Aus dem Institut für Klinische Chemie und Laboratoriumsmedizin
der Westfälischen Wilhelms-Universität Münster
Direktor: Univ.-Prof. Dr. med. Gerd Assmann, FRCP

Interactions between HDL and NAD(P)H oxidase

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Alicja Pawlak
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1. Berichterstatter: Priv. Doz.-Dr. Med. J. R. Nofer
2. Berichterstatter: Prof. Dr. rer. nat. Beate Kehrel

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ZUSAMMENFASSUNG

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Voraussetzungen und Ziel der Arbeit: Die Arteriosklerose gehört zu den häufigsten Krankheiten in den Industriestaaten. Die unkontrollierte Produktion von Sauerstoffradikalen (Reactive Oxygen Species-ROS), denen die Hyperaktivierung der NAD(P)H Oxidase zugrunde liegt, spielt in der Entwicklung und Progrendienz der Arteriosklerose eine entscheidende Rolle. Sie ist u. a. für die Sekretion oder die Aktivierung von pro-atherogenen Faktoren wie MCP-1 (monocyte chemoattractant protein-1), MMP (matrix metalloproteinases) und $p38^{MAPK}$ (p38 mitogen-activated protein kinase) verantwortlich. HDL (high density lipoprotein) erfüllt verschiedene potentielle anti-atherogene Funktionen. Mehrere dieser Funktionen sind durch die Präsenz von Lysosphingolipiden wie Sphingosylphosphorylcholin (SPC) und Sphingosin-1-phosphat (S1P) in HDL-Partikeln, bedingt. Die Wechselwirkungen zwischen der durch die NAD(P)H Oxidase vermittelten ROS Generierung und HDL wurden bisher nur unzureichend untersucht. Es war daher Ziel dieser Arbeit, den Einfluss von HDL und HDL-Lysopshingolipiden auf die Aktivierung der NAD(P)H Oxidase, die davon abhängige Produktion von ROS, die Synthese von MCP-1 und die Aktivierung von MMP`s und von $p38^{MAPK}$ zu untersuchen.
Methodik: Alle Experimente wurden an kultivierten glatten Muskelzellen der Ratte durchgeführt. Die Entstehung von ROS wurde mittels Fluoreszensspektrometrie untersucht. Die Konzentration von MCP-1 und der Aktivierungsgrad von $p38^{MAPK}$ wurden mit Hilfe von geeigneten ELISA`s untersucht. Die Expression von *mcp-1* auf der Ebene der Transkription wurde mit Hilfe der RT-PCR untersucht. Die Aktivität der MMP`s wurde unter Verwendung der Gelatine-Zymographie bestimmt. Zur Aktivitätsbestimmung der NAD(P)H Oxidase wurde ein spektrophotometrischer Assay verwendet.
Ergebnisse: Es konnte gezeigt werden, dass die Thrombin-induzierte Aktivierung der NAD(P)H Oxidase und die Generierung von ROS in glatten Muskelzellen in Anwesenheit von HDL und HDL-Lysosphingolipiden gehemmt wird. Folglich wurde die Thrombin-induzierte Aktivierung von $p38^{MAPK}$ und die davon abhängige Produktion von MCP-1 durch HDL und HDL-Lysosphingolipiden inhibiert. Es konnte dagegen keine hemmende Wirkung von HDL und HDL-Lysosphingolipiden auf die Aktivierung von MMP`s beobachtet werden.
Schlussfolgerung: Die in der vorliegenden Arbeit erzielten Ergebnisse zeigen erstmalig, dass HDL und HDL-Lysosphingolipide die Aktivierung der NAD(P)H Oxidase und die davon abhängigen pro-atherogenen Prozesse hemmen. Diese Wirkung von HDL kann substantiell zu anti-atherogenen Effekten beitragen, die diesen Lipoproteinen zugeordnet werden.

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SUMMARY

Interactions between HDL and NAD(P)H oxidase

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Working hypothesis: Atherosclerosis is one of the most common causes of death in industrial societies. The NAD(P)H oxidase-dependent generation of reactive oxygen species (ROS) plays an important role in development and progression of atherosclerosis. It induces the synthesis and/or activation of several pro-atherogenic factors such as monocyte chemoattractant protein-1 (MCP-1), p38 mitogen-activated protein kinase ($p38^{MAPK}$) or matrix metalloproteinases (MMPs). High density lipoprotein (HDL) has been demonstrated to exert several potentially anti-atherogenic effects, which are partially mimicked by lysosphingolipids associated with these lipoproteins, namely sphingosylphosphorylcholine (SPC) and sphingosine 1-phosphate (S1P). Little effort has been devoted to date to characterize the interaction between HDL and NAD(P)H oxidase. Therefore, it was the aim of the present study to investigate the effects of HDL on the agonist-triggered induction of NAD(P)H oxidase and related pro-atherogenic processes.

Methods: All experiments were performed on cultivated rat vascular smooth muscle cells (VSMCs). The ROS generation was determined by fluorescence spectroscopy. Quantitative determination of MCP-1 concentration and $p38^{MAPK}$ activity was accomplished using ELISA. The *mcp-1* gene expression was determined with a help of RT-PCR. The MMPs activity was detected by substrate gel zymography. The activity of NAD(P)H oxidase was measured with a help of spectrophotometric kinetics assay.

Results: The present results demonstrate, that the thrombin-induced NAD(P)H oxidase activation and ROS generation were inhibited in rat VSMCs in the presence of HDL or HDL-lysosphinolipids. Moreover, HDL and HDL-lysosphingolipids inhibited the thrombin-induced $p38^{MAPK}$ activation and MCP-1 production. By contrast, no effect of HDL on the thrombin-induced MMPs activation could be observed.

Conclusions: This work for the first time demonstrates, that HDL exerts inhibitory effects on the NAD(P)H oxidase and related pro-atherogenic processes. These effects may essentially contribute to anti-atherogenic properties attributed to this class of lipoproteins.

Contents

1. Abbreviations

A-II	angiotensin-II
AA	arachidonic acid
ABCA1	adenosine triphosphate-binding cassette protein A1
ACE	angiotensin II-converting enzyme
ACh	acetylcholine
ADP	adenosine diphosphate
AGEs	advanced glycation end-products
Akt	serine-threonine kinase
apo	apolipoprotein
AT_1	angiotensin II type 1 receptor
ATP	adenosine triphosphate
BAD	Bcl-2 associated death promoter
cAMP	cyclic adenosine monophosphate
CCR2	chemokine receptor 2
cDNA	complementary DNA
CETP	cholesterol ester transfer protein
cGMP	cyclic guanosine monophosphate
CHD	coronary heart disease
CMV	cytomegalovirus
CNP	C-type natriuretic peptid
COX-2	cyclooxygenase-2
CRP	C-reactive protein
DDR1	discoidin domain receptor 1
DHE	dihydroethidium
DNA	deoxyribonucleic acid
DPI	diphenylene iodinium
e.g.	exempli gratia = for instance
EDG	endothelial differentiation genes
EL	endothelial lipase

ELISA	Enzyme-Linked Immunosorbent Assay
ERK1/2	extracellular signal-regulated kinase 1/2
ET	endothelin
et al.	et alii = and others
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GTP	guanosine triphosphate
h	hour / hours
HB-EGF	heparin-binding epidermal growth factor
HDL	high density lipoprotein
HDL-C	HDL-cholesterol
HL	hepatic lipase
HMG Co	3-hydroxy-3-methylglutaryl coenzyme
H_2O_2	hydrogen peroxide
HUVECs	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IDL	intermediate density lipoprotein
IFN-γ	interferon γ
IGF-1	insulin growth factor-1
IκB	NF-κB inhibitor
IKK	NF-κB inducing kinase
IL-1	interleukin-1
JNK	c-Jun N-terminal kinase
L	Liter
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
Lp (a)	lipoprotein (a)
LPC	lysophosphatidylcholine
LPS	bacterial lypopolysaccharide
LSF	lysosulfatide
m	mili

MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
M-CSF	monocyte-colony stimulating factor
min	minute / minutes
MMPs	matrix metalloproteinases
mRNA	messenger RNA
µ	mikro
NADH	nicotamide adenine dinucleotide
NADPH	nicotamide adenine dinucleotide phosphate
NAD(P)H oxidase	nicotamide adenine dinucleotide phosphate oxidase
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
NOX	non-phagocytic NAD(P)H oxidase proteins
O_2^*	superoxide radical
OH^*	hydroxyl radical
OONO*	peroxynitrite
oxLDL	oxidized low density lipoprotein
$p38^{MAPK}$	p38 mitogen-activated protein kinase
PAF	platelet-activating factor
PAF-AH	platelet-activating factor-acetylhydrolase
PAI-1	inhibitor plasminogen activator inhibitor-1
PARs	protease-activated receptors
PDGF	platelet derived growth factor
PECAM	platelet-endothelial cell-adhesion molecule
PGE_2	prostaglandin E_2
PGI_2	prostacyclin 2
PhD	Philosophiae Doctor
PI3K	phosphatidylinositol 3-kinase
PI-PLC	phosphatidylinositol-specific phospholipase C
PKA	protein kinase A

PKC	protein kinase C
PKD	protein kinase D
PLTP	phospholipid transfer protein
PMA	phorbol 12-myristate 13-acetate
PON	paraoxonase
RANTES	regulated on activation, normal T-cell expressed and secreted chemokine
RAS	renin-angiotensin system
RCT	reverse cholesterol transport
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction analysis
SOD	superoxide dismutase
S1P	sphingosine 1-phosphate
SPC	sphingosylphosphorylcholine
SR-B 1	scavenger receptor B 1
src	tyrosine kinase
TF	tissue factor
TGF-β	transforming growth factor β
Thr	thrombin
TIMPs	tissue inhibitors of metalloproteinases
TNF-α	tumor necrosis factor α
tPA	tissue plasminogen activator
U	Units
w/v	weight per volume
VCAM-1	vascular adhesion molecule-1
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein
VSMCs	vascular smooth muscle cells
v/v	volume per volume

2. Introduction

2.1. Atherosclerosis: definition and pathogenesis

The earliest lesion of atherosclerosis is thought to be the fatty streak, which has its origin in an injury to the endothelium (response to injury hypothesis) [122]. Such an injury may be mechanical in nature (e.g. as a result of turbulent blood flow or high blood pressure), it may be caused by various chemicals such as those present in cigarette smoke [121]. Cells of the circulating blood, in particular T cells and monocytes adhere to the area of endothelial injury and then migrate through the endothelium into the sub-endothelial space [28]. There, they interact with the cells of arterial wall, in particular the smooth muscle cells, which undergo proliferation. Once the sub-intimal space, the monocytes mature to macrophages, which begin to ingest lipids and lipoprotein particle strapped in this location. In this process, the macrophages become foam cells and the fatty streak forms [41, 94]. At a later stage, platelets also adhere to the site of the incipient atherosclerotic plaque. The changes are shown schematically in Fig. 1 [149].

The processes described above are regulated by complex network of cytokines and growth factors [125]. Thus, atherosclerosis can be envisioned as a chronic inflammatory fibroproliferative process, which has become excessive [125].

"INJURY"

(mechanical, oxLDL)

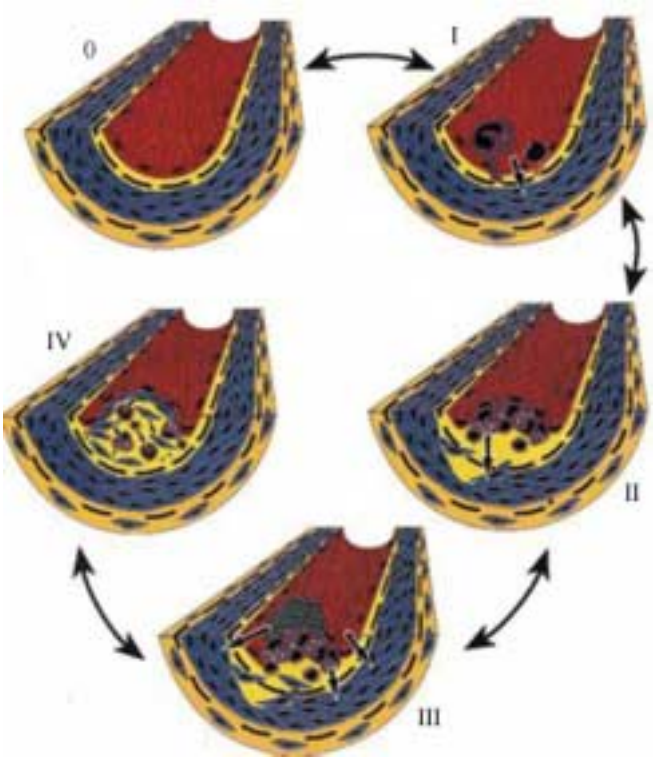


Figure 1. The response – to - injury hypothesis of atherosclerosis.
Several sources of injury to the endothelium can lead to endothelial dysfunction. Adherence of monocytes/macrophages is increased (I). These cells may migrate to subendothelial location (II). The macrophages become large foam cells because of lipid accumulation (III), and together with T cells and smooth muscle cells, form fatty streak (IV). The fatty streaks can progress to fibrofatty plaques and ultimately to fibrous plaques. Macrophages may lose their endothelial cover and platelet attachment may occur (III). At each step of lesion formation, growth factors and cytokines are released form macrophages and endothelial cells. Cells have been colour coded as follows: smooth muscle cells-blue; endothelium-red; macrophages-violet; platelet-green.

2.2. Role of cells in pathogenesis of atherosclerosis

2.2.1. Endothelial cells

Endothelial cells maintain the vascular tone by release of vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2), and vasoconstrictors such an endothelin (ET) and angiotensin-II (A-II) [40]. The endothelium also regulates the clothing status of the blood by producing anti-coagulants (heparin sulfate,

PGI_2, NO, and ectoADPase) and pro-coagulants (plasminogen activator, urokinase) [88]. The endothelium also forms a series of adhesive glycoproteins including intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and platelet-endothelial cell-adhesion molecule (PECAM), which are responsible for adhesion of leukocytes and platelets [135]. Finally, the endothelium is the source of a number of growth-regulatory molecules and cytokines including platelet derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor β (TGF-β), insulin growth factor-1 (IGF-1), and interleukin-1 (IL-1), which provide paracrine stimulation for neighboring smooth muscle cells and monocyte-derived macrophages [123].

2.2.2. Vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) have been found in most types of atherosclerotic lesions [124]. A number of chemotactic factors and mitogens released from neighboring cells and from VSMCs themselves can alter both the phenotype of the VSCMs, and determine migration, proliferation and synthesis of the extracellular matrix and be a source of a series of growth factors and cytokines such as PDGF, FGF, IGF-1, monocyte-colony stimulating factor (M-CSF), TGF-β, heparin-binding epidermal growth factor (HB-EGF), IL-1 and tumor necrosis factor α (TNF-α) [8, 118]. Both macrophages and VSMCs are capable of modifying lipoproteins to form oxidized low density lipoprotein (oxLDL) [139, 150]. The VSMCs are also responsible for the synthesis of several components of the extracellular matrix such as collagen (type I, II, IV, V) and proteoglycans [118].

2.2.3. Monocyte-derived macrophages and T lymphocytes

Monocyte-derived macrophages and T lymphocytes are a potential source of biologically active factors, including cytokines, growth and angiogenesis factors, hydrolytic enzymes, reactive oxygen species (ROS), and bioactive lipids [57, 147]. The macrophage can produce agents that induce monocyte, smooth muscle cell, and endothelial cell proliferation. It also produces growth inhibitors

such as interferon γ (IFN-γ), IL-1 and TGFβ [56]. Activated macrophages also secrete a series of chemotactic molecules for other monocytes and for smooth muscle cells [123]. Macrophages can oxidize LDL through the action of lipoxygenase, myeloperoxidase and nitric oxide synthase (NOS) [110]. The observation that monocyte derived macrophages express monocyte chemoattractant protein-1 (MCP-1), in addition to their known ability to secrete IL-1, TNF-α, and generate ROS, which can modify LDL, suggest a central role of these cells in regulating the inflammatory component of atherosclerosis [147] [Fig.2].

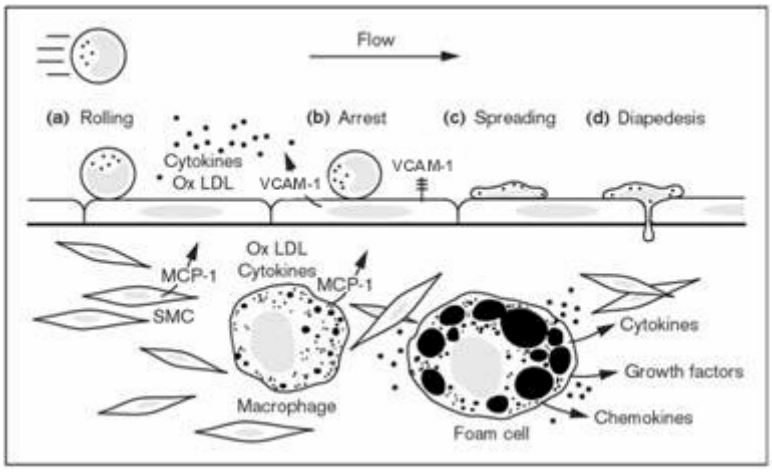


Figure 2. Cellular interactions in the initiation of atherosclerosis: monocyte-endothelial cell interactions. *Blood monocytes* ***(a)*** *roll along the vessel wall until they encounter an adhesion molecule such as VCAM-1, and then* ***(b)*** *arrest and become firmly attached to the endothelium. The monocytes then* ***(c)*** *spread out and* ***(d)*** *move into the sub-endothelial space (diapedesis), where they differentiate and take up lipids to become foam cells. Both macrophages and foam cells secrete growth factors and cytokines, such as MCP-1 and another chemokines, which might serve to set up a positive feedback loop. oxLDL, oxidized LDL; SMC, smooth muscle cells;*

2.3. Secretory components in pathogenesis of atherosclerosis

2.3.1. Oxidized low density lipoprotein

Oxidized LDL is produced by chemical modification of low density lipoprotein, probably within the sub-endothelial space [111]. LDL contains a central core with cholesterol esters and triglycerides molecules, which is surrounded by a monolayer of phospholipid molecules and unestriefied cholesterol. Embedded in

the outer layer is one apolipoprotein-B-100 (apoB-100) molecule. The oxidation of LDL may be carried out by endothelial cells, smooth muscle cells, monocytes and macrophages [62]. LDL can be oxidized by metal ions, lipooxygenases, myeloperoxidases and reactive nitrogen species (RNS) [18, 92].

High concentrations of LDL has been shown to lead to the activation of nicotamide adenine dinucleotide phosphate (NAD(P)H) oxidase and the subsequent generation of superoxide radical (O_2^*). Interaction of LDL with its cognate receptor may lead to the activation of a signal transduction pathway responsible for the direct activation of NAD(P)H oxidase. This pathway is believed to be dependent on the activity of phospholipase A_2 and the release of arachidonic acid (AA), a direct activator of NAD(P)H oxidase [93].

The sub-endothelial accumulation of foam cells is an important indicator of the initiation of atherosclerosis. These foam cells are derived from the unchecked uptake of LDL by monocytes and macrophages, which readily take up chemically modified or oxidized LDL through a scavenger pathway that can not be down-regulated [93].

Oxidatively modified LDL contributes to the inflammatory response [125, 127]. OxLDL is a chemotactic agent for monocytes and for T cells, and cytotoxic is for endothelial cells [137, 138]. The oxLDL induces an expression of the adhesion molecules as well as migration and proliferation of smooth muscle cells and the secretion of MCP-1 by endothelium. By including platelet adhesion and aggregation, decreasing production of NO, and releasing tissue factor (TF), oxLDL enhances the procoagulant activity of the endothelium [92]. OxLDL affects vasoreactivity by inducing vasoconstriction through inhibition of NO production [72], and stimulation of the expression of ET.

Apoptosis is characteristic for the more advanced atheroslerotic lesions and likely contributes to plaque rupture [75, 76, 141]. OxLDL induces the activation of a large array of signaling pathways leading to apoptosis of endothelial and smooth muscle cells [91, 127]. OxLDL also induces cellular oxidative stress by increasing of generation of ROS in different vascular cell types [127]. Finally oxLDL is able to trigger the expression of a variety of genes, such as adhesion proteins, scavenger receptors, oxidant and antioxidant enzymes, heat shock

proteins, growth factors, cytokines, chemokines and vasoactive mediators, matrix metalloproteinases (MMPs), collagen and hemostasis proteins [152].

2.3.2. Nitric oxide

Nitric oxide (NO) is an inorganic molecule with an unpaired electron (a free radical) that readily diffuses across cellular membranes to interact directly with its targets [72]. NO is produced by vascular endothelial cells and several other cell types, including phagocytes [54]. NO production from endothelial cells is stimulated by mechanical forces such as shear stress [77], and humoral factors including acetylcholine (Ach), vascular endothelial growth factor (VEGF), bradykinin, estrogen, and sphingosine 1-phosphate (S1P), which is present in large amount in high density lipoprotein (HDL). NO plays critical role in normal vascular biology and pathophysiology [11, 126]. NO derived from endothelium causes relaxation of smooth muscle cells through activation of the cyclic guanosine monophosphate (cGMP) - dependent protein kinase [97].

NO causes vasodilalation by suppression both of the synthesis of angiotensin II-converting enzyme (ACE) in the endothelium, and of A-II type 1 receptor (AT1) in smooth muscle cells [145]. NO is a potent inhibitor of platelet aggregation and adhesion, proliferation of VSMCs, and leukocyte adhesion [123]. Chronic exposure to NO down-regulates the expression of MCP-1 and adhesion molecules (such as VCAM-1) that are involved in in monocyte adhesion and infiltration [144]. NO may also influence plaque stability by its anti-apoptotic behaviour [24, 61]. Finally, NO may directly suppress the generation of oxygen-derived free radicals by nitrosating, and thereby inactivating oxidative enzymes such as NAD(P)H-oxidase [90, 126].

2.3.3. Monocyte chemoattractant protein-1

Monocyte chemoattractant protein-1 (MCP-1) is a monomeric polypeptide with a molecular weight of 9,000 to 15,000 Da. It is the prototype of the C-C chemokine β subfamily that exhibits its most potent activity toward monocytes [68]. MCP-1 is highly expressed in the initial stages of plaque formation and is postulated to play a central role in the monocyte recruitment into the arterial wall

[119]. MCP-1 mediates its chemotactic effect on peripheral blood monocytes through specific cell surface high-affinity-chemokine receptors (CCR) [114]. MCP-1 is produced by various cell types within the arterial wall, including endothelial cells, smooth muscle cells, and macrophages. [68]. Pro-atherogenic stimuli such as cytokines, oxLDL, A-II, thrombin (Thr), homocysteine, and activated platelets induce MCP-1 expression [12, 66]. While MCP-1 induction in vascular smooth muscle cells by A-II is dependent on the activation of NAD(P)H oxidase and the generation of ROS [149].

MCP-1 plays a crucial role in the early and intermediate stages of atherosclerosis. The primary effect of MCP-1 in atherogenic appears to be the recruitment of monocyte-macrophages into the atherosclerotic lesion [147]. Early expression of MCP-1 by endothelial cells in response to hypercholesterolemia or to the vessel wall is responsible for the initial influx of monocytes. Infiltrated monocytes/macrophages themselves express MCP-1, resulting in the continued influx of monocytes into the plaque as a part of a positive feed back loop [68]. In addition to a being potent monocyte chemoattractant, MCP-1 has also chemoattractant activity on activated T cells and basophils [119].

2.3.4. Angiotensin II

Angiotensin II is the effector peptide of the renin-angiotensin system (RAS), which has been implicated in the pathogenesis of atherosclerosis on various levels [132]. The classic source of angiotensin II is circulating angiotensin II [36]. However, the bulk of angiotensin II may be produced locally within the vascular wall [149].

The pro-atherogenic properties of angiotensin II include effects on endothelial function, the activation and binding of monocytes, the proliferation and migration of VSMCs and the promotion of the oxidation of LDL [36,145]. Angiotensin II may also increase the expression of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin, which promote adherence, migration and accumulation of monocytes and T lymphocytes in early atherosclerotic lesions [132]. In

addition, angiotensin II induces monocyte chemotaxis through upregulation of MCP-1.

Recent studies stress the importance of angiotensin in the production of reactive oxygen species during atherogenesis. Angiotensin II has been shown to stimulate the activity of membrane-bound NAD(P)H oxidase in VSMCs and endothelial cells [13, 149]. In macrophages, angiotensin II induces oxidative stress which also appears to be dependent on increases in NAD(P)H oxidase activity [36]. In addition, angiotensin II activates endothelial nitric oxide synthase (eNOS) to release NO*. Superoxide and NO* rapidly inactivate each other by forming the very reactive radical peroxynitrite (OONO*) which avidly modifies LDL [50].

Consequently, angiotensin II stimulates deposition of oxidized LDL within the vascular wall [37]. Angiotensin II participates, via induction of ROS, in transcriptional down-regulation of several genes involved in suppression of apoptosis. Angiotensin II increases expression of TF in endothelial cells, and in this way participates in thrombotic events by activation of the coagulation cascade [132]. Angiotensin II may also alter the extracellular matrix remodeling via activation of metalloproteinases (MMP-2, MMP-9) and thereby contribute to destabilization of fibrous plaques [149, 132]. Thus, angiotensin II appears to represent a critical, causal link in the pathogenesis of human atherosclerosis [149].

2.3.5. Matrix metalloproteinases

Clinical complications of atherosclerosis are often triggered by the rupture of unstable plaques, while thinning of the atherosclerotic vessel wall (owing to elastin and collagen degradation and media necrosis) may result in aneurysm formation and bleeding [87]. Matrix metalloproteinases (MMPs) are a family of enzymes that selectively digest individual components of the extracellular matrix. MMPs may contribute to the development of *de novo* atherosclerotic plaques by allowing smooth muscle cells to migrate from the vascular media to the intima. MMPs may also contribute to the rupture of athrosclerotic plaques by degrading the fibrous cap of the plaque [19]. Matrix metalloproteinases belong

to a group of zinc and calcium dependent proteases and are divided into four subclasses according to their substrate specificity, namely gelatinases (MMP-2 and MMP-9), collagenases (MMP-1,-8,-13, and -18), stromelysins (MMP-3,-10, and -11) and others MMPs [33]. Metalloproteinases are expressed in human atherosclerotic plaques by both VSMCs and foam cells. The activity of these enzymes is kept under tight control, which operates at three levels: transcription, activation of latent proenzymes, and inhibition of proteolytic activity. A number of cytokines and growth factors have been shown to induce or stimulate the synthesis of MMPs, including IL-1, PDGF, TNF-α, and Thr, whereas others, such as TGF-β, heparin, and corticosteroides, have an inhibitory effect. MMPs are also inhibited by a family of naturally occurring specific inhibitors known a tissue inhibitors of metalloproteinases (TIMPs) [85], and less specifically by α_2-macroglobulin [33]. Thrombin has been shown to activate purified pro-MMP-2 by proteolysis. Thus, in complicated atherosclerotic plaques, Thr may promote plaque instability by increasing the local matrix-degrading activity of MMPs. Focal degradation of collagen in the fibrous cap by MMPs produced by foam cell macrophages was demonstrated *ex vivo* in human atheroma and was associated with *in vivo* rupture of an experimental model of atherosclerotic lesions developed in the rabbit [42].

2.3.6. Thrombin

Thrombin is a multifunctional serine protease generated at sites of vascular injury, which elicits a host of cellular responses [131]. Thrombin is a pivotal component of the coagulation cascade [125, 142]. It is not only responsible for the cleavage of fibrinogen to fibrin, but is also the most powerful known platelet agonist, and is believed to play a critical role in the growth of platelet aggregates [143]. The local release of thrombin plays a significant role in the formation of proliferative vascular lesions [6]. Thrombin may also be involved in the inflammatory response in atherosclerosis [125]. It activates a variety of vascular and inflammatory cell types by interacting with so-called protease-activated receptors (PARs) [6]. At least three PARs are expressed in human cells, and have been linked to several physiological effects [143] such as the

„pro-coagulant“ initiation of platelet aggregation, the stimulation of monocyte and neutrophil chemotaxis by induction of MCP-1 expression in VSMCs, the stimulation of endothelial cells to express cell adhesion molecules such as P-selectin, and the mitogenesis of both lymphocytes and VSMCs. [6, 66].
Induction of MMP-2 and MMP-9 expression by thrombin in macrophages and other cells suggests that this protease promotes basement membrane degradation and smooth muscle migration [66]. Treatment of aortic smooth muscle cells with thrombin results in increased generation of O_2^* and hydrogen peroxide (H_2O_2) and in increased consumption of nicotamide adenine dinucleotide/nicotamide adenine dinucleotide phosphate NADH/NADPH [115].

2.3.7. Reactive oxygen species

Reactive oxygen species (ROS) are oxygen-derived molecules that have undergone univalent reduction, so that they readily react with other biological products [50]. Vascular tissues are a rich source of ROS, including superoxide (O_2^*), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^*). Virtually every cell type in the vascular wall has been shown to produce and be regulated by ROS [49]. In order for ROS to serve as a second messenger, both the production and inactivation of ROS must be tightly regulated. The half life of O_2^* is rather short (seconds) both because of its inherent instability and the efficient antioxidant defenses of the cell. Dismutation of O_2^* by superoxide dismutase (SOD) serves not only to scavenge O_2^*, but also to produce the more stable ROS, namely H_2O_2. Catalase and glutathione peroxidase are two of the most important scavengers of hydrogen peroxide, in both cases converting it into water [50].
ROS play an important role in many cardiovascular pathologies involving inflammatory processes. Several pathogenic effects of increased ROS production have been identified [96]. These include the oxidation of core lipids of lipoproteins and cell membranes, which then modify apolipoproteins and other proteins, leading to their recognition by scavenger receptors. It is also increasingly recognized that many cellular signaling pathways are oxidation-sensitive, and that ROS may provide a common link between pathways such as apoptosis or inflammation [30, 113]. A corollary of this is that pathogenic

processes that influence ROS generation affect cell proliferation, differentation, activity, and death by the balance of multiple effects on gene expression in individual cell types. Molecular targets of ROS include cell growth (hypertrophy and proliferation), apoptosis, and cell survival as well as cell migration. These processes are regulated by numerous proteins and enzymes for which ROS act as upstream regulatory factors. These include p38 mitogen-activated protein kinase ($p38^{MAPK}$), extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNKs=SAPKs), serine-threonine kinase (Akt), nuclear factor κB (NFκB) and caspases [50, 71] [Fig.3].

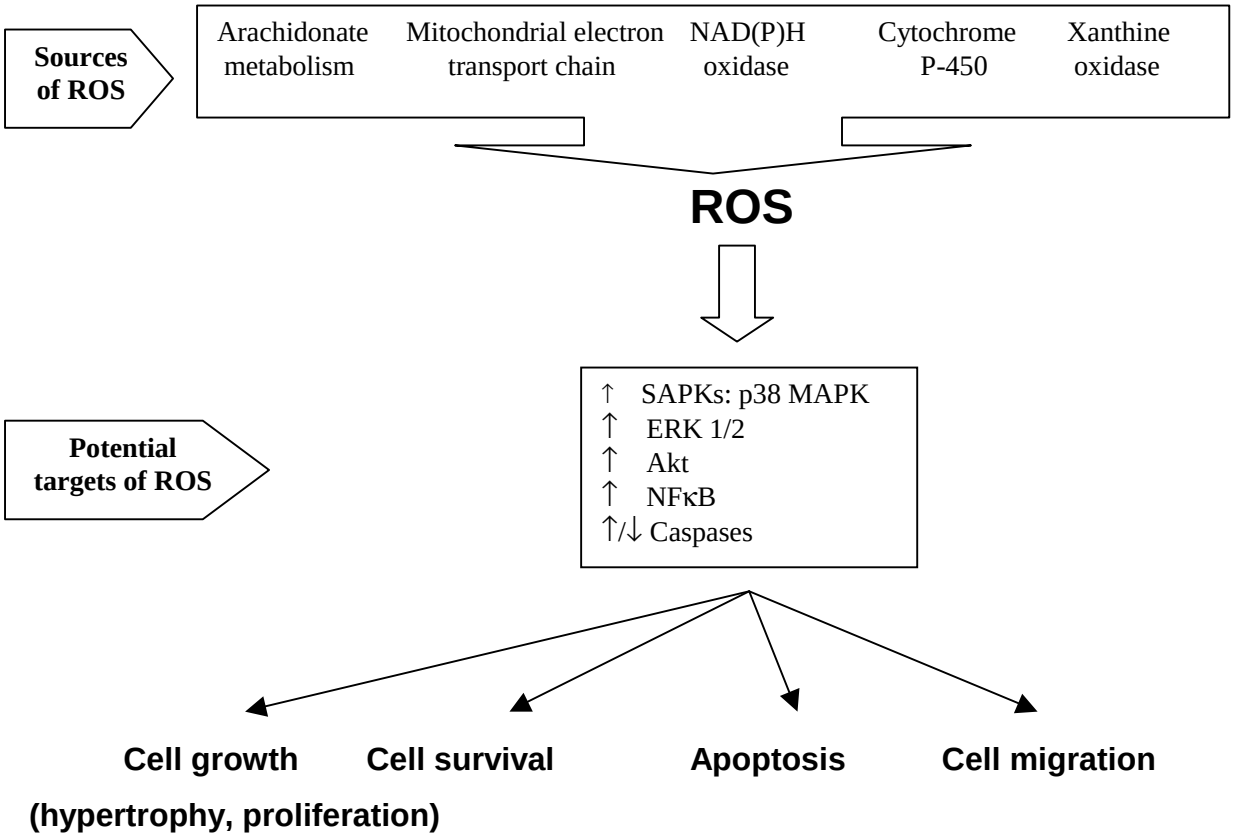


Figure 3. Schema illustrating the potential source, molecular targets, and signalling pathways mediated by ROS. *Enzymes that may generate ROS include components of the mitochondrial electron transport chain, NAD(P)H oxidase, cytochrom P-450, xanthine oxidase. ROS are also produced during arachidonate metabolism. Ultimately, ROS act on cell activities such a cell growth and survival, cell migration and apoptosis.*

2.4. Vascular NAD(P)H oxidase

2.4.1. Structure and function of NAD(P)H oxidase

ROS may be generated by various cellular sources [110]. Cellular enzymes, that are potential source of ROS, include microsomal cytochrome P-450, xanthine oxidase, the mitochondrial electron transport chain, and NAD(P)H oxidase [39, 71].

Although each of the above-mentioned enzymes can produce ROS in vascular cells, it is generally recognized that the NAD(P)H oxidase is the predominant sources of ROS in the vasculature [17, 22]. The activation of these enzymes leads to a variety of intracellular signaling events that ultimately cause dysfunction of the endothelium, proliferation of VSMCs, expression of pro-inflammatory genes and reconstruction of the extracellular matrix [17]. NAD(P)H oxidase appears to be a particulary important source of ROS production in blood vessels, where it produce relatively low levels of ROS under basal conditions, but generates higher levels in response to pro-atherogenic stimuli such as oxLDL, angiotensin II, thrombin, and others [21].

The NAD(P)H oxidases of the cardiovascular system are membrane-associated enzymes that catalyze the one electron reduction of oxygen using NADH or NADPH as electron donor [60].

$$NAD(P)H + 2O_2 \Longrightarrow NAD(P)^+ + H^+ + 2O_2^{*-}$$

The structure and function of NAD(P)H oxidase was characterized initially in neutrophils where it plays an important role in bacterial killing [93, 17]. Two membrane components, $p22^{phox}$ and $gp91^{phox}$, comprise the cytochrome *b*558. Other important components include the cytoplasmic subunits $p47^{phox}$, $p67^{phox}$ and the small guanosine triphosphate (GTP)-binding protein Rac [17, 133]. Vascular NAD(P)H oxidases differ from neutrophil NAD(P)H oxidase in several important respects. Neutrophil oxidase releases large amounts of O_2^* in bursts into the intracellular milieu, whereas vascular NAD(P)H oxidases continuously produce low levels of O_2^*, which play a role in intracellular cell signaling [17]. Messenger RNA for $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ has been found in

endothelial cells and adventitial cells. Vascular smooth muscle cells and mesangial cells appear to express $p22^{phox}$, $p47^{phox}$, and Rac1, but not $gp91^{phox}$ [49]. In the past few years, a family of $gp91^{phox}$-like proteins, termed non-phagocytic NAD(P)H oxidase (NOX) proteins, has been discovered [17]. Based on homologies with each other and their apparent evolution from ancestral NOX, these were named NOX1, NOX2 (also known as $gp91^{phox}$), NOX3, NOX4 and NOX5. Endothelial cells contain NOX1, NOX2, NOX4 and NOX5, whereas vascular smooth muscle cells express NOX1, NOX4 and NOX5 [17] [Fig.4].

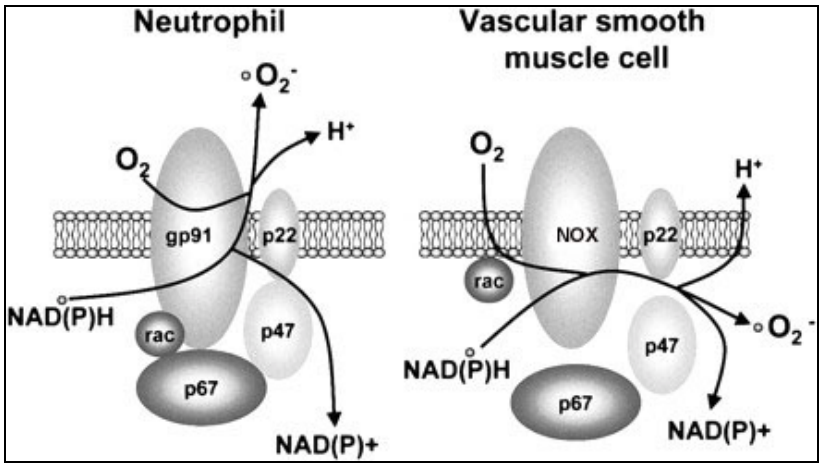


Figure 4. Structure of the NAD(P)H oxidases. *Left, structure of neutrophil NAD(P)H oxidase. $gp91^{phox}$ and $p22^{phox}$ form the electron transfer component of the oxidase, and $p47^{phox}$ and $p67^{phox}$ are cytosolic components that interact with these 2 proteins to modulate its activity. The low molecular weight G protein rac also serves a regulatory function. Right, components of the neutrophil oxidase that have been indentified in VSMCs. The functional interaction among these subunits remains to be determined. NOX is for NAD(P)H oxidase.*

2.4.2. Regulation of NAD(P)H oxidase

Cmpounds with signaling properties, such as ROS, must be rapidly synthesized and degraded. Therefore, expression of vascular oxidases is expected to be tightly regulated [82]. One of the most important attributes of the cardiovascular oxidase is its responsiveness to hormones, hemodynamic forces, and local metabolic changes. It has been demonstrated that the activity of the the vascular oxidase is increased by several pro-atherogenic agents. For instance, angiotensin II increases NADH- and NADPH-driven O_2^* production in cultured

SMCs and aortic adventitial fibroblasts, and increases diphenylene iodinium (DPI) - inhibitable ROS production in mesangial cells [49]. PDGF, and TNF-α stimulate NAD(P)H oxiadase-dependent superoxide production in SMCs. IL-1, TNF-α, and platelet-activating factor (PAF) increase NAD(P)H-dependent superoxide production in fibroblasts. Treatment of human aortic smooth muscle cells with thrombin is accompanied by increased O_2^* and H_2O_2 generation and NADH/NADPH consumption [115]. Mechanical forces such as shear stress, stimulate NAD(P)H oxidase activity in endothelial cells [32]. Regulation of NAD(P)H oxidase activity in cardiovascular cells occurs at least at 2 levels. First, activation of the oxdiase can be mediated by intracellular second messengers, including calcium. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator increases NAD(P)H oxidase in VSMCs. It has recently been shown that lipoxygenase metabolites of AA mediate A-II and Thr stimulation of the NAD(P)H oxidase in VSMCs. Second, NAD(P)H oxidase activity can also be modulated by upregulation of the component messenger RNA (mRNA). For example, TNF-α increases NAD(P)H oxidase activity in SMCs via increased transcription of $p22^{phox}$ [49].

NAD(P)H oxidase is thus regardered as a potential therapeutic target. DPI, a flavoprotein inhibitor, has been shown to inhibit NAD(P)H oxidase in non-specific fashion. Furthermore, several pharmaceutical compounds with anti-atherogenic activity have shown to inhibit NAD(P)H oxidase for instance statins and angiotensin receptor antagonists lower NAD(P)H oxidase activity.

Several studies have recently documented a direct link between NAD(P)H oxidase and atherosclerosis. Immunohistochemical studies have demonstrated the presence of a $p22^{phox}$-based oxidase in human coronary arteries in all cell types that are present in advanced atherosclerotic plaques [149]. The indentification of the NAD(P)H oxidase system as the principal source of O_2^* production in human saphenous veins from patients with atherosclerotic risk factors futher highlights the potential importance of this oxidase in human atherosclerosis [52]. Various studies demonstrated a relationship between NAD(P)H oxidase and atherosclerosis, for instance by showing that increased atherosclerotic risk factor profile (hypertension, diabetes

mellitus, smoking and hypercholesterolemia) and endothelial dysfunction are associated with increased NAD(P)H oxidase enzyme activity [52]. NAD(P)H oxidase is activated and O_2^* production is increased in vessels of rabbits with experimental atherosclerosis. In crosses between apolipoproten E (apoE)-deficient mice and $p47^{phox-/-}$ mice, atherosclerosis in the descenting aorta was diminished compared to $apoE^{-/-}$ mice. Disruption of the $p47^{phox}$ gene also resulted in decreased superoxide production in vessels and inhibition of proliferation in VSMCs. These experiments clearly establish the important role of the NAD(P)H oxidase and its components in the development of vascular lesions in atherosclerosis [82].

2.5. High density lipoprotein

2.5.1. Structure of HDL

High density lipoprotein (HDL) is a fraction of serum lipoproteins characterized by similar molecule density ($1.063 < d < 1.21$ g/ml) and size (5-17 nm in diameter) [103]. HDL contains approximately 50 % protein, 25 % phospholipid, 20 % cholesterol (mainly esterified), and 5 % triglyceride by weight [4]. The most frequently used method of lipoprotein isolation-isopycnic ultracentrifugation-separates two major HDL fractions-HDL_2 (d=1.025-1.063 g/ml) and HDL_3 (d=1.125-1.250 g/ml). Most of the HDL particles have a globular shape. Unesterified cholesterol is distributed between the surface and the core of HDL particle. Charged lipids, phospholipids, and proteins are found primarily in outer parts of lipoprotein. The most abundant protein of HDL is apolipoprotein A-I (apo A-I). Apo A-II, apo A-IV, apo C, apo E, and apo J are found in lower amounts. Some protein assiociated with HDL have enzymatic activity. The best-known are lecithin-cholesterol acyltransferase (LCAT), cholesterol ester transfer protein (CETP), phospholipid transfer protein (PLTP), platelet activating factor-acetylhydrolase (PAF-AH), and paraoxonase (PON). Nonpolar elements of HDL such as triglycerides and esterified cholesterol are localized in the center of the lipoprotein molecule. Globular HDL_3 and HDL_2 paricles migrate on agarose gel electrophoresis in a fraction with α-electrophoretic mobility designated α-LpA-I. This fraction contains cholesterol quantified as HDL-C as

well as the majority of apo A-I. The rest of apo A-I is found either lipid-free or in association with a few molecules of sphingomyelin and phosphatidylcholine in a HDL fraction with electrophoretic pre-β mobility. Similar lipid-poor particles containing apo E (γ-LpE) or apo A-IV (LpA-IV) have been also identified [34].

2.5.2. Metabolism of HDL

Lipid-poor HDL particles with pre-β mobility are produced by hepatocytes and enterocytes, or dissociate from chylomicrons and very low density lipoproteins during lipolysis, or are generated from α-HDL by CETP, PLTP or hepatic lipase (HL). Lipid-free apolipoproteins were shown to release phospholipids and unesterified cholesterol from cells or apo B-containing lipoproteins [34].

HDL has the ability to remove cholesterol from peripheral cells for delivery to the liver and excretion into the bile. This process is termed reverse cholesterol transport (RCT) [34]. In the final step of RCT, HDL is removed from the circulation by the scavenger receptor B1 (SR-B1), apo E receptors [38], or indirectly by the action of CETP and endothelial lipase. SR-BI binds HDL and mediates the selective uptake of cholesteryl esters into hepatocytes without internalizing HDL. Hepatic lipase appears to serve as a coreceptor for SR-B1. Apo E-containing HDL is internalized by hepatic apo E receptors. Cholesteryl ester transfer protein exchanges cholesteryl esters of α-HDL with triglycerides of very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and LDL, which are then removed via the LDL-receptor pathway. Endothelial lipase (EL) hydrolyzes phospholipids and generates free fatty acids taken up by endothelial cells [34] [Fig.5].

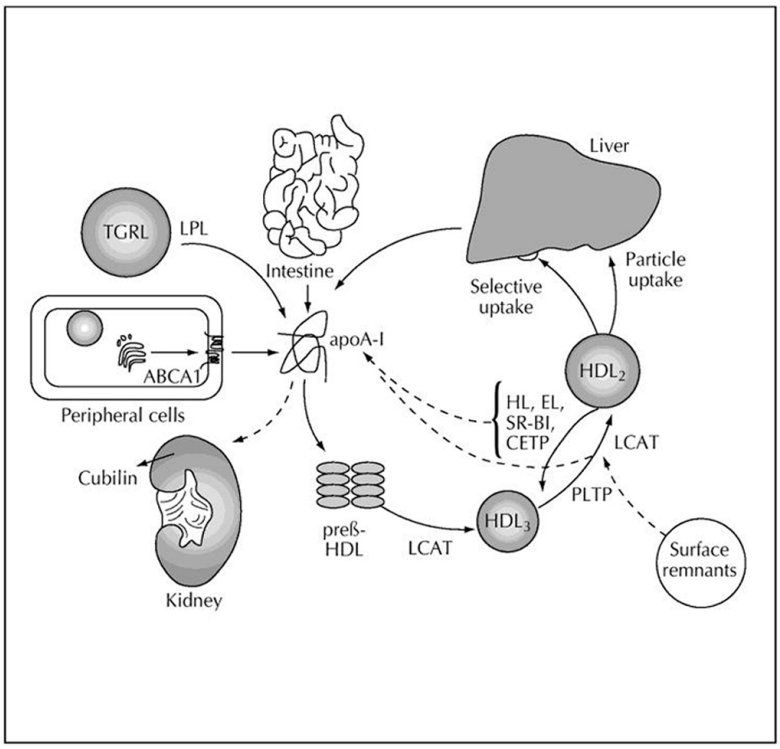


Figure 5. Pathways involved in the generation and conversion of HDL.
Mature HDL_3 and HDL_2 are generated from lipid-free apoA-I or lipo- poor pre-β_1-HDL as the precursors. These precursors are produced as nascent HDL by the liver or intestine or are released from lipolysed VLDL and chylomicrons or by interconversion of HDL_3 and HDL_2. Adenosine triphosphate-binding cassette protein A1 (ABCA)1-mediated lipid efflux from cells is important for initial lipidation; LCAT-mediated esterification of cholesterol generates spherical particles that continue to grow on ongoing cholesterol esterification and PLTP-mediated particle fusion and remnant transfer. Larger HDL_2 particles are converted into smaller HDL_3 particles on CETP-mediated export of cholesteryl esters from HDL onto apo B-containing lipoproteins, on SR-BI-mediated selective uptake of cholesteryl esters into liver and steroidogenic organs, and on hepatic lipase (HL) - and enothelial lipase (EL) -mediated hydrolysis of phospholipids. HDL lipids are catabolized either separately from HDL proteins (ie, by selective uptake or via CETP transfer) or together with HDL proteins (ie, via uptake through as-yet-unknown HDL receptors or apo E receptors). The coversion of HDL_2 into HDL_3 and the PLTP-mediated conversion of HDL_3 into HDL_2 liberated lipid-free or poorly lapidated apo A-I. A part of lipid-free apo A-I undergoes glomerular filtration in the kidney and tubular readsorption through the activation of cubilin.
Grey arrows represent lipid transfer processes, black arrows represent protein transfer processes. TGRL-triglyceride-rich lipoproteins;

2.5.3. Anti-atherogenic activities of HDL

Numerous clinical and epidemiological studies have demonstrated an inverse and independent association between HDL-cholesterol and the risk of coronary heart disease (CHD) [35]. This correlation is often explained by the involvement of HDL in reverse cholesterol transport (RTC). Distortion of RCT may favor deposition of cholesterol in arterial wall and thereby contribute to the development of arteriosclerosis. In addition to RTC several other potentially anti-atherogenic activities are exerted by HDL [103] [Fig.6].

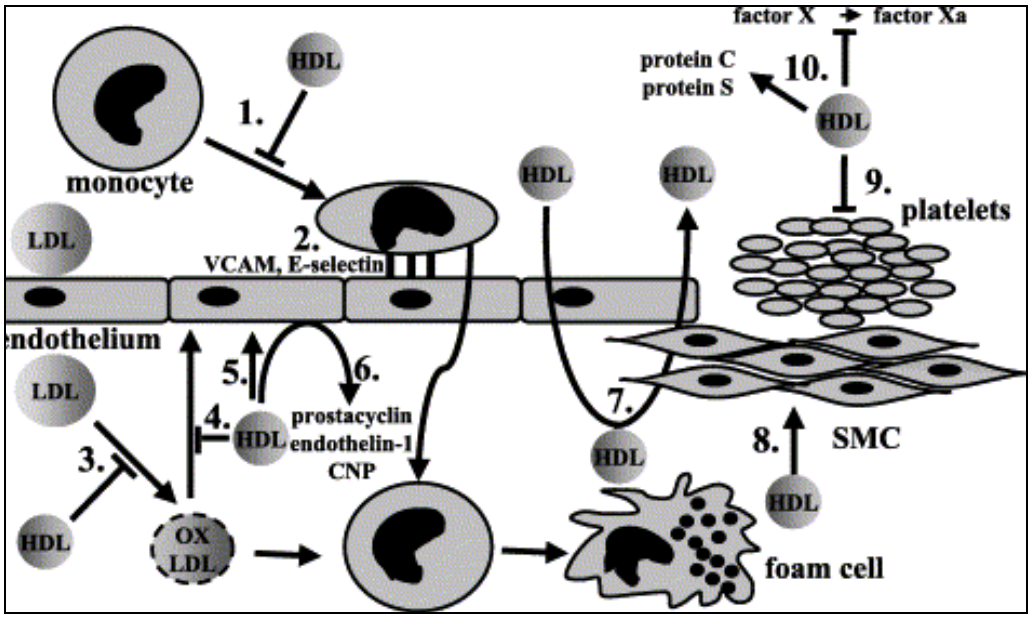


Figure 6. Pleiotropic effects of HDL in the vessel wall.

Several pleiotropic effects of HDL in the vasculature may underlie its anti-atherogenicity. These include: ***1.*** *inhibition of chemotaxis of monocytes;* ***2.*** *inhibition of monocyte adhesion to endothelial cells;* ***3.*** *inhibition of LDL oxidation;* ***4.*** *inhibition of ox-LDL-induced endothelial dysfunction and apoptosis;* ***5.*** *stimulation of endothelial cell proliferation* ***6.*** *stimulation of endothelial synthesis of prostacyclin, endothelin and c-type natriuretic peptid (CNP);* ***7.*** *stimulation of cholesterol efflux from macrophages and foam cells;* ***8.*** *stimulation of smooth muscle cells (SMC) proliferation* ***9.*** *inhibition of platelet activation;* ***10.*** *inhibition of factor X activation and stimulation of activated protein C;*

A central role of endothelial cells in pathogenesis of atherosclerosis has been described earlier. Substantially decreased concentration of HDL in plasma has

been reported to be related to endothelial cell dysfunction, which is one of the first hallmarks of atherosclerosis [103]. HDL prevents oxLDL-mediated endothelial dysfunction, reduces the chemotactic activity of LDL [92], antagonizes the inhibitory effect on vasodilatation exerted by lysophosphatidylocholine (LPC)-one of the major products of LDL oxidation, restores NO production decreased by oxLDL, diminishes the induction of cell death by oxLDL, and weakens the cytotoxic effect of oxLDL [103]. HDL may also attenuate endothelial dysfunction brought about by the complement activation. HDL also inhibits complement-mediated cell lysis [103], and protects against endothelial apoptosis induced by TNF-α [108]. Finally HDL promotes endothelial cell survival by preserving mitochondrial integrity and thus inhibiting release of cytochrom *c* and subsequent activation of the caspase cascade [105].

HDL affects several secretory functions of endothelial cells, stimulating production of PGI_2 and prostaglandin E_2 (PGE_2). HDL supplies endothelial cells in arachidonic acid, induces the expression of cyclooxygenase-2 (COX-2), and decreases ET production [103].

The regulatory effects of HDL on coagulation and fibrinolysis depend on inhibition of tissue factor synthesis. In addition, HDL antagonizes the activation of factor X induced by extrinsic tenase. HDL reverses the effect of LDL on tissue plasminogen activator (tPA) and its inhibitor plasminogen activator inhibitor-1 (PAI-1) and restores normal release of these two factors from ednothelial cells [108]. HDL is also an independent predictor of acute platelet-dependent thrombus formation. In vitro, HDL inhibits thrombin-, collagen-, ADP- and adrenalin- induced platelet agrregation [103].

Paraoxonase, a HDL-associated enzyme, prevents LDL oxidation by hydrolyzing lipid peroxides, cholesterol linoleate hydroperoxides, and hydrogen peroxide [92]. HDL is an important antioxidant and may decrease the formation of O_2^* of which main source is NAD(P)H oxidase [108].

Apolipoprotein A-I, a major apolipoprotein of HDL, accounts for a various anti-atherogenic effects attributed to these molecules [103]. Apo A-I has been

demonstrated to induce cholesterol efflux from cells [3, 78, 100,116] and to mimic some but not all of the functions exerted by native HDL. For instance, in contrast to HDL, apo A-I does not inhibit apoptosis of endothelial cells [105], stimulate proliferation of smooth muscle cells [104] and fibroblasts [100], inhibit platelet aggregation and production of PGE_2 [103] or induce NO-dependent vasorelaxation [95, 102]. Clearly, other active components must be present in HDL particles, which explain the astonishing variety of functions exerted by these lipoproteins.

2.5.4. Intracellular effects of HDL and its components

Three lysosphingolipids have been identified in the particle of HDL, namely sphingosylphosphorylcholine (SPC), lysosulfatide (LSF) and sphingosine-1-phosphate (S1P) [63]. Lysosphingolipids are known to interact with “endothelial differentation genes” (EDG), a family of heptahelical receptors coupled to several trimeric G proteins [1]. In this way lysosphingolipids generate a variety of intracellular signals. Several features of SPC, S1P and LSF are close to those seen in HDL. They exert strong mitogenic activities and induce growth-associated metabolic events such as activation of phosphatidylinositol-specific phospholipase C (PI-PLC), and liberation of intracellular calcium [103]. They have been also shown to activate mitogen activated protein kinases such as $p42/44^{MAPK1/2}$, and PKC [100, 101]. Strong mitogenic effects have been observed in endothelial cells, vascular smooth muscle cells, fibroblasts and keratinocytes [63, 134]. Similar to HDL, S1P and SPC are also the effective stimuli of endothelial cells migration and mediators of angiogenesis [63]. SPC and LSF are agonists responsible for the survival activity of HDL. They inhibit activation of caspases and biochemical features of apoptosis in endothelial cells [105]. In addition, SPC and LSF potently stimulate Akt, an ubiquitous transducer of anti-apoptotic signals [105]. Akt was also shown to restrict several signaling pathways such as intracellular generetion of ROS [101]. Concordant with the inhibitory function of HDL on endothelial adhesion (inhibition of expression of VCAM-1 and ICAM-1), exogenous lysosphingolipids inhibit expression of E-selectin in endothelial cells and thereby potentially limit transmigration of

leukocytes into arterial wall [101, 108]. Similar to HDL, SPC, LSF and S1P have been demonstrated to regulate vascular tone via EDG-3-mediated NO release. These three lysophospholipids induce intracellular Ca^{2+} mobilization and Akt-mediated eNOS phosphorylation, which result in NO release and vasodilation [102]. All these findings strongly suggest that lysosphingolipids in HDL account for some anti-atherogenic activities exerted by these lipoproteins [100, 101].

2.6. Working hypothesis

Atherosclerosis with its progression to heart disease, stroke, and peripheral vascular disease continues to be the leading cause of death in all Western civilizations /2.1/. An increasing body of evidence indicates that ROS generation plays a critical pro-atherogenic role. The NAD(P)H oxidase are a predominant source of ROS, and activation of these enzymes leads to a variety of intracellular events that ultimately cause specific response of cells and components involved in atherogenesis /2.3.7, 2.3.8/. Numerous epidemiological studies revealed an association between HDL cholesterol levels and the risk of development and progression of atherosclerosis. In addition to reverse cholesterol transport, several other potentially anti-atherogenic activities are exerted by HDL /2.3.9.3/. HDL has been shown to stimulate multiple cellular signaling pathways /2.3.9.4/. It is still unclear which components and mechanisms of activation of HDL are responsible for these activities of HDL. The subject of this work was to attempt to answer the following questions:

1) Does the anti-atherogenic HDL acts on pro-atherogenic NAD(P)H oxidase activity and ROS generation?
2) If so, which components of the HDL particle and which mechanisms of HDL-induced signal transduction are responsible for this interaction?
3) Which inflammatory components of atherogenesis are involved in the effects exerted by NAD(P)H oxidase and HDL ?

3. Materials and Methods

3.1. Materials

3.1.1. Equipment

AlphaEase FC Imaging System		Alpha Innotech, USA
AlphaEase FC software		Alpha Innotech, USA
Autoclave HV-50L		Hirayama, Japan
Bio Photometer		Eppendorf, Germany
Cell culture plates:	6 and12 wells	TPP, Switzerland
Centrifuges:	Megafuge 1,0 R	Hareus,Germany
	5417 R	Eppendorf, Germany
	5415 D	Eppendorf, Germany
	Ultracentrifuge ZU L8-70	Beckman, Germany
Centrifuge tubes Quick-Seal 25 x 89mm		Beckman, USA
CO_2, humidified incubator type B 5042 E		Hareus, Germany
Culture flasks 25 and 75 cm²		TPP, Switzerland
Culture microscope CK-40		Olympus, Japan
Easy Software		SLT,Austria
Filter Millex-GS (0,22 µm and 0,45 µm)		Millipore, USA
Fluorescence cell standard 1000 µL		Hellma, Germany
Fluorescence spectrophotometer F-2000		Hitachi, Japan
Gene Amp PCR System 9700		Perkin Elmer, USA
IScript cDNA synthesis kit		Bio-Rad, USA
Laser scanning microscope, LSM 510		Zeiss, Jena
Liquid nitrogen equipment		Westfalengas, Germany
LKB power supply 2197		LKB, Sweden
Lyophillisator DALTA 1-20 KD		Christ, Osterode
MCP-1, Elisa Immunoassay Kit		Biosource, USA
Minicon B15 concentrator		Amicon, USA
Mini Protean II, electrophoresis apparatus		Bio Rad, USA
$p38^{MAPK}$, Elisa Assay Kit		Biosource, USA
Pipette’s tips 1-5000 µL		Eppendorf, Germany

pH-meter 766	Knick, Germany
Photometer SPECTRA	SLT,Austria
Power PAC 300	Bio Rad, USA
Protein assay kit	Pierce, USA
RNase free, DNase set	Qiagen, USA
RNeasy mini kit	Qiagen, USA
Serological pipettes: 5, 10, 25 mL	TPP, Switzerland
Servapor dialysis tubing (pores 16 mm)	Serva, Germany
Spectrophotometer Uvikon 922	Kontron, Italy
Sterile-bench UVF 6.12.8 with laminar flow	BDK, Germany
TINA 2,0 software system	Raytest, Germany
Tissue culture dishes 100 x 20mm	Starstedt, USA
Tube sealer	Beckman, USA
Tubes 15, 50 mL	TPP, Switzerland
Tubes 1,5 and 2 mL	Eppendorf, Germany
Tubes 1,8 mL	Nunc, Denmark
Washer Columbus plus	Tecan,Austria
Wistar Kyoto rats	Harlan, USA

3.1.2. Reagents

Temed	Applichem, Germany
Coomasie Brillant Blue; Polyacrylamid	Bio-Rad, USA
Dulbeco's Modified Eagle's Medium; Dulbecco's Phosphate Buffered Salines with and without Ca / Mg	BioWhittaker, Belgium
Agarose	Biozym, Germany
Bovine serum albumin, fatty acids free, fraction V; PMSF; Trolox	Calbiochem,Germany
Colagenase type I; RNA ladder	GibcoBRL, USA
Sodium docedyl sulfate (SDS); Tris	ICN, USA
dNTP mix	Invitrogen, USA
Acetic acid; $CaCl_2$; chloroform; glycerol; formaldehyde sol.; formamide; KBr; KCl; KH_2PO_4; Triton X-100	Merck, Germany;

H_2DCFDA	Molecular Probes, USA
MCP-1 primer pair: FW and RW	MWG Biotech, Germany
Trypsin-EDTA (10x) 1:250; Antibiotic-Antimycotic Solution; Foetal Calf Serum	PAA, Austria
HotStarTaq DNA Polymerase, PCR Buffer	Qiagen, USA
rat/mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) PCR primers pair	R&D Systems, USA
protease inhibitor coctail	Roche, Germany
ethanol; NaCl	Roth, Germany
OTC Tissue Tek	Sakura Finetek, Netherlands
gelatine	Serva, Germany
β-Glycerophosphate; deoxycholate; diethyl pyrocarbonate; dihydroethidium (DHE); DMSO; EDTA; EGTA; elastase type I; ethidium bromide; Hank's Balanced Salt Solution (HBSS); 2-mercaptoethanol; MOPS; β-NADPH; L-α-phosphatidylcholine; L-α-phosphatidylinositol; L-α-phosphatidyl-L-serine; LSF; α-NADPH; Na_3VO_4; $Na_4P_2O_4$; sodium acetate; sodium fluoride; sodium phosphate; S1P; SPC; Tiron	Sigma; Germany
Gases and liguid nitrogen	Westfalengas, Germany

Human fresh plasma was provided from Institute of Transfusion Medicine at the Westphalen University of Münster.

3.1.3. Solutions and mediums

Antibiotic Antimycotic Solution 100x:	penicillin	10.000 U
	streptomycin sulphate	10 mg/mL
	amphotericin B	25 µg/mL

BCA protein assay kit:

BCA reagent A:	sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0,2 N sodium hydroxide
BCA reagent B:	4 % cupric sulfate

albumin standard:	2,0 mg/mL of bovine serum albumin in 0,9 % saline and 0,05% sodium azide	
Cell extraction buffer (p-38):		
	Tris-HCl	10 mmol/L
	NaCl	100 mmol/L
	EDTA	1 mmol/L
	EGTA	1 mmol/L
	NaF	1 mmol/L
	$Na_4P_2O_7$	20 mmol/L
	Na_3VO_4	2 mmol/L
	Triton X-100	1 % (v/v)
	glycerol	10 % (v/v)
	SDS	0,1 % (w/v)
	deoxycholate	0,5 % (w/v)
	PMSF	1 mmol/L
	protease inhibitor mix	1 mmol/L
	pH 7,4	
DHE solution		10 µmol/L
Dulbecco's Modified Eagle Medium:		
*inorganic salts:	$CaCl_2$	0,2 mg/mL
	$Fe(NO_3)_3 \times 9H_2O$	0,1 µg/mL
	KCl	0,4 mg/mL
	$MgSO_4 \times H_2O$	0,2 mg/mL
	NaCl	6,4 mg/mL
	$NaHCO_3$	3,7 mg/mL
	$NaH_2PO_4 \times H_2O$	0,125 mg/mL
*other components:	glucose	4,5 mg/mL
	phenol red x Na	0,015 mg/mL
	sodium pyruvate	0,11 mg/mL
*amino acids:	L-arginine x HCl	0,084 mg/mL
	L-cystine	0,048 mg/mL

	L-glutamine	0,584 mg/mL
	glycine	0,03 mg/mL
	L-histidine x HCl	0,042 mg/mL
	L-isoleucine	0,104 mg/mL
	L-leucine	0,104 mg/mL
	L-lysine x HCl	0,146 mg/mL
	L-methionine	0,03 mg/mL
	L-phenyalanine	0,066 mg/mL
	L-serine	0,042 mg/mL
	L-threonine	0,095 mg/mL
	L-tryptophan	0,016 mg/mL
	L-tyrosine	0.072 mg/mL
	L-valine	0,093 mg/mL
Dulbecco's Phosphate Buffered Salines:		
	NaCl	8 mg/mL
	KCl	0,2 mg/mL
	KH_2PO_4	0,2 mg/mL
	Na_2HPO_4 x $7H_2O$	2,16 mg/mL
*{with Ca/Mg}	$CaCl_2$ x $2H_2O$	0,13 mg/mL
	$MgCl_2$ x $6H_2O$	0,1 mg/mL
Foetal Calf Serum „Gold“:	Albumin	1,8 - 2,45 g/dL
Hank's Balanced Salt Solution (HBSS):		
	$CaCl_2x2H_2O$	0,185 mg/mL
	$MgSO_4$	0,097 mg/mL
	KCl	0,4 mg/mL
	KH_2PO_4	0,06 mg/mL
	$NaHCO_3$	0,35 mg/mL
	NaCl	8,0 mg/mL
	Na_2HPO_4	0,047 mg/mL
	D-glucose	1,0 mg/mL
	phenol red Na	0,011 mg/mL

H_2DCFDA stock solution:		10 mmol/L
Non-reducing loading-gel buffer:		
	Tris pH 6,8	50 mmol/L
	SDS	2 % (w/v)
	glycerol	10 % (v/v)
	bromophenol blue	0,1 % (v/v)
PBS buffer:		
	NaCl	140 mmol/L
	KCl	2,7 mmol/L
	KH_2PO_4	1,5 mmol/L
	Na_2HPO_4 x 2 H_2O	8,1 mmol/L
	pH 7,4	
Resolving gel (zymography):		
	Tris pH 8,8	1,5 mmol/L
	Polyacrylamide	40 % (v/v)
	gelatine	1 % (w/v)
	APS	10 % (v/v)
	SDS	10 % (v/v)
	Temed	1 % (v/v)
Smooth muscle cells – complete medium:		
	DMEM	500 mL
	FCS	10 % (v/v)
	Antibiotic-Antimycotic solution	2 % (v/v)
Smooth muscle cells – serum-free medium:		
	DMEM	500 mL
	Antibiotic-Antimycotic solution	2 % (v/v)
Stacking gel (zymography):		
	Tris pH 6,8	0,5 mol/L
	Polyacrylamide	40 % (v/v)
	SDS	10 % (w/v)

	APS	10 % (v/v)
	Temed	2 % (v/v)
Trypsin-EDTA (1:250) – 10x (PAA):		5 g/L

3.2. Methods

3.2.1. Culture isolation and primary culture of rat VSMCs

Cells were maintaned in a humidified atmosphere containing 5 % CO_2 at 37 °C under sterile conditions.

Cultured vascular smooth muscle cells (VSMCs) were prepared from aortas of adult rats [58]. Animals were sacrificed by cervical dislocation and the thoracic aorta was removed and placed in DMEM with penicillin (100 U/mL) and streptomycin (0,1 mg/mL) [83]. Each aorta was opened longitudinally, and the endothelium was scraped away with a teflon spatula [46]. The aortic segments were than placed in mixture of 0,1 % collagenase and 0,05 % elastase in Hank's Balanced Salt Solution (HBSS) for 1 h at 37 °C. The supernatant was discarded thereafter and the tissue fragments were rinsed with 0,05 %-0,1 % Trypsin-EDTA in HBSS without Ca/Mg using wide-mouth pipette to aid the tissue dissociation. After adequate dispersion, trypsin was inactivated by adding fetal calf serum (20 % v/v). The cells were centrifuged (200 x g for 7 minutes) [46,20].The trypsin-serum supernatant was discared and the cells were resuspended in 2 ml of the complete DMEM medium to a concentration of about 10^5 per 35 mm dish. Cells were initially cultured on the 35 mm dish and than transferred into the 75 cm^2 flasks.

Cells were cultivated in 10-12 mL of DMEM supplemented with 10 % (v/v) of FCS and Antibiotic-Antimycotic sol. 2 % (v/v) [26]. Every second day cells were washed with 5-10 mL of DPBS and the medium was changed. All media, buffers and reagents were warmed to 37 °C before use.

3.2.2. Cultivation of rat VSMCs

Stock cells at subconfluence were washed with DPBS without Ca/Mg (BioWhittaker) and subcultured by trypsinization with 0,5 g/L (v/v) of Trypsin-EDTA (1:250 (PAA)) for 1 minute at 37 °C. After trypsinization cells were

suspended in 8 mL of complete medium and centrifuged for 10 min at 250 x g, to remove trypsin. Then cell pellet was suspended in the fresh complete medium and dispensed in bottles, dishes or plates. All experiments were performed on monolayer cultures grown to 70 – 90 % confluence. Passages 5 to 17 were used [14, 84].

Seeding densities of monolayer cultured smooth muscle cells on surface areas were:

- Flask 75 cm^2 : 2,1 x 10^6
- 6 wells plate : 0,3 x 10^6
- 12 wells plate : 0,1 x 10^6
- 100 mm dish: 2,2 x 10^6

3.2.3. Cells preparation for the experiments

Before each experiment cells were made quiescent by incubation in serum-free DMEM for 24 and 48 h at 37 °C.

3.2.4. Isolation of HDL

HDL_3 fraction was isolated from human plasma by discountinous centrifugation on KBr gradients [59, 106]. The fresh human serum from unknown donnors was obtained from Institute of Transfusion Medicine at the Westfalian University of Münster.

The density of serum or infranates was adjusted to a desired density by the addition of KBr according to a formula:

$$M_{KBr} = V_{plasma} \times \text{K-factor}$$

where K-factor was calculated as follows:

$$\text{K-factor} = \frac{\text{initial density} - \text{desired density}}{1 - (0{,}312 \times \text{desired density})}$$

The density of plasma (1,006 g/mL) was adjusted to 1,125 g/mL for isolation of VLDL, IDL, LDL, and HDL_2. The density of the infranate from first centrifugation was adjusted to 1,210 g/mL for isolation of HDL_3. The ultra-centrifugation was carried out in 25 x 89 mm centrifugation tubes (Beckman), using the 70 Ti-rotor (Beckman) and ultra-centrifuge ZU L8-70 (Beckman). The first centrifugation was performed at 226000 x g for 48 h at 4 °C. The supernate containing VLDL, IDL, LDL and HDL_2 was removed and the infranate was adjusted to a desired density with KBr and the centifugation was repeated. After second centrifugation, supernatant containing HDL_3 was collected, transferred to dialysis tubes (Serva) and dialyzed against 0,3 mmol/L Tris-HCl / 0,14mol/L NaCl, pH 7,2 for at least 24 h at 4 °C. When necessary HDL was concentrated using Minicon B15 concentrator (Amicon) [106]. HDL solution was then sterilized using Millex filter (Millipore), with 0,22 µm pores. The protein concentration was determined and lipoproteins were stored at 4 °C.

3.2.5. Protein quantification

The protein quantification was performed using commercially available protein assay kit (Pierce) [7]. The method combines reduction of Cu^{+2} to Cu^{+1} in alkaline medium with chelation of molecules of bicinchoninic acid by the cuprous cation:

$$\text{Protein} + Cu^{+2} \xrightarrow{OH^-} \text{tetradentate-}Cu^{+1}\text{ complex}$$

$$Cu^{+1} + 2\ \text{Bicinchonic Acid (BCA)} \longrightarrow \text{BCA-}Cu^{+1}\text{ complex}$$

The water-soluble complex exhibits absorbance at 562 nm.

The protein assay kit contains:

- BCA reagent A: sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tetrate in 0,2 N sodium hydroxide
- BCA reagent B: 4 % cupric sulfate
- Bovine serum albumin (BSA) as a standard: 2 mg/mL in 0,9 % saline and 0,05 % sodium azide

To prepare working reagent 50 parts of reagent A was mixed with one part of reagent B. Than 2 µL of each albumin standard dilution or unknown protein sample were added. After incubation for 30 min at 37 °C, absorbance at 562 nm was read.

3.2.6. Total RNA isolation from rat VSMCs and cDNA synthesis

Quiescent rVSMCs were pre-incubated with various agonists for 30 min, and than stimulated with thrombin for 6 h at 37 °C.

After stimulation the cells were washed twice with DPBS and treated with 0,5 g/L (v/v) of Trypsin-EDTA (1:250) (PAA) for 3 min at 37 °C. The cell suspension was centrifuged at 250 x g for 10 min and the cell pellet was collected. Total RNA isolation was performed using RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. The cells were lysed in 600 µL of lysis buffer supplied with β-mercaptoethanol. The lysates were carefully mixed by triturating to homogenize the sample, transferred onto QIAshredder spin column placed in a collection tube, and centrifuged for 2 min at 8000 x g. Next, 600 µl of 70 % ethanol was added to the lysate and mixed well by pipetting. The sample (700 µL) was applied to a RNeasy column placed in a collection tube to bind total RNA to the column-membrane, and centrifuged for 15 s at 8000 x g. Then, DNase solution (80 µL) was applied and incubated for 15 min at room temperature. Three washing steps, one with 350 µL of washing buffer and two with 500 µL of buffer containing ethanol were performed to remove contaminants from RNA sample. Finally, to elute RNA, RNeasy column was transferred to a new collection tube and 40 µL of RNase-free water was pipetted onto the column. The column was centrifuged for 1 min at >8000 x g, and the eluate was collected. To verify RNA integrity, 5 µL of eluate was heated for 4 min at 65°C and run on the 1 % agarose (Biozym) gel containing 0,5 µg/mL of ethidium bromide (Sigma), using 18 S + 28 S ribosomal RNA ladder (Gibco) as a positive control. The quantity of the extracted RNA was determined by spectrophotometry (BioPhotometer, Eppendorf) [148].

First-strand cDNA was synthesized from 1 µg of total RNA with the use of 1 µL of iScript Reverse Transcriptase, 4 µL of 5x iScript Reaction Mix containing

oligo (dT) and random hexamer primers (iScript cDNA Synthesis Kit, Bio-Rad) in a total volume of 20 µL water [128]. The incubation protocol using a PCR cycler (Perkin Elmer) comprised time of denaturation at 25°C for 5 min, elongation at 42 °C for 90 min, and the inactivation of reverse transcriptase at 85 °C for 5 min.

3.2.7. Determination of MCP-1 gene expression with RT-PCR

The resulting cDNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with gene specific primers for rat MCP-1 gene and mouse/rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (R&D Systems) [74]. The primers used were as follows: 5' – CTC TTC CTC CAC CAC TAT GC – 3' (sense) and 5' – GGC ATC ACA TTC CAA ATC ACA C-3' (antisense) for MCP-1, and 5' – CCC TTC ATT GAC CTC AAC TAC AAT GGT –3' (sense), and 5' – GAG GGG CCA TCC ACA GTC TTC TG –3' (antisense) for GAPDH. 2 µL of cDNA sample or positive control (R&D Systems) were amplified with 2,5 U of HotStarTaq DNA Polymerase (Qiagen) in a 50 µL reaction mixture containing 1,5 mmol $MgCl_2$, 0,2 mmol dTNP mix, and each of the Primers Pair (R&D Systems) at 7,5 µmol [129]. The amplification protocol comprised 25 cycles of denaturation at 95°C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR for *gapdh* was performed to rule out the possibility of RNA degradation and was used to control the variation of mRNA concentration in the RT-PCR reaction. PCR products were visualized by electrophoresis on a 1% agarose gel containing 0,5 µg/mL of ethidium bromide, electrophoresis. Gel was photographed and scanned, and the PCR products were quantified using the AlphaEase FC software system (Alpha Innotech, USA) and were standardized against the level of their respective *gapdh* control [74].

3.2.8. Quantitative determination of rat MCP-1

The rat MCP-1 Immunoassay Kit (Biosource) was applied for the quantitative detection of MCP-1 protein in culture media. The principle of the method is a solid phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA).

Quiescent rat VSMCs were incubated in serum-free medium with agonists for indicated time. The supernatants were then centrifuged at 10.000 x g for 3 min to remove remaining cells and immediately used or frozen at -75 °C for further experiments.

Samples and control specimens were pipetted (50 µL) into wells coated with anti-MCP-1 antibody (capture antibody), followed by the addition of a biotinylated secondary antibody (50 µL). The plates were incubated for 90 min at room temperature. After removal of secondary antibody excess by washing 4 times with a supplied buffer, 100 µL of straptavidin-peroxidase enzyme was added. After a second, incubation (30 min) at room temperature and washing to remove the unbound enzyme, the 100 µL of substrate solution was added. The third incubation (30 min) was performed in the dark at room temperature and the reaction was stopped thereafter by adding 100 µL of stop solution. The absorbance (optical density) of the solution was then read at 450 nm using a photometer SPECTRA (SLT). The results are shown as a ratio of the optical density of the sample to the absorbance of the control sample (supernatant from non-stimulated cells).

3.2.9. Fluorescent detection of intracellular ROS generation in rat VSMCs

Intracellular ROS levels were measured using the fluorescence dye 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes) in the concentration of 10 µmol/L, which is a non-polar compound converted into nonfluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is rapidly oxidized to the highly fluorescent 2'7'-dichlorofluorescein (DCF) in the presence of intracellular ROS [130].

Quiescent rVSMCs on 100 mm dishes were washed two times with DPBS buffer with Ca/Mg and treated with 0.05 % Trypsin-EDTA (PAA). After 5 min of incubation at 37 °C, 5 mL of 0,1 % Bovine serum albumin (Calbiochem) was added. The cell suspension was transferred to 50 mL tubes and centrifuged twice at 250 x g for 10 min with 20 mL of DPBS. The 2 mL of DPBS was added to the cell pelet and gently mixed. The cells were incubated at 37 °C for 30 min with 10 µmol/L H_2DCFDA. The rat VSMCs were then centrifuged at 250 x g for

10 min to remove the extraneous dye. Cells were resuspended in fresh DPBS and a baseline fluorescence reading was taken prior treatment [44]. Fluorescence was measured at excitation and emission wavelengths of 488 nm and 534 nm, respectively. Measurements were taken 0 min, 15 min, 30 min, 60 min, 120 min after the treatment using a Fluorescence spectrophotometer F-2000 (Hitachi).

Results were shown as a percent change from the baseline:

$$[Ft_{exp} / Ft_{base}] \times 100\%$$

where: Ft_{exp} = fluorescence at any given time during the experiment in sample and

Ft_{base} = baseline fluorescence of the same sample [15].

3.2.10. Detection of ROS production in aortas wall using DHE fluorescence

In situ O_2^* levels were assessed by the fluorescence probe dihydroethidium (DHE) (Sigma) [9]. Aortae were harvested from Wistar Kyoto rats (Harlan) and cut into 1 cm rings. The aortae were stimulated with thrombin (4 U/mL) for 6 h at 37 °C and incubated for 1 h with either HDL (0,5 mg/mL), S1P (10 µmol/L), SPC (10 µmol/L) or saline. The aorate were embedded in OTC Tissue Tek (Sakura Finetek Europe, Zoeterwonde) and frozen using liquid nitrogen-cooled isopantane. Cryosections (20 µm thick) of the rings were then placed on a glass, slide and DHE (10 µmol/L) were applied topically to each slide and incubated for 30 min, during which hydroethidine was oxidized to the fluophore ethidium, at 37 °C. Subsequently, the sections were washed, coverslipped and fluorescence images were obtained (λ Ex: 520 nm, Em: 605 nm) using a laser scanning microscope (Model LSM 510, Zeiss, Jena). To exclude an influence of the embedding procedure on fluorescence, all samples from treatment groups (Control, HDL, S1P, SPC) were embedded in the same block analyse simultaneously.

3.2.11. Measurement of NAD(P)H oxidase activity

Quiescent rVSMCs were incubated for 30 min with various agonists and stimulated with 2 U/mL thrombin for 10 min at 37 °C. Cells were than washed with DPBS and 350 µL of HBSS was added to each dish. Cells were scrapped and cell-extracts were prepared by three freeze-thaw cycles. Cells were centrifuged for 2 min at 250 x g. The protein concentration of each supernatant sample was determinated as described above. NADPH (Sigma) was added to the concentration of 1 mmol. DPI, a specific inhibitor of the NAD(P)H oxidase - was added immediately before assaying at the concentration of 10 µmol. The assay sample contained 500 µL of HBSS, NAD(P)H and 50 µg of homogenate. The absorbance of NAD(P)H at 340 nm was recorded continuously during 60 min. The changes in the absorbance obtained after 60 min in the presence of agonist or DPI were compared with changes obtained under control conditions. Use of DPI, a specific flavoprotein inhibitor, confirmed that under these experimental conditions the decrease of NAD(P)H absorbance was the result of the NAD(P)H oxidase activity [45].

3.2.12. Quantitative detection of $p38^{MAPK}$

The $p38^{MAPK}$ [pTpY180/182] Immunoassay Kit was used to estimate the quantity of phosphorylated isoform of $p38^{MAPK}$ protein in smooth muscle cells. The principle of the method is a solid phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA).

The quiescent rat VSMCs were stimulated with agonists for indicated times. Cells were washed twice with cold DPBS and collected by scraping. Samples were centrifuged at 250 x g for 10 minutes and pellets were suspended in 1 mL of cell extraction buffer (p38). The cells were lysed for 30 min on ice with vortexing in 10 min intervals. The cell extract was centrifuged at 10.000 x g for 10 min at 4 °C. The clear lysates were aliquoted and used immediately or frozen at –75 °C for longer storage.

The samples were diluted 1:10 with standard diluent buffer to reduce the matrix effect of the cell lysis buffer. Diluted samples (100 µL) and controls specimens were pipetted into wells coated with a capture antibody against $p38^{MAPK}$ and

incubated at room temperature for 2 h. After washing 4 times with the washing buffer, the antibody specific for $p38^{MAPK}$ phosphorylated at threonine 180 and 182 (detection antibody) was added for 1 h. After removal of the excess of detection antibody, 100 µL of horseradish peroxidase-labeled anti-rabbit IgG (anti-IgG-HRP) was added for 30 min and after washing, 100 µL of the substrate solution was added and incubated at room temperature for 30 min at dark. To stop the reaction, 100 µL of stop solution was added and the absorbance was read at 450 nm using photometer SPECTRA (SLT). The results are shown as ratio of the absorbance of the sample to the absorbance of the control specimen.

3.2.13. Gelatine zymography

MMP activity in the conditioned medium of rVSMCs was analysed by substrate-gel electrophoresis (zymography). To visualize the lysis of the substrate, the discontinous 10 % SDS - polyacrylamide gel containing 1 mg/mL gelatine (Serva) was used. The quiescent rVSMCs were incubated in serum-free medium with agonists for 24 h. Supernatants were then centrifuged at 10.000 x g for 3 min to remove remaining cells and used immediately or stored in –75 °C for further experiments. The amount of protein in samples was estimated as described above. 20 µg of each sample was mixed with 2 % (v/v) SDS - sample buffer containing 0,1 % bromophenol blue and loaded on to the gel (resolving gel: 80 mm x 50 mm x 0,5 mm). Gels were run using Mini Protean II electrophoresis apparatus (BioRad) at 24mA until the Bromophenol Blue dye run out of the gel. The gels were then washed in a buffer containing 2,5 % Triton X-100 at room temperature for 30 min. Gels were developed in a solution containing 50mmol Tris (pH 7,8) / 10mmol $CaCl_2$ for 18 h at 37 °C, stained with 1 % coomassie blue and destained in 5 % acetic acid / 10 % ethanol. Migration of proteins was compared with that of prestained low-MW range markers (Bio-Rad). Gelatinolytic activity appears as a clear band on a blue background. To compare intensity of lytic bands within a stained gel, gel was photographed using AlphaEase FC imaging system (Alpha Innotech) and analyzed with appropriate software [43, 81].

4. Results

4.1. Effects of HDL and its correlated lysosphingolipids on the induction of rat MCP-1

An increasing body of evidence unequivocally suggests that the monocyte chemoattractant protein-1 (MCP-1) plays a central role in the pathogenesis of atherosclerosis [55]. Thrombin, a coagulation factor inherently involved in the formation of atherosclerotic lesions, is one of the strongest stimuli for MCP-1 production [64, 66]. Thus, as a first step the effect of native HDL and lysosphingolipids associated with HDL on the thrombin-induced accumulation of MCP-1 in rat VSCMs was investigated. The production of MCP-1 was examined both at the gene expression and protein level. The abundance of MCP-1 mRNA was determined by reverse transcriptase-polymerase chain reaction analysis (RT-PCR) using mRNA isolated from rat VSMCs as a template and MCP-1 specific primers. As shown in Fig.7 thrombin added to rVSMCs at a concentration of 2 U/ml induced MCP-1 gene expression in a time dependent fashion. The maximal response to thrombin was observed after 6 h and remained stable afterwards. Therefore, incubation of rat VSMCs with thrombin for 6 h has been used in ensuing experiments.

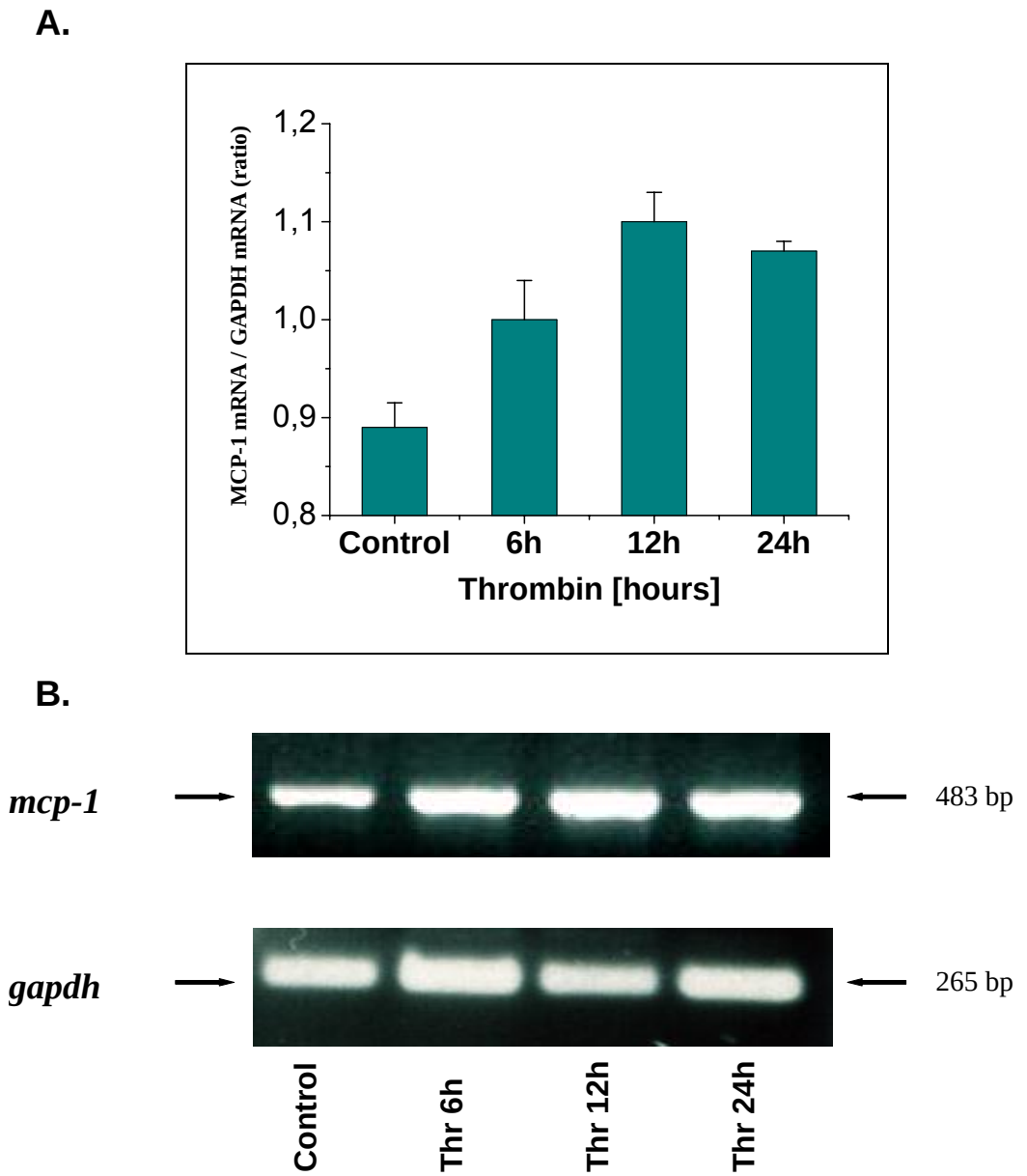


Figure 7. Effect of thrombin on MCP-1 expression. (A). *Rat vascular smooth muscle cells were incubated for indicated times with 2 U/ml of thrombin. The cells were washed and total RNA was isolated as described in 'Materials and Methods' section. The abundance of MCP- mRNA was estimated using RT-PCR with primers specific for MCP-1. GAPDH mRNA was used as a control. The ratio of MCP-1 mRNA to GAPDH mRNA was calculated from three experiments and used as a measure of thrombin stimulated MCP-1 expression in rat VSMCs. The data show means ± SD from three independent experiments.* ***(B).*** *Shown is a representative RT-PCR analysis of MCP-1 gene expression. The reaction products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. The 483-base pair (bp) band represents the mcp-1 product and the 265 bp band represents the gapdh product.*

To assess the effect of HDL on thrombin-induced the *mcp-1* expression, rat VSMCs were incubated with 1mg/mL HDL for 30 min prior to the treatment with 2 U/mL thrombin for 6 h. A total RNA isolation and cDNA synthesis were performed as described in the *'Materials and Methods'* section. The resulting cDNAs were subjected to RT-PCR with primers specific for rat *mcp-1* and mouse/rat glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) as a control. The reaction products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. As shown in Fig.8 HDL markedly attenuated thrombin-induced expression of *mcp-1*. Subsequent quantification of the results of electrophoresis using densitometric analysis revealed that in three independent experiments HDL reduced thrombin stimulated *mcp-1* expression by 54,3 % ± 16,02 %.

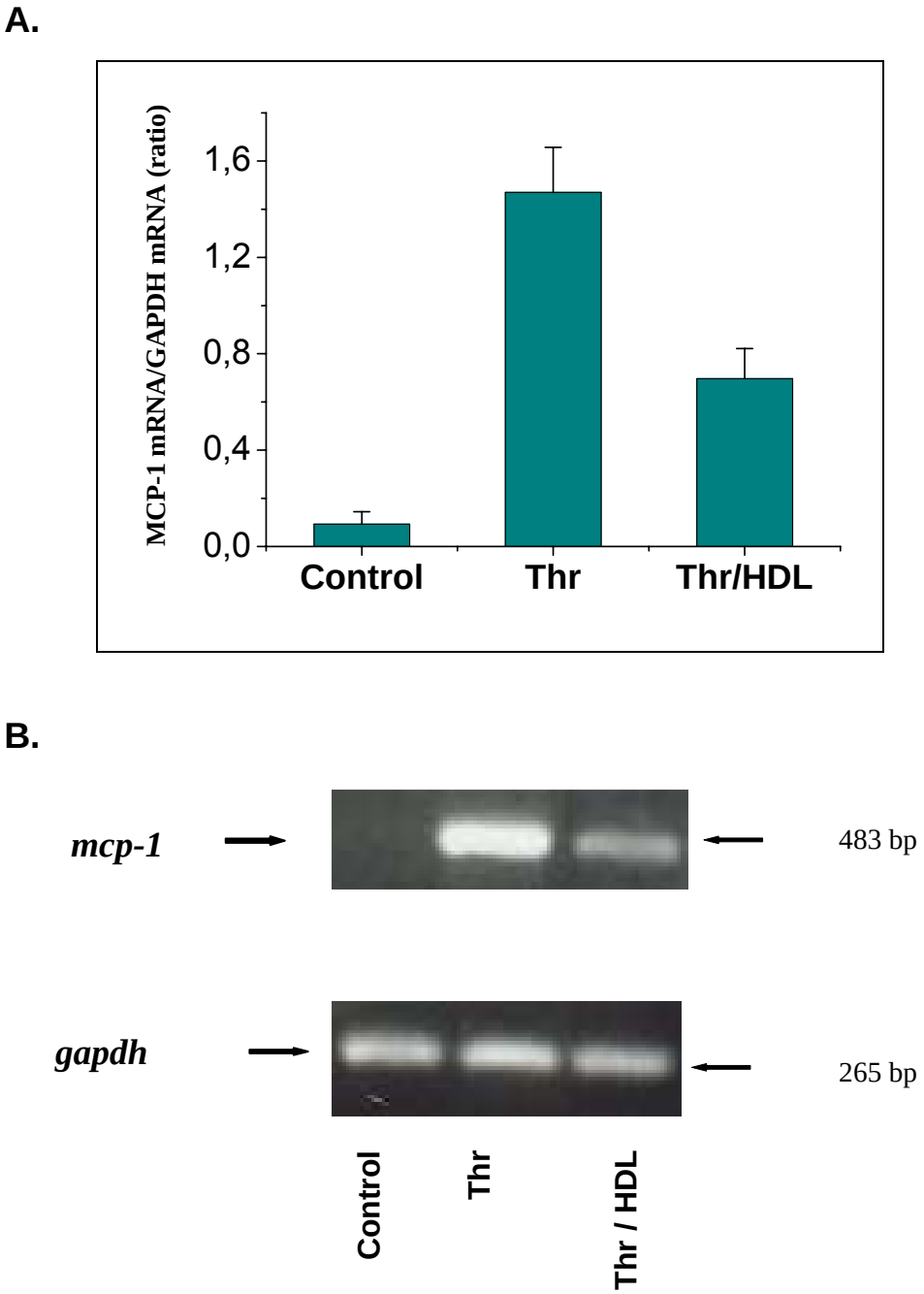


Figure 8. HDL inhibits thrombin-induced MCP-1 expression in rat VSMCs. *Rat vascular smooth muscle cells were incubated with 1mg/ml of native HDL for 30 min prior to stimulation with 2 U/ml of thrombin for 6 h. The cells were washed and total RNA was isolated as described in the 'Materials and Methods' section. The abundance of MCP-1 mRNA was estimated using RT-PCR with primers specific for MCP-1.* ***(A).*** *Bar graph shows the ratio of MCP-1 mRNA to GAPDH mRNA. The data show means ± SD from three independent experiments.* ***(B).*** *Shown is a representative RT-PCR analysis of mcp-1 gene expression. The reaction products were electrophoresed on a 1 % agarose gel and stained with ethidium bromide. The 483 bp band represents the mcp-1 product, and the 265 bp band represents the gapdh product.*

Next, the effect of lysosphingolipids associated with HDL particles on the *mcp-1* expression in rVSMCs has been examined. To this purpose, quiescent rat VSMCs were treated with 5 µmol/L of S1P for 30 min prior to stimulation with 2 U/mL of thrombin for 6 h. As shown in Fig.9, exposure of cells to S1P led to a marked reduction of MCP-1 mRNA levels induced by treatment with thrombin. In three independent experiments, S1P reduced MCP-1 expression by 36,8 % ± 26,7 %, respectively.

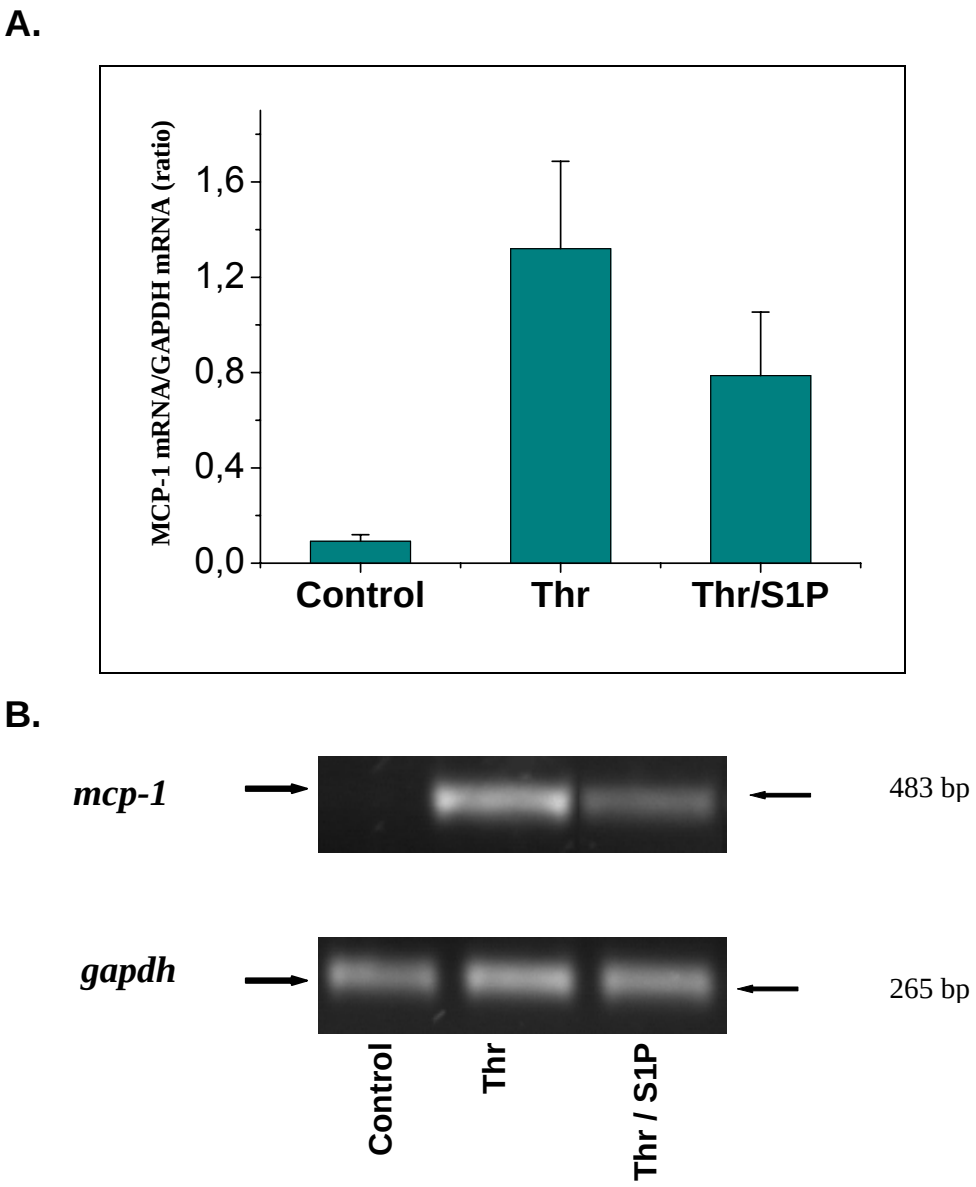
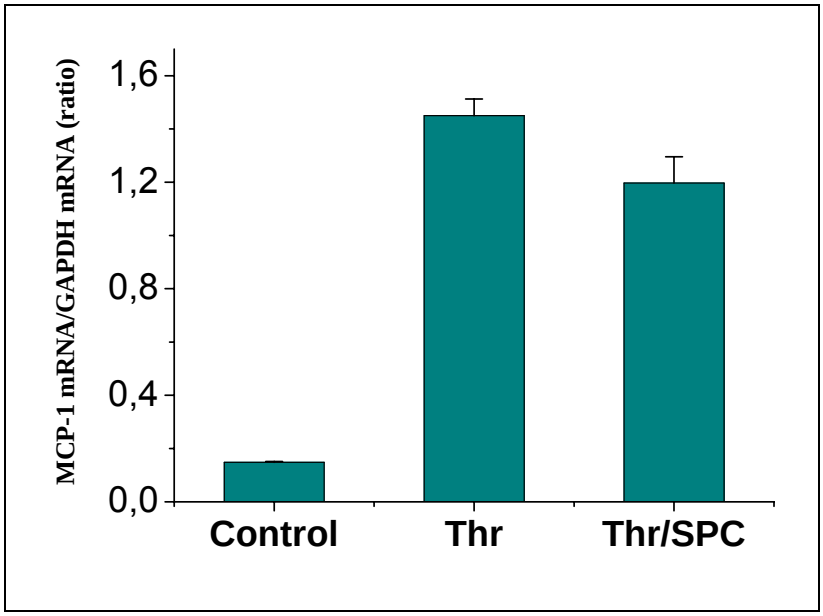


Figure 9. Effect of S1P on thrombin-induced mcp-1 expression. (A). *Bar graph shows mcp-1 expression levels in rat VSMCs stimulated with 2 U/mL of thrombin for 6 h, following to the pretreatment with 5 µmol/L of S1P for 30 min. The ratio of MCP-1 mRNA to GAPDH mRNA was used as an estimation of MCP-1 expression. The data show means ± SD from three independent experiments.* ***(B).*** *Shown is a representative RT-PCR analysis of mcp-1 gene expression. The reaction products were electrophoresed on a 1 % agarose gel and stained with ethidium bromide. The 483 bp band represents the mcp-1 product, and the 265 bp band represents the gapdh product.*

The same procedure was performed with another lysosphingolipid associated with HDL, namely SPC. The quiescent rat VSMCs were exposed to 5 µmol/mL of SPC for 30 min prior to stimulation with 2 U/mL of thrombin for 6 h. As shown

in Fig.10, exposure of cells to SPC reduced a MCP-1 mRNA levels induced by treatment with thrombin. In three independent experiments, SPC reduced *mcp-1* expression by 17,2 % ± 9,8 %, respectively.

A.



B.

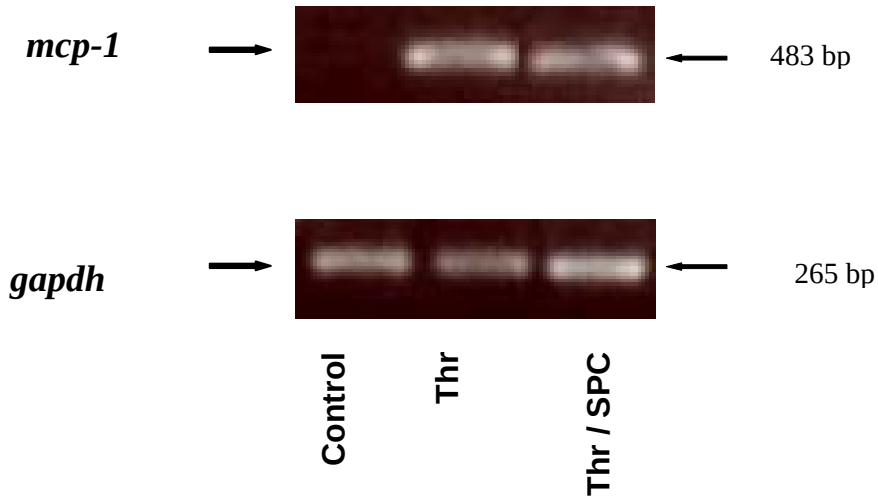


Figure 10. Effect of SPC on thrombin-induced MCP-1 expression. (A). *Bar graph shows mcp-1 expression levels in rat VSMCs stimulated with 2 U/mL of thrombin for 6 h, following to the pretreatment with 5 µmol/L of SPC for 30 min. The ratio of MCP-1 mRNA to GAPDH mRNA was used as an estimation of MCP-1 expression. The data show means ± SD from three independent experiments.* ***(B).*** *Shown is a representative RT-PCR analysis of mcp-1 expression. The reaction products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. The 483 bp band represents the mcp-1 product, and the 265 bp band represents the gapdh product.*

In the next series of experiments the effects of HDL and HDL-associated lysosphingolipids on thrombin-induced production of MCP-1 protein were examined. As MCP-1 is secreted by smooth muscle cells, the MCP-1 concentration was determined in the cell culture medium instead of cell extracts. Commercially available Enzyme-Linked Immunosorbent Assay (ELISA) specific for MCP-1 has been used to estimate the protein concentration. Initially, the kinetic of MCP-1 secretion under influence of thrombin was determined. As shown in Fig.11, stimulation of cells with 2 U/mL of thrombin led to progressive accumulation of MCP-1 in the culture medium. After 6 and 24 h the concentrations of accumulated MCP-1 were 0,04 ± 0,014 O. D. and 0,161 ± 0,034 O. D. respectively. Thus, in the subsequent experiments the effect of HDL and lysosphingolipids on the thrombin-induced MCP-1 expression were determined after 6 and 24 h of incubation with the agonist.

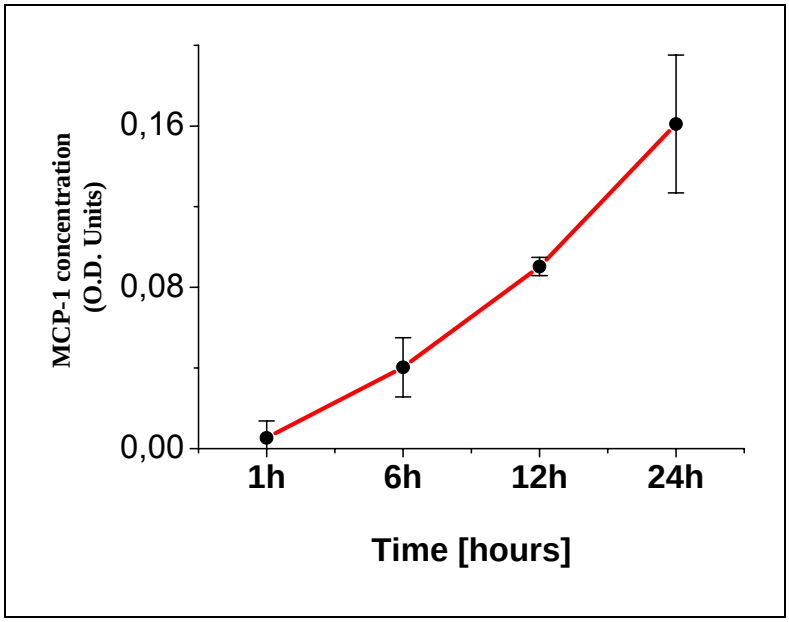


Figure 11. Effect of thrombin on MCP-1 concentration. *Rat VSMCs were incubated for indicated times with 2 U/mL of thrombin. The medium was collected and MCP-1 concentration was determined using ELISA as described under 'Materials and Methods' section. Results show means ± SD from three independent experiments.*

Next, the effect of HDL and lysosphingolipids on the MCP-1 secretion was investigated. rVSMCs were exposed to 1 mg/mL HDL, 10 μmol/L S1P,

20 µmol/L of SPC and 500 µmol of Trolox for 30 min at 37°C before addition of 2 U/mL of thrombin for 6 and 24 h. Trolox, a vitamin E derivative is known to suppress agonist-induced MCP-1 production and was used as a control in these experiments. The absorbance in cell supernatants was taken to estimate the MCP-1 concentration [Fig.12]. Under control condition, thrombin (2 U/mL) significantly stimulated the MCP-1 accumulation from 0,105 ± 0,04 O.D. to 0,707 ± 0,15 O.D. within 6 h after treatment, and from 0,130 ± 0,05 O.D. to 1,83 ± 0,2 O.D. within 24 h after treatment. Exposure of the cells to HDL or HDL-lysosphingolipids prior to stimuli did not affect the concentrations of MCP-1 in cell supernatants. By contrast, all tested compound severely suppressed thrombin-induced MCP-1 secretion in rat VSMCs. In cells pre-incubated for 30 min with 1mg/mL of HDL, the thrombin-induced MCP-1 concentration was significantly decreased to 0,381 ± 04 O.D. after 6 h and to 0,897 ± 0,12 O.D. after 24 h of incubation with the agonist. Furthermore, MCP-1 concentrations were significantly diminished to 0,367 ± 0,07 O.D. after 6 h, and to 0,962 ± 0,32 O.D. after 24 h of incubation with 10 µmol/L S1P and to 0,325 ± 0,06 O.D. after 6 h, and to 0,842 ± 0,16 O.D. after 24 h incubation with 10 µmol/L SPC. As expected, Trolox effectively reduced MCP-1 secretion by thrombin-stimulated rat VSMCs. The concentrations of MCP-1 in cell supernatants decreased to 0,304 ± 0,05 O.D. after 6 h, and to 0,453±0,03 O.D. after 24 h of thrombin treatment in the presence of this compound.

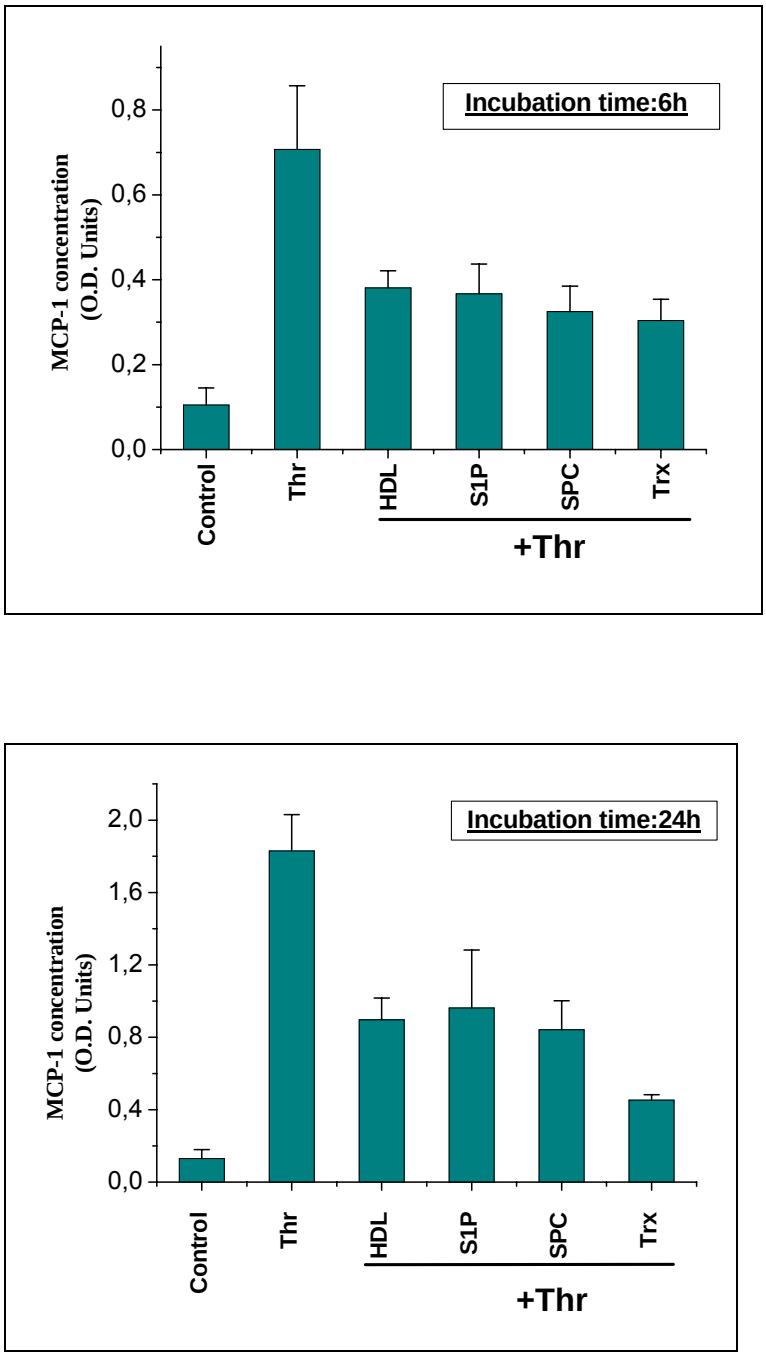


Figure 12. Accumulation of thrombin-induced MCP-1 after HDL, S1P, SPC, and Trolox treatment. *Quiescent rat VSMCs were pre-incubated with 1 mg/mL of HDL, 10 µmol/L of S1P, 10 µmol/L of SPC and 500µmol of Trolox for 30 min and then stimulated with 2 U/mL of thrombin for 6h (upper panel) and 24h (lower panel). The concentration of MCP-1 was determined in cell supernatants using MCP-1 specific ELISA as described under 'Material and Methods' section. The optical density at 450 nm was used to measure .for relative MCP-1 concentration. The results show means ± SD from four to six independent experiments.*

The lysosphingolipids are present in HDL in concentrations lower than these used in the described experiments. To examine, whether S1P or SPC affect MCP-1 production in concentrations close to physiological ones, the concentration-dependent inhibition of thrombin-induced MCP-1 secretion by each compound was examined. To this purpose, rat smooth muscle cells were pre-treated with either S1P or SPC in increasing concentrations for 30 min prior to addition of 2 U/mL thrombin. Fig.13 demonstrates the effects of S1P and SPC on MCP-1 concentrations in supernatants from thrombin-treated rat VSMCs. The MCP-1 concentration in cells stimulated with thrombin for 24 h was set as 100%. It is evident that the inhibitory effects of S1P and SPC were already observed at agonist concentrations as low as 0,5 µmol/L. These concentrations of sphingolipids are likely to be achieved in the circulating HDL fraction.

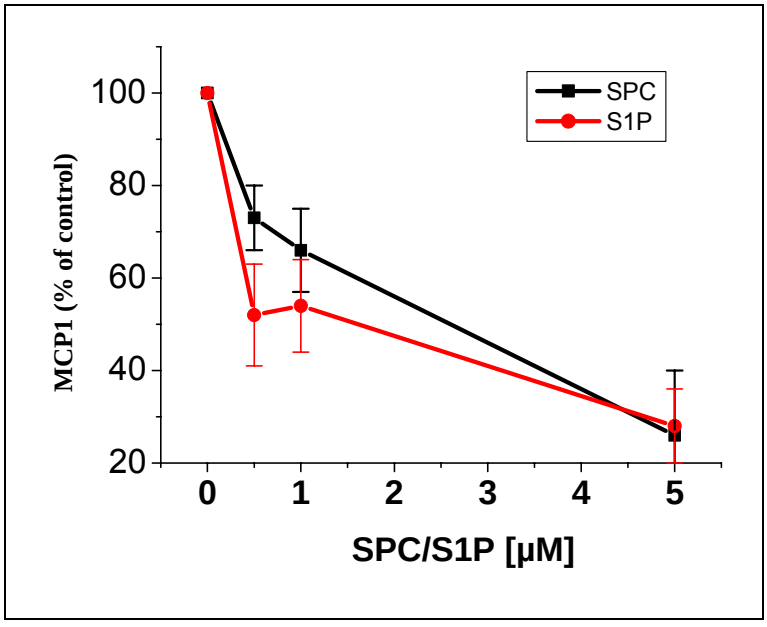


Figure 13. Effects of S1P and SPC on the MCP-1 concentration. *Quiescent rat VSMCs were exposed for 30 min to increasing concentrations of S1P (-●-) or SPC (-■-) prior to treatment with 2U/mL of thrombin. The concentration of MCP-1 was determined in cell supernatants using MCP-1 specific ELISA as described under 'Materials and Methods' section. The optical density at 450 nm was used to measure for relative MCP-1 concentration. The results shown means ± SD from three independent experiments.*

4.2. Effects of HDL, SPC, and S1P on ROS formation in rat VSMCs

A large body of evidence accumulated over recent years present that production of MCP-1 in smooth muscle cells and other cell lines is under tight control of NAD(P)H-oxidase-generated reactive oxygen species (ROS), which act as an intracellular messenger [12,48]. HDL was previously shown to scavenge oxygen free radicals by increasing intracellular content of antioxidants. There is also some evidence that HDL counteracts intracellular generation of ROS induced by oxidized LDL [120,136]. It was thus of great interest to investigate whether HDL exerts the inhibitory effects on thrombin-induced MCP-1 synthesis via modulation of NAD(P)H-oxidase activity and intracellular generation of ROS. To test this hypothesis, the level of intracellular ROS production was monitored fluorometrically in rVSMCs loaded with H_2DCFDA (10 µmol/L)-a ROS-sensitive dye. As shown in Fig.14, H_2DCFDA-associated fluorescence increased gradually in rat VSMCs over a period of 120 min indicating a – steady - state generation of ROS in culture cells. Addition of thrombin in a concentration of 2 U/mL enhanced intracellular ROS production. In the presence of this agonist, the H_2DCFDA - associated fluorescence increased by 18,8 % and 12,5 % after 60 min and 120 min, respectively. Addition of HDL completely inhibited the thrombin-induced enhancement in ROS production. Under these experimental conditions, the increase of H2DCFDA fluorescence remained slightly under the control levels in rat VSMCs exposed to HDL regardless of stimulation with thrombin [Fig.14]. This experiment provides thus the evidence that HDL inhibits intracellular ROS generation in rat VSMCs.

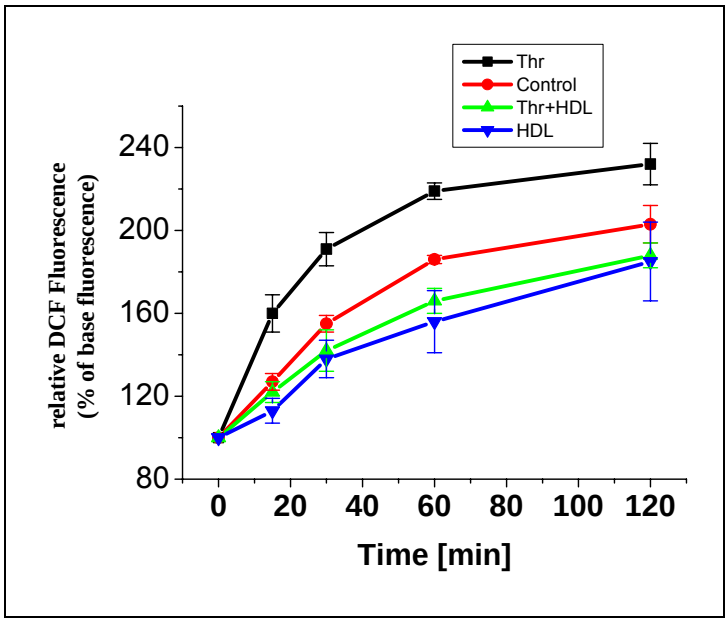


Figure 14. Effect of HDL on thrombin-induced ROS generation in rat VSMCs. *The graph represents percentage increase of fluorescence in cells loaded with the redox-sensitive dye H_2DCFDA (10 µmol/L), which is converted to the highly fluorescent oxidation product DCF. In the presence of ROS the changes in fluorescence were recorded after 15, 30, 60, and 120 min in rat VSMCs in the absence (-●-) or in the presence of 1 mg/mL HDL (-▼-), 2 U/mL thrombin (-■-), and thrombin and HDL (-▲-). The results shown means ± SD from three to six independent experiments.*

Next, the effects of HDL on thrombin-induced ROS generation were compared with the effects of the HDL-associated lysosphingolipids, S1P and SPC. To this end rat VSMCs were loaded with the ROS-sensitive fluorescent dye H_2DCFDA as described in the *'Materials and Methods'* section. Thereafter, cells were incubated for 30 min with 1mg/mL HDL, 10 µmol/L S1P, or 10 µmol/L SPC and ROS generation was triggered by adding 2 U/mL of thrombin. The measurements of fluorescence were taken after 30 and 60 min. The results are shown in Fig. 15, where the fluorescence corresponding to ROS generation in the presence of thrombin was set as 100 %. As expected, the pre-treatment the cells with HDL for 30 and 60 min reduced thrombin-induced ROS generation to 49,8 ± 7,6 % and to 48,4 ± 6,8 % respectively. Likewise, in the presence of S1P, the thrombin induced ROS generation was decreased to 77,7 ± 4,1 % and 72,2 ± 2,6 % after 30 and 60 min of stimulation, respectively. The pre-incubation of rat VSMCs with SPC also suppressed the production of ROS, which under

these experimental conditions amounted to 71,8 ± 5,4 % and 66,9 ±3,1 % after 30 and 60 min, respectively.

Collectively, these data clearly demonstrate that HDL and HDL-associated lysosphingolipids exert inhibitory effects on thrombin-induced ROS generation in rat VSMCs.

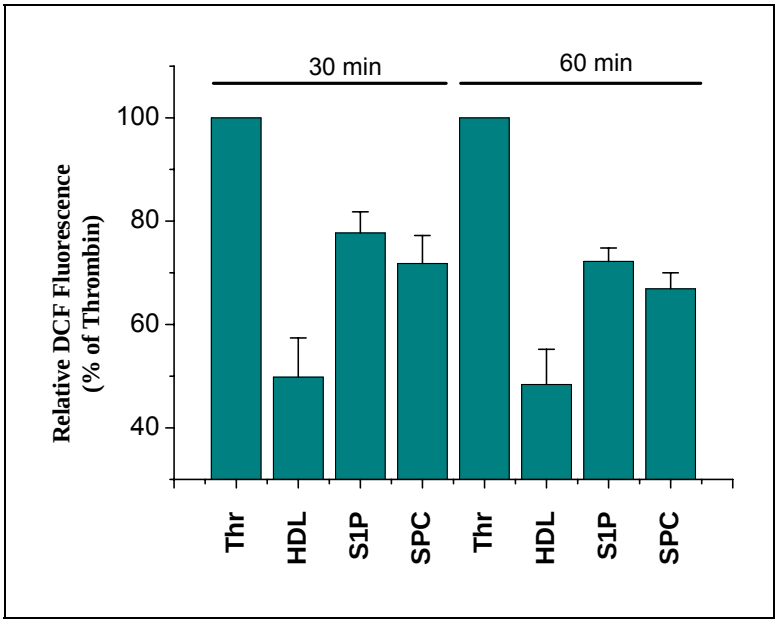


Figure 15. Effects of HDL, S1P and SPC on the thrombin-induced ROS generation in rat VSMCs. *The rat VSMCs were loaded with 10 µmol/L of H_2DCFDA for 30 min as described in the 'Materials and Methods' section. Thereafter, cells were incubated with 1 mg/mL HDL, 10 µmol/L S1P or 10 µmol/L SPC, and ROS production was triggered by addition of 2 U/mL thrombin. Shown is a change in DCF fluorescence seen after 30 and 60 min of stimulation. The DCF fluorescence associated with the ROS generation induced by 2 U/mL thrombin was set as 100 %.*

To confirm the effect of HDL and HDL-associated lysopshingolipids on ROS production, the dihydroethidium (DHE) fluorescent microscopy *in situ* was performed. The aortas were prepared as described in the *'Materials and Methods'* section. The sections of aortas were treated then for 30 min with 1mg/mL HDL, 10 µmol/L S1P or 10 µmol/L SPC before incubation with 2 U/mL thrombin for 10 min. After addition of the ROS-sensitive fluorescence dye, DHE(10 µmol/L) for 30 min, the sections were washed and the overlays of fluorescence were obtained. As shown in Fig. 16, thrombin markedly enhanced

DHE fluorescent intensity compared to control (A and B). The increase in fluorescence was profoundly reduced by HDL and by HDL-associated lysosphingolipids S1P and SPC (images from C to E).

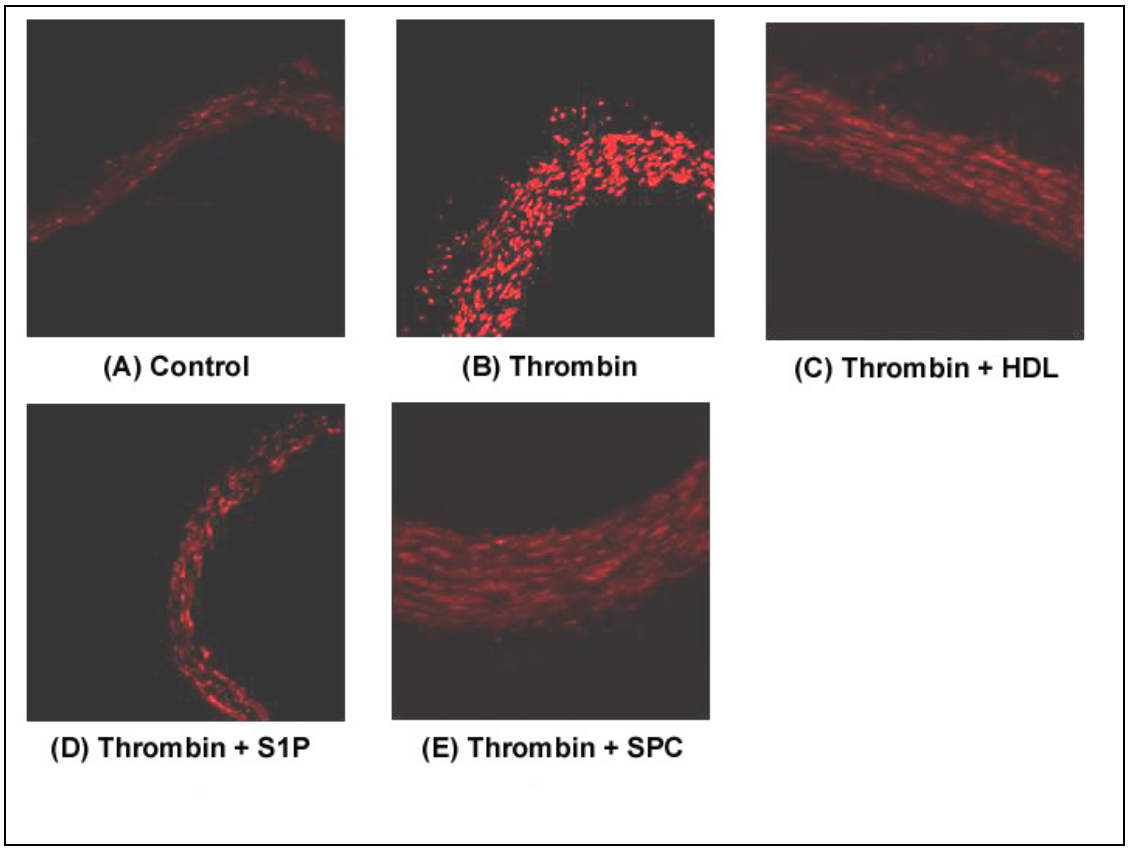


Figure 16. ROS formation in aortas wall after HDL, SPC and S1P treatment prior to thrombin stimulation. *In situ ROS generation was determined using fluorescence microscopy of aortas incubated with ROS-sensitive dye, DHE (10 µmol/L).* ***(A)*** *Weak ROS signals were detected in the control aorta.* ***(B)*** *Intense production of ROS was observed in thrombin (2 U/mL) treated aorta.* ***(C,D,E)*** *Compared to thrombin-treated aorta, the ROS production was markedly inhibited by addition of HDL (1 mg/mL), S1P (10 µmol/L), and SPC (10 µmol/L) respectively. Shown is a representative fluorescence image from three independent aortas sections.*

4.3. Effects of HDL and HDL–associated lysosphingolipids on the activity of NAD(P)H oxidase

The inhibitory effect of HDL and HDL-associated lysosphingolipids on the thrombin-triggered ROS generation could be explained by their direct effect on the activity of NAD(P)H-oxidase, which is the major source of ROS in VSMCs. However, HDL could also affect ROS generation indirectly, for instance, by

supplying the cells with antioxidants such as α-tocopherol. It was, therefore, next sought to determine the effects of HDL and HDL-associated lysosphingolipids on the activity of NAD(P)H-oxidase. To this end, the turnover rate of NADPH was determined photometrically in rat VSMCs homogenates and used as a raw estimation of NAD(P)H-oxidase activity. As shown in Fig.17, incubation of rat VSMCs homogenates with NADPH led to the continuous consumption of NADPH as indicated by the decrease of the absorbance at the NADPH-characteristic wavelength of 340 nm. The substantial increase in NADPH consumption indicating stimulation of NAD(P)H-oxidase activity could be observed in homogenates of rat VSMCs pre-treated with 2 U/mL of thrombin. Pre-treatment of VSMCs with HDL did not change the consumption of NADPH. By contrast, the thrombin-induced increase in the NADPH turnover rate was much decreased in homogenates pre-incubated with HDL prior to the addition of the agonist.

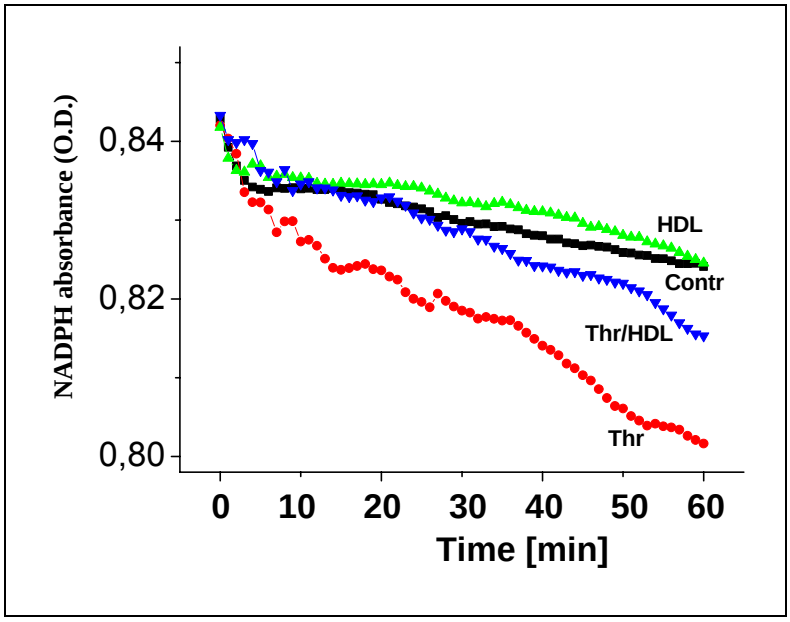


Figure 17. Effect of HDL on the NAD(P)H-oxidase activity in rVSMCs. *The rat VSMCs were pre-treated with 1 mg/mL of HDL for 30 min, prior to the addition of 2 U/mL thrombin. Cell homogenates were collected and incubated with 1 mmol of NADPH. Absorbance was monitored continuously measured at 340 nm over 60 min. Shown are original tracing from three to five independent experiments. Tracings were superimposed for comparison.*

To express the NAD(P)H-oxidase activity in quantitative terms, NADPH consumption rates were calculated as described in the *'Material and Methods'* section. In un-stimulated cells, the basal NAD(P)H-oxidase activity amounted to 3,3 ±1,4 O.D. Units/min. In thrombin-stimulated cells, the NADPH consumption rate increased to 8,8 ± 2,0 O.D. Units/min. By contrast the pre-treatment of cells with HDL prior to addition of thrombin reduced the NADPH consumption rate to 4,6 ± 1,3 O.D. U/min [Fig.18]. Similar effects could be observed in rVSMCs pre-incubated with 10 µmol/L of S1P or 10 µmol/L of SPC. Whereas these lysosphingolipids alone did not affect the NAD(P)H turnover under basal conditions, they reduced the thrombin-induced NAD(P)H consumption rate to 3,9 ± 0,7 O.D. U/min and 5,0 ± 1,1 O.D. U/min, respectively. To demonstrate, that the recorded changes in the NAD(P)H consumption were indeed related to NAD(P)H-oxidase activity, the effect of its specific inhibitor- diphenylene iodinium (DPI) has been additional examined. As shown in Fig. 18, the pre-treatment of rVSMCs with 10 µmol/L of DPI almost completely abolished changes of the absorbance at 340 nm. These results strongly support the motion that the changes in the NADPH consumption observed in this study are attributable to actual changes of NAD(P)H-oxdiase activity.

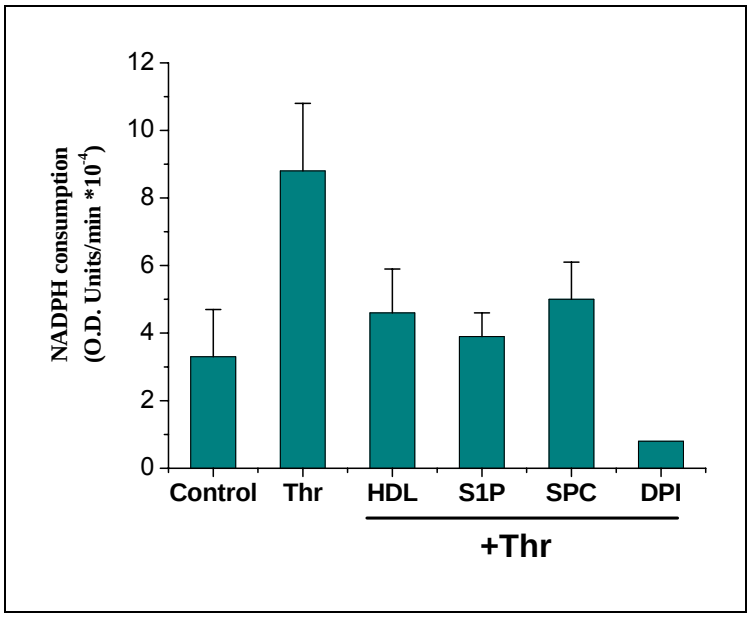


Figure 18. Effects of HDL and HDL- associated lysosphingolipids on NAD(P)H oxidase activity. *Bar graph shows effects of HDL, S1P, SPC and DPI on the thrombin-induced NADPH consumption which is a raw estimate of the NAD(P)H oxidase activity. The rVSMCs were pre-incubated with 1 mg/mL HDL, 10 µmol/L of S1P or 10 µmol/L of SPC and then stimulated with 2 U/mL of thrombin. The absorbance changes at 340 nm associated with the consumption of NADPH were monitored photometrically. The NADPH consumption rate was calculated as described in the 'Materials and Methods' section. Shown are means ± SD from three to five independent experiments.*

4.4. Effects of HDL, S1P, and SPC on the thrombin-induced activation of the p38^{MAPK}

Several studies demonstrated that the p38^{MAPK} activity is directly regulated by NAD(P)H-oxidase in smooth muscle cells [12, 48]. It was, therefore, logical to assume that the inhibitory effects of HDL and HDL-associated phospholipids exerted on the NAD(P)H–oxidase activity should be reflected by changes in the p38^{MAPK} activity in thrombin-stimulated rat VMSCs.

To investigate the effect of HDL and HDL-associated phospholipids on p38^{MAPK} activation, antibodies specifically reacting with phosphorylated epitopes of p38^{MAPK} were used. This approach takes advantage of the fact that the phosphorylation of these epitopes by upstream-located kinases is associated with activation of p38^{MAPK} in rat VSMCs. Intracellular levels of the phosphorylated isoform of p38^{MAPK} were determined using specific ELISA as described in the *'Material and Methods'* section, and were directly proportional

to the level of activation of this kinase. As shown in Fig.19, addition of thrombin (2 U/mL) to rat VSMCs led to a substantial increase in the active form of $p38^{MAPK}$ within 1 min after stimulation. The levels of active $p38^{MAPK}$ remained elevated 5 and 10 min after stimulation. Pre-treatment of rVSMCs with 1 mg/mL of HDL much reduced the levels of active $p38^{MAPK}$ at all time-points examined.

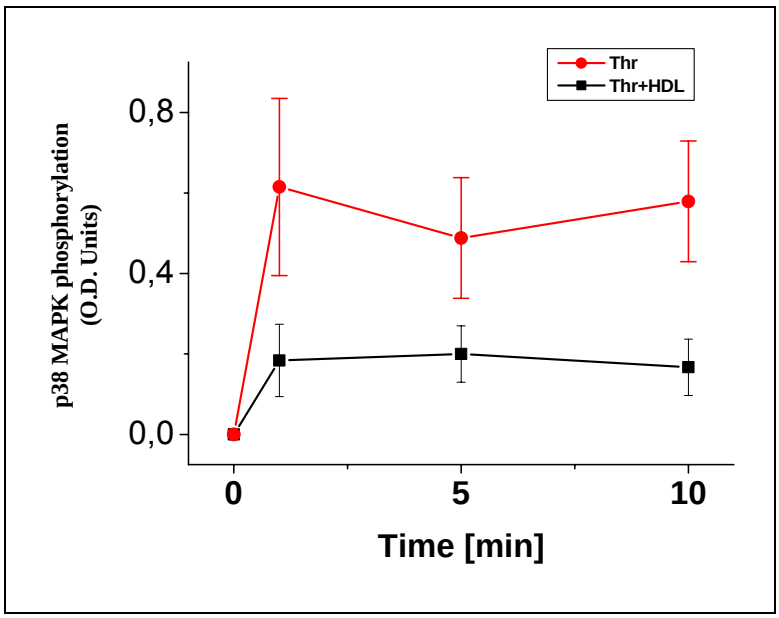


Figure 19. Effects of HDL on the thrombin-induced phosphorylation of the $p38^{MAPK}$. *The rat VSMCs were pre-incubated with 1 mg/ml of HDL prior to stimulation with 2 U/ml of thrombin. The absorbance at 450 nm reflecting the level of active $p38^{MAPK}$ was measured using ELISA with antibody specific for phosphorylated $p38^{MAPK}$ as described in the 'Material and Methods' section. Line graph shows phosphorylation of $p38^{MAPK}$ after pretreatment with 1 mg/mL of HDL prior to stimulation with 2 U/mL of thrombin for 1, 5 and 10 min. Shown are means ± SD from three to five independent experiments.*

To assess whether S1P and SPC contribute to inhibitory effects of HDL on the thrombin-induced activation of $p38^{MAPK}$, the experiment described above was performed using these two agents. Rat VSMCs were treated with 1 mg/mL of HDL, 10µmol/L of S1P, and 10 µmol/L of SPC for 30 min prior to stimulation with 2 U/mL of thrombin for 10 min. As shown in Fig. 20, both S1P and SPC markedly reduced $p38^{MAPK}$ activation in smooth muscle cells. Under these conditions the $p38^{MAPK}$ phosphorylation was reduced by 44,6 % and 46,7 %, respectively.

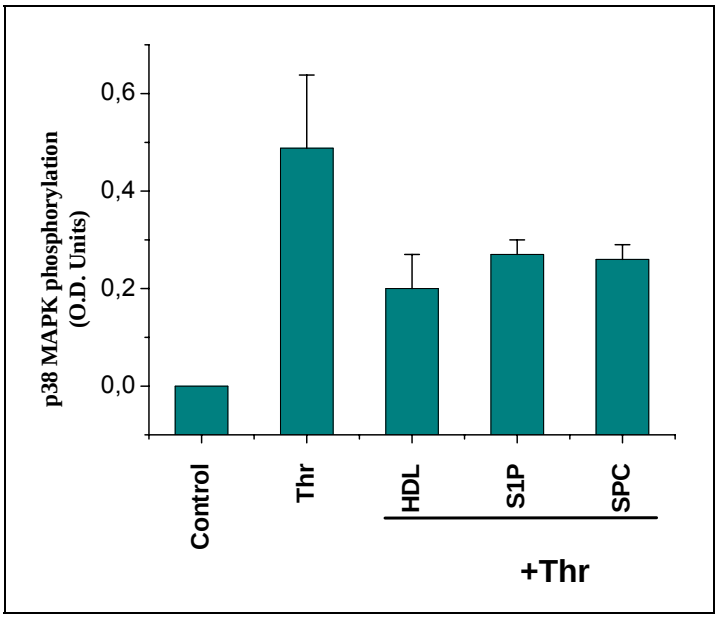


Figure 20. Effects of HDL, S1P, and SPC on thrombin-induced p38^{MAPK} phosphorylation. *The rat VSMCs were treated with 1 mg/mL of HDL, 10 µmol/L of S1P, and 10µmol/L of SPC prior to stimulation with 2 U/mL of thrombin. Bar graph shows the absorbance at 450 nm of an activated p38^{MAPK} in cells extracts measured using the p38^{MAPK} ELISA kit. The absorbance is directly related to the activation of p38^{MAPK}. The results shown means ± SD from three to six independent experiments.*

4.5. Effects of HDL on thrombin-induced MMP-2 expression by rat VSMCs

MMPs are involved in the degradation of the extracellular matrix during atherogenesis and intracellular signaling via ROS is potentially involved in this process [51, 69]. Therefore, it was next sought to determine whether HDL or HDL-associated lysopshingolipids are able to inhibit metalloproteinase secretion via their inhibitory effect on NAD(P)H-oxidase activity and ROS generation. As smooth muscle cells are potentially able to secrete MMPs in response to the thrombin, it was used as agonist in this series of experiments [33]. Gelatine zymography was performed to evaluate MMP-2 activities in the conditioned media obtained from rat vascular smooth muscle cells stimulated for various time intervals. As shown in Figure 21, the most pronounced MMP-2 activation could be observed after 24 h exposure of rVSMCs to 2 U/mL of thrombin. Therefore, in subsequent experiments the effect of HDL on the thrombin-

induced MMP-2 activation was investigated after 24 h stimulation with the agonist.

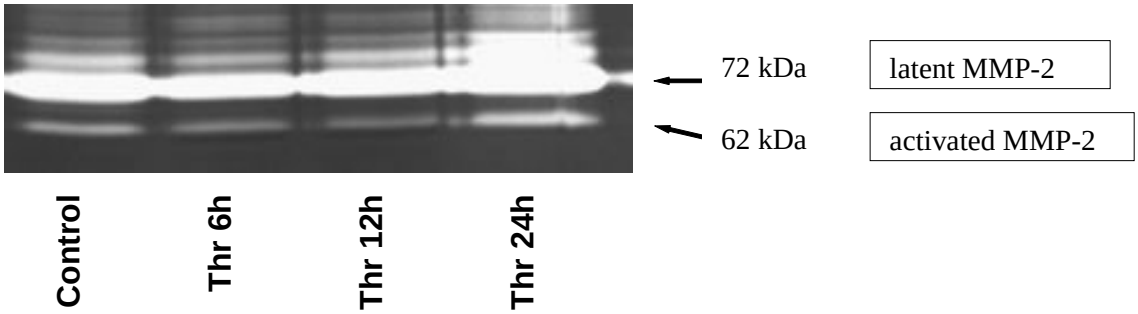


Figure 21. Effect of the thrombin treatment on MMP-2 activity. *Rat VSMCs were incubated with 2 U/mL of thrombin for 6, 12 and 24 h. The cells lysates were collected and MMP activity was determined using gelatine zymography as described in the 'Materials and Methods' section. The results are representative for one independent experiment out of three. Latent form (72 kDa) and 62 kDa form which corresponds to the active form of MMP-2 are shown by the arrows.*

Next, the effects of HDL on the thrombin-induced MMP-2 activity were examined. To this purpose, cells were treated with 1 mg/mL of HDL for 30 min prior to induction with thrombin. The data in Fig.22 demonstrate lack of the inhibitory effect of HDL on the conversion of MMP-2 from the latent to the activated form. It appears, therefore, that there is no inhibitory effect of HDL on thrombin-induced MMP-2 activity in rat vascular smooth muscle cells.

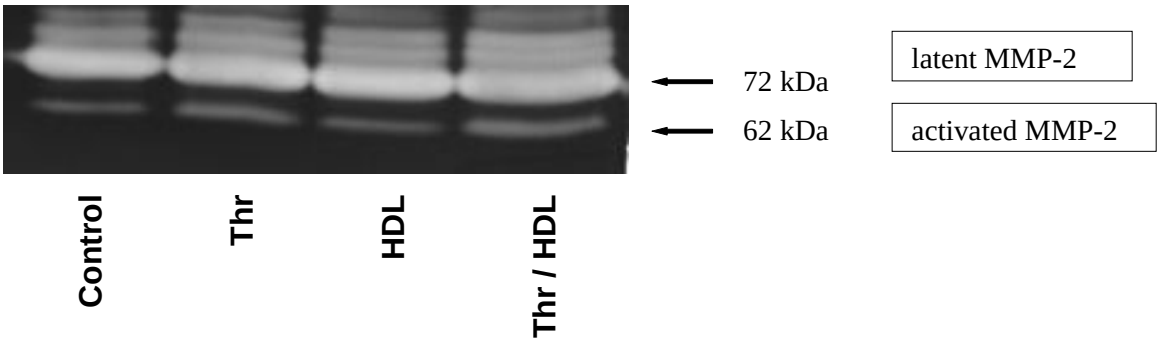


Figure 22. Effect of HDL on MMP-2 activity. *Rat VSMCs were treated with 1mg/ml of HDL for 30 min prior to stimulation with 2 U/mL of thrombin for 24 h. Cell lysates were collected and MMP-2 activity was examined using gelatine zymography as determined under 'Materials and Methods' section. Zymogram shows band of 62 kDa which corresponds to the active form of MMP-2. The results are representative for one independent experiment out of three.*

5. Discussion

The results of the present work unequivocally demonstrate that:

1) HDL exerts a profound inhibitory effect on the thrombin-induced MCP-1 synthesis.
2) The inhibitory effect of HDL is associated with the suppression of the NAD(P)H oxidase - dependent ROS generation.
3) The inhibitory effects of HDL on MCP-1 production and NAD(P)H oxidase - dependent ROS generation depends on the presence of biologically active lysophospholipids such as S1P and SPC in these lipoproteins.

A large body of evidence accumulated over recent years showing that monocyte chemoattractant protein-1 (MCP-1) exerts strong pro-atherogenic effects [23]. Mice that had a deletion in the MCP-1 gene and therefore expressed no *mcp-1*, had a reduced susceptibility to atherosclerosis. Consistent with this, significantly smaller lesions were observed in mice apo $E^{-/-}$ mice lacking chemokine receptor CCR2, which is a receptor for MCP-1 [114]. Conversely, overexpression of MCP-1 reversed this effect in apo $E^{-/-}$ mice and accelerated progression of atherosclerosis [119]. Genetic studies in human populations revealed several polymorphisms both in MCP-1 and CCR2 associated with premature atherosclerosis or severity of coronary heart disease. Some of these polymorphisms were associated with increased levels of MCP-1 in plasma and/or elevated levels of lipoprotein (a) (Lp(a)), which is a well-known risk factor for coronary heart disease (CHD) [23 ,114, 119]. Investigations *in vitro* demonstrated that the production of MCP-1 is induced in response to many established pro-atherogenic factors such as minimally modified LDL, oxidized LDL [138], thrombin [12, 66], angiotensin II, IL-1β, IL-4, TNF-α, IFN-γ [114, 119, 147], and PDGF [89]. In addition, increased MCP-1 levels were observed in patients with metabolic syndrome and/or diabetes, which are known to precede development of manifest atherosclerosis. Accordingly, high glucose [53] or leptin [153], increased concentrations of which are typically encountered

in diabetes, were identified as MCP-1 inducing factors *in vitro*. In contrast to pro-atherogenic factors, which almost uniformly stimulate MCP1-synthesis, little is known about the influence of anti-atherogenic factors on MCP-1 production. Few reports demonstrated suppressing effects of such anti-atherogenic factors as prostaglandin E_1 and E_2 [68], nitric oxide [151], insulin [27] and estrogen [80], on MCP-1 production *in vitro*. Although HDL is widely recognized as a potent anti-atherogenic factor and hypo-alphalipoproteinemia is a dyslipidemia most frequently encountered among patients with premature CHD, little effort has been devoted towards investigating the direct influence of HDL on the MCP-1 production or degradation. There is, however, some circumstantial evidence that processes driven by the excessive MCP-1 release are influenced by HDL. For instance, HDL was shown to counteract the LDL-induced monocyte transmigration, which is contingent on stimulation of MCP-1 synthesis [98]. Moreover, HDL reverts the effects of LDL on the expression of MCP-1 receptors in monocytes isolated from hypercholesterolemic patients [55]. The present results provide for the first time unequivocal evidence that MCP-1 synthesis in vascular smooth muscle cells is directly inhibited by HDL. This has been shown both at the transcription (mRNA) and translation (protein) levels. As the HDL-mediated inhibition of MCP-1 secretion was seen in the physiological HDL concentration range, it seems reasonable to argue that this process may be pertinent to the situation under *in vivo* conditions.

The enhanced MCP-1 production is an integral part of a larger response, by which human organism reacts to various pathological situations. Concerted liberation of other inflammatory mediators such as interleukines 1, 2, 6, and 8, interferons β and γ, chemokines such as regulated on activation, normal T-cell expressed and secreted chemokine (RANTES) and fraktalkin, as well as increased expression of adhesive proteins such as E-selectin, ICAM-1 and VCAM-1 represent other atherosclerosis-relevant components of this response [16, 85, 119]. Interestingly, HDL was previously shown to inhibit various aspects of pro-inflammatory processes. Several lines of evidence point to the *in vivo* relevance of HDL as an anti-inflammatory agent. First, infusion of reconstituted HDL was found to correct endothelial dysfunction in patients with either familial

hyper-cholesterolemia or hypo-alphalipoproteinemia due to heterozygosity for Tangier disease [3]. Second, in animal studies, infusion of HDL reduced organ damage and mortality in response to endotoxic or hemorrhagic shock [25]. Third, in isolated heart and kidney models application of HDL reduced ischemia-reperfusion injury, which is also considered as a model of inflammation [25]. Fourth, the combined knock-out of apoA-I and the LDL-receptor led to the occurrence of fatal systemic inflammation in some mice with adrenal cholesterol depletion in the adrenals and leukocyte accumulation in the liver [154]. Finally, patients with familial hypo-alphalipoproteinemia were found to have higher serum levels of C-reactive protein (CRP) than normo-alphalipoproteinemic controls [117]. Investigations *in vitro* shown that HDL inhibits expression of adhesive molecules such as VCAM-1 and E-selectin in endothelial cells and thereby limits penetration of leukocytes into the arterial wall [85, 101]. The adhesion of polymorphonuclear leukocytes to endothelial cells induced by bacterial lipopolysaccharide (LPS) was likewise inhibited in the presence of HDL [67]. The present work extends these observations showing that another important pro-inflammatory pathway – stimulation of MCP-1 production - is inhibited by HDL. It may be thus speculated that HDL not only restricts adhesion of leukocytes to the endothelial lining but also reduce the chemoattractant stimulus and thereby the transmigration of leukocytes into the arterial wall. In this way HDL may further contribute to the restriction of local inflammation in the arterial wall and eventually to the inhibition of the progression of atherosclerosis.

As yet it is not possible to define the chronological or hierarchical order of the pro-inflammatory effects of cytokines, chemokines and adhesion molecules as well as the anti-inflammatory effects of HDL. Cytokines and chemokines were found to stimulate the expression of selectins and adhesion molecules [85]. These may in turn initiate extravasation of leukocytes, which then produce cytokines and chemokines thus accelerating the vicious cycle of inflammation. The observation, however, that HDL effectively interrupts this circle at several points to the presence of a common mechanism that underlies the inhibitory effects of that lipoprotein. Activation of nuclear factor (NF-κB), an ubiquitously

expressed transcription factor, may represent an important link between inflammation and atherosclerosis and thus a potential target of HDL action. Multiple genes whose products are putatively involved in the atherosclerotic process are regulated by NF-κB [120]. These include leukocyte adhesion molecules, chemokines such as MCP-1 and IL-8, tissue factor (TF) that tip the pro/anti-coagulant balance on the endothelial cell surface towards coagulation and cyclin D1, that may induce cell proliferation at the sites of lesion formation [27]. Collectively, the coordinate induction of NF-κB – dependent genes may exert a substantial atherogenic effect on the vessel wall and several pro-atherogenic factors (*e.g.* LDL, oxLDL, angiotensin II, hyperglycemia and advanced glycosylation end products (AGEs) [53], infectious agents such as Cytomegalovirus (CMV) and *Chlamydia pneumonie,* and anti-atherogenic factors (*e.g.* polyunsaturated fatty acids, antioxidants) were shown to activate or inhibit NF-κB activity, respectively [103]. However, available evidence cast doubts about the direct effect of HDL on NF-κB function. Neither NF-κB binding to its responsive DNA element nor NF-κB translocation to the nucleus were found to be affected in the presence of HDL [105]. Furthermore, HDL exerted no effects on the activity of IKK (NF-κB inducing kinase), which is the upstream kinase obligatorily regulating NF-κB function. It is of interest, however, that NF-κB is one of the transcription factors that may be controlled by the redox status of the cell. Indeed, generation of reactive oxygen species (ROS) may be a common step in all of the signaling pathways that lead to IKK-mediated IκB (NF-κB inhibitor) phosphorylation and NF-κB nuclear accumulation. Support for this concept comes from a variety of studies showing that the diverse agents that can activate NF-κB also elevate levels of ROS and that chemically distinct antioxidants as well as overexpression of antioxidant enzymes can inhibit NF-κB activation [140]. It appears, therefore that whereas HDL does not affect NF-κB directly it might exert indirect inhibitory effect on the activity of this transcription factor via modulation of intracellular ROS generation and the redox status of the cell. Recent observations give some credence to this notion. For instance, Robbesyn et al. shown that the following sequence of signaling events triggered by oxLDL: intracellular ROS generation, proteasome activation, NF-κB inhibitor

degradation, and subsequent NF-κB activation is antagonized by HDL [120]. Other authors described the modulatory effect of HDL on the oxidative metabolism and bactericidal activity of polymorphonuclear leukocytes [2]. However, direct effects of HDL on intracellular ROS generation were not investigated to date. The present work for the first time demonstrates that HDL suppresses agonist-induced ROS production at the cellular level. This has been demonstrated both *in vitro* in a culture cell mode and *in situ* in isolated aortas. As modulation of the redox status of the cell is considered to be an integral element of signal transduction processes, inhibition of ROS generation by HDL may represent a novel mechanism by which this lipoprotein influences intracellular signaling pathways.

The present results shed some light on the molecular mechanisms underlying the inhibitory effect of HDL on intracellular ROS generation. The 'antioxidant' mechanisms of HDL result (in part) from the associated antioxidant enzymes, namely platelet activating factor - acetylhydrolase (PAF-AH) and paraoxonases (PON). HDL-associated PAF-AH is able to reduce oxidative stress and subsequent macrophage homing to endothelium [86] and PON may play a role in the antioxidant and cytoprotective effects of HDL [5]. Another HDL-associated enzyme LCAT prevents the accumulation of oxidized lipids in LDL [92]. An anti-oxidative activity of HDL also includes non-enzymatic mechanisms such as removing of lipids peroxidation products by apoA-I and apoJ present in HDL [73, 92, 99]. HDL are also able to catalyze the non-enzymatic conversion of hydroperoxides into hydroxides [5]. All these antioxidant effects of HDL, however, occur in the extracellular space and are unlikely to affect generation of ROS within the cell. As HDL is known to carry large amounts of α-tocopherol, which is a major lipophilic antioxidant in plasma, the supplementation of cells with this compound could account for the inhibitory effect of HDL on the ROS generation. Actually, several mechanisms were proposed, which could specifically facilitate transfer of α-tocopherol between HDL and cell interior. For instance, plasma-borne enzyme phospholipid transfer protein (PLTP) was demonstrated to transfer α-tocopherol from HDL to endothelial cells [29, 67, 79, 92]. ATP-binding cassette protein A1 (ABCA1)

transporter, which serves as apo A-I receptor, was shown to mediate trans-plasma membrane movement of α-tocopherol [109]. Both enzymes could co-operate to enrich cell with HDL-derived α-tocopherol. However, the present data and those by Robbesyn *et al.* argue strongly against the contention that HDL acts by supplying cells with vitamin E. First, in both experimental settings the inhibitory effect of HDL on ROS generation and NF-κB activation was seen within minutes after treatment. By contrast, the inhibitory effects of α-tocopherol on membrane lipid peroxidation are usually evident after few hours of incubation. Second, α-tocopherol - depleted HDL was still able to influence the redox status of the cell, whereas pure α-tocopherol, even when applied in large concentration, failed to attenuate ROS generation. Third, purified lipid components of HDL (S1P, SPC) without antioxidative properties mimicked HDL in its ability to reduce intracellular ROS generation. Robbesyn *et al.* in addition demonstrated that oxLDL-induced ROS generation is effectively prevented by several hydrophilic antioxidants. This suggests that ROS implicated in NF-κB activation are probably not generated in the membrane proximity, where vitamin E is located, and are independent of lipid peroxidation (which is inhibited by vitamin E). ROS are rather generated and activated in the cytosol, where hydrophilic antioxidants operate more effectivelly than the hydrophobic vitamin E. This also suggests that the 'antioxidant' effect of HDL is not mediated by its content in vitamin E.

Several enzymatic systems may account for cytosolic production of ROS. These include NAD(P)H oxidase, xanthine oxidase, glutathion peroxidase, lipooxygenases and myeloperoxidase. Evidence accumulated over recent years unequivocally shows that NAD(P)H oxidase is a major source of intracellular ROS in biological systems [71]. Moreover, this enzyme was implicated in the development of several pathologies including atherosclerosis, diabetes mellitus, hypertension, inflammation and neurodegenarative syndromes to name a few [32, 70]. The present study demonstrates that HDL directly suppresses NADPH consumption attributable to flavin-dependent enzymes, to which NAD(P)H oxidase counts. Moreover, HDL inhibits agonist-induced stimulation of $p38^{MAPK}$, the activity of which – as it has been shown by several laboratories –

is directly controlled by NAD(P)H oxidase [12, 48, 146]. Thus, it seems reasonable to assume that HDL exerts its antioxidative effects by directly influencing NAD(P)H oxidase activity. Basing on the present results no firm conclusion can be drawn as to which mechanism underlies this inhibitory effect. However, it may be of interest to note that HDL stimulates serine/threonine kinase (Akt), which phosphorylates Rac1, a small G protein and an activating component of NAD(P)H oxidase complex [10]. Recent study demonstrated that Akt-mediated phosphorylation of Rac1 prevents agonist-induced activation of NAD(P)H oxidase and generation of ROS [48]. It would be thus tempting to speculate that the inhibitory properties of HDL on NAD(P)H oxidase activity is mediated by activation of Akt. As activation of Akt accounts for several anti-atherogenic effects of HDL such as inhibition of endothelial apoptosis [24], and induction of NO-dependent vasorelaxation [11], inhibition of NAD(P)H oxidase by this kinase would further underscore the pivotal role of Akt for the anti-atherogenic effects of HDL.

The direct inhibitory effect of HDL on the agonist-induced intracellular ROS generation and NAD(P)H oxidase activation for the first time demonstrated in the present study is of immense importance for understanding the role played by this lipoprotein in the prevention of atherosclerosis. Uncontrolled ROS generation is believed to be a distinguished feature of a virtually every pro-atherogenic process [22]. One way that ROS are presumed to participate in atherogenesis is through the formation of oxidized lipids, particularly oxLDL [54]. Among the pro-atherogenic properties of oxLDL is the characteristic unregulated uptake of this modified lipoprotein by macrophages that thereby contributes to the formation of foam cells. Numerous other pro-atherogenic biologic activities have been attributed to oxLDL. It is a chemotactic factor for monocytes and a cytotoxic agent for various vascular cells. It can also induce endothelial cells to express pro-atherogenic MCP-1 or adhesion molecules, inhibits endothelium-dependent relaxation [111, 152], and promotes in plaque instability via regulation of matrix metalloproteinases (MMPs) production [2, 107]. ROS, in addition to mediating LDL oxidation, may contribute to the pathogenesis of atherosclerosis in a variety of other ways [32]. Among them,

the production of ROS induces stress responses that alter cell function, including adhesion, proliferation, and motility. ROS are also very effective scavengers of nitric oxide (NO) and can thereby not only regulate endothelial relaxation but also generate highly reactive peroxynitrite ($OONO^*$) [50]. Increased ROS production was demonstrated in atherosclerotically changed human arteries and appears to be a marker of unstable plaques [21]. However, ROS production may play more direct roles in modulating plaque stability. Superoxide (O_2^*), and $OONO^*$ formed by the interaction between O_2^* and nitric oxide, are proinflammatory radicals, leading to activation of redox-sensitive transcription factors such as NF-κB and activating protein-1 in endothelial and VSMCs, and in macrophages [113]. O_2^* induces expression of matrix-degrading proteases, including MMP-2 and MMP-9 in foam cells that directly contribute to plaque instability [48]. The role of ROS and NAD(P)H oxidase has been directly assessed for its contributions to the development of atherosclerosis in mice. In one study, in which total aortic lesions were evaluated, dramatic decrease in lesion extent was observed in animals deficient in the cytoplasmic subunit $p47^{phox}$ – of the NAD(P)H oxidase [82]. The observations in human and in animal models of atherosclerosis collectively point up the role of NAD(P)H oxidase - mediated ROS generation for the development and progression of atherosclerosis. Inhibition of NAD(P)H oxidase activity by HDL may thus constitute an important mechanism by which that lipoprotein exert its potent anti-atherogenic effect.

As pointed above, decrease plaque stability is accompanied by both increased activation of matrix metalloproteinases and uncontrolled generation of ROS mediated by activation of NAD(P)H oxidase. Recently, the causal relationship between MMPs and NAD(P)H oxidase activity and expression has been convincingly demonstrated in vascular smooth muscle cells (VSMCs) [51]. Thus, the inhibition of NAD(P)H oxidase - dependent ROS formation by HDL should predictably result in a reduced expression and, consequently, activity of matrix metalloproteinases. However, no effect of HDL on the activity of MMP-2 could be observed in this study. Several explanations may account for this astonishing observation. First, the cellular signaling events located upstream to

MMP-2 expression are complex and include several mutually redundant pathways. Thus, in addition to the cellular redox balance, matrix metalloproteinase expression appears to be under the control of cAMP - protein kinase A (PKA) pathway as well as several kinases such as Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), protein kinase C zeta (PKC zeta), c-Jun N-terminal kinase (JNK), and discoidin domain receptor 1 (DDR-1) tyrosin kinase. The exact contribution of each of these pathways to the expression of MMP-2 is unknown and may vary contingent upon conditions such as cell type, agonist, etc. It cannot, therefore, be excluded, that in the experimental setting applied in the present study the contribution of NAD(P)H oxidase to regulation of MMP-2 expression is minor. Second, matrix metalloproteinases are regulated at several levels, which in addition to expression include intra- and extracellular processing as well as secretion into the extracellular space. It cannot be, thus, entirely excluded that the decreased expression of MMP-2 in thrombin-stimulated VSMCs was compensated by the increased processing and/or secretion rate at another regulatory level.

HDL is a complex molecule with various lipid and protein components, which potentially contribute to the wide anti-atherogenic spectrum of activities attributed to this lipoprotein [136]. Different entities of HDL are potentially involved in triggering a multitude of intracellular signals and conceivably interact with functionally different receptors [38]. Previous results from several laboratories demonstrated that HDL serves as a carrier of several bioactive lipids such as sphingosine 1-phosphate (S1P), sphingosylphosphorylcholine (SPC), and lysosulfatide (LSF) [47]. The identification of SPC, S1P and LSF in HDL suggests that these entities may account for at least some of its biological effects. Actually, all three compounds were previously demonstrated to emulate HDL with respect to several pleiotropic anti-atherogenic effects exerted by this lipoprotein. For instance, S1P, SPC and LSF were shown to protect human umbilical vein endothelial cells (HUVECs) from apoptosis induced by serum or growth factor deprivation by suppressing apoptotic markers like cytochrome *c* release or caspase-3 activation [65, 105]. Both HDL- and lysophospholipid-mediated cell survival occurred through activation of the protein kinase Akt and

phosphorylation of the mitochondrial protein Bcl-2 - associated death promoter (BAD), a major Akt substrate [105]. Other report demonstrated that similarly to HDL, all three lysophospholipids induced phosphatidylinositol (PI3) kinase/Akt-dependent phosphorylation of Akt at Ser^{473} and endothelial nitric oxide synthase (eNOS) at Ser^{1177}, respectively, in cultured endothelial cells and, consequently, promoted vasorelaxation in isolated rat arteries [102]. Finally, both HDL and lysophospholipids were shown to inhibit TNF-α-induced expression of E-selectin on the surface of endothelial cells in Akt-dependent manner [101]. Current findings extend these previous observations by showing that similarly to native HDL lysophospholipids inhibit thrombin-induced MCP-1 expression in VSMCs. Furthermore, the suppression of ROS generation and NADPH-oxidase activation underlying the inhibitory effects of HDL on MCP-1 production were reduced in the presence of both S1P and SPC. Thus, the present study demonstrates that yet another important anti-atherogenic function of HDL can be attributed to the presence of biologically active lysophospholipids in this lipoprotein. Recently, it has been demonstrated that the lysophospholipid-dependent effects of HDL on NO generation and NO-dependent vasorelaxation are critically dependent on the interaction of HDL with two receptors – scavenger receptor B1 (SR-B1) and endothelial differentiation gene 3 (EDG 3) receptor [102]. Future work will help to clarify whether the inhibitory effects of HDL on ROS-dependent production of MCP-1 are also dependent on the interaction of HDL with these receptors.

In conclusion, the present study delineates the previously unknown mechanisms by which HDL exerts its potent anti-atherogenic effects. In this way, it brings an important contribution to our understanding of the pathophysiology of this fascinating class of lipoproteins.

6. References

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8. I. Lebenslauf

Name	Pawlak
Vorname	Alicja Joanna
Geburtsdatum/-ort	20.12.1975, Łódź, Polen
Familienstand	ledig
Nationalität	polnisch

Mutter: Małogorzata Pawlak, geb. Herzog, M.A.
Vater: Jan Pawlak, Dipl. Ingenieur

Ausbildung und Berufserfahrung:

1982-1990	Grundschule Nr. 35, Łódź
1990- 1994	Mikołaj Kopernik-Gymnasium, Łódź, Polen
1994	Abitur
1994 – 2000	Medizinstudium an der Medizinischen Akademie Łódź, Polen
2000	Anerkennung als Ärztin durch Kreisärztekammer Łódź, Polen
2000 – 2001	Ärztin im Praktikum am Uni - Klinikum Łódź
2001 – 2002	Medizinische Außerdienst bei pharmazeutischen Firma POLON, Łódź
seit 01.05.2002	Zweitstudium der Humanmedizin and der Westfälische Wilhelms-Universität, Münster. Wissenschaftliche Tätigkeit und Anfertigung der Promotionsarbeit am Institut für Arteriosklerosеforschung an der WWU Münster (Direktor: Univ.-Prof. Dr. Gerd Assmann, FRCP)

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2. Pawlak A. (2000) Neue Fakten über die Behandlung von Krebs in Eierstöcken *Ann Acad Med Lodz* Vol. 41: 82

Alicja Pawlak

8. II. Chronological Resume

Name:	Alicja Joanna Pawlak
Date and place of birth:	20th of December 1975 in Łódź, Poland
Marital status:	single
Nationality:	polish

Mother: Małogorzata Pawlak, (neé) Herzog, chemist
Father: Jan Pawlak, engineer

Education and Medical Experience:

1982-1990	Primary school 35 in Łódź, Poland
1990- 1994	Comprehensive School No. I in Łódź; Poland A-levels in math and physics
1994	Matriculation Diploma
1994 – 2000	Graduate Diploma in medicine at Medical University of Łódź, Poland
2000 – 2001	Postgraduate residence at Medical University of Łódź, Poland
2001 – 2002	Medical representative in pharmaceutical company POLON, Łódź, Poland
from 1st of May 2002	Postgraduate medical study at Westphalia University of Münster, Germany; research and preparing of dissertation at Institute for Clinical Chemistry and Laboratory Medicine of the University of Münster (Director: Univ.-Prof. Dr. Gerd Assmann, FRCP)

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Alicja Pawlak