ACIDIC pH AND ACIDIC ENZYMES IN ATHEROSCLEROSIS

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ACADEMIC DISSERTATION

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To my family

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I. **Riia Kaakinen**, Ken Lindstedt, Mia Sneck, Petri T. Kovanen and Katariina Öörni (2007) Angiotensin II increases expression and secretion of cathepsin F in cultured human monocyte-derived macrophages: an angiotensin II type 2 receptor-mediated effect. *Atherosclerosis*, **192**, 323-7

II. **Riia Plihtari**, Eva Hurt-Camejo, Katariina Öörni and Petri T. Kovanen (2010) Proteolysis of LDL particles sensitizes them to phospholipolysis by secretory phospholipase A2 group V and secretory sphingomyelinase - a novel mechanism of enhanced LDL retention. *J Lipid Res*, **51**, 1801-9

III. Katariina Lähdesmäki, **Riia Plihtari**, Pasi Soininen, Eva Hurt-Camejo, Mika Ala-Korpela, Katariina Öörni and Petri T. Kovanen. (2009) Phospholipase $A_{(2)}$-modified LDL particles retain the generated hydrolytic products and are more atherogenic at acidic pH. *Atherosclerosis*, **207**, 352-9

IV. **Riia Plihtari**, Petri T. Kovanen and Katariina Öörni (2010) Acidity increases the uptake of native LDL by human monocyte-derived macrophages. Submitted to *Atherosclerosis*

ABBREVIATIONS

ACE	angiotensin converting enzyme
AngII	angiotensin II
apoB-100	apolipoproteinB-100
$apoE^{-/-}$	apoE knockout
AT_1-receptor	angiotensin type 1 receptor
ATP	adenosine triphosphate
CS	chondroitin sulfate
DS	dermatan sulfate
FFA	free fatty acid
GAG	glycosaminoglycan
GM-CSF	granulocyte-macrophage colony-stimulating factor
HL	hepatic lipase
HS	heparan sulfate
IF	interferon
IL	interleukin
LDL	low density lipoprotein
$LDLR^{-/-}$	LDL receptor knockout
LPL	lipoprotein lipase
M-CSF	macrophage colony-stimulating factor
MMP	matrix metalloproteinase
NO	nitric oxide
PLA_2	phospholipase A_2
ROS	reactive oxygen species
SMase	sphingomyelinase
SMC	smooth muscle cell
$sPLA_2$-V	secretory PLA_2 group V
sSMase	secretory sphingomyelinase
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alfa
VLDL	very low density lipoprotein
VSMC	vascular smooth muscle cell

ABSTRACT

Atherosclerosis is an inflammatory disease characterized by accumulation of lipids and fibrous connective tissue in the arterial wall. Recently, it has been suggested that decrease in the pH of extracellular fluid of the arterial intima may enhance LDL accumulation by increasing binding of the LDL to matrix proteoglycans and also by making the plaque more favorable for acidic enzymes to be active.

Many lysosomal acidic enzymes have been found in atherosclerotic plaques. In this thesis, we were able to induce secretion of lysosomal acidic cathepsin F from human monocyte-derived macrophages by stimulation with angiotensin II. We also showed that LDL pre-proteolyzed with cathepsin S was more prone to subsequent hydrolytic modifications by lipases. Especially acidic secretory sphingomyelinase was able to hydrolyze pre-proteolyzed LDL even at neutral pH. We also showed that the proteolyzed and lipolyzed LDL particles were able to bind more efficiently to human aortic proteoglycans. In addition, the role of extracellular acidic pH on the ability of macrophages to internalize LDL was studied. At acidic pH, the production of cell surface proteoglycans in macrophages was increased as well as the binding of native and modified LDL to cell surface proteoglycans. Furthermore, macrophages cultured at acidic pH showed increased internalization of modified and native LDL leading to foam cell formation.

This thesis revealed various mechanisms by which acidic pH can increase LDL retention and accumulation in the arterial intima and has the potential to increase the progression of atherosclerosis.

INTRODUCTION

Cardiovascular diseases are the leading cause of death in Western countries. Since their prevalence is rapidly increasing in the developing countries, it has been suggested that in the future they become a main cause of death throughout the world (Lopez 1998). Atherosclerosis, the most common form of cardiovascular disease, is a slow, chronic inflammatory disease characterized by extracellular and intracellular accumulation of lipids from the circulating blood and the thickening of the innermost layer of the arterial wall, the intima. The earliest type of atherosclerotic lesions, the so called fatty streaks, can be seen as early as the first decade of life; however, these are only precursors of clinically significant disease (Stary 1994). In advanced atherosclerosis, the walls of arteries are thickened due to the formation of large plaques, which consist of a lipid-rich necrotic core covered by fibrous cap composed of smooth muscle cells and extracellular matrix. The severity of atherosclerosis can be determined by the different features of the advanced plaque architecture. Plaques that have a small or even a large lipid core but that have a thick fibrous cap are stable. However, these plaques may slowly narrow the arterial lumen, causing decreased blood flow for example, to the myocardium (Lusis 2000). In contrast, plaques with a large lipid core and a thin fibrous cap are unstable and are more prone to rupture, especially in the shoulder areas of the plaque, where the blood flow is turbulent and has been determined to cause shear stress (Gimbrone, Jr. 1999) and where the circumferential stress is highest (Richardson 1989). In the case of a rupture, local thrombus formation occurs and may result in myocardial infarction or ischemic stroke.

Inflammation has been considered to have a major role in atherosclerosis, and this has led to suggestions of many novel mechanisms involved in the progression of the disease (Ross 1999, Hansson 2009). In addition to lipids, inflammatory cells and proinflammatory mediators are important players in the establishment of atherosclerosis. Recently, the extracellular pH of the lesion has been shown to decrease causing an activation in several proatherogenic mechanisms (Öörni 2006). In the present work, the effects of acidic pH of the extracellular fluids in atherosclerotic plaques and secretion of inflammation-induced acidic enzymes are the main areas of interest. We studied modification of LDL by acidic enzymes and also the influence of acidic pH on the internalization of the native and modified LDL by macrophages.

LITERATURE REVIEW

1. The arterial wall

1.1. Structure of the healthy arterial wall

The arterial wall consists of three different layers, intima, media, and adventitia (Stary 1992, Stary 2000, Sims 1989). The intima is the innermost layer and is separated from the lumen by the monolayer of endothelial cells. The normal intima is thin and is composed mainly of extracellular connective tissue matrix, that is composed primarily of proteoglycans, but that also contains collagens, hyaluronan, fibronectin and laminin. The intima can be separated into two layers based on its composition. The inner layer is composed mainly of proteoglycans and is therefore called the proteoglycan layer, whereas the outer layer is called the musculoelastic layer due to the abundance of smooth muscle cells (SMCs) and elastic fibers. In addition to SMCs, a small number of macrophages, T-cells, and even occasional mast cells are also located in the normal intima. Intimal SMCs are derived from the medial layer and lose their ability to contract upon migration to the intima. These SMCs are capable of synthesizing and secreting different components of the extracellular matrix such as proteoglycans and therefore are classified as SMCs of the synthesis phenotype.

The medial layer is well organized, and consists of layers of SMCs embedded in an extracellular matrix (ECM) consisting mainly of small elastic fibers. These SMCs are of the contractile phenotype and are essentially responsible for the proper artery strength and constriction. The adventitial layer is more loose and it is formed from collagen and proteoglycan synthesized by local fibroblasts. The adventitia also contains clusters of mast cells as well as SMCs around the vessel. In addition, the adventitia contains nerve fibers and networks of small vessels called *vasa vasorum*, which supply blood to the adventitia and also contain lymphatic vessels responsible for the disposal of the entered substances such as LDL. Fenestrated layers of elastic tissue called the internal and external elastic lamina lie between the intima and media and between the media and adventitia, respectively.

1.2. The atherosclerotic arterial wall

During atherogenesis, the structure of the arterial wall changes, mainly in the intimal layer. Advanced atherosclerotic plaques contain high amounts of retained LDL due to binding to the extracellular matrix components (Williams 1995, Williams 1998, Tabas 2007). Retained LDL is more prone to modification by extracellular enzymes and following modifications, modified LDL particles can cause an inflammatory response, which then leads to activation of the endothelial cells and other cells present in the intima. Stimulated endothelial cells produce adhesion molecules and growth factors, which recruit more inflammatory cells

such as monocytes, T-cells and progenitors of mast cells from the circulation (Hansson 2009, Libby 2009). In the arterial wall, monocytes differentiate into macrophages and mast cell progenitors become mature mast cells. The mature mast cells contain cytoplasmic granules consisting of histamine, neutral proteases, and heparin proteoglycans, which are easily secreted upon degranulation when mast cells are activated (Kovanen 2007). Since macrophages are capable of massive internalization of modified LDL, atherosclerotic plaques contain large numbers of cholesterol-loaded macrophages, which are called foam cells due to their foamy appearance. The mechanisms of foam cell formation are discussed in more detail later (section 3.1).

The foam cells, together with extracellular lipid deposits, that may originate either from the retained lipids or from lipids released by apoptotic foam cells, form areas called fatty streaks. Over time, these lesions can progress and large necrotic lipid cores with cholesterol crystals can be formed (Stary 1994, Pasquinelli 1989). Growth factors and cytokines secreted by macrophages and T-cells stimulate medial SMCs to migrate into the intima and to secrete components of the extracellular matrix (Lusis 2000). The lipid core is separated from the endothelium by a fibrous cap formed of accumulated SMCs, macrophages, and extracellular matrix components, mainly collagen type I secreted by the SMCs. Due to the increased amount of collagen, fibrous caps are rigid. They become prone to rupture if elevated levels of enzymes capable of degrading extracellular

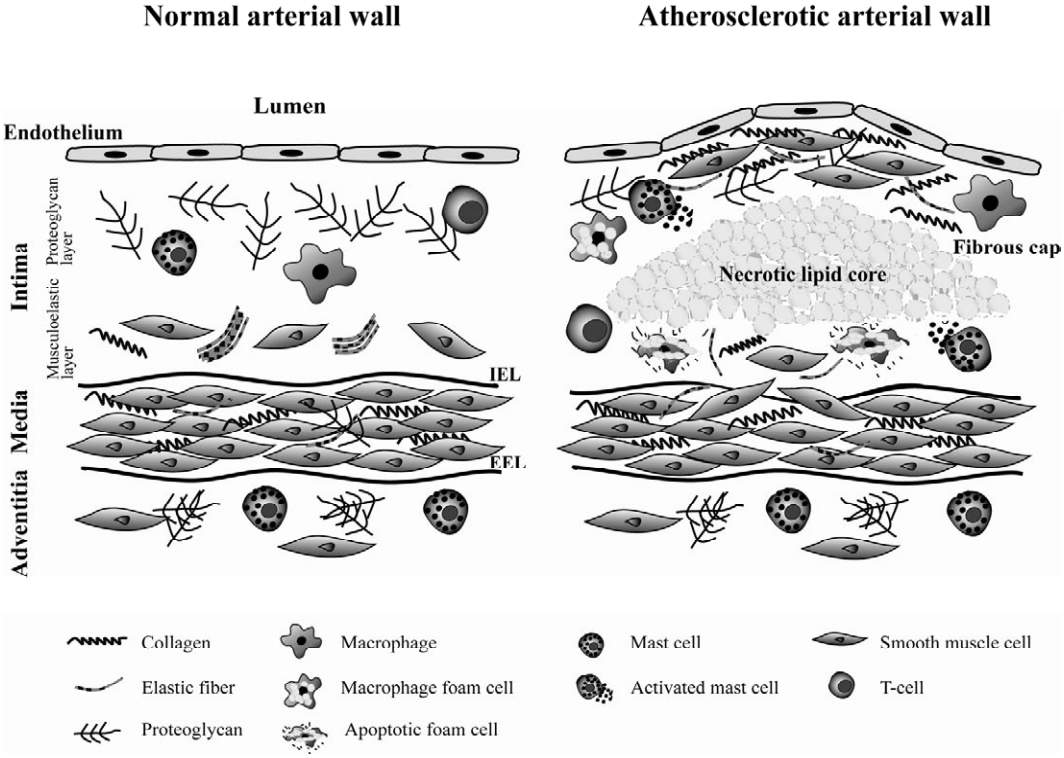

Figure 1. Schematic view of a normal healthy arterial wall and an atherosclerotic arterial wall. Abbreviations: IEL; internal elastic lamina, EEL; external elastic lamina

matrix are present (Libby 1995, Libby 2009). The lesions initially expand towards the adventitia, but at the late stages, lesions grow also towards the lumen thus obstructing circulation.

1.3. The extracellular matrix

The extracellular matrix is a mixture of different macromolecules including collagen, elastin, glycoproteins, and proteoglycans. The main role of the extracellular matrix in the arterial wall is to provide structural integrity, but the matrix also participates in several important events in atherogenesis, such as cell migration and proliferation, lipoprotein retention, and thrombosis (Katsuda 2003).

Proteoglycans

According to the Response-to-Retention theory, binding of LDL to the extracellular matrix proteoglycans is an initial event in atherogenesis (Williams 1995, Williams 1998, Tabas 2007). The finding that mice expressing proteoglycan-binding-defective LDL develop significantly less atherosclerosis than do mice expressing normal LDL illustrates the importance of the binding of LDL to extracellular matrix proteoglycans in lesion development (Skålen 2002). Further support for the Response-to Retention theory is provided by the finding that arterial proteoglycans co-localize with retained LDL in early and advanced lesions (O'Brien 1998, Nakashima 2008).

Proteoglycans (PGs) are macromolecules composed of a core protein covalently linked to one or many glycosaminoglycan (GAG) chains, which are formed from repeating disaccharide units having negatively charged sulfate or carboxyl-groups (Katsuda 2003).Vascular cells have been shown to synthesize three major families of proteoglycans, which are distinguished by their predominating GAG chains. Proteoglycans are enriched in chondroitin sulfate (CS), dermatan sulfate (DS) or heparan sulfate (HS). In the arterial wall, endothelial cells produce HSPGs, such as perlecan, which can contain also CS, and also produce CS/DSPGs, such as biglycan, whereas SMCs produce mainly CSPGs, such as versican as well as biglycan and decorin (CS/DSPG). In the arterial wall, proteoglycans have many important roles in maintenance of the extracellular matrix structure and viscoelastic properties, in cell adhesion, migration, and proliferation, and in hemostasis, thrombosis, and lipid metabolism (Camejo 2002). This thesis focuses mainly on the interactions of LDL with the extracellular matrix proteoglycans and with macrophage cell surface proteoglycans.

Over 20 different proteoglycans are found in the vascular extracellular matrix (Järveläinen 2003, Nakashima 2008). Versican is shown to be the most abundant proteoglycan, with biglycan and decorin being quantitatively the next most significant. Versican interacts with a very long GAG chain, nonsulfated hyaluronan, to form proteoglycan complexes. These complexes then form a tight

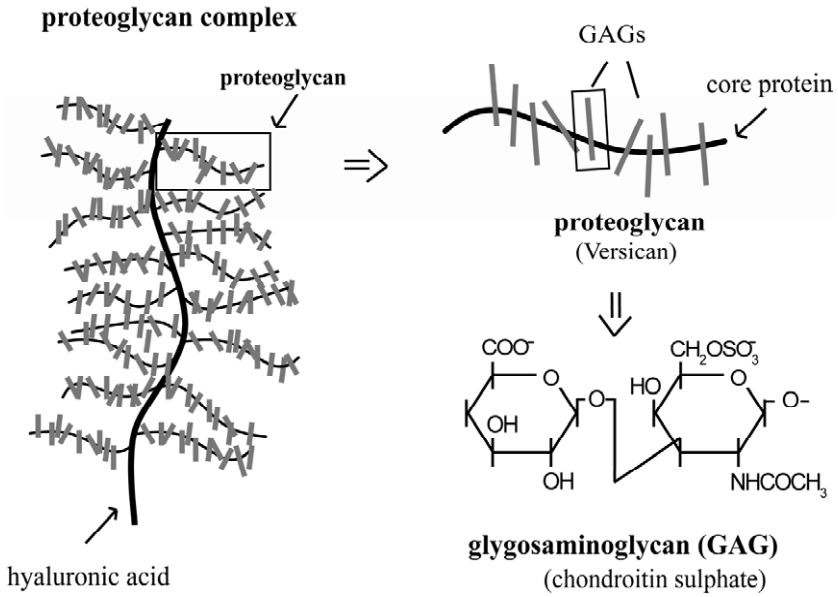

Figure 2. Schematic representation of the structure of the proteoglycan complex. The proteoglycan complex is formed of proteoglycans that are linked to a nonsulfated glycosaminoglycan (GAG), called hyaluronic acid. Proteoglycan consists of a core protein and varying numbers of different sulfated GAG chains attached to it. GAGs are formed of repeating disaccharide units that contain negatively charged sulfate groups. The GAG illustrated here is chondroitin sulfate. In the arterial intima, versican is present in such proteoglycan complex.

network in the extracellular matrix that is required for the arterial SMC proliferation and migration (Wight 2002). Versican has also been suggested to have roles in lipid accumulation, inflammation, and thrombosis.

Most of the intimal proteoglycans are produced by SMCs, but macrophages also produce a number of different proteoglycans, such as syndecan, glypican, serglycin, versican and perlecan (Wegrowski 2006, Asplund 2010). It has been shown that the secretion of the serglycin-related chondroitin sulfate GAGs increases after inflammatory stimulus (Uhlin-Hansen 1989). In addition, other components in the arterial wall, such as lipoprotein lipase and apoE are known to enhance cellular proteoglycan production (Obunike 2000).

The role of proteoglycans in LDL retention

As discussed above, interaction of LDL with proteoglycans is largely responsible for the retention of LDL in the arterial intima. With regard to LDL retention, the role of versican is somewhat ambiguous. Versican contains many potential binding sites for LDL, but it is not often present in the same areas of atherosclerotic lesions as is LDL (O'Brien 1998). Biglycan, in contrast, co-localizes with apolipoproteins, such as apoE, apoA-1 and apoB-100 in early and advanced atherosclerotic lesions (O'Brien 1998) and dermatan sulfates have also been shown to have stronger binding strengths towards LDL than do chondroitin

sulfates (Little 2008, Cardoso 1994). Taken together, these facts suggest that biglycan has an important role in LDL retention in the arterial intima.

Table I. Proteoglycans located in extracellular matrix or cell surface.

Proteoglycan	GAGs	ECM	Cell surface
Versican	CS	+	-
Biclycan	CS/DS	+	-
Decorin	CS/DS	+	-
Serglycin	CS/HS/heparin	+	-
Perlecan	HS/CS	+	+
Syndecan	HS/CS	-	+
Glypican	HS/CS	-	+

The predominant proteoglycans in the arterial wall are presented in this table. Abbreviations: GAG; glycosaminoglycan, ECM; extracellular matrix, CS; chondroitin sulfate, DS; dermatan sulfate, HS; heparan sulfate. (Wight 2004, Williams 2001, Williams 1997, Nakashima 2008, Wegrowski 2006)

Many factors in the atherosclerotic intima, such as transforming growth factor-β (TGF-β), angiotensin II, oxidized LDL, and fatty acids, can activate SMCs to produce proteoglycans with longer GAG chains, which increases affinity towards LDL (Little 2002, Figueroa 2002, Chang 2000, Olsson 1999). Since, many LDL particles can bind to a single CS chain, the length of the GAG chain also becomes an important determinant for the LDL binding capacity of the proteoglycans. Growth factors such as platelet-derived growth factor can further regulate the sulfation pattern of the GAGs by increasing sulfation of the disaccharide units at the 6 position of the ring (Schonherr 1993, Little 2007). These proteoglycans have a higher affinity for LDL than those, in which the position 4 is mostly sulfated (Cardoso 1994). GAGs isolated from human atherosclerotic cerebral arteries may contain oversulfated disaccharide repeat sequences (Murata 1989), which again may markedly increase the affinity of PGs for LDL (Sambandam 1991). Interestingly, native LDL, but not VLDL or oxidized LDL, stimulates macrophages to secrete proteoglycans, that are mainly small-sized (120 kDa) with long GAG chains and predominantly chondroitin-6-O-sulfated (Lindholm 2005).

1.4. Extracellular enzymes in atherosclerotic lesion

Atherosclerotic plaques contain high amounts of enzymes that are involved in extracellular matrix remodeling, LDL modification and many other proinflammatory events and biological processes. Various proteases are found to be present in the plaques, such as matrix metalloproteases, lysosomal proteases, mast cell-derived tryptase and chymase, and plasma-derived plasmin (Torzewski

2004, Lutgens 2007, Kaartinen 1994). In addition, atherosclerotic plaques contain many lipases, such as sphingomyelinase, phospholipases, lysosomal acid lipase, and lipoprotein lipase (Marathe 1999, Hurt-Camejo 1997, Kimura-Matsumoto 2008, Hakala 2003, O'Brien 1992). In this thesis, the primary enzymes of interest are cathepsins F and S, acidic sphingomyelinase (SMase), and secretory phospholipases A_2 ($sPLA_2$).

Cathepsins

Proteases can be divided into four major groups, the cysteine, serine, aspartate, and metallo-proteases (Chapman 1997). These four groups can be distinguised by their different strategies for catalyzing the irreversible hydrolysis of peptide bonds. Cathepsins F and S, which belong to a group of lysosomal cysteine proteases, are only weakly expressed in normal human arteries. However, in advanced human atherosclerotic plaques their mRNA and protein levels are increased and they become localized in macrophages and SMCs as well as extracellularly (Sukhova 1998, Öörni 2004). Cathepsins are synthesized as inactive proenzymes and are activated after cleavage of an N-terminal segment (Buhling 2000). They are normally localized inside the lysosomes, but they may also be present extracellularly in atherosclerotic lesions (Öörni 2004). Most cathepsins have an acidic pH optimum and are relatively unstable at neutral pH. However, despite their acidic pH optima, cathepsin F still shows weak activity and cathepsin S is fully active at neutral pH (Wang 1998, Kirschke 1989). Thus, besides degrading proteins in lysosomes, some cathepsins may also have a role outside of the lysosome both intra- and extracellularly (Turk 2000).

The release of cathepsins into the cytoplasm has been suggested to be induced by lysosomal permeabilization caused by oxidized LDL and reactive oxygen species (ROS), such as oxygen ions (Li 2004, Chwieralski 2006). In addition, macrophages and SMCs, after stimulation by proinflammatory cytokines, have been shown to secrete lysosomal cysteine proteases such as cathepsin S, K, and L (Reddy 1995, Sukhova 1998, Punturieri 2000). In addition to the active forms of cathepsin F, S, and K, macrophages have also been shown to secrete the fairly stable proenzyme forms of these cathepsins (Öörni 2004). Extracellularly, the secreted proenzymes can then be activated by other extracellular proteolytic enzymes, such as cathepsin D (Turk 2000).

Cathepsins can degrade proteins such as elastins, collagens and proteoglycans i.e., all components of the extracellular matrix. Therefore, they can contribute to pathophysiological processes in plaques, such as matrix remodeling, neovascularisation, and plaque rupture (Lutgens 2007). In $LDLR^{-/-}$ mouse models, cathepsin S deficiency led to a significant attenuation of the development of atherosclerosis, as indicated by reduced plaque size (Sukhova 2003). Furthermore, cathepsin S-deficiency in apoE-/- mice reduced atherosclerotic plaque size by

Table II. Lysosomal cysteine proteases in atherosclerosis.

	pH optim.	Expr.	In atherosclerosis	Deficiency/inhibition	References
Cath B	Acidic	human MΦ	• Induces apoptosis	ApoE$^{-/-}$ mice ↓ Inflammasome activation Cathepsin B inhibition ↓ LDL degradation	(Turk 2000, Chen 2002, Li 2001, Li 2004, Duewell 2010, Rajamäki 2010, Tertov 1997)
Cath F	Acidic	human MΦ, SMC, EC	• Degrades apoB-100, apoA-1 • Proteolyses preβ-HDL • Degrades MHC class II-associated Ii		(Öörni 2004, Lindstedt 2003, Shi 2000)
Cath H	Acidic	human mono-cytes	• Supports generation of atherogenic LDL		(Han 2003)
Cath K	Acidic	human MΦ, SMC, EC	• Degrades apoB-100, apoA-1 • Degrades type I collagen, elastin	ApoE$^{-/-}$ mice ↓ Plaque size/progression ↓ Elastin breaks ↑ Collagen content ↑ Macrophage foam cell formation ↑ Plaque stability	(Garnero 1998, Yasuda 2004, Sukhova 1998, Öörni 2004, Lutgens 2006, Lindstedt 2003)
Cath L	Acidic	human MΦ, SMC, EC	• Induces apoptosis • Degrades type I collagen, elastin • Degrades MHC class II-associated Ii	LDLR$^{-/-}$ mice ↓ Plaque size ↓ Elastin breaks ↓ Collagen content ↓ MΦ, SMC, CD4^{+} cells ↓ Inflammasome activation	(Li 2001, Liu 2006, Hsieh 2002, Kitamoto 2007, Duewell 2010)
Cath S	Neutral	human MΦ, SMC, EC	• Degrades apoB-100, apoA-1 • Proteolyses preβ-HDL • Degrades type I collagen, elastin • Degrades MHC class II-associated Ii	LDLR$^{-/-}$ mice ↓ Plaque size ↓ Elastin breaks ↓ Collagen content ↓ MΦ, SMC, CD4^{+} cells ApoE$^{-/-}$ mice ↓ Plaque size ↑↑ Plaque stability ↓ Elastin content	(Liu 2004, Sukhova 1998, Öörni 2004, Sukhova 2003, Rodgers 2006, Cheng 2006, Lindstedt 2003, Shi 1999)
Cath V	Acidic	human MΦ	• Degrades elastin		(Yasuda 2004)

Abbreviations: MΦ; macrophage, cath; cathepsin, SMC; smooth muscle cell, EC; endothelial cell, MHC; major histocompatibility complex,

46 % and also reduced the number of plaque ruptures (Rodgers 2006). In addition to matrix remodeling, cathepsin F and S appear also to be involved in lipoprotein modifications. This is discussed in more detail in the section 2.3.

Phospholipases

Secretory phospholipase A_2 ($sPLA_2$) groups IIA, IID, IIE, IIF, III, V and X are all found in the atherosclerotic arterial intima, where they are expressed in SMCs and macrophages (except for $sPLA_2$-V that is found only in SMCs) (Kimura-Matsumoto 2008). The expression of all $sPLA_2$s increases with the progression of atherosclerosis. In addition, lipoprotein-associated PLA_2 ($Lp\text{-}PLA_2$) has been located in the atherosclerotic lesions and it is also considered to have important functions in the development of atherosclerosis (Häkkinen 1999).

Table III. Secretory phospholipase A_2s in human atherosclerotic lesion.

	Expression in:		**Binding to PGs**	**Hydrolytic potency**
	Normal intima	Advanced atherosclerotic lesion		
$sPLA_2$-IIA	+	++	Weak	+
$sPLA_2$-IID	+	+++	Weak	
$sPLA_2$-IIE	-	+	Very weak	+
$sPLA_2$-IIF	-	+	Very weak	++
$sPLA_2$-III	+	+++	Not reported	+++
$sPLA_2$-V	+	++	Strong	+++
$sPLA_2$-X	+	++	No binding	++++

Expression of different secretory PLA2s in normal intima and in advanced atherosclerotic lesions and their binding affinity to proteoglycans as well as hydrolytic potency. (Kimura-Matsumoto 2008, Sato 2008, Rosengren 2006, Suzuki 2000, Murakami 2002a, Murakami 2002b)

Of all the PLA_2 enzymes, $sPLA_2$-V hydrolyzes native LDL most efficiently. In addition, it has been shown that $sPLA_2$-V is capable of hydrolyzing lipoproteins in serum (Rosengren 2006), which indicates a lack of natural inhibitors for this enzymes. Since blood is a precursor for interstitial fluid, this finding suggests the absence of inhibitors in the intimal fluid, which makes $sPLA_2$-V a very potent candidate enzyme responsible for the modification of LDL in the lesions (Öörni 2007). Recently, Bostrom et al. have shown the *in vivo* contribution of $sPLA_2$-V in the development of atherosclerosis (Bostrom 2007). They showed an increase in the size of atherosclerotic lesions as well as in the amount of collagen in the

arterial wall of $LDLR^{-/-}$ mice that overexpressed $sPLA_2$-V. They suggested that PLA_2-V may regulate the signaling pathway that leads to the increased deposition of collagen. Indeed, PLA_2 enzymes have been implicated in the development of atherosclerosis in several ways (Öörni 2007). First, the generated lipolytic products may have vasoactive, chemotactic, and proinflammatory effects on many cells types (Hurt-Camejo 2001, Boyanovsky 2010). Second, PLA_2-induced modification induces LDL particle aggregation and fusion and increases the binding of LDL to proteoglycans (Öörni 2005). Third, macrophages avidly internalize PLA_2-modified LDL and the uptake is mediated by cell surface proteoglycans (Wooton-Kee 2004, Boyanovsky 2005, Boyanovsky 2009).

Sphingomyelinases

Three main types of sphingomyelinases (SMases) that are involved in cardiovascular physiology include the lysosomal and secretory acidic SMases and the intracellular neutral SMase (Pavoine 2009). The same gene encodes acidic SMases, but differential protein trafficking generates the two distinct forms of acidic SMases: the secretory and the lysosomal enzymes (Schissel 1996a). Both types have acidic pH optimum and require Zn^{2+} for activity. Secretory sphingomyelinase (sSMase) is mainly secreted from macrophages and endothelial cells after stimulation with inflammatory cytokines, such as interleukin-1β (IL-1β), and interferon-γ (IF-γ) (Marathe 1998) and is found in the atherosclerotic plaques associated with many components of the extracellular matrix (Marathe 1999, Schissel 1996a). The *in vivo* relevance of acidic sSMase in atherosclerosis was recently shown in atherosclerotic $ApoE^{-/-}$ and $LDLR^{-/-}$ mice having acidic sSMase deficiency (Devlin 2008). In these mice, a clearly impeded lesion development was shown with a striking decrease in the trapping of atherogenic lipoproteins in the arterial wall. SMases can contribute to both extra- and intracellular accumulation of LDL since LDL particles modified by SMases have been shown to have increased affinity for proteoglycans and to induce foam cell formation by macrophages (Xu 1991, Öörni 2000). Compared to acidic lysosomal and secretory SMases, the role of neutral SMase in atherosclerosis has been much less studied, but it does appear to be involved in ceramide-dependent apoptosis and growth of VSMC (Pavoine 2009).

2. LDL in atherosclerosis

Low density lipoprotein (LDL) is the main cholesterol transporter in the blood (Brown 1986). Dietary as well as liver synthesized cholesterol is packed into very low density lipoproteins (VLDLs) in the liver and transported into the bloodstream, where LDL is formed from VLDL after sequential lipolysis by lipoprotein lipase (LPL) and hepatic lipase (HL). The uptake of LDL into cells is mediated by specific receptors majority the LDL receptor, but also by the

scavenger receptors, such as SR-B1. In the cells, LDL is delivered to lysosomes, where LDL components such as cholesterol esters are hydrolyzed.

2.1. LDL structure

LDL is heterogeneous and varies in its buoyant density, size, and chemical composition (Chapman 1988, Chapman 1998). In general, LDL has a globular shape with a size range of 18-25 nm and an average particle diameter of 22 nm. The characteristic differences of the particles affect the atherogenic potential of LDL for example, small, dense LDL is for instance oxidatively modified more easily and binds more strongly to the proteoglycans than do larger LDL particles (Chen 1994, Hurt-Camejo 2000).

LDL particles contain a hydrophobic core and a monolayer surface composed of amphipathic lipids, free cholesterol and a major structural protein, apolipoprotein B-100 (Olofsson 1987, Esterbauer 1992). The core consists of about 170 triglyceride, 1600 cholesteryl ester, and 200 unesterified cholesterol molecules, whereas the surface is composed of about 700 phospholipid molecules and 400 unesterified cholesterol molecules and one apoB-100 molecule. The main phospholipids in the surface are phosphatidylcholine, sphingomyelin and lysophosphatidylcholine. Different phospholipids have a tendency to separate into local molecular nanodomains, enriched either in phosphatidyl choline or in sphingomyelin and unesterified cholesterol (Sommer 1992, Hevonoja 2000). These different nanoenvironments of the lipids can facilitate the diffusion of core lipids toward the surface, making it possible, for example, for water-soluble enzymes and transferproteins, such as cholesteryl ester transfer protein (CETP) to attack hydrophobic core lipids.

ApolipoproteinB-100

An important part of the LDL surface is apolipoprotein B-100 (Knott 1986), the only protein component in LDL. It constitutes approximately 20 % of the particle weight and covers about 30 % of the particle surface (Baumstark 1990). The molecular mass of apoB-100 is about 550 kDa (4536 amino acids) making it one of the largest monomeric glycoproteins known. It circles the surface of the LDL particle and stabilizes the structure of the protein-lipid complex (Yang 1989). The N-terminal side of the apoB-100 molecule contains areas that interact with lipases and scavenger receptors, whereas most of the binding sites for glycosaminoglycans are located close to the C-terminus (Sivaram 1994, Kreuzer 1997, Camejo 1998). The C-terminus also contains the LDL-receptor binding motif, which is rich in cationic amino acids with lysine and arginine residues (Yang 1986). Interestingly, this is the same area of apoB-100 that interacts with both proteoglycans and the LDL-receptor. Nevertheless, selective inhibition of the

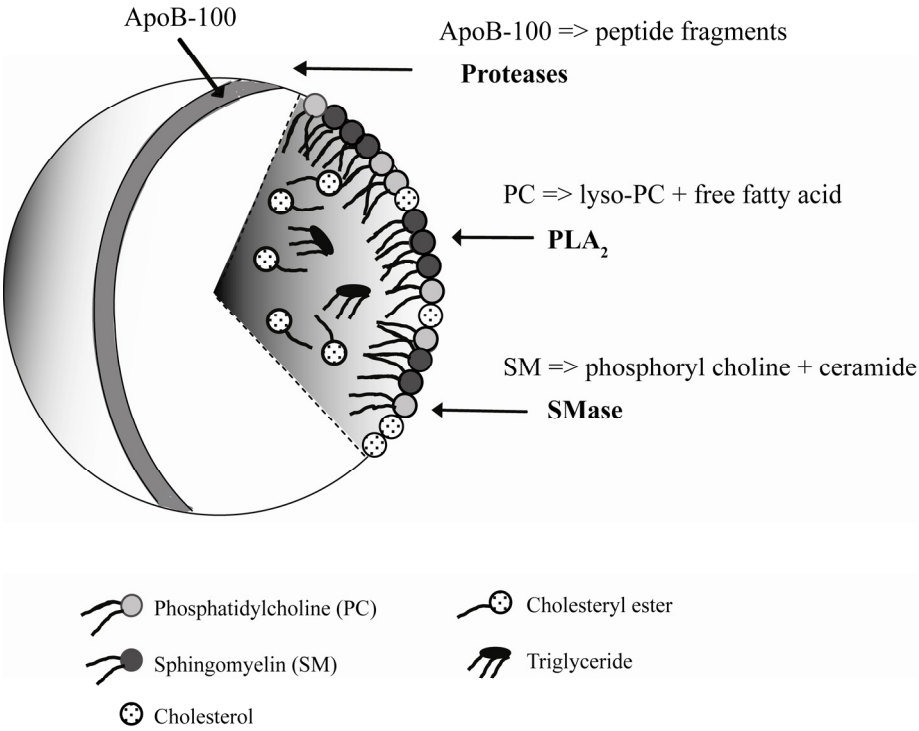

Figure 3. Schematic picture of the structure of LDL and modifications by various enzymes

binding of LDL to the proteoglycans is possible by changing the charge of the sequence. This finding suggested that proteoglycan-binding is mainly mediated via electrostatic interactions, while the conformation of amino acids seems to be more important for the binding of LDL to the LDL-receptor (Boren 1998).

2.2. LDL interaction with proteoglycans

LDL binds to proteoglycans via electrostatic interactions between negatively charged sulfate and carboxyl groups of the GAGs and positively charged amino acids of the apoB-100 in LDL (Borén 1998a). In addition, some accessory molecules, such as LPL, can mediate the binding (Pentikäinen 2002). Eight specific proteoglycan-binding sites in apoB-100 have been discovered and two of these (site A at residues 3148-3158 and site B at residues 3359-3369) can act co-operatively in the binding to proteoglycans. A disulphide link between Cys-3167 and Cys-3297 of apoB-100 has been suggested to facilitate the binding of apoB-100 to proteoglycans by bringing the two proteoglycan-binding sites close to each other (Olsson 1997). To examine the role of the various putative proteoglycan-binding sites in the proteoglycan-LDL interaction, human recombinant LDL that had mutations in various sites was expressed in transgenic mice (Borén 1998b). Of the sites tested, site B appeared to be primarily responsible for interaction with proteoglycans. However, PLA_2-treatment (be venom) of LDL was able to alter the conformation of apoB-100 in a way that site A is also able to mediate the interaction of LDL with proteoglycans, co-operatively with site B (Flood 2004).

The ability of LDL to bind to proteoglycans is influenced by the different characteristics of LDL. For example, small dense LDL has a higher affinity for artery wall proteoglycans than does the more buoyant LDL (Anber 1997). Since smaller LDL has fewer surface phospholipids, more GAG-binding sequences in apoB-100 may be exposed for binding (Camejo 1998). Indeed, small dense LDL has been found in human blood and elevated amounts of small LDL have been shown to correlate with the severity of atherosclerosis (Krauss 1982, Rizzo 2006). The formation of LDL-proteoglycan complexes is also increased following removal of sialic acids from the LDL surface (Millar 1999).

Lund-Katz and colleagues (Lund-Katz 1988) have found that apoB-100 contains two types of lysine residues that have different pKa values, at 8.9 and 10.5. These more unusual lysines with the lower pKa values are called active lysines and are thought to form as a result of conformational differences on the surface of LDL. These active lysines have been suggested to be located in the proteoglycan-binding areas of apoB-100 and their amounts are increased in proteolyzed LDL despite the loss of apoB-100 fragments. Therefore, active lysines have been suggested to be involved in the increased binding of proteolyzed LDL to proteoglycans (Paananen 1995).

2.3. Modified LDL in atherosclerosis

Arterial intima contains several proteolytic and lipolytic enzymes as well as oxidants capable of modifying LDL (Öörni 2000). When the modifications of LDL particles are sufficiently extensive, the surface structure of the particles can lose its stability and this allows interaction between the modified LDL particles that may lead to particle aggregation and fusion. Aggregation can be a reversible reaction, but after even more extensive modifications, LDL will lose its energetic stabilization, which can lead to irreversible particle fusion (Kokkonen 1989, Piha 1995, Öörni 2000).

Extracellular lipid particles can be isolated from atherosclerotic lesions. These particles are of two types: apoB-100 containing particles and cholesterol linoleate-rich lipid particles lacking apoB-100. Although the apoB-100-containing particles resemble plasma LDL, they are enriched in lysophosphatidylcholine and ceramide molecules, which strongly suggests hydrolysis of phosphatidylcholine and sphingomyelin on the LDL surface (Schissel 1996b, Ylä-Herttuala 1989). In the same way, the size and composition of the cholesterol linoleate-rich particles supports the hypothesis that they are formed during atherogenesis from LDL modified in multiple ways (Morton 1986, Chao 1990). For example, the ratio of protein content to cholesterol content in the particles is decreased compared to plasma LDL and the fragmentation of apoB-100 induced by modifications is suggested to lead to loss of apoB-100 immunoreactivity of the particles. The sizes of the cholesterol linoleate-containing particles can range from 40 nm to 200 nm,

which has been suggested to result from fusion of LDL particles. Proteolytic and lipolytic modifications of LDL are discussed in more detail in the following paragraphs.

Proteolytic modifications of LDL

Proteases modify LDL by degrading the apoB-100 protein of the particles, which leads to reorganization of the LDL surface. Loss of peptide fragments allows core lipids to penetrate into the surface, which then enhances the surface hydrophobicity (Öörni 2000). Proteolytic fragmentation of apoB-100 can lead to particle aggregation; however, for the initiation of fusion, some of the formed peptide fragments need to be released from the surface (Piha 1995). Cathepsin F extensively degrades apoB-100 (60 %), while cathepsin S induces less extensive degradation (20 %) at pH 6.0 (Öörni 2004). The ability of the cathepsin S and F to degrade apoB-100 decreases as the pH increases, especially with cathepsin F. Proteolytic degradation of apoB-100 with cathepsin F, (but not cathepsin S), induces aggregation and fusion of LDL particles and increases LDL binding to proteoglycans.

Lipolytic modifications of LDL

PLA_2 enzymes catalyze the hydrolysis of the sn-2 ester of phosphatidylcholine on the LDL surface to generate free fatty acids (FFAs) and lysophosphatidylcholine (lyso-PC). Secretory PLA_2 groups IIA and V are capable of hydrolyzing lipoproteins *in vitro*, although $sPLA_2$ group V shows much higher activity towards LDL than group IIA (Pruzanski 2005). LDL hydrolyzed by $sPLA_2$s has an enhanced affinity for proteoglycans and hydrolysis of LDL phosphatidylcholines has been shown to induce LDL aggregation and fusion (Hakala 2001). In addition, $sPLA_2$ modification induces tighter packing of the particle surface, which then decreases the size of the particle (Hevonoja 2000). Lipolytic modifications of LDL particles also cause changes in the composition of the surface and core lipids, which lead to conformational changes in the apoB-100 (Flood 2004). As discussed above, these changes expose more proteoglycan-binding sites in apoB-100.

Lyso-PC and FFA, the two $sPLA_2$-generated lipolysis products, have been shown to be involved in many proatherogenic actions, such as inducing smooth muscle cells to synthesize proteoglycans with increased affinity for LDL (Rodriguez-Lee 2006, Olsson 1999). The lipolytic products have also been shown to stimulate the expression and production of many proinflammatory cytokines and chemokines and in high concentrations they may even contribute to cell death in the atherosclerotic plaques (Haversen 2009, Hsieh 2000, Peter 2008).

SMases hydrolyze sphingomyelin on the LDL surface to ceramide and phosphocholine. The hydrophilic phosphocholines are then released from the LDL

surface, whereas ceramide molecules accumulate and tend to cluster, forming hydrophobic spots on the LDL surface. When the majority of the sphingomyelin molecules is hydrolyzed, LDL particles start to aggregate and fuse, presumably due to hydrophobic interactions between ceramide spots on different LDL particles (Schissel 1996b, Öörni 1998). An interesting finding was that, although sSMase has an acidic pH optimum, it is active at neutral pH towards LDL particles, that have been rendered more susceptible to hydrolysis by other modifications, such as oxidation and PLA_2-treatment (Schissel 1998).

3. LDL retention and accumulation

LDL enters the arterial intima either by crossing the endothelium in transcytotic vesicles or by passing through between the endothelial cells (Vasile 1983, Kao 1995). Endothelial permeability to plasma lipoproteins can be locally enhanced, for instance, by histamine released from the granules of the activated mast cells (Langeler 1989, Ma 1997). Since the intima lacks lymphatic vessels, LDL particles have to pass through the intima to reach the nearest lymphatic vessel located in the medial layer (Groszek 1980). As discussed previously, in the intima, LDL can bind to many components of the extracellular matrix, such as proteoglycans, which makes the passage of LDL slower and lengthens its retention time in the intima. Essentially, more LDL particles enter the intima than are removed from it, with a result being an increase in the concentration of LDL in the arterial intima. Indeed, LDL concentration in the intima is twice that in circulation and even 10 times higher than in other tissues (Smith 1990). Retained LDL particles are subject to attacks by many different enzymes and hence become modified. Modified LDL is often bound more tightly to the extracellular matrix, but oxidation of LDL, for example, can reduce its binding to the aortic proteoglycans (Öörni 1997). Modified LDL particles can aggregate and fuse, which can further increase LDL retention in the intima.

Bone-marrow-derived monocytes are recruited to the intima from circulation by inflammatory signals (chemokines) and then differentiated into macrophages. They start to internalize modified LDL and once become filled with cholesterol ester droplets, they turn into foam cells (Pasquinelli 1989). Areas of the intima, where the accumulation of foam cells is increased are known as fatty streaks and are the precursors for the formation of clinically more significant atherosclerotic plaques (Lusis 2000). Eventually, in advanced atherosclerotic plaques, a lipid core develops from extracellular lipid droplets derived from accumulated LDL particles and dead foam cells (Guyton 1994, Stary 2000).

3.1. The role of macrophages in atherosclerosis

Large numbers of macrophages are found especially in the shoulder areas of the atherosclerotic plaques (Li 2002). After being stimulated by agents such as lipopolysaccharide (LPS), macrophages undergo changes in their functional

properties. The stimulated macrophages have enhanced capacity for phagocytosis and they become highly secretory (Uhlin-Hansen 1993). Macrophages influence the extracellular matrix remodeling and wound repair by secreting many different cytokines such as IL-1β and tumor necrosis factor α (TNF-α), growth factors, and proteases such as matrix metalloproteases (MMPs) (Boyle 2005). In addition, human macrophages synthesize and secrete many proteoglycans, such as the chondroitin sulfate proteoglycans whose secretion is increased after macrophage activation (Uhlin-Hansen 1993). A crucial role of macrophages in atherosclerosis has been shown in apoE$^{-/-}$ mice having deficiency in macrophage colony-stimulating factor (M-CSF) or in chemokines. These mice have decreased numbers of macrophages in the arterial wall, and their atherosclerotic lesion size is considerably decreased (Smith 1995, Boring 1998).

Receptor-mediated pathways for the LDL internalization

One important aspect of macrophages in atherosclerotic plaques is their role in internalization and metabolism of the subendothelial lipoproteins. In lesions, this leads to intracellular accumulation of lipoprotein-derived cholesterol. The LDL receptor is the most important receptor for LDL in many tissues. However, it is expressed at very low level in the arterial intima, which is likely due to down-regulation of the receptor by high LDL cholesterol concentration in the arterial extracellular fluid (Brown 1986). Thus, there is no reason to believe that LDL receptors are involved in the lesion development. Rather, macrophages express high levels of scavenger receptors (SR), which are not inhibited by the increasing cellular cholesterol (Hoff 1990, Steinberg 1997). These are defined by their ability to endocytose modified LDL (acetylated or oxidized), and were first described by Goldstein and Brown in 1979 (Goldstein 1979). Several forms of scavenger receptors have been identified, but Class A, B and D are thought to be the most important for foam cell formation. Kunjathoor et al. have shown that SR-A and CD36 (a member of the SR-B family) are responsible for the majority of the uptake of modified LDL by macrophages and also that no other scavenger receptors can compensate for their absence (Kunjathoor 2002). Moreover, reduction in the size of atherosclerotic lesion in apoE $^{-/-}$ mice has been demonstrated after disruption of the macrophage SR-A gene (Suzuki 1997).

The LDL receptor-related protein (LRP) is also found to be highly expressed in macrophages and SMCs in the atherosclerotic lesions (Lupu 1994, Hiltunen 1998) and its expression is up-regulated by accumulation of intracellular cholesteryl esters (Llorente-Cortes 2002). Macrophages have been shown to be able to internalize aggregated LDL trough LRP (Llorente-Cortes 2000). The family of LRPs together with cell surface proteoglycans are also involved in the internalization of various other ligands to SMCs, such as TNF-α, apoE-enriched

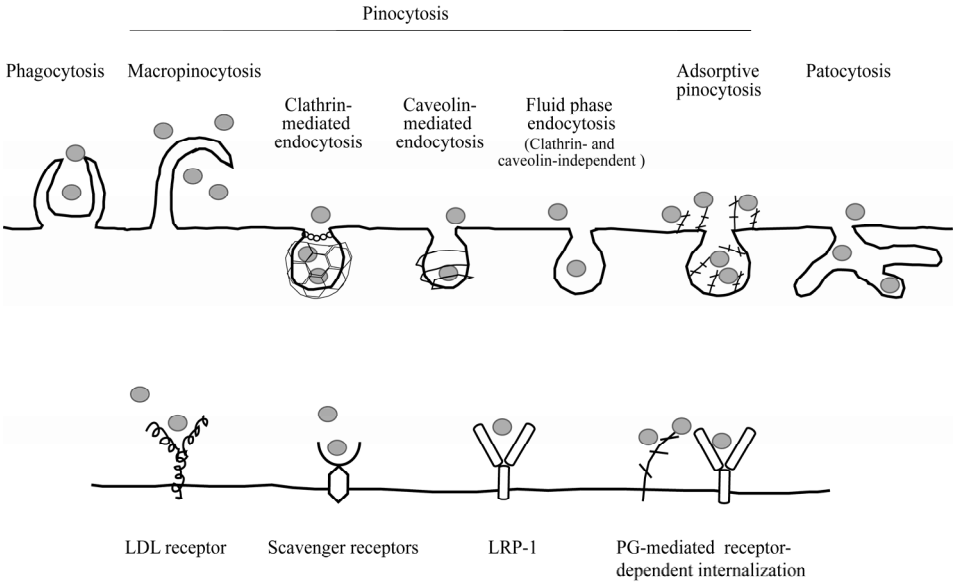

Figure 4. Non-receptor-mediated and receptor-mediated pathways for LDL internalization. (Conner 2003, Kruth 1999, Boyanovsky 2009, Brown 1986, Goldstein 1979, Lupu 1994)

remnants, and thrombospondin-1 (Andres 1989, Ji 1994, Godyna 1995). Thus, heparan sulfate proteoglycans serve as the initial binding sites for the ligands, after which the ligands are presented to LRPs for internalization within the cell.

Non-receptor-mediated pathways for LDL internalization

Macrophages can also take up cholesterol, without specific receptors, via different forms of fluid phase endocytosis. Endocytosis can be divided into two broad categories, phagocytosis and pinocytosis (Conner 2003). Phagocytosis is used for uptake of large particles, whereas pinocytosis used for uptake of fluids and solutes. Phagocytosis is a highly regulated process often involving specific cell-surface receptors. Pinocytosis can occur via at least four different basic mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin- and caveolin-independent endocytosis. Like phagocytosis, macropinocytosis triggers the actin-dependent formation of membrane protrusions, whereas the three other forms of pinocytosis are actin-independent. Activated macrophages have been shown to be able to take up native LDL by macropinocytosis (Kruth 2005). Macrophages can also internalize aggregated LDL by either actin-independent pinocytosis or by actin-dependent patocytosis (Shashkin 2005). The patocytosis pathway has been described by Kruth et al., and in this pathway the internalized aggregated LDL accumulates within surface-connected compartments (SCC) from where it is gradually transported to lysosomes (Kruth 1999, Anzinger 2010). Haka et al. have also studied the SCCs in macrophages and found that after aggregated LDL was internalized into the SCC, the pH inside the compartment decreased and the

lysosomal content was secreted into it (Haka 2009). They also showed that some of the LDL was actually hydrolyzed extracellularly.

Many different proteoglycans are located on the macrophage cell surface and these have been suggested to play a role in the binding of LDL to the cells (Wegrowski 2006). Monocytes in the blood circulation express low levels of heparan sulfate proteoglycans, but their expression is increased in the activated macrophages. Surface proteoglycans are mainly members of the syndecan, glypican, and perlecan families, which are composed primarily of heparan sulfate, as well as, a number of chondroitin sulfate glycosaminoglycans (Bernfield 1999, Fuki 2000). The core protein of syndecans is a transmembrane protein, whereas glypicans are bound to the cell surface with a glycosyl phosphatidylinositol (GPI) anchor (Williams 1997). Upon secretion, perlecan incorporates into the basement membrane or becomes attached to the cell surface via integrins. Syndecan-4 has been shown to mediate the uptake of $sPLA_2$-V-modified LDL by macrophages (Boyanovsky 2009). Lipoprotein lipase has been shown to act as a bridging molecule between native and oxidized LDL and heparan sulfates on the macrophage (murine macrophage cell line J774) cell surface, thus mediating their uptake by macrophages (Hendriks 1996).

4. Angiotensin II in atherosclerosis

Angiotensin II has an important role in the regulation of blood pressure, but it also affects the development of vascular wall inflammation and remodeling (Strawn 2002). The pharmacological inhibition of angiotensin II has been used therapeutically for a long time and its use has improved the prognosis of patients with cardiovascular diseases. Angiotensin II is formed from angiotensin I by the angiotensin-converting enzyme (ACE), which is normally found mostly on the surface of the endothelial cells in lung capillaries. ACE is also expressed in macrophages and its production in macrophages is up-regulated when the cells are located in areas of inflammation (Fukuhara 2000). In vascular tissues, mast cell derived chymase is also capable of catalyzing the conversion of angiotensin I to angiotensin II (Takai 2004). Angiotensin II mediates its activities primarily through its binding to angiotensin II type 1 (AT_1) or type 2 (AT_2) receptors.

4.1. Angiotensin II type 1-receptor-mediated effects of angiotensin II

The AT_1 receptor is widely expressed in different cells involved in atherosclerosis, but is particularly prevalent in the shoulder regions of the atherosclerotic plaques, which are the rupture sites of the plaques. The AT_1 receptor has classically been thought to mediate the proatherogenic effects of angiotensin II (Schieffer 2000). Angiotensin II causes endothelial dysfunction and vasoconstriction by increasing the production of reactive oxygen species (ROS) that inactivate endothelium-derived nitric oxide (NO) (Strawn 2002). Enhanced oxidative stress also induces apoptosis of endothelial cells (Choy 2001).

Angiotensin II increases the expression of various proinflammatory adhesion molecules, chemokines, and cytokines. Angiotensin II acts also as a vascular SMC growth factor and stimulates the production of MMPs and plasminogen activator inhibitor 1 (PAI-1), thus contributing to plaque rupture and thrombosis (Montecucco 2009). In addition, angiotensin II increases LDL oxidation by macrophages and also the internalization of oxidized LDL by up-regulating the expression of lectin-like oxidized LDL receptors (LOX-1) and scavenger receptors (SR-A, CD36) (Strawn 2002). In VSMCs, angiotensin II stimulates production of fibronectin and collagen as well as proteoglycans that have high affinity for LDL (Figueroa 2002). Recently, rat SMCs stimulated with angiotensin II have been shown to induce enhanced $sPLA_2$-IIA protein expression and activity, which suggests that angiotensin II may elicit proatherosclerotic effects via $sPLA_2$-IIA dependent LDL-modification (Divchev 2008).

4.2. Angiotensin II type 2-receptor-mediated effects of angiotensin II

AT_2 receptor expression is dramatically decreased after birth and although it remains low throughout the lifespan, it is re-expressed after cardiac and vascular injury (Horiuchi 1999). The role of the AT_2 receptor in atherosclerosis is still poorly understood, but it is thought to antagonize at least some of the AT_1 receptor-mediated effects (Henrion 2001). Interestingly, data from many experiments suggest that the AT_2-receptor exerts its protective action in atherosclerosis only when the AT_1-receptor is blocked (Carey 2003). Nevertheless, the effects of AT_2 receptor stimulation are somewhat controversial. Most of the effects mediated via the AT_2 receptor are shown to be antigrowth, antifibrotic, and proapoptotic, whereas, in contrast, stimulation of the AT_2-receptor also increases collagen synthesis in VSMCs (Mifune 2000). The stimulation of the AT_2 receptor with angiotensin II has been shown to induce production of vasoactive substances such as NO, which could produce vasodilation and an ensuing lowering of the blood pressure (Ichiki 1995, Tsutsumi 1999).

5. Acidic pH and hypoxia

5.1. Formation of local acidic areas

In atherosclerosis, the existence of acidic extracellular areas is still poorly characterized, despite the fact that extracellular fluid in inflammatory sites is known to be acidic (Rotstein 1988, Grinstein 1991). Atherosclerosis is an inflammatory disease and, in fact, it has been described, that vulnerable areas of human atherosclerotic plaques show heterogeneity in temperature, which correlates with the density and the proximity of inflammatory cells, notable the macrophages (Casscells 1996). Furthermore, extracellular pH has been measured both in rabbit and in human atherosclerotic plaques and the plaques were found to contain areas in which the pH is significantly decreased (Naghavi 2002). Indeed,

in the acidic areas of the plaque, the hydrogen ion concentration was up to 8-12 times higher than in normal areas of the intima.

Macrophages are known to be able to acidify their extracellular surroundings by extruding protons using different mechanisms such as vacuolar-type H^+-ATPase, Na^+/H^+ antiport, Na^+-dependent $HCO3^-/Cl^-$ exchanger and a proton conductive pathway (Leake 1997). An interesting finding is the correlation between the up-regulated expression of vacuolar-type H^+-ATPase components in the human monocyte-derived macrophages and secretion of lysosomal cysteine proteases (cathepsin K, S and L) with acidic pH optimum by the cells (Punturieri 2000). This suggests that macrophages acidify the extracellular fluids in their surroundings in order to sustain the activity of the acidic enzymes also outside the cell.

The main cause of low extracellular pH in atherosclerotic plaques is most likely hypoxia. The formation of hypoxia and its effects on atheroclerosis are discussed later (see sections 5.3 and 5.4). Due to decreased amounts of oxygen under hypoxic conditions in atherosclerotic plaques, macrophages start to use anaerobic glycolysis to generate ATP by converting glucose to lactate, which yields two ATP molecules. Macrophages are metabolically very active cells and consume large amounts of ATP. Hydrolysis of one ATP molecule results in generation of one ADP, one phosphate ion, and one hydrogen ion. Under normoxia, the formed hydrolysis products are transported into the mitochondria and used as substrates for oxidative phosphorylation. However, if the oxygen level in the cell is low, oxidative phosphorylation is decreased and ADP molecules and phosphate ions will be used for anaerobic cytosolic glycolysis. Thus, hydrogen ions will accumulate in the cytosol. Excess amounts of hydrogen ions acidify the cytosol and macrophages begin to extrude hydrogen ions for the stabilization of the pH of the cytosol. Hydrogen ions are transferred out of the cells via Na^+/H^+ exchangers and H^+/lactate symporters, resulting in a decrease in the pH of the extracellular fluid. The acidic areas are usually local, which probably reflects the tendency of macrophages to be located in clusters in the lesions (Leppänen 2006). The amounts of hydrogen ions in the extracellular fluid are determined not only by the rate of the ions released from the cells, but also by the rate of removal of the ions from the extracellular fluid and the buffering capacity of the extracellular fluid (Leake 1997).

The shift to anaerobic glycolysis due to the hypoxia, however, is fairly inefficient, since it consumes 15 times more glucose per ATP molecule than does oxidative phosphorylation (Leppänen 2006). Using anaerobic glycolysis, cells maintain their energy production, but this may also result in ATP depletion in the cells, which can eventually lead to impaired cell functions and even cell death (Björnheden 1987). Interestingly, advanced rabbit atherosclerotic plaques have

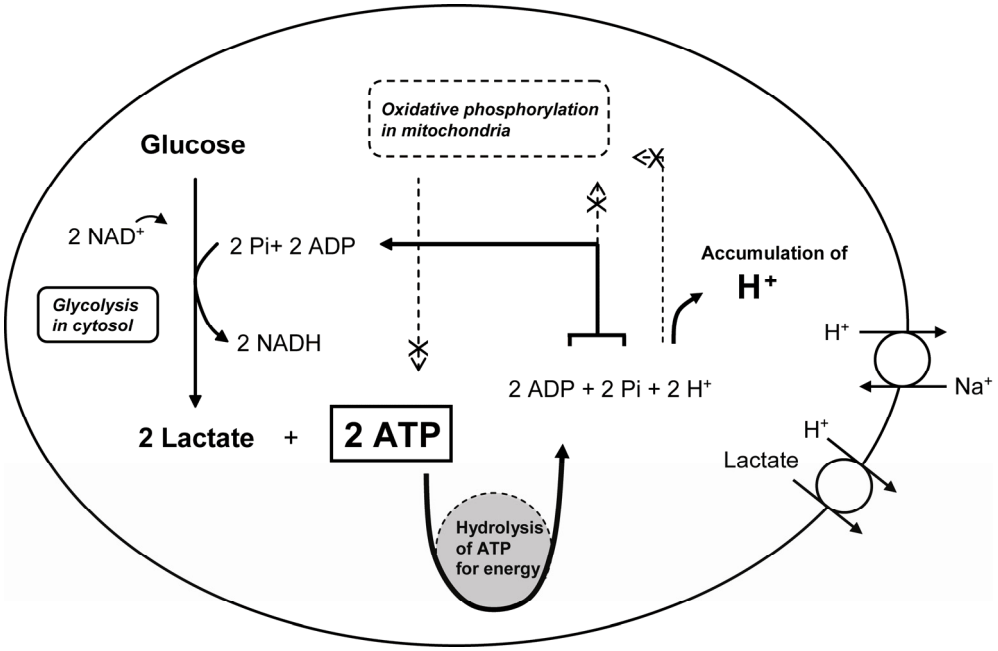

Figure 5. Schematic picture representing energy metabolism in cells during hypoxic conditions, which will lead to a decrease in the pH in the extracellular fluid. Oxidative phosphorylation in mitochondria is reduced due to the lack of oxygen and the cell starts to form ATP using anaerobic glycolysis in its cytosol. Under normoxic conditions, the end products of ATP hydrolysis are reused in mitochondria, whereas during anaerobic glycolysis only ADP and Pi can be reused, leading to the accumulation of H+. When being overloaded with H+ -ions, cells start to transport them to the extracellular fluid using, for example, H+/Na+ exchanger and H+/lactate transporter. Increased concentration of H+ ions in the extracellular fluid will locally decrease the pH. Abbreviations: ATP; adenosine triphosphate, ADP; adenosine diphosphate, Pi; inorganic phosphate, H+; hydrogen ion, Na+; sodium ion, NAD; nicotinamide adenine dinucleotide.

been shown *in vivo* to contain low concentrations of ATP and glucose and high concentrations of lactate (Leppänen 2006).

5.2. The effects of acidic pH

In atherosclerosis, the effects of acidic pH are still poorly understood. However, in cancer, for example, the effects of acidic conditions have been under investigation since the 1930s (Kraus 1996). Interestingly, many cell types have been shown to contain receptors that function particularly at acidic pH, but these have not yet been well characterized. Cell surface annexin VI, for example, has been suggested as an acidic receptor for the ligands of the neutral LRP-1 receptor, such as TGF-β and α_2-Macroglobulin (Ling 2004).

In studies with neutrophils, Trevani et al. showed that lowering the extracellular pH to 6.5 clearly increased neutrophil activation, which may intensify acute inflammatory responses (Trevani 1999). They showed that acidic pH causes an increase in the intracellular calcium levels and promotes H_2O_2 production by neutrophils. Furthermore, at acidic pH, the surface expression of an adhesion

molecule (β2-integrin CD18), which is involved in the binding of neutrophils to endothelial cells, was up-regulated and also apoptosis of neutrophils was delayed. Metabolic acidosis has been shown to activate the complement system, probably by inactivating complement protease inhibitors in the plasma (Emeis 1998). When human melanoma cells are cultured at acidic pH, they begin to secrete increased amounts of proteases, such as MMP-2, MMP-9, and cathepsins B and L, as well as proangiogenic factors such as vascular endothelial growth factor-A (VEGF-A) and IL-8 (Rofstad 2006).

Already in the 1990s, LDL oxidation was demonstrated to proceeds faster at acidic pH (Morgan 1993). Recently, it has also been shown that even a small reduction in the extracellular pH considerably inhibits oxidized LDL-induced apoptosis of macrophages, which is possibly partly due to the reduced endocytosis of oxidized LDL (Gerry 2008). However, LPS-activated alveolar macrophages suppress the release and the activity of TNF-α at a lower extracellular pH, thus impairing the response of the cells to ongoing infection (Bidani 1998). Interestingly, acidic pH has also been connected to angiotensin II by the finding that the expression of the AT_1-receptor is upregulated in the tubule cells of the kidney at acidic pH, thus amplifying the effects of angiotensin II (Nagami 2010).

Recently, it was demonstrated that acidic pH strongly increases the binding of native, proteolyzed, lipolyzed, and oxidized LDL to human aortic proteoglycans (Sneck 2005). Even oxidized LDL binds efficiently to proteoglycans at acidic pH, although oxidation itself decreases the binding. Oxidation neutralizes the basic amino acids, lysine and arginine, causing attenuated binding affinity of the oxidized LDL particles. However, at acidic pH at least some of the amino acids remain in the basic form and mediate the residual binding of oxidized LDL to proteoglycans. Interestingly, it has been shown that as the pH decreases, the binding of native and sphingomyelinase-treated LDL, VLDL, and IDL to proteoglycans increases (Öörni 2006).

It has also been shown that the acidic enzyme, cathepsin F, is secreted by macrophages, and is capable of intensively modifying LDL at acidic pH. Cathepsin F-induced modification causes an increase in LDL binding to proteoglycans and it can induce formation of LDL aggregates and fusion of LDL particles at a magnitude, previously only seen with certain neutral proteases (Öörni 2004). Interestingly, acidic sphingomyelinase at acidic pH has been shown to induce formation of large lipoprotein aggregates of sizes up to 1 μm (Sneck 2007). Furthermore, LDL that has been incubated with macrophage-conditioned medium at acidic pH also aggregates and fuses massively, due to the hydrolytic enzymes (cathepsin D and lysosomal acid lipase, LAL) that are secreted by the macrophages into the medium (Hakala 2003).

5.3. Hypoxia

Hypoxic areas have been shown to be present in advanced atherosclerotic lesions (Bjornheden 1999, Sluimer 2008). Hypoxia develops when the amount of oxygen in the plaque markedly decreases. Normally, in healthy tissue the oxygen tension is between 20 and 70 mmHg, which is equivalent to 2.5-9 % oxygen. In contrast, in diseased tissue, oxygen tension can be as low as 10 mmHg (less than 1 % oxygen) (Lewis 1999). Low oxygen tension could be a result of either decreased oxygen supply or increased oxygen demand. The intima is an avascular tissue and therefore its supply of oxygen is via diffusion from the lumen. The thickness of the normal intima ranges from 150-500 μm and atherosclerosis can increase this thickness up to 1500 ± 350 μm (Sluimer 2008). Since the maximal oxygen diffusion distance is approximately 200 μm, even the thickness of the normal intima can exceed this maximal distance, leading to decreased oxygen tension in the tissue layer beyond this distance (Torres, I 1994, Nissen 2001).

Macrophages are metabolically active inflammatory cells and thus consume high amounts of oxygen. Indeed, there is a correlation between the presence of macrophages and the hypoxic areas in human atherosclerotic plaques (Sluimer 2008). Hypoxic foam cells have been found even in the subendothelial areas at a distance of 20-30 μm from the endothelial layer, i.e. even if they are located within the oxygen diffusion distance. Angiogenesis does occur in the plaques and this generation of new microvessels should restore the oxygen level in the deep hypoxic areas of the plaques. However, besides oxygen, microvessels also deliver inflammatory cells to the plaque, which could even perpetuate the hypoxic state of the plaque (Sluimer 2009). The importance of macrophages in the development of hypoxia is illustrated by the finding of hypoxic areas in the mouse intima (Sluimer 2009). The thickness of the mouse intima is generally much smaller than the maximal oxygen diffusion distance, yet hypoxic areas are still found in mouse atherosclerotic lesions. In this case, plaque hypoxia is mostly a result of the inflammatory cell content rather than the distance to the lumen.

5.4. The effects of hypoxia on atherosclerosis

For this thesis, the most important effect of hypoxia is the lowering of the extracellular pH, but hypoxia also induces lipid accumulation and several pro-inflammatory and anti-fibrotic functions. Thus, hypoxia can be viewed as being one of the pro-atherogenic players in the development of atherosclerosis (Hulten 2009). Hypoxia increases the formation of triglyceride-containing lipid droplets in macrophages, which differ from the more generally found cholesterol-containing droplets in that the triglyceride-rich lipid droplets are formed by the accumulation of fatty acids (Bostrom 2006). To continue the formation of lipid droplets under hypoxia, macrophages increase the biosynthesis of triglycerides and the expression of adipose differentiation-related protein (ADRP), while reducing β-

oxidation of fatty acids by macrophages (Bostrom 2006). Hypoxia contributes to lipid metabolism in macrophages also by up-regulating the expression of VLDL receptors (Nakazato 2001).

Hypoxia promotes inflammation in atherosclerotic plaques by increasing the production of the T-cell attractant IL-8 by macrophages and the expression of 15-lipoxygenase-2 (15-LOX-2), which again increases secretion of chemokines (Rydberg 2003). In addition, 15-LOX-2 has been suggested as an enzyme mediating hypoxia-induced LDL oxidation (Rydberg 2004). In macrophages, hypoxia also induces increased cytokine production and the secretion of MMPs (Sluimer 2009). Interestingly, hypoxia has been shown to increase macrophage motility, which is possibly due to the decreased synthesis of heparan proteoglycans. However, hypoxia decreases only the synthesis of heparan sulfate , while the production of chondroitin sulfate and dermatan sulfate remains unchanged (Asplund 2009). Later, the same group showed that hypoxia increases the expression of versican and perlecan core proteins (Asplund 2010).

Cells need to adapt to a hypoxic environment and they do so by expressing the hypoxia-inducible transcription factor (HIF-1α), which regulates cellular responses to low oxygen levels (Semenza 2009). Indeed, HIF-1α has been found in the hypoxic and macrophage-rich regions of human atherosclerotic lesions (Sluimer 2008, Vink 2007). HIF-1α mediates, for example, the increased phagocytosis of macrophages under hypoxic conditions (Anand 2007). Hypoxia has also been shown to activate a local angiotensin-generating system by increasing the activity of ACE (Lam 2004).

AIMS OF THE STUDY

In atherosclerosis, LDL enters the arterial wall where it binds to the extracellular matrix proteoglycans. This leads to LDL retention and accumulation in the subendothelial layer of the arterial wall, the intima. Many enzymes are also present in atherosclerotic lesions, most of which are capable of modifying LDL. Modified LDL binds more tightly to the aortic proteoglycans and macrophages are also capable of internalizing modified LDL, thus increasing the development of atherosclerosis. Recently, acidic areas have been shown to be present in advanced atherosclerotic lesions and acidic enzymes are also found in the extracellular matrix of the intima.

On the basis of the above findings, we studied the effects of the acidic extracellular matrix and the acidic enzymes in the atherosclerotic plaque. The specific aims of this study were:

1. To study the capability of macrophages to secrete acidic lysosomal cathepsin F.
2. To study the effect of modifications of LDL by the acidic proteolytic enzyme, cathepsin S, on the susceptibility of LDL to subsequent lipolytic modifications with sPLA$_2$-V and sSMase.
3. To study binding of double-modified LDL (cathepsin S with either sPLAs-V or sSMase) to extracellular matrix proteoglycans and also to examine the effect of acidic pH on the binding of LDL to cell surface proteoglycans.
4. To study the effect of an acidic environment on the accumulation of LDL by macrophages.

METHODS

The methods used in this thesis are summarized in Table IV and the techniques have been described in more detail in the original publications listed in Table IV. The methods used most extensively are described briefly in this chapter.

Table IV. List of methods used in this thesis.

Method	Original publications	References
Isolation and labeling of LDL	II, III, IV	(Havel et al., 1955)
Isolation of human aortic proteoglycans	II, III	(Hurt-Camejo et al. 1990, Öörni et al. 1997)
Preparation of macrophage monolayers	I, III, IV	(Saren et al. 1996)
Modifications of LDL with:		
Plasmin	II	
Chymase	II	
Cathepsin S	II	
α-chymotrypsin	II	
$sPLA_2$-IIA	II	
$sPLA_2$-V	II, III	
sSMase	II	
TCA-precipitation	II, III, IV	(Goldstein et al. 1983)
Lowry protein assay	I, II, III, IV	(Lowry et al. 1951)
NEFA kit for FFAs	II, III	Waco Chemicals, Neuss
Phosphorylcholine assay	II	AmplexRed, Molecular Probes
Electron microscopy	II	(Pentikäinen 1996)
Gel filtration chromatography	II	
Proteoglycan binding assay	II, III	
Cholesterol assay	II, III	AmplexRed, Molecular Probes
High performance thin-layer chromatography	III	
RNA isolation	I	
RT-PCR	I	
Western blotting	I	
Lactate dehydrogenase activity	I, III, IV	
Trypan blue staining	III, IV	
Oil Red O staining	III, IV	

Abbreviations: LDL; low density lipoprotein, $sPLA_2$; secretory phospholipase A_2, sSMase; secretory sphingomyelinase, TCA; trichloroacetic acid, FFA; free fatty acids, RT-PCR; reverse-transcriptase polymerase chain reaction.

Isolation and labeling of LDL

Human LDL (d = 1.019–1.050 g/ml) was isolated from plasma of healthy volunteers (plasma obtained from Finnish Red Cross Blood Transfusion Center, Helsinki, Finland) by sequential ultracentrifugation in the presence of 3 mM EDTA (Havel 1955, Radding 1960). The amount of LDL particles was expressed as total protein, that was determined by the method of Lowry *et al.* (Lowry 1951). ApoB-100 of LDL was labeled with a ^{3}H-labeling reagent (N-succinimidyl-^{3}H-propionate, Amersham Biosciences) according to the Bolton-Hunter procedure (Bolton 1973).

Modifications of LDL

1. LDL proteolysis with cathepsin S

^{3}H-radiolabeled LDL (2 mg/ml) was first incubated for 0.5–18 hours at 37 ˚C in 150 mM NaCl with 20 mM PIPES (pH 7.0) and containing 35 µg/ml of human recombinant cathepsin S (Calbiochem). Proteolysis by cathepsin S was stopped by adding E-64 (SigmaAldrich) to give a final concentration of 10 µM. The degree of proteolysis was determined by the trichloroacetic acid (TCA) precipitation method (Piha 1995). Proteolyzed samples were also analyzed by SDS-polyacrylamide gel electrophoresis on 4-20 % gradient gels, which were stained with SimplyBlue™ SafeStain (Invitrogen).

2. LDL lipolysis with phospholipases

Proteolyzed LDL was incubated for 0–24 hours at 37 ˚C with the selected phospholipases in a buffer containing 150 mM NaCl, 2 % (w/v), fatty acid-free bovine serum albumin (BSA; MP chemicals, Ohio, USA), 6 mM $CaCl_2$, 0.005 mM $ZnCl_2$, and 20 mM PIPES (pH 7.0), which contained 10 µg/ml of human recombinant PLA_2 group IIa, 0.1 µg/ml of human recombinant $sPLA_2$ group V, or 40 µg/ml of human recombinant acidic sphingomyelinase (a kind gift from Genzyme, USA). Lipolytic reactions were stopped by addition of EDTA to give a final concentration of 10 mM.

The degree of PLA_2-induced LDL lipolysis was determined by measuring the released fatty acids with a NEFA-C-kit (Wako Chemicals, Neuss). The degree of SMase-induced lipolysis was determined by measuring the amounts of phosphorylcholine with an Amplex Red phosphorylcholine assay (Molecular Probes, Eugene, Oregon, USA). The average sizes of native and modified LDL were measured using “Zetasizer Nano” apparatus (Malvern Instruments, USA).

3. Preparation of $sPLA_2$-V-modified LDL for macrophage experiments

LDL (1.8 mg/ml) was incubated with human recombinant $sPLA_2$-V in phosphate-buffered saline, at pH 7.5, 6.5, or 5.5 in the presence of 2 % (w/v) fatty acid-free human serum albumin. Lipolysis was stopped by addition of EDTA to give a final concentration of 10 mM. Albumin was separated from the $sPLA_2$-V–modified

LDL particles by ultracentrifugation in a self-forming density gradient of Optiprep™.

Preparation of macrophage monolayers

Human monocytes were isolated from buffy coats (Finnish Red Cross Blood Transfusion Center, Helsinki, Finland) by centrifugation in a Ficoll-Paque gradient as described previously (Saren 1996). Washed cells were suspended in DMEM supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, counted, and seeded in 24 wells. After one hour, non-adherent cells were removed and the medium was replaced with macrophage-SFM medium (Gibco) supplemented with 1% penicillin-streptomycin and 10 ng/ml of granulocyte-macrophage colony stimulating factor (Biosite, USA). The culture medium was replaced with fresh macrophage-SFM medium after 24 h and after 48-65 h thereafter. Experiments were initiated when monocytes had been cultured *in vitro* for eight days, during which time the monocytes had differentiated into macrophages.

Analysis of macrophages

1. Macrophages treated with Angiotensin II

Angiotensin II (100 nM) (Sigma-Aldrich) with or without Losartan (10 µM) (Merck,) or PD123319 (1 µM) (Sigma- Aldrich) was added to the human monocyte-derived macrophages in RPMI 1640 medium containing 5% penicillin-streptomycin and L-glutamine. After incubation for 3-24 h the media and cells were collected for further analysis.

2. Macrophages treated with LDL at different pH values

Prior to the experiments, the culture medium was replaced with custom-made HyQ DME/HIGH Glucose medium (HyClone) having a pH of either 7.5, 6.5, or 5.5 and containing 5% penicillin-streptomycin and L-glutamine. After incubation for 1 h, 0.1 mg/ml of native or modified LDL were added to cells at each pH value. Control cells were incubated in the absence of LDL. In some experiments, uptake was inhibited by incubating cells with either heparinase and chondroitinase or lactoferrin, or $NaClO_3$ for 1 h before LDL addition. After incubation for the indicated times, the media and cells were collected for further analysis.

3. Measurement of proteoglycan synthesis by macrophages

^{35}S radiolabeled sulfate (0.8 µCi) (PerkinElmer) was added to the macrophages and incubated for 4 h at 37 °C. The medium was collected and the cells were rinsed three times with PBS containing Ca^{2+} and Mg^{2+}. After the final rinse, macrophages were lysed with 0.2 M NaOH. Radioactivity of media and cell lysates were measured.

4. LDL binding to macrophages

To determine the effect of pH on the binding of native and modified LDL by macrophages, 0.01-0.2 mg/ml of LDL was added to the cells and incubated for 2-3 h at +4 °C. After the incubation, the cells were rinsed three times with a buffer containing 50 mM NaCl, and either 50 mM HEPES (pH 7.5), 50 mM PIPES (pH 6.5), or 50 mM MES (pH 5.5), after which the cells were lysed with 0.2 M NaOH and their radioactivities were measured.

5. Determination of foam cell formation

The uptake of ^{3}H-labeled LDL

To determine the uptake of ^{3}H-LDL, cell-associated and degraded ^{3}H-LDL were measured (Goldstein 1983). Macrophages were first rinsed three times with PBS containing Ca^{2+} and Mg^{2+}, then heparin (10 mg/ml in PBS) was added to the cells and incubated for 1 h at 4 °C to remove any cell-surface-bound LDL particles. The heparin solutions and the cells were collected and their radioactivities were measured. Lipoprotein degradation was quantified by measuring TCA acid-soluble ^{3}H-radioactivity in the incubation media.

Lipid staining with Oil Red O

To visualize the formation of foam cells, some macrophages were plated onto 13 mm glass coverslips. After the incubation, the cells were washed with PBS, fixed with 3.7 % formalin for 3 min at room temperature, stained with 0.7 % Oil Red O, and counterstained with hematoxylin. The coverslips were mounted on glass microscope slides with Aquamount (BDH Laboratory Supplies) and photographed.

Thin layer chromatography of lipids

To determine the cellular cholesteryl ester contents, lipids were extracted from the cells with hexane-isopropanol (3:2, vol/vol). Cholesteryl ester content of the cells was then measured by using HP-TLC and using hexane/diethyl ether/concentrated acetic acid/H_2O (130:30:2:0.5, vol/vol/vol/vol). Individual lipid classes were visualized by dipping the TLC plate into $CuSO_4$ (3%)/H_2PO_4 (8%) and then heating the plate for 10–20 minutes at 150°C. The bands were scanned with an automatic plate scanner (CAMAG TLC Scanner No.3).

6. Western blot analysis of macrophage media and lysates

Western blot analysis was used to determine the presence of cathepsin F in the cells and in the media. Non-adherent cells were removed from the media by centrifugation. Protease inhibitors (5 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM benzamidine, 1 mM PMSF, 1 mM orthovanadate) were added, and the samples were concentrated into 1/5 using Vivaspin concentrators (Vivascience) (molecular weight cut off 10 kD). Cells were lysed with RIPA-buffer with the protease inhibitors. Aliquots of 10-100 μg of the lysates and media were loaded under reducing conditions onto a 4-20 % gradient SDS-

polyacrylamide gel and transferred to nitrocellulose membrane. The membranes were then incubated with monoclonal cathepsin F antibody (Novo Castra Laboratories Ltd.) followed by horseradish peroxidase-conjugated detection antibody (Dako, Sigma-Aldrich).

7. Cell viability

Cell viability was determined by Trypan blue staining and by measuring the lactate dehydrogenase activities in the media and in the cells during the 24 h-incubation using a commercial kit (Roche Diagnostics). The level of lactate dehydrogenase activity in the media was less than 5 % of the total cellular activity at any pH value. Of the macrophages, typically 3-5 % at pH 5.5 and typically 0-2% at pH 6.5 and at pH 7.5 were Trypan blue-positive after incubation for 24 h.

Isolation of human aortic proteoglycans

Human aortas for the proteoglycan isolation were obtained at autopsy within 24 h after accidental death. The proteoglycans from the intima-media were isolated essentially as described previously (Hurt-Camejo 1990, Öörni 1997). Glycosaminoglycans were quantified by the reported method (Bartold 1985), and the amounts of proteoglycans are expressed in terms of their glycosaminoglycan content. Our preparation of proteoglycans, isolated from human aortas, contained chondroitin-6-sulfates, chondroitin-4-sulfates, and dermatan sulfates.

Binding of LDL to proteoglycans

The wells of polystyrene 96-well plates (Thermo Labsystems, Finland) were coated with 100 µl of human aortic proteoglycans (50 µg/ml in PBS) or bovine serum albumin (5 mg/ml) by incubation at 4°C overnight. Wells were blocked with 3 % BSA, 1 % fat-free milk powder, and 0.05 % Tween20 in PBS for 1 h at 37°C as described earlier (Sneck 2005). The proteoglycan-coated wells contained about 0.25 µg proteoglycan/well. Wells coated with BSA alone served as controls.

Differentially hydrolyzed LDL was added (range 30-100 µg) to proteoglycan- or BSA-coated wells and incubated overnight at 37°C in a buffer containing 1 % BSA, 50-140 mM NaCl, 2-5 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM either HEPES (pH 7.5), PIPES (pH 6.5, 7.0) or MES (pH 5.5). The wells were washed three times with a buffer containing 50 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and the same pH buffers. The amount of proteoglycan-bound LDL was determined by the cholesterol assay (Amplex Red cholesterol assay, Molecular Probes).

Electron microscopy

LDL particles were negatively stained using 2 % uranyl acetate, pH 7.4, and then viewed and photographed under a JEOL 1200-EX II transmission electron microscope using the standard method available at the Electron Microscopy Unit, Institute of Biotechnology, University of Helsinki. The diameters of 150 randomly selected lipoprotein particles were measured from the electron micrographs.

Statistical analysis

Statistical analysis of the data was performed with the SPSS software, version 17.0, either using Student's *t* test, the Mann-Whitney *U* test, the Wilcoxon signed rank paired test, or the Friedman test. Differences between sample variances were analyzed with one way ANOVA with a post-hoc Bonferroni test. Results are given as the mean ± SD or SEM. The data were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

1. The effect of lysosomal acidic enzymes on extracellular environment

Both proteolytic and lipolytic enzymes have been found in atherosclerotic plaques, where they may contribute to the early and later steps of atherogenesis by degrading LDL or extracellular matrix. Lysosomal proteases are generally considered to be found only in lysosomes where they are responsible for protein degradation and turnover. However, lysosomal enzymes such as cathepsin F have been shown to also be present extracellularly in atherosclerotic lesions (Öörni 2004). In addition, when stimulated, these cells have been shown to be able to secrete various lysosomal enzymes, which typically have an acidic pH optimum and therefore have reduced activity in a neutral environment. Interestingly, macrophages have been shown to acidify their surroundings while secreting lysosomal enzymes and furthermore, atherosclerotic plaques have been found to contain acidic areas (Punturieri 2000, Naghavi 2002, Haka 2009). Thus, lysosomal enzymes may be able to retain their activity also extracellularly within atherosclerotic lesions.

1.1. Angiotensin II increases secretion of cathepsin F in macrophages

Cathepsin F is a member of cysteine protease family and shows high affinity for peptide substrates (Turk 2000). It is capable of degrading the extracellular matrix, as well as degrading apoB-100 in LDL particles, and inducing LDL aggregation, fusion, and enhanced retention to proteoglycans (Wang 1998, Öörni 2004). Angiotensin II is present in atherosclerotic plaques and is known to be involved in different anti- and proatheroclerotic processes (Schieffer 2000). Here, we have shown that the expression of cathepsin F in monocyte-derived macrophages was increased after stimulation with angiotensin II (Study I, Fig.1). Furthermore, we also showed an angiotensin II-induced increase in the secretion of cathepsin F (Study I, Fig. 2 and 3). Since angiotensin II did not have any effect on the intracellular amount of cathepsin F, the cathepsin F produced after angiotensin II stimulation was majorly secreted from the cells. Angiotensin II mediates its reactions through two different receptors, angiotensin type I or type II receptor. Traditionally, the AT_1- receptor is thought to be involved in all proatherogenic reactions induced by angiotensin II. We treated macrophages either with an AT_1-receptor inhibitor, losartan, or with an AT_2- receptor inhibitor, PD123319, prior to angiotensin II stimulation (Study I, Fig. 4). Unexpectedly, the AT_2-receptor inhibitor was able to inhibit cathepsin F secretion by macrophages more efficiently than did the AT_1-receptor inhibitor. Thus, angiotensin II-induced cathepsin F secretion from macrophages appears to be one of the few proatherogenic actions that is mediated via the AT_2-receptor. Besides its ability to modify LDL, cathepsin F can participate in degradation of the extracellular

matrix, thus contributing to the weakening of the plaque. Interestingly, angiotensin II has been shown to stimulate Na^+/H^+ exchangers system in VSMCs (Berk 1987), and to increase the amount of released H^+ ions into the extracellular fluid (see also Figure 5). This phenomenon might be connected to the finding that macrophages extrude protons concurrently with secretion of lysosomal acidic enzymes (Punturieri 2000).

1.2. Proteolysis increases lipolysis of LDL

ApoB-100-containing lipid droplets isolated from atherosclerotic lesions show signs of proteolytic and lipolytic modifications (Öörni 2000). In addition to many proteases, atherosclerotic plaques contain also two types of extracellular phospholipolytic enzymes, $sPLA_2$ and sSMase (Hurt-Camejo 1997, Marathe 1999). $sPLA_2$-V is capable of complete degradation of the LDL surface phospholipids at neutral pH, whereas sSMase, being an acidic enzyme, shows only a limited capacity to degrade LDL surface sphingomyelins at neutral pH. However, at acidic pH, sSMase extensively degrades sphingomyelin to ceramide and phosphorylcholine (Schissel 1998).

In the present study, it was investigated whether pre-proteolysis of LDL particles might influence the efficiency of lipases toward LDL particles. Specific aim was to test whether pre-proteolysis of LDL would affect the ability of acidic SMase to hydrolyze LDL at neutral pH. Therefore, we first treated LDL with cathepsin S, which induced about a 13 % degradation of apoB-100. This was followed by subjecting the pre-proteolyzed LDL to hydrolysis by $sPLA_2$-V and sSMase at neutral pH (Study II, Fig. 1B, 1C and 2). Pre-proteolysis with cathepsin S markedly increased the ability of sSMase to hydrolyze LDL at neutral pH, leading to as much as 40 % hydrolysis of the sphingomyelin (Study II, Fig. 1C). Similarly, pre-proteolysis of LDL increased the hydrolytic efficiency of $sPLA_2$-V toward LDL, so that complete hydrolysis of LDL phospholipids was achieved with a smaller amount of the enzyme (Study II, Fig. 1B). Apparently, proteolysis of LDL by cathepsin S altered the lipid surface structure of LDL in such a way that LDL surfaces became more prone to hydrolysis by sSMase and $sPLA_2$-V.

To evaluate this possibility, similar studies were performed using the mast cell-derived neutral serine protease chymase, instead of cathepsin S. Even though chymase and cathepsin S induced a similar degree of proteolysis, sSMase was able to hydrolyze more avidly the cathepsin S-treated LDL particles than the chymase-treated LDL (Study II, Fig. 1C). These proteases are known to hydrolyze apoB-100 in different ways; cathepsin S favors branched hydrophobic residues, whereas chymase hydrolyzes apoB-100 on the carbonyl side of aromatic amino acids. Therefore, a difference in the residual lipoprotein-bound apoB-100 fragments may explain the stronger activity of sSMase toward cathepsin S-treated LDL. Interestingly, the activity of sSMase has been shown to be strongly

dependent on the interaction efficiency between the enzyme and the LDL particle surface (Goni 2002). Pre-proteolysis certainly modifies the surface of LDL, and does so in an enzyme-dependent fashion, which may result in differences in the binding efficiencies between sSMase and LDL, and which could explain the differences in the amount of lipolysis observed in the present study.

We showed also that proteolytic fusion did not increase the susceptibility of the lipoproteins for the sSMase; instead, fusion rather stabilized the LDL particles (Study II, Fig. 5A). Previously, it has been shown that an increase in substrate vesicle size decreases SMase activity (Linke 2001), which fully supports our present findings. Thus, we suggest that LDL destabilization induced by proteolysis rendered the LDL particles more susceptible to hydrolysis by lipases; for example, proteolysis could have induced disordering of the lipids in the LDL surface monolayer. Recently, the propensity of the substrate efflux (i.e. vertical movement of the phospholipids) from the bilayer has been shown to be engaged with the active site cavity of the PLA_2 enzyme as an important determinant in the specificity of the enzyme (Haimi 2010). Proteolysis of LDL may thus have increased the propensity of the phospholipid substrate to efflux and this could be one explanation for the observed increase in lipolysis of pre-proteolyzed LDL with PLA_2. Furthermore, the finding that proteolysis enabled the sSMase to be active also at neutral environment, emphasized the important role of protease function in LDL modification in early atherosclerosis.

2. Effect of acidic pH on proteoglycans

2.1. Acidic pH induces cell surface proteoglycan synthesis and binding by macrophages

Smooth muscle cells are the primary source of proteoglycans in the arterial intima, but macrophages also substantially contribute to their production. In this study, incubation of macrophages at acidic media increased the production of cell surface proteoglycans by almost 4-fold and the acidity-induced increase in proteoglycan production was achieved already at pH 6.5 (Study IV, Fig. 3). It has been suggested that TGF-β1 can induce the utilization of the hexosamine pathway, which is a necessary step in the synthesis of GAGs (Schonherr 1993, Singh 2004). Under hypoxic conditions, macrophages produce increased amounts of TGF-β1 (Jackson 2007) and importantly, annexin VI, a receptor for TGF-β1, has been demonstrated to be active also at acidic pH (Ling 2004). Together, these findings suggest that TGF-β1 could be one of the major factors dictating the increase in the production of proteoglycans at acidic pH.

In the present study, the binding of native and $sPLA_2$-V-modified LDL to macrophages was increased considerably at acidic pH (Studies IV, Fig. 1 and III, Fig. 4A). Native LDL has already been shown to bind more tightly to extracellular human aortic proteoglycans at acidic pH (Sneck 2005), and therefore this thesis

suggests that LDL could also bind to the proteoglycans on the macrophage cell surface. Thus, the increased binding to cells could have been due to acidity-induced enhanced binding efficiency of LDL to cell surface proteoglycans as well as due to increased amount of proteoglycans produced at acidic pH.

2.2. Acidic pH increases binding of native and modified LDL to matrix proteoglycans

The binding of LDL to extracellular matrix proteoglycans at acidic pH was studied as well as the influence of modifications of LDL on this binding. LDL proteolysis by cathepsin S increased the binding of LDL to human aortic proteoglycans over two-fold. After sSMase-treatment of the pre-proteolyzed LDL, the binding increased further and was five-fold higher than the binding of native LDL (Study II, Fig. 6). Fused particles are known to bind to the proteoglycans more tightly because of the increased number of apoB-100 molecules on their surfaces, most likely suggesting polyvalent binding of LDL to proteoglycans (Öörni 2000). Modification of LDL with cathepsin S alone and with both $sPLA_2$-V and sSMase induced notable particle fusion (Study II, Fig. 4). By calculating the relative number of proteoglycan-bound particles, we observed that the increased binding of LDL modified with cathepsin S alone or LDL modified with cathepsin S and $sPLA_2$-V was related to the increased particle size (Study II, Table I). The combined modification of LDL with cathepsin S and sSMase increased the number of bound LDL particles to proteoglycans. Accordingly, cathepsin S and sSMase combination may have exposed new effective proteoglycan-binding sites in LDL apoB-100. Interestingly, this double-modified LDL resembled the extensively modified LDL particles found especially in the areas of the atherosclerotic arterial intima, where LDL is known to be more tightly bound to the extracellular matrix (Hoff 1978, Smith 1977, Camejo 1985).

The amount of LDL binding to proteoglycans was much smaller at neutral than at acidic pH (Study III, Fig. 3B). Here, we observed a further 5-fold increase in the binding of $sPLA_2$-V-modified LDL to proteoglycans at acidic pH compared to that at neutral pH (Study III, Fig. 3B). Similarly, treatment of LDL with α-chymotrypsin and SMase has been shown to enhance the binding of LDL to proteoglycans at acidic pH (Sneck 2005). LDL binds to proteoglycans via electrostatic interactions between negatively charged sulfate and carboxyl groups of the GAGs and the positively charged amino acids of apoB-100, and therefore the number of positively charged and exposed amino acids will be a relevant factor in the binding efficiency. The protonation state of the amino acids is determined by their pKa values, so a decrease in pH introduces more positive charges to amino acids having pKa values above the pH value in question. Histidines with a pKa value of 6.0, and active lysines with a pKa value of 8.9 are plausible amino acids to become positively charged at acidic pH. Therefore, these

residues may participate in the proteoglycan binding, particularly if additional proteoglycan-binding sites in apoB-100 become functional.

3. Effect of acidic pH on LDL accumulation in macrophages

3.1. Uptake of native LDL

The ability of macrophages to internalize modified LDL (i.e. acetylated LDL) leading to foam cell formation was discovered by Brown and Goldstein already in the 1970s (Brown 1986). Only recently, it was demonstrated that macrophages, stimulated with PMA are able to become foam cells by internalizing native LDL (Kruth 2002). In the present study, we have investigated human monocyte-derived macrophages, differentiated with GM-CSF, and examined the influence of acidic environment particularly on their ability to internalize native and modified LDL. First, macrophages were incubated with native LDL at different external pH and the amount of accumulated cholesterol was determined (Study IV, Fig. 1). At neutral pH, unstimulated macrophages did not usually internalize native LDL, but the uptake of native LDL was increased, if the pH of the medium was decreased. We were able to decrease the uptake of native LDL either by removing the proteoglycans from the surface of macrophages by heparinase and chondroitinase treatment or by inhibiting the sulfation of cell surface proteoglycans (Study IV, Fig.2). These results suggested that cell surface proteoglycans of macrophages are important players in the uptake of native LDL.

Cell surface proteoglycans may serve as the initial binding sites for various ligands and then present the ligands to receptors for internalization to the cell. Since LRP-1 has been shown to use cell surface proteoglycans for mediating the uptake of ligands, the possible role of LRP-1 was tested. Macrophages were incubated with lactoferrin, a small protein capable of binding to LRP-1 and inhibiting the reactions through this binding. Lactoferrin was able to inhibit the uptake of LDL by about 50 % at pH 5.5 and 40 % at pH 6.5, indicating involvement of LRP-1 in the acidity-induced increase in the uptake of LDL (Study IV, Fig. 3). However since, it has also been shown that lactoferrin can bind to heparan sulfate proteoglycans (Ji 1994), it was possible that the lactoferrin-induced inhibition could also have been independent of LRP-1.

Indeed, cell surface proteoglycans have been shown to be involved in receptor-independent adsorptive pinocytosis of PLA_2-modified LDL particles (Boyanovsky 2009). Principally, nonspecific internalization is directly proportional to concentration of the solutes in the medium and the volume encased by the transporting vesicles (Conner 2003). Nonspecific binding of the solutes to the cell membrane can increase the efficiency of the solute endocytosis, a process termed adsorptive pinocytosis. Thus, the increased uptake of native LDL seen in our study could have resulted from enhanced adsorptive pinocytosis, which would depend on both the increased affinity of LDL to cell surface proteoglycans and the

increased production of cell surface proteoglycans at acidic pH. The present results showed that proteoglycans played a critical role in LDL uptake, either in trapping the LDL particles and then transferring the particles to LRP for internalization, or by acting as direct binders mediating internalization of LDL.

3.2. Uptake of secretory sphingomyelinase and phospholipase A_2 group V-modified LDL

In addition to native LDL, the uptake of sSMase- and sPLA$_2$-V-modified LDL by human monocyte-derived macrophages was investigated. At neutral pH, the uptake of sSMase-modified LDL by macrophages derived from apoE$^{-/-}$ mouse involves the interaction of apoB-100 with the same cell-surface receptor that is unique for VLDL (Marathe 2000).

On the other hand, SMase-induced enrichment of ceramide in lipoprotein particles, but not the particle aggregation, has been shown to increase LDL uptake by macrophages, and this uptake is mediated via heparan sulfate proteoglycans and LRP (Morita 2004). In this thesis, the uptake of sSMase-modified LDL by human monocyte-derived macrophages was much more efficient when compared to that of native LDL, both at neutral and at acidic pH. Moreover, at acidic pH, the uptake of sSMase-modified LDL was further increased, i.e. it was 3-fold higher than at neutral pH (unpublished observation Fig.6).

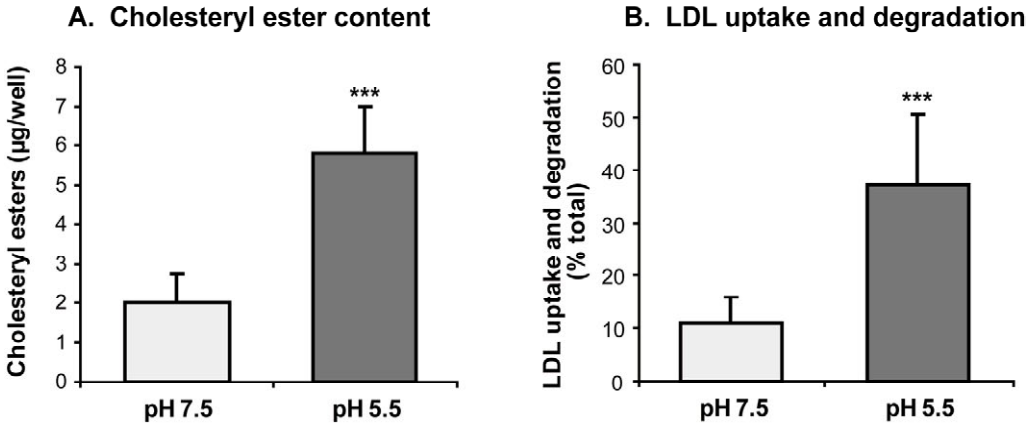

Figure 6. sSMase-treated LDL uptake by macrophages. Human monocyte-derived macrophages were incubated 4 h with sSMase-treated LDL at pH 7.5 and 5.5. Panel A shows cholesteryl ester content in cells measured with TLC. The uptake was also determined by measuring the cell-associated and degraded 3H-LDL (panel B). The values shown are means ± SEM of incubations with macrophages from four different donors.

The uptake of PLA$_2$-modified LDL by macrophages at neutral pH has been shown to be mediated via cell surface proteoglycans, and scavenger receptors seemed not to be involved in LDL internalization (Boyanovsky 2005). Here, it was similarly illustrated that PLA$_2$-V modification of LDL increased LDL uptake by macrophages at neutral pH and that the uptake was increased up to 3-fold as the

pH was decreased (Study III, Fig. 4). The uptake was decreased with heparinase and chondroitinase treatment, which indicated an important role for cell surface proteoglycans in the internalization of PLA_2-V-LDL by macrophages.

PLA_2-V hydrolyzes LDL phospholipids to free fatty acids and lysophospholipids. If this occurs in the extracellular fluid of a tissue, then the hydrolysis products are majority transferred back to the circulation by albumin. However, at acidic pH, the transfer of lipolytic products to albumin was significantly decreased and increased amount of free fatty acids and lyso-PCs were accumulated in the PLA_2-V-modified LDL particles (Study III, Fig. 1). Thus, the hydrolysis products may be retained together with modified LDL in the extracellular matrix of atherosclerotic lesions; moreover, increased amounts of bioactive FFAs and lyso-PCs can be internalized by macrophages at acidic pH. In the macrophages, these may act as intracellular second messengers and may be further metabolized into proinflammatory lipid mediators. Interestingly, excess amounts of FFAs in SMCs have been shown to stimulate synthesis of GAG by these cells, affecting the amount of proteoglycans in the extracellular matrix (Camejo 2002).

At acidic pH, the role of cell surface proteoglycans appeared to be very significant in the uptake of both native and modified LDL. Acidic pH increased the binding of LDL to cell surface proteoglycans, leading thus to an increased effective concentration of native or modified LDL on the surface of macrophages, from where LDL could easily be internalized by adsorptive pinocytosis.

SUMMARY AND CONCLUSION

In this thesis, the effects of acidity on the development of atherosclerosis have been discussed. The main focus was to study the effects of acidic pH and acidic enzymes on LDL modifications and on the extracellular and the intracellular accumulation of LDL.

Due to an inflammatory response, monocytes migrate to the arterial wall, where they differentiate into macrophages. Macrophages produce ACE, which catalyzes the conversion of angiotensin I into angiotensin II. Angiotensin II, again, influences in many different ways on the development of atherosclerosis and in this thesis it was demonstrated that macrophages stimulated with angiotensin II start to secrete a lysosomal acidic enzyme, cathepsin F. Since most lysosomal cathepsins are most active at acidic pH, they are likely to be rapidly inactivated after being secreted to a neutral extracellular fluid. However, macrophages are able to acidify their surroundings in many ways and, indeed, acidic areas have been found in advanced atherosclerotic plaques. It was further demonstrated that LDL proteolyzed with another acidic cathepsin, cathepsin S, is more prone to subsequent hydrolytic modifications by lipases. Interestingly, pre-proteolysis of LDL renders the LDL particles to become more susceptible to hydrolysis by acidic sSMase even at neutral pH.

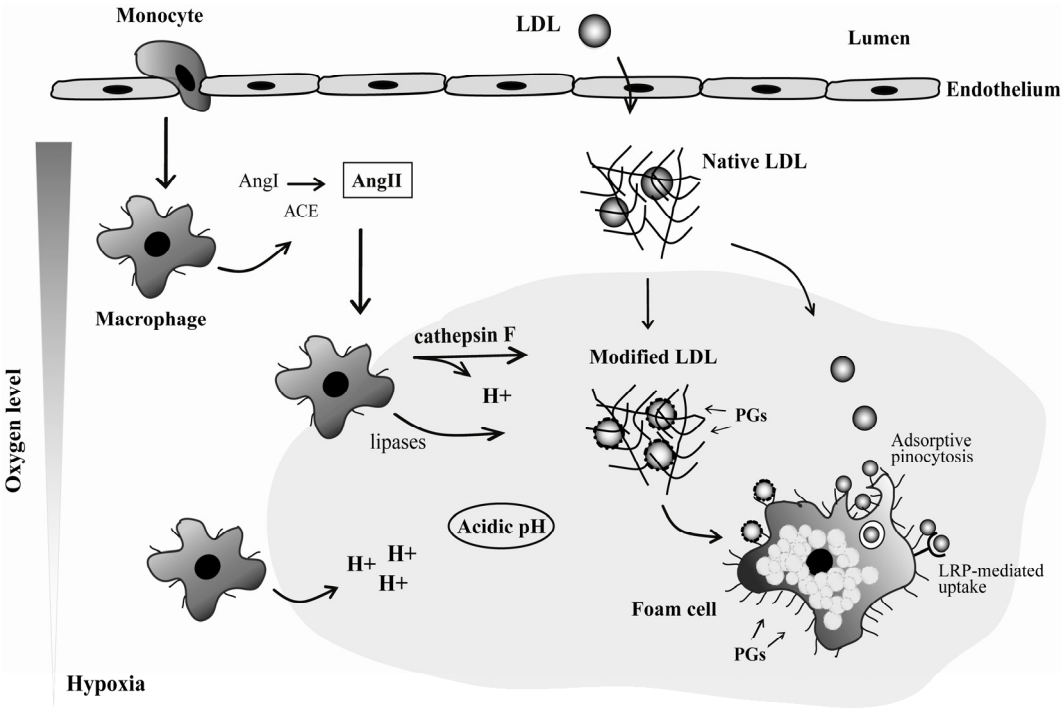

Figure 6. Schematic representation of the effects of acidic enzymes and acidic extracellular matrix in the progression of atherosclerosis. Abbreviations: Ang; angiotensin, ACE; angiotensin converting enzyme, PG; proteoglycans, LRP; LDL receptor related protein.

Also an increased binding of these double-modified LDL particles to human aortic proteoglycans was verified, a finding that may indicate an increase in LDL retention in the arterial wall. In addition, increased production of cell surface proteoglycans and increased binding of native and modified LDL to the proteoglycans on the surface of macrophages at acidic pH were observed. Furthermore, macrophages avidly internalized modified and even native LDL at acidic pH, a phenomenon that is suggested to be dependent on cell surface proteoglycans. For native LDL, cell surface proteoglycans seemed to mediate the uptake of LDL either through LRP-1 function or by adsorptive pinocytosis.

These results strongly suggest an important role of extracellular acidic pH and the acidic enzymes in the extra- and intracellular accumulation of LDL in the atherosclerotic lesions, and consequently, in the progression of atherosclerosis.

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