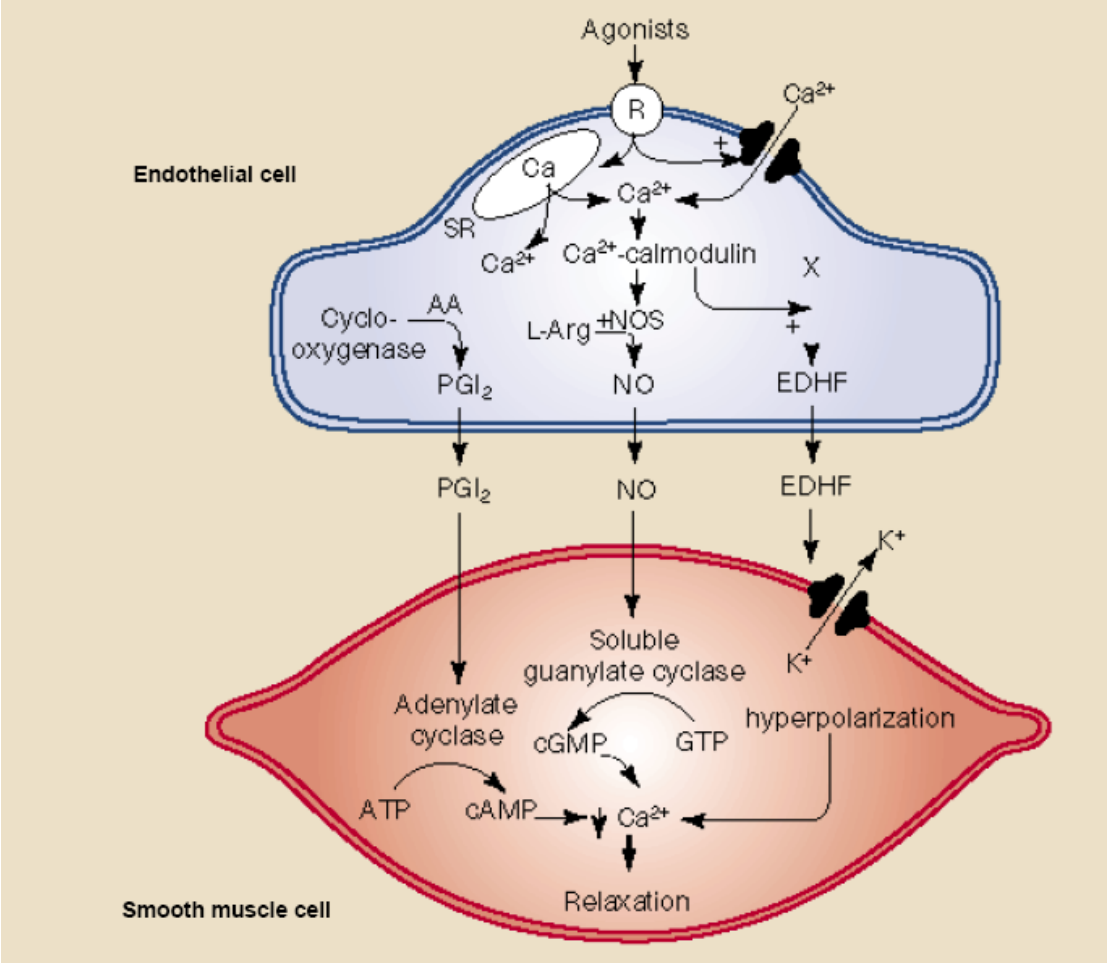


Figure .4. Relaxation of vascular smooth muscle cells by diffusable vasodilator substances from endothelial cells

Nitric oxide (NO) activates soluble guanylate cyclase, yielding increased levels of cyclic GMP (cGMP). Prostacyclin (PGI_2) activates adenylate cyclase, leading to increased production of cyclic AMP (cAMP). Endothelium-derived hyperpolarizing factor (EDHF) causes Ca2+-dependent K+ channels in vascular smooth muscle cells to open, leading to their hyperpolarization. AA, arachidonic acid; NOS, NO synthase; L-Arg, L-arginine; R, membrane receptor; SR, sarcoplasmic reticulum; X, unknown precursor. Adapted from (58).

1.5.2. Endothelium derived contracting factors

1.5.2.1. Endothelin-1

Cultured endothelial cells were found to synthesize and secrete a vasoconstrictor peptide named endothelin-1 (ET-1), which was later identified as one of the most potent vasoconstrictors (59). There are three known isoforms of endothelin ET-1, ET-2, ET-3. Endothelins interact with two G-protein coupled receptors, ET_A and ET_B. ET-1 is the preferred substrate for the ET_A receptor subtype (60). ET-1 is a vasoactive peptide, but it also has other atherogenic functions. It is involved in enhancing mitogenesis, inducing extracellular matrix formation and contributing to the formation of inflammation in the vessel wall (61). ET_A and ET_B receptors are localized in the vascular smooth muscle cells where they induce their vasoconstrictive, proliferative and hypertrophic actions. ET_A is the most predominant vasoconstrictor receptor in arteries (62). The contractions elicited by ET_A receptor activation develop slowly but last longer and cause contraction even after washing out the peptide. This could be because of the almost irreversible binding of the peptide to the receptor and persistent association even after internalization. The signal transduction following ET_A receptor stimulation involves several G-protein-dependent and -independent pathways, including phospholipase C, phospholipase A_2, adenylate cyclase, Rho kinase, transactivation of receptor tyrosine kinases, beta-arrestin and mitogen activated protein kinase cascades. These pathways contribute to the increase in $[Ca^{2+}]_i$, linked to facilitation of calcium influx and calcium mobilization, and the changes in calcium sensitivity, essential for ET-1-induced contraction of vascular smooth muscle cells (63). ET-1 is stored in storage vesicles in endothelial cells. The constitutive release of ET-1 helps to maintain basal endothelial tone and hence blood pressure as observed in genetically modified mice (64). In normotensive human beings too it contributes to maintaining endothelial tone. Never the less, it has been demonstrated that in response to thrombin, endothelial cells can produce an acute synthesis and release of ET-1 in aortic rings of aging rats, eventually contributing to the contraction of underlying smooth muscle cells (65).

1.5.2.2. Angiotensin II

The renin-angiotensin system (RAS) consists of renin derived by the kidney, which cleaves angiotensinogen produced by the liver to form angiotensin I in the circulation.

Furthermore, angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II, which is the main effector peptide of RAS. Angiotensin II exerts its effects by activating angiotensin II receptors, AT_1 and AT_2. AT_1 receptor subtype is localized in smooth muscle cells. Angiotensin II mediates vasoconstriction by binding to AT_1 receptors. These vasoactive responses can partly be counteracted by the AT_2 receptor, which causes vasodilatation in some vascular beds of rats and human (66) (67). However, nearly 25 years ago, it was suggested that, besides the circulating RAS, angiotensin II could be produced locally by a vascular RAS (68). In isolated arteries, a renin substrate could be metabolized to form angiotensin II, however, whether or not endothelial cells express renin still remains to be determined. In addition to this angiotensin II generation may occur on endothelial cell surface as well as within the cells. Hence, angiotensin II could be involved in the autocrine and paracrine control of vascular resistance (69).

1.5.2.3. Thromboxane A2 (TXA_2)

TXA_2 is enzymatically produced from PGH_2 as a substrate by a specific synthase, the thromboxane synthase (70). TXA_2 elicits diverse pathophysiological reactions, like platelet aggregation, as well as contraction and proliferation of vascular smooth muscle. Additionally, thromboxane A_2 is involved in allergies, modulation of acquired immunity, atherogenesis, neovascularization and metastasis of cancer cells (71). TXA_2 acts via TP receptors which are known to couple to at least nine different G proteins, which in turn activate numerous downstream effectors, including second messenger systems such as inositol trisphosphate (IP3)/ diacylglycerol (DAG), cAMP, small G proteins (Ras, Rho), PI3 kinase, as well as PKC and PKA (72). Mice in which TP receptors are deleted are normotensive but do not show normal vascular response to TXA_2 . They also have an increased tendency to bleed (73). Depletion of TP receptors also delays atherogenesis in $apoE^{-/-}$ mice and also prevents angiotensin II and L-NAME-induced hypertension and cardiac hypertrophy. The TXA_2 synthase knock out mice do not have such a prominent phenotype. This can be explained by the fact that TXA_2 is just one of the agonists for TP receptor and that deletion of this enzyme could redirect the AA pathway to a less atherogenic synthases (37). TP receptors can be involved in cross talks with receptor tyrosine kinases, such as the EGF receptor, to induce cell proliferation and differentiation as well as with the IP

receptor. PGH_2 is a potent agonist of TP receptor and higher concentrations of other prostaglandins, isoprostanes and hydroxyeicosatetraenoic acids can also activate it with various ranges of potency (74). Reactive oxygen species play a role by increasing the density and stability of functional TP receptors at the cell membrane. In endothelial cells, the activation of TP receptors inhibits NO production (75). The generation of harmful eicosanoids, the post-transcriptional stabilization of TP receptors and the decreased production of NO are factors that work together leading to endothelial dysfunction. Taken together, experimental evidence indicates that TP receptors are likely to play a pivotal role in cardiovascular diseases (75).

1.5.2.4. Reactive oxygen species (ROS)

ROS production by reduction-oxidation reactions is important chemical processes that control signal transduction. Increased ROS may be a risk factor for cardiovascular events and hence, understanding the biological processes that generate ROS and the intracellular signals induced by ROS is important to understand the pathogenesis of diseases. ROS consists of free radicals as well as species like H_2O_2, that act as oxidants. Free radicals include, O_2^- (superoxide anion), ·OH (hydroxyl radical) and ·NO (nitric oxide). Mitochondria, endoplasmic reticulum, and nuclear membranes produce superoxide as a by-product due to oxidative phosphorylation. Another important source of ROS are the metabolic by-products of AA metabolism by cyclooxygenase, lipoxygenase, and cytochrome p450 mono-oxygenase. Two other O_2^--generating enzyme systems are xanthine oxidase and NADH/NADPH oxidase. Xanthine dehydrogenase is converted to xanthine oxidase and mediates metabolism of nucleotides such as xanthine to form O_2^-, H_2O_2, and uric acid. NADH/NADPH oxidase may be the major regulated source of O_2^- production in many tissues. Dismutation of O_2^- spontaneously or enzymatically by superoxide dismutase produces H_2O_2, which is scavenged either by catalase or by peroxidases. Superoxide and H_2O_2can also undergo further reactions with each other or with iron-containing molecules (Haber-Weiss or Fenton reaction) to generate the highly reactive hydroxyl radical (·OH) (76). On the other hand, ROS directly causes vasoconstriction by releasing isoprostanes, which are produced from AA via a free radical catalyzed-COX-independent mechanism (77). Isoprostanes are recognized as an indicator of oxidative stress. Isoprostanes act as partial agonists at the TP receptor and exert

numerous biological effects, including vasoconstriction and platelet activation (78). ROS also favor macrophage infiltration of the vessel wall, and SMC proliferation, migration, and apoptosis.

In aortic vessel experiments it was found that increased oxidation products enhanced atherosclerosis and restenosis. This was observed in aortas from hyperlipidemic rabbits and coronary arteries of balloon-injured pigs, which produced higher levels of O2- as compared to control vessels. Increased ROS is always associated with decreased NO bioavailability. Hence, increase in ROS causes diminished endothelium-dependent relaxation, and antioxidant treatment has been shown to improve such responses in vitro and in vivo (79). However, the use of antioxidants as therapy has not been successful so far.

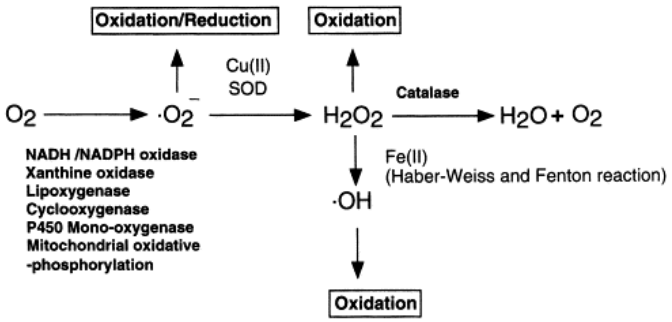


Figure .5. Sources of reactive oxygen species (ROS) produced endogenously by the vessel wall and key metabolic pathways for these species

Multiple enzymes may stimulate ROS production in vascular smooth muscle cells (VSMC) and endothelial cells. These include NADH/NADPH oxidase, xanthine oxidase, lipoxygenases, cyclo-oxygenase, p450 mono-oxygenases, and the enzymes of mitochondrial oxidative phosphorylation. ($\cdot O_2$, superoxide anion; H_2O_2, hydrogen peroxide; $\cdot OH^-$, hydroxyl radical; SOD, superoxide dismutase. Adapted from (76).

1.6. LPC and Endothelial dysfunction

The endothelium controls vascular smooth muscle tone by controlled release of vasodialators and vasoconstrictors. It has been experimentally shown that LPC inhibits EDR. Pretreatment of tissues with LPC strongly attenuates Ach mediated relaxation. It was also reported that LPC produced further contraction, in addition to the contraction produced by PGF2 in pig coronary artery. It was suggested that this could be due to LPC induced inhibition of NO release (80). In rat mesenteric arteries, LPC inhibits hyperpolarization and relaxation mediated by EDHF (12). All these results suggest that LPC may cause endothelial dysfunction by reduced production or impaired signaling of EDRF. Other reports suggest that LPC also cause intervention at various endothelial-signaling processes. For example, LPC disrupts arginine uptake by impairing high-affinity arginine uptake in endothelial cells (81). It can also block G-protein dependent signal-transduction pathways by selective uncoupling of G-protein from its receptor (82). LPC has been found to stimulate Phospholipase D (PLD) activity, which is related to inhibition of EDR (83). LPC may also affect endothelial function by activating PKC pathway, leading to vasoconstriction of tissue when treated with agonists like phenylephrine or thrombin. PKC activation can also induce ROS production. LPC can also activate NAD(P)H oxidase, enhancing ROS production (18). The direct vascular effects of LPC have been studied in isolated vessel strips ex vivo and in vivo. On incubating tissues within the physiological and pathophysiological concentration range, LPC on its own produced no significant change on vascular tone. In addition, LPC does not alter the vasoconstriction resulting from K+-induced direct depolarization of vascular smooth muscle. It was concluded from a number of studies that LPC potentiates receptor-operated vasodilator and vasoconstrictor responsiveness in conduit arteries. For instance, in large artery ring preparations (from rats, rabbits, cattle, and pigs), LPC impairs EDR (12, 84), probably by inhibiting EDHF and NO. Some agonist-induced contractions in isolated blood vessels were also potentiated by LPC, including contractile responses induced by UK14(5-bromo-N-(2-imidazolin-2- yl)-6-quinoxalinamine, an **α**2-adrenoceptor agonist), and Ag II (85). Further studies are needed to elucidate the mechanisms leading to LPC mediated endothelial dysfunction.

1.7. Rational for the Hypothesis

Based on results obtained from various studies that LPC produces altered vascular reactivity, as well as the fact the LPC levels are found to be elevated in various cardiovascular diseases, it is reasonable to assume that LPC is an important mediator that regulates the altered vascular tone that is associated with these diseases. Although the role of LPC in various inflammatory conditions had been extensively studied, the contribution of LPC in regulating vascular tone has not been completely elucidated. Previously we found that the capacity and underlying mechanisms of palmitoyl-LPC (16:0 LPC), oleoyl-LPC (18:1 LPC), linoleoyl-LPC (18:2 LPC) and arachidonoyl-LPC (20:4 LPC) to modulate endothelial prostanoid production were remarkably different and related to the acyl-chain length as well as degree of saturation. At present only the impact of 16:0 LPC on vascular reactivity has been investigated. Here we tested the hypothesis of acyl chain dependency of LPC in altering vascular reactivity. To this end we compared the effects of LPC 18:1, 18:2 and 20:4 with LPC 16:0 on ACh-induced vasorelaxation in an ex vivo system using mouse aortic rings and myography. We found that the tested LPC attenuate ACh-induced relaxation through induction of proconstricting prostanoids and superoxide anions whereby the potency of attenuating relaxation and the relative contribution of underlying mechanisms are strongly related to LPC acyl-chain length and degree of saturation.

2. Materials and Methods

2.1. LPC

LPC 16:0, 18:1, 18:2 and 20:4 were purchased from Avanti Polar Lipids, Alabaster, AL or prepared as described (13). LPC were dissolved in chloroform/methanol and stored at -20°C under argon atmosphere. Required amounts of LPC were dried under a stream of nitrogen or argon and re-dissolved in PBS (pH7.4) before the experiment.

2.2. Mice and tissue preparation

Mice received care in accordance with the Austrian law on experimentation with laboratory animals, which is based on the U.S. National Institutes of Health guidelines. Male C57BL/6 mice (10-12 weeks old) provided by Himberg, Austria, were killed by cervical dislocation. The descending thoracic aorta was isolated and dissected free of adherent tissue.

2.3. Organ Chamber Experiments

Aortic rings approximately 2 mm in length were cut from descending thoracic aorta. The arterial rings were positioned in small wire myograph chambers (Danish MyoTechnology, Aarhus, Denmark), which contained Physiological salt solution (PSS) (114 mM NaCl, 4.7 mM KCl, 0.8 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11 mM D-glucose pH 7.4) aerated with 5% CO_2/95% O_2 at 37°C. The myograph chambers were connected to force transducers for isometric tension recording (PowerLab, ADInstruments). The rings were heated in PSS buffer to 37°C. An initial preload of 10 mN was applied, and the rings were allowed to stabilize for 30 min. PSS containing 60 mM KCl was used to determine maximum contractility of the tissue. When the developed tension attained its peak value, the rings were relaxed by rinsing with the buffer. Next, the rings were pre-contracted with increasing concentrations of norepinephrine (NE) (1 nM – 0.3 μM) (Sigma-Aldrich) to produce 80% of the maximum contraction achieved by 60 mM KCl, followed by endothelium-dependent relaxation to cumulatively increasing concentrations of acetylcholine chloride (ACh) (1 nM – 0.3 μM) (Sigma-Aldrich). After washout and equilibration, the rings were preincubated with 10 μM LPC in the presence or absence of inhibitors for 30 minutes, followed by contraction (NE) – relaxation (ACh) cycle as described above. Relaxation values were expressed as a percentage of the NE-induced

contraction. The endothelium-independent relaxation was examined by exposure of rings to increasing concentrations (0.1 nM to 30 nM) of sodium nitroprusside (SNP) (Sigma-Aldrich), a nitric oxide (NO)-donor.

2.4. Pharmacological inhibitors

The pharmacological inhibitors used included indomethacin (non-selective COX inhibitor; 20 μM), N5-[imino(nitroamino)methyl]-L-ornithine (L-NNA; 200 μM) (eNOS inhibitor; 200 μM), Tempol (superoxide dismutase (SOD)- mimetic; 200 μM), SQ29548 (TXA_2 receptor antagonist; 10 μM), Furegrelate (TXA_2 synthase inhibitor; 10 μM), Tranylcypromine (PGI_2 synthase inhibitor; 10 μM), Diethyldithiocarbamic acid diethylammonium salt (DETCA) (SOD inhibitor; 10 μM) and TIRON (superoxide ion scavenger; 100 μM). All inhibitors except Furegrelate (Cayman Chemicals, Ann Arbor, Michigan, USA) were from Sigma (Saint Louis, MO). Stock solutions (10 mM) of Indomethacin and Tranylcypromine were made in dimethylsulfoxide (DMSO) and further diluted with distilled water. The stock solution (10 mM) of SQ29548 was prepared in ethanol and further diluted with distilled water. All other drugs were dissolved in distilled water.

2.5. Prostanoid measurement

Mouse aortic rings (approximately 2-mm in length) were incubated in 200 μl aerated PSS under cell culture conditions at 37°C for 1 h. Thereafter, buffer was replaced with fresh PSS supplemented with PSS (control) or LPC (10 μM) followed by further incubation under cell culture conditions at 37°C for 1 h. The buffers were flash frozen in liquid nitrogen for subsequent prostanoid quantification and rings were homogenized for protein quantification. Protein concentration was determined with the BCA protein assay kit (Novagen, Darmstadt, Germany). The concentrations of 6-Keto-$PGF_{1\alpha}$, TXB_2, PGE_2 and $PGF_{2\alpha}$ were measured by corresponding correlate-EIA kits (Cayman, Ann Arbor, MI) according to the manufacturer's protocol.

2.6. Superoxide anions measurement

Superoxide anions were measured as described (86) with some modifications. Mouse aortic rings were equilibrated in 100 μl PSS buffer containing 10 μM DETCA, an

SOD-inhibitor and 10 µM lucigenin (Sigma) at 37°C for 30 minutes. LPC (10 µM) was added to the tubes immediately before measurements. The luminometer (Lumat LB9501, Berthold technologies, Germany) was set up to report arbitrary units of emitted light (RLU). Measurements were taken in triplicates every 10 seconds. In addition, blank measurements with and without aortic rings were collected in the same way to subtract background emission. The amounts of released superoxide anions (chemiluminiscence units) were normalised to protein content of respective aortic rings. The RLU obtained in control incubations with PSS were set to 100% and the RLU obtained by LPC's were expressed as percentage of the control.

2.7. Nitrite determination

Nitrite as an indicator of NO production was determined according to a previously described fluorometric HPLC method (87) utilizing the reaction of nitrite with 2,3-diaminonaphthalene (DAN). In brief, the nitrite levels were determined in the myography incubation buffers. Samples (500 µl) were taken and snap frozen in liquid nitrogen. After thawing 100 µL of the sample (incubation buffer) was incubated at 24°C with 10 µL of 316 µmol/L DAN (in 0.62 mol/L HCl) for 10 min, followed by addition of 10 µL of 2.8 mol/L NaOH. This reaction mixture was directly used for chromatographic separation (injection volume: 20 µL) of the formed 2, 3-naphthotriazole (NAT). Nitrite standards (range: 0 – 2 µmol/L) were derivatized accordingly. NAT was isocratically separated on a 5-µm ODS hypersil column (150 x 4.6 mm) guarded by a 5-µm ODS hypersil column (10 x 4.6 mm; Uniguard holder) with a 30 mmol/L sodium phosphate buffer (pH 7.5) containing 50% methanol (flow rate: 0.8 mL/min). Fluorescence was monitored at an excitation wavelength of 375 nm and an emission wavelength of 415 nm. The HPLC apparatus consisted of an L-2200 autosampler, L-2130 HTA pump and L-2480 fluorescence detector (VWR Hitachi, Tokyo, Japan). Detector signals were recorded with a personal computer. The EZchrom Elite (Scientific Software Inc., San Ramon, CA USA) was used for data requisition and analysis. The detection limit for nitrite was 10 pmol/mL.

Statistical Analysis

EC50 values (the ACh concentrations required to achieve 50% of maximal relaxation) are expressed as mean with 95% confidence intervals. Data are otherwise expressed

as mean ± SEM. The significance of the difference between group means was analyzed by two-way analysis of variance and the Bonferroni-post test for samples. For prostanoid, nitrite and ROS measurements control and LPC treated aortic rings were compared by student's T-test. Values of $P<0.05$ (*), $P< 0.01$ (**), and $P< 0.001$ (***) were taken as statistically significant. Statistical analysis was performed by Prism Version 4.0 (GraphPad Software, USA).

3. Results

3.1. LPC impair ACh-induced endothelium-dependent relaxation

All tested LPC evoked impairment of aortic ring relaxation to cumulatively increasing concentrations of ACh with rank order of potency as follows: 18:2>20:4>16:0>18:1 (Fig. 1). EC_{50} values for LPC 18:2, 20:4, 16:0 and 18:1 were 354 nM (261-479), 298 nM (222-401), 214 nM (143-314) and 115 nM (86.2-155), respectively. The subsequent relaxation of the same rings to SNP, following precontraction with NE, was not affected by either of the tested LPC, indicating that responsiveness of aortic smooth muscle layers to NO was not impaired by prior exposure to LPC.

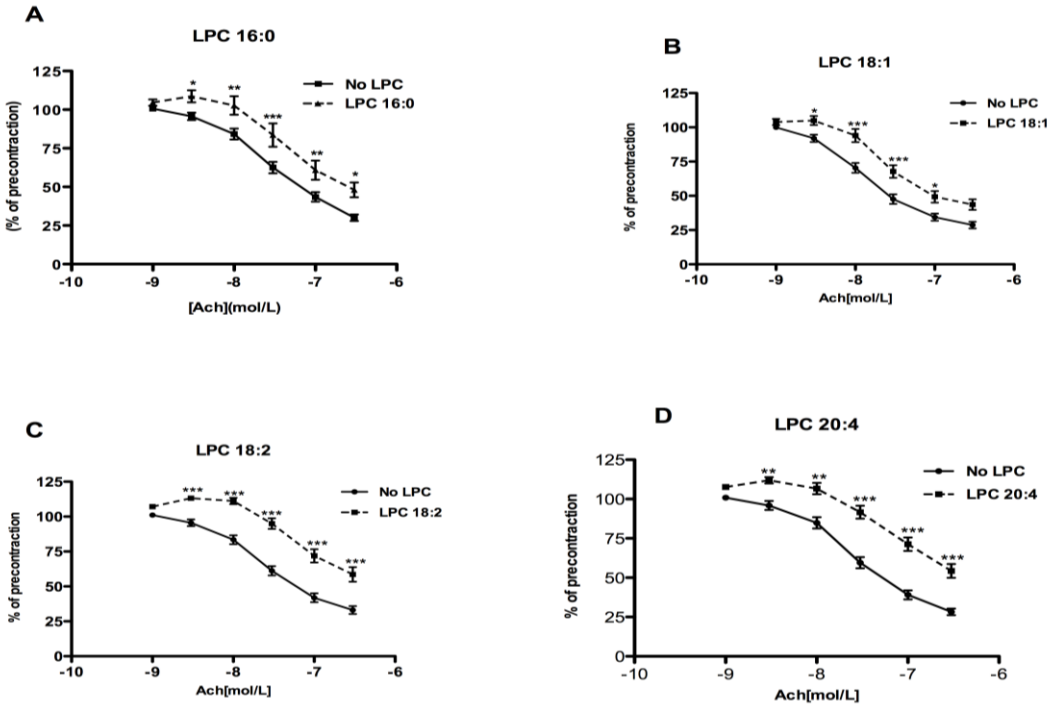


Figure. 6. ACh-induced relaxation of mouse aortic rings is impaired by LPC

The rings were preincubated without (no LPC) or with 10 μM LPC 16:0 (A), 18:1 (B), 18:2 (C) or 20:4 (D) for 30 minutes, followed by precontraction with NE and cumulative addition of ACh. Relaxation values were expressed as a percentage of the NE-induced contraction. Results of each experimental condition are mean ± SEM of 24 rings for each case from 6 mice. *P<0.05, **P<0.01***P<0.001.

3.2 SNP induced smooth muscle relaxation is not affected by LPC treatment

LPC was found to cause impairment of endothelium mediated tissue relaxation as observed on relaxing the tissue with ACh. To test weather the impaired relaxation was due to direct effect of LPC on smooth muscle cells we used SNP as a vasorelaxing agent. SNP is a nitric oxide donor that leads to smooth muscle relaxation even in the absence of a functional endothelium.

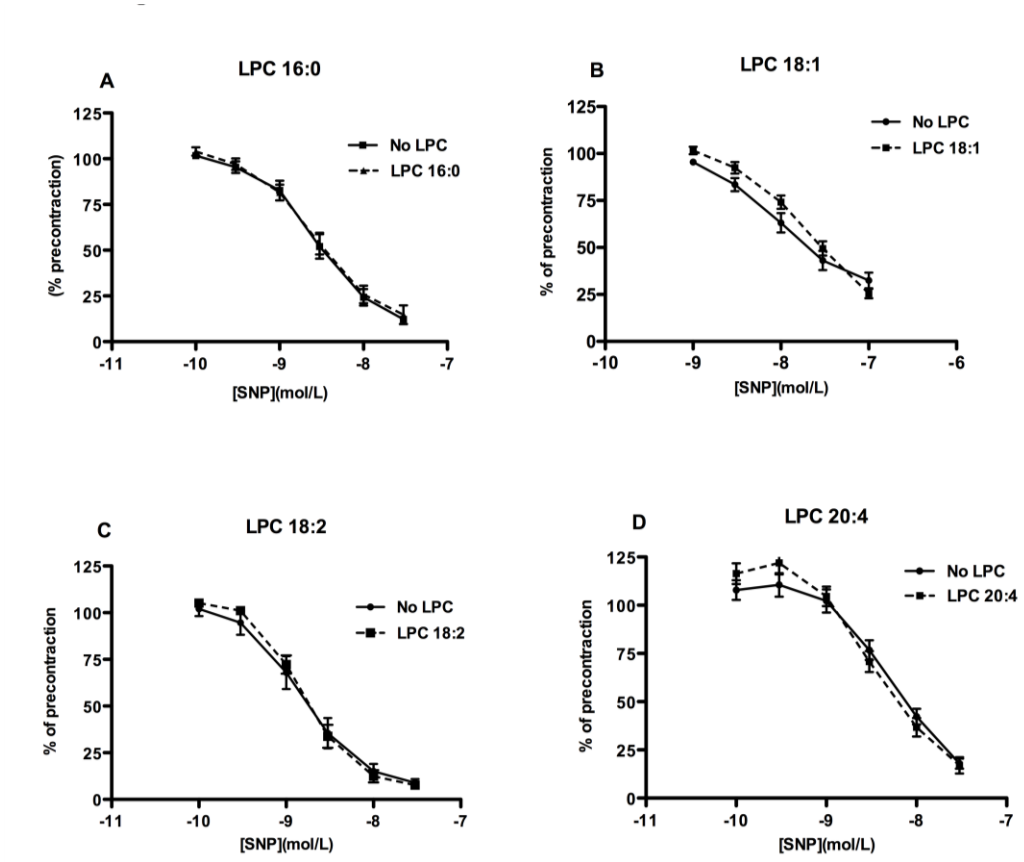


Figure. 7. SNP-induced relaxation is not affected by prior exposure of rings to LPC

The rings were preincubated without (no LPC) or with 10 μM LPC 16:0 (A), 18:1 (B), 18:2 (C) or 20:4 (D) for 30 minutes, followed by precontraction with NE and cumulative addition of ACh. Rings were rinsed thoroughly with PSS. Thereafter, the rings were precontracted with NE, followed by cumulative addition of SNP (0.1nM to 30nM). Relaxation values were expressed as a percentage of the NE-induced contraction. Results of each experimental condition are mean ± SEM of 16 rings for each case from 4 mice.

3.3. Impact of L-NNA and indomethacin on Ach-induced relaxation

Nitric oxide being the major vasodialator in main conduit arteries, we tested the impact of L-NNA on our mouse aortic tissues. Pre-treating tissues with L-NNA led to almost complete abolition of tissue relaxation when treated with ACh. This indicated that NO is the major contributor to tissue relaxation. In another separate experiment we tested the impact of indomethacin on ACh mediated tissue relaxation and found no effect of indomethacin on tissue relaxation.

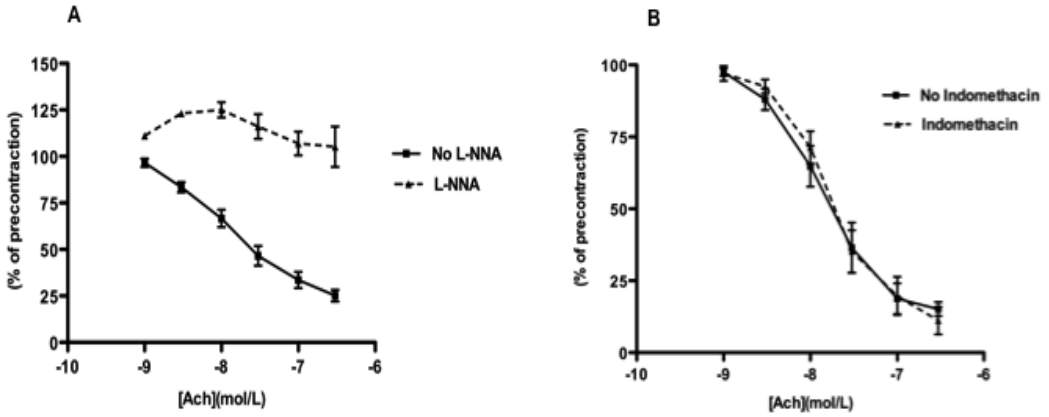


Figure. 8. Impact of L-NNA and indomethacin on ACh-induced relaxation

The rings precontracted with NE were relaxed by a cumulative addition of ACh in the absence (no L-NNA) or presence of 200 μM L-NNA (A) or 20μM indomethacin (B). Relaxation values were expressed as a percentage of the NE-induced contraction. Results are mean ± SEM of 8 rings for each case from 4 mice.

3.4. The role of COX in LPC- mediated impairment of vascular relaxation

Considering the capacity of LPC to supply COX enzymes with arachidonic acid (4), we assumed that COX-derived vasoconstricting prostanoids might be responsible for the observed LPC-induced impairment of relaxation. To test our assumption we examined the effect of LPC on vasorelaxation in the absence or presence of the non-selective COX inhibitor, indomethacin. Ach relaxation impaired by LPC 16:0 was exaggerated by indomethacin (Fig. 9A). Similarly, tranylcypromine, a PGI_2 synthase inhibitor exaggerated LPC 16:0-induced impairment of relaxation without reaching statistical significance (Data not shown). An IP receptor antagonist CAY10441also caused significant exaggeration of LPC 16:0 mediated impaired tissue relaxation (Fig.

9B). Indomethacin improved the impairment of relaxation caused by LPC 18:2 (Fig. 9C) and 20:4 (Fig. 9D), respectively, but had no effect on the impairment caused by LPC 18:1 (Fig.10).

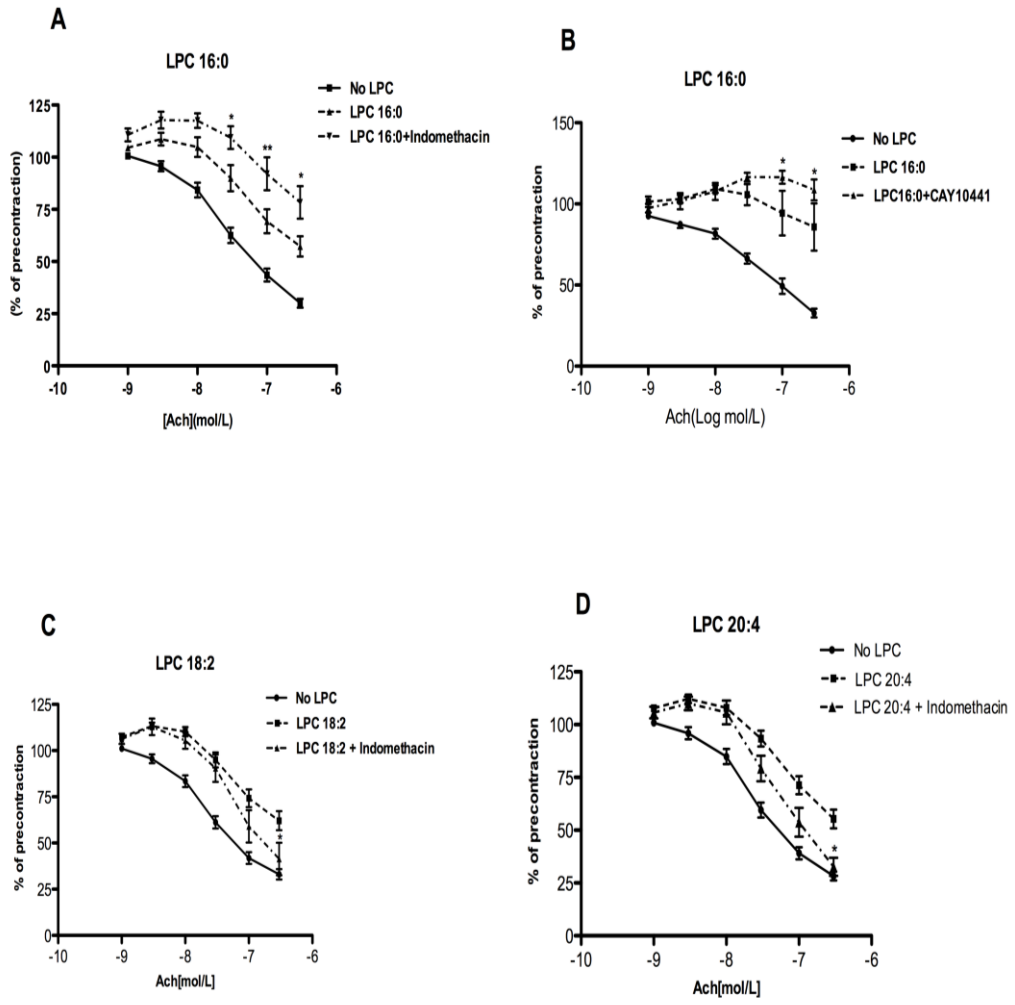


Figure. 9. Inhibition of COX improves relaxation impaired by LPC 18:2 and 20:4

The rings were preincubated without (no LPC) or with 10 μM LPC 18:2 (C) or 20:4 (D) in the absence or presence of indomethacin (20 μM) for 30 minutes, followed by precontraction with NE and cumulative addition of ACh. Results are mean ± SEM of 12 rings for each case from 6 mice. *P<0.05

3.5. No impact of Indomethacin on LPC 18:1-mediated impairment of tissue relaxation

Pre-treatment of LPC 18:1 treated tissues with Indomethacin did not produce any change in LPC18:1 mediated impaired tissue relaxation (Fig. 5) This indicated that LPC 18:1 did not cause impaired relaxation by activating the production of any proconstricting eicosanoids via COX-2 activation.

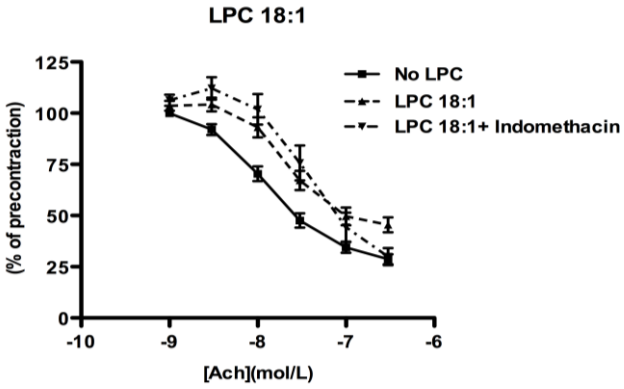


Figure. 10. No impact of Indomethacin on LPC 18:1-mediated impairment of tissue relaxation

The rings were preincubated without (no LPC) or with 10 μM LPC 18:1 in the absence or presence of indomethacin (20 μM) for 30 minutes, followed by precontraction with NE and cumulative addition of ACh. Results are mean ± SEM of 9 rings for each case from 3 mice. *P<0.05

3.6. TP receptors are involved in LPC 18:2- and 20:4- mediated impairment of vascular relaxation

Considering the well-established importance of TP receptors in mediating endothelium-dependent contractions (88, 89), we examined whether SQ29548, a TP receptor antagonist could attenuate the inhibitory effect of LPC 18:2 and 20:4 on vasorelaxation. As shown in (Fig. 11A), SQ29548 markedly attenuated the inhibitory

effect of LPC 18:2, yielding relaxation similar to that obtained in the absence of LPC. The relaxation impairment caused by LPC 20:4 was also improved by SQ29548 (Fig. 6B). However, the effect was less pronounced as compared with LPC 18:2.

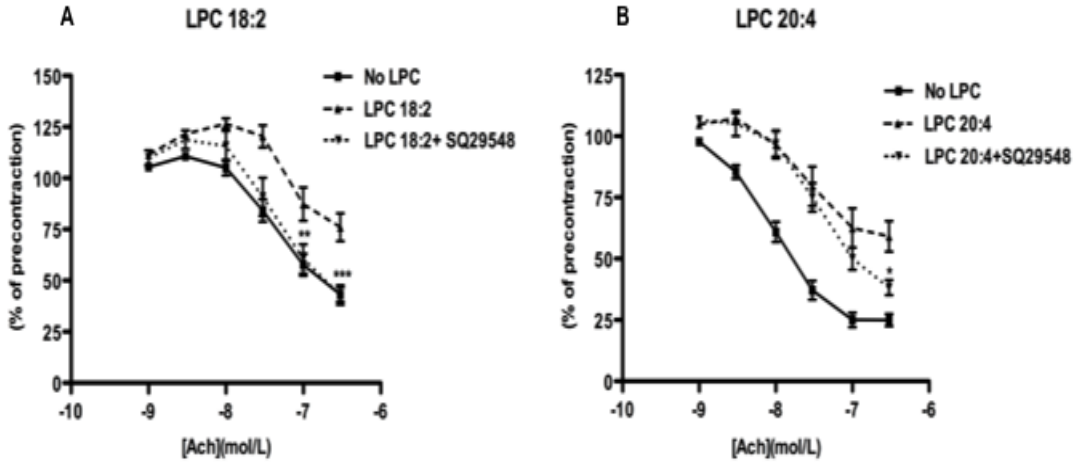


Figure.11. Blocking of TP receptor improves LPC 18:2- and 20:4-induced impairment of relaxation
The rings were preincubated without (no LPC) or with 10 μM LPC 18:2 (A) or 20:4 (B) in the absence or presence of 10 μM SQ29548 for 30 minutes, followed by precontraction with NE and cumulative addition of ACh. Results are mean ± SEM of 12 rings from 6 mice. *P<0.05, **P<0.01***P<0.001.

3.7. The role of TXA_2 and PGI_2 in LPC 18:2- and 20:4- mediated impairment of vascular relaxation

Since TP receptors can be activated by both TXA_2 and PGI_2 (90, 91), we tested the involvement of both prostanoids in LPC 18:2- and 20:4-induced impairment of relaxation. Relaxation impaired by LPC 20:4 was markedly improved upon inhibition of TXA_2-synthase by furegrelate (Fig. 12A) as well as upon inhibition of PGI_2-synthase by tranylcypromine (Fig. 12B). The combination of both inhibitors yielded further improvement of relaxation (Fig. 12C) when compared with situations in the presence of single inhibitors.

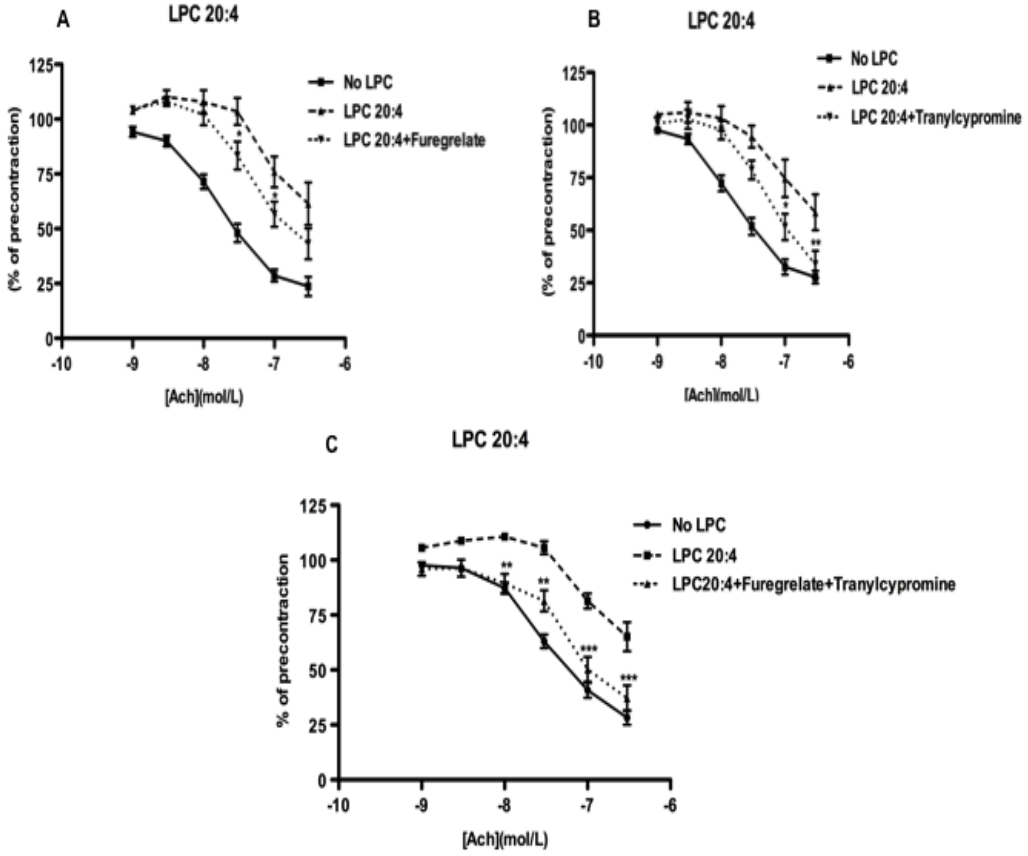


Figure. 12. Inhibition of TXA_2- and PGI_2- synthase improves relaxation impaired by LPC 20:4

The rings were preincubated without (no LPC) or with LPC 20:4 in the absence or presence of 10 μM furegrelate (A) or 10 μM tranylcypromine (B) or in the presence of both inhibitors (C) for 30 minutes, followed by precontraction with NE and cumulative addition of ACh. Results are mean ± SEM of 12 rings from 6 mice. *P<0.05, **P<0.01.

3.8. Furegrelate and tranylcypromine fail to recover relaxation induced by LPC 18:2

LPC 18:2 was found to produce impaired tissue relaxation by activating TP receptors as observed when tissues were treated with SQ29548. But when treated with Furegrelate or Tranylcypromine no improvement in relaxation was observed. This suggests that apart from TXA2 and PGI2 some other prostanoid may be acting via the TP receptors and causing impaired vasorelaxation in case of LPC 18:2.

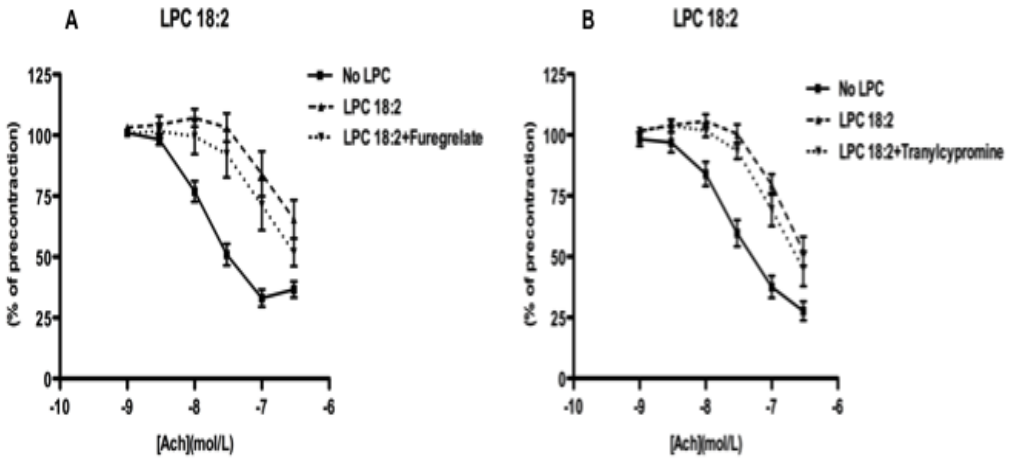


Figure. 13. Furegrelate and tranylcypromine fail to recover relaxation induced by LPC 18:2

The rings were preincubated without (no LPC) or with LPC 18:2 in the absence or presence of 10 μM furegrelate (A) or 10 μM tranylcypromine (B) for 30 minutes, followed by precontraction with NE and cumulative addition of ACh. Relaxation values were expressed as a percentage of the NE-induced contraction. Results are mean ± SEM of 12 rings for each case from 6 mice.

3.9. Prostanoid release from LPC-treated aortic rings

To examine whether the production of prostanoids implicated in LPC-induced impairment of relaxation (Figs. 1-8) was increased by LPC, we measured concentrations of prostanoids produced and secreted by aortic rings upon incubation with LPC. Compared with PSS-treated control incubations, LPC 20:4 was the most potent inducer of PGI_2 production (measured as 6-Keto $PGF_{1\alpha}$, a stable degradation product of PGI_2), followed by 18:2 and 16:0 (Fig 14A). The effect of LPC 18:1 concerning prostanoids did not reach statistical significance (Fig 14A). The release of TXB_2 was significantly increased only with LPC 20:4 (Fig. 14B). The levels of $PGF_{2\alpha}$ were significantly increased with LPC 20:4 and 18:2, respectively (Fig. 14C), whereas PGE_2 production was significantly increased only upon incubation with LPC 20:4 (Fig. 14D).

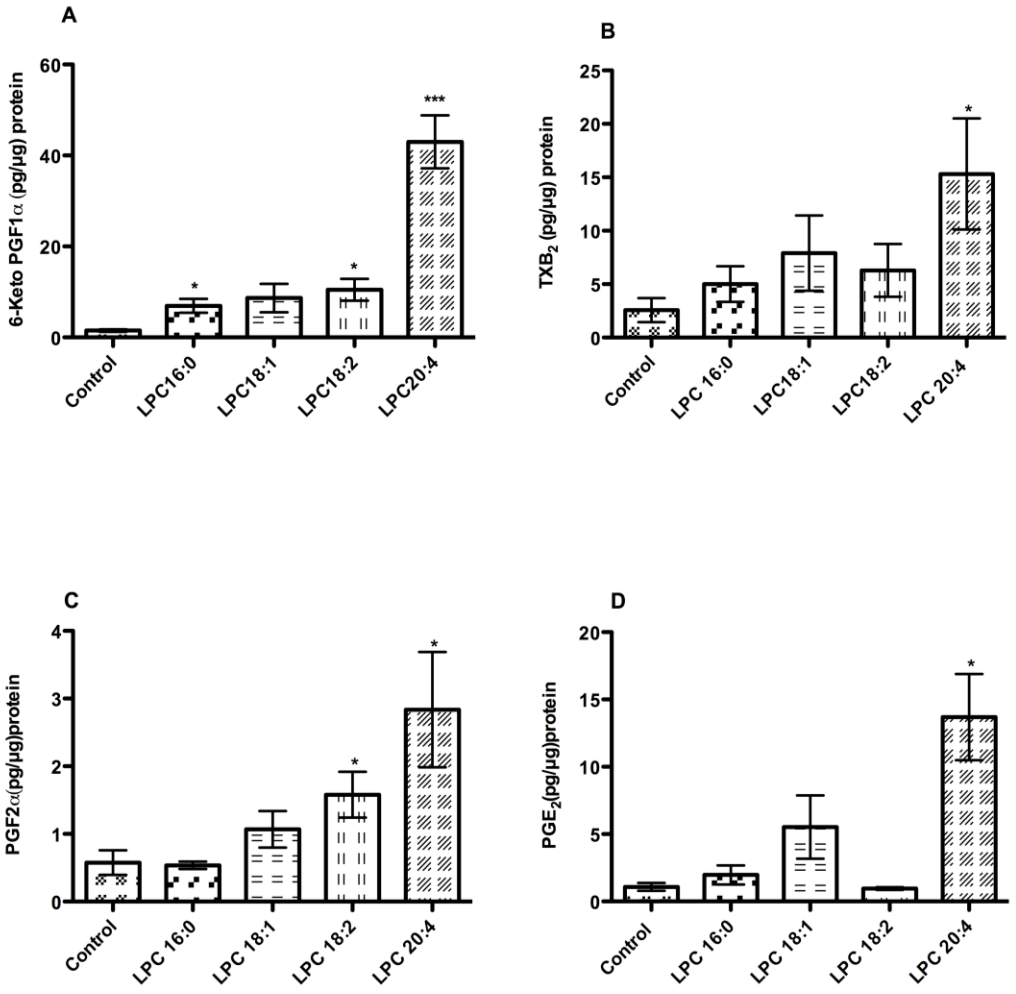


Figure.14. Prostanoid release from LPC-treated aortic rings

The rings were incubated in 200 µl aerated PSS under cell culture conditions at 37°C for 1 h. Thereafter, buffer was replaced with fresh PSS supplemented with PSS (control) or 10 µM LPC followed by further incubation under cell culture conditions at 37°C for 1 h. (A) 6-keto $PGF_{1\alpha}$ (B) TXB_2, (C) $PGF_{2\alpha}$ and (D) PGE_2 were quantified by EIA assays and rings were solubilized for protein quantification. Results shown in A and B are means ± SD of four experiments and those in C and D of two experiments, done in triplicates. *P<0.05, **P<0.01***P<0.001.

3.10. LPC-induced oxidative stress contributes to impairment of vascular relaxation

Since superoxide anions are established EDCF (90), we examined whether their production is triggered by LPC and whether they contribute to the observed LPC-induced impairment of relaxation. As shown in Fig. 15A all LPC induced superoxide anion production in mouse aortic rings with the following order of potency:

16:0>18:2>20:4>18:1. The SOD mimetic tempol (92) significantly improved relaxation impairment caused by LPC 18:1 (Fig. 15B), 18:2 (Fig, 15C) and 20:4 (Fig. 15D). In contrast, the relaxation impairment evoked by LPC 16:0 was not improved by tempol. However, it caused a slight but not significant exaggeration at low ACh concentrations, and a slight, but not significant, improvement at high ACh concentrations (Fig.15E).

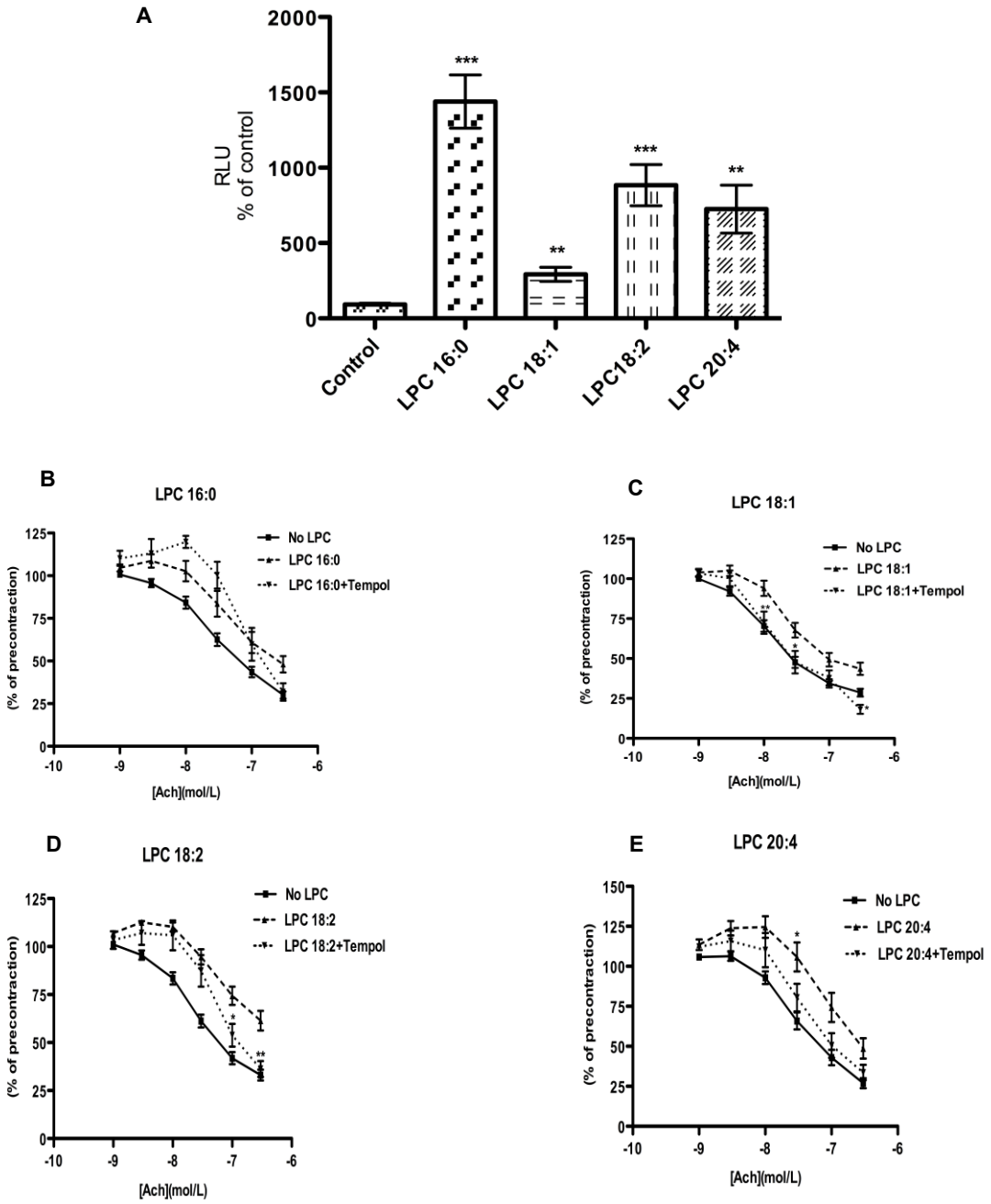


Figure.15. ROS are induced by LPC and contribute to LPC-induced impairment of relaxation

A) The rings were equilibrated in 100 µl PSS buffer containing 10 µM DETCA and 10 µM lucigenin at 37°C for 30 minutes, followed by addition of PSS (control) or LPC (10 µM). Emitted light (RLU) was recorded every 10 seconds for 30 seconds. The RLU were normalized to protein content of respective aortic rings and expressed as percentage of control set to 100%. Results are means ± SEM of three separate experiments.

The rings were preincubated without (no LPC) or with 10 µM LPC 18:1 (B), 18:2 (C), 20:4 (D) or 16:0 (E) in the absence or presence of 200 µM Tempol for 30 minutes, followed by precontraction with NE and cumulative addition of ACh. Results for each condition are mean ± SEM of 12 rings from 6 mice. *P<0.05, **P<0.01.

4. Discussion

We found that all LPC caused a pronounced impairment of endothelium-dependent relaxation (EDR) to ACh with remarkable acyl-chain dependent differences regarding the potency and underlying mechanisms. LPC did not alter the SNP-induced relaxation. This demonstrates that LPC are not toxic to smooth muscle cells and that the observed LPC-mediated impairment of relaxation is an endothelium-dependent effect. Neither of the tested LPC modified the contractile response to NE, nor did they induce contraction when co-applied with the eNOS inhibitor L-NNA (not shown). L-NNA completely inhibited, whereas indomethacin had no effect on ACh-induced relaxation of mouse aortic rings. Thus, NO seems to be the major mediator of ACh-induced relaxation in our experimental model. This is in line with the inability of ACh to alter vascular tone in aortic rings from eNOS deficient mice (93). Accordingly, the observed impairment of relaxation caused by LPC may reflect increased production and activity of EDCF, with concomitant counteraction of ACh-induced NO-mediated relaxation. This is similar to the inability of endothelial NO to curtail the effect of EDCF observed in arteries of aging and diseased (essential hypertension, diabetes) animals and humans (37, 52, 94). In spontaneous hypertensive rats (SHR) and in essential hypertensive patients, impaired vasodilatation was almost normalized by the COX-inhibitor indomethacin, indicating that COX-derived vasoconstrictors are key EDCF responsible for impaired endothelial function and blunted vasorelaxation (74).

Improvement of LPC 18:2- and 20:4-induced impairment of relaxation by inhibition of both COX (Fig. 2 A, B) and TP receptors (Fig. 3 A,B) indicated the involvement of COX-derived EDCF, which are capable of inducing contraction by acting via TP receptors (74, 90, 95). These receptors are highly expressed in mouse aortic smooth muscle cells (96). Both TXA_2 and PGI_2, were markedly induced by LPC 20:4 (Fig 6 A,B). These prostanoids are capable of activating TP receptors and may, therefore, have a major contribution to the LPC 20:4-effect (Fig. 4 A,B).

Promotion of contraction by PGI_2, induced by LPC 20:4 most likely reflects markedly higher levels of TP compared with IP receptors in mouse aortic smooth muscle cells (96). Additionally, the decreased ability of IP receptors to promote relaxation, as found in vascular smooth muscles of SHR (74), might at least in part also contribute to increased contraction, despite increased PGI_2 production. Future experiments should reveal whether responsiveness of IP receptors to a stable PGI_2 analogue,

iloprost, is altered by LPC. Neither TXA_2 nor PGI_2 were involved in LPC 18:2-induced relaxation impairment. However, the robust counteracting effect of the TP receptor antagonist on the LPC 18:2-induced relaxation impairment (Fig. 3A) strongly suggests the existence and action of some LPC 18:2-induced TP-receptor agonists. Because LPC 18:2 induced $PGF_{2\alpha}$ (Fig. 5C) which can activate TP receptors (90), the LPC 18:2-induced impairment of relaxation might at least in part be due to the EDCF-activity of induced $PGF_{2\alpha}$. Furthermore, isoprostanes formed non-enzymatically by ROS induced peroxidation of cell membrane polyunsaturated fatty acids, as well as PGH_2, a direct product of COX, might by acting via TP receptors, contribute to LPC 18:2-mediated impairment of relaxation (95, 97). Likewise, $PGF_{2\alpha}$ and PGE_2 (88) both markedly increased with LPC 20:4 (Fig.5 C,D) might as well contribute to the relaxation impairment caused by LPC 20:4 by acting via the TP receptors. Due to the negligible expression of PGE_2 receptors EP1 and EP3 (receptors associated with contraction) in mouse aortic smooth muscle cells, their contribution to contraction seems unlikely (93, 96).

Besides vasoconstricting prostanoids, ROS are established EDCF. Because ROS are formed as by-products during the generation of prostanoids by COX (42), LPC-mediated provision of arachidonic acid to COX can lead to increased COX activity resulting in elevated ROS levels in aortic rings exposed to LPC (Fig. 6A). We have previously shown that LPC increase cytosolic calcium concentration and thereby activate phospholipase-mediated release of arachidonic acid from membrane phospholipids and in turn PGI_2 production (4). In contrast to LPC 16:0, 18:1 and 18:2, LPC 20:4 not only induces arachidonic acid release, but also provides its own arachidonic acid to COX. This resulted in the highest induction of PGI_2, relative to the other studied LPC, both in cultured endothelial cells (4) and aortic rings (Fig. 5A). Because the capacity of ROS induction by LPC 20:4 and 16:0 was inversely related to their capacity to increase prostanoid production (Fig.5 and 6 A), the LPC-induced COX activity does not seem to account for increased ROS levels in aortic rings exposed to LPC. Additional LPC-mediated effects such as activation of NADPH oxidase (98), or inhibition of superoxide dismutase (SOD)-1 (99) might contribute to the increased ROS levels.

Although LPC 16:0 was the most potent inducer of ROS, tempol, a SOD mimetic (92), failed to improve the LPC 16:0 induced impairment of relaxation (Fig. 6E).

Similarly, LPC 16:0 also promoted ROS production in rat aortic rings, but $MnCl_2$, another SOD mimetic, failed to restore the impaired relaxation (100). In contrast to LPC 16:0, relaxation impairment caused by LPC 18:1, 18:2 and 20:4 was very efficiently restored by tempol (Fig. 5), as well as by a combination of DETCA (SOD inhibitor) and TIRON (ROS scavenger) (not shown). This indicates that the removal of superoxide anions and not the generation of hydrogen peroxide, a potential vasorelaxing factor (92) was responsible for restored vasorelaxation. Whether superoxide anions induced by these LPC had direct constricting effect on smooth muscle cells (101) or potentiated contraction by reducing bioavailability of NO (102) remains to be determined.

Previous studies have shown opposite effects of LPC 16:0 on ACh-induced relaxation: i) increased relaxation in bovine pulmonary artery (103, 104) as well as in coronary and renal circulation in the rabbit (105), ii) impaired relaxation in rat aortic rings due to interference with NO- and endothelium derived hyperpolarizing factor-mediated relaxation mechanisms (22, 84) and iii) impaired relaxation in mouse aortic rings (100). These contradictory findings can probably be attributed to vascular bed- and species- specific differences in tissue responsiveness to LPC.

Improvement of relaxation observed in mouse aortic rings exposed to LPC 16:0 upon supplementation of L-arginine, a substrate for eNOS, implicated a reduced NO bioavailability as the cause of impaired relaxation (100). However, LPC 16:0 increased the nitrite levels (indicative of NO levels) in organ bath of aortic rings exposed to ACh arguing against decreased NO as underlying mechanism. Despite the capacity of LPC 16:0 to upregulate eNOS expression (106) the increased NO/nitrite may not, due to the short exposure of aortic rings to LPC (45 min) in our experiments, reflect increased eNOS protein. The observed augmenting effect of LPC 16:0 on NO production is rather a consequence of an acute effect of LPC 16:0, such as an LPC-induced increase in cytosolic calcium with concomitant activation of eNOS (107). Importantly, SNP-induced relaxation was not altered by the presence of LPC 16:0, indicating that responsiveness of smooth muscle cells to NO was not impaired by this LPC. Whether endothelin-1 or some pro-constricting non-COX-derived eicosanoids, mediate 16:0 LPC-induced impairment of relaxation remains to be determined.

Based on our results, LPC 16:0, 18:1, 18:2 and 20:4 emerge as important triggers of endothelial dysfunction. The major players responsible for the blunted endothelium-dependent relaxation in aged vessels and in various pathologies (essential

hypertension, diabetes and atherosclerosis) are vasoconstricting prostanoids and ROS. The fact that the studied LPC promote the production of these established EDCF strongly argues for the role of LPC 16:0, 18:1, 18:2 and 20:4 as important contributors to endothelial dysfunction in aging and aforementioned pathologies. Future experiment should reveal the relationship between plasma levels of those LPC and the incidence and degree of endothelial dysfunction in humans and animal models of hypertension.

Chapter II

1. Introduction

1.1. Lipoproteins: Structure and Metabolism

Lipoproteins are soluble complexes of proteins and lipids that make lipids compatible with the aqueous nature of body fluids and also help transport lipids throughout the body. Lipoproteins are synthesized in the liver and intestine, they may also arise from metabolic changes of precursor lipoproteins. In the circulation lipoproteins exist in a constant flux, as surrounding tissues take up various components of the lipoproteins and change their composition and physical structure. The remnants return to the liver. The most abundant lipid constituents are triacylglycerols (TG), free cholesterol (FC), cholesterol esters (CE) and phospholipids (PL). Lipoproteins are classified as chylomicrons (CM), very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL), based on the relative densities of the aggregates on ultracentrifugation. The different classes may be further refined by improved separation procedures, and intermediate-density lipoproteins (IDL) and subdivisions of the HDL (e.g. HDL_1, HDL_2, HDL_3) are often defined. Density is determined mainly by the relative concentrations of TG and proteins and by the diameters of the broadly spherical particles, which vary from about 6000Å in CM to 100Å or less in the smallest HDL (108).

Physical properties and lipid compositions of lipoprotein classes				
	CM	**VLDL**	**LDL**	**HDL**
Density (g/ml)	< 0.94	0.94-1.006	1.006-1.063	1.063-1.210
Diameter (Å)	6000-2000	600	250	70-120
Total lipid (wt%) *	99	91	80	44
Triacylglycerols	85	55	10	6
Cholesterol esters	3	18	50	40
Cholesterol	2	7	11	7
Phospholipids	8	20	29	46

Table 1: Showing physical properties and lipid compositions of different lipoprotein classes

1.2. Lipoprotein metabolism

Metabolism of lipids can be divided into the exogenous pathway, which refers to the metabolism of intestinally derived lipids, and the endogenous pathway, which refers to hepatic-derived lipids. In case of cholesterol about 10 - 20 % is derived from the diet and 75 % is endogenously synthesized (109).

1.2.1. Exogenous pathway

Epithelial cells that line the small intestine absorb lipids from diet. Dietary fats are made up of TG, PL, cholesterol and bile salts, which are emulsified with bile acids in the intestine to form mixed micelles. Pancreatic lipases bind to micelles and digest TG to yield fatty acids (FA) and 2- monoglyceride (2-MG). FA and 2-MG are taken up by fatty acid transporters. Short chain FA and cholesterol are also taken up via passive diffusion. Once inside the enterocytes, FC and FA are re- esterified, packaged with TG, PL and apolipoprotein B-48 into chylomicrons (110). They circulate through lymph vessels and nascent chylomicrons bypass liver circulation to be drained via the thoracic duct into the bloodstream. In the blood stream chylomicrons mature after acquiring apolipoprotein C-II (apoC-II) and apolipoprotein E (apoE). Through apoC-II activate lipoprotein lipase (LPL), an enzyme that hydrolyzes triacylglycerols and ultimately releasing glycerol and fatty acids from chylomicrons. Peripheral tissues then take up the glycerol and fatty acids, especially by muscles and adipose tissues for energy and storage. The hydrolyzed chylomicrons are now called as chylomicron remnants, which continue circulating until they interact via apoE with chylomicron remnant receptors, present in the liver. This interaction causes the endocytosis of the chylomicron remnants, which are subsequently hydrolyzed within lysosomes. Lysosomal hydrolysis releases glycerol and fatty acids into the cell, which can be used for energy or stored for later use (108).

1.2.2. Endogenous pathway

The liver is the principal source of VLDL. Triacylglycerol and cholesterol along with apolipoprotein B-100 (apoB-100) combines to form VLDL particles, which are further released into the blood stream. As in case of chylomicrons, VLDL also acquires apoC-II and apoE to be converted into the mature form. ApoC-II activates LPL, hydrolyzing the VLDL particle and releasing of glycerol and fatty acids that can be absorbed by peripheral tissues, like adipose and muscle. The hydrolyzed VLDL particles are now called VLDL remnants or intermediate density lipoproteins (IDLs). VLDL remnants can circulate and, via an interaction between apoE and the remnant receptor, are absorbed by the liver. They can also be further hydrolyzed by hepatic lipase (HL). Hydrolysis by HL releases glycerol and fatty acids, leaving behind IDL remnants, called LDL, containing high cholesterol content. LDL circulates and is absorbed by the liver and peripheral cells. Binding of LDL to its target tissue occurs through an interaction between the LDL receptor and apoB-100 or apoE on the LDL particle. Absorption occurs through endocytosis, and the internalized LDL particles are hydrolyzed within lysosomes, releasing lipids, mainly cholesterol (111, 112).

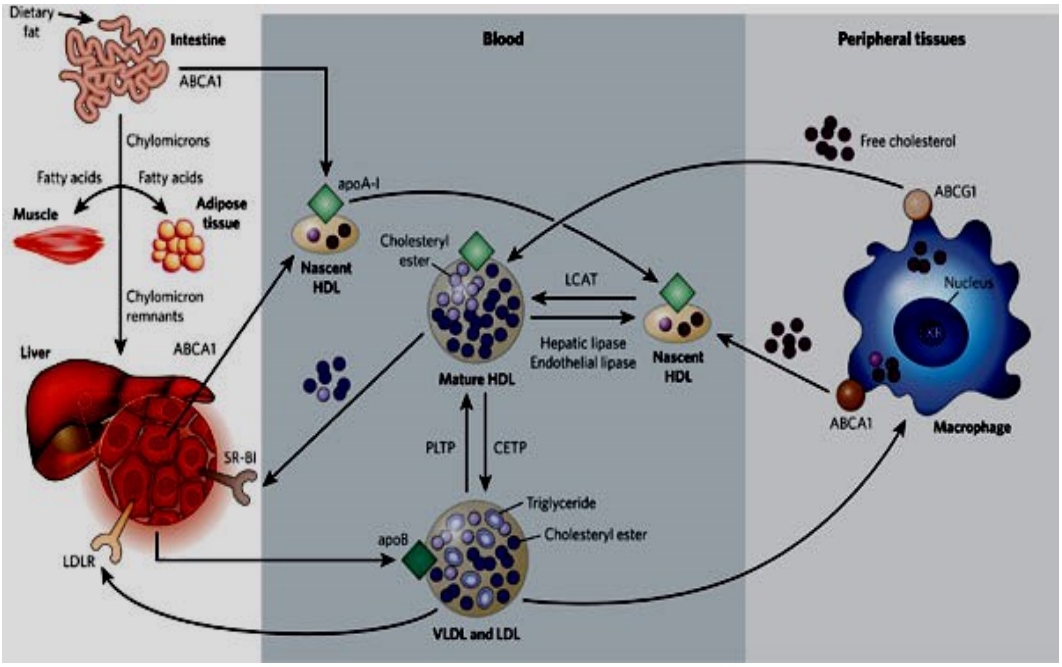


Figure 1: Endogenous pathway of lipid metabolism
Adapted from (113).

1.3. HDL structure

HDL is made up of a heterogenous population differing in shape, size, density and composition (114). HDL is a protein rich particle (~60%). ApoA-I is the most abundant protein component followed by apoA-II. HDL also consists of about 70 other proteins, which have been identified only recently (115). These HDL-associated proteins have been implicated in lipid metabolism, complement activation, growth-factors regulation and proteolysis regulation, indicating that HDL exerts multiple biological activities.

HDL can be separated based on its density into two subclasses, the less dense HDL_2 (1.063–1.125 g/ml) and the denser HDL_3 (1.125–1.21 g/ml) (115). Initially, spherical HDLs are small and lipid-poor. These are called HDL_{3a}, HDL_{3b} and HDL_{3c}. As HDL continues to grow, large cholesterol-rich HDLs are generated, which are termed HDL_{2a} and HDL_{2b}. When classified using two-dimensional gel electrophoresis, nascent HDL occurs as pre-β HDL, whereas HDL_3 and HDL_2 are found as α or pre-α HDL. Non-denaturating gel electrophoresis produces five subclasses of HDL according to size: HDL2b (~10.6 nm), HDL2a (~9.2nm), HDL3a (~8.4 nm), HDL3b (~8.0 nm) and HDL3c (~7.6 nm). In addition, HDL has been classified depending on its protein composition (presence of apoA-I or apoA-I and apoA-II) or its surface charge (116). Accumulating evidence suggests that HDL functionality varies between subclasses (117, 118). Nevertheless, the relationship of HDL subclass quantity and quality to cardiovascular diseases is not fully understood.

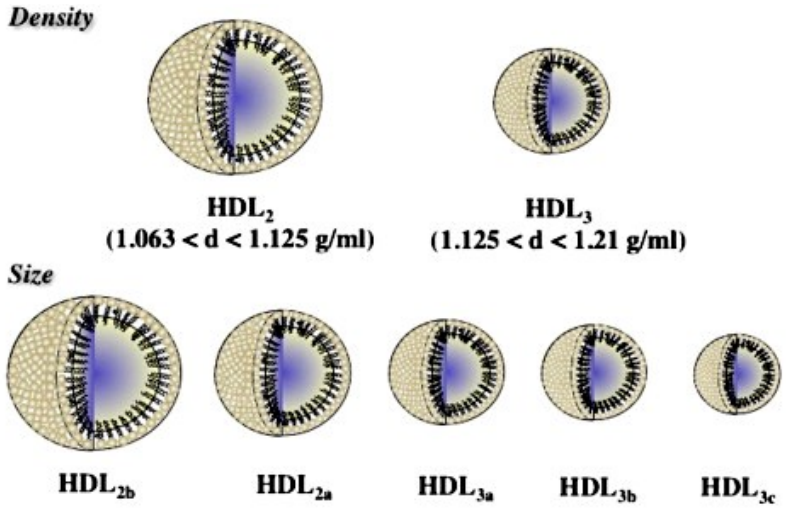


Figure 2: Structure and classification of HDL species according to their densities

Non-denaturating gelelectrophoresis produces five subclasses of HDL according to size: HDL2b (~10.6 nm), HDL2a (~9.2nm), HDL3a (~8.4 nm), HDL3b (~8.0 nm) and HDL3c (~7.6 nm). Adapted from (119).

1.4. HDL mediated Reverse cholesterol transport

Liver and intestine secrete lipid free apoA-I. ApoA-I takes up cholesterol from organs by the help of transporter proteins ATP binding cassette transporter A1 (ABCA1) to form nascent HDL. This nascent HDL, in peripheral tissues promotes cholesterol efflux through ABCA1. Macrophages in particular are mainly dependent upon this pathway because in the absence of a functional ABCA1 transporter, as occurs in Tangier disease, there is a marked accumulation of cholesteryl esters in macrophages. Other transporters, in particular ABCG1 and scavenger receptor class B type I (SR-BI), may also participate in the efflux of excess cholesterol from cells to mature HDL. In addition, there is a spontaneous bidirectional exchange of cholesterol between cell membranes and extracellular lipoproteins. These alternative pathways differ from ABCA1 in that they largely promote cholesterol efflux to mature lipid rich forms of HDL, which are spherical in shape and migrate in the α-position during electrophoresis (120). The free cholesterol in nascent HDLs is esterified to cholesteryl ester by the enzyme lecithin cholesterol acyltransferase (LCAT), creating mature HDLs. The cholesterol in HDL is returned to the liver both directly, through uptake by the receptor SR-BI and indirectly, by transfer to LDLs and VLDLs through the cholesteryl ester transfer protein (CETP). The lipid content of HDLs is altered by the enzymes HL and endothelial lipase (EL) and by the transfer proteins CETP and phospholipid transfer protein (PLTP), affecting HDL catabolism (113). EL and HL are important in HDL metabolism. Both belong to the triglyceride lipase gene family, but have different functions. EL is secreted mainly from vascular endothelial cells and acts at site of secretion. It is bound to endothelial cells by heparin sulfate proteoglycan molecules (121). EL has high phospholipase A1 activity and remodels HDL into small particles. It has very little triglyceride lipase activity. In contrasts to EL, HL is produced by the liver and is a triglyceride lipase. It also causes the remodelling of HDL into smaller particles, but additionally causes the release of lipid-poor apoA-I (121). The mature HDL particle can deliver cholesterol directly via hepatic SR-BI to

the liver or indirectly via CETP mediated CE-transfer to LDL/VLDL followed by subsequent liver uptake, where it can be excreted in form of bile acids. Besides clearance through the liver, a non-biliary RCT pathway has emerged, where HDL-cholesterol can be released into the intestine via the enterocytes with the help of ABCG5 and ABCG8 (122, 123). In summary, the net effect of reverse cholesterol transport is the removal of excess cholesterol from the peripheral tissue by HDL and its excretion.

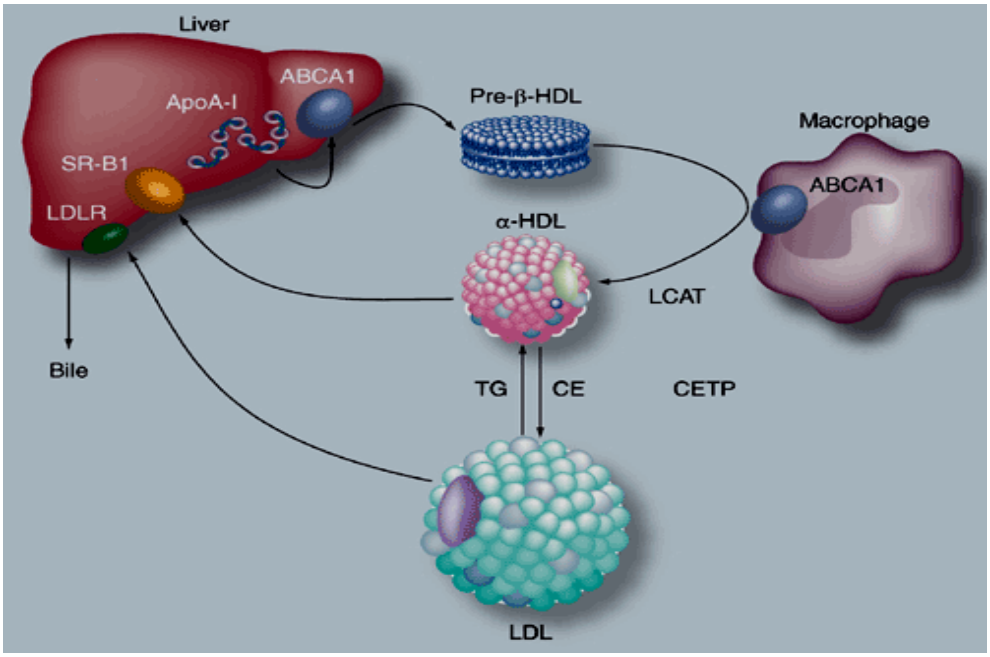


Figure. 3: Reverse cholesterol transport by HDL. Adapted from (124)**.**

Apart from reverse cholesterol transport HDL has other atheroprotective effects. It prevents oxidation of LDL, inhibits expression of proinflammatory cytokines by macrophages and also prevents expression of adhesion molecules by endothelial cells. HDL also prevents cell apoptosis and promotes endothelial cell proliferation and migration. It is also known to stimulate nitric oxide (NO) release from endothelial cells causing vasodilation. Due to these properties of HDL it is thought to protect the endothelium and prevent events that may lead to atherosclerosis or other inflammatory diseases (125, 126).

1.5 HDL and its role in maintaining endothelial function

The atheroprotective properties of HDL are partly due to its role in maintaining the endothelial function (127). HDL has a number of beneficial effects on endothelial cells, which include, stimulation of cell proliferation, cell survival, migration and NO synthesis as well as inhibition of the expression of adhesion molecules (128, 129). Several mechanisms underlying the effects of HDL on endothelial reactivity have been suggested, including synthesis of vasorelaxing prostanoids such as prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2) as well as activation of endothelial nitric oxide synthase (eNOS) via SR-BI (130).

A B

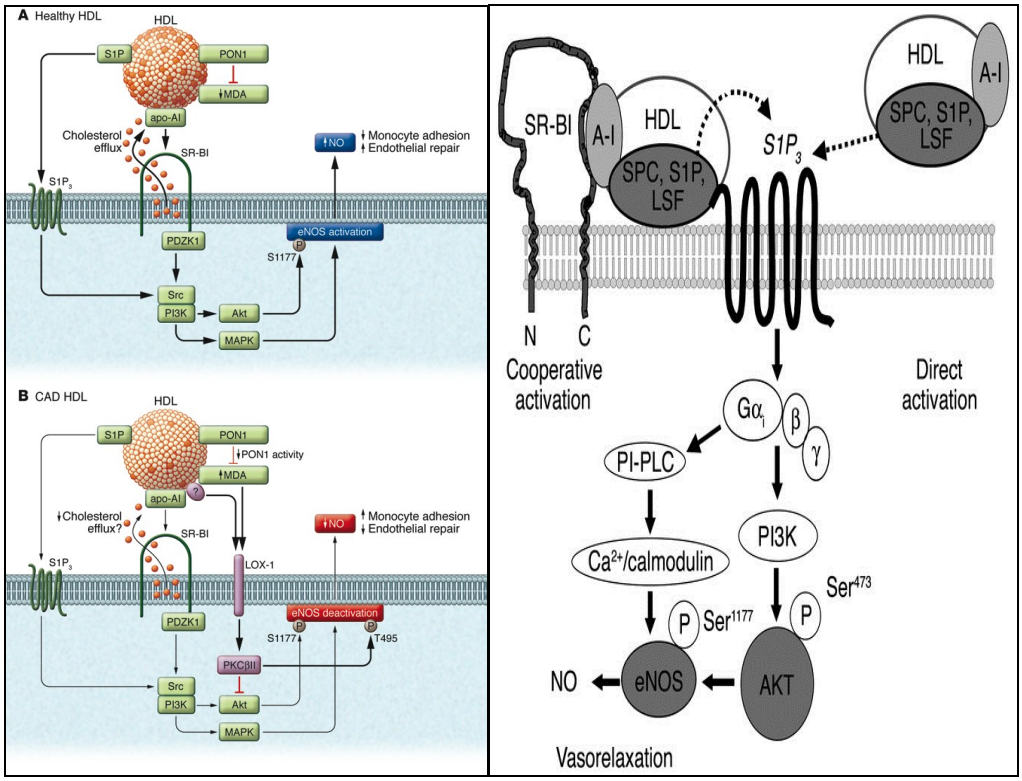


Figure 4: (A) HDL stimulates eNOS through multiple mechanisms. ApoAI allows HDL to bind to SR-BI. SR-BI is involved in the cholesterol efflux from HDL. It then causes activation of PDZK1- dependent activation of Src kinase, PI3K and Akt, which phosphorylates eNOS Ser1177 and thereby increases enzyme activity. SR-BI–, Src-, and PI3K-dependent Erk MAPK activation is also required for eNOS activation

by HDL. HDL-associated S1P and related molecules may also be involved in the activation of lysophospholipid receptor $S1P_3$ to stimulate eNOS. HDL contains active PON1, which suppresses the formation of oxidized lipids and lipoproteins such as MDA. The net effect of eNOS activation by HDL is to blunt endothelial cell–monocyte adhesion and promote endothelial repair (131).
(B) Model of HDL-induced eNOS activation and vasodilation by the lysophospholipid receptor S1P3. PI-PLC, phosphatidylinositol-specific phospholipase C. A-I, apoAI; N and C, amino- and carboxyterminus of SR-BI (130).

The ability of HDL to cause vasorelaxation in isolated ring preparations is shown to be impaired in SR-BI deficient mice. SR-BI is also reported to produce increased eNOS activity in cultured endothelial cells, in response to HDL (129). A recent study shows that ABC transporters and specifically ABCG1 play a very important role in maintaining the eNOS dimer (active form) when activated by HDL (132). A lot of different mechanisms have been proposed to explain the property of HDL to protect and increase the activity of arterial eNOS. HDL appers to be able to produce eNOS dependent vascular relaxation when directly added on aortic rings obtained from rats and mice (133, 134). The vasorelaxing property of HDL has been attributed to apoAI, SR-BI, S1P and also minor lysophospholipids and proteins associated with it(25). Therefore, further studies need to be carried out to assess the relative contribution of different potential mechanisms in HDL induced eNOS activity *in vivo*.

1.6. HDL dysfunction

Inflammation causes dramatic changes in HDL composition and function, which can turn HDL profile from anti-atherogenic to pro-atherogenic in nature. Acute phase HDL lost its ability to protect LDL against oxidation and to inhibit cytokine-induced adhesion molecule expression (135). The ability of HDL to promote cholesterol efflux was also markedly reduced (136). The fact that HDL can turn dysfunctional was further proved in patients with coronary artery disease (CAD). HDL from those patients (CAD-HDL), having similar HDL levels as healthy, exhibited decreased anti-inflammatory properties and diminished endothelial protective effects (22). CAD-HDL had also increased malondialdehyde levels and decreased PON1 levels thereby

rendering HDL pro-atherogenic (131). Altered composition and functionality of HDL were also found in end-stage renal disease (137), psoriasis (138) and type 2 diabetes mellitus (139). Another study with HDL from CAD patients showed that this HDL caused activation of endothelial pro-apoptotic pathways instead of being anti-apoptotic (26). This was due to proteome remodeling of HDL where HDL associated clusterin levels were decreased and apoC-III levels were elevated (140). HDL may protect against atherosclerosis by several mechanisms, but under certain inflammatory conditions it may also promote atherosclerosis. This brings forth the issue that most likely, total plasma HDL levels may be a crude measure of the protective effects provided by HDL, since HDL are both structurally and functionally heterogenous. This functional heterogeneity can be observed in the fact that transgenic mice expressing high ApoAII have high HDL levels but are more prone to atherosclerosis. PON1 knockout mice on the other hand have increased atherosclerosis despite of showing no change in HDL levels (141). Hence, along with the quantity it is also important to determine the quality of HDL to estimate antiatherosclerotic capacity of HDL.

Although, these results are very intriguing, there is still a need for better and more physiological assay to assess functional properties of HDL. HDL particles are very heterogeneous in composition, and particle size or density might have direct effects on its properties. In recent years, it was revealed that HDL has a substantial list of HDL-associated proteins (>70) (115, 142). These associated proteins are involved in lipid metabolism, complement activation, growth-factors regulation and proteolysis regulation, reflecting that HDL exerts multiple biological activities. The impact of HDL protein cargo is evident as HDL from CAD patients had an altered proteome (143).

1.7. Endothelial Lipase (EL)

Two separate research groups discovered the EL gene in 1999 (144, 145). The human genome database shows that EL locus is on chromosome 18 (18q21.1) (146). It has 45% homology with LPL, 40% with HL and 27% with pancreatic lipase. The mature EL protein consists of 482 amino acids and has a molecular weight of about 68 kDa. EL is a member of triacylglycerol family synthesized by endothelial cells. EL has

mainly phospholipase activity and very low triglyceride lipase activity. It shows very distinct lipoprotein preference as it plays a physiological role in HDL metabolism. EL hydrolyses HDL - phospholipids to produce LPC and free fatty acids. EL expression can be upregulated by inflammatory cytokines such as TNF-α and IL-1β as well as shear stress (147, 148). EL may influence atherogenic process by being expressed at the atherosclerotic lesions and also by hydrolysing phospholipids in lipoproteins to produce lysophosphatidylcholines (149). Hepatic over- expression of EL in mice using adenoviral gene transfer markedly reduced HDL-C levels. In EL knockout mice model, HDL-C levels were significantly increased. Published data suggests EL to be an atherogenic enzyme but results obtained from atherosclerosis studies using EL knockout models were conflicting (150, 151).

1.8. EL expression and Regulation

Endothelial cells such as human umbilical vein endothelial cells; human coronary artery endothelial cells and murine endothelial- like yolk sac cells produce EL. However, EL expression is not restricted to endothelial cells, as EL expression was also observed in the placenta, lung, liver, kidney, testis, thyroid and corpus luteum of the ovary (152). Additionally, EL expression was also reported in different macrophage cell lines like, human THP-1, mouse RAW 294.7 cells and also primary macrophages (145, 153).

1.9. Factors impacting EL expression levels

The expression of EL can be increased by a number of triggers. EL expression was significantly increased after treatment with ox-LDL (153) as well as inflammatory cytokines TNFα and IL-1ß (154). EL mRNA levels were found to be higher in tissues of SHR rats as compared to control rats, and the highest expression was found to be in the aorta (155). EL expression was also found to be higher in patients with type I diabetes mellitus (156). Lipopolysaccharide (LPS) could also induce an increase in EL mRNA and protein levels in mouse tissues (157).

1.10. EL and HDL metabolism

EL mainly has phospholipase activity and mature HDL is the favoured substrate for EL (145). It cleaves the PL in HDL generating a number of saturated and unsaturated fatty acids as well as a mixture of lysophosphatidylcholines (LPC). Experimental evidence indicates that EL is a major determinant of HDL plasma levels. In C57BL/6 mice EL was overexpressed by injecting intravenously EL encoding adenoviruses. This led to more than 90% decrease in plasma HDL levels. This decrease in HDL levels remained up to 30 days after injection (153). Similarly, EL overexpression in human apoAI transgenic mice caused a decrease in HDL cholesterol and apoAI levels. When EL polyclonal antibodies were injected into HL-/- and human apoAI transgenic mice, an increase in HDL cholesterol, PL and total cholesterol was observed within 24-48 hours (158). This emphasized the role if EL in HDL metabolism. In C57Bl/6 mice, HDL cholesterol was found to be increased in EL-/- and EL+/- mice, whereas, EL transgenic mice had about 19% decreased HDL cholesterol levels (159). The phospholipase activity of EL remodels HDL to produce smaller HDL particles. When EL was inhibited using antibodies HL-/- and human apoAI transgenic mice produced larger HDL particles, which were rich in phospholipids (154). The same was also observed in EL-/- mice (160). The bridging function of EL which is bound via heparan sulfate proteoglycan (HSPG) molecules to the cell surface facilitates binding of lipoproteins independent of EL enzymatic activity (161, 162). Accordingly, dissociation of EL from HSPG resulted in decreased HDL binding (162). There is a possibility that EL may also regulate other factors in HDL metabolism. EL-/- mice have increased levels of hepatic LCAT mRNA and increased LCAT protein plasma levels. Despite this, the EL-/- mice have about 50% impaired LCAT esterification rate, suggesting the role of EL in modulating LCAT activity. Furthermore, HL and LPL are upregulated in EL-/- mice, whereas, PLTP is downregulated (160, 163), suggesting an overall indirect effect of EL on HDL metabolism (163).

1.11. Metabolism of ApoB containing lipoproteins by EL

EL regulated the level of ApoB- containing lipoproteins to a much lesser extent than HDL. It was observed in chow-fed LDLR-/- mice, injection of EL by adenovirus produced about 50% decrease in VLDL/LDL levels (153). EL mediated apoB-containing lipoprotein metabolism depends on the catalytic activity of EL and not on its bridging function. EL knockout caused an elevation of lipoproteins like chylomicrons, VLDL and IDL levels, in addition to increased HDL levels (151).

1.12. Impact of EL on RCT

There are only a few studies addressing the effect of EL on HDL- mediated cellular cholesterol efflux, but there are no conclusive results. A decrease in EL expression *in vitro* in macrophages caused decreased efflux towards apoAI, but overexpression of EL produced an opposite effect 47. It was also found that the bridging and catalytic functions of EL are equally important for this process (164).

Contrastingly, one group found no effect of EL on ABCA1 dependent cholesterol efflux(165), while another group found 63% increase in ABCA1 mediated cholesterol efflux to HDL from EL overexpressing mice (166). Another study demonstrated increased efflux towards HDL from EL-KO mice was also observed (167). Regarding SR-BI mediated efflux, it was found that EL modified HDL had reduced capacity for mediating cholesterol efflux via SR-BI (165, 166). EL was shown to modulate cholesterol efflux variably, but it was consistently shown that EL enhanced the uptake of HDL cholesterol into the liver (168, 169). Despite observed modulation of the capacity of HDL to mediate cholesterol efflux or delivery of cholesterol ester to cells the major pathway of cholesterol excretion from the body, the biliary sterol secretion, was found to be unchanged in EL overexpressing mice (170).

1.12. The Role of EL in Atherosclerosis

The role of EL in atherosclerosis is controversial as all studies performed so far have been inconclusive. According to one study, disruption of EL in apoE-/- mice, reduced atherosclerotic lesions by 70%, when mice were kept on a chow diet. On a western type diet the decrease in atherosclerotic lesions was less significant. The lack of EL lead to an increase in HDL as well as LDL-levels. The authors explained the decrease in atherosclerotic lesions as a result of reduced monocyte adhesion to vascular wall via HSPG molecules (151). In contrast, in another study, carried out independently, no difference in atherosclerotic lesion size and lesion macrophage content was detected in mice expressing or lacking EL either on an apoE-/- or LDLR-/- background (150). Atherosclerosis is known as an inflammatory disease and a link between EL and atherosclerosis has been suggested. The expression of EL was found significantly increased in response to proinflammatory cytokines in cultured

endothelial cells (154). EL is also upregulated in humans during acute experimental inflammation (171) LPC formed by the action of EL were also shown to have proinflammatory effects (172). EL was shown to increase expression of adhesion molecules in the vessel wall (151) whereas another group suggested that EL decreased TNFα-induced expression of endothelial adhesion molecule expression by generating perioxisome proliferator- activated receptor α (PPAR α) ligands from HDL phospholipids (126). Due to these opposing results the conclusion that can be drawn is that EL expression is upregulated by inflammatory stimuli, but the role of EL itself as a pro- or anti- inflammatory molecule is controversial.

In humans, the relation between atherosclerotic disease and EL levels has been investigated. As indirect evidence, a positive correlation was found in clinically healthy individuals, between coronary artery calcification scores and EL mass levels in pre- and post-heparin plasma (173). In patients with end- stage renal disease a two-year follow up study showed higher serum EL levels in patients who experienced cardiovascular disease events as compared to patients without any event (174). Two separate studies showed that Thr111Ile EL variant was related to a reduced risk of acute myocardial infarction and coronary artery disease (169). However, another study on a larger study population could not confirm the impact of Thr111Ile variant on cardiovascular risk (175). A recent Mendelian randomization analysis in 20913 myocardial infarction casas was compared with 95407 control subjects. It was found that only one single nucleotide polymorphism in the EL gene significantly increased HDL-C levels but this increase in HDL did not confer any protection against myocardial infraction (176). These conflicting data raise doubts about the link between HDL levels per se, and protection against atherosclerosis. It also does not clarify the relation between the function of EL and weather it exhibits pro- or anti-atherosclerotic properties

1.13.Rational for the Hypothesis

Among various cellular and plasma factors, endothelial lipase (EL) is a strong negative regulator of HDL plasma levels (159) and a modulator of HDL lipid composition (165, 177). Of particular interest, EL plasma levels and activities are increased in the pathologies associated with impaired HDL functionality such as type 2 diabetes, subclinical atherosclerosis (173, 178), end-stage renal disease (174) or acute inflammation (171).

Although the role of EL in HDL metabolism is well established (159), nothing is known whether and how EL modification of HDL impacts the endothelial function of HDL, in particular the capacity to promote NO and vasorelaxation.

Hypothesis:

Considering capacity of EL to provoke structural and compositional alterations in HDL, we hypothesized that EL induced alterations of the lipid and protein composition of HDL might yield dysfunctional HDL, with dramatically impaired endothelial function.

2. Materials and Methods

2.1. Mice and tissue preparation

Mice received care in accordance with the Austrian law on experimentation with laboratory animals, which is based on the U.S. National Institutes of Health guidelines. Male C57BL/6 mice (10-12 weeks old) provided by Himberg, Austria, or mice generated in our facility were killed by cervical dislocation. The descending thoracic aorta was isolated and dissected free of adherent tissue.

2.2. Adenovirus injection in mice

Adenoviruses (Ad) encoding human EL and bacterial ß-galactosidase (LacZ) were prepared as described previously (161). C57BL/6 mice (9 to 12 weeks old) were injected i.v. with a total of $2.4x10^9$ particles of EL- or LacZ- adenovirus. After 48 hour aortae were collected for myography. In a separate set of experiments mice were injected with different concentrations of EL-Ad followed by bleeding after 48 hours to obtain serum. HDL was further isolated from serum by ultracentrifugation.

2.3. EL- and LacZ-HDL isolation from mouse serum

Mice that were injected with EL-Ad or LacZ-Ad were bled by retro-orbital puncturing to obtain serum. HDL was isolated from pooled mouse serum (179). The density of serum was adjusted to 1.24g/ml using KBr. A density gradient was generated by layering this serum below PBS in centrifuge tubes (Beckman 16X76 mm). The tubes were then sealed and centrifuged at 90,000 rpm for 4 hours in a Ti90 fixed angle rotor (Beckman). Centrifugation caused HDL to separate from the remaining serum as a distinct band. HDL was collected by puncturing the centrifuge tubes with a syringe. The HDL thus obtained was desalted by gel filtration by passing through PD-10 columns, layered with argon and freshly used or stored at -70 °C.

2.4. Protein and lipid analyses

Protein concentrations were determined with the BCA reagent according to the manufacturer's instructions (Pierce, Rockford, IL). Lipid concentrations (total cholesterol, HDL-cholesterol, triglyceride, and phospholipid) were measured using commercially available assay kits (Diagnostic systems, Germany).

2.5. Lipid extraction, HPLC and MS

The procedure was done by Center for Medical Research, Core Facility Mass Spectrometry, Medical University Graz, by Dr. Harald Koefeler..

300 µg of mouse LacZ and EL-HDL protein was extracted according to Bligh and Dyer (180), in the presence of an internal standard (PC 12:0/12:0) and dried under a stream of nitrogen. Dried lipid extracts were resuspended in 200 µl $CHCl_3$/MeOH (1:1, v/v) containing 1 pmol/µl of each LPC 17:1, LPE 17:1, PE 12:0/13:0 and PC 12:0/13:0 respectively, serving as internal standards. Chromatographic separation of lipids was performed by an Accela HPLC (Thermo Scientific) on a Thermo Hypersil GOLD C18, 100 × 1 mm, 1.9 µm column. Solvent A was a water solution of 1% ammonia acetate (v/v) and 0.1% formic acid (v/v) and solvent B was acetonitrile/2-propanol (5:2, v/v) supplemented with 1% ammonia acetate (v/v) and 0.1% formic acid (v/v), respectively. The gradient was run from 35% to 70% B for 4 min, then to 100% B in additional 16 min with subsequent hold at 100% for 10 min. The flow rate was 250 µl/min. Phospholipid species were determined by a TSQ Quantum ultra (Thermo Scientific) triple quadrupole instrument in positive ESI mode. The spray voltage was set to 4500 V and capillary voltage to 35 V. The PC species were detected in a precursor ion scan on m/z 184 at 34 eV. Acquisition of triglyceride species was performed on a LTQ-FT in FT full scan mode at a resolution of 200,000. Phospholipid peak areas were calculated by QuanBrowser for all lipid species and the calculated peak areas for each species were expressed according to the amount of internal standard. TG was quantified by Lipid Data Analyzer (181).

2.6. Non-denaturing gradient –gel electrophoresis

Electrophoresis was performed using 4-20% non-denaturing polyacrylamide gels

(Nusep, Australia) at 4^{o}C.After a pre-run (30 minutes at 125V), 10µg of EL- and LacZ-HDL were loaded on to the gel and run for 30 minutes at 150V and finally for 3 hours at 125V. The gels were fixed in sulphosalicylic acid for 5 min and stained with Coomassie R 250 overnight. Destaining was performed in a mixture containing 20% acetic acid / 20% methanol (v/v). The particle size of HDL was determined by comparison with standard proteins (Invitrogen).

2.7. Paraoxonase/Arylesterase assay

Ca2+-dependent arylesterase activity assay was performed by Dr. M. Holzer, Institute of Experimental and Clinical Pharmacology by a photometric assay using phenylacetate as substrate (119). HDL samples (10 µg protein) were added to 200 µl buffer containing 100 mmol/L Tris, 2 mmol/L CaCl2 at pH 8.0, either containing 1 mmol/L phenylacetate. The rate of hydrolysis of phenylacetate was monitored in the increase of absorbance at 270 nm and readings were taken every 30 seconds to generate a kinetic plot. Form the kinetic chart, the slope was used of the determine **Δ**Ab270nm / min. Enzymatic activity was calculated with the Beer-Lambert Law from the molar extinction coefficient of 1310 L*mol-1*cm-1 for phenylacetate.

2.8. EL-Ad overexpression and HDL re-isolation from HepG2 cells

Human liver hepatoma cell line, HepG2 cells were maintained in DMEM medium supplemented with 2 mmol/L glutamine, 1% PS (100 units/ml penicillin, 100 µg/ml streptomycin) and 10% FBS. HepG2 cells were plated on 60mm cell-culture dishes (Corning) so as to have 2X10^{6} cells per dish. The cells were then infected with either EL- or LacZ-Ad in DMEM without FCS for 2 hours. After 2 hours the medium was removed and replaced with fresh DMEM with FCS for the next 20 hours. The cells were then incubated with 2.5 mg human HDL protein per dish. The HDL was incubated with cells for 16 hours after which the medium was collected into centrifuge tubes. Density was adjusted with KBr to 1.24g/ml. HDL was re-isolated by centrifuging at 100,000 rpm for 4 hours. The HDL band was collected and desalted by gel filtration through Sephadex PD-10 columns (Amersham Biosciences), and concentrated using vivaspin columns (Sartorious). The HDL was layered with argon

and used fresh or stored at -70 °C.

2.9. Organ chamber experiments

Organ chamber experiments were performed as described in materials and methods Chapter I (2.3).

2.10. Nitrite Measurements

Nitrite was measured by HPLC and was performed as described in materials and methods Chapter I (2.7).

2.11. SDS-PAGE and Western Blotting

Ten µg of the HDL protein was mixed with equal volume of SDS sample buffer (5 % β-mercaptoethanol added freshly) and boiled at 95 °C for 5-10 min. 12 % SDS polyacrylamid gel separating gel and stacking gel were prepared. Running buffer was added on the top of the assembly and in the tank. Boiled samples were loaded onto the stacking gel and run at 150 V for 1.5 h (SDS-PAGE). Transfer was done using PVDF membrane. The transfer was performed at 150 mA for 90 min. Protein transfer was confirmed by coomassie staining. The membrane was blocked with 10 % skim milk for 1 hour at room temperature. Thereafter, primary antibody (Cell Signalling) in a 1:1000 dilution in 10 % skim milk was incubated with the membrane at 4 °C overnight. The membrane was washed for 1 hour with the washing buffer. Then, the membrane was incubated with the secondary antibody (Dako, Denmark) in a 1:5000 dilution for 2 h at room temperature. The membrane was incubated with super ECL mixture (Amersham Bioscience, Piscataway, USA) and developed by placing in chemi doc instrument (Biorad). Image doc software was used for performing densitometry.

Statistical Analysis

Data are expressed as mean ± SEM. The significance of the difference between group means for multiple comparisons was analyzed by two-way analysis of variance and

the Bonferroni-post test for tissue relaxation experiments using myography. Lipid parameters and densitometry were compared by student's T-test. Values of $P<0.05$ (*), $P< 0.01$ (**), and $P< 0.001$ (***) were taken as statistically significant. Statistical analysis was performed by Prism Version 4.0 (GraphPad Software, USA)

3. Results

3.1. Adenovirus-mediated EL over-expression in mice causes dose-dependent decrease in serum cholesterol levels

Compared with LacZ-control mice EL overexpression decreased total cholesterol serum levels in mice in a virus dose-dependent manner, 48 h after adenovirus injection. Considering the major portion of cholesterol in mice being accommodated in HDL, the decrease in cholesterol was indicative of decreased HDL.

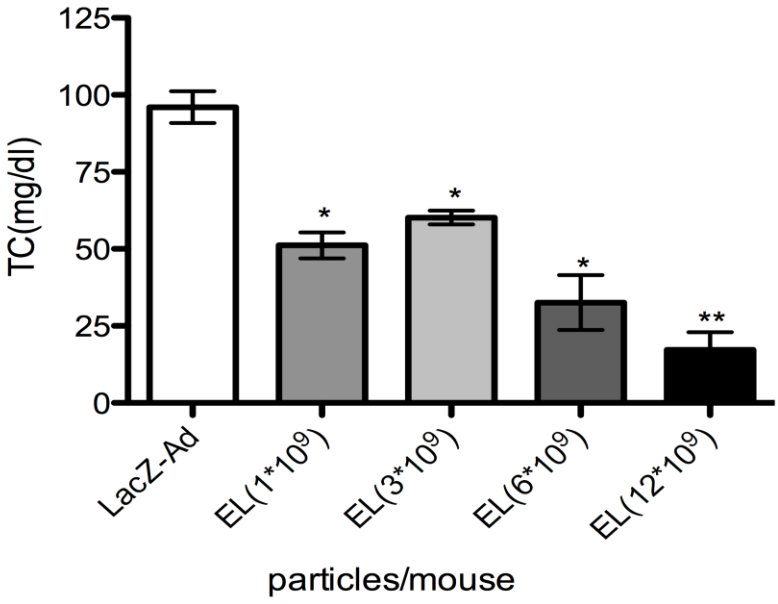


Figure.5. Dose –dependent decrease in cholesterol levels in mice over-expressing EL using adenovirus

Mice were injected with LacZ-Ad ($2.4X10^9$) particles/mouse or different doses of EL-Ad (calculated as active virus particles/ml). Increasing concentrations of EL-Ad caused corresponding decrease in cholesterol levels. N=4 mice for each concentration, values were compared by student's T-test should have been Anova because multiple comparison. Values of $P<0.05$ (*), $P< 0.01$ (**), and $P< 0.001$ (***) were taken as statistically significant.

3.2. Effect of EL over-expression on ACh-induced vasorelaxation

The decrease in HDL levels could have some functional implications. It is known that HDL in addition to RCT has a number of other positive functions. One of those is to induce nitric oxide (NO) production, thereby promoting vasorelaxation. We wanted to

study if HDL depletion in EL-Ad overexpressing mice caused any changes in vascular reactivity and the response of tissues to ACh. To examine the impact of EL over-expression on vascular reactivity, mice were injected with adenoviruses encoding EL or LacZ (control) and aortas were isolated after 48 hours. Aortas were cut into rings and pre-contracted with norepinephrine (NE) followed by relaxation with cumulative concentrations of ACh. As shown in Fig. 6 EL over-expression decreased the ACh-induced vasorelaxation of the aortic rings when compared with LacZ treated mice.

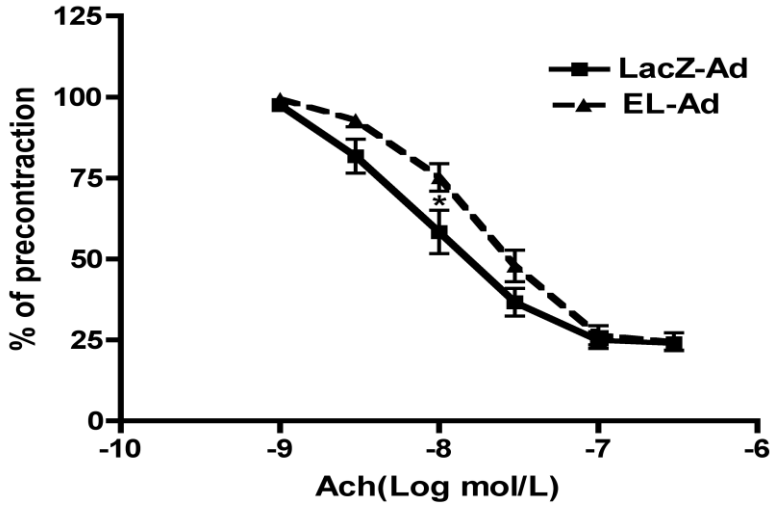


Figure.6. EL overexpression in mice impairs vascular reactivity

Mice (C57Bl6; male 10-12 week old) were injected with EL- or LacZ-adenovirus. Aortic rings were collected after 48 h and used for the measurements of ACh-induced vasorelaxation. Results are mean ± SEM of measurements with n=12 rings of n=4 mice. P value indicates statistical comparison via 2-way ANOVA and post hoc t test with Bonferroni correction.

3.3 Effect of EL over-expression on SNP-induced vasorelaxation

EL –Ad overexpressing aortic rings had impaired vasorelaxation as compared to the LacZ controls. To elucidate weather the observed impairment of relaxation was endothelium dependent or due to reduced functionality of the smooth muscle cells, we relaxed NE precontracted tissues with sodiumnitropruside (SNP). SNP is a nitric oxide donor and directly causes smooth muscle cell relaxation independent of the endothelial cell release of NO. As shown in the figure both EL – and LacZ-Ad overexpressing aortic tissues relaxed to a similar extent. This indicates that EL-overexpression causes endothelium dependent impairment of tissue vasorelaxation.

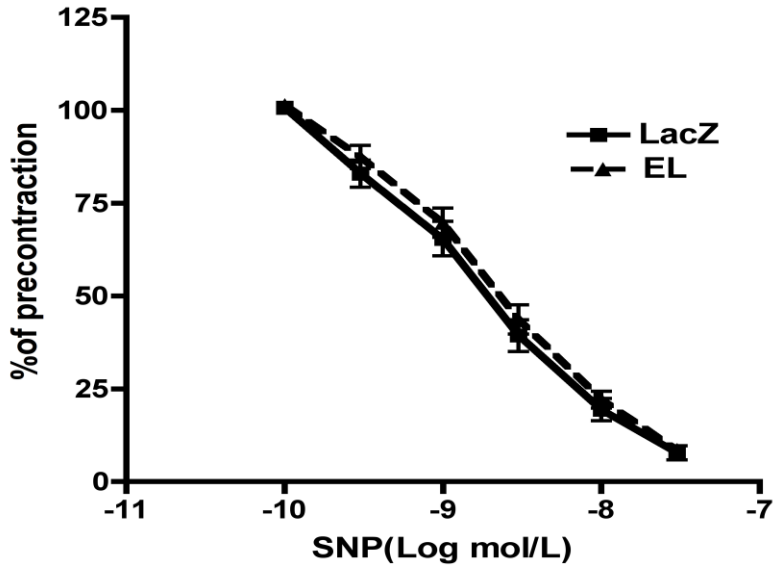


Figure.7. EL mediated impaired vasorelaxation is an endothelium dependent effect

Mice (C57Bl6; male 10-12 week old) were injected with EL- or LacZ-adenovirus. Aortic rings were collected after 48 h and used for the measurements of SNP-induced vasorelaxation. Results are mean ± SEM of measurements with n=12 rings of n=4 mice. P value indicates statistical comparison via 2-way ANOVA and post hoc t test with Bonferroni correction.

Attenuated vasorelaxation in rings from EL overexpressing mice led us to assume that either decreased HDL serum levels or decreased functionality of EL-modified HDL or

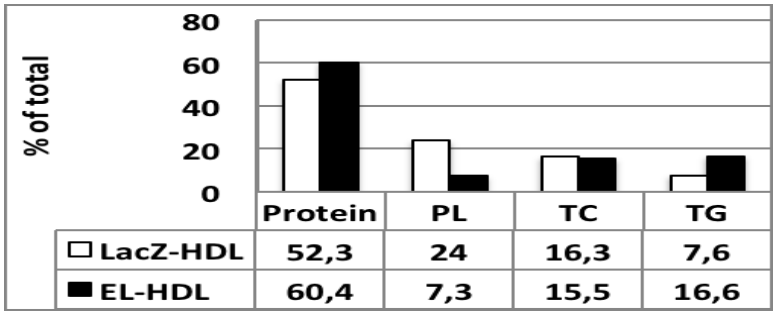
both might be the reason for observed effects. Therefore we decided to examine the composition and functionality of mouse and human EL-HDL.

3.4. Impact of EL on structure, composition and functionality of HDL

3.4.1. Composition of mouse EL-HDL

Mice were injected with EL- or LacZ–Ad, followed by isolation of HDL from serum after 48 hours. The lipid parameters and protein content were measured in the HDL using kit- based assays. The HDL composition revealed a decrease in choline-containing phospholipid content (PC and SM) of EL-HDL as compared to control as well as increased relative TG content (Fig. 8A). As shown in Fig. 8B. the mass spectrometry analysis revealed that the content of all PC species was significantly lower in EL-HDL as compared with control HDL. It is known that EL cleaves HDL to generate one molecule of free fatty acid and one molecule of LPC. The LPC content of EL-HDL was also quantified by mass spectroscopy. As shown in Fig. 8C. LPC were decreased in EL-HDL arguing against accumulation of LPC in EL-HDL. This excludes the possibility that EL-generated LPC associated with HDL are responsible for dysfunctionality of EL-HDL.

A



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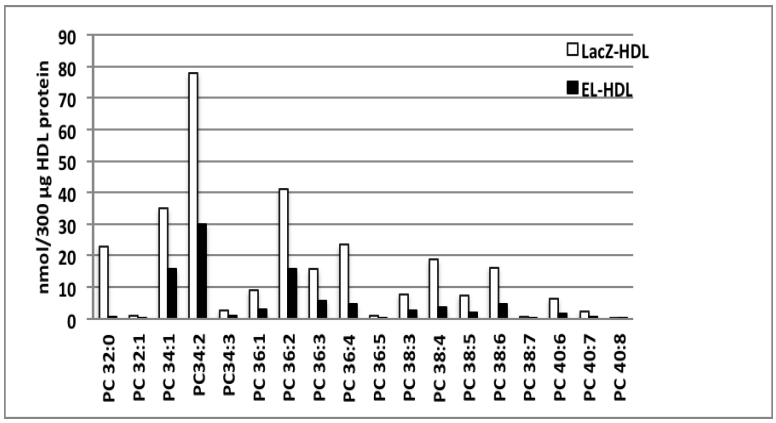

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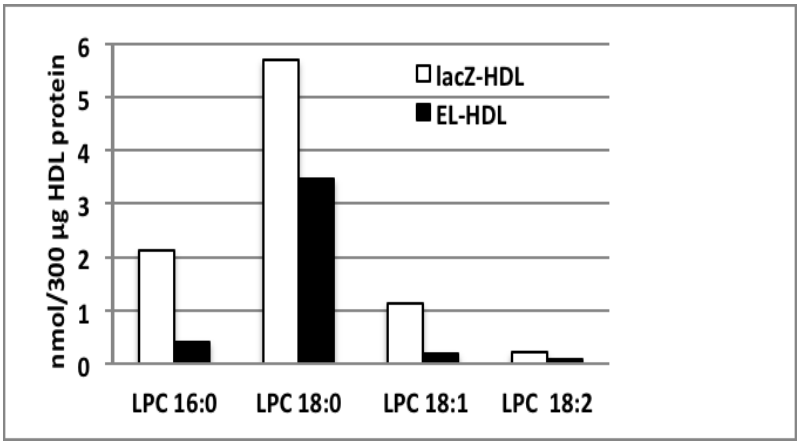

Figure.8. Composition of EL-HDL compared to LacZ-HDL

HDL obtained from EL- and LacZ-Ad overexpressing mice was used to detect differences in composition. The composition of EL-HDL was measured using kits, expressed as a relative abundance of each component (A). The PC (B) and LPC (C) content of LacZ- and EL-HDL were measured by mass spectrometry.

3.4.2. Unaltered size of mouse EL-HDL

A difference in the composition of EL-HDL prompted us to study the size of HDL particles as well. A non- denaturing gradient gel electrophoresis was performed to determine size of HDL particles. As shown in Fig. 9 no change in particle size was observed as compared to control. This suggests that in EL-HDL, despite of PC-depletion the HDL still retained its original size.

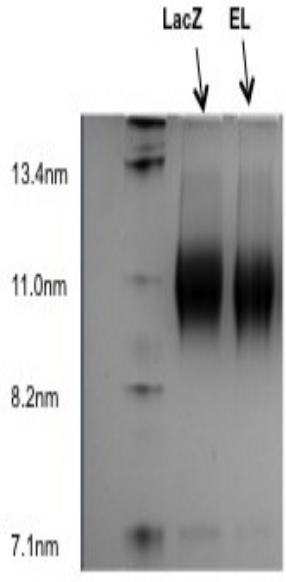

Figure.9. Non- denaturing polyacrylamide gel electrophoresis of LacZ – and EL-HDL
HDL was isolated from serum of EL-Ad or LacZ-Ad injected mice by ultracentrifugation procedure. HDL protein (15μg) was loaded on a native gel. Proteins were stained with coomassie. EL–HDL particles appeared to be of about the same size as LacZ-HDL.

3.4.3. Vasorelxing capacity of mouse EL-HDL

From results shown above it was clear that EL alters composition of HDL, with decreased PC content as a hallmark of that compositional alteration. Maintenance of the normal vascular tone and the capacity of blood vessels to undergo relaxation upon physiological stimuli is a prerequisite for vascular health. It is well established that HDL increases bioavailability of NO, a potent vasorelaxing factor. The HDL-mediated promotion of NO production has been shown to be accomplished by endothelial nitric oxide synthase (eNOS) in SR-BI-, S1P receptor- and ABCG1-dependent manner (132-134). Importantly, in humans, HDL plasma levels are correlated with flow-mediated vasodilation responses of the brachial artery (127) and in general, endothelium-dependent vasorelaxation is directly associated with HDL levels (130). Because our previous study showed that EL modification of HDL attenuates interaction of HDL with SR-BI, an important mediator of HDL –induced vasorelaxation, we tested the capacity of EL-HDL to promote relaxation of mouse aortic rings precontracted with NE. As shown in Fig 10, compared with a profound relaxation induced by LacZ-HDL, the capacity of EL-HDL to evoke tissue relaxation was dramatically reduced.

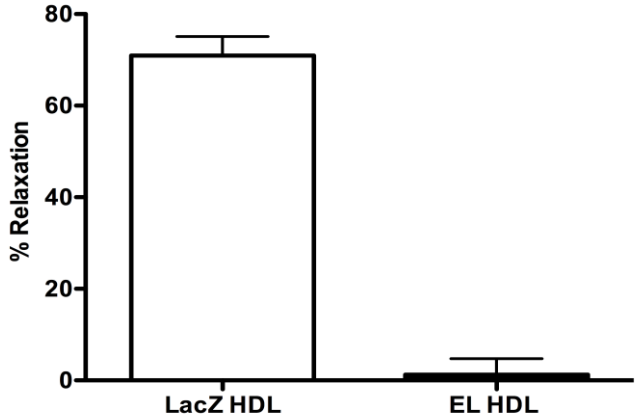

Figure.10. Mouse EL-HDL exhibits attenuated vasorelaxing capacity

Mouse aortic rings were precontracted with NE followed by addition of EL- or LacZ-HDL (250µg protein per ring). EL-HDL failed to produce significant relaxation of tissues as compared to the LacZ control. N=2 rings for each condition.

3.4.4. Arylesterase activity of mouse EL-HDL

A recent study showed attenuated vasorelaxing capacity, decresed PON1- and increased manolndialdehyde (MDA)-content of HDL isolated from patients with coronary artery disease (131) argues for PON1 being a crucial component of HDL required for vasorelaxing capacity of HDL. Therefore, we examined whether arylesterase activity, that reflects partially the activity of PON1 is altered in EL-HDL; this might at least in part explain attenuated vasorelaxing capacity of EL-HDL. As shown in (Fig. 11) EL-HDL exhibited reduced arylesterase activity as compared to control HDL, strongly suggesting that decreased PON1 in EL-HDL contributed to the observed decreased vasorelaxing capacity of EL-HDL.

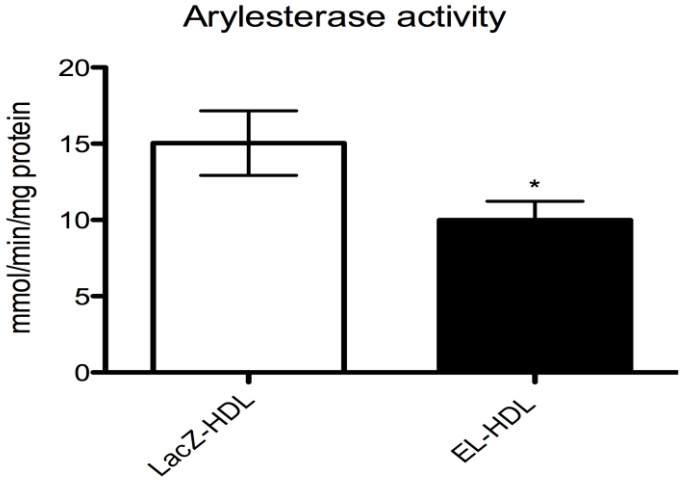

Figure.11. Mouse EL-HDL has reduced arylesterase activity as compared to control HDL

HDL (10µg protein) obtained from serum of EL-and LacZ-Ad overexpressing mice was used to perform arylesterase activity assay. All measurements were performed in triplicates. Values were compared by student's T-test. Values of $P<0.05$ (*), $P< 0.01$ (**), and $P< 0.001$ (***) were taken as statistically significant.

3.5. Structural and functional properties of human EL-HDL

To study the impact of EL on the functionality of human HDL and to mimic at least in part EL modification of HDL *in vivo*, human HDL isolated from plasma of healthy male volunteers was incubated with HepG2 cells infected with EL- or LacZ-Ad. This was performed in DMEM without FCS, under cell culture conditions for 16 hours. HDL was then reisolated from conditioned media by one- step ultracentrifugation as mentioned in the methods section and designated as human EL- or LacZ-HDL.

3.5.1. Composition of human EL-HDL

The lipid parameters and protein content were measured using kit- based assays. The measurements revealed a decrease in relative phospholipid content and an increase in the TC content of EL-HDL as compared to control.

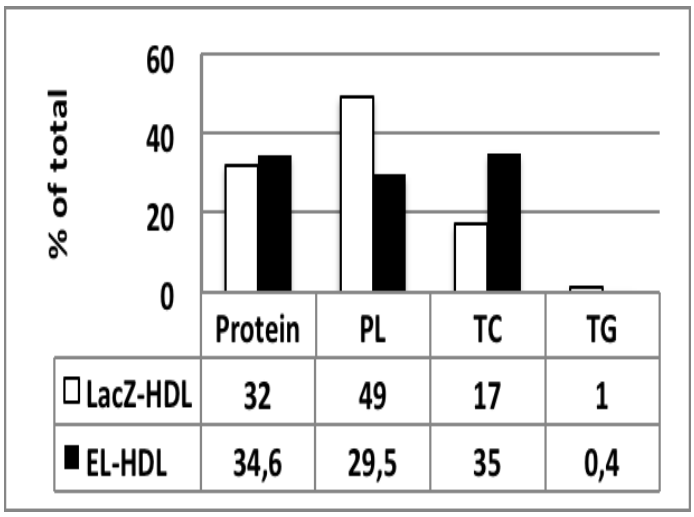

Figure.12. Composition of human EL-HDL

The mass of HDL-lipids and protein was measured by appropriate kits and the relative abundance of each constituent expressed as % of total mass.

3.5.2 Particle size of *in vitro* generated EL-HDL

A non- denaturing gradient gel electrophoresis was performed to determine size of HDL particles. A shift in particle size of both HDL_2 and HDL_3 was observed in EL-HDL, accompanied with appearance of bands smaller than 8.2 nm.

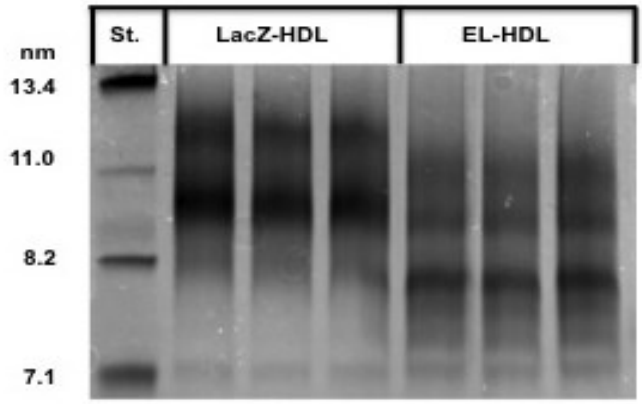

Figure.13. Non- denaturing polyacrylamide gel electrophoresis of human LacZ – and EL-HDL

HDL protein (10µg) was loaded on a native gel. Proteins were visualized upon staining with Coomassie as described in Methods.

3.5.3. Vasorelaxing capacity of human EL-HDL

To examine the impact of EL-induced compositional alterations of HDL we tested the capacity of human EL-HDL to relax precontracted mouse aortic rings. As shown in Fig. 14, the vasorelaxing capacity of human EL-HDL was markedly decreased when compared with control human LacZ-HDL

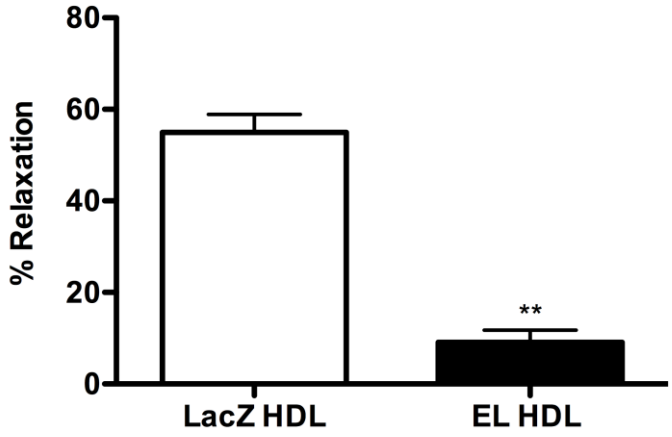

Figure.14. EL-HDL

Mouse aortic rings were precontracted with NE followed by addition of EL- or LacZ-HDL (250µg protein/5 ml buffer/ ring). EL-HDL failed to produce significant relaxation of tissues as compared to the LacZ control. N=2 rings for each condition.

3.5.4. Arylesterase activity of human EL-HDL

To further examine whether in human, like in mouse EL-HDL PON1 activity is decreased in comparison to control human LacZ-HDL, arylesterase activity was evaluated for human EL- and LacZ-HDL. Arylesterase activity of human EL-HDL was decreased compared with control HDL, however, the difference did not reach statistical significance.

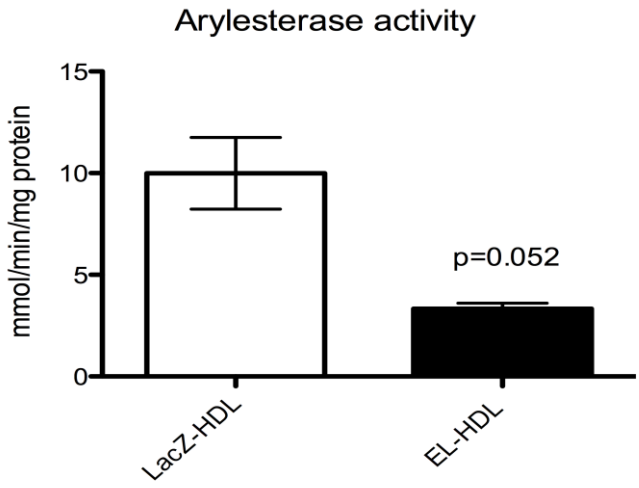

Figure.15. Human EL-HDL has reduced arylesterase activity

HDL (10µg protein) was used to perform arylesterase activity assay. All measurements were performed in triplicates. Values were compared by student's T-test. Values of $P<0.05$ (*), $P< 0.01$ (**), and $P< 0.001$ (***) were taken as statistically significant.

3.5.5. apoAI-, apoM- and PON1- content in human EL-HDL

ApoAI is the major protein associated with HDL. It is crucial for the normal HDL function (182). ApoAI is essential for binding to SR-BI, which is considered as the first step for eNOS activation (133). ApoM and PON1 are known as essential antioxidants associated with the HDL particle and are largely responsible for antioxidant properties to HDL (183).

As revealed by Western blot, EL-HDL contained markedly lower amounts of apoM- and PON1- and slightly decreased apo AI- as compared with LacZ-HDL. This implies

that attenuation of vasorelaxing capacity of human EL-HDL might be at least in part due to decreased content of those proteins.

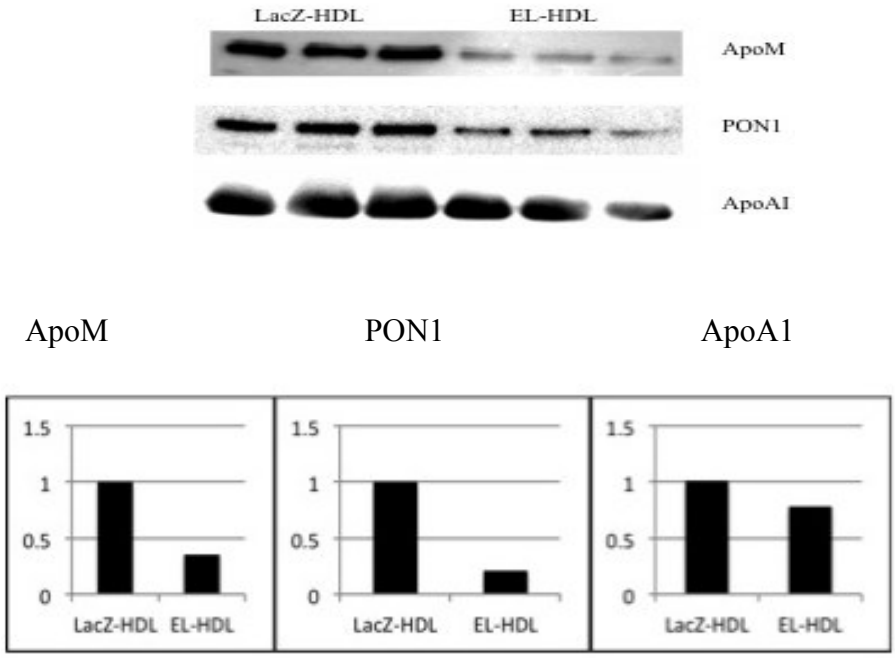

Figure.16. Human **EL-HDL has reduced amount of ApoM and PON1 proteins**

10µg HDL protein was allowed to separate on an SDS-PAGE gel. Proteins were transferred on PVDF membranes followed by incubating with respective antibodies and developing the blot. ApoM and PON1 are significantly depleted in EL-HDL as compared to LacZ-HDL. The blot is a representative of three experiments. The densitometry is done using the representative blot.

4. Discussion

We found in mice overexpressing human EL by use of adenoviruses a marked decrease in total cholesterol plasma levels. This is in accordance with the fact that EL cleaves HDL-PL thereby reducing HDL content, a principal carrier of cholesterol in mice (184, 185). HDL is known to be an important determinant to maintain the vascular tone by inducing the release of NO. We observed that aortas of EL overexpressing mice were unable to relax as efficiently to ACh as compared to their LacZ controls. This reduced relaxation to ACh could be due to: i) the EL-mediated decrease in HDL levels due to EL overexpression, ii) the generation of cleavage products of HDL which might have an adverse effect on ACh mediated relaxation (127, 132, 186), or iii) the alterations in structural and functional properties of HDL. Attenuated relaxation in aortic rings from EL-overexpressing mice was found to be an endothelium dependant effect as the aortic tissues from EL-overexpressing mice relaxed to the same extent as tissue from control LacZ-mice, in response to NO donor, SNP. It is important to stress that adenoviral overexpression leads to EL overexpression almost exclusively in the liver. We found by RT-PCR no human EL mRNA in aortic preparations. Accordingly, the effect of EL overexpression on aortic tissue is a systemic one. Studies using cultured endothelial cells have shown that HDL positively modulates eNOS levels. Both apoAI and SR-BI were shown to be colocalized with eNOS, which is associated with increased NO production. We found that mouse EL-HDL had significantly depleted phospholipid content (Fig. 8A) without the accumulation of LPC within the HDL particle (Fig. 8C). ApoAI transgenic mice overexpressing EL showed large reduction in PL/apoAI ratio (about -60%), total cholesterol (-89%) and HDL cholesterol (-91%) as compared to serum before EL overexpression. SR-BI mediated cholesterol efflux capacities of serum from EL overexpressing ApoAI transgenic mice were found to be reduced by 90% while ABCAI mediated efflux increased by 63%. The decrease in SR-BI mediated cholesterol efflux was due to more than 91% decrease in HDL cholesterol, whereas in case of ABCA1 mediated increased cholesterol efflux was due to formation of small pre beta HDL that are easily lipidated by ABCA1 (166). In vivo overexpression of human EL by means of recombinant adenovirus resulted in generation of small pre-HDL particles in wild- type mice, detected by analysis of mouse plasma by both HPLC and agarose gel electrophoresis followed by Western blot for detection of apo

AI (177). In contrast, we did not observe any change in EL-HDL particle size analysed by a native gel. However, because we analyzed HDL and not plasma of EL-overexpressing mice it is possible that smaller HDL particles were formed but we lost them during HDL preparation due to their higher density compared with collected EL-HDL (Fig. 9). The effect of EL on HDL can be different when performed *in vitro* and *in vivo* in mice. One can speculate that in mice EL could cause decrease in HDL particle size but a number of smaller HDL particles could cluster together to form normal sized HDL particles as observed on the native gel. In a study when EL was inhibited using antibodies, HL-/- and human apoAI transgenic mice produced larger HDL particles, which were rich in phospholipids (159). The same was also observed in EL-/- mice (160). In the present study, EL-HDL isolated from mice was applied on pre-contracted mouse aortic rings in myography to test its vasorelaxing capacity. EL-HDL had markedly attenuated capacity to induced tissue relaxation in comparison to LacZ-HDL used as a control (Fig. 10). This led us to conclude that EL cleavage of HDL phospholipids, causes the HDL to undergo certain structural and functional alterations that renders it dysfunctional. There are various recent studies that show that due to inflammation and disease conditions HDL can turn dysfunctional and could also have pro-atherosclerotic functions. The fact that HDL can turn dysfunctional was proved in patients of coronary artery disease (CAD) (131). These patients, despite of having similar HDL levels as normal subjects had decreased anti-inflammatory properties, and diminished endothelial protective effects. HDL from CAD-patients had also increased MDA levels and decreased PON1 levels thereby rendering HDL pro-atherogenic (131). Another study with HDL from CAD patients showed that this HDL caused activation of endothelial pro-apoptotic pathways instead of being anti-apoptotic (140). This was due to proteome remodeling of HDL where HDL associated clusterin levels were decreased and apoC-III levels were elevated (140). Since HDL has specific antioxidant properties we measured the arylesterase activity and found it was significantly reduced in case of EL-HDL from mice (Fig. 11). This supports our myography data. Nitrite levels, indicative of NO production, measured in buffer obtained after treatment of mouse aortic rings with EL or LacZ-HDL did not produce conclusive results (data not shown). It still remains to be elucidated whether the impaired relaxation due to EL-HDL is due to the inability of HDL to induce eNOS and eventually NO production or the impaired relaxation is due to some other mechanisms.

We employed HepG2 cells to test the impact of EL on human HDL. Similar to our mouse data we found that human EL-HDL had significantly depleted phospholipid content (Fig. 12). The most striking difference that we observed was that on a native gel human EL-HDL produced by incubation with EL-overexpressing HepG2 cells was smaller in size than control human LacZ-HDL. Organ bath experiments revealed that human EL-HDL behaved comparable to mouse EL-HDL in terms of vasorelaxing capacity (Fig. 14). The arylesterase activity of EL-HDL was also significantly lower as compared to the control HDL. The HDL associated apolipoproteins are essential for the function of HDL. ApoAI is the major protein associated with HDL. It is crucial for the normal HDL function (182). ApoAI is essential for binding to SR-BI, which is considered as the first step for eNOS activation (133). ApoM and PON1 are known as essential antioxidants associated with the HDL particle and confer important antioxidant properties to HDL as such (183). By western blotting we observed a pronounced decrease in, apoM and PON1 proteins associated with human EL-HDL (Fig. 16), which may be responsible for the reduced capacity of human EL-HDL to produce tissue relaxation. About 65% of plasma S1P is associated with HDL and 35% with albumin (187). It is shown that S1P that is associated with HDL can cause aortic tissue relaxation via Akt activation followed by NO release (134). ApoM mainly resides in the plasma HDL fraction (188). ApoM is a major carrier for S1P and is required for delivering S1P to the SIP1 receptors on the endothelium to have its vasculoprotective effect (189). It is possible that in EL-HDL due to reduced apoM levels the levels of S1P are also diminished thus being responsible for diminished ability of EL-HDL to relax precontracted aortic rings. There are also studies showing that ABCG1 and HDL maintain endothelial cell function in mice fed with high cholesterol diet, by promoting cholesterol efflux and preserving active eNOS dimer levels (132). Further studies need to be carried out to determine the exact mechanisms responsible for attenuated vasorelaxing capacity of EL-HDL.

From the results that we have obtained so far we can conclude that EL modification of HDL *in vivo* and *in vitro* alters HDL lipid and protein composition thereby attenuating its vasorelaxing capacity.

5. References

Chapter I

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