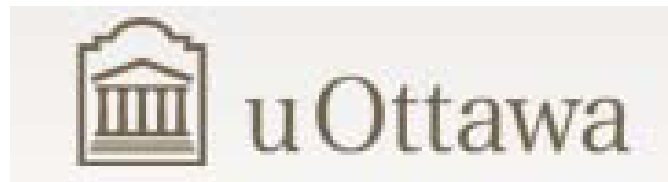


Role of cathepsin G in atherosclerosis

By

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Abstract

Angiotensin II (Ang II) is an important modulator for development of atherosclerosis from early stage foam cell formation to advanced stage plaque rupture. Recently, the importance of locally generated Ang II, especially in macrophages, has become more evident. Generation of Ang II by several enzymes other than ACE and renin has been shown mainly *in vitro*. Cathepsin G is one these enzymes which is expressed in neutrophils and macrophages. Macrophages are one of the primary and crucial cells in atherosclerotic lesions which become lipid-laden foam cells through lipoprotein uptake. We hypothesized that activation of nuclear factors in foam cells increases Ang II by modulation of the renin angiotensin system (RAS) genes and cathepsin G. We also hypothesized that cathepsin G, through its Ang II generating activity and its other catalytic functions, promotes atherosclerosis.

The present study assessed the Ang I and II levels and expression of the RAS genes in THP-1 cells, a human acute monocytic leukemia cell line, and in peritoneal and bone marrow-derived macrophages after exposure to acetylated LDL (ac-LDL). I also evaluated how RAS blockade would affect foam cell formation in THP-1 cells. In parallel, I assessed the role of cathepsin G in Ang II generation and in the progression of atherosclerosis in cathepsin G heterozygous knockout mice on an *ApoE*^{-/-} background (*CtsG*^{+/-}*ApoE*^{-/-} mice).

Ac-LDL treatment increased Ang I and Ang II levels in cell lysates and media from THP-1 cells but not in peritoneal or bone marrow-derived macrophages from wild type *C57BL/6* mice. In ac-LDL-treated THP-1 cells, ACE and cathepsin G mRNA levels and

activities were elevated. Angiotensinogen mRNA also is increased but not the angiotensinogen protein concentration. Renin mRNA level and activity were not altered by ac-LDL treatment. Blocking RAS by an AT₁ receptor blocker, ACE inhibitors or a renin inhibitor decreased cholesteryl ester content of THP-1 cells after exposure to ac-LDL. To confirm that the Ang II effect on foam cell formation was not unique to ac-LDL, we treated the THP-1 macrophages with a renin inhibitor or an AT₁ receptor inhibitor after exposure to oxidized LDL (ox-LDL). RAS blockade in ox-LDL-treated cells also abolished cholesteryl ester formation. To see how Ang II plays a role in foam cell formation we assessed the effect of RAS inhibitors on SR-A, the principal receptor for mediating ac-LDL entry into the cells and on acyl-CoA:cholesterol acyl transferase (ACAT-1), the enzyme responsible for intracellular cholesterol esterification. RAS blockade in both ac-LDL- and ox-LDL-treated cells decreased SR-A and ACAT-1 protein levels.

Cathepsin G partial deficiency on an *ApoE*^{-/-} background did not change Ang II levels in peritoneal or bone marrow-derived macrophage cell lysates or media. This deficiency also did not affect immunoreactive angiotensin peptide levels in atherosclerotic lesions. After 8 weeks on a high fat diet *Ctsg*^{+/-}*ApoE*^{-/-} mice were similar to *Ctsg*^{+/+}*ApoE*^{-/-} mice in terms of lesion size and serum cholesterol levels but the *Ctsg*^{+/+}*ApoE*^{-/-} mice had more advanced lesions with more collagen and smooth muscle cells and fewer macrophages. Moreover, *Ctsg*^{+/+}*ApoE*^{-/-} mice had more apoptotic cells than their *Ctsg*^{+/-}*ApoE*^{-/-} littermates.

Overall, our findings indicate that Ang II is increased in foam cells and this endogenous Ang II is involved in cholesteryl ester formation, possibly by regulating the levels of

ACAT-1 and SR-A. We did not find any role for cathepsin G in generation of Ang II in mice but cathepsin G does, nevertheless, promote the progression of atherosclerotic lesions to a more advanced stage.

List of abbreviations

ABCA-1	ATP-binding cassette sub type A1
ACAT-1	acyl-CoA cholesterol acyl transferase-1
ACE	angiotensin converting enzyme
ac-LDL	acetylated low density lipoprotein
AIM	apoptosis inhibitory molecule
Ang II/ AngI/ Ang IV/ Ang 3-7/ Ang1-7	angiotensin II/ angiotensin I/ angiotensin IV/ angiotensin 3-7/ angiotensin 1-7
AP-1	activating protein
Apo	apolipoprotein
<i>ApoE</i> ^{-/-}	apolipoprotein E-knockout
AT ₁ R	angiotensin receptor type 1
AT ₂ R	angiotensin receptor type 2
bFGF	basic fibroblastic growth factor
Cdk	cyclin-dependent kinase
CEH	cholesterol ester hydrolase
DAG	diacylglycerol

DAMP	damage-associated molecular patterns
DDR	discoidin domain receptors
EC	endothelial cell
ECM	extracellular matrix
EGR	early growth response
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
FABP	fatty acid binding protein
HDL	high density lipoprotein
HIF	hypoxic inducing factor
HMG Co A	3-hydroxy-3-methyl glutaryl Coenzyme A
HNE	Na ⁺ /H ⁺ exchanger
HODE	hydroxy-y,z-octadecadienoic acid
HUVEC	human umbilical vein endothelial cells
ICAM-1	intracellular adhesion molecule 1
IFN	interferon
IGF	insulin-like growth factor

IL	interleukin
IRAP	insulin-regulated aminopeptidase
JAK	janus kinase
LDL	low density lipoproteins
<i>Ldlr</i> ^{-/-}	low density lipoprotein receptor- knockout
LOX-1	lectin -like receptor
LPS	lipopolysaccharide
LXR	liver X receptor
MCP-1	macrophage chemoattractant protein 1
M-CSF	macrophage colony stimulating factor
MIF	migratory inhibitor factor
MMP	matrix metallo protease
NAC	N-acetyl cysteine
NF-κB	nuclear factor κ B
ox-LDL	oxidized low density lipoprotein
P38 MAPK	P38 mitogen activated protein kinase
PDGF	platelet-derived growth factor

PI3	inositol 1 4 5-trisphosphate
PKC	protein kinase C
PL	phospholipase
PPAR	peroxisome proliferator-activated receptors
RAGE	receptor for advance glycation end
RAS	renin angiotensin system
RBC	red blood cell
RIA	radioimmunoassay
ROS	reactive oxygen species
SMC	smooth muscle cell
SR	scavenger receptor
STAT3	signal transducer and activator of transcription 3
TEM	transmission electron microscopy
TGF- β	tumor growth factor β
TIMP	tissue inhibitors of matrix metalloprotease
TLR	toll- like receptor
TNF α	tumor necrosis factor alpha

UTR	untranslated region
VCAM-1	vascular endothelial cells adhesion molecule-1
VLDL	very low density lipoprotein

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1GENERAL INTRODUCTION

1.0 Overview

Cardiovascular diseases are the prominent cause of morbidity and mortality in western society. Atherosclerosis is characterized by an accumulation of cholesterol deposits in macrophages in large- and medium- sized arteries. These deposits lead to proliferation of certain cell types within the arterial wall and this expansion can gradually impinge on the lumen of the vessel and impede blood flow. Atherosclerosis is the major cause of stroke and myocardial infarction (Stocker and Keaney, 2004, Roger et al., 2012).

The renin angiotensin system (RAS) is a key modulator of atherosclerosis. AngII the main player of this system is involved in all steps of atherosclerosis from initiation to progression and complications (Sata and Fukuda, 2010). Ang II is present in human and mouse atherosclerotic lesions. Ang II peptide is co-localized with CD68⁺ cells which are considered to be active macrophages (Schieffer et al., 2000, Daugherty et al., 2004). All components of the RAS are present in macrophages *in vitro* and they are co-localized with macrophages *in vivo* (Okamura et al., 1999a, Daugherty et al., 2004). The presence of renin in bone marrow- derived cells enhances the progression of atherosclerotic lesions, which emphasizes the importance of locally produced Ang II in macrophages in the development of atherosclerosis(Lu et al., 2008a). Other than renin and angiotensin converting enzyme (ACE), cathepsin G is one of the enzymes that is able to generate Ang II directly from angiotensinogen or from angiotensin I (Ramaha and Patston, 2002b). This enzyme is expressed in neutrophils and monocytes (Wintroub et al., 1984, Panush,

1989). In human atherosclerotic lesions cathepsin G mRNA level is higher than in normal carotid artery (Legedz et al., 2004a).

The pattern of gene expression in macrophages present in atherosclerotic lesions due to uptake of lipids is changed. This is the result of activation of several transcription factors like peroxisome proliferator-activated receptors (PPARs) and hypoxia inducing factor (HIF) (Hung et al., 2006b, Whatling et al., 2004). The activated nuclear factors can potentially enhance the Ang II level in macrophages. The angiotensinogen gene has a PPAR α site in its promoter and HIF also regulates cathepsin G expression during hematopoiesis (Shimamoto et al., 2004a, van den Beucken et al., 2007) .

Macrophages within lesions take up lipids in different forms and via different pathways and, as a result they form lipid droplets composed primarily of cholesterol esters. Formation of lipid droplets depends on activity of the endoplasmic reticulum enzyme, acyl-CoA cholesterol acyl transferase (ACAT) and efflux of cholesterol via ATP-binding cassette sub type A1 (ABCA-1)(Moore and Tabas, 2011). ACAT expression is enhanced by exogenous Ang II (Kanome et al., 2008a). Ang II also decreases ABCA1 protein level. Together Ang II can elevate cholesterol ester which is the main component of the lipid droplets of macrophages.

We hypothesized that lipoprotein uptake increases endogenous Ang II production in macrophages by affecting the enzymes and substrates responsible for Ang II generation which, in turn, enhances lipid droplet formation. This suggests a positive feed- back loop in foam cell formation with Ang II being a major player. We also have evaluated the role of cathepsin G in Ang II production and its role in atherosclerosis development *in vivo*.

1.1 Atherosclerosis

Atherosclerosis is defined by cholesterol deposition in large and medium size arteries, which initiates an inflammatory reaction leading to the presence of macrophages and other inflammatory cells in the sub-endothelial space which over years blocks the vessel lumen and impedes blood flow. Several theories exist in terms of initiation of atherosclerosis: the very first theory from the 19th century did not assume any active role for cells and related atherosclerosis to intimal thickening and deposition of lipid – mucopolysaccharides complexes in the sub-endothelial space (Virchow, 1989). A second subsequent theory purposed that endothelial denudation leads to recruitment of inflammatory cells and passage of lipoproteins. Inflammatory cells secrete cytokines and vasoactive molecules that initiate inflammatory cascades. This theory is called “response to injury” (Ross and Glomset, 1976b, Ross and Glomset, 1976a). The problem with this theory is that endothelial cell (EC) denudation is not essential for lipoproteins and inflammatory cell passage into the intima and both lipoproteins and inflammatory cells can pass through intact endothelium. This led to a third theory called “response to retention”. Based on this theory, apolipoprotein B (apo B)-containing lipoproteins are retained in the sub-endothelial space and react with proteoglycans. Retained lipoproteins form aggregates and start a chemotactic inflammatory reaction and eventually aggregates are taken up by macrophages and smooth muscle cells (SMC)s (Tabas et al., 2007). Another theory proposes an essential role for lipoprotein modification. In this model low density lipoproteins (LDL) enter the sub-endothelial space and become chemically modified which initiates a chemotactic reaction. Based on this theory modified, but not native LDLs, are taken up by cells (Stocker and Keane, 2004). The presence of

lipoproteins in ECM changes the integrin signaling to EC and activates P38 mitogen activated protein kinase (p38MAPK) which in turn activates nuclear factor- κ B(NF- κ B). Activated NF- κ B up-regulates expression of endothelial adhesion molecules, such as vascular endothelial cells adhesion molecule-1 (VCAM-1) and selectins. Elevated adhesion molecules recruit inflammatory cells and initiate atherosclerosis (Orr et al., 2005) . Regardless of which mechanisms initiate the process, it is now apparent that different types of inflammatory cells play pivotal roles in the development and progression of atherosclerosis. Besides different inflammatory cells, the respective roles of vascular SMCs and endothelial cells (ECs) in development of atherosclerotic lesions will be discussed here:

1.1.1 Inflammatory cells

While different types of myeloid and lymphoid cell types are involved in progression of atherosclerosis, macrophages are the predominant cell type in atherosclerotic lesions.

1.1.1.1 Monocyte/ macrophage

The presence of monocytes is important for the initiation of atherosclerosis whereas, in the established lesions, macrophages have a more limited role in determining lesion size and the cellular and intercellular matrix composition of the lesions. Depletion of CD11b monocytes by administration of diphtheria toxin to apolipoprotein E-deficient (*ApoE*^{-/-}) mice which are recipient of CD11b–diphtheria toxin receptor transgenic bone marrow cells reduces the size of early atherosclerotic lesions and this is accompanied by a decrease in collagen content and a diminished necrotic core. However depletion of monocytes in *ApoE*^{-/-} mice after 22 weeks of a high fat diet reconstituted with CD11b–diphtheria toxin receptor transgenic bone marrow for a period of 10 weeks does not affect

lesion progression, cellularity, collagen or necrotic core size (Stoneman et al., 2007). The first step in the entry of monocytes and other leukocytes into the sub-endothelial space consists of their rolling over the endothelium, a process mediated primarily in atherosclerosis by P-selectin and E-selectin. Combined deficiency of P/E selectins in low density lipoprotein receptor- deficient (*Ldlr*^{-/-}) mice decreased atherosclerotic lesion size by 80 % at early stages and by 40% at later stages. P-selectin expression on the luminal side of ECs is increased by an atherogenic diet (Johnson-Tidey et al., 1994, Dong et al., 1998) . The next step in the influx of leukocytes into the lesion area is their firm adhesion to ECs facilitated by the interaction of the leukocyte, intracellular adhesion molecule 1 (ICAM-1) with VCAM-1 on the ECs. Lipoproteins, especially lipoproteins containing apolipoprotein CIII (ApoC III) increase ICAM-1 and VCAM-1 expression (Kawakami et al., 2006, Verna et al., 2006) . Deficiency in ICAM-1 and VCAM-1 in *Ldlr*^{-/-} mice leads to attenuated leukocyte adhesion to vascular ECs and homing, but only VCAM-1 deficiency decreases lesion size by gene dosage (Nageh et al., 1997, Dansky et al., 2001, Cybulsky et al., 2001). Entrance of monocytes into the sub-endothelial space is also under control of several chemokines. The most important chemokines in the influx of leukocytes are macrophage chemoattractant protein 1(MCP-1), CX3CL1 and CCL5. Combined deficiency of these chemokines or their respective receptors reduces bone marrow monocytoisis and atherosclerotic lesion development by 90%. Each of these chemokines is shown to have partially independent roles in the entry of monocytes into atherosclerotic lesions (Combadiere et al., 2008) .

1.1.1.2 Dendritic cells

Dendritic cells are present in the vascular wall and their number is increased in atherosclerosis-prone areas. Dendritic cells are localized within the areas which have high numbers of T cells. Unstable lesions have more dendritic cells in their shoulder regions (Bobryshev, 2005).

1.1.1.3 Neutrophils

The presence of neutrophils in atherosclerotic lesions was first shown in monkeys. There is a positive correlation between blood neutrophil count and the incidence of heart disease (Kawaguchi et al., 1996, Sweetnam et al., 1997); however, an increase in the relative number of neutrophils in murine blood due to disrupted CXCL12 and CXCR4 signaling enhances atherosclerotic lesion development in both *Apoe*^{-/-} and *Ldlr*^{-/-} mice. Depletion of neutrophils in the same model reversed the enhanced hematopoiesis-induced exacerbation of atherosclerosis (Zernecke et al., 2008). Despite the existence of many molecules in neutrophils that could potentially promote atherogenesis, the actual mechanisms which enhance atherosclerosis progression have yet to be defined. Neutrophils may pave the way for monocyte entry at early stages of atherosclerosis. Neutrophils may be detected in the shoulder region of rupture-prone lesions and may decrease SMCs and collagen and increase the volume of the necrotic core due to their short life-span and increase the chance of secondary necrosis in advanced atherosclerotic plaques (Bjorkbacka, 2011).

1.1.1.4 T lymphocytes

Recruitment of T cells to atherosclerosis-prone sites of vessels is accelerated. CD4⁺ T lymphocytes which are predominant in lesions are proatherogenic, whereas lesions-associated regulatory T-cells seem to be athero-protective. Natural killer (NK) T cells are also present in atherosclerotic lesions (Mallat et al., 2003, Robertson and Hansson, 2006). Chimeric *Ldlr*^{-/-} mice which have been reconstituted with bone marrow having functionally deficient NK T-cells develop 70 % and 40% less atherosclerotic lesions in the aortic root and aortic arch , respectively (Whitman et al., 2004, Galkina and Ley, 2009) .

1.1.1.5 B- lymphocytes

B cells are present in atherosclerotic lesions. B1 cells due to their secretion of IgM against oxidized low density lipoprotein (ox-LDL) have a protective role in lesion development, whereas transfer of B2 cells into immunodeficient *ApoE*^{-/-} mice has proatherogenic activity (Lewis et al., 2009, Kyaw et al., 2010).

1.1.1.6 Mast cells

Mast cells are present in vulnerable plaques. Mast cells secrete chymase, a neutral serine protease, which activates matrix metalloproteinase, degrades collagen and may contribute to Ang II formation. Therefore, chymase enhances plaque vulnerability(Lindstedt et al., 2007) . Mast cells decrease collagen contents and the fibrotic cap of atherosclerotic lesions. They also increase inflammation by Il-6 and IFN γ secretion in an atherosclerosis milieu (Leskinen et al., 2006, Sun et al., 2007).

1.1.1.7 Platelets

They are found at different stages of atherosclerosis and by forming a bridge through their P-selectins can attract inflammatory cells to the atherosclerotic lesions (Galkina and Ley, 2009) .

1.1.2 Smooth muscle cells:

In contrast to humans, some animal models of atherosclerosis such as mice and rats do not have SMCs in their intimal layer in the non-diseased state (Stary et al., 1992) . The SMCs in intima and media have different phenotypes. Whereas the SMCs in media have a contractile phenotype with a high contraction capacity those in the intima have a synthetic phenotype with greater motility, proliferation and secretion (Worth et al., 2001). In vitro studies show that, in response to stimuli such as growth factors (eg; TGF β) , extracellular matrix proteins, shear stress and reactive oxygen species, SMC with a contractile phenotype can become synthetic (Hedin et al., 1989, Corjay et al., 1989, Reusch et al., 1996, Su et al., 2001) . SMCs, by taking up lipids, can become foam cells. Diet-induced atherosclerosis and cytokines such as tumor necrosis factor alpha (TNF α) and interleukin 1 beta (IL1- β) enhance the expression of LDL receptors, very low density lipoprotein (vLDL) receptors and scavenger receptors on SMC which favors the uptake of native, acetylated, aggregated and enzymatically modified LDLs and chylomicron remnants. CXCL16/SR-PSOX which is only expressed by SMCs in atherosclerotic vessels might be responsible for uptake of ox-LDL (Li et al., 1995, Yu and Mamo, 1997, Klouche et al., 2000, Wagsater et al., 2004, Ruan et al., 2006). SMCs in lesion- prone areas of vessels in both humans and mice express adhesion molecules ICAM-1, VCAM-1 and CX₃CL₁. In atherosclerotic lesions these adhesion molecules are co-localized with

macrophages and monocytes and are involved in the retention of leukocytes in the sub-endothelial space. In vitro data suggests that the SMC-monocyte interaction leads to the activation of the Akt ,PI3 kinase pathway and down-regulates pro-apoptotic proteins and thus promotes macrophage survival. Besides maintaining monocyte viability this interaction also enhances secretion of chemokines and cytokines by SMCs and macrophages including Il-6 and MCP-1(O'Brien et al., 1993, Endres et al., 1997, Braun et al., 1999, Wong et al., 2002, Cai et al., 2004, Chen et al., 2009). Other cytokines , chemokines and growth factors are secreted by SMCs in atherosclerosis including interleukin 1 (IL-1), interleukin 18 (IL-18), interferon gamma (IFN γ), MCP-1, migratory inhibitor factor (MIF), TGF β and platelet-derived growth factor (PDGF) but, due to the redundancy in origin of these factors the relative contribution of SMC to lesion progression is not clearly defined. Overall, SMC- derived bioreactive factors are thought to increase in adhesion molecule expression, leukocyte recruitment, extracellular matrix (ECM)-remodeling and SMC proliferation and migration (Raines and Ferri, 2005).

SMCs are the main producer of ECM in both the healthy and atherosclerotic vessel wall. Whereas, in the healthy vessel walls, the ECM consists mainly of collagen type I and III, in the atherosclerotic vessel walls, the ECM contains proteoglycans and scattered type I collagen and fibronectin. This composition leads to increased lipoprotein retention in the ECM and LDL uptake by macrophages. Ox-LDL in turn regulates ECM synthesis by SMCs; enhances biglycan synthesis with elevated lipoprotein retention in the ECM. Modified ECM stimulates VSMC proliferation by down-regulation of the cyclin-dependent kinase 2 (Cdk2) inhibitor p27Kip1 (Hurt et al., 1990, Koyama et al., 1996, Chang et al., 2000, Roy et al., 2002) .

There is some evidence that SMCs in human lesions can come from progenitor cells in blood. Approximately, 20% of SMCs in patient who have undergone bone marrow transplantation show markers of the donor genotype. In mouse atherosclerosis-models there is conflicting evidence concerning the contribution of blood-borne cells to the population of lesion-associated SMCs (Sata et al., 2002, Bentzon et al., 2006, Metharom et al., 2008).

The presence of SMCs in lesions seems to be plaque stabilizing. Targeted diphtheria toxin- induced killing of SMCs in *ApoE*^{-/-} mice with established lesions reduces the ECM content and the fibrotic cap and increases the necrotic core and inflammation. In another study, conditional SM22 α knockout mice showed that the presence of SMCs with α -actin expression limits lesion development and inflammation (Feil et al., 2004, Clarke et al., 2006).

1.1.3 Endothelial cells

ECs are responsible for vascular homeostasis by regulating vascular tone, controlling vascular permeability, leukocyte and platelet adhesion, aggregation and thrombosis. Most of these functions depend on NO production by endothelial nitric oxide synthase (eNOS). Superoxide anions scavenge NO and make peroxynitrite which reduces NO bioavailability. By increasing NO reactive oxygen species formation hypercholesterolemia and inflammation decrease NO and lead to endothelial dysfunction (Marletta, 1989, Matoba et al., 2000, Boger et al., 2004). Usually atherosclerosis-prone regions are sites with low shear stress which initiates endoplasmic reticulum (ER)-stress leading to endothelial apoptosis. Regeneration of endothelium by proliferation of adjacent ECs and by recruitment of blood-borne endothelial progenitor cells is not always

complete. Damaged endothelium facilitates the entrance of lipid. and inflammatory cells and enhances atherosclerotic lesion formation (Xu, 2009).

1.1.4 Foam cell formation

ApoB containing lipoproteins and especially LDL have a primary role in atherogenesis. LDL, VLDL, lipoprotein (a) and remnant lipoproteins enter the sub-endothelial space by non-receptor mediated endocytosis and transcytosis (Vasile et al., 1983, Twickler et al., 2005, Tabas et al., 2007, Nordestgaard et al., 2010) . The most important factor which influences the entry of atherogenic lipoproteins into the sub-endothelium is their concentration in the circulation, but other factors such as particle size, charge and cholesterol contents also affect their entry and retention (Berneis and Krauss, 2002). In the sub-endothelium, lipoproteins interact with the ECM (Camejo et al., 1975). This is an ionic interaction between the negatively charged ECM proteoglycans and positively charged region of apoB including the LDL receptor- binding site. Mutation of specific lysine and arginine residues of apoB impairs LDL retention in the vessel wall and strongly reduces atherosclerosis. These *in vivo* experiments provide strong support for the "response to retention" model of atherogenesis (Skalen, Gustafsson et al. 2002) Other sites on apoB that can interact with proteoglycans may be unmasked following LDL modification. Interaction with proteoglycans in the ECM enhances lipoprotein oxidation and aggregation (Skalen et al., 2002, Fogelstrand and Boren, 2012, Cyrus et al., 2001). *In vivo*, LDL can be oxidized by enzymatic or non-enzymatic pathways. 12/15 lipoxygenase deficiency leads to reduced plasma anti-ox-LDL antibody titer which would be consistent with the lower numbers of ox- LDLs *in vivo*. 12/15 lipoxygenase-deficient mice have reduced atherosclerosis likely due to a lower incidence of lipoprotein oxidation (Cyrus et

al., 2001). Free or heme-associated iron in erythrocytes also increases LDL oxidation. Besides oxidation, LDL can be modified by enzymes such as s-sphingomyelinase or lipoprotein lipase. Enzymatic modification increases LDL aggregates (Miller et al., 2010).

The LDL receptor is not responsible for foam cell formation since LDL receptor deficiency does not prevent foam cell formation and the LDL receptor is generally down-regulated in patients with atherosclerosis (Yla-Herttuala et al., 1991). Modified lipoproteins are usually taken up by other sets of receptors called scavenger receptors such as SRA, CD36 and LOX-1 (Greaves and Gordon, 2009). Scavenger receptors have redundancy in function in terms of modified lipoprotein uptake. Deficiency in SR-A or CD36 decreases modified lipoproteins uptake by peritoneal macrophages. SR-A is the only receptor on macrophages that is involved in acetylated lipoprotein uptake. While the pro-atherogenic role of SR-A *in vivo* is controversial, CD36 deficiency decreases lesion development in aortas of *ApoE*^{-/-} mice. Combined deficiency of CD36 and SR-A in *ApoE*^{-/-} mice did not have additive effects in reduction of lesion size but decreased the size of necrotic core (Moore et al., 2005, Manning-Tobin et al., 2009).

There is considerable evidence that scavenger receptor-mediated lipoprotein uptake might not be the main pathway for foam cell formation. Ox-LDL extracted from atherosclerotic plaques is not sufficiently oxidized to be a ligand for scavenger receptors and multiple scavenger receptor deficiency did not decrease atherosclerotic lesion development in some animal models (Steinbrecher and Lougheed, 1992). Moreover, the concentration of LDL in vessel walls is much higher than *in vitro* experiments; therefore lipoprotein pinocytosis might be an important pathway for lipid uptake by cells. In fluid

phase pinocytosis minimally ox-LDL binds to CD14, a co-receptor for the toll- like receptor 4 (TLR-4) (a member of the pattern recognition receptor family), and activates TLR-4. TLR-4 activation induces membrane ruffling and cytoskeletal rearrangement. Aggregated LDL also needs cytoskeleton rearrangement to be taken up by phagocytosis. Aggregated LDL together with VLDL and remnant lipoproteins can also be taken up by LDL receptor-related proteins (Miller et al., 2010, Kruth, 2011) .

Cholesterol is stored in macrophages in the form of cholesterol ester droplets. Cholesterol, primarily in the form of cholesterol esters are taken up by cells via lipoprotein endocytosis. The cholesterol esters are hydrolyzed by acidic cholesterol esterase in the late endosome and lysosome compartments. Foam cell formation is the result of an imbalance between lipoprotein uptake and cholesterol efflux. In the absence of adequate cholesterol efflux, cholesterol should be re-esterified to decrease cytotoxicity of free cholesterol. This process is catalyzed in the ER by ACAT-1. To efflux cholesterol from lipid droplets, cholesterol ester needs to be hydrolyzed to free cholesterol by a cholesterol ester hydrolase associated with lipid droplets (Ghosh et al., 2010). Another mechanism for formation of free cholesterol is fusion of lipid droplets and autophagosomes. In this pathway acid lipase involved in autophagy are responsible for formation of free cholesterol (Ouimet et al., 2011). Several transporters in macrophages are involved in cholesterol efflux from macrophages. ABC transporters are a family of integral membrane proteins that mediate transport against a concentration gradient. ABCA1 seems to be the most important transporter in efflux which transfers cellular cholesterol to lipid-poor apoA-I (Voloshyna and Reiss, 2011). The actual mechanism of ABCA1- mediated efflux is still controversial. According to a recent model, a small

portion of apo A-I binds directly to ABCA1; whereas, the majority of the apoA-I interacts with membrane lipids. Binding of apoA-I to ABCA1 acts as a regulatory element that enhances phospholipid translocation to the plasma membrane exofacial leaflet which makes a bend in the plasma membrane. Formation of exovesicles relieves the membrane bending. Attachment of apoA-I to the vesicles help them to be released from membrane in the form of nascent high density lipoprotein (HDL). Binding of apoA-I to ABCA-1 activates Janus kinase 2 (JAK2) which in turn regulates lipoprotein binding and lipid export by ABCA-1. (Vedhachalam et al., 2007, Vaughan et al., 2009). Others working models have been proposed for ABCA-1 function in cholesterol efflux. One of them suggests that apoA-I and ABCA-1 form a complex on the cell surface which can then internalize. The apoA-I in this complex undergoes lipidation and is secreted from the cell (Takahashi and Smith, 1999, Hassan et al., 2008). However, some studies showed that internalized apoA-I becomes degraded in the cells (Lu et al., 2008b). ABCA-1-null mice have extremely low plasma HDL levels and highly lipid-loaded macrophages (Aiello et al., 2003). ABCG-1 and ABCG-4 work together to transport more cholesterol to pre- β HDL which results in the formation of mature HDL. ABCG-1 deficiency impairs mature HDL formation (Kennedy et al., 2005). However, more recent studies shows that ABCG-1 deficiency does not affect HDL level in plasma instead this deficiency impairs redistribution of cholesterol intracellularly from endocytic vesicles to ER (Tarling and Edwards, 2011). Scavenger receptor BI (SR-BI) is involved in bidirectional lipid transport. SR-BI deficiency enhances atherosclerosis which demonstrates that it has an important role in reverse cholesterol transfer in mice (Trigatti et al., 1999). In addition to the above mentioned ABC transporters, ABCA-5 seems to regulate ABCA-1 activity in

efflux of cholesterol. ABCA-5 knockout mice have reduced efflux to HDL but enhanced efflux to apoA-I due to an increase in expression of ABCA-1 (Ye et al., 2010). ApoE is also involved in efflux, as it can absorb lipid at the plasma membrane and is subsequently released after lipidation. In addition, proteoglycan bound-apoE stimulates release of lipid from the plasma membrane. It can also act as an acceptor for cholesterol released from transporters like ABCA-1 (Greenow et al., 2005).

Foam cell formation increases the fatty acid and cholesteryl ester content of the cells. The presence of fatty acid peroxides like 9-hydroxy-10,12-octadecadienoic acid (9-HODE) and 13-hydroxy-9,11-octadecadienoic acid (13-HODE) is able to activate PPAR γ (Nagy et al., 1998). Similarly PPAR α is activated by fatty acids. These two PPARs are able to induce expression of liver x receptor (LXR) (Brun et al., 1996, Chinetti et al., 2001). An increase in three acyl glycerol inside macrophages impairs mitochondrial electron transfer which lead to enhanced reactive oxygen species (ROS) formation (Aronis et al., 2009). Excess free cholesterol can also exaggerate generation of ROS by induction of ER stress. ER stress elevates intracellular Ca²⁺ which results in impaired electron transfer in mitochondria (Jacobson and Duchon, 2002). Failure in proper electron transfer generates ROS. ROS activates HIF-1 α through induction of NF- κ B (Kabe et al., 2005, Bonello et al., 2007). Activation of these transcription factors is responsible for a major change in the profile of gene expression after foam cell formation. Whatling et al (2004) report a large difference between the protease repertoires of foam cells and macrophages. Moreover, Hung et al (2006) showed that 447 common genes are differentially expressed in THP-1 cells following treatment with either ac-LDL or ox-LDL. This is likely the result of the activation of different nuclear factors in foam cells compared to macrophages.

LXR enhances expression of ABCA-1, ABCG-1 and ABCG-4. PPAR α elevates expression of ABCA-1 and PPAR γ through an LXR-dependent pathway and increases

the expression of ABCA-1 and ABCG-1. By increasing other genes participating in efflux like SR-BI or cholesterol ester hydrolase (CEH), these nuclear factors further enhance cholesterol efflux (reviewed by (Chinetti-Gbaguidi and Staels, 2009)).

1.1.5 Lesion stability

Most of atherosclerosis complications are related to thrombosis. Thrombosis occurs due to rupture, erosion or calcified nodules in lesions. Lesions which are less vulnerable to thrombosis are usually called stable lesions whereas those with higher susceptibility to thrombosis are unstable or vulnerable. It is generally accepted that an increase in the necrotic core size to more than one third of the atherosclerotic lesion area, results in a decrease in fibrotic cap thickness, degradation of ECM components and an enhanced chronic inflammatory state all of which contribute to lesion vulnerability. Besides intrinsic lesion characteristics which determine vulnerability of lesions, extrinsic factors are also involved in thrombosis (Virmani et al., 2005, Yazdani et al., 2010). As it is difficult to differentiate between inter- and intra-plaque factors, they are discussed together in the following sections

1.1.5.1 Biomarkers and stimulant of lesion instability

Lesion instability is the result of inter-plaque and intra-plaque conditions. Many of the makers for the vulnerable plaque are both the stimulant and the biomarker. Low shear stress or high tensile stress not only increases lesion formation in vessel walls but also decreases lesion stability (Cheng et al., 2006, Jankowski, 2006). Low shear stress induces apoptosis in ECs. Apoptotic cells release microparticles which contain phosphatidylserine. These microparticles increase lesion instability since they are

prothrombotic, procoagulant, angiogenic, proinflammatory and proapoptotic (Morel et al., 2006). Patients with familial hypercholesterolemia have high levels of plasma microparticles and tissue factor. Owens et al recently showed that in hypercholesterolemic *Ldlr*^{-/-} mice and in African green monkeys expression of tissue factors is induced through TLR4/ TLR6. Simvastatin treatment in hypercholesterolemic animals reduced tissue factor expression and coagulation (Owens et al., 2012).

There is an established correlation between visceral fat tissue and atherosclerosis progression. Fat tissue secretes adipokines and cytokines that are pro- and anti-atherosclerotic. Adiponectin is one of the anti-atherogenic adipokines. There is an inverse relation between plasma adiponectin and mixed and non-calcified vulnerable plaques in humans (Broedl et al., 2009). In lipid or lipoprotein-activated macrophages and T- cells fatty acid binding protein (FABP) is expressed. Expression of FABP4/ aP2, which is coordinating inflammatory reactions, cholesterol trafficking and ER stress, is a marker of plaque instability (Agardh et al., 2011). Symptomatic plaques from human have higher free cholesterol contents relative to asymptomatic ones. Therefore, high concentration of extracellular free cholesterol in atherosclerotic lesions is a marker of plaque vulnerability (Chen, Ichetovkin et al. 2010). However, an older study suggests that due to stiffness of free cholesterol crystals the difference between the fibrotic cap and underneath lipid layer is decreased by the presence of cholesterol crystals therefore the incidence of rupture become attenuated (Loree et al., 1994), however the clinical significance of cholesterol crystal is unknown. In the intima minimally modified LDL primes monocytes by inducing NF- κ B through TLR4/TLR6 and CD36 pathways. Internalization of minimally ox-LDL forms small cholesterol crystals inside the cells. These crystals are able to

damage lysosome and release its content including cathepsin L or cathepsin B into cytosol. These proteases activate inflammasome and increase IL-1 in atherosclerosis which in turn chemotactically recruits monocytes and neutrophils (Dewell et al., 2010).

The inflammatory state of lesions and plasma not only enhances lesion instability but also, in some cases, may predict the presence of vulnerable plaques in the asymptomatic patients. MCP-1 promotes an inflammatory state of the lesions since it is upstream of most inflammatory pathways. Blocking the MCP-1 – CCR-2 interaction decreases inflammation and reverses plaque progression and inhibits plaque rupture in established rabbit atherosclerotic lesions (Zhong, Chen et al. 2008). Since inflammation is one of the primary features of atherosclerosis, autoantibodies against different molecules like HSP60, ox-LDL and apoA-I can be found in atherosclerotic patients. Anti- apoA-I autoantibodies are another marker of plaque instability. This antibody enhances MMP-9 function and neutrophil chemotaxis. apoA-I antibody titers are inversely related to the collagen content of lesions (Montecucco et al., 2011). Moreover high expression of some cytokines such as interleukin-17 (IL-17) in plaques is another marker of lesion vulnerability (Erbil et al., 2011). Plaque rupture induces expression of CD40 ligand (CD40L) on the cell surface of platelets. The cleaved CD40L forms soluble CD40 L (sCD40L), which is a marker of unstable plaque with high lipid content. Binding of sCD40L to CD40 initiates a proinflammatory cascade by enhancing the expression of chemokines, adhesion molecules, matrix metallo proteases (MMPs) and growth factors (Koenig and Khuseyinova, 2007).

Intraplaque hemorrhage is another sign of rupture-prone lesions and plaque vulnerability. Hemorrhage increases red blood cells (RBCs) in the lesion area. Phagocytosis of RBCs

by macrophages enhances foam cells formation. Increased numbers of foam cells under the fibrotic cap due to difference between fluidity of fibrotic cap and foam cells make the atherosclerotic lesion unstable. Moreover, foam cells due to ER and oxidant stress and hypoxia become apoptotic or necrotic. The increase in necrotic core area is also another sign of lesion vulnerability. Expansion of the necrotic core is combined with an increase in the free cholesterol: cholesteryl ester ratio. Part of this free cholesterol is coming from membrane of dead foam cells and the other portion of free cholesterol belongs to the erythrocyte membrane. Progression of the necrotic core induces hypoxia within the atherosclerotic lesions; hypoxia in turn activates angiogenesis. Newly formed microvessels are inherently leaky which leads to a further discharge of erythrocytes into the plaque. RBCs increase macrophages infiltration, free cholesterol and iron deposition in atherosclerotic lesions. Intraplaque hemorrhage creates a vicious cycle which enhances plaque instability. RBCs also release heme. Heme modifies lipids and makes them more susceptible to oxidation. Heme-associated iron can also increase formation of reactive oxygen species (Virmani et al., 2005, Lin et al., 2008).

Hemoglobin, through induction of the CD163 receptor on monocytes and activation of ERK, p38 MAPK pathways, act as chemotactic factor. Hemoglobin stimulate adhesion and trans-endothelial migration of monocytes and monocyte-derived dendritic cells (Buttari et al., 2011). Hemoxigenase 1 is a cyto-protective enzyme against oxidative injury .It is produced by CD163 positive monocytes which are responsible for clearance of hemoglobin, and while, HO-1 is anti-inflammatory its presence in atherosclerotic lesions is, nevertheless, a marker of vulnerable lesions (Cheng, Noordeloos et al. 2009).

Overall, it seems that inflammatory state of lesions, the amount cholesterol ester crystals and the RBC content of lesions indicates the stability state of atherosclerotic lesions.

1.1.5.2 Extracellular matrix:

In the normal vessel wall there is a highly ordered ECM and cell structure. A basement membrane which consists of type IV collagen, fibronectin and laminin separates the media and intima layers. SMCs in the media are surrounded by types I, III, V and XVIII collagen. Besides different types of collagen, fibronectin and proteoglycan are also present. SMCs interact with the ECM through their integrins and discoidin domain receptors (DDR). DDRs only bind to fibrillar collagens. The type of collagen interacting with SMC defines their proliferative state. Whereas, fibrillar type I collagen restricts SMC proliferation and migration, interaction of SMC through DDRs with type VIII collagen which is found in atherosclerotic and injured vessels stimulates their proliferation by masking the intact type I collagen. This interaction can influence the ECM synthesis and accumulation of SMC in atherosclerotic plaques (Adiguzel et al., 2009). Degraded collagens signal through different type of integrins, for example degraded type I collagen signals through $\alpha_v\beta_3$ but not through $\alpha_1\beta_1$ or $\alpha_2\beta_1$ and stimulates production of tenascin-C. This glycoprotein stimulates proliferation and migration of SMCs (Chapados et al., 2006). Synthesis of newly made collagen is necessary for SMC migration. Cells, which are unable to make collagen, cannot migrate on pre-existing collagen (Rocnik et al., 1998). The inflammatory state of atherosclerotic lesions can regulate collagen synthesis by SMCs. Whereas anti-inflammatory cytokines like TGF- β enhance the expression of Type I, III, IV and V collagen by SMCs, inflammatory cytokines like TNF α , Il-1 decrease collagen synthesis (Amento et al., 1991).

In addition to the different types of collagen whose expression is altered during atherogenesis several glycoprotein and proteoglycans are newly expressed. In the atherosclerotic vessel wall

thrombospondin , vitronectin , tenascin and osteopontin are expressed in the basement membrane. The presence of proteoglycans like hyaluronan and versican are markers of plaque vulnerability. Hyaluronan detaches EC and elicits platelet migration and induces aggregation and polymerization of fibrin (Kolodgie et al., 2002).

Macrophages and SMCs inside lesions can produce versican, perlecan, syndecan, glypican-1, thrombomodulin and biglycan (Makatsori et al., 2003). ECM molecules can also affect the differentiation and polarization of the monocyte – macrophage. Type I collagen increases phagocytic capacity of macrophages and foam cell formation (Wesley et al., 1998). The ECM – macrophage interaction enhances superoxide production through induction of arachidonic acid metabolites (Gudewicz et al., 1994).

Collagen and elastin compose the fibrous cap. Fibrous cap thickness determines plaque stability. Thickness of the cap is influenced by collagen synthesis and degradation. ECM and mainly collagen can be degraded by MMPs. The ECM- macrophage interaction also enhance MMP 1 and 9 secretion. Collagen is degraded by MMP-1 ,8 and 13 to shorter form ($\frac{1}{4}$ and $\frac{3}{4}$ length of normal collagen size) (Miller et al., 1976). The short forms of collagen become denatured to gelatin which is a substrate for MMP-2 and MMP-9. The increase in expression of several MMPs including MMP- 1, 2, 3, 7, 8, 9, 12, 13, 14, 16 and 17 in human and experimental models has been reported (Newby, 2005). MMPs contribute to infiltration of macrophages and SMCs migration in to atherosclerotic lesions. They also regulate ECM deposition which controls atherosclerotic lesion size (Adiguzel et al., 2009) . Despite the increase in expression of MMPs in hypercholesterolemia and atherosclerotic lesions, they may have pro-atherogenic, anti-atherogenic or neutral effects on lesion development, MMP-9 and MMP-2 are atherogenic as MMP-9 or MMP-2 deficiency on an *ApoE*^{-/-} background reduced lesion size in mice. MMP-2 enhances infiltration of SMCs and macrophages, whereas there is a discrepancy in terms of MMP-9 effect on macrophages and SMCs migration (Silence et al., 2002, Lessner et al., 2004, Kuzuya et al., 2006). MMP-1 and MMP-3 appear to be athero-protective as their overexpression

and deletion respectively, decreased and increased atherosclerotic lesion size (Silence et al., 2001, Lemaitre et al., 2001). MMP-13 deficiency in *ApoE*^{-/-} mice did not change the lesion size, however the collagen content of lesions was increased in MMP-13- deficient mice (Deguchi et al., 2005). Despite the studies which showed the pro or anti-atherogenic role of several different MMPs, the contribution of MMPs to plaque rupture is not clear. For example studies with overexpression and deletion of MMP-9 showed contradictory results. Overall it seems that MMPs 2, 8, 12 and 14 are involved in plaque rupture and its vulnerability (Ketelhuth and Back, 2011). MMP activities are regulated at the level of transcription, activation through cleavage of their propeptides by other MMPs and serine proteases and inactivation by tissue inhibitors of matrix metalloprotease (TIMPs)(Ra and Parks, 2007). There are no definitive conclusions concerning the role of TIMPs in atherosclerosis development. Over expression of TIMP-1 in *ApoE*^{-/-} mice decreased atherosclerotic lesion size in the brachiocephalic artery but did not have any effect in the aorta. Deletion of TIMP-1 in *ApoE*^{-/-} mice decreased lesion development in one study but did not have any effects on lesion development in another (Silence et al., 2002, Cuaz-Perolin et al., 2006) . TIMP- 2 overexpression stabilized lesions in *ApoE*^{-/-} mice by decreasing macrophage infiltration and increase in SMC and collagen deposition (Elezkurtaj et al., 2004, Johnson et al., 2006)

Besides MMPs, other proteases in atherosclerotic lesions can also destabilize atherosclerotic lesions. Cathepsins and especially cysteine proteases like cathepsin K, L and S through ECM remodeling enhance atherosclerotic lesion progression (Cheng et al., 2011) It seems that the cellular origin of cathepsin K influences its role in atherosclerosis development. Cathepsin K from SMCs, by degrading collagen within the lesion, increases the vulnerability of plaques. However, cathepsin K from bone marrow-derived cells leads to an increase in number of SMCs within the lesions (Samokhin et al., 2008, Guo et al., 2009b). Serine proteases like plasmin and elastase also activate MMPs and indirectly or directly modulate ECM and make plaques vulnerable. Intra- plaque microvessels release neutrophils into the lesion area. Proteases

released by neutrophils like elastase activates MMP-9. As discussed above, MMPs by degrading ECM may destabilize plaque (Leclercq et al., 2007).

Mast cells are present in unstable plaque and their chymase can activate procollagenase (Bot et al., 2011).

Whereas primary modulation of ECM leads to SMCs migration to intima has stabilizing effects on lesions. Extensive degradation of ECM by various proteases makes lesions vulnerable to rupture.

1.1.5.3 Apoptosis

Apoptosis is involved in lesion vulnerability in several aspects. All cellular components of lesions undergo apoptosis and depending on cell type and the stage of the atherosclerotic lesions, the consequence of apoptosis on lesion stability is different. Decreased macrophage apoptosis following transplantation of p53 knockout bone marrow cells into APOE*3-Leiden transgenic mice results in enhanced atherosclerosis and increased lesion size after 12 weeks on a high fat diet (van Vlijmen et al., 2001). In a similar manner, decreased macrophage apoptosis following transplantation of Bax-deficient bone marrow cells into irradiated *Ldlr*^{-/-} recipients leads to accelerated lesion progression (Liu et al., 2005). Enhanced macrophage apoptosis in apoptosis inhibitory molecule (AIM)-deficient mice on an *Ldlr*^{-/-} background reduces atherosclerotic lesion size both after 5 weeks and 12 weeks of a high fat diet (Arai et al., 2005). These studies suggest that apoptosis in macrophages in early atherosclerotic lesions is a limiting factor for lesion development. In contrast to early atherosclerotic plaques, the incidence of apoptosis in advanced lesions increases the vulnerability of plaques.

The necrotic core is a large lipid core which results from dying macrophage foam cells. Whereas some markers of macrophages including immunoreactive CD68 and ceroid are found in the necrotic core, there is an absence of markers of SMCs (Ball et al., 1995). Macrophages undergo apoptosis in advanced lesions mainly due to ER stress, as the markers of ER stress and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive macrophages are co-localized (Myoishi et al., 2007). Several ER stress inducers including oxidized phospholipids, hypoxia, oxidant stress, free cholesterol, homocysteine and aggregated and modified lipoproteins are present in atherosclerotic lesions (Seimon and Tabas, 2009). A deficiency in ER stress mediators such as CHOP in both *LDLr^{-/-}* and *ApoE^{-/-}* mice reduces the incidence of macrophage apoptosis and the size of the necrotic core size (Thorp et al., 2009). Similarly, bone marrow deficiency in STAT1 a nuclear factor which is active during ER stress reduces apoptosis and necrotic core size in *Ldlr^{-/-}* mice (Lim et al., 2008). Another theory to describe ER-induced apoptosis is that a combination of physiological levels of ER stress with a second hit triggers apoptosis. The second hit could be pathogens, foreign antigens, endogenous proteins, and modified lipids which bind to pattern recognition receptors (PRR). Examples of PRR are toll-like receptors (TLRs) and scavenger receptors (SR) (Seimon et al., 2006). In line with this mechanism, Seimon et al found that macrophages undergo apoptosis in high saturated fat-fed mice through collaboration of CD36 and TLR-2 pathways. In the same study, they showed that macrophages in advanced lesions of *Ldlr^{-/-}* mice with bone marrow deficiency in TLR 2/4 are protected from apoptosis. These mice show smaller necrotic core size compared with their littermates having TLR2/4 wild type bone marrow (Seimon et al., 2010). An increase in macrophage

apoptosis enhances necrotic core formation possibly due to reduced engulfment of apoptotic bodies, a process called “efferocytosis”. Efferocytosis depends on the ligands on apoptotic bodies, receptors on phagocytes, and bridging molecules that link the two latter molecules; defects in any of the components impairs efferocytosis. Inefficient efferocytosis leads to secondary necrosis. A number of studies assessed the effects of each of the efferocytosis mediators in formation of necrotic core in atherosclerotic lesions. Deficiency in functional Mertk , a receptor of apoptotic bodies on phagocytes, lactadherin and the complement 1q bridging molecules all increase necrotic core size in atherosclerosis-prone mouse models. An increase in soluble lysophosphatidyl choline which is a “find me” ligand on apoptotic bodies also enhances necrotic core formation (Thorp and Tabas, 2009). The exact mechanism of defective efferocytosis in atherosclerotic lesions is not clear but some defects in efferocytosis mediators under inflammatory conditions, such as cleavage of MertK by sheddase or down-regulation of lactadherin expression, might explain ineffective efferocytosis in advanced atherosclerosis lesions. Binding of ox-LDL to CD14 and TLR4 also changes actin polymerization and impairs efficient efferocytosis (Miller et al., 2003, Sather et al., 2007, Komura et al., 2009). Human SMCs are efficient phagocytes able to clear apoptotic SMCs. The process of secondary necrosis as mentioned previously is common in advanced plaques; secondary necrosis induces IL-1 α and β formation. IL-1-induced MCP-1 and IL-6 release from surrounding SMC which impairs SMC phagocytosis and enhance inflammation (Clarke et al., 2010)

Besides impaired efferocytosis which induces secondary necrosis, various factors including highly ox-LDL, high level oxidative stress, increased intracellular calcium and

ATP depletion inside atherosclerotic lesions triggers programmed necrosis. High numbers of necrotic cells have been detected in advanced atherosclerotic lesions by transmission electron microscopy (TEM). Programmed necrosis is a regulated cell death that shares some pathways with apoptosis but does not induce caspases and is regulated by receptor interacting kinases (Martinet et al., 2011). Necrotic cells release various molecules that act as damage-associated molecular patterns (DAMP). One of the DAMPs that can be found in atherosclerotic lesions is high mobility group box 1 (HMGB1) which reacts with pattern recognition receptors like TLR 2 and 4 and the receptor for advanced glycation end (RAGE) products and initiates inflammatory reactions by releasing IL-1 α and β , TNF α and IL-6. It is also chemotactic for SMC migration. Necrotic cells also release cyclophilin A prior to HMGB1, which induces secretion of IL-6 and MMP-9 by macrophages, migration of SMCs and activation of ECs (Martinet et al., 2011, Zheng et al., 2011).

Besides macrophages in atherosclerotic lesions, SMCs and ECs also become apoptotic. Induction of SMC apoptosis in *ApoE*^{-/-} mice did not change the lesion size but decreased the fibrotic cap, collagen content, the number of SMCs and increased inflammation, macrophage number and necrotic core (Clarke et al., 2006). SMC apoptosis in the atherosclerotic lesion is usually due to increased sensitivity to death receptor-induced apoptosis and a decreased response to insulin-like growth factor (IGF-1). The presence of macrophages in the vicinity of SMCs is necessary for SMC apoptosis since macrophages can present FAS L and TNF α . They also decrease p53 in SMCs by their NO production. ox-LDL having hydroperoxide modifications increases p53 activity and decreases IGF-R and promotes apoptosis in SMCs. Mechanical stress also induces apoptosis in SMCs

through activation of p38 MAPK (Mayr and Xu, 2001). Overexpression of SMC-specific IGF-1 did not change plaque burden but enhanced plaque stability by increasing SMCs and collagen content and, as a result, fibrotic cap thickness (Shai et al., 2010). Endoplasmic reticulum stress induced SMC apoptosis by mediators such as 7-ketocholesterol, free cholesterol, homocysteine and glucosamine have been reported *in vitro* but the *in vivo* significance of such mediators is not clear (Tabas, 2010).

Enhanced SMC apoptosis by induction of FAS-mediated apoptosis through overexpression of Fas ligand or P53 decreases lesion cellularity and the fibrotic cap and increases incidence of plaque rupture in established lesions by 30-40 % (von der Thusen et al., 2002, Zadelaar et al., 2005). However, studies using induction of acute apoptosis suggested that SMC apoptosis is not involved in plaque progression whereas chronic SMC apoptosis in *ApoE*^{-/-} mice accelerates plaque progression. These lesions show characteristics of vulnerable plaques with a thin fibrous cap, large necrotic core and calcifications (Clarke et al., 2008). Disperse calcification is another sign of unstable plaques. The numbers of matrix vesicles which are precursors of matrix calcification are higher in plaques with thinner fibrotic caps (Bobryshev et al., 2008).

SMCs can undergo apoptosis by losing their anchorage to adjacent cells or ECM, a process called anoikis (Gilmore, 2005). MMPs and other proteases like cathepsins which are present in atherosclerotic plaques potentially can induce apoptosis in SMCs. MMP-7 increases SMC anoikis and apoptosis by cleavage of N-cadherin, a cell-cell anchorage molecule (Williams et al., 2010). Loss of interaction with ECM not only induces apoptosis in SMC but also in macrophages. Cathepsin S-deficient mice on an *Ldlr*^{-/-} background had less apoptotic macrophages in their lesions. They also had reduced

necrotic core size and SMCs and collagen in their lesions (de Nooijer et al., 2009). Cathepsin K deficiency in *ApoE*^{-/-} mice also increases fibrous cap thickness and collagen content after 8 weeks of a high fat diet without any change in plaque size. After 16 weeks of high fat diet in addition to changes in collagen and fibrotic cap content the plaque size in cathepsin K- deficient mice is also decreased. Cathepsin K deficiency also reduces the number of apoptotic cells (Samokhin et al., 2008). Mast cells and SMCs are in close proximity in vulnerable atherosclerotic lesions. In vitro studies have shown that mast cell chymase through inactivation of NF- κ B, a transcription factor that can provide, a pro-survival signal, induces apoptosis in SMCs (Leskinen et al., 2006).

Endothelial cell turnover in atherosclerotic mice is significantly greater than in normal mice. The area with higher turnover rate shows signs of endothelial dysfunction. Areas with a high turnover rate and dysfunctionality are sites with disturbed blood flow (Foteinos et al., 2008). Disturbed blood flow activates integrin α 2 β 1 that in turn activates p38 MAPK and induces apoptosis (Mayr et al., 2000). Disturbed blood flow also activates markers of ER stress in endothelial cells. The marker of ER stress, XBP1, down-regulates VE cadherin and causes detachment of ECs and activation of caspases (Zeng et al., 2009). Besides mechanical stress proteases also induce EC apoptosis. For example mast cell chymase through inactivation of focal adhesion kinase which is a survival signal activates effector caspase 8 and 9 and triggers apoptosis in ECs (Heikkila et al., 2008). Endothelial cell apoptosis increases thrombosis by exposure of thrombogenic phosphatidyl serine on the outer leaflet of the plasma membrane and also enhances inflammatory cell endothelial migration. Inflammatory cells produce MMPs and other proteinases which degrade ECM and decrease lesion stability. Moreover

cytokines produced by inflammatory cells like $\text{IFN}\gamma$ and IL-1 can modulate ECM production by decreasing or increasing smooth muscle proliferation, respectively (Mitra et al., 2004).

The impact of apoptosis on atherosclerotic lesion stability is different depending to cell time and stage of lesions. Generally, apoptosis in SMCs and EC makes lesions unstable, but only macrophage apoptosis in late stage apoptosis increases lesions vulnerability. The importance of late stage apoptosis in atherosclerosis might be due to impaired efferocytosis.

1.1.6 Animal models of atherosclerosis

Human atherosclerotic samples usually contain final stage plaques and only provide a snap shot from the moment the sample is obtained from either atherectomy after surgery or from a patient who has died from cardiac arrest or non-cardiac death. To experimentally assess the factors that are involved in the initiation of atherosclerosis and its progression and to have a better insight into the mechanism of thrombosis and its modulators, animal models which mimic human-like atherosclerotic lesions are necessary. Some animals like pigs and non-human primates spontaneously develop atherosclerosis; and have the highest similarity to humans both in lipoprotein metabolism and vessel wall anatomy. However, due to the high cost and, in the case of non-human primates, ethical issues, their use is usually limited to pre-clinical drug trial. Other animals like avians, rabbits, guinea pigs, rats and mice are also used as models of human atherosclerosis. Most animals do not develop atherosclerosis spontaneously and require a modified diet containing a high level of fat and cholesterol and /or genetic modification

(Bond and Jackson, 2011). Due to the ease of handling and genetic manipulation, the availability of inbred strains, a wealth of genetic information, a short breeding-time and low cost, the mouse has become a popular model for atherosclerosis.

1.1.6.1 Mouse models of atherosclerosis

The mouse is an atherosclerosis-resistant animal. It only develops atherosclerosis after feeding with a high fat diet. Different strains of mice have different susceptibility to atherosclerosis. Whereas C57BL/6 mice represent the strain most susceptible to diet-induced atherosclerosis, the C3H strain is the most resistant, with BALB/c mice showing an intermediate susceptibility (Paigen et al., 1987). The relative resistance to development of atherosclerosis is often due to differences in lipoprotein metabolism. As a result, the generation of lines of mice having genetic modifications that result in the over-expression or deletion of genes encoding proteins involved in lipoprotein metabolism have given rise to atherosclerosis-prone mice. The first type of genetically engineered line of athero-susceptible mice has a deficiency in apolipoprotein E. *ApoE*^{-/-} mice were generated by two separate groups. This was followed by LDL receptor knockout mice that were generated as a model of human familial hypercholesterolemia. Other genetically modified mouse models for atherosclerosis also exist such as genetic replacement models (replacement of ApoE with human Apo E2,3 or 4), and transgenic models(expressing human apo B100, human apoE2 or apoE3 Leiden (Whitman, 2004b). The most commonly used mouse models of human atherosclerosis are *Ldlr*^{-/-} and *ApoE*^{-/-} mice.

1.1.6.1.1 Apolipoprotein E knockout mice

ApoE is a glycoprotein synthesized by the liver and several other tissues and cell types including macrophages. It is present in all lipoproteins except LDL and it is a ligand for the LDL receptor and other members of the LDL receptor family (Greenow et al., 2005). The primary defect in *ApoE*^{-/-} mice is an impaired hepatic clearance of VLDL and chylomicron remnants which accumulate in the plasma.

ApoE^{-/-} mice on a chow diet develop severe hypercholesterolemia, with plasma cholesterol concentrations 5 to 8 times those of wild type littermates. Feeding *ApoE*^{-/-} mice with a high fat diet (western-like diet) further enhances plasma cholesterol levels to 10 times that of wild type mice on a high fat diet. While HDL is the major lipoprotein species in wild type mice, HDL is reduced in *ApoE*^{-/-} mice. The VLDL and IDL fraction of *ApoE*^{-/-} mice are increased relative to normal mice and are enriched in apo B48, apo AI and apo AIV. The exaggerated increase in plasma cholesterol and the difference in distribution amongst lipoprotein classes compared to humans are the major drawbacks of this mouse model. In terms of atherosclerosis, *ApoE*^{-/-} mice develop fatty streaks in the proximal aorta at 3 months of age on a chow diet, and the lesions become more advanced and include SMCs and fibrotic cap by 8 months of age. While *ApoE*^{-/-} mice develop atherosclerosis spontaneously, when placed on a western diet atherogenesis is accelerated and larger lesions develop. Mice heterozygous for the apoE gene disruption are susceptible to diet-induced atherosclerosis but do not develop spontaneous lesions. In addition to the aorta, *ApoE*^{-/-} mice on a high fat diet also develop plaques in the coronary, pulmonary, carotid, brachiocephalic and iliac arteries (Zhang et al., 1992, Plump et al., 1992, Zhang et al., 1994, Rosenfeld et al., 2000). By 42-60 weeks of age ,

lesions in the brachiocephalic artery of *ApoE*^{-/-} mice show intraplaque hemorrhage and a fibrotic conversion of the necrotic zone accompanied by a loss of the fibrotic cap making apoE-deficient mice a valuable model for studying advanced stage atherosclerosis (Rosenfeld et al., 2000).

Expression of apo E in bone marrow-derived cells can influence on development of atherosclerosis in mice. Whereas apo E deficiency in bone marrow enhances atherosclerosis susceptibility in wild type mice, the presence of functional apoE in cells derived from bone marrow transplanted into *ApoE*^{-/-} mice reduces plasma cholesterol and attenuates atherosclerosis development. (Mazzone and Reardon, 1994, Fazio et al., 1997, Van Eck et al., 2000).

ApoE deficiency is pro-atherogenic in several ways other than its effects on lipoprotein metabolism. ApoE deficiency appears to decrease endothelial nitric oxide synthase (eNOS) activity, to elevate pro-inflammatory helper T1 cells, to promote T-cell activation, to decrease efferocytosis and to enhance secondary necrosis and inflammation (Sacre et al., 2003, Tenger and Zhou, 2003, Grainger et al., 2004, Ali et al., 2005).

1.1.6.1.2 LDL receptor knockout (*Ldlr*^{-/-}) mice

LDL deficiency in mice, in contrast to humans, only modestly changes the plasma cholesterol concentration, but similar to humans, the increased cholesterol is mainly in the LDL and IDL fractions (Ishibashi et al., 1993). *Ldlr*^{-/-} mice do not develop atherosclerosis unless they are fed a high fat diet. Lesions are located throughout the aorta and coronary arteries. (Ishibashi et al., 1994). Compared to *ApoE*^{-/-} mice at the same age and fed the same atherogenic diet, *Ldlr*^{-/-} mice develop simpler, smaller lesions with a

foamy appearance and a smaller necrotic core (Roselaar et al., 1996). The presence or absence of the LDL receptor on bone marrow-derived cells does not affect the lipoprotein profile nor atherosclerosis development (Linton et al., 1999). *Ldlr*^{-/-} mice that carry a human apoB100 transgene develop spontaneous atherosclerosis on a chow diet comparable to that of chow-fed *ApoE*^{-/-} mice. These mice have severe hypercholesterolemia with most of the cholesterol carried by LDL particles (Sanan et al., 1998).

1.1.6.2 Lipoprotein metabolism and atherosclerotic lesions in mice and humans

Atherosclerosis in humans commonly occurs due to a moderate increase in plasma apo B containing lipoproteins together with the presence of other factors including smoking, hypertension and genetic predisposition and only in minority of cases, it is related to the genetic mutations that are found in *Ldlr*^{-/-} and *ApoE*^{-/-} mice (Bentzon and Falk, 2010).

There are important differences in lipoprotein metabolism between mice and humans. In wild type mice, plasma cholesterol is carried primarily by HDL particles whereas, in humans, the highest concentration of cholesterol is in the LDL fraction. ApoB48 is secreted uniquely by intestinal enterocytes in humans but, in mice, apoB48-containing triglyceride-rich particles are secreted by both the intestine and the liver. ApoB48 lipoprotein remnants are rapidly cleared by the liver in mice and are not converted to LDL which, in part, accounts for the low LDL levels in wild type mice. Humans, unlike mice, can transfer cholesteryl esters from HDL to triglyceride-rich lipoproteins in exchange for triglyceride, a process mediated by cholesteryl ester transfer protein (CETP). CETP activity tends to reduce HDL levels. This lipid transferase protein does not expressed in mice (Westerterp et al., 2006).

Similar to humans, mice develop atherosclerotic plaque in areas with low shear stress or high tensile stress. Unlike mice the primary site of lesions in humans is the coronary artery and not the aortic root; however, humans with type II (defective LDL receptor) and type III (homozygous Apoe2 plus additional risk factor(s)) hypercholesterolemia can develop aortic lesions similar to those seen in *Apoe*^{-/-} mice. Thus a further limitation in mouse models of atherosclerosis is that they mimic only a subgroup of human atherosclerotic patients. Besides genetic susceptibility, murine anatomy also dictates the area of lesion development (Bentzon and Falk, 2010, Getz and Reardon, 2012). Unlike the human vessel wall the intimal layer in mice only contains SMCs that have migrated from the media once atherosclerosis is in relatively advanced state. In humans, there is a resident intimal population of SMCs and the numbers of SMCs in the intima can be increased by SMC migration from the media and by recruitment of blood-borne progenitors (Stary et al., 1992, Sata et al., 2002, Bentzon et al., 2006, Metharom et al., 2008).

Although old *Apoe*^{-/-} mice do develop advanced lesions with intraplaque hemorrhage in the brachiocephalic artery they do not show final stage lesions with plaque rupture and thrombosis. However, it has been argued that this could, in part, reflect the difficulties in detecting thrombosis in mice (Rosenfeld et al., 2000, Bond and Jackson, 2011)

1.2 Renin angiotensin system (RAS)

The crucial role of the RAS in controlling blood pressure, electrolyte balance and fluid volume has been known for decades. In the classical view, renin, a highly specific aspartyl protease, secreted by juxtaglomerular cells in the kidney into the circulation cleaves angiotensinogen which is produced and secreted from the liver to form Ang I.

Ang I, an inactive decapeptide, is cleaved mainly by ACE, which is produced by endothelial cells of the pulmonary system to form Ang II, the active octapeptide (Oparil and Sanders, 1970) . This classical view of the circulatory RAS has been expanded by the presence of a local RAS in most organs and tissues including the brain , heart and vasculature (Dzau, 1987). The recognition of renin- prorenin, the identification of other enzymes involved in production of angiotensin peptides, other active angiotensin peptides and the intracellular RAS have extended the concept of the classical systemic RAS (Paul et al., 2006). The components of the systemic and vascular RAS and their contribution to disease states, specifically atherosclerosis, will be addressed.

1.2.1 Physiology of the RAS

1.2.1.1 Angiotensinogen

In humans, angiotensinogen is a glycoprotein with 452 amino acids and different glycosylation levels. The gene for angiotensinogen is located on chromosome 1 and contains 5 exons. In the angiotensinogen gene, exon 2 contains the start codon and most of the angiotensinogen coding sequence. Cell-specific expression of angiotensinogen is determined by exon 5 cis elements (Brasier et al., 1999) . The essential promoter sequence for expression of angiotensinogen is located within the first 25 bp upstream of the start codon (Yanai et al., 1996). Angiotensinogen is a unique precursor for angiotensin peptides and its deficiency leads to severe hypotension (Wu et al., 2011, Tanimoto et al., 1994). Plasma angiotensinogen mainly comes from the liver, where hepatic nuclear factor 4 is involved in angiotensinogen transcription whereas, in other tissues PPAR α promotes angiotensinogen expression (Shimamoto et al., 2004a).

Inflammation , glucocorticoid hormone , thyroid hormone, estrogen and Ang II upregulate angiotensinogen expression (Brasier et al., 1999). In an inflammatory state, NF- κ B is activated. The activated NF- κ B translocates to the nucleus and can bind to acute phase response element (APRE) in the angiotensinogen promoter and enhance angiotensinogen expression (Li and Brasier, 1996). The APRE is surrounded by two glucocorticoid response elements. Upregulation of angiotensinogen expression by glucocorticoids, estrogen and thyroid hormone needs NF- κ B binding to APRE (Brasier et al., 1990). Ang II increases angiotensinogen expression in a positive feedback control by induction of reactive oxygen species (ROS) and NF- κ B (Brasier et al., 1996). Among the extra-hepatocyte sources of angiotensinogen only adipocyte angiotensinogen contributes to the plasma angiotensinogen (Stec et al., 1999, Yiannikouris et al., 2012). Circulating angiotensin peptides are generated by hydrolysis of angiotensinogen by kidney renin. Renin is the rate-limiting factor in generation of Ang II in plasma of most animals except mice (Wu et al., 2011, Lum et al., 2004). Expression of angiotensinogen mRNA in the vessel wall is mostly located in periadventitia adipose tissue, but it is also expressed in the medial layers of the vessel wall. Angiotensinogen generated in periadventitia and medial layer migrates through the vessel wall to reach renin, ACE and other enzymes in the endothelial layer to generate Ang I and II (Cassis et al., 1988, Naftilan et al., 1991).

1.2.1.2 Renin and prorenin

Renin is an aspartyl protease with molecular weight of 36-40 KD. Its gene in humans is located on chromosome 1 with 10 exons. Renin mRNA is expressed in juxtaglomerular cells of kidney; and to a lesser extent, it is expressed in renal proximal tubules, collecting

ducts and connecting tubules(Castrop et al., 2010). cAMP is the main transcription activator for renin transcription; other factors such as thyroid hormone , PPAR γ or LXR α enhance the cAMP effects(Gilbert et al., 1994, Morello et al., 2005, Desch et al., 2010). Renin mRNA primarily forms prorenin, a protein containing the signal sequence. After removal of the signal sequence, prorenin can be secreted from the golgi apparatus or stored in cell granules. Whereas, in human cells expressing renin *in vitro*, cathepsin B removes the propeptide from prorenin to generate renin in secretory granules; *in vivo* cathepsin B does not seem to be the enzyme responsible for removing the propeptide, at least in rodents. Cathepsin B deficiency and cathepsin B inhibitor treatment did not affect renin concentration in rodents. The actual enzyme responsible for propeptide removal in rodents is not yet known (Percival et al., 2010, Mercure et al., 2010). Synthesis and secretion of renin from the juxtaglomerular apparatus is regulated by interrelated factors such as blood pressure, sodium balance, Ang II, sympathetic stimulation, hormones and autocooids (Castrop et al., 2010). Blood pressure controls renin synthesis and release via intrarenal as well as extrarenal mechanisms. Increased renal blood pressure is sensed by baroreceptors. A decrease in blood pressure increases renin secretion and vice versa (Castrop et al., 2010). The nature and location of these baroreceptors have not yet been clarified. They might be in the form of connexin 40 gap junction in juxtaglomerular apparatus cells (Gomez and Sequeira Lopez, 2009). The Na⁺: K⁺: 2Cl cotransporter and Na⁺/H⁺ exchanger (NHE) at the apical layer of the macula densa are involved in sensing salt. Activity of these channels together with channels in the basolateral layer such as the cation channel and K⁺/H⁺ /ATPase change the intracellular calcium and pH of the cells. The change in pH that results from a low salt diet induces extracellular regulated signal 1/2

(ERK1/2) activity which in turn increases COX-2 expression. Activation of the prostaglandin receptor PE4 by prostaglandin E (PGE) , which is generated by COX-2, elevates cAMP level which in turn increases renin expression and release in juxtaglomerular cells (Komlosi et al., 2004, Hanner et al., 2008, Facemire et al., 2011) Sympathetic activity also controls renin synthesis and secretion via cAMP-mediated pathways (Castrop et al., 2010). Sympathetic nerve input innervates juxtaglomerular cells to release renin (Holmer et al., 1994). One mode of sympathetic activation is sensing blood pressure by arterial baroreceptors in carotid sinus of the left and right internal carotid arteries and aortic arch. β -adrenergic receptors are expressed in juxtaglomerular granular cells. β -adrenergic receptor antagonists or neuronal denervation decrease renin mRNA and protein levels in rat kidney compared to controls (Castrop et al., 2010). Moreover, β -adrenergic receptor knockout mice have an 85% reduction in plasma renin concentration (Kim et al., 2007). β -adrenergic receptors are G-coupled transmembrane receptors; their stimulation activates adenylyl cyclase and increases cAMP. Elevated cAMP transduces a cAMP/ PKA cascade which leads to opening of ionic channels like Ca^{2+} - sensitive voltage-activated potassium channels. As a result, the membrane becomes hyperpolarized and renin is released. PKA activity also phosphorylates cAMP response element binding protein which results in enhanced expression of the renin gene (Kopp, 2011). Ang II also controls renin secretion by increasing the activity of both the NHE and the $\text{Na}^+ : \text{K}^+ : 2\text{Cl}$ cotransporter and enhancing the uptake of sodium by macula densa which lead to decreased expression of COX- 2 in macula densa (Komlosi et al., 2004) and negative regulation of renin secretion from juxtaglomerular cells (Harris et al., 2004).

Renin mRNA is not present in healthy vasculature. Samani et al (1987) and Hilgers et al (2001) did not detect any mRNA in aorta of Wistar-Kyoto and Sprague-Dawley rats, but renin mRNA is expressed in rat carotid artery after balloon injury (Samani et al., 1987, Iwai et al., 1997, Hilgers et al., 2001). Renin protein is present in the vascular system but probably is taken up by prorenin/ renin receptors (Nguyen and Contrepas, 2008b). Presence of renin protein in endothelial cells of aorta of rat is shown by immunohistochemistry (Okamura et al., 1992). The renin amount decreases after nephrectomy and renin-like activity in the aorta increases after injection of mouse renin into nephrectomized rats (Loudon et al., 1983, Hilgers et al., 2001).

1.2.1.3 (Pro) Renin receptor

The (pro) renin receptor is a 350 amino acid protein. Its gene is located on chromosome 1. This membrane protein has equal binding affinity for renin and prorenin. The (pro)renin/ renin receptor is involved in tissue renin uptake (Nguyen and Contrepas, 2008a). Hypertensive rats have higher numbers of (pro) renin receptors in their vasculature which increases renin activity and Ang II production in their vessel wall (Krebs et al., 2007). Binding to (pro) renin receptor conformationally changes prorenin so that it becomes catalytically active without losing its pro-segment (Mercure et al., 1995). Besides prorenin activation, this receptor also increases the activity of renin (Batenburg et al., 2007). Prorenin binding to the (pro) renin receptor enhances proliferation and the profibrotic process. This function of prorenin is triggered through MAPK p42/p44 and ERK 1/2 (Huang et al., 2007, Feldt et al., 2008). Renin also binds to the mannose 6-phosphate / insulin like growth factor 2 receptors in myocytes and endothelial cells. This

general receptor for mannose-containing glycoproteins internalizes prorenin and removes pro signal peptide. However this does not lead to intracellular angiotensin generation and renin eventually becomes degraded (van Kesteren et al., 1997, van den Eijnden et al., 2001).

1-2-1-4 Angiotensin converting enzyme (ACE)

ACE is a 130 -180 KD dipeptidyl carboxylase metallo enzyme, which generates Ang II from Ang I and hydrolyzes the vasodilator substance, bradykinin. The ACE gene consists of 26 exons and is located on chromosome 17. The ACE gene has both proximal and distal promoter involved in basal expression of ACE. The proximal ACE promoter is located between -122 and- 288 bp upstream of the transcription start site and the distal promoter is between -415 and -1303 bp. These two promoters have two cAMP response elements which can be transactivators or transrepressors depending on the cell types (Xavier-Neto et al., 2004). Cell-type specific expression of ACE is regulated by ACE promoter methylation and histone acetylation levels (Riviere et al., 2011). Aldosterone through its binding to the mineralocorticoid receptor induces JAK2 and increases ACE expression (Sugiyama et al., 2005). PPAR α and γ activation suppresses the expression of ACE (Takai et al., 2007). Various stimuli such as basic fibroblastic growth factor (bFGF) and vascular endothelial growth factor enhance ACE expression. In most of these cases protein kinase C (PKC) is activated. Active PKC, through induction of ERK1/2, increases binding of early growth response (Egr-1) to GC rich consensus sequences in the proximal promoter. PKC activation also enhances binding of activating protein 1 (AP-1) to a 21bp cAMP-responsive element in the distal promoter (Eyries et al., 2002b). In the vascular wall, ACE is prominently expressed on the endothelial cell membrane. ACE expression

in adventitia of different species has been reported; however, in all cases it is confined to endothelial cells of vasa vasorum. Arnal et al (1994) showed ACE expression in the media layer of the aorta from rats but most studies suggest ACE expression in SMC from media is only induced in pathological states (Arnal et al., 1994, Fernandez-Alfonso et al., 1997, Zhuo et al., 1998, Gosgnach et al., 2000).

ACE2 is a metalloprotease with 42 % similarity with somatic ACE and generates the vasodilator peptide, Ang 1-7, from Ang I and Ang II. ACE2 is expressed in various tissues along with somatic ACE. ACE2 is expressed in endothelial cells of rat arteries (aorta arch, pulmonary vein and coronary arteries). ACE2 is also expressed ubiquitously in endothelial cells and SMCs from human veins and arteries (Hamming et al., 2004, Riviere et al., 2005).

1.2.1.5 Angiotensin peptides

Ang 1-12 is a recently discovered precursor for Ang I, II and other angiotensin peptides. Ang 1-12 formation is renin-independent. The enzyme(s) that is (are) involved in the formation of angiotensin 1-12 is not yet known. Angiotensin 1-12 is cleaved to Ang II by ACE or chymase (Bujak-Gizycka et al., 2010). Angiotensin I is an inactive decapeptide which can be converted to other angiotensin peptides. Ang II is the most important effector of the RAS mainly generated from angiotensin I by ACE and by other enzymes like chymase or cathepsin G (Paul et al., 2006). Both Ang I and II can be hydrolyzed to Ang 1-7 by ACE2 and other enzymes including prolyl endopeptidase and neutral endopeptidase (Welches et al., 1993, Santos et al., 2002, Reudelhuber, 2005). Ang 1-7 is then degraded to Ang 1-5 and 3-5. Ang II can also be converted to Ang III and IV by aminopeptidase A and aminopeptidase N, respectively. Ang 3-7 is generated from Ang II,

Ang IV and Ang 1-7 by carboxypeptidases or aminopeptidases (Nguyen Dinh Cat and Touyz, 2011).

Whereas Ang I is mainly generated in the medial layer of vessel wall and then diffuses to other layers, Ang II is generated in the endothelial layer. Both angiotensin I and II are mainly located in the medial layer of the vessel wall (Gohlke et al., 1992). Ang III is generated in endothelium and SMCs (Palmieri et al., 1989). Immunohistochemistry data showing the location of Ang III in vessel walls is not available. Ang IV can be formed in the endothelial cells since aminopeptidase N is present in the endothelial layer of aorta. Whether Ang IV diffuses to other layers or it is only located in the endothelial cells is not clear (Papapetropoulos et al., 1996). Ang1-7 is highly present in the periadventitial tissue but not the medial or the endothelial layer of aorta in Wistar rats (Lee et al., 2009a).

1.2.1.6 Angiotensin receptors

Ang II and III bind to two 7- transmembrane receptors, angiotensin receptor type 1(AT₁R) and 2 (AT₂R), which have different functions, tissue specificities and genomic organizations. The AT₁R gene consists of 4 exons and it is located on human chromosome 3. The first 3 exons form the 5' untranslated region and the open reading frame is encoded by exon 4. Human AT₁R protein expression is regulated by alternative splicing. Depending on inclusion or exclusion of exon 2 and/or 3 the translation rate of AT₁R is different. The inclusion of exon 2 in mRNA due to alternative splicing down-regulates AT₁R protein level. Several modulators including growth factors, Ang II and PPAR γ decrease AT₁R expression. Stimulators like inflammatory cytokines (TNF α and IL-6) and insulin-like growth factor enhance AT₁R expression (reviewed by (Elton and Martin, 2007)). AT₁R is expressed in many organs throughout the body including brain,

peripheral nervous system, male and female reproductive tracts, adrenal gland , heart, parts of digestive organs, eye , adipose tissue, lymphatic tissue and kidney (Paul et al., 2006). In the vessel wall, AT₁R is highly expressed in the SMCs of arteries. It is also expressed at low level in the adventitial layer of the vessel walls (Allen et al., 2000). AT₁Rs after activation undergo endocytosis; a mechanism for desensitization. The other mechanism for desensitization is serine/threonine phosphorylation. Phosphorylation triggers G protein uncoupling (Srivastava, 2002; Yan et al, 2003).

The AT₂R gene is located on chromosome X. The AT₂R promoter sequence contains a suppressor sequence between -1208 bp and -749 bp .Sequences between -749 bp and -216 bp have positive regulatory elements. The basal promoter activity is located between -44 bp and +58 bp. Efficient expression of AT₂R needs a 123 bp intron 1 fragment. Some negative regulators of AT₁R are positive regulators of AT₂R expression. Whereas cAMP is a positive regulator of AT₁R, it is a negative regulator for AT₂R. Similarly, in contrast to AT₁R, interferon γ induces AT₂R expression via a JAK/ STAT pathway (Ichiki et al., 1996, Murasawa et al., 1996, Warnecke et al., 1999, Horiuchi et al., 2000). AT₂R opposes AT₁R function by either activating serine/ threonine phosphatases that uncouple AT₁R and G protein or by heterodimerization with AT₁R. (Kaschina and Unger, 2003). Expression of the AT₂R declines after birth and it is upregulated in pathological states, such as vascular injury and cardiac remodeling (Horiuchi et al., 1999, Adachi et al., 2003). AT₂R is expressed in heart, vasculature, kidney, brain, male and female reproductive tracts, parts of the gastrointestinal tract, adipose tissue and lymphatic tissue (Yvan-Charvet et al., 2005, Paul et al., 2006). AT₂ is only expressed in some arteries including mesenteric artery, the adventitial layer of inter-

and intra-lobular arteries, arcuate arteries in kidney, the medial layer of the anterior cerebral artery and the endothelial layer of coronary arteries (Tsutsumi and Saavedra, 1991, Zhuo et al., 1996, Wang et al., 1998, Allen et al., 2000).

Other angiotensin peptides, such as Ang 3-8 or Ang IV act through the AT₄R (Paul et al., 2006, Vanderheyden, 2009). AT₄R or insulin-regulated aminopeptidase is a multimeric protein with a molecular weight around 160-190 kDa. The AT₄R gene is located on chromosome 5 and it has 18 exons. A GC- rich promoter/ enhancer sequence is located – 621 bp upstream of the site of transcription initiation which can bind to Sp1 and retinoic acid inducible components. Hypoxia is one of the inducer for the expression of AT₄R in the carotid artery (Rasmussen et al., 2000, Albiston et al., 2001, Fung et al., 2007). AT₄R is present in brain, heart, vasculature, kidney, adrenal gland, prostate and colon.(Paul et al., 2006). In the normal vessel wall, AT₄R is expressed in SMCs and vasa vasorum (Moeller et al., 1999). Ang 1-7 binds to the Mas receptor. The Mas receptor gene is located on chromosome 6 and it only has a single open reading frame which is not interrupted by introns. Oxidative stress reduces the expression of the Mas receptor and a PPAR γ -associated pathway elevates the expression of Mas (Lakshmanan et al., 2011). Mas receptor expression is also under negative feedback control of its ligand Ang 1-7 (Tan et al., 2011). This 325-amino acid protein is expressed in various tissues including brain, heart, kidney, lung, liver, spleen, tongue and skeletal muscle (Alenina et al., 2008). In vasculature, the endothelial layer expresses the Mas receptor (Sampaio et al., 2007a).

1.2.1.7 Angiotensin peptides action

1.2.1.7.1 Vascular tone: Ang II and III can trigger vasoconstriction through AT₁R. Ang II vasoconstrictive effects are G protein-dependent. G protein coupling activates a series of phospholipases (PL) including PLC. PLC generates inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to its ER receptor and releases Ca²⁺, which in turn, by binding to calmodulin kinase, phosphorylates myosin and triggers muscle contraction (Yan et al., 2003). Ang 3-7 also induces vasoconstriction but through AT₄R (Ferreira et al., 2007). Vasodilation is induced when Ang II or III binds to AT₂R or angiotensin IV binds to AT₁R (Haulica et al., 2005). Ang 1-7, by binding to the Mas receptor, also triggers vasodilation in the endothelial cells via two pathways: either by stimulating eNOS activity causing nitric oxide (NO) release or by counter-regulating the vasoconstrictive action of the AT₁R (Sampaio et al., 2007b, Sampaio et al., 2007a).

1.2.1.7.2 Growth: Binding of Ang II and III to AT₁R is growth-promoting either by inducing mitosis or hypertrophy. AT₁R activation of G protein coupling produces DAG through activation of PLC. DAG by activation of protein kinase C (PKC) activates a downstream cascade of Ras/ Raf/MEK / ERK and triggers the growth-promoting effects of Ang II (Srivastava, 2002). Binding of Ang II and III to AT₂R has opposing effects including both growth inhibition and apoptosis. AT₂R activates protein phosphotyrosine phosphatases for a rapid attenuation of uncontrolled growth. Activation of MAP kinase phosphatase by AT₂R, suppresses ERK1/2 activation. Inactivation of ERK leads to upregulation of Bax and dephosphorylation of BCL-2 which eventually facilitates apoptosis. AT₂R inhibits growth by inhibition of cyclin D1 and cyclin-dependent kinases that are involved in the cell cycle (Haulica et al., 2005, Mehta and Griendling, 2007).

1.2.1.7.3 Water and sodium retention: Ang II and III through AT₁R increase sodium reabsorption and water retention. They are also involved in aldosterone release from the adrenal cortex (Paul et al., 2006). Angiotensin 1-7 has an opposing effect and acts to promote natriuresis or diuresis (Dilauro and Burns, 2009).

1.2.1.7.4 Neurotransmission: Brain has a local RAS system; angiotensinergic neurons in some parts of the brain synthesize angiotensin peptides and following action potential-triggered calcium influx transfer them to their axon synapses. Excitation of the angiotensinergic neurons leads to long-duration post-synaptic excitation (Ferguson et al., 2001, Haulica et al., 2005). Some data suggest that Ang II is converted to Ang III and then acts as a neurotransmitter. Blocking Ang II to Ang III conversion by an aminopeptidase A inhibitor reduces the response to intracerebroventricular injection of Ang II. The neurotransmission of Ang III or II can be blocked by AT₁R blockers (Wright et al., 1990, Speth and Karamyan, 2008).

1.2.1.7.5 Memory: Ang IV through its receptor AT₄R may be involved in memory recall. The exact mechanism is not yet known but it is possible that Ang IV interacts with the dopamine receptors in brain regions such as hippocampus. AT₄R is an insulin-regulated aminopeptidase (IRAP) which can hydrolyze oxytocin. Oxytocin participates in memory recall and social memory. Some data suggest that angiotensin IV binding to AT₄R inhibits IRAP degrading effects on oxytocin (Gard, 2008).

(Zaman et al., 2002, Ferreira et al., 2007, Nguyen Dinh Cat and Touyz, 2011).

1.2.2 RAS in atherosclerosis

Several strategies have been applied to elucidate different aspects of the RAS and its main functional effector Ang II in atherosclerosis including the infusion of Ang II to animal models of atherosclerosis, Ang II treatment in cellular models, the overexpression or the inactivation of RAS genes in animal models and the application of RAS inhibitors in animal models and in humans.

1.2.2.1 Ang II effects in vitro

Ang II increases de novo cholesterol biosynthesis in macrophages. Intraperitoneal injection of Ang II increases cholesterol synthesis at a cellular level in peritoneal macrophages of the *ApoE*^{-/-} mice (Keidar et al., 1999). In vitro experiments also confirm cholesterol biosynthesis in monocyte-derived macrophages from human and BALB/c mice and also the RAW-7, macrophage cell line. This is an AT₁R-mediated effect. Ang II increases 3-hydroxy-3-methyl glutaryl Coenzyme A (HMG CoA) reductase mRNA levels, the rate limiting enzyme in cholesterol biosynthesis (Keidar et al., 1999).

Foam cells are one of the hall marks of atherosclerosis and are formed by uptake of modified LDL. One of the physiologically relevant modifications is oxidation. Ang II is able to increase 12- and 15-lipoxygenase and NADPH oxidase activity through AT₁R. Enhanced enzymes activity increases the LDL modification and its chemotactic activity which exaggerate foam cell formation (Keidar et al., 1995, Scheidegger et al., 1997). Foam cell formation is the interplay between lipid uptake and lipid efflux. Ang II modulates expression of several genes which eventually enhances foam cell formation.

Ang II augments the expression of some ECM proteins, like syndecan, which enhance LDL retention and oxidation. Ang II also increases the expression of scavenger receptors involved in uptake of modified LDL such as CD36. Increased modified LDL in macrophage milieu and elevated number of scavenger receptors promote foam cell formation (Keidar and Attias, 1997, Khalil et al., 2004). Moreover, Ang II decreases lipid efflux. ABCA1 is one of the main transporters of lipid efflux in cells. Ang II decreases ABCA1 expression in a dose-dependent manner in peritoneal macrophages from C57BLmice. This is an AT₁R-dependent effect which is blocked by an AT₁R blocker, losartan, but not by the AT₂R inhibitor, PD-123319. Ang II induces c-fos-related binding protein to ABCA1 promoter and suppresses its expression (Kaplan et al., 2002, Takata et al., 2005a).

Foam cell formation is not only restricted to macrophages. Endothelial cells and SMCs can also become foam cells. Ang II enhances foam cell formation in SMCs by upregulating the expression of the low density lipoprotein- related receptor (LRP). LRP, a member of the LDL receptor protein family, is involved in uptake of modified LDL. Losartan or LRP antagonists reduce Ang II- induced foam cell formation (Sendra et al., 2008). In endothelial cells, Ang II increases lectin -like receptor (LOX-1) expression and enhances foam cell formation (Li et al., 1999b). Scavenger receptor B-I (SR-BI) is involved in lipid efflux. Ang II through PI3-kinase/Akt/FoxO1 suppresses SR-BI expression in human umbilical vein endothelial cells (HUVEC), which again facilitates foam cell formation (Yu et al., 2007).

Inflammation is an important modulator of atherosclerosis. Ang II enhances inflammatory cascades in different cell types. In endothelial cells and VSMCs, Ang II

through AT₁R activates NF-κB transcription factor and triggers inflammatory gene expression. AT₂R is also involved in Ang II- induced pro-inflammatory cascades in VSMCs since an AT₂R blocker reduces NF-κB activation in AT₁R knockout VSMCs (Ruiz-Ortega et al., 2001).

1.2.2.2 Ang II administration to animal models

Subcutaneous infusion of Ang II at 500 ng/kg / min in wild type mice on an atherogenic or normal diet for 12 weeks increases blood pressure by 30-45 mm Hg but does not induce atherosclerosis development (Weiss et al., 2001). However, subcutaneous infusion of Ang II 500-1000 ng/kg/ min into 6 month-old *ApoE*^{-/-} mice for one month does not affect tail cuff measured blood pressure but increases the severity of atherosclerotic lesions. Ang II- infused animals have larger established lesions with a necrotic core and SMCs. They also develop more newly formed lesions in the thoracic aorta (Daugherty et al., 2000). Enhanced atherosclerosis development is independent of Ang II blood pressure- increasing effects. Subcutaneous administration of Ang II at a dose of 500ng / kg/ min for 8 weeks in *ApoE*^{-/-} mice both on a normal chow diet and a high fat diet increases atherosclerotic lesion size in the ascending and descending aorta; however, administration of norepinephrine increases blood pressure 30-40 mmHg, but does not have such an effect on the atherosclerotic lesion size (Weiss et al., 2001). Ang II has differential effects on vessels from different lesions. Though lesion development in the aortic root is more intense in mice, Ang II accelerates lesion development in the descending aorta more than in the aortic root in *ApoE*^{-/-} mice (Zhou et al., 2005). Atherogenic effects of Ang II are not through induction of the aldosterone pathway since

infusion of aldosterone does not accelerate atherosclerosis in *ApoE*^{-/-} mice. Moreover, Ang II effects are not attenuated by co-administration of Ang II and spironolactone, an aldosterone receptor blocker (Cassis et al., 2005).

Cell aging, senescence, and vascular disease are related. Arteries with a lower degree of telomere shortening, a hallmark of senescence, are resistant to atherosclerosis. Ang II enhances vascular senescence. Subcutaneous infusion of Ang II at dose of 1000 ng/kg/min for 4 weeks, which increases blood pressure by 30% in *ApoE*^{-/-} mice, enhances senescence in SMC by sustained induction of ERK1/2. ERK1/2 induces cell cycle regulator p53 which in turn increases cyclin- dependent kinase inhibitor p21. P21 inhibits cell division and keeps the cells in G phase which makes cells senescent. Mediators of senescence cyclin-dependent kinase inhibitor and p53 are also mediator of vascular inflammation. In P21-deficient *ApoE*^{-/-} mice Ang II-induced SMC senescence is decreased (Kunieda et al., 2006, Kovacic et al., 2011).

Ang II is involved in progression and destabilization of atherosclerosis through an enhanced inflammatory state. Monocytes are necessary for Ang II- induced enhanced atherosclerosis. Osteopetrotic mice are deficient in macrophage colony stimulating factor (M-CSF). Infusion of Ang II at 1000 ng/kg /min in osteopetrotic mice on *ApoE*^{-/-} background reduces the size of atherosclerotic lesions compared to their wild type cohorts (Babamusta et al., 2006). Many inflammatory effects of Ang II are triggered through MCP-1, since MCP-1 deficiency attenuates the expression of other inflammatory molecules (Ni et al., 2004). Ang II administration also increases the pro-inflammatory cytokines and chemokines in different cell types present in atherosclerotic lesions. In

macrophages and fibroblasts of *Ldlr*^{-/-} mice, Ang II increases cytokines such as interleukin-6 (IL-6) by induction of Jak-STAT3 pathway (Recinos et al., 2007).

Disintegration of ECM protein destabilizes atherosclerotic lesions. Various proteases are involved in degradation of ECM proteins. Increased inflammation exaggerates ECM hydrolysis due to the elevated presence of proteases. One of the important families of proteases that are involved in ECM degradation is MMPs. Ang II increases various proteases which in turn activate other enzymes like MMPs and cytokines. One example of this type of Ang II effects on atherosclerosis is the increase in calpain, a cysteine neutral protease that activates MMP-12 and IL-6. Ang II can also increase expression of various MMPs including MMP-1, 2, 8, 9 and 13 in SMCs. These Ang II effects are ROS-dependent and are mostly mediated through NF- κ B and AP1 transcription factors (Browatzki et al., 2005, Luchtefeld et al., 2005, Cheng et al., 2009, Subramanian et al., 2012).

1.2.2.3 RAS activity in atherosclerosis

Hypercholesterolemia increases plasma angiotensinogen and Ang II in *Ldlr*^{-/-} after 12 weeks on a high fat diet. Ang II is the predominant peptide in plasma but other angiotensin peptides including Ang III, IV and 3-8 and 4-8 are also significantly increased. At the end of study, Ang II in plasma from hypercholesterolemic mice is increased around 5 times, from 100pg/ml to 500pg/ml. In plasma from normocholesterolemic mice Ang III, IV, 4-8 and 5-8 are not detectable but in hypercholesterolemic mice they reach levels around 100,300, 75 and 50 pg/ml, respectively (Daugherty et al., 2004). Hypercholesterolemia does not modify ACE

activity in plasma of 10- week cholesterol fed rabbits (Hoshida et al., 1997). The exact mechanism of the increase in angiotensinogen is yet unknown. Certain cholesterol metabolites increase angiotensinogen expression through induction of PPAR α in kidney and other cell types but not in hepatic cells (Shimamoto et al., 2004b, Shimamoto et al., 2004a, Putnam et al., 2012). Immunohistochemical analysis detected components of RAS including renin, angiotensinogen, ACE, AT₁R and angiotensin peptides in atherosclerotic lesions and vessel walls from humans, mice and rabbits. Most of the staining co-localized with macrophages and inflammatory cells inside the atherosclerotic lesions (Diet et al., 1996, Fukuhara et al., 2000, Daugherty et al., 2004, Johansson et al., 2008, Ohishi et al., 2010). In hypercholesterolemic New Zealand White rabbits which develop atherosclerosis, autoradiographic analysis shows the expression of AT₁R in aortic media and to some extent aortic intima is increased by five-fold. The enhanced AT₁R expression in atherosclerotic aorta is both at the protein and mRNA level (Yang et al., 1998). Whereas ACE expression in normal artery is mostly confined to endothelial cells, hypercellular lesions and atheromatous plaques from human coronary artery show ACE expression in SMCs and macrophages. There is a strong association between atherosclerotic plaque stability and enhanced ACE expression in lesions. Lesions with a thick fibrotic cap have lower ACE expression even in their endothelial cells (Ohishi et al., 1997).

RAS in macrophages and dendritic cells is also upregulated. Monocyte to macrophage differentiation, which occurs in atherosclerotic lesions upregulates RAS. Renin is only expressed in macrophages. Monocyte differentiation does not change angiotensinogen expression but it increases ACE activity and AT₁R and AT₂R expression by 3- and 6-

fold, respectively (Okamura et al., 1992). Dendritic cells differentiated from peripheral blood monocytes of patients having coronary artery disease express more ACE and AT₁R than those from normal healthy individuals. This enhanced expression correlates with increased T cell recruitment and progression of disease (Sun et al., 2009).

1.2.2.4 Overexpression of RAS and atherosclerosis

Whereas wild type mice on a high fat diet do not show atherosclerotic lesions, transgenic mice on a C57BL/6J background expressing human renin and angiotensinogen are hypertensive and when placed on an atherogenic diet, they develop atherosclerosis. This suggests an interaction between Ang II and hypercholesterolemia in induction of atherosclerosis (Sugiyama et al., 1997).

Overexpression of AT₂R shows an anti-atherogenic role for AT₂R. AT₂R transgenic *Ldlr*^{-/-} mice have reduced atherosclerotic lesions relative to their wild type littermates by minimizing the increase in NADPH oxidase and NF- κ B which results in reduced lectin-like oxidized-LDL receptor expression on endothelial cells (Hu et al., 2008).

Overexpression of ACE2 in hypercholesterolemic rabbits confirms the atheroprotective effect of ACE2. These rabbits have more stable atherosclerotic lesions (Dong et al., 2008). ACE2 overexpression also has anti-inflammatory effects through reduction of MCP-1 expression in monocytes (Guo et al., 2008). ACE2 by modulation of NADPH oxidase subunits decreases ROS and as a result attenuates anti-inflammatory state in atherosclerosis (Lovren et al., 2008)

1.2.2.5 Inhibition of RAS activity and atherosclerosis

1.2.2.5.1 ACE

One of the first experiments showing the effect of RAS inhibitors in attenuation of atherosclerosis development was done in cynomolgus monkeys. Gross morphological analysis of intima and lumen area together with total tissue cholesterol show captopril-treated monkeys have less atherosclerotic lesions relative to placebo-treated animals (Aberg and Ferrer, 1990). Captopril also decreases lesion development in Watanabe heritable hyperlipidemic rabbits. The captopril effect is more prominent in the descending aorta. Attenuation of atherosclerosis development is accompanied by a decrease in lesion cellularity and increase in ECM content (Chobanian et al., 1990). The beneficial effects of several ACE inhibitors in various other atherosclerotic animal models like mini pig and mouse have been reported (Andersson et al., 1994, Hayek et al., 1999b). Some of these studies provide mechanistic insights to the anti-atherosclerotic effects of ACE inhibitors. ACE inhibitors attenuate atherosclerosis through reducing inflammatory cytokine expression in SMCs, tissue factor in monocyte and macrophages, elastin degradation, and macrophage recruitment (Kranzhofer et al., 1999, Napoleone et al., 2000, Wojakowski et al., 2001, Schmeisser et al., 2004, Abd Alla et al., 2010).

ACE-deficient mice on an *ApoE*^{-/-} background have also been used to assess the role of ACE in atherosclerosis. Total body ACE heterozygous mice have reduced oxidative stress and attenuated atherosclerotic lesion development relative to their wild type littermates. The extent of reduction in atherosclerotic lesion and oxidative stress is even greater enhanced in their ACE knockout littermates (Hayek et al., 2003).

Locally produced ACE in the vascular wall and inflammatory cells appears not to play a role in development of atherosclerosis; bone marrow transplantation into *ApoE*^{-/-} mice with restricted kidney and liver ACE expression have a similar extent of atherosclerotic lesions as their ACE wild type *ApoE*^{-/-} littermates (Weiss et al., 2010).

1.2.2.5.2 ACE-2

ACE-2 opposes the ACE function by reducing Ang II and formation of angiotensin 1-7. ACE-2 deficient mice show increased inflammatory markers such as Il-6, MCP-1 and VCAM-1. Crossing these mice with *ApoE*^{-/-} mice shows that ACE2 deficiency enhances the pro-inflammatory state and is pro-atherogenic. Consistent with *ApoE*^{-/-} / *ACE2*^{-/-} mice, *ACE2*^{-/-} / *Ldlr*^{-/-} mice also have enhanced atherosclerosis relative to their *Ldlr*^{-/-} littermates (Thomas et al., 2010, Thatcher et al., 2011). Transplantation of ACE-2-deficient bone marrow cells into *Ldlr*^{-/-} mice increases the monocytes adhesion to the endothelium and accelerates atherosclerosis (Thatcher et al., 2011).

1.2.2.5.3 Angiotensin receptors

AT₁R blockers also attenuate lesion progression in *ApoE*^{-/-} mice. Losartan limits lesion progression by decreasing lipid oxidation; malondialdehyde LDL, a marker lipid oxidation is reduced by 55% (Keidar et al., 1997). Fatty streak lesions in losartan treated monkeys are confined to the branching areas of aorta and they do not show elastic lamina disruption (Strawn et al., 2000). Various AT₁R blockers in atherosclerotic animals decrease the inflammatory state of atherosclerotic lesions. Specifically they decrease macrophage content of lesions and reduce lesion size and increase lesion

stability by elevation of the collagen content (Strawn and Ferrario, 2008, Fukuda et al., 2010). Blocking AT₁R also attenuates inflammatory cascades by down-regulation of inflammatory receptors such as Toll-like receptors (TLR) 2 and 4, members of a family of pattern recognition receptors that can influence inflammatory cytokine expression in atherosclerosis (Dasu et al., 2009).

Several AT₁R blockers reduce the inflammatory state and ROS formation in atherosclerosis by decreasing NADPH oxidase subunits, i.e p22phox and p47phox expression (Li et al., 2004, Takaya et al., 2006). They suppress atherosclerosis in *ApoE*^{-/-} mice by attenuating oxidative stress and inflammatory pathways through reducing the active molecular mediators such as signal transducer and activator of transcription 3 (STAT3), ERK1/2, NF-κB and JNK2. As a result of this attenuation, the expression of inflammatory genes like MCP-1 is reduced (Doran et al., 2007, Yao et al., 2010). Besides ROS and enzymes related to ROS formation, other enzymes like secretory phospholipase A2 are also involved in lipid oxidation. Losartan is able to block this type of lipid modification in atherosclerosis (Luchtefeld et al., 2007).

Losartan improves Ang II-induced plaque instability in *ApoE*^{-/-} mice by reducing expression of cathepsin S which decreases ECM degradation (Sasaki et al., 2010). One of the features of unstable atherosclerotic lesions is activation of platelets. Losartan reduces platelet activation through the thromboxane pathway (Schwemmer et al., 2001). Azilsartan decreases plasmin-activating inhibitor (PAI) in SMC, which promotes lesion stability (French et al., 2011). Ang II induces c-fos and MAPK in SMCs to promote their growth and migration. SMC migration and growth promote formation of more advanced plaques. Irbesartan completely blocked DNA synthesis, migration of VSMCs (Xi et al.,

1999). As mentioned previously, calcified nodules in atherosclerotic lesions accelerate thrombosis which is one feature of unstable lesions. Olmesartan, markedly reduces calcification in hypercholesterolemic rabbits by inhibiting markers of osteoblast-like cells such as bone morphogenetic protein 2, osteocalcin in SMCs (Arishiro et al., 2007, Armstrong et al., 2011).

AT₁R deficiency in *ApoE*^{-/-} mice confirms the results of AT₁R blockers. These double knockout mice have reduced atherosclerotic lesions, endothelial dysfunction and oxidative stress in their vascular wall (Wassmann et al., 2004). Similar results are obtained from *Ldlr*^{-/-} / *AT_{1a}R*^{-/-} mice; these mice have attenuated atherosclerosis (Daugherty et al., 2004). Long term AT₁R deficiency completely abrogates the age-related increase in the atherosclerotic lesion size. Lesion size in 25-week old and 60-week old AT₁R deficient *ApoE*^{-/-} mice is similar (Eto et al., 2008).

There is no conclusive evidence about the role of AT₁R in bone marrow cells in promotion of atherosclerosis. *Ldlr*^{-/-} chimeric mice with AT₁R deficiency in total body, irrespective of the presence or absence of AT₁R in the bone marrow of donors are protected from Ang II- induced atherosclerosis (Cassis et al., 2007). Another study by Lu et al also fails to show any effects for AT₁R presence in bone marrow cells in development of atherosclerosis in *Ldlr*^{-/-} mice (Lu et al., 2008a). In contrast to these results, mice expressing human renin and angiotensinogen which have AT₁R deficiency in bone marrow have enhanced atherosclerosis and accelerated lethality. It seems that in this model, RAS pathway in macrophage is athero-protective (Kato et al., 2008). AT₁R deficiency in bone marrow cells does not influence the extent of atherosclerotic lesion size in Ang II- induced atherosclerosis in *ApoE*^{-/-} mice but AT₁R deficiency in vascular

wall suppresses atherosclerosis development. Whereas AT₁R deficiency in bone marrow also does not affect the expression of important inflammatory factors involved in the acceleration of atherosclerosis such as MCP-1 and VCAM-1, AT₁R deficiency in the vascular wall impairs MCP-1 and VCAM-1 expression in vessel wall. In the *ApoE*^{-/-} model, the presence or absence of AT₁R in vascular wall is crucial for Ang II-induced atherosclerosis development (Koga et al., 2008).

In terms of elucidation of the AT₂R role in atherosclerosis, there are contradictory results, which are listed below. AT₂R is increased in atherosclerotic lesions but the AT₂R antagonist, PD123319, either enhances atherosclerosis suggesting AT₂R has anti-atherogenic properties (Daugherty et al., 2001) or does not affect atherosclerosis development (Johansson et al., 2005). Studies with AT₂R knockout mice also do not clarify the role of AT₂R in development of atherosclerosis. *AT₂R*^{-/-} mice on an *Ldlr*^{-/-} background develop the same extent of lesions as their wild type littermates on an *Ldlr*^{-/-} background (Daugherty et al., 2004). In another study, *AT₂R*^{-/-} *ApoE*^{-/-} mice have exaggerated atherosclerosis relative their *AT₂R*^{+/+} *ApoE*^{-/-} littermates after 10 weeks of a high fat diet. The enhanced atherosclerosis is partly due to increased oxidative stress and expression of NADPH oxidase which is blocked by valsartan therapy (Iwai et al., 2005). In contrast to this result, another study reported that AT₂R deficiency in *ApoE*^{-/-} mice does not affect lesion size; however, it increases the lesion cellularity due to AT₂R pro-apoptotic effects (Sales et al., 2005).

Stimulation of AT₂R with Ang II in *AT₁R*^{-/-} *ApoE*^{-/-} mice reduced atherosclerosis whereas its blockade enhanced atherosclerosis (Tiyerili et al., 2012b).

1.2.2.5.4 Renin

Only a few studies assessed the anti-atherosclerotic effects of renin inhibitors. Piperidine, a non-peptide renin inhibitor showed similar inhibitory effect to those of AT₁R blockers and ACE inhibitors on transgenic atherosclerotic mice expressing human renin and angiotensinogen genes (Major et al, 2008). Aliskiren administration to *ApoE*^{-/-} mice reduces atherosclerosis by decreasing NADPH oxidase activity (Poss et al., 2010). Aliskiren administration in the atherosclerotic mouse model, APOE*3Leiden-CETP transgenic mice, reduces atherosclerotic lesion size, necrotic core size, T-cell and macrophage content. Renin inhibition also increases collagen content in these mice. Aliskiren attenuates atherosclerosis possibly by a T-cell- mediated mechanism in this mouse model (Kuhnast et al., 2012).

Renin deficiency in bone marrow of *LDLr*^{-/-} mice attenuates atherosclerosis development whereas AT₁R deficiency did not affect atherosclerosis progression, suggesting there is not an autocrine /paracrine Ang II pathway in monocytes in mice (Lu et al., 2008a).

1.3 RAS alternative pathways

Various in vitro studies showed that the vasoconstrictive action of tissue lysates incubated with angiotensinogen or angiotensin I is not completely blocked by ACE inhibitors and that AT₁R antagonists are more effective than ACE inhibitors. These studies imply that enzymes other than ACE are involved in Ang II generation (Balcells et al., 1997, Takai et al., 2001). Several enzymes that are able of generation of Ang II independently of renin and ACE have been described, and may potentially participate in an alternative RAS pathway. These enzymes either produce Ang II directly from angiotensinogen or convert angiotensin I to Ang II.

Tonin extracted from rat submaxillary gland, was the first identified enzyme which can produce Ang II from Ang I and angiotensinogen (Grise et al., 1981, Schiller et al., 1976). Trypsin, tissue kallikrein and cathepsin G also generate Ang II directly from angiotensinogen (Arakawa and Maruta, 1980, Arakawa et al., 1976, Wintroub et al., 1981) and chymase, elastase and cathepsin G can convert Ang I to Ang II (Paula et al., 1998, Urata et al., 1990, Klickstein et al., 1982). Most of these enzymes are serine proteases. Based on their sensitivities to inhibitors, they are divided into two groups of chymostatin-sensitive e.g. chymase and elastase and aprotonin-sensitive e.g. trypsin (Becari et al., 2011). Another enzyme which could participate in a RAS alternative pathway, cathepsin D, has renin-like activity and produces Ang I from angiotensinogen (Morris and Reid, 1978).

The Ang II-generating capacity of these enzymes has been demonstrated primarily *in vitro* and the significance of these enzymes in production of Ang II *in vivo* in physiological and pathological states is not clear (Becari et al., 2011). Chymase the most studied has two isoforms α and β . The α -chymase is an Ang II-forming enzyme and β -chymase is an Ang II-degrading enzyme (Caughey et al., 2000). In hearts from humans, chymase is present in mast cells, endothelial cells, and mesenchymal cells, but in the rodent heart it is only located in mast cells (Urata et al., 1993, Huntley et al., 1990). *In vitro* studies suggest chymase is responsible for up to 80% of Ang II production in heart and arterial lysates from humans and dogs (Urata et al., 1990, Balcells et al., 1996), whereas, in rodents almost all of the Ang II-forming ability of heart lysates is blocked by ACE inhibitors (Okunishi et al., 1993). Variability in the relative contribution of

classical RAS and alternative pathways to Ang II generation may reflect differences in isoform, and cell type-specific gene expression (Becari et al., 2011).

Inhibition of chymase release by mast stabilizer Tranilast in hypertensive rats reduces hypertrophy and fibrosis. However this inhibition does not specify the exact role of chymase in Ang II generation and its consequences since mast cell stabilization also reduces cytokine secretion (Umemura et al., 1998). In hamsters, the specific chymase inhibitor, 4-[1-(naphthylmethyl) benzimidazol-2-ylthio] butanoic acid (TEI-E548), decreases sudden cardiac death, hypertrophy and ventricular pressure after infarction (Hoshino et al., 2003). Oral administration of a chymase inhibitor, BCEAB, in cardiomyopathic hamsters does not reduce hypertrophy but it decreases collagen formation (Takai et al., 2003). In a dog model of heart failure a chymase inhibitor also reduces collagen deposition in left ventricle (Matsumoto et al., 2003) .

Alternative pathways are unlikely to have a major role in the generation of Ang II in the circulation due to the presence in plasma of large and small molecular weight serine protease inhibitors like α 2-macroglobulin. Therefore, these enzymes would presumably function primarily in tissue and more specifically in intracellular Ang II generation. Whereas in the physiological state, 90% of Ang II formation is blocked by ACE inhibitors, in SMC from diabetic rats the importance of non-ACE enzymes is elevated and an ACE inhibitor is able to block only 20% of Ang II generation in these cells (Lavrentyev et al., 2007, Kumar and Boim, 2009).

1.3.1 Cathepsin G

Wintroub et al purified a neutral protease that generates vasoactive Ang II from angiotensinogen, and they subsequently identified the neutral peptidase as cathepsin G (Tonnesen et al., 1982, Wintroub et al., 1981, Wintroub et al., 1984). Cathepsin G at physiological pH hydrolyzes Ang I to Ang II without further degradation of Ang II (Klickstein et al., 1982). Cathepsin G is a serine protease which converts both angiotensin I and angiotensinogen to Ang II in vitro. However, studies to verify the importance of these activities in vivo have not yet been done. Since a major portion of my PhD research has been devoted to a determination of the role of cathepsin G in atherogenesis and Ang II formation in a mouse model of atherosclerosis , over the next several pages, I will describe in details the biochemistry and biology of cathepsin G.

Human cathepsin G is a 225-aminoacid cationic, neutral serine protease. The cathepsin G gene is located on chromosome 14 and includes 5 exons. The cathepsin G gene has a TATA box , PU.1 binding sequence and a GC rich enhancer in its 5'untranslated region (UTR) (Korkmaz et al., 2008). Cathepsin G is synthesized in neutrophils, monocytes and mast cells. Cathepsin G has a propeptide which is cleaved by a cysteine protease, dipeptidyl peptidase (cathepsin C), to yield the mature enzyme. The mature enzyme is kept in its active form in azurophilic granules of neutrophils and in peroxidase-sensitive granules of macrophages (Korkmaz et al., 2008). Karlsson et al. reported the expression of cathepsin G in adipose tissue (Karlsson et al., 1998). In vascular disease, SMCs transform to a synthetic phenotype and this is accompanied by expression of cathepsin G (Hu et al., 2003). In human atheroma plaques from coronary arteries cathepsin G mRNA expression is 2-fold higher than in the normal vessel wall. Whereas in stage I, II and III

atherosclerotic lesions cathepsin G protein is located in SMCs in both media and neointima layers, in more advanced lesions (atheroma plaques) cathepsin G protein is associated with both SMCs and macrophages and there is an enhanced cathepsin G staining in the neointimal layer (Legedz et al., 2004a).

Cathepsin G has a broad range of catalytic activities and a part of this promiscuity is due to flexibility in the active site as a result of the absence of a disulfide bond. Cathepsin G can hydrolyze the bond between any hydrophobic large amino acids such as Met and Phe at P1 site and small amino acids like Ser at the P'1 site which preferably able to make hydrogen bond with Ser 40 of the active site (figure 1). However, there is a marked preference for the presence of Pro at the P2 site, there is wide range of flexibility for amino acid choices at P2', P3 and P3' sites (Korkmaz et al., 2008). Therefore, cathepsin G has multiple potential substrates in vivo including Ang I and angiotensinogen that could be important in atherogenesis.

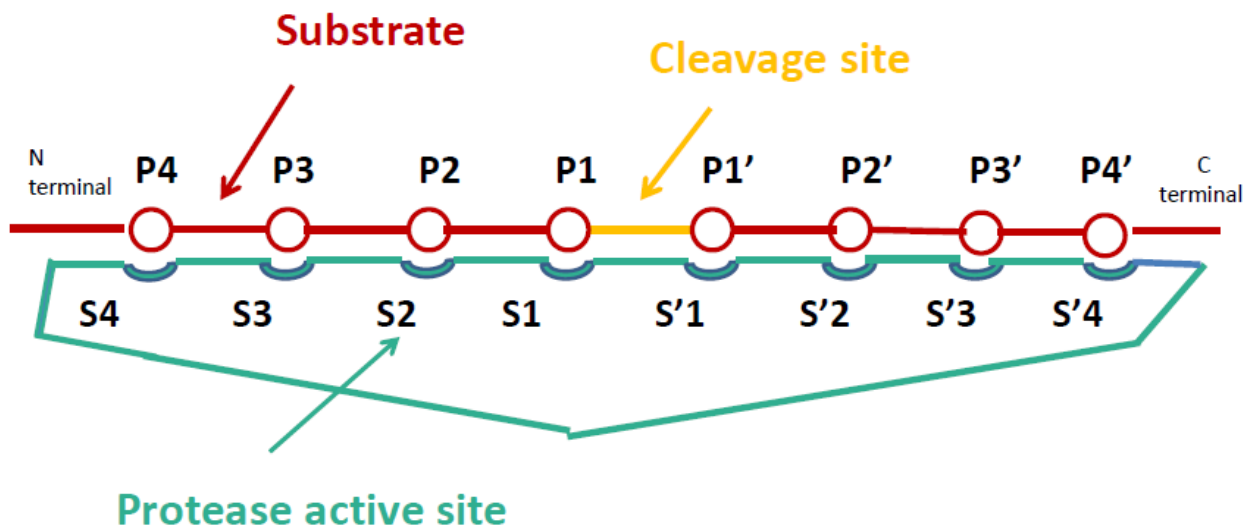


Figure1-1 The nomenclature of the substrate specificity for proteases

Amino acids in the N-terminal side of the cleavage site (the bond to be cleaved) are named P_1 , P_2 , P_3 , and so forth. Likewise, amino acids on the C-terminal of cleavage site are labeled P_1' , P_2' , P_3' , and so forth. The matching sites on the enzyme are known as S_1 , S_2 or S_1' , S_2' , and so forth (Hedstrom, 2002).

1.3.1.1 Cathepsin G and Ang II formation

Activated inflammatory cells express cathepsin G on their membrane and membrane-bound cathepsin G is resistant to plasma inhibitors such as α_1 -antichymotrypsin. The specificity constant (k_{cat}/K_m) which reflects the propensity of an enzyme to catalyze a substrate for membrane bound human cathepsin G for Ang I is higher than for any of its other known substrates, ($6.8 \times 10^3 M^{-1} S^{-1}$). However, it is significantly lower than the

specificity constant of either ACE and or human heart chymase for Ang I ($1.9 \times 10^5 \text{ M}^{-1} \text{ S}^{-1}$ and $2.7 \times 10^6 \text{ M}^{-1} \text{ S}^{-1}$, respectively) (Owen et al., 1995, Owen and Campbell, 1998b). Klickstein et al (1982) reported that cathepsin G does not degrade Ang II but Ramaha and Patston (2002) used higher concentrations of purified cathepsin G with molar ratio of 2:1 relative to angiotensinogen or angiotensin I and showed that cathepsin G is able to degrade Ang II. They concluded that cathepsin G not only produces Ang II but also by degrading Ang II controls neutrophil chemotaxis. The only study that supports the role of cathepsin G in formation of Ang II in live cells showed that suppression of cathepsin G expression in synthetic smooth muscle cells by cathepsin G antisense oligonucleotides decreases Ang II in conditioned media by one-half (Hu et al., 2003).

1.3.1.2 Extracellular matrix degradation and its consequences

Cathepsin G activity can cause the degradation of various ECM molecules either directly or indirectly. Cathepsin G hydrolyzes glycoproteins such as elastin, fibronectin, thrombospondin and von Willebrand factor (Bonney and Legrand, 2000a). Cathepsin G also increases degradation of elastin enhancing elastase activity (Boudier et al., 1981a). Collagen is degraded by collaboration of collagenase and cathepsin G. Cathepsin G activates collagenase which can hydrolyze and denature collagen that, in turn, can be further degraded by cathepsin G (Capodici and Berg, 1989). Cathepsin G can indirectly degrade ECM by activating MMPs such as MMP-1, MMP-9 and MMP-10. MMP-10 itself also activates MMP-1 (Saunders et al., 2005a, Han et al., 2008). Cathepsin G in cardiomyocytes induces apoptosis as well by activating protein phosphatase, SHP2, which dephosphorylates focal adhesion kinases (FAK) and results in down-regulation of the pro-survival signal, protein kinase B (AKT) (Rafiq et al., 2008b). Cathepsin G

cleaves ICAM-1 and VCAM-1 which facilitates migration of inflammatory cells (Robledo et al., 2003, Levesque et al., 2001) and contributes to sustained neutrophil action and cell and tissue damage after ischemia and reperfusion injury (Shimoda et al., 2007).

1.3.1.3 Inflammatory cascades

1.3.1.3.1 Inhibitors

Cathepsin G is involved in both pro and anti-inflammatory actions. Due to its broad range of catalytic activity, it is able to activate some cytokines and inactivate others. Cathepsin G enhances neutrophil and monocyte chemotaxis by removing the N-terminal pro-peptide from CCL15 resulting in its activation (Richter et al., 2005b). Hydrolytic cleavage of connective tissue activating factor by cathepsin G in the vicinity of platelets forms neutrophil activating peptide 2 which also enhances neutrophil chemotaxis (Car et al., 1991, Brandt et al., 1991, Cohen et al., 1992). Another pro-inflammatory function of cathepsin G is maturation of Il-33, which drives cytokine release from various inflammatory cells (Lefrancais et al., 2012).

On the other hand, the hydrolytic activity of cathepsin G degrades some cytokines which might be anti-inflammatory or pro-inflammatory. Membrane bound cathepsin G not only is able to inactivate IL-6 by proteolytic cleavage but also promotes the shedding of the cell surface Il-6 receptor (Laouar et al., 1993, Bank et al., 1999a, Bank et al., 1999b). Cathepsin G proteolysis of Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) or CCL5 renders it less efficient following binding to its receptor, CCR5. Therefore, due to inactivation of T-cell chemotactic factor there would be fewer

T-cell in the inflammation milieu (Lim et al., 2006). Cathepsin G can temporarily suppress T-cell cytotoxicity by cleaving of T- lymphocyte cell antigens CD2, CD4 and CD8 (Doring et al., 1995). An in vitro study by Scuderi et al.(1991) suggests that cathepsin G degrades recombinant TNF α into 11 and 7.6 KD fragment without biological activity. A more recent study by Mezyk-Kopec et al. (2005) demonstrates that cathepsin G cleaves membrane-bound TNF α from fibroblast cells expressing TNF α to yield active soluble TNF α that can potentially induce cytotoxicity and enhance iNOS activity (Scuderi et al., 1991). The anti- inflammatory function of cathepsin G is not restricted to the inactivation of cytokines, as it can also inhibit adhesion of neutrophils to endothelial cells (Renesto et al., 1996).

1-3-1-3-2 Studies with cathepsin G- deficient models

Due to similarity in structure, catalytic activity and storage organelle many of cathepsin G activities are accompanied by elastase activity and a genetic model of cathepsin G/ elastase double knockout has been developed. In this model, Il-18 interferon γ - inducing activity is enhanced due to reduced proteolytic cleavage of Il-18 (Robertson et al., 2006).

Cathepsin G and elastase-deficient mice are protected against collagen autoantibody-induced arthritis at early stages of disease and they have fewer neutrophils in their joints but when the inflammation wanes, they have the same degree of joint damage (Adkison et al., 2002, Schalkwijk et al., 1988). In a mouse model of nephritis, cathepsin G / elastase deficiency reduces glomerular basement membrane damage and albuminuria (Schrijver et al., 1989). Lipopolysaccharide (LPS) from gram-negative bacteria by induction of an inflammatory cascade involving cytokine and complement activation

triggers endotoxin shock. Cathepsin G / elastase- deficient mice are resistant to the lethal effects of endotoxin shock (Tkalcevic et al., 2000).

Although the presence of cathepsin G and elastase accelerates some inflammatory diseases, their presence is protective in mice infected with bacteria such as *Streptococcus pneumoniae* and *Mycobacterium bovi*. The protective role of neutrophil serine proteases in lung infection is due enhanced intra-alveolar immunoreactivity which limits the bacterial outgrowth (Hahn et al., 2011, Steinwede et al., 2012).

Some studies used cathepsin G single-knockout mice to assess the role of cathepsin G *in vivo* more specifically. Cathepsin G- deficient mice have normal hematopoiesis and blood clotting and neutrophils from these mice have normal chemotactic responses, phagocytosis and superoxide production (MacIvor et al., 1999) Cathepsin G is involved in processing of soluble mediators of neutrophil chemotaxis, and deficient mice also have attenuated wound- healing at the early stages of wound formation (Abbott et al., 1998).

1.4Rationale

Lipid- laden macrophages are one of the primary and main components of atherosclerotic lesions .Ang II is expressed in macrophages and it is one of the most important modulator of atherosclerosis. Uptake of cholesterol and modified fatty acids from lipoproteins activates various nuclear factors such as PPAR α , HIF1- α and NF- κ B which can greatly alter the profile of gene expression between foam cells and macrophages (section 1-1-4). No previous study assessed how the changes in gene expression after foam cell formation would affect the Ang II levels. We propose that the transformation of macrophages to

foam cells leads to increased production of endogenous Ang II. Exogenous Ang II enhances the progression of atherosclerosis in different ways, including promoting foam cell formation (section 1-2-2). The importance of locally produced Ang II especially in macrophages has been shown by reconstitution of irradiated *Ldlr*^{-/-} mice with bone marrow from renin-deficient donors (Lu et al., 2008a). Therefore, we propose that uptake of modified LDL by macrophages leads an increase in Ang II which, in turn, promotes further modified LDL uptake and intracellular cholesterol accumulation leading to a proatherogenic vicious cycle. Several enzymes other than renin and ACE are able to generate Ang II in vitro including cathepsin G which can produce AngII both from Ang I and from angiotensinogen. While the specificity constant of cathepsin G for Ang I is not as high as that of ACE , it can nevertheless potentially contribute to Ang II formation in vivo. Cathepsin G is expressed mainly in inflammatory cells such as neutrophils and macrophages that are present in atherosclerotic lesions (section 1-3-1) and cathepsin G has higher expression in human atheroma plaques relative to healthy carotid artery (Legedz et al., 2004a). Cathepsin G is an enzyme with broad catalytic activity able to degrade ECM and modulate inflammatory cytokines and chemokines (sections 1-3-1-2 and 1-3-1-3). To our knowledge the importance of cathepsin G in generation of AngII has not been assessed in vivo. We therefore proposed that cathepsin G could influence the development and progression of atherosclerosis by contributing to Ang II generation and by modulation of ECM and cytokines.

1.5 Hypothesis

1. Ang II production is increased during foam cell formation.
2. Cathepsin G is involved in the production of the Ang II in foam cells and progression of atherosclerosis.
3. Increased endogenous Ang II increases foam cell lipid loading.

1.6 Objectives

- 1.6.1 To compare RAS components and Ang II production as a function of foam cell formation.
- 1.6.2 To determine the effect of cathepsin G on Ang II production in macrophages and foam cells.
- 1.6.3 To define the role of the endogenous renin angiotensin system in foam cells formation.
- 1.6.4 To determine the role of cathepsin G in the development and progression of diet-induced atherosclerosis by comparing cathepsin G heterozygous knockout and cathepsin G wild type mice on Apo E knockout background.

1.7 Outline of approach to problem

The following manuscripts summarize our experimental approach in testing my hypotheses.

- 1) Manuscript #1 Role of Renin Angiotensin System in Activation of Macrophages by modified Lipoproteins. (American Journal of Physiology, Heart and circulation submitted)
- 2) Manuscript #2 Cathepsin G deficiency decreases complexity of atherosclerotic lesions in apolipoprotein E deficient mice (American Journal of Physiology, Heart and circulation submitted).

2 . Role of Renin Angiotensin System in Activation of Macrophages by modified Lipoproteins.

Short title: Ang II and foam cells: a two-way relation

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submitted

Abstract

Objective: Angiotensin II favours the development and progression of atherosclerosis. Our goal was to determine if foam cell formation increases angiotensin II generation by the endogenous renin-angiotensin system (RAS) and if endogenously produced angiotensin II promotes lipid accumulation in macrophages.

Methods and Results: Differentiated THP-1 cells were treated with acetylated low density lipoproteins (ac-LDL), native LDL (n-LDL) or no LDL for 1, 2 and 4 days. Expression of RAS genes was assessed and angiotensin I and II levels were quantified in media and cell lysate. Ac-LDL treatment increased angiotensin I and II levels and the angiotensin II/I ratio in cells and media after foam cell formation. Renin mRNA expression or activity did not change, but renin blockade completely inhibited the increase in angiotensin II. Angiotensinogen mRNA but not protein level was increased. Angiotensin converting enzyme (ACE) and cathepsin G mRNA levels and activities were enhanced by exposure of cells to ac-LDL. Inhibition of renin, ACE or the angiotensin II receptor 1 (AT₁ receptor) largely abolished cholesteryl ester formation in cells exposed to ac-LDL and decreased scavenger receptor A (SR-A) and acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT-1) protein levels. Inhibition of renin or the AT₁ receptor in cells treated with oxidized LDL also decreased SR-A and ACAT-1 protein and foam cell formation.

Conclusions: These findings indicate that, during foam cell formation, angiotensin II generation by the endogenous RAS is stimulated and that endogenously generated angiotensin II is crucial for cholesterol ester accumulation in macrophages exposed to modified LDL.

Key Words: THP-1 cells, angiotensin II, ac-LDL, foam cells and cholesteryl ester.

Angiotensin II (Ang II) is an important modulator of the development and progression of atherosclerosis. Infusion of Ang II at pressor and non-pressor doses increases lesion size and complexity in mouse models (Daugherty et al., 2000, Weiss et al., 2001, Ayabe et al., 2006). Inhibition of the effects of Ang II by blocking angiotensin converting enzyme (ACE) or the angiotensin II receptors decreases lesion progression in Apo E knockout (*ApoE*^{-/-}) mice (Keidar et al., 1997, Hayek et al., 1999a, Hayek et al., 2002). Angiotensin II receptor 1 (AT₁ receptor) deficiency in *ApoE*^{-/-} and *LDLR*^{-/-} atherosclerotic mice attenuates progression of atherosclerotic lesions, suggesting that AT₁ receptors mediate most of Ang II functions (Daugherty et al., 2004, Wassmann et al., 2004, Tiyerili et al., 2012a). All components of the renin angiotensin system (RAS) are present in atherosclerotic lesions (Daugherty et al., 2004). Ang II in atherosclerotic lesions co-localizes with macrophages (Potter et al., 1998). Human macrophages express all components of the RAS and release Ang II (Okamura et al., 1999b). Macrophages also express other enzymes which are able to produce Ang II such as cathepsin G which can generate Ang II from either angiotensin I (Ang I) or angiotensinogen (Ramaha and Patston, 2002a). Low density lipoprotein (LDL) receptor-deficient atherosclerotic mice with renin deficiency in their bone marrow have reduced atherosclerotic lesion size, suggesting the importance of macrophage-derived Ang II in atherosclerosis (Daugherty et al., 2008).

Lipid-laden macrophages are the predominant cell type in early stages of atherosclerosis (Daugherty et al., 2008). Increased Ang II in the macrophage milieu

enhances foam cell formation through induction of enzymes involved in biosynthesis of cholesterol (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, HMG CoA reductase) and cholesterol ester formation (acyl-coenzyme A cholesterol acyl transferase 1, ACAT-1) (Keidar et al., 1999, Kanome et al., 2008b). Ang II also decreases cholesterol efflux by suppression of the ATP-binding cassette, sub-family A (ABCA1) transporter in macrophages (Zuo et al., 2009). Monocyte-derived macrophages from losartan-treated patients have lower oxidized LDL (ox-LDL) uptake. Similarly, peritoneal macrophages from ramipril-treated *ApoE*^{-/-} mice take up less ox-LDL compared to placebo-treated mice. Both studies related this reduction in ox-LDL uptake to reduced scavenger receptor CD36 expression (Hayek et al., 2002, Hayek et al., 2000).

The expression pattern of several genes is changed after modified lipoprotein uptake in macrophages (Hung et al., 2006a, Whatling et al., 2004). Although the expression of the RAS genes and Ang II release have been shown in macrophages (Okamura et al., 1999b), changes in the expression of these genes and their effects on the final product of the system (Ang II) have not yet been studied in foam cells. Several studies assessed the effects of exogenous Ang II on foam cell formation (Takata et al., 2005b, Keidar et al., 2001), but none has elucidated the role of locally generated Ang II in foam cell formation independently of circulating Ang II. In the present study we examined the hypothesis that 1) foam cell formation leads to an increase in expression of RAS genes and an increase in Ang II levels in lipid-laden macrophages, and 2) inhibition of Ang II formation decreases foam cell formation. We therefore evaluated 1) the effects of acetylated LDL treatment on expression of RAS genes and on Ang II levels, 2) the

effects of renin inhibition on intra and extracellular Ang II levels in acetylated LDL treated samples and 3) the consequence of RAS inhibitor treatment on foam formation in phorbol-ester differentiated THP-1 cells co- incubated with acetylated or oxidized LDL.

Methods

Cell culture

THP-1 cells (a human acute monocytic leukemia cell line) from the American Type Culture Collection (ATCC TIB-202) were cultured in complete [Roswell Park Memorial Institute medium](#) 1640 (RPMI-1640) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol, and 2 mM glutamine (Invitrogen, Burlington, ON, Canada). To differentiate these cells into macrophages, the monocytes were seeded in 6-well plates at a density of $2\text{-}2.5 \times 10^6$ cells/well in the same medium containing 100 nm phorbol 12-myristate 13-acetate (PMA) (Sigma, St Louis, MO, USA) and incubated for 24 h. Differentiated macrophages were incubated for 24, 48 and 96 hours in serum-free medium containing native LDL (n-LDL) or acetylated LDL (ac-LDL) (Biomedical Technologies. Inc, Stoughton, MA, USA) at 50 µg/mL (Ouimet et al., 2008).

Cholesteryl ester formation was also assessed in differentiated THP-1 cells treated with 50 µg/ mL oxidized LDL (ox-LDL) (Biomedical Technologies Inc., Stoughton, MA, USA) (Ouimet et al., 2008). To estimate the contribution of the scavenger receptor A (SR-A) to cholesteryl ester accumulation in ac-LDL-treated THP-1 cells, THP-1 cells were exposed to ac-LDL in the presence of 50 µg/ml/day of the SR-A ligands, dextran sulphate or fucoidan (Sigma St.Louis, MO, USA) (Seimon et al., 2006).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

THP-1 cells were washed with ice-cold PBS and homogenized in TRIzol Reagent solution (Invitrogen, Burlington, ON, Canada) by repeated pipetting. Total RNA was isolated from cells according to the manufacturer's instructions. To eliminate potential

genomic DNA contamination, total RNA was treated with DNase I (Ambion, Austin, TX, USA) before the reverse transcription reaction. cDNA was synthesized using Superscript II RNase H Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) at 42°C for 50 min. Real-time PCR amplifications were performed with a Roche Light Cycler by using Fast Start DNA Master SYBRGreen I (Roche Diagnostics, Penzberg, Germany). Two microliters of the RT product from each sample was used as a template at 20 µl reaction. The specific primer sequences for angiotensinogen, ACE, renin, cathepsin G, SR-A, ACAT-1 and cyclophilin A are listed in Table 1. The ACAT-1 primers that were used do not allow the differentiation between long and short ACAT transcripts (Kanome et al., 2008b). The real-time PCR conditions were as follows: an initial step of 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 5 s, and annealing of primers to the target for 5 s at 65°C for the renin primers, or 62°C for other primers. The extension step was performed at 72°C, and the extension time was determined by the formula: amplicon length/25 s. The specificity of real-time PCR products was documented with a melting curve analysis and high-resolution gel electrophoresis. The RT-PCR products of genes of interest were sub-cloned into pCRII TA vector (Invitrogen, Burlington, ON, Canada), followed by restriction endonuclease and sequencing analysis. The vector was transformed into *E.coli* bacteria strain DH5α for replication. The concentration of each construct was quantified by absorbance at 260 nm. Serial 10-fold dilutions of each plasmid clone were used to generate individual external standards with the same real time PCR condition as described above. Expression of all genes was normalized to the cyclophilin A mRNA level as reference gene.

Angiotensin I and II assay

Cell media were collected into 15 ml tubes containing EDTA-Na₂ and 1, 10-phenantroline (inhibitor cocktail) (Sigma, St Louis, MO, USA). After centrifugation, the media were immediately extracted on Sep-Pak C18 cartridges (Waters, Milford, MA, USA). Cells were collected and washed with PBS then lysed in PBS containing the same inhibitor cocktail by sonication. Cell lysates were centrifuged and extracted on Sep-Pak 18 cartridges. Angiotensin peptides were assessed by radioimmunoassay (RIA) after separation on high performance liquid chromatography (HPLC) (Leenen et al., 1999, Li et al., 1999a).

Renin activity

Five million cells were washed with PBS and lysed by sonication in 250 µl of 0.2 M Tris buffer (containing 0.5 mM of EDTA-Na₂, phenylmethylsulfonyl fluoride, and sodium tetrathionate dihydrate and 0.1% Triton X-100). Lysates were centrifuged at 14000 rpm and 4°C in an Eppendorf microfuge. 200 µl of each supernatant was incubated for 1 h at 37°C with 250 µl of renin substrate-enriched plasma from rats nephrectomized for 24 hours. RIA for Ang I was done in duplicate on 200 µl of the incubation mixture (Leenen et al., 2001).

Cathepsin G activity

Cells were harvested in PBS and washed with PBS. Protein was extracted from the cells by sonication of re-suspended cells in assay buffer (1.5 M NaCl, 0.1 M Tris HCl, pH 7.4) plus Triton x-100 (Sigma, St Louis, MO, USA). The reaction was carried out at 25°C in total volume of 90 µl: 20 µl of sample plus 60 µl of buffer (160 mM Tris/HCl and 1.6 M NaCl, pH 7.4) and 10 µl N-succinyl-Ala-Ala-Pro-Phe-pNA as a substrate (20 mM in

DMSO) (Sigma, St Louis, MO, USA). The increase in absorbance at 410 nm due to the release of paranitroanilide per unit time is a measure for cathepsin G activity (Barrett, 1981).

Angiotensinogen concentration

Angiotensinogen concentrations were determined by an in vitro enzyme kinetic assay (Genain et al., 1984). Human angiotensinogen was completely exhausted by cleavage with excess human recombinant renin. Twenty-five microliters of lysate prepared from 5 million cells lysate was incubated together with 450 μ L of 1X inhibitor cocktail (0.2 M Tris buffer containing: 0.5 mM of EDTA-Na₂, phenylmethylsulfonyl fluoride, and sodium tetrathionate dihydrate and 0.1% Triton X-100) plus 1.0 pmol/mL of human recombinant renin (Sigma, St. Louis, MO, USA) for 1 hour at 37°C. To measure angiotensinogen in media 250 μ l of media from 0.5 million cells were incubated with 250 μ l of 2X-concentrated inhibitor cocktail plus 1.0 pmol/mL human recombinant renin. Ang I was measured by direct RIA. Angiotensinogen concentrations are expressed as picomoles per million cells based on an equimolar production of Ang I from angiotensinogen.

ACE activity

Total protein from 1.25×10^6 cells in 25 μ l of Tris-NaCl buffer containing 0.5% Triton X-100 (Sigma, St. Louis, MO, USA) was preincubated at 37°C for 20 min in the presence or absence of 100 μ M captopril (Sigma, St.Louis, MO, USA). To quantify ACE activity in media 25 μ l of media was used instead of cell lysate. Substrate, N-Hippuryl-His-Leu hydrate (Sigma, St.Louis, MO, USA), was added to the mixture and, after incubation at 37°C for 1 hr, the reaction was stopped with NaOH, and then *o*-phthaldialdehyde and

HCl were added. Samples were centrifuged at 1900g at 4°C and 325 µl of the supernatants were added to an opaque 96-well plate. His-Leu concentration was determined by measuring the fluorescence at wavelengths 360 nm excitation and 480nm emission, using a Fluostar Galaxy fluorometer (BMG LABTECH, Durham, NC). The fluorescence of captopril-containing tubes was subtracted from those without captopril for each sample to have specific ACE activity. Activity was expressed as nanomoles His-Leu per minute per million cells (Zhao et al., 2000).

Cholesteryl ester measurement

Cells were washed with PBS and cholesterol was extracted by a 2-hour incubation with isopropanol containing 1% Triton X100. Dried isopropanol extracts were dissolved in the reaction mixture from Cholesterol/Cholesterol Ester Quantitation Kit (Biovision, Mountain view, CA, USA). Free and total cholesterol were quantified based on the manufacturer's protocol. Cholesteryl ester concentration was quantified by deduction of free cholesterol from total cholesterol and is expressed as µg cholesterol/mg protein.

Renin, ACE, cathepsin G and AT₁ receptor inhibition

To block the function of renin in THP-1 cells, lipoprotein-treated samples and controls were treated with 0, 100, 200 or 400 nM aliskiren, added once daily. Previous studies showed that a concentration of 100 nM aliskiren is required to block renin activity by 50% in human mast cells (Krop et al., 2008). Ang II and cholesteryl ester were quantified in drug- and vehicle-treated samples.

Losartan at the concentration of 100 µM was added once daily to block AT₁ receptors. At this concentration losartan inhibits external Ang II-induced THP-1 cells migration toward endothelial cells (Kintscher et al., 2001). Control and ac-LDL-treated

samples were simultaneously treated with 100 μM losartan or vehicle for 2 or 4 days. Cholesteryl ester formation was assessed in losartan- and vehicle-treated samples.

To block ACE activity differentiated THP-1 cells were treated with 1 mM captopril or 5 μM enalapril added once daily. Captopril at the concentration of 1mM completely blocks Ang II generation induced by MCP-1 in rat mast cells (Chao et al., 2011). 150 μM enalapril completely inhibits tissue factor up-regulation in co-culture of human monocytic cells and human coronary artery endothelial cells (Lindmark and Siegbahn, 2002).

To block cathepsin G activity, the cathepsin G-specific inhibitor, CK-08, at a concentration of 5 μM was used (MP biomedical, Solon, OH, USA). At this concentration the proteolytic activity of cathepsin G for degrading, a human developmental nuclear factor is completely abolished. The inhibitor was added to the media every day (Biggs et al., 2001).

To confirm that drug treatments do not affect cell viability and consequently other results the number of viable cells after all drug treatments were counted with cell viability analyzer (Vi cell) (Beckman Coulter).

Western blots

THP-1 cells were extracted by RIPA buffer and protein concentrations were measured using the BCA assay (Fisher, Pittsburgh, PA, USA). 20 μg of protein was separated by non-reducing 10% SDS–polyacrylamide gel electrophoresis for SR-A and a reducing 10% SDS–polyacrylamide gel electrophoresis for ACAT-1. Migrated proteins were transferred to polyvinylidene fluoride (PVDF) a membrane (300 mA for two hours at 4° C). Membranes were blocked with 5% milk for one hour and then incubated with rabbit

anti- human ACAT-1 sc 20951 (Santa Cruz, CA, USA), mouse anti-human SR-A MAB27081 (R&D System, Burlington , ON, Canada) or monoclonal β actin antibody produced in mouse A2228 (Sigma, St. Louis, MO, USA) overnight at 4°C. Membranes were washed and based on the type of primary antibody, they were incubated with goat anti-rabbit HRP conjugated Sc 2004 (Santa Cruz, CA, USA) or anti-mouse Ig horseradish peroxidized linked whole antibody from sheep GRP NXA931 (GE Healthcare, UK) for one hour at room temperature. The conversion of horse radish peroxidase substrate, western lightning Plus ECL NE 104001EA (Perkin Elmer, MA, US), was detected by digital imaging and analysis system (AlphaEase, Alpha Innotech Corporation; San Leandro, CA, USA). Densitometric analysis of bands was performed with AlphaEase software. The density of ACAT-1 and SR-A bands was normalized to that of β -actin.

Statistical analysis

Values are means \pm SE. Comparisons were made using one-way ANOVA to compare different treated samples at a given time point or two-way ANOVA to compare treated samples at two different time points. All tests were performed using SigmaStat software (SPSS, Chicago, IL, USA). The level of statistical significance was set at $p < 0.05$.

Results

Ac-LDL increases Ang II levels in THP-1 cells

To examine the effects of modified lipoprotein uptake on Ang II levels in macrophages, differentiated THP-1 cells were treated with no LDL, n-LDL or ac-LDL. After 2 days of treatment with ac-LDL lipid droplets became visible in THP-1 cells (figure 2-1I). Ang I levels did not change after 1 day but significantly increased in both cell lysates and media after 2 days of treatment with ac-LDL by 100% in media and 80% in cells. Ang II levels remained elevated after 4 days of treatment with ac-LDL. N-LDL did not affect Ang II levels (figure 2-1A and 2-1B). Ang I levels increased by 50% after 2 days of treatment with ac-LDL and the increase persisted after 4 days (figure 2-1C and 2-1D). The ratio of Ang II/I was elevated in ac-LDL-treated samples around 1.5-fold both in media and cells after 2 days. At 4 days, the ratio of Ang II/I was still elevated with a slight but non-significant decrease relative to 2 days (figure 2-1E and 2-1F).

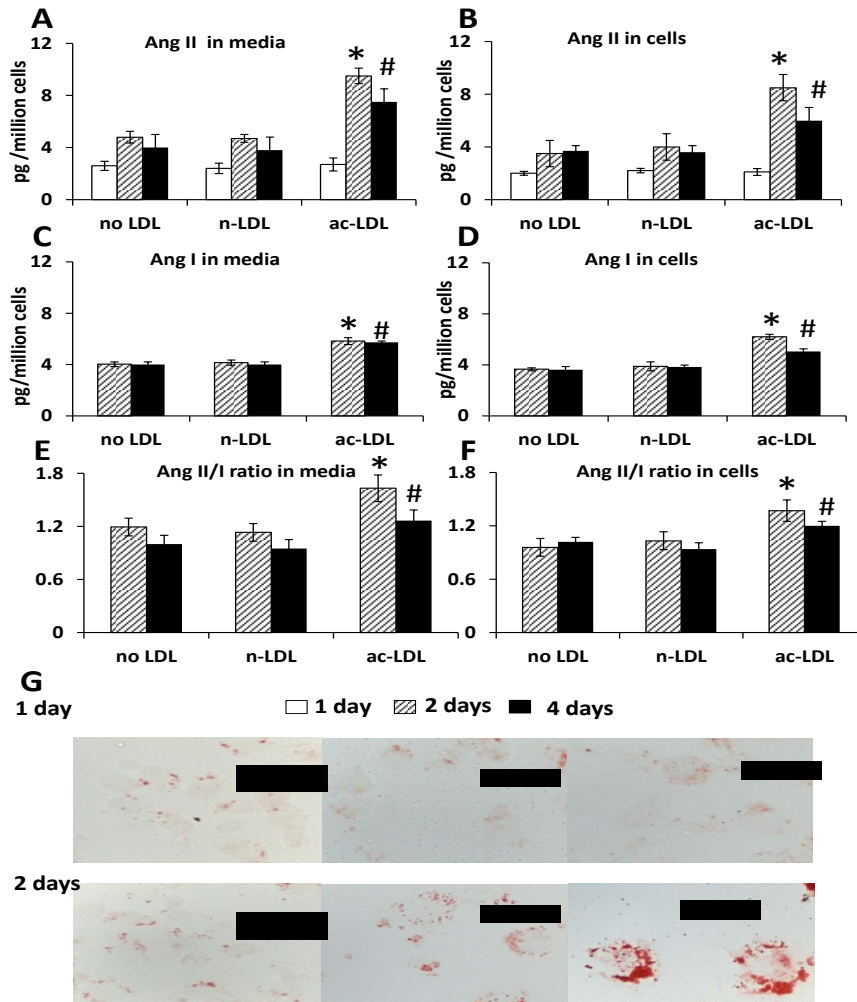


Figure 2-1

Effects of n-LDL and ac-LDL on Ang I and II levels in THP-1 cells and bone marrow derived macrophages after 1, 2 or 4 days. Ang I and II were assessed in media and cell lysate of 5×10^6 cells. A) Ang II in media; B) Ang II in cells. C) Ang I in media ; D) Ang I level in cells ; E) Ang II /I ratio in media ; F) Ang II / I ratio in cells; G) THP-1 cells stained with oil red o after 1 and 2 days of lipoproteins treatment. Values are mean \pm SEM (n = 4-5 samples per group): * $p < 0.05$ ac-LDL vs. no LDL and ac-LDL vs. n-LDL after 2 days; # $p < 0.05$ ac-LDL vs. no LDL and ac-LDL vs. n-LDL after 4 days. Data were analyzed by one-way repeated measures ANOVA.

Expression of RAS genes

Renin. Renin mRNA levels did not change after one or two days of treatment with ac-LDL (figure 2- 2A). Renin activity in THP-1 cell lysates also was not different (figure 2- 3A).

Angiotensinogen. One-day treatment with ac-LDL did not change the mRNA level of angiotensinogen in THP-1 cells, but 2 days of treatment increased angiotensinogen mRNA levels significantly (by 90%) compared with non-treated and n-LDL-treated samples (figure 2- 2B). N-LDL did not affect mRNA levels. The increase in the mRNA level in ac-LDL treated cells did not, however, result in a higher angiotensinogen concentration in media or cells, as assessed by the kinetic assay (figure 2-3C and 2-3D).

ACE. Ac-LDL treatment for one day caused a significant increase in ACE mRNA levels (by ~60%) in THP-1 cells relative to non-treated and n-LDL-treated samples. ACE mRNA levels remained elevated after 2 days of treatment with ac-LDL (figure 2-2C). N-LDL did not change ACE expression. ACE activity was increased in media and cell lysates after 2 days of ac-LDL by 60 % and 40 % relative to controls, respectively (figure 2-3E and 2- 3F).

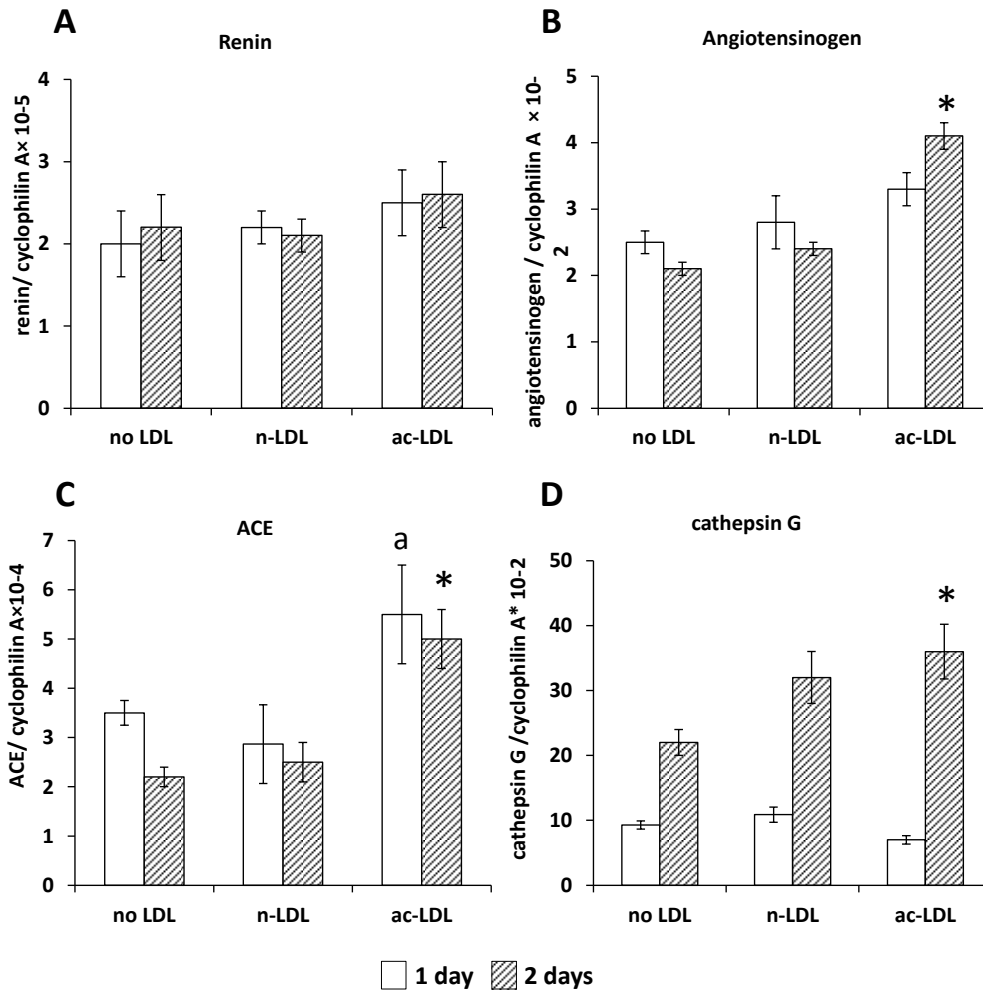


Figure 2-2

mRNA levels of RAS genes in control cells and in THP-1 cells exposed to n-LDL or ac-LDL for 1 and 2 days. Trizol extracted RNA was reverse transcribed and subjected to real time RT-PCR. The mRNA concentration of each gene of interest was normalized to mRNA concentration of a housekeeping gene (cyclophilin A). A) renin; B) angiotensinogen ; C) ACE; D) cathepsin G.

Values are mean \pm SEM (n = 6-7 samples per group): * $p < 0.05$ ac-LDL vs. no LDL and ac-LDL vs. n-LDL after 2 days; ^a $p < 0.05$ ac-LDL vs. n-LDL and ac-LDL vs. no LDL after 1day. Data were analyzed by one-way repeated measures ANOVA.

Cathepsin G. mRNA levels in treated and non-treated samples were similar after 1 day of treatment, but both ac-LDL and n-LDL significantly increased cathepsin G expression in THP-1 cells after 2 days of treatment (figure 2-2D). There was also no difference in cathepsin G activity in cell lysates after 1 day, but cathepsin G activity was significantly increased after 2 days in ac-LDL-treated THP-1 cells versus control and n-LDL-treated cells (figure 2-3B).

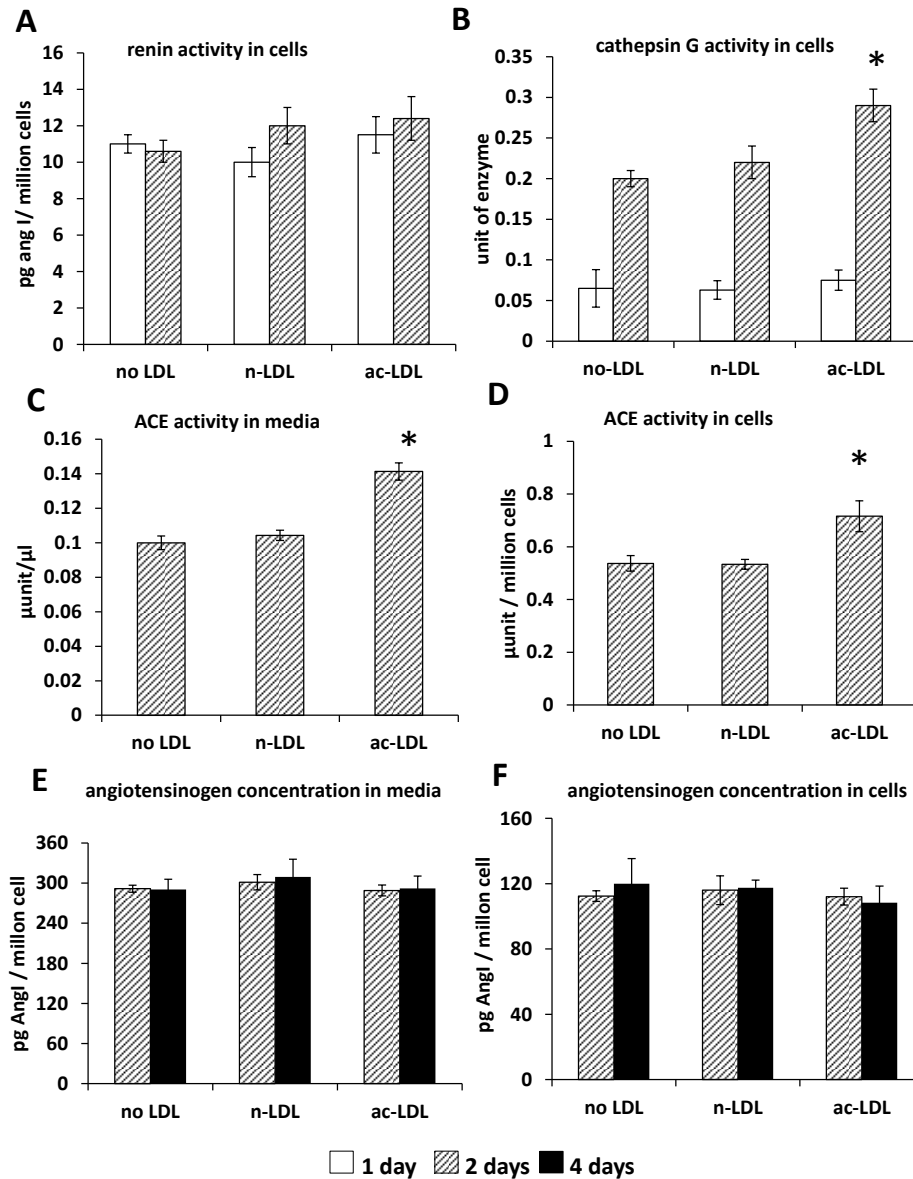


Figure 2- 3

Renin, ACE and cathepsin G activity and angiotensinogen concentration after treatment with no LDL, n-LDL or ac-LDL for 1, 2 or 4 days. Activity in cell lysates and media was assessed by adding specific substrate. Values are expressed as product per number of cells applied for each assay. Values are mean \pm SEM (n=4-5 samples per group): * $p < 0.05$ ac-LDL vs. n-LDL and ac-LDL vs. no LDL. Data were analyzed by one-way repeated measures ANOVA.

Role of renin in Ang II production

The renin inhibitor, aliskiren, at concentrations of 100 and 200 nM reduced Ang II production by 60 and 70%, respectively. The concentration of 400 nM completely blocked Ang II production in non-treated and ac-LDL-treated samples (figure2- 4). This finding is consistent with a key role of renin in production of Ang II from angiotensinogen in THP-1 cells. We could not assess the effect of ACE inhibition on Ang II production in our model since the inhibitors interfered with the radioimmunoassay measurement. We were also not able to determine the role of cathepsin G in Ang II production due to toxicity of long term exposure of cells to the cathepsin G inhibitor.

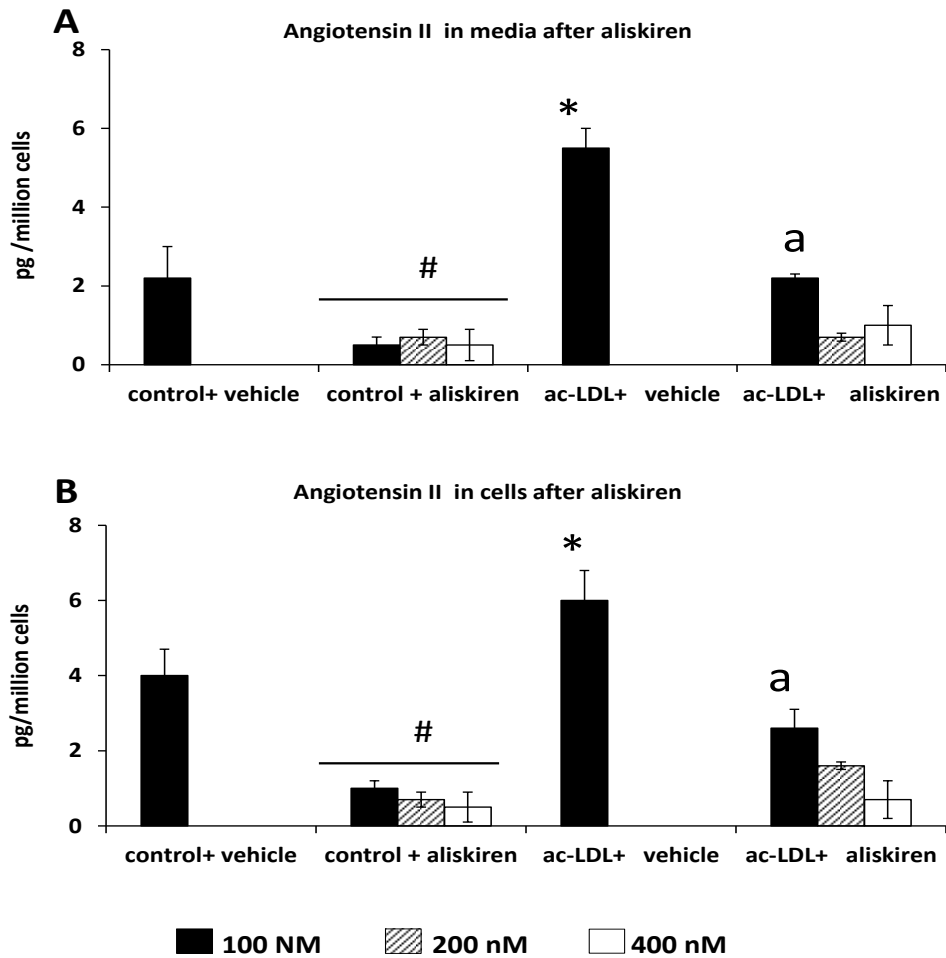


Figure 2-4

Inhibition of Ang II production in ac-LDL and none- treated THP-1 cells by aliskiren at 100, 200 or 400 nM for 2 days. Ang II was assessed in cell lysate and media from 5×10^6 cells treated with different concentrations of aliskiren. Amounts are expressed per million cells applied in the assay. Values are mean \pm SEM (n=4-5 samples per group): * $p < 0.05$ ac-LDL + vehicle vs. others; ^a $p < 0.05$ acLDL + 100 nM aliskiren vs. ac-LDL +200 or 400 nM aliskiren; [#] $p < 0.05$ control + vehicle vs. aliskiren-treated cells. Data were analyzed by two-way ANOVA.

Ang II and foam cell formation

Blockade of Ang II formation by treatment of THP-1 cells with ac-LDL and the renin inhibitor aliskiren decreased cholesteryl ester formation after 2 days by 95% without changing cell viability (figure 2-5A). The cholesteryl ester content of cells was further increased by 4 days of ac-LDL treatment compared with 2 days of treatment, but in cells exposed to 4 days of ac-LDL with aliskiren cholesteryl content was reduced by 96% relative to non-aliskiren-treated cells (figure 2-5A). Blocking AT₁ receptors by losartan also abolished cholesteryl ester formation in ac-LDL-treated samples both at 2 days and 4 days (figure 2-5B). The ACE inhibitors captopril and enalapril reduced cholesteryl ester content of the ac-LDL-treated cells by 80% (figure 2-5C and 2-5D).

To assess whether Ang II is also involved in cholesteryl ester accumulation in ox-LDL-treated THP-1 cells, THP-1 cells were exposed to ox-LDL, either alone, or together with losartan or aliskiren. Both losartan and aliskiren prevented the cholesteryl ester formation in ox-LDL-treated cells (figure 2-6A) suggesting a general role of Ang II in accumulation of cholesteryl ester in modified LDL-treated macrophages.

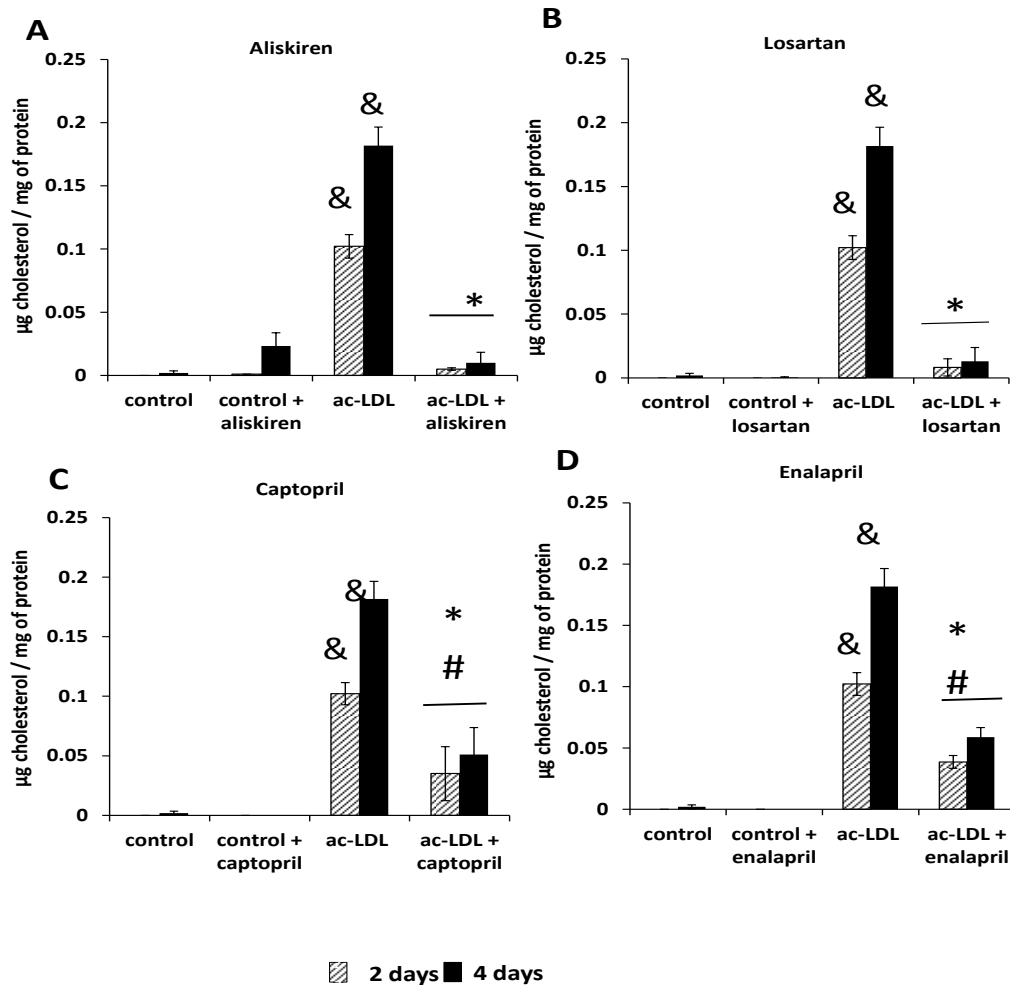


Figure 2-5

RAS inhibition and cholesteryl ester content in ac-LDL-treated THP-1 cells. THP-1 cells were cultured with or without ac-LDL in the presence of RAS inhibitors or vehicle. Cholesteryl ester content of cells was measured after 2 and 4 days and values normalized to cellular protein. A) Aliskiren 400 nM, B) Losartan 100 µM C) Captopril 5 µM and D) Enalapril 150 µM. Values are mean \pm SEM (n = 5-6 samples per group): * $p < 0.05$ RAS inhibitor vs. vehicle in cells exposed to ac-LDL; # $p < 0.05$ ac-LDL-treated cells exposed to ACE inhibitors vs controls; & $p < 0.05$ ac-LDL vs. controls. For each time point data were analyzed by one-way repeated measures ANOVA.

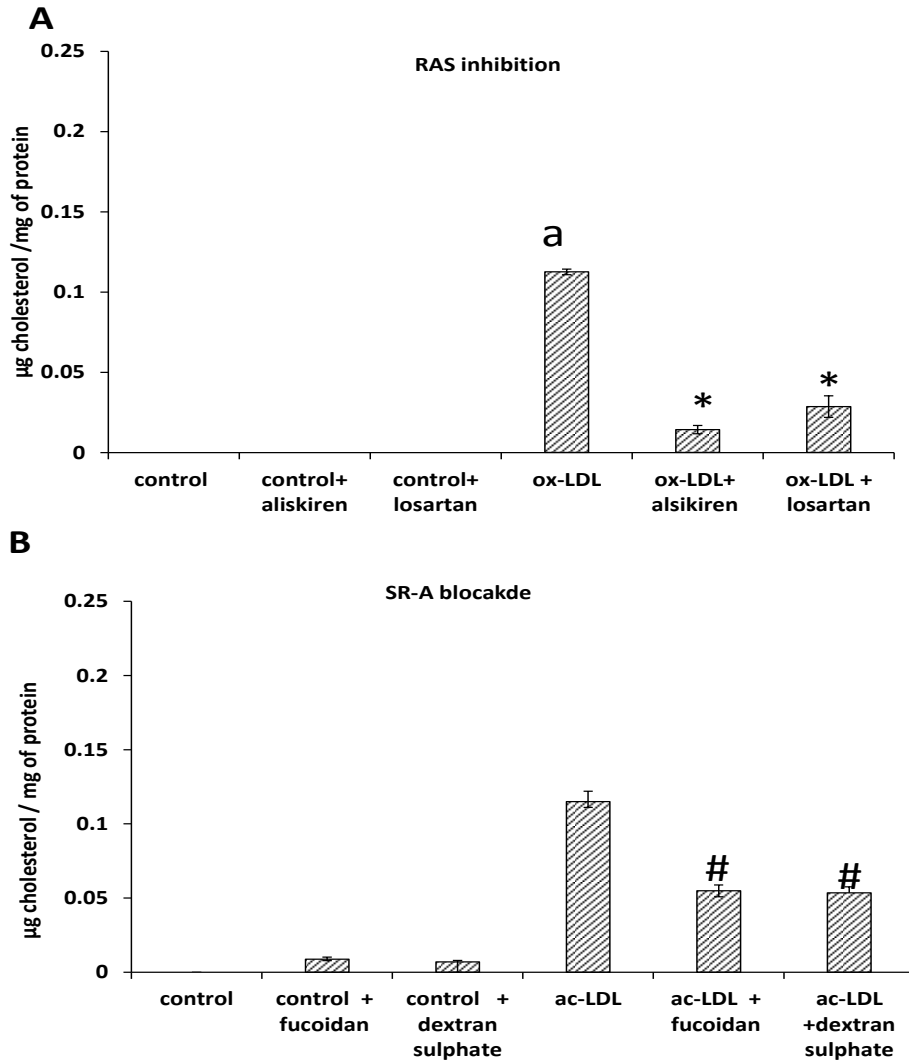


Figure 2-6

Effects of RAS inhibition and SR-A blockade on accumulation of cholesteryl ester in THP-1 cells exposed for 2 days to ox-LDL or ac-LDL. A) renin and AT₁R inhibition; 400 nM Aliskiren 100 µM losartan in ox-LDL treated THP-1 cells and B) SR-A blockade by fucoidan (50 µg/ml) and dextran sulphate (50 µg/ml) in ac-LDL treated cells. Values are mean ±SEM (n=5-6 samples per group): * $p < 0.05$ RAS inhibitor vs. vehicle; # $p < 0.05$ SR-A blocker vs. vehicle. Data were analyzed by one-way ANOVA repeated measurement.

RAS inhibitors and expression of SR-A and ACAT-1

Blocking SR-A by an excess of the SR-A ligands, fucoidan or dextran sulphate, significantly reduced the cholesteryl ester content of ac-LDL-treated cells which suggests that SR-A is a major pathway for ac-LDL uptake (figure2-6B). To assess the effects of RAS inhibitors on SR-A and ACAT-1, the enzyme responsible for cholesteryl esterification in macrophages, cell extracts from THP-1 cells treated with ac-LDL with or without aliskiren, losartan, captopril or enalapril were subjected to western blot. Treatment with ac-LDL for 2 days significantly increased SR-A, and ACAT-1 protein levels by ~30% (figure2-7 A and B). All RAS blockers similarly prevented the increase in SR-A protein. For ACAT-1, the blockers not only prevented the increase in ACAT-1 immunoreactive protein induced by ac-LDL, but significantly decreased ACAT-1 protein levels below those of the control (figure2-7B). In contrast to ac-LDL treatment, exposure of THP-1 cells to ox-LDL decreased immunoreactive SR-A but did not change ACAT-1. SR-A protein in ox-LDL-treated cells was further reduced by both losartan and aliskiren (Figure 2-7C). Losartan and aliskiren also reduced immunoreactive ACAT-1 protein in THP-1 cells treated with ox-LDL (Figure2- 7D).

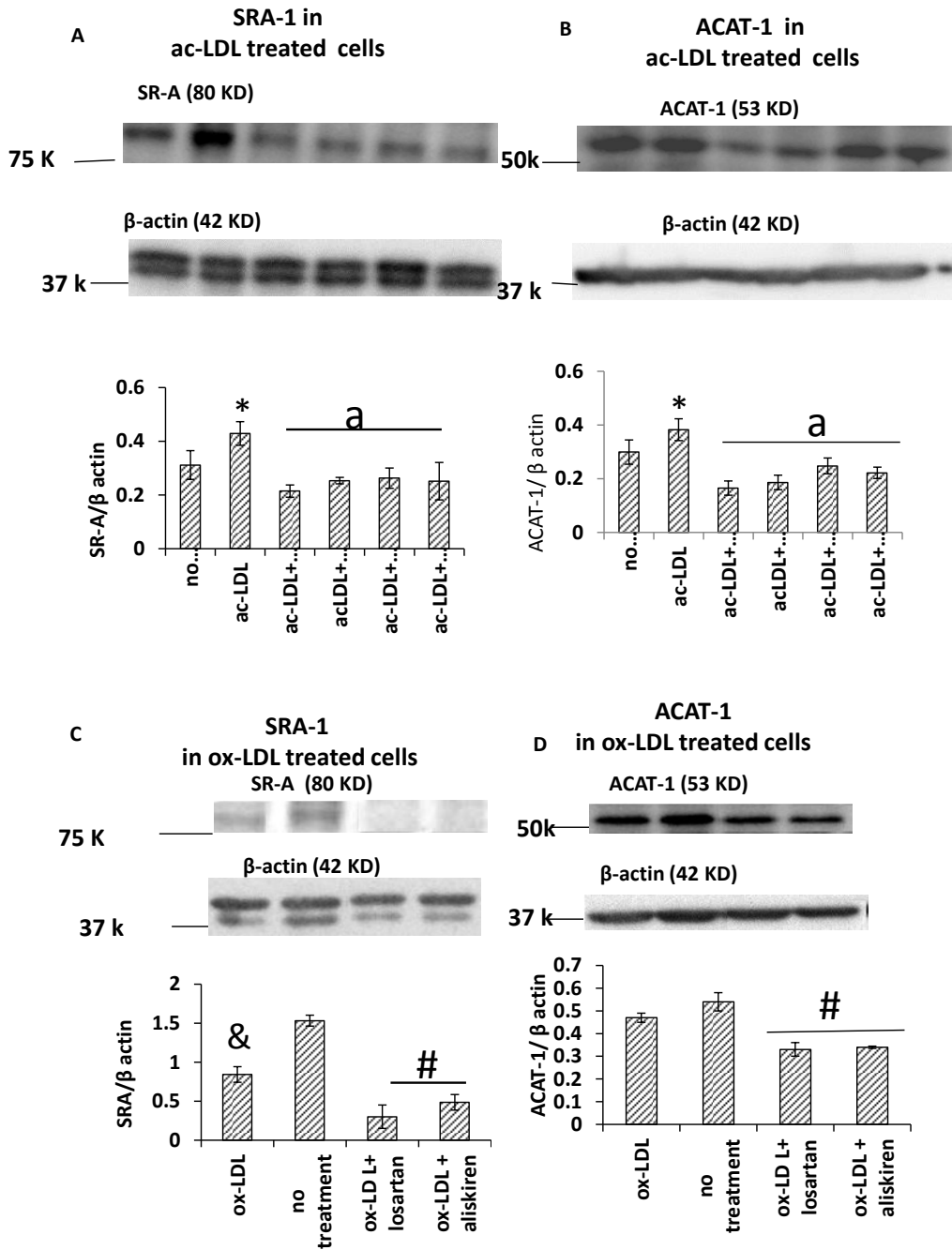


Figure 2-7

The effects of renin, ACE and AT₁R blockade on levels of immunoreactive SR-A and ACAT-1 in THP-1 cells exposed to ac-LDL or ox-LDL. Cell lysates from THP-1 cells that had been cultured for 2 days in the presence of ac-LDL (A,B) or ox-LDL (C,D) in the presence or absence of inhibitors were subjected to western blotting and

densitometry. The monomeric SR-A and ACAT-1 protein bands was normalized to that of β -actin. For doses of drugs, see figure 5. Values are presented as mean \pm SEM (n=3 samples per group): * $p < 0.05$ ac-LDL vs. others; ^a $p < 0.05$ RAS inhibitors vs. control; # $p < 0.05$ losartan and aliskiren vs. vehicle in ox-LDL-treated samples; & $p < 0.05$ ox-LDL vs. non-treated. Data were analyzed by two-way ANOVA.

To assess whether RAS inhibition down-regulates SR-A and ACAT-1 also at the mRNA level, the mRNA levels were measured using real time qRT-PCR. Neither ac-LDL nor ox-LDL affected SR-A or ACAT-1 mRNA levels (figure 2- 8). Losartan or aliskiren did not change the SR-A mRNA level in ac-LDL treated THP-1 cells but, in ox-LDL-treated cells, the RAS inhibitors reduced SR-A mRNA (Figure 8B). Unexpectedly, ACAT-1 mRNA was increased by RAS inhibitors in ac-LDL-treated cells (figure 2-8C). ACAT-1 mRNA in ox-LDL-treated cells was not measured.

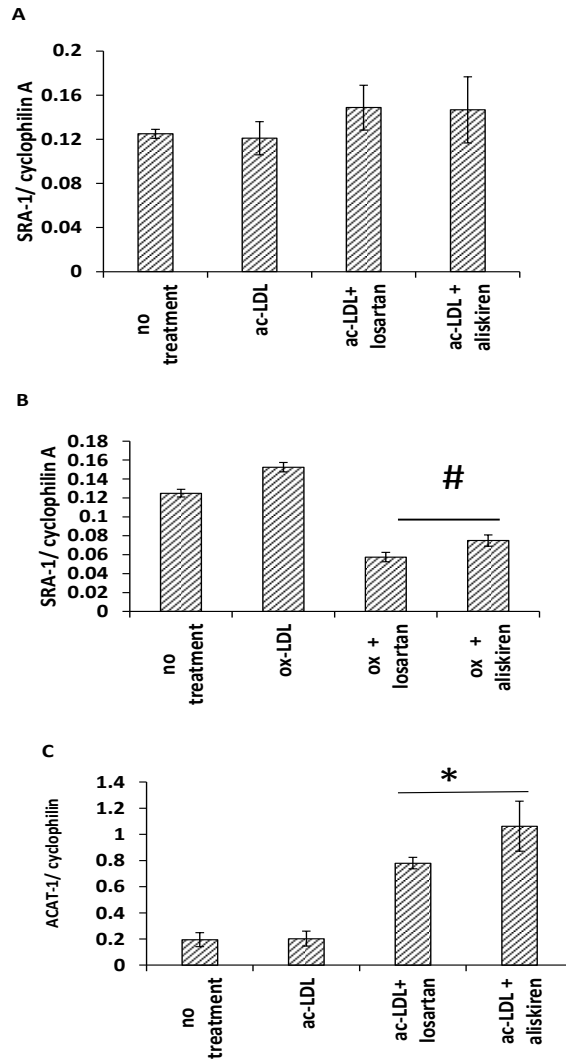


Figure 2-8

The effects of renin or AT₁R blockade on levels of SR-A mRNA (A and B) and ACAT-1 mRNA(C) in THP-1 cells exposed to (A and C) ac-LDL or to (B) ox-LDL. Values are presented as mean \pm SEM (n=5-6 samples per group): # $p < 0.05$ RAS inhibitors vs others for SR-A: * $p < 0.05$ drug vs. vehicle for ACAT-1. Data analysis was done by one-way ANOVA repeated measurement.

Discussion

The present study shows as new findings that treatment of THP-1 macrophages with ac-LDL increases Ang I and II levels and the Ang II to Ang I ratio both in cell lysates and in the media. This is likely a consequence of up-regulation of mRNA levels of key components of the RAS, angiotensinogen and ACE, as well as that of cathepsin G, a protease that can generate Ang II from both angiotensinogen and Ang I. The increased levels of ACE and cathepsin G mRNA are accompanied by augmented ACE and cathepsin G enzyme activities in ac-LDL-treated THP-1 cells. Although renin mRNA and activity levels do not change in cells exposed to ac-LDL, renin is essential for Ang II formation as the renin inhibitor, aliskiren completely abolishes Ang II production in both ac-LDL-treated and non-treated cells. Thus, increased production of Ang II occurs during foam cell formation. Furthermore, it appears that this endogenously generated Ang II plays an essential role in cholesteryl ester accumulation in THP-1 cells. Inhibition of Ang II with renin, ACE or AT₁ receptor blockers attenuates cholesteryl ester formation in ac-LDL-treated cells. Importantly, Ang II is not only required for ac-LDL-induced foam cell formation but also for the stimulation of cholesteryl ester formation in THP-1 cells exposed to the more physiologically relevant, modified lipoprotein, ox-LDL. Ang II may promote both lipoprotein uptake by increasing SR-A protein and cholesterol esterification by increasing ACAT-1 protein. In a milieu where macrophages are exposed to modified lipoproteins such as the arterial wall, the combination of increased generation of Ang II and the ability of Ang II to promote the accumulation of cholesteryl esters through binding to the AT₁ receptor may amplify foam cell formation and thus contribute to atherogenesis.

Modulation of endogenous RAS during foam cell formation

Treatment of differentiated THP-1 cells with ac-LDL increased angiotensinogen, cathepsin G and ACE mRNA levels whereas renin expression was not changed. While we did not explore the molecular mechanisms responsible for the increase of these mRNAs in the presence of ac-LDL, a number of transcription factors and signaling pathways have been implicated in regulation of genes encoding components of RAS including early growth response-1 which is important in both foam cell formation (Khachigian, 2006a) and ACE gene expression (Eyries et al., 2002a) and hypoxia inducible factor -1 α (Shatrov et al., 2003), whose activation in human monocytes can be triggered by ox-LDL and which activates ACE gene expression (Zhang et al., 2009). In addition, binding of Ang II to the AT₁ receptor triggers a signaling pathway leading to nuclear factor kappa B-activated transcription of the angiotensinogen gene initiating a positive feed-back loop (Ruiz-Ortega et al., 2000a). Any or all of these pathways could be implicated in the increased mRNA level encoding proteins involved in Ang II formation.

Ac-LDL treatment increased Ang I and II content of THP-1 cells and media by 50 and 100%, respectively. Increases in angiotensinogen protein, renin, ACE, cathepsin G and D enzyme activities could potentially explain the increased production of Ang I and II in THP-1 cells. As noted above, renin mRNA expression and activity did not change in the treated cells, but renin activity is essential for Ang II generation since renin blockade completely abolished Ang II in ac-LDL-treated and non-treated THP-1 cells. The complete blockade of Ang II production in aliskiren-treated samples rules out a role of cathepsin D in conversion of angiotensinogen to Ang I and the direct generation of

Ang II from angiotensinogen by cathepsin G or by other enzymes. Moreover, Whatling et al have reported that cathepsin D is not increased in foam cells(Whatling et al., 2004). Angiotensinogen mRNA was increased 2 days after ac-LDL treatment, but this was not reflected in the angiotensinogen protein level. This dissociation might be due to increased turnover of angiotensinogen protein. Angiotensinogen might be a rate-limiting step in production of angiotensin peptides in THP-1 cells and the absence of an increase in angiotensinogen protein might be due to faster consumption although we cannot exclude the possibility of reduced translation of the angiotensinogen mRNA or co-translational degradation of the nascent angiotensinogen polypeptide. For ACE and cathepsin G, both mRNA and enzyme activities increased in ac-LDL-treated THP-1 cells. The ~50% increase in cathepsin G and ACE activities likely promotes the conversion rate of Ang I to Ang II resulting in the ~1.5-fold higher ratio of Ang II to Ang I in cells to ac-LDL. Diet et al had previously shown that ac-LDL treatment increases ACE activity in THP-1 cells (Diet et al., 1996). Overall, increased renin-dependent conversion of angiotensinogen together with enhanced ACE and cathepsin G activities appear to be responsible for the augmented Ang II generation in THP-1 cells during their transformation to foam cells. Unfortunately, due to toxicity of cathepsin G inhibitors, we did not directly test the contribution of cathepsin G to Ang II generation.

Role of Ang II in foam cell formation

Our results suggest that macrophage-derived Ang II binds to the AT₁ receptor and activates a signaling pathway that is required for cholesteryl ester accumulation in cells exposed to ac-LDL. Interference with the generation of Ang II by renin and ACE inhibitors or blockade of Ang II binding to the AT₁ receptor markedly decreases ac-

LDL-induced cholesteryl ester accumulation in THP-1 cells. Whereas ac-LDL is taken up primarily by the SR-A, the more physiologically relevant ox-LDL is taken up by both the SR-A and other macrophage surface receptors such as CD36 (Agrawal et al., 2007). We show that the renin inhibitor, aliskiren, and the AT₁R blocker, losartan, also reduce the cholesteryl ester accumulation in ox-LDL-treated THP-1 cells. Blocking Ang II by an ACE inhibitor or AT₁R blocker reduces cholesteryl ester formation after ox-LDL treatment by attenuating CD36 expression in patients or mice treated with losartan and ramipril, respectively (Hayek et al., 2002, Hayek et al., 2000). Thus, the endogenous RAS appears to be required for foam cell formation mediated by multiple cell surface receptors for modified lipoproteins.

Exposure of THP-1 cells to ac-LDL increases immunoreactive SR-A and this is prevented by inhibition of renin, ACE or the AT₁R. The modulation of SR-A protein level by ac-LDL and by RAS inhibitors does not appear to be a function of altered SR-A gene expression as these treatments do not change mRNA levels. It is possible that SR-A protein stability is increased upon exposure of the THP-1 cells to ac-LDL and this requires a functioning RAS. Exposure of THP-1 cells to ox-LDL decreases immunoreactive SR-A and SR-A protein is further reduced by aliskiren and losartan treatment. The down-regulation SR-A protein by ox-LDL could reflect the reciprocal regulation of SR-A and CD36 expression or by the degree of oxidation of the LDL and its content of specific oxidized lipids, both of which can modulate SR-A expression (Makinen et al., 2010, Hayden et al., 2002, Yoshida et al., 1998).

Kanome et al (Kanome et al., 2008b) reported that exogenous Ang II increases the shorter form of ACAT-1 mRNA and elevates ACAT-1 protein level in human

peripheral blood monocyte-derived macrophages. The increase in ACAT-1 mRNA exaggerates cholesteryl ester formation with no change in the level of SR-A protein. Our results also suggest a possible role for Ang II in regulating cholesteryl ester accumulation in macrophages through ACAT-1 as we show that the level of immunoreactive ACAT-1 in THP-1 cells is decreased by blocking either the formation of Ang II or Ang II binding to AT₁ receptor. However, it is difficult to compare directly the observations of Kanome et al to those reported here as they did not expose cells to ac-LDL and only assessed the effect of supra-physiological doses of exogenous Ang II (Kanome et al., 2008b). Surprisingly, we observed that aliskiren and losartan both increase rather than decrease ACAT-1 mRNA levels in ac-LDL treated cells. It should be noted, however, that we used primers that would detect all ACAT-1 transcripts and would not differentiate the shorter transcript that was shown to be increased by Ang II treatment in the study of Kanome et al (Kanome et al., 2008b). While we were preparing this manuscript, Osada-Oka et al (Osada-Oka et al., 2012) reported that blocking AT₁R receptors by candesartan and losartan at 10 and 20 μ M decreased foam cell formation in ox-LDL treated THP-1 cells by attenuating ACAT-1 expression. In this study ACE inhibitors, captopril and enalapril at 100 μ M, were ineffective and the authors concluded that the reduction in foam cell formation by losartan and candesartan was independent of endogenously generated Ang II and was due to the ability of the AT₁R blockers to reduce epidermal growth factor (EGF) receptor activation by ox-LDL. The discrepancy with our observation in terms of the ability of ACE inhibitors to prevent cholesteryl ester accumulation might be due to the shorter incubation time with ox-LDL and the lower dose of inhibitors used by

Osada-Oka (Osada-Oka et al., 2012). While losartan-mediated inhibition of EGF receptor activation may contribute to its capacity to suppress foam cell formation, our finding that the inhibition of renin or ACE prevents accumulation of cholesteryl ester in THP-1 cells exposed to modified LDL underlies the importance of Ang II generation by endogenous RAS in foam cell formation.

In addition to altering modified lipoprotein uptake and cholesteryl esterification, Ang II may also influence cholesteryl ester accumulation in macrophages by altering cholesterol efflux or cholesterol synthesis. In apolipoprotein E-null mice, plasma Ang II is increased and macrophages from these animals have lower ATP-binding cassette protein A1 (ABCA1) expression.(Zuo et al., 2009) Ang II has also been shown to increase cholesterol biosynthesis in macrophages through up-regulation of HMGCoA reductase (Keidar et al., 1999). However, under the experimental conditions used in the present study (the absence of cholesterol acceptors and exposure of cells to ac-LDL), it is unlikely that either decreases in cholesterol efflux or increases in cholesterol synthesis could explain the profound effects of endogenous Ang II on ac-LDL-induced cholesteryl ester accumulation in THP-1 cells.

Based on our results, we propose a model in which exposure of THP-1 cells to ac-LDL up-regulates the RAS leading to increased generation of Ang II. Ang II, in turn, binds to the AT₁ receptor and activates a signaling cascade that results in increased synthesis or reduced catabolism of SR-A and ACAT-1 and, as a consequence, enhanced cholesteryl ester accumulation. The ability of renin and ACE inhibitors and AT₁ blockers

to break this positive feedback loop may contribute to their well-documented anti-atherogenic properties.

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Naimeh Rafatian: experimental procedures, experimental design, data analysis, manuscript writing

Ross W. Milne: experimental design, data analysis, manuscript writing

Frans H.H. Leenen: project conception, experimental design, data analysis, manuscript writing

Stewart C. Whitman: project conception, experimental design, data analysis

3. Cathepsin G deficiency decreases complexity of atherosclerotic lesions in apolipoprotein E-deficient mice

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Running Head: Cathepsin G and atherosclerotic plaque complexity

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Abstract

Cathepsin G is a serine protease with a broad range of catalytic activities including production of angiotensin II, degradation of extracellular matrix and cell-cell junctions, modulation of chemotactic responses and induction of apoptosis. Cathepsin G mRNA expression is increased in human coronary atheroma versus the normal vessel. To assess whether cathepsin G modulates atherosclerosis, cathepsin G knockout (*Cstg*^{-/-}) mice were bred with apolipoprotein E knockout (*ApoE*^{-/-}) mice to obtain *Cstg*^{+/-}*ApoE*^{-/-} and *Cstg*^{+/+}*ApoE*^{-/-} mice. Heterozygous cathepsin G deficiency led to a 70 % decrease in cathepsin G activity in bone marrow cells but this reduced activity did not impair generation of angiotensin II in bone marrow-derived macrophages. Atherosclerotic lesions were compared in male *Cstg*^{+/-}*ApoE*^{-/-} and *Cstg*^{+/+}*ApoE*^{-/-} mice after 8 weeks on a high fat diet. Plasma cholesterol levels and cholesterol distribution within serum lipoprotein fractions did not differ between genotypes nor did the atherosclerotic lesion areas in either the aortic root or aortic arch. *Cstg*^{+/-}*ApoE*^{-/-} mice, however, showed a lower percentage of complex lesions within the aortic root, and a smaller number of apoptotic cells compared to *Cstg*^{+/+}*ApoE*^{-/-} littermates. Thus, cathepsin G activity appears to accelerate the progression of early atherosclerotic lesions to more complex lesions in *ApoE*^{-/-} mice.

Key words: atherosclerosis, cathepsin G, Apo E, lesion progression

Introduction

Cathepsin G is found in azurophilic granules of monocytes and neutrophils. It is a neutral serine protease expressed at the promyelocytic and promonocytic stage of hematopoiesis (Grisolano et al., 1994, Hanson et al., 1990b) and its expression on the neutrophil cell surface is induced by chemoattractants (Owen and Campbell, 1998a). Cathepsin G has a broad range of functions, many of which can potentially enhance development and progression of atherosclerotic lesions. It might participate in several steps in the renin angiotensin system. Purified cathepsin G from human neutrophils activates prorenin (Dzau et al., 1987). Cathepsin G is expressed in non-contractile smooth muscle cells (synthetic phenotype) where it is involved in angiotensin II production (Hu et al., 2003). Membrane-bound cathepsin G from human neutrophils converts angiotensin I to angiotensin II at a relatively high rate and can also generate angiotensin II from angiotensinogen at a lower rate (Owen and Campbell, 1998a). Angiotensin II is a key modulator of atherosclerosis by increasing inflammation and migration of immune cells, and causing endothelial dysfunction and smooth muscle cell proliferation (Mazzolai and Hayoz, 2006).

Cathepsin G has an important role in regulation of chemotaxis. It cleaves N-terminal residues of some chemokines like CCL15 to generate more potent chemotactic factors for monocytes and neutrophils (Richter et al., 2005a). On the other hand, cathepsin G can inactivate cytokines such as tumor necrosis factor- α (TNF- α) and interleukin (IL)-8 that can enhance development of atherosclerotic lesions (Abbott et al., 1998a). TNF- α enhances endothelial dysfunction and stimulates activation of other cytokines (Boisvert et

al., 2000) and Il-8 increases macrophage retention and promotes the expansion and activation of intimal macrophages (McKellar et al., 2009).

Cathepsin G changes extracellular matrix composition in many tissues due to its various hydrolytic activities. Cathepsin G, like other serine proteases, is able to activate matrix metalloproteinase (MMP)-1 directly or through activation of MMP-10. MMPs degrade basement membrane and collagen (Saunders et al., 2005b). Activated collagenase transforms collagen to its denatured form which is, in turn, a substrate for cathepsin G (Capodici and Berg, 1989). Cathepsin G not only hydrolyzes elastin but also modulates elastase and makes it a more potent elastin-degrading enzyme (Boudier et al., 1981b). Besides elastin, cathepsin G degrades glycoproteins such as fibronectin, thrombospondin and von Willebrand factor (Bonney and Legrand, 2000b). Cathepsin G is also able to disrupt cell-matrix and cell-cell adhesion. This has been demonstrated for both endothelial and cardiac smooth muscle cells with the result that the detached cells become susceptible to apoptosis (Boehme et al., 2002, Rafiq et al., 2008a).

Cathepsin G mRNA is more abundant in human atheromata (stage IV lesions) relative to the normal vessel wall (Legedz et al., 2004b, Whitman, 2004a). Although the presence of cathepsin G has been shown in human atherosclerotic lesions and cathepsin G has a variety of functions that could potentially enhance atherosclerosis development, the role of cathepsin G has never been studied in an animal model of human atherosclerosis. In the present study we compared development of atherosclerotic lesions in male heterozygous cathepsin G knockout mice on an apolipoprotein E (apo) E-deficient background (*Ctsg*^{+/-} *ApoE*^{-/-} mice) with lesion development in apo E-deficient littermates that have two functional *Ctsg* alleles (*Ctsg*^{+/+} *ApoE*^{-/-} mice).

Materials and Methods

Animals

Ctsg^{-/-} mice were purchased from (stock number: FESA: 001531; Medical Research Council Harwell). *ApoE*^{-/-} mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in the Animal Care Facility at the University of Ottawa Heart Institute. Both strains of mice have been backcrossed to the C57BL/6 background. F1 heterozygotes were mated with *ApoE*^{-/-} mice to have an F2 generation of breeding mice that were *Ctsg*^{+/-}*ApoE*^{-/-}. The *Ctsg*^{-/-}*ApoE*^{-/-} mice were embryonically lethal. Therefore, the study was designed for cathepsin G heterozygous knockout mice and cathepsin wild type mice both on an *ApoE*^{-/-} background. This study was approved by the University of Ottawa Animal Care Committee and conforms to the *Guide for the Care and Use of Laboratory Animals* of the Canadian Council on Animal Care.

For atherosclerosis studies, *Ctsg*^{+/+}*ApoE*^{-/-} or *Ctsg*^{+/-}*ApoE*^{-/-} male mice at 8 weeks of age were fed a standard laboratory mouse diet supplemented with 21% (wt/wt) butterfat, 0.15% (wt/wt) cholesterol and 19.5% (wt/wt) casein but without sodium cholate (Harlan Teklad; Madison, Wisconsin, USA) for 8 weeks. Mice were then anaesthetized by intraperitoneal injection of somnitol and blood was collected by puncture from the left ventricle. Mice were perfused with PBS via a cannula placed in the left ventricle, with the perfusate exiting from the right atrium. Hearts were separated from the aorta at the base, embedded in optimum cutting temperature medium (OCT, Fisher Scientific, Ottawa, On, Canada) and snap-frozen on a pre-cooled metal plate immersed in liquid nitrogen. The imbedded hearts were then kept at -80°C.

Cathepsin G assay: Bone marrow cells were harvested in, and washed with, PBS. Protein was extracted by sonication following re-suspension of the cells in assay buffer (1.5 M NaCl, 0.1 M Tris HCl, pH 7.4) plus 1% Triton x-100 (Sigma, St Louis, MO, USA). The reaction was in a total volume of 90 μ l : 20 μ l of sample plus 60 μ l of buffer (160 mM Tris/HCl and 1.6 M NaCl, pH 7.4) and 10 μ l of the substrate, N-succinyl-Ala-Ala-Pro-Phe-pNA (20 mM in DMSO) (Sigma, St Louis, MO, USA) at 25°C. The increase in absorbance at 410 nm due to the release of paranitroanilide per unit time was used as a measure of cathepsin G activity (Barrett, 1981).

Angiotensin II measurement in bone marrow derived macrophages

Bone marrow derived macrophage culture: Cells were extracted by aspiration of bone marrow cells from mouse femurs and tibias with DMEM containing 10% foetal bovine serum, 15% L929 conditioned media and 1% penicillin and streptomycin (Invitrogen, Burlington, ON, Canada). Cells were counted and plated at a concentration of 2×10^6 cells/ml. Cells were differentiated to macrophages after 7 days incubation at 37°C. On day 7, cells were washed and medium was replaced by serum-free RPMI 1640 for 2 days, either without acetylated low density lipoprotein (ac-LDL) or with ac-LDL (Biomedical Technologies Inc, Stoughton, MA) at a cholesterol concentration of 50 ng/ml (Ouimet et al., 2008).

Angiotensin I and II assays: Two days after changing the media, cell media were collected in 15 ml tubes containing 10% of total volume EDTA- Na_2 and 1,10-phenanthroline (inhibitor cocktail) (Sigma, St Louis, MO, USA). After centrifugation, the media were immediately extracted on Sep-Pak C18 cartridges (Waters, Milford, MA, USA). Cells were collected, washed with PBS and then lysed by sonication in PBS

containing the same inhibitor cocktail. Cell lysates were centrifuged and extracted on Sep-Pak 18 cartridges. Angiotensin peptides were assessed by radioimmunoassay after separation by high pressure liquid chromatography (Leenen et al., 2001).

Quantification of atherosclerotic lesions

Aortic root :The method for quantification of atherosclerotic lesions in tissue sections of the aortic root has been described in detail (Daugherty and Whitman, 2003). Briefly, lesion size in the ascending aorta was determined from four, 10 μ m Sudan IV-stained serial sections, taken at 100 μ m intervals with the first section (level 0) being defined as that including the ostia for the coronary arteries. Lesion area was defined as intimal tissue within the internal elastic lamina and was quantified using Image-Pro Plus software (V6.2, Media Cybernetics, Silver Springs, MD, USA) on images that were created using a digital CoolSNAP cf camera (Roper Scientific Inc., Duluth, GA, USA).

Aortic arch: Adventitial tissue was removed from formaldehyde-fixed aortas. Aortas were cut open longitudinally through the inner curvature of the aortic arch that extends down the whole length of the aortic tree. The tissue was laid out on a black background and an image was taken. The extent of grossly discernible lesions versus total aorta was quantified (Daugherty and Whitman, 2003).

Complexity analysis

Lesions with different layers of macrophages were considered simple lesions whereas lesions with layers of macrophages together with smooth muscle cells, collagen within the intima and necrotic cores were counted as complex lesions. Macrophages were identified by immunohistochemistry based on CD68 (ABD Serotec, Raleigh, NC, USA) immunoreactivity. Area covered by smooth muscle cells was determined by

immunohistochemistry based on α -smooth muscle actin (Abcam, Toronto, On, Canada). Collagen was stained with Gomeri Trichrome. Necrotic core area was analyzed with hematoxylin and eosin staining.

Lipids and lipoproteins

Plasma (50 μ l) was subjected to Sepharose 6 fast protein liquid chromatography (FPLC). Cholesterol was measured in total plasma and in 0.5 ml FPLC fractions using commercial enzyme-based kits (Genzyme and Wako Bioproducts, respectively) (Whitman et al., 2000).

Apoptosis

Aortic root cryo-sections were fixed and post-fixed in 1% paraformaldehyde in PBS and acetic acid–ethanol mixture. Slides were stained according to the protocol for cryo-sections provided with the Apop® Tag Red *in situ* apoptosis detection kit (Millipore Inc., Billerica, MA).

Cleaved caspase 3 (Cell Signaling Technologies , Danvers, MA) was detected in acetone-fixed aortic root cryosections by immunohistochemistry

Statistical analysis

Values are reported as means \pm SE. One way repeated measures analysis of variance (ANOVA) followed by Newman-Keuls was used. The level of significance was set at a P value of 0.05.

Results

Characterization of cathepsin G-deficient mice

Cathepsin G deficiency in mice on a wild type background did not influence either fetal or post-natal development. However, cathepsin G homozygote knockout mice on an *ApoE*^{-/-} background died *in utero* between 15 and 18 days of gestation. *Ctsg*^{+/-}*ApoE*^{-/-} were born at the expected frequency and developed normally. Bone marrow leukocytes were isolated from *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} littermates and cathepsin G activity was measured in cell lysates. Cathepsin G activity in bone marrow of *Ctsg*^{+/-}*ApoE*^{-/-} was decreased by almost 70 % relative to bone marrow from *Ctsg*^{+/+}*ApoE*^{-/-} littermates (figure 3-1).

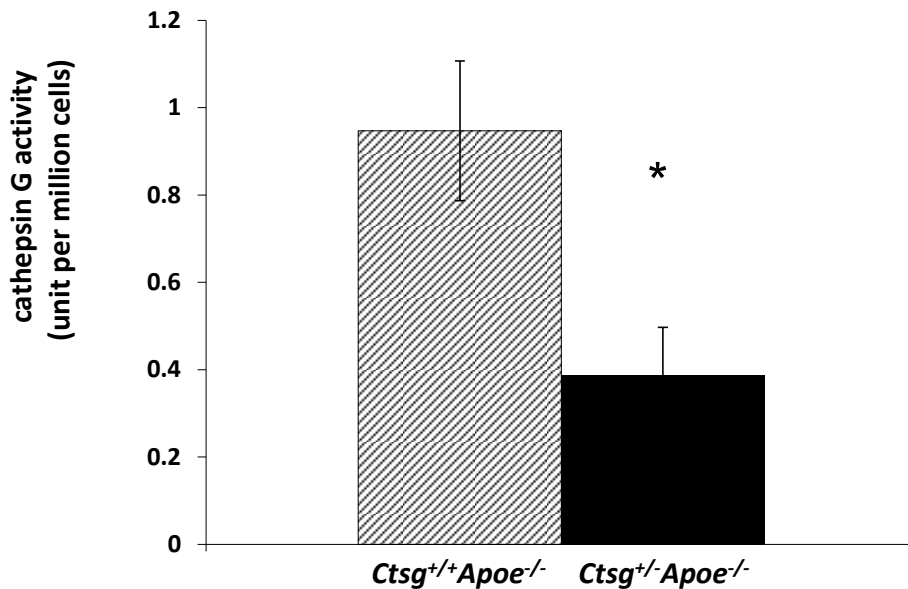


Figure 3-1. Cathepsin G activity in bone marrow cell lysates from *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} mice after 8 weeks on a high fat diet. Values are mean \pm SEM, n= 10 per group.* $p < 0.05$ *ctsg*^{+/-}*ApoE*^{-/-} vs *ctsg*^{+/+}*ApoE*^{-/-}.

Circulating lipoprotein and cytokines

After 8 weeks on the high fat diet plasma cholesterol concentrations were similar in *Ctsg^{+/-}ApoE^{-/-}* (1348 ± 69 mg / dl) and *Ctsg^{+/+}ApoE^{-/-}* (1281 ± 69 mg / dl) mice. The FPLC serum lipoprotein profiles of the two strains also did not differ (not shown) with the cholesterol being found primarily in fractions containing very low density, intermediate and low density lipoproteins.

Quantification and characterization of atherosclerotic lesions

No differences were observed between *Ctsg^{+/-}ApoE^{-/-}* and *Ctsg^{+/+}ApoE^{-/-}* mice in either the number of atherosclerotic lesions (not shown) or the area occupied by the lesions within the aortic root (figure 3-2A). *En face* analysis of atherosclerosis in the aortic arch also revealed no differences between the two groups (figure3-2B).

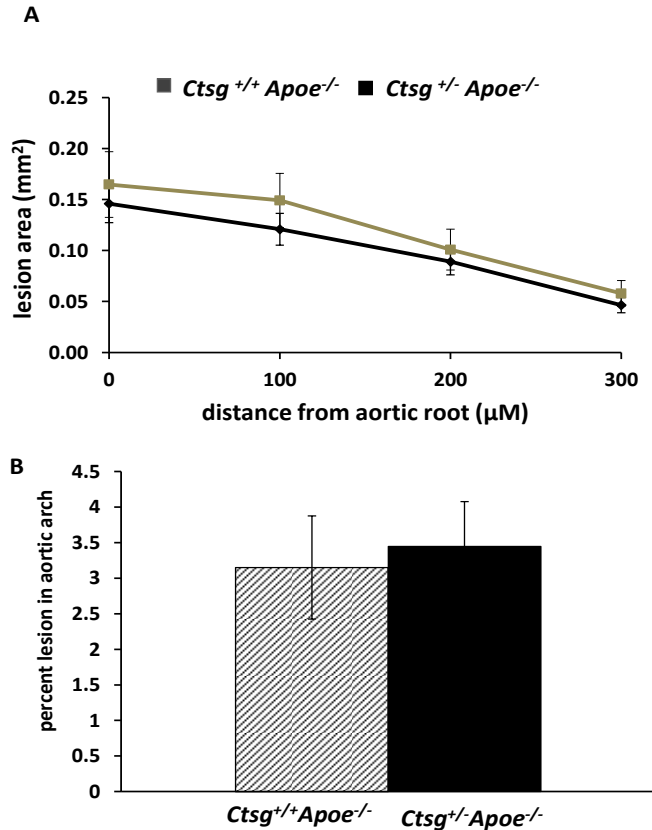


Figure3-2. Quantification of atherosclerotic lesion development in two vascular beds of *Ctsg*^{+/-} *Apoe*^{-/-} and *Ctsg*^{+/+} *Apoe*^{-/-} mice. A) Atherosclerotic lesion area in aortic root. Values are mean ± SEM at each level. n=18 per group; B) *En face* quantification of lesions in the aortic arch. Values are mean ± SEM, n= 15 per group.

Partial cathepsin G deficiency did, however, result in a decreased percentage of lesions that were classified as complex at all levels of the aortic root (figure 3-3A). Lesions in *Ctsg*^{+/-} *Apoe*^{-/-} mice had less collagen (figures 3-3B -3-3D), fewer smooth muscle cells (figure 3K-M), a higher number of macrophages (figures 3-3E-3-3G) and a smaller volume of necrotic core (figures 3-3H-3-3J) compared to *Ctsg*^{+/+} *Apoe*^{-/-} mice. It is

notable however that, when only early lesions were compared, *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} mice did not differ in their respective numbers of CD68-positive macrophages per unit area (181 ± 4 vs 182 ± 6). While few lesions in the *Ctsg*^{+/-}*ApoE*^{-/-} mice showed a complex morphology with a fibrous cap and smooth muscle cells, complex lesions for both mouse lines are shown in figure 3 to illustrate that, even when complex lesions of similar size are compared, those of *Ctsg*^{+/-}*ApoE*^{-/-} mice appear to have smaller necrotic cores, less collagen, and fewer smooth muscle cells. Consistent with the greater numbers of CD68-positive macrophages in lesions of *Ctsg*^{+/-}*ApoE*^{-/-} mice and the smaller necrotic cores, there were also fewer TUNEL-positive nuclei compared to lesions of *Ctsg*^{+/+}*ApoE*^{-/-} mice (figure 3-4A-3-4C). The result of cleaved caspase 3 immunohistochemistry confirm the lower incidence of apoptosis in *Ctsg*^{+/-}*ApoE*^{-/-} mice (figure 3-4D).

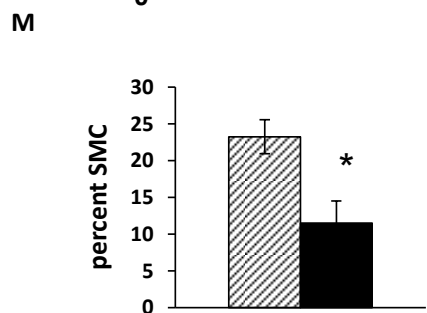
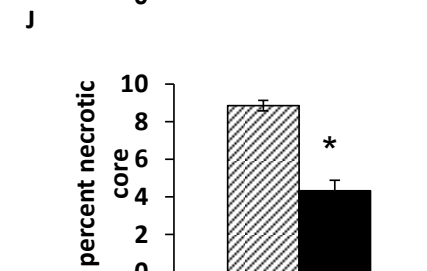
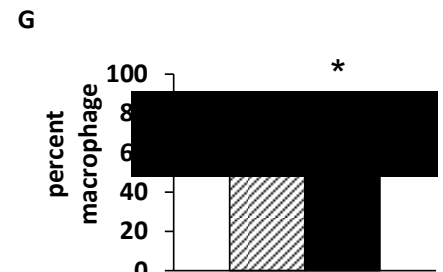
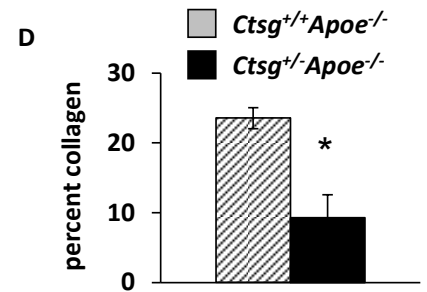
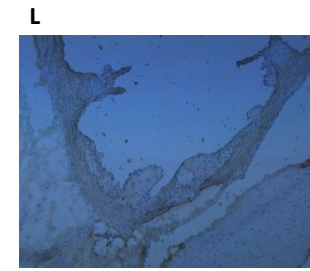
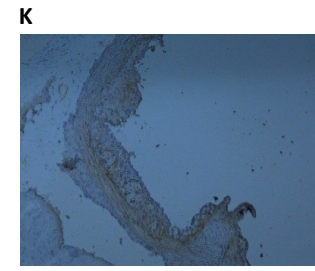
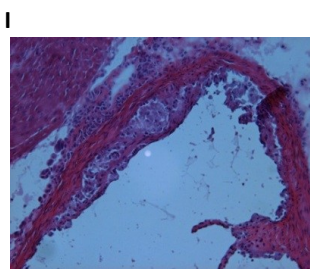
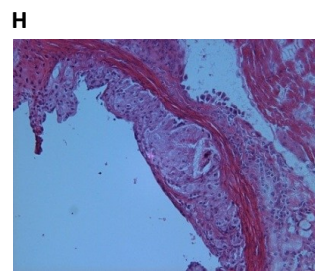
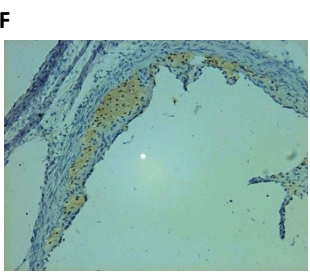
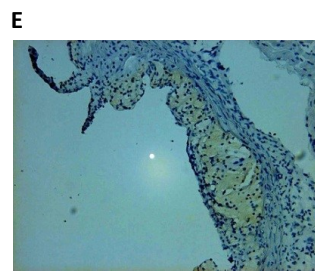
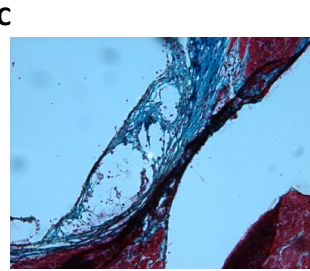
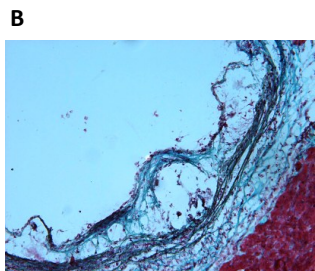
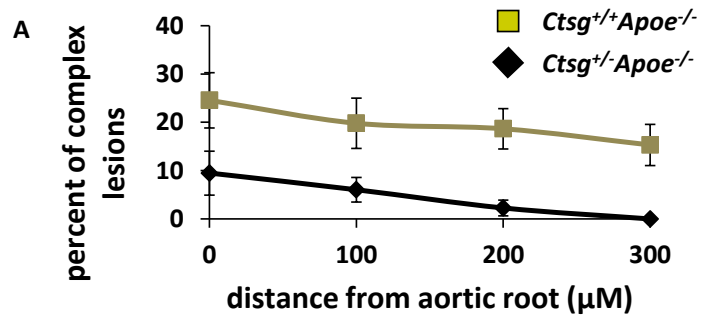


Figure 3-3. Analysis of the complexity and composition of atherosclerotic lesions in aortic roots of *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} mice. A) Percent of area covered by complex lesions vs total lesion area. Values are mean ± SEM, n=18 per group,* *p*<0.05. B) and C) Collagen content of aortic root lesions of (B) *Ctsg*^{+/+}*ApoE*^{-/-} and (C) *Ctsg*^{+/-}*ApoE*^{-/-} mice . D) The percent of lesion area covered by collagen. Values are mean ± SEM, n=8 per group,* *p*<0.05. E) and F) CD68⁺ macrophages in aortic root lesions of E) *Ctsg*^{+/+}*ApoE*^{-/-} and (F) *Ctsg*^{+/-}*ApoE*^{-/-} mice. G) The percent area covered by lesion-associated CD68-positive macrophages. Values are mean ± SEM, n=8 per group,* *p*<0.05. H) and I) Aortic root lesions stained with hematoxylin and eosin in H) *Ctsg*^{+/+}*ApoE*^{-/-} and I) *Ctsg*^{+/-}*ApoE*^{-/-} mice. J) Percent necrotic area versus total aortic root lesion area. Values are mean ± SEM, n=8 per group,* *p*<0.05. K) and L) smooth muscle α-actin in aortic root lesions of (K) *Ctsg*^{+/+}*ApoE*^{-/-} and (L) *Ctsg*^{+/-}*ApoE*^{-/-} . M) The percent lesion area covered by smooth muscle cells indicated by staining for smooth muscle α-actin. Values are mean ± SEM, n=6 per group,* *p*<0.05. Magnification 200X.

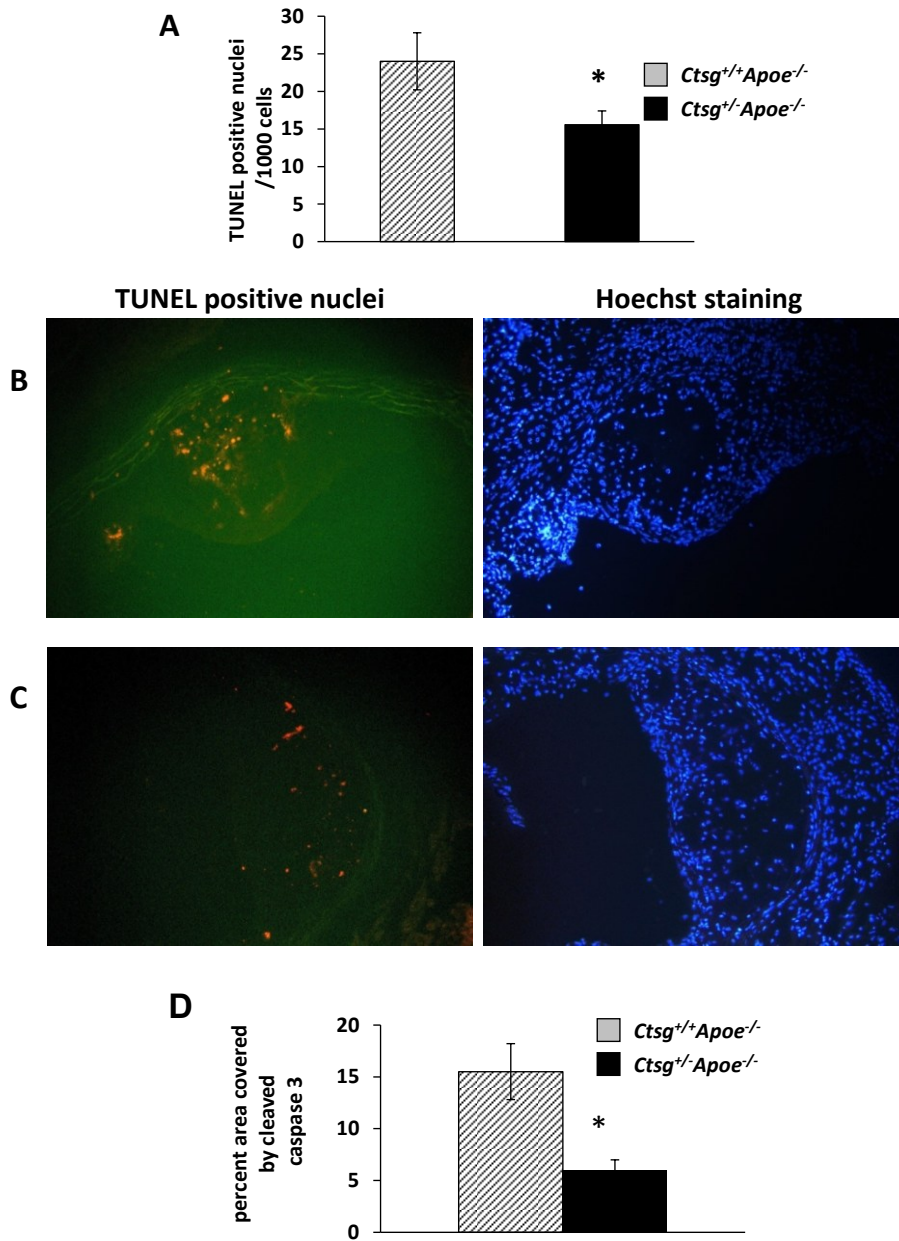


Figure 3-4. Apoptosis in atherosclerotic lesions of *Ctsg*^{+/-}*Apoe*^{-/-} and *Ctsg*^{+/+}*Apoe*^{-/-} mice. A) The number of TUNEL-positive nuclei per 1000 nuclei. Values are mean \pm SEM, n= 8 per group * $p < 0.05$ *Ctsg*^{+/-}*Apoe*^{-/-} vs *Ctsg*^{+/+}*Apoe*^{-/-}. B) and C) TUNEL-positive and total nuclei (Hoechst-staining) in aortic root lesions of B) *Ctsg*^{+/+}*Apoe*^{-/-} and C) *Ctsg*^{+/-}*Apoe*^{-/-} mice (100X magnification). D) percent area covered by cleaved caspase 3. Values are mean \pm SEM, n= 6 per group * $p < 0.05$ *Ctsg*^{+/-}*Apoe*^{-/-} vs *Ctsg*^{+/+}*Apoe*^{-/-}.

Angiotensin II levels in media and cell.

Angiotensin II levels were quantified in media and cell lysates from *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} bone marrow-derived macrophages which had, or had not, been exposed to ac-LDL. No differences in the levels of angiotensin II were detected between *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} independently of whether the cells were, or were not, treated with ac-LDL (figure 3-5). Likewise, lesion-associated immunoreactive angiotensin II appeared to be similar in aortic sections of *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} mice (not shown).

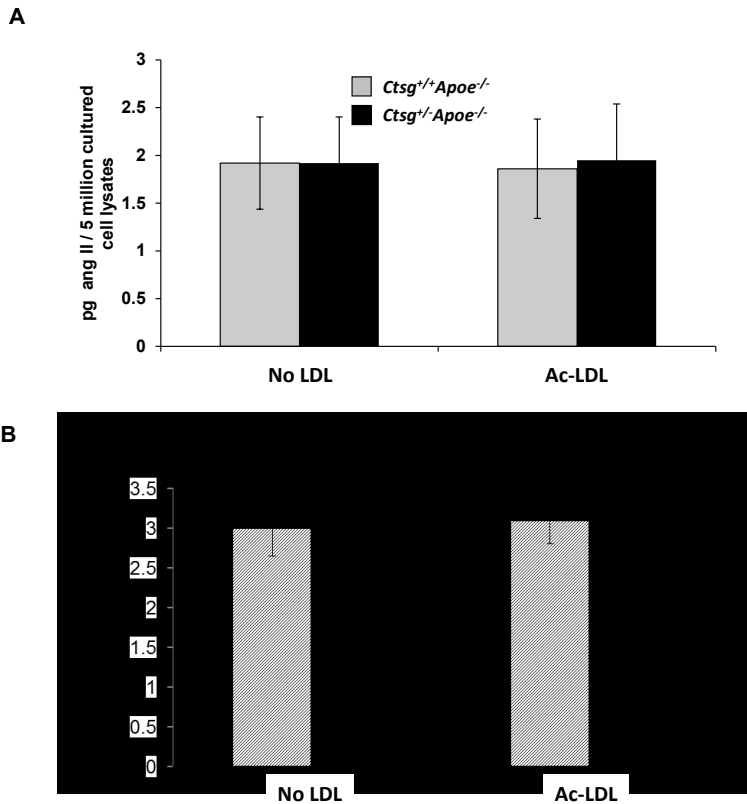


Figure 3- 5. Angiotensin II levels in cell lysates and conditioned media from bone marrow-derived macrophages of *Ctsg*^{+/+}*ApoE*^{-/-} and *Ctsg*^{+/-}*ApoE*^{-/-} mice .Values are mean \pm SEM, n = 5 per group.

Discussion

The present study shows that a 70% reduction in cathepsin G activity does not affect the development of atherosclerotic lesions in the aorta of *ApoE*^{-/-} mice fed a high fat diet for 8 weeks but it does impede the progression of early atherosclerotic lesions to more advanced complex lesions. Compared to lesions in the aortic root of *Ctsg*^{+/+}*ApoE*^{-/-} littermates, those of *Ctsg*^{+/-}*ApoE*^{-/-} mice are characterized by a paucity of fibrotic caps, less collagen and smooth muscles cells, more CD68-positive macrophages, fewer numbers of apoptotic cells and reduced necrotic cores. The difference between *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} mice occurs in the absence of differences in plasma cholesterol concentrations or differences in cholesterol distribution amongst lipoprotein sub-fractions. Cathepsin G can activate cytokines such as TNF- α and IL-8 which could potentially contribute to the progression of atherosclerotic lesions. However, we saw no differences between *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-}*Ctsg* mice in the serum levels of either of the cytokines (results not shown).

By limiting the cellularity of the lesion during the early stages of atherosclerosis, apoptosis is considered beneficial whereas, in the later stages of atherosclerosis, apoptosis can lead to instability and greater vulnerability to rupture (Tabas, 2009, Clarke et al., 2006). The reduced number of TUNEL-positive nuclei and cleaved caspase 3-stained area within lesions of *Ctsg*^{+/-}*ApoE*^{-/-} mice that was observed could reflect a decrease in apoptosis and / or more efficient clearance of apoptotic bodies. When total lesions (early and complex) were analyzed, *Ctsg*^{+/-}*ApoE*^{-/-} mice had increased numbers of lesion-associated CD68-positive macrophages compared to *Ctsg*^{+/+}*ApoE*^{-/-} mice but this difference was not apparent when early lesions were analyzed in isolation. Apoptosis of

macrophages may, therefore, occur coincident with, and possibly contribute to, the transformation of simple lesions into complex lesions. Cathepsin G activity has been shown to promote apoptosis by several mechanisms. Due to its proteolytic activity, cathepsin G can cause the detachment of adhesion-dependent cells from the extracellular matrix and from surrounding cells resulting in anoikis, an anchorage-dependent programmed cell death (Bonney and Legrand, 2000b, Boehme et al., 2002). In the case of cardiomyocytes, activation of protein phosphatase, SHP2, by cathepsin G leads to dephosphorylation of focal adhesion kinase (FAK) and impaired activation of the downstream protein kinase B (AKT)-dependent survival pathways (Rafiq et al., 2008a). Cathepsin G has also been shown to impede the engulfment of apoptotic cells by macrophages. Efferocytosis, the process of engulfment of apoptotic bodies, becomes impaired in the later stages of atherosclerosis. With deficient efferocytosis, apoptotic bodies undergo secondary necrosis and more apoptotic nuclei are detected in atherosclerotic lesions (Vandivier et al., 2002, Thorp and Tabas, 2009). Cathepsin G has been shown to destroy an “eat me” signal on apoptotic neutrophils and thus prevents their engulfment by macrophages (Guzik et al., 2007). However, it is unknown if apoptotic macrophages in atherosclerotic lesions present a cathepsin G-sensitive “eat me” signal similar to that of apoptotic neutrophils.

The lesions of *Ctsg*^{+/-}*ApoE*^{-/-} mice show a decreased content of collagen and decreased numbers of smooth muscle cells, and this appeared to be the case even when complex lesions of a similar size were compared. Decreased cathepsin G-mediated degradation of intracellular matrix or decreased cathepsin-G-mediated activation of other proteases capable of degrading extracellular matrix in lesions of *Ctsg*^{+/-}*ApoE*^{-/-} mice may impede

migration of smooth muscle cells into the intima. As synthetic smooth muscle cells are major producers of collagen in atherosclerotic lesions (Barnes and Farndale, 1999), the decreased collagen may reflect the decreased numbers of lesion-associated smooth muscle cells. Although collagen is a substrate for cathepsin G, the reduced cathepsin G activity in *CtsG*^{+/-}*ApoE*^{-/-} mice did not lead to collagen accumulation in lesions.

The role of several broad range cysteine protease members of the cathepsin family has been investigated in mouse models of human atherosclerosis. Cathepsin S deficiency in bone-marrow-derived cells of LDL receptor-deficient (*CatS*^{-/-} x *Ldlr*^{-/-}) mice fed a high fat diet for 12 weeks resulted in atherosclerotic lesions of the aortic root that did not differ in size from those in *CatS*^{+/+} x *Ldlr*^{-/-} mice but showed increased numbers of macrophages, decreased TUNEL-positive cells, decreased collagen and a reduction in the necrotic core (de Nooijer et al., 2009). The absence of an effect of leukocyte-specific cathepsin S deficiency on lesion size and changes in plaque morphology shows a remarkable similarity to what we observed with partial cathepsin G deficiency in *ApoE*^{-/-} mice. It is possible that the two proteases participate sequentially in a pathway that promotes the evolution of the atherosclerotic lesion. Likewise, cathepsin K deficiency in bone marrow-derived cells also altered the morphology of aortic root lesions in *Ldlr*^{-/-} mice fed a high fat diet for 12 weeks without changing lesion size (Guo et al., 2009a). Again, lesions were characterized by an increased number of macrophages and decreased collagen but differed in having an increase in both the necrotic core and in TUNEL-positive nuclei. Thus, these three members of the protease family appear to influence the progression of the atherosclerotic plaque but not the development of the initial fatty streak and may accomplish this through both mutual and distinct mechanisms. It is also

possible that partial cathepsin G deficiency may modulate the expression or activation of other members of the cathepsin family and this could further contribute to the observed phenotype.

We initially postulated that cathepsin G could potentially promote atherogenesis through its ability to activate the renin angiotensin system (Dzau et al., 1987, Hu et al., 2003, Owen and Campbell, 1998a) and the slower progression of atherosclerosis in *Ctsg*^{+/-} *ApoE*^{-/-} mice could therefore result from reduced angiotensin II levels. However, when we compared bone marrow-derived macrophages from *Ctsg*^{+/-} *ApoE*^{-/-} and *Ctsg*^{+/+} *ApoE*^{-/-} mice for their respective production and secretion of angiotensin II, no differences were observed. This was also the case when macrophages were converted to foam cells by incubation with ac-LDL. Likewise, no differences were apparent between *Ctsg*^{+/-} *ApoE*^{-/-} and *Ctsg*^{+/+} *ApoE*^{-/-} mice in lesion-associated immunoreactive angiotensin II when monitored by immunohistochemistry. Thus, if cathepsin G has an important role in the generation of angiotensin II by mouse macrophages, when cathepsin G activity is limited as in the case of macrophages from *Ctsg*^{+/-} *ApoE*^{-/-} mice, other mechanisms for angiotensin II production, such as the renin-angiotensin-converting enzyme pathway, can compensate. It is, therefore, unlikely that the slower progression of atherosclerosis in *Ctsg*^{+/-} *ApoE*^{-/-} mice can be attributed to impaired cathepsin G-mediated generation of angiotensin II.

The intrauterine death of the *Ctsg*^{-/-} *ApoE*^{-/-} fetuses was unanticipated given the viability of *Ctsg*^{-/-} mice when on a wild type background. This could suggest a functional cathepsin G-apolipoprotein E interaction during fetal development. Alternatively, cathepsin G deficiency in the context of hyperlipidemia may be incompatible with normal growth of

the fetus. Consistent with the latter, we have been unsuccessful to date in obtaining *Ctsg*^{-/-} pups on an LDL receptor-deficient background. Experiments are underway to identify the mechanisms that are involved.

Taken together, our findings indicate that cathepsin G activity may not have a major role in the development of early atherosclerotic lesions but can promote the progression of these early lesions to more complex lesions. Over longer periods of time, the larger necrotic zones associated with cathepsin G activity could favour the development of unstable plaques, whereas the increased numbers of lesion-associated smooth muscle cells and increased collagen content could provide a stabilizing influence. Additional experiments will be required to elucidate the biological mechanisms and pathways that are involved, as well as the potential clinical consequences.

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Disclosures

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Author Contributions

Naimeh Rafatian: experimental procedures, experiment design, data analysis, manuscript writing

Frans H.H. Leenen: experimental design, data analysis, manuscript writing

Ross W. Milne: experimental design, data analysis, manuscript writing

Stewart C. Whitman: project conception, experimental design, data analysis

Endnotes

At the request of the authors, readers are herein alerted to the fact that additional materials related to this manuscript (cytokine assays, additional photomicrographs) may be found at the institutional website of one of the authors, which at the time of publication they indicate is:

[http://www.ottawaheart.ca/content_documents/Rafatian_et_al_%281%29_supplementary_figures.pdf]. These materials are not a part of this manuscript, and have not undergone peer review by the American Physiological Society (APS). APS and the journal editors

take no responsibility for these materials, for the website address, or for any links to or from it.

3.1 Online supplement

Supplementary data for the manuscript entitled:

Cathepsin G deficiency decreases complexity of atherosclerotic lesions in apolipoprotein E-deficient mice

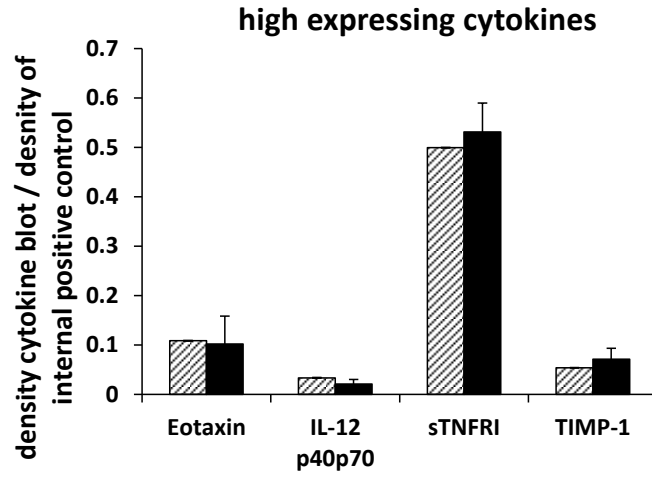
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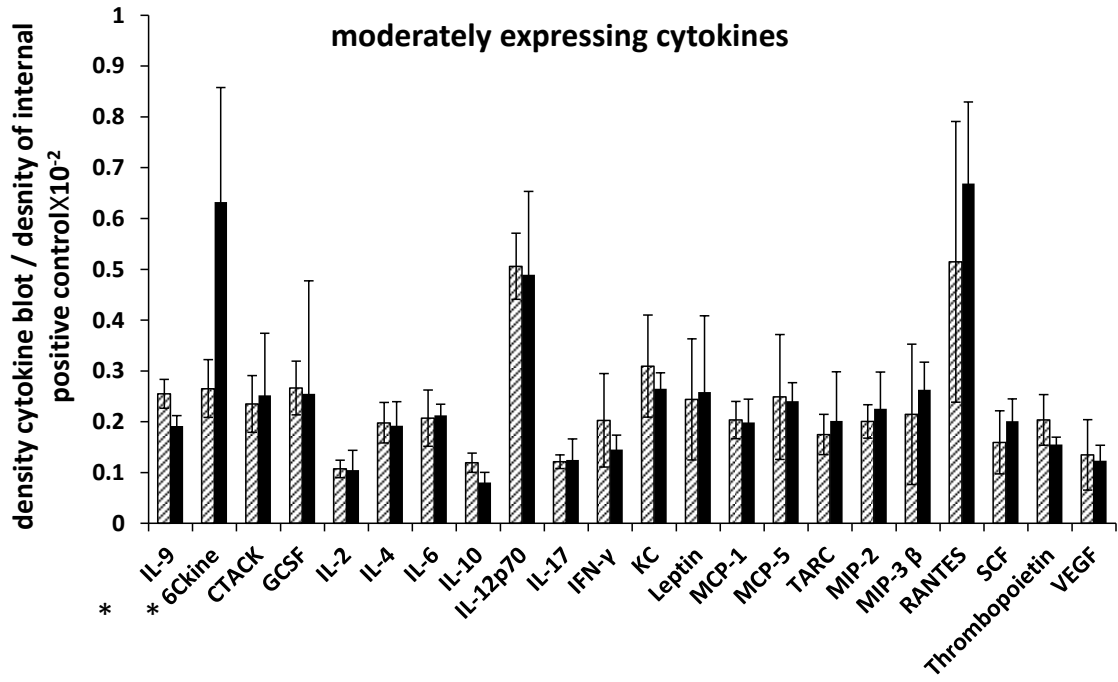
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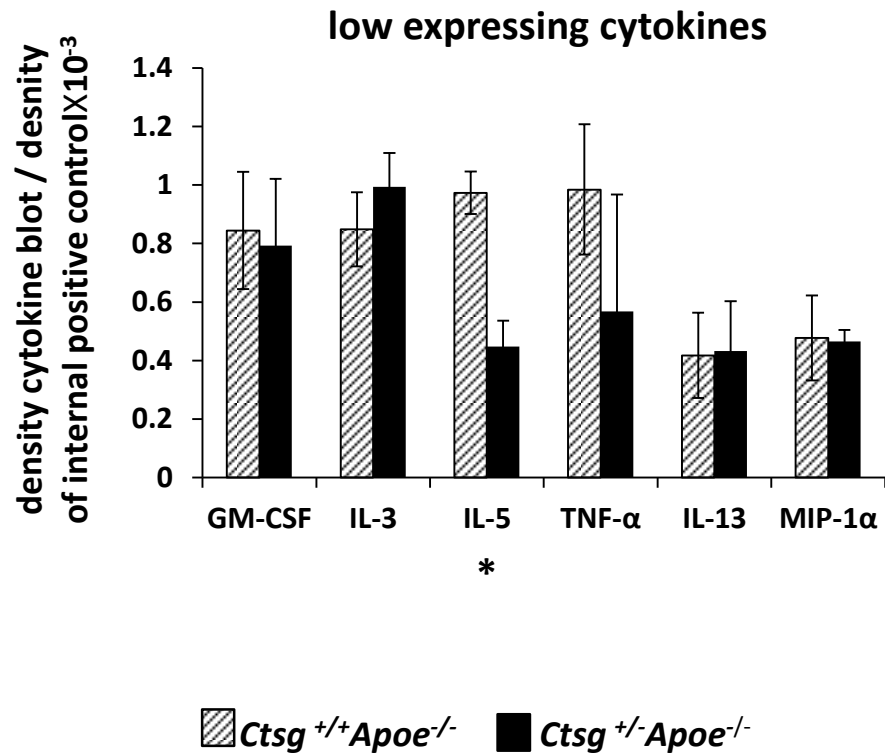
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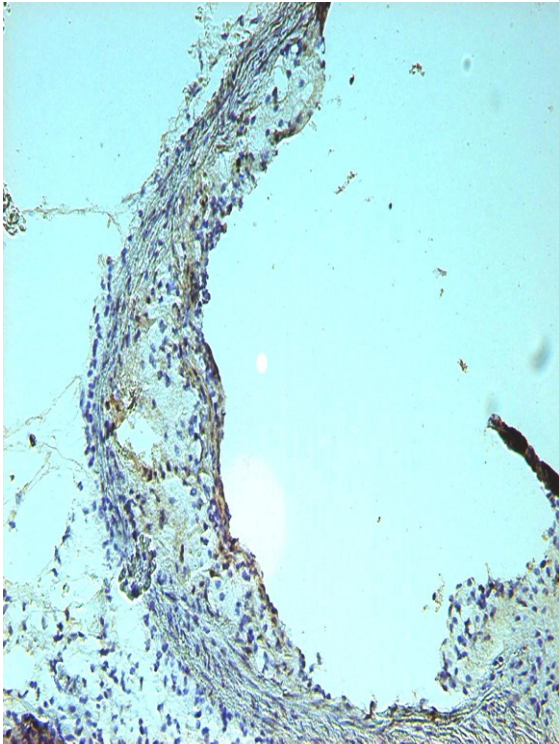
C



Relative cytokine levels in serum of *Ctsg*^{+/+}*Apoe*^{-/-} and *Ctsg*^{+/-}*Apoe*^{-/-} mice after 8 weeks on a high fat diet. Serum cytokines were assayed using the RayBio® Mouse Cytokine Antibody Array G series 2 (Norcross, GA, USA) following the manufacturer's protocol.

Angiotensin II immunohistochemistry

Ctsg^{+/-} Apoe^{-/-}



Ctsg^{+/+} Apoe^{-/-}



Immunoreactive angiotensin II in lesions of *Ctsg^{+/-}Apoe^{-/-}* and *Ctsg^{+/+}Apoe^{-/-}* mice after 8 weeks on a high fat diet. Acetone-fixed frozen aortic root sections, after quenching the endogenous peroxidase and blocking with 2% BSA, were incubated with a 1/100 dilution of an anti-rabbit Ang II antibody, a generous gift from Drs. Schalekamp and Danser (Erasmus University Rotterdam, The Netherlands) for 90 min. After washing, bound antibody was revealed using the Vectastain ABC horse radish peroxidase system (Vector Labs) according to the manufacturer's recommendation.

4. GENERAL DISCUSSION

4.1 Summary of main findings

My first goal was to define the effect of foam cell formation on the renin-angiotensin system. To this end, I measured mRNA and protein level and enzyme activity of components of the RAS in THP-1 cells after exposure to ac-LDL. I showed that treatment of THP-1 macrophages with ac-LDL elevates Ang I and II levels as well as the Ang II to Ang I ratio in cell lysates and media. In contrast, exposure of mouse peritoneal macrophages or bone marrow-derived macrophages to ac-LDL does not have such an effect. Renin activity is crucial for Ang II formation as the renin inhibitor, aliskiren, completely abolishes Ang II production in both ac-LDL- and non-treated THP-1 cells. Exposure to ac-LDL does not, however, change either renin mRNA levels or its enzyme activity. Angiotensinogen mRNA increases after ac-LDL treatment, but its protein level does not change whereas the enhanced levels of ACE and cathepsin G mRNA are associated with higher ACE and cathepsin G enzyme activities in ac-LDL-treated THP-1 cells. Due to technical reasons, I could not assess the role of enhanced cathepsin G activity in Ang II generation in THP-1 cells during foam cell formation. I did observe that Ang II levels were similar in cell lysates and media of bone marrow-derived macrophages from *Ctsg*^{+/-} *Apoe*^{-/-} and *Ctsg*^{+/+} *Apoe*^{-/-} mice whether the cells had or had not been exposed to ac-LDL. I next set out to establish if the RAS contributed to foam cell formation in THP-1 macrophages. I found that blocking endogenously formed Ang II decreases cholesteryl ester accumulation in THP-1 cells, and this is associated with a decrease in SR-A and ACAT-1 proteins.

My third aim was to determine if cathepsin G activity has a role in the development and progression of atherosclerosis using cathepsin G-deficient *ApoE*^{-/-} mice. While *Ctsg*^{-/-} *ApoE*^{-/-} died in utero, *Ctsg*^{+/-} *ApoE*^{-/-} mice developed normally and had around a 70% reduction in cathepsin G activity in bone marrow leukocytes. After two months on high fat diet *Ctsg*^{+/-} *ApoE*^{-/-} and *Ctsg*^{+/+} *ApoE*^{-/-} mice did not differ in terms of serum cholesterol levels, the distribution of cholesterol amongst lipoprotein subclasses, or the atherosclerosis lesion area in the aortic root and aortic arch. However, *Ctsg*^{+/-} *ApoE*^{-/-} mice have fewer stage IV lesions; their atherosclerotic plaques contain more CD68⁺ macrophages and are without SMC or collagen deposition. Moreover fewer apoptotic bodies are found in atherosclerotic lesions from *Ctsg*^{+/-} *ApoE*^{-/-} mice.

4.2 Modulation of RAS gene expression during foam cell formation

Treatment of THP-1 cells with ac-LDL increases the mRNA level of angiotensinogen, ACE and cathepsin G but not the renin mRNA level. We did not explore the mechanisms for the increase in expression of these genes. However, the enhanced expression of angiotensinogen, ACE or cathepsin G can be explained to some extent by previous studies. In foam cell formation, cholesterol loading causes activation of various transcription and nuclear factors that potentially can change the expression pattern of several genes. Cholesterol loading increases free cholesterol inside the cells. Free cholesterol inserts itself into the ER and plasma membrane. Excess cholesterol in the ER induces ER stress which in turn releases Ca²⁺ into the cytosol (Feng et al., 2003, Malhotra and Kaufman, 2007). Ca²⁺ by modulating different pathways which are listed below enhances ROS formation by mitochondria. Ca²⁺ opens mitochondrial pores and as a result

cytochrome C is released into the cytosol. This inhibits complex III in the mitochondrial electron transport chain and enhances ROS formation. Ca^{2+} also stimulates the tricarboxylic acid cycle and nitric oxide synthase and both result in exaggerated ROS generation (Jacobson and Duchon, 2002). ROS modifies the cholesterol inside the foam cells to yield oxysterol which are activating ligands for liver X receptors (LXR). Active LXR induce fatty acid synthetase to help the esterification of free cholesterol in cells by providing fatty acids. The newly synthesized fatty acids together with fatty acids released from endocytosed modified LDL are ligands for the PPAR family of transcription factors including PPAR α and γ (Li and Glass, 2002, Zandbergen and Plutzky, 2007, Dushkin, 2012). ROS induces expression of HIF-1 α in murine macrophages. ROS also activates NF- κ B in the cytosol. Ang II through AT₁R can also trigger NF- κ B by generation of ROS(Kabe et al., 2005). ROS might activate HIF-1 α transcription through NF- κ B since HIF-1 α has an NF- κ B elements in its promoter(Bonello et al., 2007). Blocking Ang II by losartan or ROS by NAC attenuates NF- κ B activation (Li et al., 2011). Activation of these nuclear factors induces differential expression of genes in foam cells and macrophages. Enhanced expression of oxidative stress-related- genes and a variety of proteases in foam cells are examples of differences in the profile of gene expression in macrophages and foam cells.

No previous study assessed the effects of ac-LDL on RAS gene expression in macrophages. Kohlstedt et al (2011) showed that treatment of human blood monocyte-derived macrophages with conditioned media from adipocyte cultures increases ACE expression. They showed that a lipid moiety in the conditioned media is responsible for this increase and activates AMP-activated protein kinase (AMPK) (Kohlstedt et al.,

2011). Moreover, activation of HIF-1 α and early growth response 1(Egr-1) in foam cells (Shatrov et al., 2003, Hung et al., 2006b, Khachigian, 2006b) can also increase ACE expression (Eyries et al., 2002b, Zhang et al., 2009).

The activation of PPAR α and NF- κ B in foam cells can increase expression of angiotensinogen mRNA since both nuclear factors can bind to the angiotensinogen promoter and act as a positive regulator of gene expression. (Ruiz-Ortega et al., 2000b, Shimamoto et al., 2004a)

We had expected an increase in renin mRNA due to activation of peroxisome PPAR α under conditions of cholesterol loading(Zandbergen and Plutzky, 2007). A PPAR-binding site is present in the first intron of the renin gene (Di Nicolantonio et al., 1998, Yu and Di Nicolantonio, 1998) and PPAR α knockout mice have attenuated renin expression (Tordjman et al., 2007). Absence of an increase in renin expression despite activation of PPAR α might be the result of activation of LXR α . Activation of LXR α by lipid ligands attenuates renin expression in cardiac and kidney tissues(Kuipers et al., 2010).

Microarray data did not show changes in cathepsin G expression after 2-4 days treatment of THP-1 cells with ac-LDL and ox-LDL(Hung et al., 2006b). The apparent contradiction with our demonstration of increased cathepsin G expression in THP-1 cells following exposure to ac-LDL might be due to lower sensitivity of the microarray in detection of small changes. HIF is activated in foam cells. HIF-1 α might also be involved in enhanced expression of cathepsin G in foam cells since HIF-1 α activates a transcription factor, Cited 2, which binds to a C/EBP site in the cathepsin G promoter and increases cathepsin G gene expression(Chen et al., 2007, van den Beucken et al., 2007).

4.3 AC-LDL treatment and Angiotensin I and II

Ac-LDL treatment elevates angiotensin I and II levels in THP-1 cells and their media by 50 and 100%, respectively. No previous study reported Ang II generation in macrophages after modified LDL treatment. Increases in angiotensinogen as well as the enzymes that are involved in the conversion to Ang II including renin, ACE or cathepsin G or D enzymes can potentially elevate angiotensin I and II. Ac-LDL does not change the renin activity. However, renin activity plays an essential role in generation of Ang II, since renin inhibition completely abolishes Ang II generation in treated and non-treated THP-1 cells. The complete blockade of Ang II generation in aliskiren-treated THP-1 cells excludes the role of cathepsin D in conversion of angiotensinogen to Ang I and the direct generation of Ang II from angiotensinogen by cathepsin G or the other enzymes. Whatling et al (2004) showed that cathepsin D is not increased in THP-1 foam cells. Angiotensinogen mRNA is increased 2 days after ac-LDL treatment, but not the protein level. This dissociation might be due to increased turnover of angiotensinogen protein. Although plasma renin usually is considered as the rate limiting factor for Ang II generation, in THP-1 foam cells angiotensinogen might be a rate-limiting step in production of angiotensin peptides and the absence of an increase in angiotensinogen protein might be due to higher consumption and degradation of angiotensinogen.

Ac-LDL treatment increased both ACE and cathepsin G mRNA and activity by 50%. Diet et al (1996) showed that ac-LDL treatment increased ACE activity in THP-1 cells. No previous study assessed the effect of modified LDL on cathepsin G activity. The ratio of Ang II to angiotensin I was increased 1.5 fold after a 2 day-exposure of cells to ac-LDL. The higher conversion rate of angiotensin I to II might be a consequence of

enhanced cathepsin G and ACE activity. We were not able to assess the role of ACE in this pathway since traces of ACE inhibitors in our radioimmunoassay were interfering with quantification of Ang II. Similarly we could not evaluate the role of cathepsin G in the increased Ang II levels since applying cathepsin G inhibitor for a long period in cell culture had toxic effects. In cells and media from cultured peritoneal and bone marrow-derived macrophages of cathepsin G heterozygous knockout and cathepsin G wild type mice on *apoeE*^{-/-} background the Ang II level is similar, indicating that a 70% decrease in cathepsin G activity does not result in any significant changes in Ang II level. Similarly, cathepsin G partial deficiency does not affect Ang II levels detected by immunohistochemistry in atherosclerotic lesions of *Ctsg*^{+/-} *Apoe*^{-/-} and *Ctsg*^{+/+} *Apoe*^{-/-} mice. However, these findings do not rule out a role for cathepsin G in generation of Ang II in human cells. In vitro experiments using human and dog heart and arterial lysates an ACE inhibitor only partially (20-80%) blocked AngII generation (Urata et al., 1990, Balcells et al., 1996), whereas almost all of the Ang II-forming ability of rodents heart lysates is blocked by ACE inhibitors (Okunishi et al., 1993). Our results are consistent with the existence of species differences between humans and mice. Not only we did not see any difference between cathepsin G deficient and wild type macrophage Ang II levels which suggests the alternative pathway seems to be more important in humans but also because we failed to observe an enhancement in Ang II generation in mouse peritoneal and bone marrow-derived macrophages that had been exposed to modified LDL.

Overall, it appears that in our model the increased renin-dependent conversion of angiotensinogen together with elevated activity of ACE and cathepsin G activity are responsible for the increase in angiotensin I and II formation in ac-LDL treated samples.

4.4 Ang II and foam cell formation

RAS blockade reduced cholesterol ester accumulation in both ac-LDL and ox-LDL treated cells. Consistent with our study Hayek et al (2000 and 2002) showed that in mice and in human patients treated with ramipril or losartan, respectively, cholesterol ester formation was attenuated. Recently, Osada-Oka et al (2012) also showed that in THP-1 cells exposed to ox-LDL losartan and candesartan decreased foam cell formation.

Our results suggest that macrophage derived Ang II activates a signaling pathway through AT₁R that is essential for cholesteryl ester accumulation in THP-1 cells exposed to ac-LDL or ox-LDL. Blocking Ang II by inhibiting renin, ACE or AT₁ receptors decreases ac-LDL-induced cholesteryl ester accumulation in THP-1 cells. While cholesterol loading in ac-LDL-treated THP-1 cells is basically abolished by the renin inhibitor, aliskiren, and the AT₁ receptor antagonist, losartan, suppression of cholesteryl ester accumulation by the ACE inhibitors, captopril and enalapril, is around 80%. One possibility for the incomplete suppression of cholesteryl ester accumulation by captopril and enalapril is that the dose of inhibitor used did not achieve total inhibition of ACE activity. Alternatively, the difference between ACE inhibitor and renin and AT₁R blocker might be due to alternative pathways such as cathepsin G that can participate in the conversion of angiotensin I to Ang II (Klickstein et al., 1982). Unfortunately, due to toxicity of cathepsin G inhibitors, we could not directly test the contribution of cathepsin G to Ang II generation.

Foam cell formation is the result of an imbalance between cholesterol influx and efflux and is represented by formation of cholesterol ester droplet inside the cells (Gkretsi et al., 2011). Atherogenic lipoproteins are modified lipoproteins carrying apoB. These lipoproteins are not entering cells through the tightly controlled LDL receptor but the influx is either through scavenger receptors such as SR-A, CD36 or LOX-1 or non-receptor mediated endocytosis and transcytosis (Vasile et al., 1983, Yla-Herttuala et al., 1991, Tabas et al., 2007, Greaves and Gordon, 2009). Free cholesterol inside the cells is converted to cholesteryl ester by the enzyme acyl-coenzymeA cholesterol acyl transferase 1 (ACAT-1) for storage. Cholesteryl esters can be subsequently hydrolyzed by neutral cholesterol ester hydrolase and the resulting cholesterol can be effluxed from the cells to extracellular receptors (eg. Apolipoprotein A-I, HDL), a process mediated by ABCA1 and ABCG1 (Sekiya et al., 2011). Zhou et al (2009) showed that Ang II, by attenuating ABCA1 expression exaggerates foam cell formation. In *ApoE*^{-/-} mice plasma Ang II increases and macrophages from these animals had lower ABCA1 expression (Zuo et al., 2009). We did not assess the efflux pathway since under our experimental condition, an extracellular cholesterol acceptor was absent and so it was unlikely that the efflux pathway can affect cholesteryl ester accumulation in the THP-1 cells.

Ac-LDL is taken up primarily by the SR-A, whereas the more physiologically relevant ox-LDL is taken up by both the SR-A and other macrophage receptors such as CD36 (Agrawal et al., 2007). We show that blocking renin and AT₁R by aliskiren and losartan, respectively, reduce the cholesteryl ester accumulation in ox-LDL-treated cells. Blocking Ang II by an ACE inhibitor or AT₁R blocker attenuates cholesteryl ester formation

after ox-LDL treatment by decreasing CD36 expression in mice or human patients treated with ramipril or losartan, respectively(Hayek et al., 2000, Hayek et al., 2002). Thus, the endogenous RAS by modulating multiple cell surface receptors for modified lipoproteins appears to have an essential role in foam cell formation. Treatment of THP-1 cells with ac-LDL elevates SR-A protein level which and this can be prevented by inhibition of renin, ACE or AT₁R. As, these treatments do not change SR-A mRNA levels the alterations in SR-A protein levels appear to be independent of changes in gene expression and to occur at a translational or post translational level. Exposure of THP-1 cells to ox-LDL decreases SR-A protein and it is further reduced by aliskiren and losartan treatment. The down-regulation of SR-A protein by ox-LDL could be the result of the mutual regulation of SR-A and CD36 expression and /or the degree of oxidation of the LDL and its content of specific oxidized lipid(Yoshida et al., 1998, Hayden et al., 2002, Makinen et al., 2010) .

Ang II by induction of NADPH oxidase through AT₁R increases ROS formation. The exact mechanism through which Ang II induced ROS formation in macrophages is not well understood. Two of the NADPH oxidase subunits, gp91phox and p22phox are located on cell membranes and a number of cytosolic subunits including p47 and p67 are recruited to assemble the active enzyme (Garrido and Griendling, 2009). Ang II also activates protein kinase C activation which phosphorylates the P47 and p67 NADPH cytosolic subunits (Zafari et al., 1999). In macrophages activation of ERK or p38 MAP kinase phosphorylates phospholipase A2, which in turn promotes the translocation of phosphorylated P47 and p67 to the membrane(Hazan-Halevy et al., 2005). Ang II through guanine nucleotide exchange factor activates the small G protein Rac1 (Zuo et

al., 2005). Rac1 acts as a regulatory factor for NADPH oxidase. Fully functional NADPH generates ROS which can trigger a second oxidative burst by activation of mitochondrial ROS pathways or other cytosolic enzymes (Zhang et al., 2007). Consistent with the concept that ROS contributes to the effect of the RAS on foam cell formation, blockade of ROS by the anti-oxidant, N-acetyl cysteine, decreases cholesteryl ester accumulation in THP-1 cells exposed to either ac-LDL or ox-LDL. On the other hand ROS-associated signalling pathway is not involved in SR-A expression in ox-LDL-treated samples since NAC treatment did not change SR-A expression. Previous studies showed that attenuation of ROS by N-acetyl cysteine decreases foam cell formation by decreasing CD36 and increasing apo E in ox-LDL treated THP-1 cells (Sung et al., 2012, Lee et al., 2009b, Schmitz and Grandl, 2007).

Our results suggest a role for Ang II in regulation of cholesteryl ester accumulation in macrophages through ACAT-1. We show that the level of immunoreactive ACAT-1 in THP-1 cells is attenuated by inhibiting the formation of Ang II or by blocking Ang II binding to AT₁Rs. This is consistent with Kanome et al (2008) who showed that exposure of human peripheral blood monocyte-derived macrophages to exogenous Ang II increases the expression of the shorter forms of ACAT-1 transcripts and enhances ACAT-1 protein level. This elevation in ACAT-1 expression exaggerates cholesteryl ester formation in the cells without any changes in the SR-A protein level. However, it is difficult to compare the observations of Kanome et al to the result of our study as they did not treat the cells with ac-LDL and they only assessed the effect of supra-physiological doses of exogenous Ang II. Surprisingly, aliskiren and losartan-treated THP-1 cells exposed to ac-LDL show an increase rather than decrease

in ACAT-1 mRNA levels. This might be due to the use of primers that detect all ACAT-1 transcripts and do not differentiate between various transcripts. Osada-Oka et al (2012) recently reported that exposure of THP-1 cells to ox-LDL and AT₁R blockade by candesartan and losartan at 10 and 20 μ M decreased foam cell formation by attenuating ACAT-1 expression. In their study ACE inhibition by captopril and enalapril at 100 μ M, was ineffective and the authors concluded that the decrease in foam cell formation by AT₁R blockers was independent of endogenously generated Ang II. Instead they proposed that the ability of the AT₁R inhibitors to attenuate foam cell formation is due to decrease epidermal growth factor (EGF) receptor activation by ox-LDL. The discrepancy between our study and their observation in terms of the inability of ACE inhibitors to prevent cholesteryl ester formation might be due to the lower dose of inhibitors they used and the shorter incubation time with ox-LDL (Osada-Oka et al., 2012). While losartan-mediated blockade of EGF receptor activation may contribute to attenuation of foam cell formation, our findings that renin or ACE blockade prevents accumulation of cholesteryl ester in THP-1 cells treated with modified LDL underline the essential role of Ang II generation by endogenous RAS in foam cell formation.

In addition to the afore mentioned mechanisms for the effects of AngII on foam cell formation, Ang II can enhance cholesterol biosynthesis in macrophages through up-regulation of HMGCoA reductase (Keidar et al., 1999). However, the presence of excess cholesterol in the form ac-LDL in our experimental conditions makes this possibility unlikely.

Based on our results, we propose a model in which treatment of THP-1 cells with ac-LDL up-regulates the RAS leading to enhanced production of Ang II. Ang II binds to the AT₁R and activates a signaling cascade which leads to enhanced synthesis or reduced catabolism of SR-A and ACAT-1 and, as a result, exaggerated cholesteryl ester accumulation. The ability of RAS blockers to interfere with this Ang II positive feedback loop may contribute in the anti-atherogenic properties of these drugs.

4.5 Cathepsin G promote atherosclerotic lesion complexity

Ctsg^{+/-}*ApoE*^{-/-} mice fed a high fat diet for 8 weeks show a delayed progression of fatty streaks into complex lesions compared to *Ctsg*^{+/+}*ApoE*^{-/-} littermates. As cathepsin G can generate Ang II from either angiotensinogen or Ang I and there is ample evidence that increased Ang II is atherogenic, it is possible that reduced cathepsin G activity in the *Ctsg*^{+/-}*ApoE*^{-/-} mice could slow progression of atherosclerosis through decreased Ang II production. However, we did not observe differences in levels of Ang II generated intracellularly or extracellularly by peritoneal or bone marrow-derived macrophages isolated from *Ctsg*^{+/-}*ApoE*^{-/-} or *Ctsg*^{+/+}*ApoE*^{-/-} mice. The similar generation of Ang II in cathepsin G wild type and heterozygous is not affected by ac-LDL treatment. Moreover, cathepsin G partial deficiency does not affect immunoreactive angiotensin peptides in atherosclerotic lesions within the aortic root. Thus, it appears unlikely that the enhanced progression of atherosclerosis in *Ctsg*^{+/+}*ApoE*^{-/-} relative to their *Ctsg*^{+/-}*ApoE*^{-/-} littermates can be attributed to modulation of cathepsin-G-mediated generation of Ang II.

Apoptosis in atherosclerotic lesions has two sides in early atherosclerotic lesion by limiting the cellularity of the plaques, apoptosis is considered beneficial, whereas in the

later stages of atherosclerosis, apoptosis may promote instability and vulnerability to rupture (Clarke et al., 2006, Tabas, 2009). The decrease in the number of TUNEL-positive nuclei in *Ctsg*^{+/-}*ApoE*^{-/-} mice could reflect a reduction in apoptosis and/ or more efficient clearance of apoptotic bodies. Anoikis an anchorage-dependent programmed cell death might happen as a consequence of cathepsin G proteolytic activity which causes the detachment of adhesion-dependent cells from the extracellular matrix and from adjacent cells (Boehme et al., 2002, Bonnefoy and Legrand, 2000a) . Activation of the protein phosphatase, SHP2, in cardiomyocytes, by cathepsin G leads to dephosphorylation of focal adhesion kinase (FAK) and impaired activation of the downstream pro-survival pathway of protein kinase B (AKT) (Rafiq et al., 2008b). While degradation of ECM by cathepsin G, could potentially promote cell detachment and anoikis, the atherosclerotic plaques within the aortic root of *Ctsg*^{+/+}*ApoE*^{-/-} mice showed an increase rather than a decrease in the collagen content compared to those of *Ctsg*^{+/-}*ApoE*^{-/-} littermates. Efferocytosis, the process of engulfment of apoptotic bodies, is impaired in the later stages of atherosclerosis. Under conditions of impaired efferocytosis, apoptotic bodies undergo secondary necrosis therefore more apoptotic bodies are detected in atherosclerotic lesions (Vandivier et al., 2002, Thorp and Tabas, 2009). Cathepsin G has been shown prevent neutrophil engulfment by macrophages because it can destroy an “eat me signal” on apoptotic neutrophils (Guzik et al., 2007). If apoptotic macrophages were to express such a cathepsin G-sensitive “eat me” signal, the reduced number of apoptotic bodies in lesions of *Ctsg*^{+/-}*ApoE*^{-/-} could reflect more efficient efferocytosis.

The role of several other members of the cathepsin family in the development and progression of atherosclerosis has been investigated in mouse models. Cathepsin S deficiency in bone marrow of *Ldlr*^{-/-} mice after 12 weeks of a high fat diet do not change the area occupied by atherosclerotic lesions in the aortic root compared to *Ldlr*^{-/-} mice with a wild type cathepsin S gene but they show elevated numbers of macrophages, a reduced number of TUNEL-positive cells, a decrease in collagen content and a reduced necrotic core size (de Nooijer et al., 2009). This is a phenotype that is strikingly similar to that which we observed with partial cathepsin G deficiency in *ApoE*^{-/-} mice after 8 weeks on a high fat diet. It is possible that these two proteases are targeting some common substrates and activating common mechanisms to promote the progression of early atherosclerotic lesions to more advanced plaque forms. Cathepsin K deficiency in bone marrow-derived cells also alters plaques morphology without changing the lesion size in the aortic root lesion of *Ldlr*^{-/-} mice fed a high fat diet for 12 weeks (Guo et al., 2009b). Again, the lesions have an increased number of macrophages and decreased collagen but, in contrast to what we observed in *Ctsg*^{+/-}*ApoE*^{-/-} mice lesions have an increased necrotic core size and more TUNEL-positive nuclei. Thus, these three members of the cathepsin family, though not from the same protease type (ie, cathepsin K and cathepsin S are cysteine proteases whereas cathepsin G is a serine protease) appear to influence the progression of the atherosclerotic lesion but not the development of the initial fatty streak. This ability to alter plaque morphology likely occurs through both shared and distinct pathways.

Cathepsin G potentially is able to change the active concentration of various inflammatory modulators. The serum levels of three cytokines/ chemokines, IL-5, IL-9

and 6Ckine differed between *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} after 8 weeks of a high fat diet. The biological significance of these changes is unclear; as anti-inflammatory cytokines IL5 and IL9 are lower in plasma of cathepsin G heterozygous mice and the levels of 6Ckine is increased. 6Ckine level usually is higher in atherosclerosis and especially when there are unstable atherosclerotic plaques (Binder et al., 2005). It is unlikely that the altered levels of these factors in the circulation directly contributes to the difference between atherogenesis in *Ctsg*^{+/-}*ApoE*^{-/-} and *Ctsg*^{+/+}*ApoE*^{-/-} but may possibly be a consequence of the more rapid progression of atherosclerosis in the *Ctsg*^{+/+}*ApoE*^{-/-} mice.

4.6 Intra uterine death of *Ctsg*^{-/-}*ApoE*^{-/-} mice

We were forced to use *Ctsg*^{+/-}*ApoE*^{-/-} for our experiments as we were unable to obtain viable *Ctsg*^{-/-}*ApoE*^{-/-} pups. As *Ctsg*^{-/-} mice on a wild type background show normal foetal development and adult *Ctsg*^{-/-} mice have no morphological abnormalities (Abbott et al., 1998b, MacIvor et al., 1999), the intrauterine death of the *Ctsg*^{-/-}*ApoE*^{-/-} mice was unanticipated. The death of the *Ctsg*^{-/-}*ApoE*^{-/-} fetuses may reflect the existence of parallel, alternative pathways that require cathepsin G and apo E, respectively. The two pathways may be mutually compensatory in the absence of either apoE or cathepsin G but non-functional if the foetus lacks both cathepsin G and apoE. Another possibility is that cathepsin G deficiency in the context of hyperlipidemia is incompatible with normal growth of the fetus. In support of this we were unable to obtain cathepsin G knockout pups on an *Ldlr*^{-/-} background. It has recently been shown that proteoglycan-bound apoE has an important role in regulating the proliferation of hematopoietic stem cells (HSCs)

by promoting cholesterol efflux mediated by ABCA1 and ABCG1 (Murphy et al., 2011). HSCs from *ApoE*^{-/-} mice show defective cholesterol efflux that leads to cholesterol accumulation. This, in turn, causes an up-regulation of the interleukin 3 (IL-3) / granulocyte-macrophage colony stimulating factor (GM-CSF) receptor and hypersensitivity to IL-3 / GM-CSF signaling. As a consequence there is unregulated proliferation and expansion of HSCs. This could be reversed by infusion of recombinant HDL which would promote cholesterol efflux from the HSCs. Cathepsin G is expressed in early myeloid cells (Hanson et al., 1990a, Grisolano et al., 1994) and expression decreases during differentiation. The role of cathepsin G in early myeloid cells is unknown but one possible function, in concert with related proteases, could be cleavage and inactivation of cytokines and growth factors (Zhao et al., 2005, El-Ouriaghli et al., 2003). Given the increased sensitivity of HSCs from *ApoE*^{-/-} mice to IL-3 / GM-CSF signaling, the effects on the proliferation of *ApoE*^{-/-} HSC could be further amplified by decreased cytokine / growth factor degradation due to cathepsin G deficiency. This could result in a lethal defect in hematopoiesis. A detailed morphological, hematological and biochemical study of the *Ctsg*^{-/-}*ApoE*^{-/-} mice during foetal development is required to get further clues to the mechanisms involved in their intrauterine lethality.

4.7 Conclusions

Ang II has been shown to accelerate atherosclerosis and my studies provide evidence that locally produced Ang II could be important in this process. In this study, I have shown that ac-LDL increases the Ang II level in THP-1 cells and media but not murine macrophages. While renin has an essential role in generation of Ang II in the THP-1

cells, the increase in AngII can be explained to some extent by enhanced expression and activity of ACE and cathepsin G. The relative contribution of these two enzymes to the elevated level of AngII generated by THP-1 cells exposed to ac-LDL is not clear. Although partial deficiency of cathepsin G did not modulate Ang II level in mouse macrophages, there may be specific difference in the pathways responsible for Ang II generation between humans and mice. As noted above, mouse macrophages did not show increased Ang II in response to ac-LDL exposure. I have further shown that Ang II produced endogenously plays a role in accumulation of cholesteryl ester in THP-1 macrophages exposed to modified LDL (both ac-LDL and ox-LDL) since blocking formation of AngII or its activity reduces cholesteryl ester content of these cells. RAS blockade in THP-1 cells exposed to modified LDL attenuates foam cell formation which could in part, be due to causing a decrease in the SR-A protein without changing its mRNA. Moreover, a reduction in ACAT-1 protein level as a consequence of RAS blockade could also contribute to the attenuation of cholesteryl ester accumulation in modified LDL-treated THP-1 cells. Together, my results show that, in an environment where macrophages are exposed to modified lipoproteins, a positive feedback loop exists in which macrophages take up modified lipoproteins and this stimulates endogenous Ang II generation. By binding to AT₁R the endogenously generated Ang II then activates pathways that promote further cholesteryl ester accumulation , possibly by increasing modified LDL uptake due to higher level of cell surface SR-A and increasing cholesteryl esterification due to augmented ACAT-1 protein. This is illustrated in the model shown in figure 4-1.

My studies further provide novel evidence that cathepsin G does not have a major, unique role in the development of early atherosclerotic lesions but contributes to their progression to more complex lesions characterized by a large necrotic core, extensive collagen deposition and the presence of smooth muscle cells within the lesion.

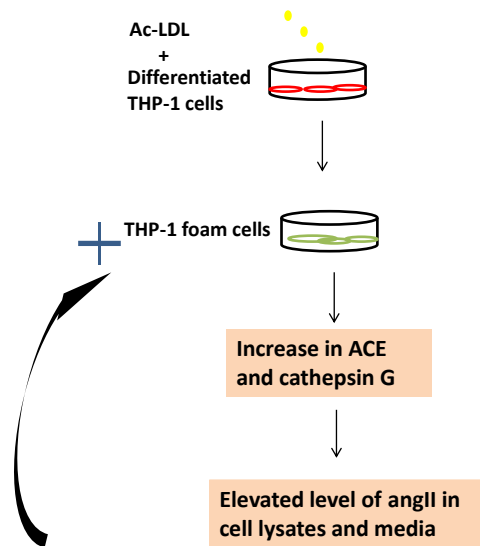


Figure 4-1 schematic summary of Ang II and foam cell formation.

4.8 Future directions

In this study we did not assess how RAS genes and cathepsin G are affected by ac-LDL treatment. The actual role of proposed nuclear factors in modulation of the RAS could be assessed by treatment with the suggested nuclear factors agonists and antagonists. Moreover, the role of cathepsin G in Ang II formation in macrophages and foam cell was not assessed due to toxicity of the cathepsin G inhibitor; attenuating cathepsin G

expression by siRNA should provide an alternative method to evaluate the role of cathepsin G in Ang II generation in human macrophages.

It would also be important to extend the studies on the role of cathepsin G in atherosclerosis to look at later stages of atherosclerosis by keeping animals on high fat diet for a longer period for example 16-18 weeks. Likewise one could monitor another vascular bed which would give a more comprehensive understanding of how cathepsin G contributes to atherosclerotic lesion complexity. By looking at longer periods on high fat diet, one could determine if partial cathepsin G deficiency prevents; or merely delays, the progression of early to advanced lesions and whether it influences both the size and composition of more advanced plaques. One might also be able to test the effects of complete cathepsin G deficiency in hematopoietic cells on atherogenesis in *Ldlr*^{-/-} mice using a bone marrow transplantation model.

One of the most intriguing observations from my studies was the intrauterine death of the *Ctsg*^{-/-}*ApoE*^{-/-} fetuses. A detailed histological and hematological study of *Ctsg*^{-/-}*ApoE*^{-/-} embryos/foetuses could reveal the morphological defects that result from the presence of combined apoE and cathepsin G deficiency which in turn could give clues to the mechanisms involved. As *Ctsg*^{-/-}*Ldlr*^{-/-} fetuses were also not viable this suggests a complex interaction between cathepsin G and the LDL receptor pathway or that complete cathepsin G deficiency is only lethal in the context of hyperlipidemia. Although statins are less efficient in lowering circulating cholesterol levels in mice than in humans, they do inhibit HMG-CoA reductase and modulate the components of the LDLr pathway. It could be informative to treat pregnant mothers with statins to determine if this could allow the survival of *Ctsg*^{-/-}*ApoE*^{-/-} fetuses.

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