

Carboxyl-Terminal Cleavage of Apolipoprotein A-I by Human Mast Cell Chymase Impairs Its Anti-Inflammatory Properties

Su Duy Nguyen, Katariina Maaninka, Jani Lappalainen, Katariina Nurmi, Jari Metso, Katariina Öörni, Mohamad Navab, Alan M. Fogelman, Matti Jauhiainen, Miriam Lee-Rueckert, Petri T. Kovanen

Objective—Apolipoprotein A-I (apoA-I) has been shown to possess several atheroprotective functions, including inhibition of inflammation. Protease-secreting activated mast cells reside in human atherosclerotic lesions. Here we investigated the effects of the neutral proteases released by activated mast cells on the anti-inflammatory properties of apoA-I.

Approach and Results—Activation of human mast cells triggered the release of granule-associated proteases chymase, trypsin, cathepsin G, carboxypeptidase A, and granzyme B. Among them, chymase cleaved apoA-I with the greatest efficiency and generated C-terminally truncated apoA-I, which failed to bind with high affinity to human coronary artery endothelial cells. In tumor necrosis factor- α -activated human coronary artery endothelial cells, the chymase-cleaved apoA-I was unable to suppress nuclear factor- κ B-dependent upregulation of vascular cell adhesion molecule-1 (VCAM-1) and to block THP-1 cells from adhering to and transmigrating across the human coronary artery endothelial cells. Chymase-cleaved apoA-I also had an impaired ability to downregulate the expression of tumor necrosis factor- α , interleukin-1 β , interleukin-6, and interleukin-8 in lipopolysaccharide-activated GM-CSF (granulocyte-macrophage colony-stimulating factor)- and M-CSF (macrophage colony-stimulating factor)-differentiated human macrophage foam cells and to inhibit reactive oxygen species formation in PMA (phorbol 12-myristate 13-acetate)-activated human neutrophils. Importantly, chymase-cleaved apoA-I showed reduced ability to inhibit lipopolysaccharide-induced inflammation in vivo in mice. Treatment with chymase blocked the ability of the apoA-I mimetic peptide L-4F, but not of the protease-resistant D-4F, to inhibit proinflammatory gene expression in activated human coronary artery endothelial cells and macrophage foam cells and to prevent reactive oxygen species formation in activated neutrophils.

Conclusions—The findings identify C-terminal cleavage of apoA-I by human mast cell chymase as a novel mechanism leading to loss of its anti-inflammatory functions. When targeting inflamed protease-rich atherosclerotic lesions with apoA-I, infusions of protease-resistant apoA-I might be the appropriate approach. (*Arterioscler Thromb Vasc Biol.* 2016;36:274-284. DOI: 10.1161/ATVBAHA.115.306827.)

Key Words: apolipoprotein A-I ■ carboxyl-terminal cleavage ■ chymase ■ endothelial cells ■ inflammatory ■ mast cell ■ proteases

Circulating high-density lipoprotein (HDL) comprises a spectrum of lipoproteins ranging from nascent discoidal to mature spherical particles, the former having pre β - and the latter α -electrophoretic mobility.¹ Irrespective of their shape, size, or composition, all HDL particles contain either a single copy or multiple copies of apolipoprotein A-I (apoA-I), a polypeptide with an apparent molecular weight of 28 000 kDa. Both lipid-free apoA-I and the nascent lipid-poor pre β -HDL are the primary acceptors of cholesterol effluxed via the

ATP-binding cassette transporter A1 (ABCA1) from macrophage foam cells,² and so play critical roles in promoting reverse cholesterol transport in vivo. Although the circulating blood contains only minute amounts of pre β -HDL, these particles are enriched in human interstitial fluids.³ This appears also to apply to the arterial intimal fluid, with a concentration of HDL almost 40% of that in plasma, and in which most of the HDL particles have a density comparable to the very high-density lipoprotein subclass and contain only apoA-I.⁴

Received on: March 21, 2014; final version accepted on: November 18, 2015.

From the Wihuri Research Institute, Biomedicum Helsinki, Helsinki, Finland (S.D.N., K.M., J.L., K.N., K.Ö., M.L.-R., P.T.K.); National Institute for Health and Welfare, Genomics and Biomarkers Unit, Biomedicum Helsinki, Helsinki, Finland (J.M., M.J.); and Division of Cardiology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles (M.N., A.M.F.).

The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.115.306827/-/DC1>.

Correspondence to Petri T. Kovanen, MD, Wihuri Research Institute, Biomedicum Helsinki, Haartmaninkatu 8, FI 00290 Helsinki, Finland. E-mail petri.kovanen@wri.fi

© 2015 The Authors. *Arteriosclerosis, Thrombosis, and Vascular Biology* is published on behalf of the American Heart Association, Inc., by Wolters Kluwer. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial-NoDerivs License, which permits use, distribution, and reproduction in any medium, provided that the original work is properly cited, the use is noncommercial, and no modifications or adaptations are made.

Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.115.306827

Nonstandard Abbreviations and Acronyms	
(A-I)rHDL	apoA-I-containing reconstituted HDL
ABCA1	ATP-binding cassette transporter A1
apoA-I	apolipoprotein A-I
EC	endothelial cells
HCAECs	human coronary artery endothelial cells
HDL	high-density lipoprotein
IL	interleukin
NF-κB	nuclear factor-κB
TNFα	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule-1

Current data suggest that by regulating cellular cholesterol homeostasis, HDL can also regulate inflammatory responses in various types of cells that have been activated by proinflammatory stimuli in the arterial wall.⁵ Importantly, proinflammatory activation of the endothelium is regarded critical for the initiation and progression of atherosclerosis. Mechanistically, dysfunctional endothelium may arise when activated endothelial cells (ECs) express the vascular cell adhesion molecule-1 (VCAM-1) or the intercellular adhesion molecule-1 that trigger leukocyte adhesion to the activated ECs.⁶ Both lipid-free apoA-I and HDL particles have been shown to exert potent anti-inflammatory effects on activated cultured ECs of human, bovine, or murine origin⁷⁻⁹ and also on other cell types involved in atherogenesis, such as human monocytes¹⁰ and monocyte-derived macrophages.^{11,12} The anti-inflammatory actions of apoA-I and HDL have been shown to involve attenuation of nuclear factor-κB (NF-κB) activation in various types of human ECs when they are exposed to proinflammatory stimuli, such as tumor necrosis factor (TNF-α), lipopolysaccharide (LPS), or palmitic acid.^{8,13-15} ApoA-I exhibits anti-inflammatory functions also in vivo, as demonstrated by injecting into rabbits apoA-I in the lipid-free form, or as a component of discoidal reconstituted HDL (rHDL) or of mature spherical HDL.^{16,17}

In atherosclerotic lesions, the infiltrating inflammatory cells include mast cells, which upon activation and ensuing degranulation release neutral serine proteases, among them chymase and tryptase, both capable of cleaving the various apolipoproteins present in HDL particles.¹⁸ Importantly, mast cell chymase efficiently cleaves lipid-free apoA-I and depletes preβ-HDL particles, and so blocks their ability to promote ABCA1-dependent cholesterol efflux from macrophage foam cells in vitro and in vivo.¹⁹⁻²² Here we hypothesized that proteolytic cleavage of apoA-I by chymase could also influence its anti-inflammatory activities. Our data demonstrate that C-terminal cleavage of apoA-I by mast cell chymase impairs its ability to suppress proinflammatory responses in vitro and in vivo. Similarly, plasmin-treated apoA-I also failed to exert anti-inflammatory action on activated human coronary artery endothelial cells (HCAECs) in culture. The present study discloses a pathophysiological link between proteolytic cleavage of apoA-I and vascular inflammation.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Proteolysis Impairs the Ability of ApoA-I to Attenuate TNF-α-Induced VCAM-1 Expression in HCAECs

HCAECs were preincubated with lipid-free apoA-I, then exposed to TNF-α, and the expression of VCAM-1 protein, as a cell surface marker of proinflammatory activation,²³ was analyzed by flow cytometry. Stimulation with TNF-α shifted the fluorescence peak from left to right, indicating upregulation of VCAM-1, whereas pretreatment with apoA-I partially prevented it, as indicated by the lesser right-shift of the fluorescence peak (Figure 1A). To evaluate the effect of proteolysis on this anti-inflammatory function of apoA-I, we incubated apoA-I with recombinant human chymase and compared the abilities of the untreated and chymase-treated apoA-I to inhibit TNF-α-dependent VCAM-1 expression. As shown in Figure 1B, addition of increasing concentrations of untreated apoA-I to the cultured HCAECs suppressed VCAM-1 upregulation in a dose-dependent fashion, with maximal suppression observed at 50 μg/mL. In contrast, chymase-treated apoA-I totally failed to prevent the TNF-α-dependent induction of VCAM-1 expression.

Analysis of the apoA-I degradation products by Western blotting indicated that chymase completely depleted the full-length apoA-I and generated a large-sized polypeptide band which had lost immunoreactivity against a monoclonal antibody recognizing a C-terminal epitope encompassing the amino acid residues 211–220 (Figure 1C, left). In agreement with a previous report, chymase did not modify apoA-I reactivity with a monoclonal antibody specific for the N-terminal region (amino acids 2–8).²⁰ A similar degradation pattern was observed when apoA-I was treated with conditioned culture medium in which human mast cells had been activated to degranulate and release a complex mixture of granule neutral proteases, including chymase.²⁴ Accordingly, incubation of apoA-I with such preconditioned medium resulted in loss of C-terminal reactivity in a concentration-dependent manner (Figure 1C, right). We further determined the individual contribution of each of the 5 released proteases (chymase, tryptase, cathepsin G, carboxypeptidase A3, and granzyme B) to apoA-I degradation. For this purpose, specific protease inhibitors were used. Regarding inhibition of the 2 chymotryptic enzymes, that is, chymase and cathepsin G, the chymase inhibitor specifically inhibited chymase, whereas the cathepsin G inhibitor was found to inhibit both chymase and cathepsin G. We observed that selective inhibition of chymase almost completely blocked apoA-I degradation, whereas the combined inhibition chymase and cathepsin G completely blocked it. In contrast, inhibition of tryptase, carboxypeptidase A3, or granzyme B only slightly inhibited proteolysis or had no effect (Figure I in the online-only Data Supplement). These results indicated that among the neutral proteases released by activated human mast cells, chymase was the main protease responsible for the mast cell-dependent loss of the anti-inflammatory function of apoA-I in HCAECs. This finding prompted us to perform subsequent experiments with chymase alone.

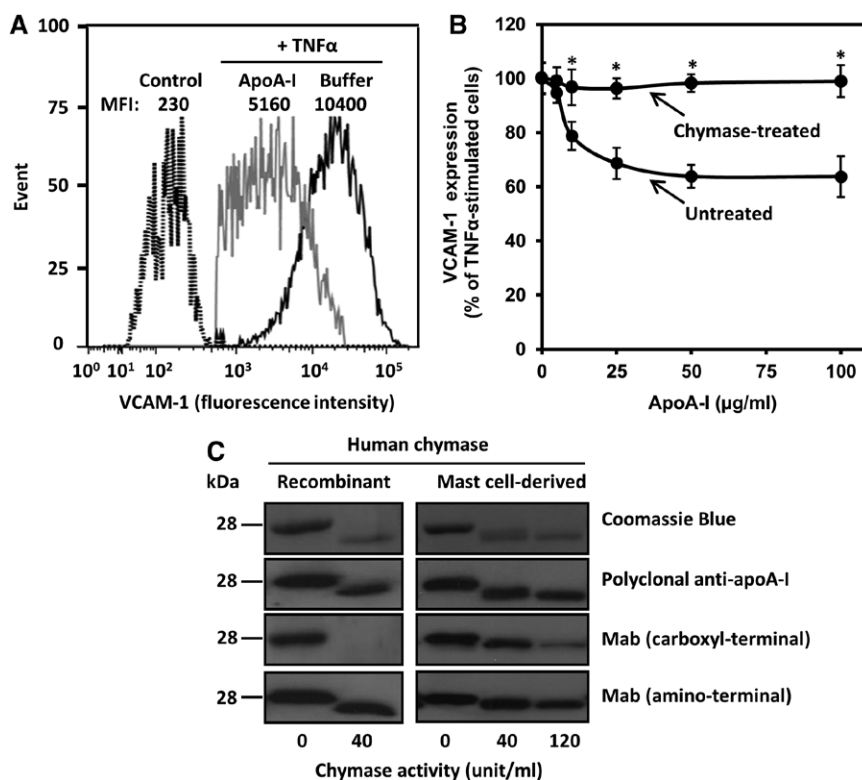


Figure 1. Chymase treatment abolishes the anti-inflammatory activity of apolipoprotein A-I (apoA-I) on activated human coronary artery endothelial cells (HCAECs). **A**, HCAECs were preincubated for 16 h in the absence (buffer) or presence of apoA-I (50 μ g/mL) and then activated with tumor necrosis factor- α (TNF- α ; 10 ng/mL) for 5 h. Nonactivated cells were incubated in medium alone for 21 h (control). Cell surface vascular cell adhesion molecule-1 (VCAM-1) protein was determined by flow cytometry. Data are representative of 6 independent experiments. **B**, ApoA-I (1 mg/mL) was treated for 6 h in the absence (untreated) or the presence (chymase-treated) of chymase (0.5 μ g=40 BTEE units/mL). HCAECs were preincubated with increasing concentrations of the untreated or chymase-treated apoA-I and then activated with tumor necrosis factor- α (TNF- α), as described in **A**. TNF- α -induced VCAM-1 surface protein expression was analyzed by flow cytometry and expressed as percentage of its expression levels in TNF- α -activated cells preincubated in the absence of apoA-I, which was set as 100%. Data represent the means \pm SD from 3 to 4 independent experiments performed in duplicate. * P <0.01 denotes statistical significance between cells preincubated with untreated or chymase-treated apoA-I. **C**, ApoA-I was incubated with the indicated activities (BTEE units) of recombinant human chymase or chymase-containing human mast cell-conditioned medium for 6 h, after which the incubation was stopped by adding soybean trypsin inhibitor. Proteins in the incubation mixtures were resolved in 12.5% SDS-PAGE and detected by Coomassie Blue or immunoblotted with anti-human apoA-I polyclonal antibody or with anti-human apoA-I monoclonal antibodies recognizing either a C-terminal (amino acids 211–220) or an N-terminal (amino acids 2–8) region of apoA-I. MFI indicates median fluorescence intensity.

Lipidation of ApoA-I Renders It Partly Resistant to Chymase-Dependent Loss of Its Anti-Inflammatory Effects on HCAECs

To examine the effect of apoA-I lipidation on the ability of chymase to attenuate its anti-inflammatory properties, we studied apoA-I-containing reconstituted HDL ((A-I)rHDL) particles that have been widely used to mimic the naturally occurring pre β -HDL.²⁵ For this purpose, we prepared 3 species of discoidal (A-I)rHDL with increasing amounts of phospholipids and constant amounts of cholesterol and apoA-I: rHDL-1, rHDL-2, and rHDL-3 (Table I in the online-only Data Supplement). Similar to proteolysis of lipid-free apoA-I with chymase, proteolysis of lipidated apoA-I generated a polypeptide band with an apparent molecular weight slightly lower than that of intact apoA-I (Figure 2A, top). Yet, the degree of lipidation was inversely related to the degree of proteolysis (Figure 2A, bottom), suggesting that lipidation progressively masked the chymase cleavage sites of apoA-I. Next, we examined the effect of chymase on the anti-inflammatory properties of the various (A-I)rHDL preparations. When activated HCAECs were preincubated with any of the untreated (A-I)rHDL species, the

levels of VCAM-1 mRNA and protein were reduced to levels comparable to those observed when lipid-free apoA-I was used (Figure 2B, top and middle). Moreover, any of the untreated (A-I)rHDL reduced adhesion of THP-1 monocytes to the activated HCAECs to the levels observed in the nonactivated control cells (Figure 2B, bottom). Cleavage of apoA-I in (A-I)rHDLs affected the ability of these particles to inhibit surface VCAM-1 protein expression, and it appeared that the stronger apoA-I degradation (rHDL-1>rHDL-2>rHDL-3), the weaker its inhibitory effect on VCAM-1 protein expression (P <0.05 for trend). However, all of the proteolyzed rHDLs had lost to a similar extent their capacity to reduce VCAM-1 mRNA and monocyte adhesion to TNF- α -activated HCAECs (Figure 2B, top and bottom), suggesting that even a minor degree of proteolysis may have significantly affected particle integrity and the specific conformational characteristics of apoA-I in (A-I)rHDL particles that were essential for these particular apoA-I-dependent effects. Importantly, analysis with nonreducing electrophoresis revealed that incubation of (A-I)rHDLs with chymase resulted in significant changes in protein staining pattern and particle size distribution (Figure IIA and IIB in the online-only

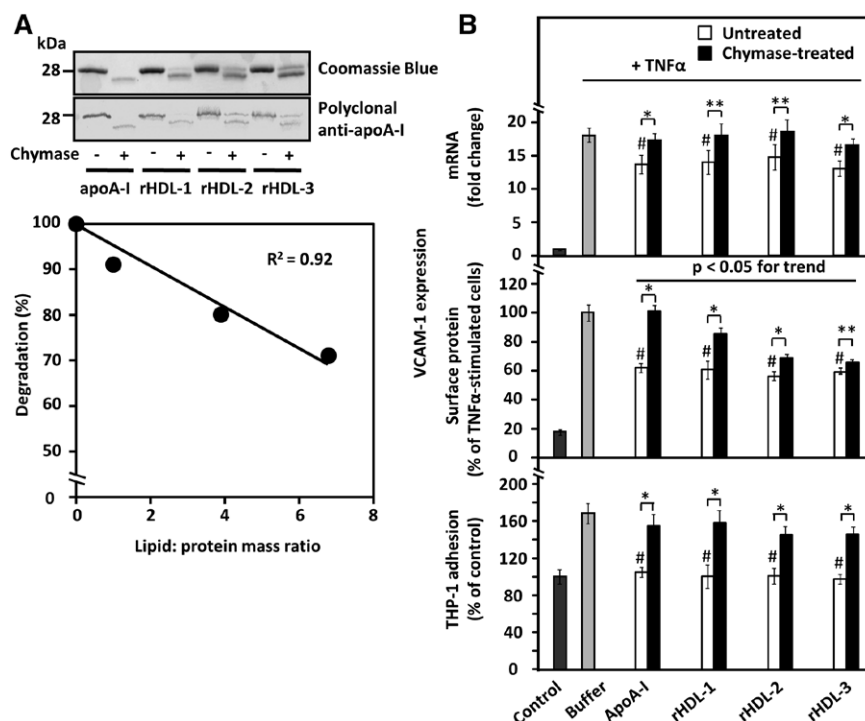


Figure 2. Effect of apolipoprotein A-I (apoA-I) lipidation on its sensitivity to proteolysis and on the ability of untreated and chymase-treated apoA-I to influence vascular cell adhesion molecule-1 (VCAM-1) expression in and THP-1 adhesion to tumor necrosis factor- α (TNF- α)-activated human coronary artery endothelial cells (HCAECs). **A, Top**, ApoA-I and discoidal apoA-I-containing reconstituted high-density lipoprotein ((A-I)rHDLs) with 3 different degrees of lipidation (see Table I in the online-only Data Supplement) were incubated in the absence or presence of recombinant chymase, and the proteins in the incubation mixtures were analyzed, as described in Figure 1. **Bottom**, ApoA-I degradation was expressed as a percentage of the intensity of the intact band in the proteolyzed samples relative to the corresponding untreated sample and plotted against the lipid/protein mass ratio of the various (A-I)rHDL preparations. **B**, HCAECs were preincubated for 16 h with the various untreated or chymase-treated apoA-I or (A-I)rHDL species (50 μ g/mL, each) and then activated with tumor necrosis factor- α (TNF- α), as described in Figure 1. Nonactivated cells (control) and TNF- α -activated cells preincubated in only medium (buffer) acted as references. Vascular cell adhesion molecule-1 (VCAM-1) mRNA levels (**top**, fold change relative to control) and surface protein expression (**middle**, % of buffer) were evaluated in the TNF- α -activated cells. In a separate experiment, HCAECs that had been preincubated with the various apoA-I-containing preparations were further incubated for 1 h with fluorescently labeled THP-1 cells, and the fluorescence of HCAECs-bound THP-1 cells was then measured (**bottom**). The fluorescence signal of the TNF- α -activated HCAECs was expressed as percentage of the basal signal from the nonactivated cells (control) which was set as 100%. Data shown in each panel represent the means \pm SD from 3 to 4 independent experiments performed in triplicate wells. ** $P < 0.05$; * $P < 0.01$; # $P < 0.01$ (untreated apoA-I-containing preparations vs buffer).

Data Supplement). Consistent with previous reports,^{20,26} and in contrast to lipid-free apoA-I and rHDLs, apoA-I in mature HDL particles isolated from human plasma (HDL₂ and HDL₃) was poorly degraded by chymase, and the particles maintained their ability to inhibit TNF- α -induced VCAM-1 expression, despite treatment with chymase (Figure IIIA and IIIB in the online-only Data Supplement). It is plausible that the insensitivity of apoA-I in mature HDL particles is because of inaccessibility of proteases to the apolipoprotein, which is partially embedded in the surface phospholipid layer of the particles. Interestingly, neither chymase nor cathepsin G, but rather α -1 antitrypsin, has been found to be a component of total (unfractionated) HDL and the HDL₃ fraction derived from circulating plasma or from atherosclerotic carotid tissue,²⁷ and so providing an additional explanation for the protease insensitivity of apoA-I in these particles.

Proteolysis of ApoA-I by Plasmin or Cathepsin S Impairs Its Anti-Inflammatory Effects on HCAECs

We evaluated the effect of other proteases known to be present in atherosclerotic lesions, namely plasmin and

cathepsin S,^{28,29} on the anti-inflammatory activity of apoA-I in cultured HCAECs. Similar to chymase, plasmin cleaved the C-terminal region, resulting in the formation of a fragment containing the N-terminal region, whereas cathepsin S completely degraded apoA-I (Figure IVA in the online-only Data Supplement). Importantly, similar to chymase, treatment of apoA-I with either of the 2 proteases resulted in complete loss of its ability to inhibit VCAM-1 expression in TNF- α -activated HCAECs and to prevent the adhesion of THP-1 monocytes to these cells (Figure IVB in the online-only Data Supplement).

Chymase Treatment of ApoA-I Impairs Its Ability to Attenuate NF- κ B Activation, to Bind to HCAECs, and to Prevent Transendothelial Migration of THP-1 Monocytes

Next we examined the effect of chymase treatment of lipid-free apoA-I on NF- κ B activation, a dominant signal transduction pathway upregulating a variety of chemokines and adhesion molecules in human ECs.³⁰ Pretreatment of HCAECs with untreated apoA-I significantly attenuated TNF- α -mediated

NF- κ B activation, as indicated by suppression of nuclear translocation of the p65 subunit of NF- κ B (Figure 3A), a finding in agreement with previous reports.^{13,14} In contrast, treatment with chymase abolished the inhibitory effect of apoA-I on NF- κ B activation, indicating that the intactness of apoA-I is a prerequisite for the inhibition of this signaling pathway. To further investigate the mechanisms involved in the chymase-dependent loss of the anti-inflammatory effects of apoA-I, we compared binding of untreated and chymase-treated ¹²⁵I-labeled apoA-I to HCAECs. We found that chymase treatment severely blunted ($\geq 80\%$) the high-affinity binding of apoA-I to HCAECs (Figure 3B), strongly suggesting that reduced interaction of apoA-I with the cell membrane was necessary for loss of the anti-inflammatory effect. The above finding also agrees with the observed strong reduction of specific binding to bovine aortic endothelial cells of C-terminal deletion mutants of apoA-I.³¹

We further examined the effect of untreated and chymase-treated apoA-I on transmigration of THP-1 monocytes across monolayers of TNF- α -activated HCAECs using the transwell system, which mimics the human endothelium in vivo. We found that, regardless of whether adding to the apical or basolateral compartment, chymase-treated apoA-I failed to attenuate THP-1 transmigration across the activated HCAECs (Figure 3C). Interestingly, untreated apoA-I significantly prevented the endothelial transmigration of THP-1 when placed in the apical compartment, whereas a slight, but nonsignificant decrease was observed when apoA-I was added to the basolateral compartment. The smaller effect of untreated apoA-I could be possibly because of much lower basolateral than apical binding of apoA-I, which may be partly caused by sterical hindrance binding to ECs because of the presence of a porous membrane and coated matrix in the transwell insert.³²

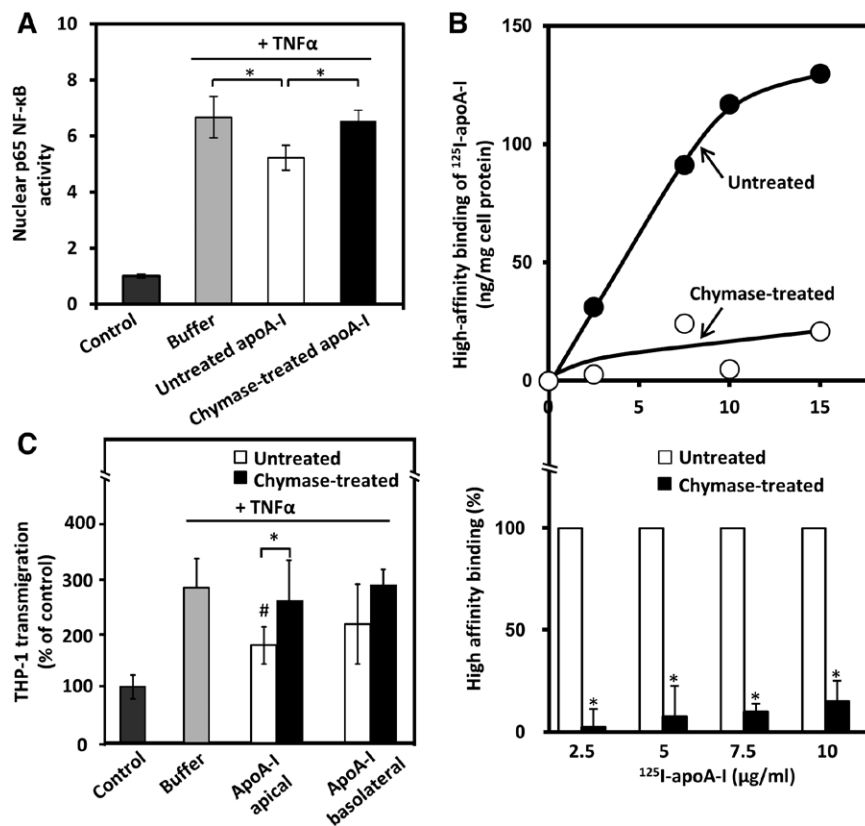


Figure 3. Proteolysis of apolipoprotein A-I (apoA-I) impairs its ability to attenuate nuclear factor- κ B (NF- κ B) activation to bind to human coronary artery endothelial cells (HCAECs) and to prevent transmigration of THP-1 monocytes across HCAEC monolayer. **A**, HCAECs were incubated for 16 h with untreated or chymase-treated apoA-I and then activated with tumor necrosis factor- α (TNF- α) for 15 min. Nonactivated cells (control) and TNF- α -activated cells preincubated in only medium (buffer) acted as a reference. Nuclear NF- κ B p65 activity was determined by measuring nuclear translocation of the NF- κ B p65 subunit. The basal activity of the nonactivated HCAECs (control) was set as 1. Data represent the means \pm SD from triplicate wells and are representative of 2 independent experiments. * P <0.05. **B**, ApoA-I was labeled with ¹²⁵I and then treated with chymase as described in Figure 1B. HCAECs were incubated with the indicated concentrations of ¹²⁵I-labeled untreated or chymase-treated apoA-I in the absence or presence of a 40-fold excess of untreated apoA-I for 2 h at 4°C. High-affinity binding of ¹²⁵I-apoA-I to HCAECs was calculated by subtracting the values of the nonspecific binding from the total binding. A representative pair of binding curves is shown in the **top** panel. Data (mean \pm SD) from 4 independent experiments from duplicate wells are expressed as percentages of untreated apoA-I (**bottom**). * P <0.01. **C**, Confluent HCAEC monolayers grown on a transwell insert were first incubated with untreated or chymase-treated apoA-I added either to the apical or basolateral compartment, then activated with TNF- α for 5 h, and finally incubated with fluorescently labeled THP-1 cells added to the apical compartment. Migration across the endothelial monolayer of the cells was quantified by measuring the fluorescence signal of the transmigrated cells. Migration in the absence of any additions was designated as 100% (control). Data shown represent the means \pm SD from 3 independent experiments performed in triplicate wells. * P <0.05; # P <0.05 (untreated apoA-I vs buffer).

Chymase Treatment of ApoA-I Reduces Its Ability to Inhibit Expression of Proinflammatory Genes in HCAECs and in Macrophages and to Induce Cholesterol Efflux From Macrophage Foam Cells

We next examined the effect of chymase-treated lipid-free apoA-I on selected proinflammatory genes in TNF- α -activated HCAECs. TNF- α strongly increased mRNA levels of intercellular adhesion molecule-1, cyclooxygenase-2 (COX-2), and interleukin (IL)-8, although only slightly affected that of IL-6 (Figure 4A–4D). Treatment of HCAECs before activation with untreated, but not with chymase-treated, apoA-I significantly decreased the TNF- α -dependent induction of COX-2, IL-6, and IL-8 mRNA expression. In contrast, neither untreated nor chymase-treated apoA-I had any effect on intercellular adhesion molecule-1 expression. We also determined the effect of the chymase-treated apoA-I on the expression of cytokines in LPS-activated macrophage foam cells. For this purpose, human monocytes were allowed to differentiate into macrophages either in the presence

of GM-CSF (granulocyte-macrophage colony-stimulating factor) or M-CSF (macrophage colony-stimulating factor) to obtain the GM-Mac and M-Mac subpopulations, which have their counterparts in human atherosclerotic lesions,³³ and finally incubated with acetyl-LDL. To avoid any LPS binding to apoA-I,³⁴ the foam cells were preincubated with untreated or chymase-treated apoA-I, then extensively washed, and finally challenged with LPS. Similar to the effects observed in HCAECs, preincubation with the untreated apoA-I, but not with the chymase-treated apoA-I, significantly suppressed LPS-induced upregulation of mRNA levels of proinflammatory cytokines, namely of TNF- α , IL-1 β , IL-6, and IL-8 in both macrophage foam cell subtypes (Figure 4E–4L). To investigate how the loss of the anti-inflammatory effect of apoA-I by chymase modification was related to the known chymase-dependent loss of the cholesterol efflux ability of apoA-I, we also determined cholesterol efflux capacity of chymase-treated apoA-I in the GM-Mac- and M-Mac-derived foam cells. Consistent with previous studies,^{19,20} treatment of

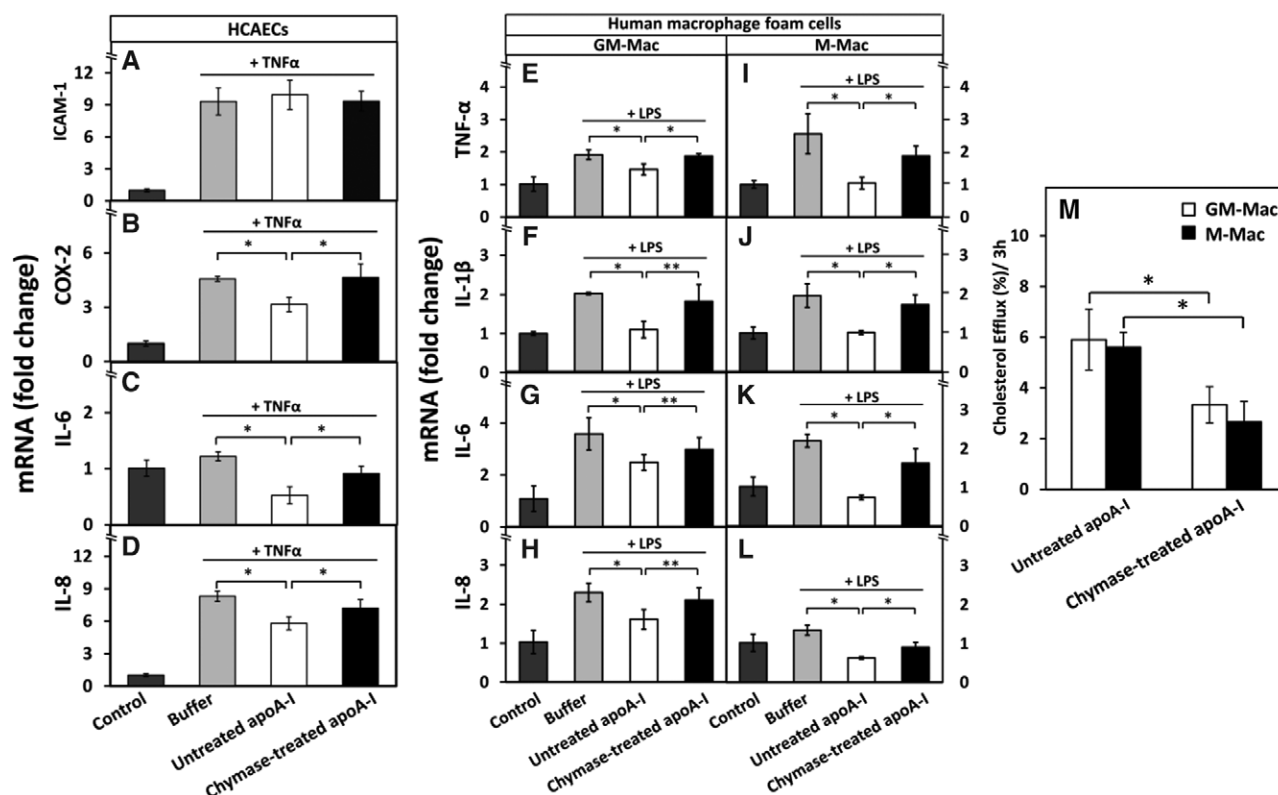


Figure 4. Proteolysis of apolipoprotein A-I (apoA-I) reduces its ability to downregulate the expression of proinflammatory genes in human coronary artery endothelial cells (HCAECs) and in human GM-Mac and M-Mac macrophage subpopulations and to promote cholesterol efflux from the macrophages. **A–D**, HCAECs were preincubated with untreated or chymase-treated apoA-I and then activated with tumor necrosis factor- α (TNF- α), as described in Figure 1. Nonactivated cells (control) and TNF- α -activated cells preincubated in only medium (buffer) acted as a reference. The mRNA levels of intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), interleukin (IL)-6, and IL-8 in HCAECs were measured by quantitative real-time polymerase chain reaction (qRT-PCR) and expressed as fold changes relative to the control cells. Data represent the means \pm SD from 3 independent experiments performed in duplicate. * P <0.01. **E–L**, Human monocyte-derived macrophages were differentiated in the presence of GM-CSF (granulocyte-macrophage colony-stimulating factor) or M-CSF (macrophage colony-stimulating factor) into GM-Mac and M-Mac subtypes, respectively, and then converted into foam cells by incubation with acetyl low density lipoprotein (LDL). The cells were then incubated for 3 h with untreated or chymase-treated apoA-I (50 μ g/mL, each), washed, and activated for 3 h with lipopolysaccharide (LPS). Nonactivated cells (control) and LPS-activated cells preincubated in only medium (buffer) acted as references. LPS-induced mRNA levels of TNF- α , IL-1 β , IL-6, and IL-8 in GM-Mac and M-Mac foam cells were evaluated by qRT-PCR and expressed as fold changes relative to the control cells. * P <0.01; ** P <0.05. **M**, GM-Mac- and M-Mac-derived foam cells were incubated with untreated or chymase-treated apoA-I (50 μ g/mL, each) for 3 h, the media were collected, centrifuged to remove cellular debris, and the radioactivity of each supernatant was determined by liquid scintillation counting. Cells were solubilized, and radioactivity was determined in the cell lysates. Cholesterol efflux was calculated as $\text{dpm}_{\text{medium}} / (\text{dpm}_{\text{cells}} + \text{dpm}_{\text{medium}}) \times 100$. Data represent the means \pm SD from triplicate wells and are representative of 2 independent experiments. * P <0.01.

apoA-I with chymase decreased its ability to induce cholesterol efflux from macrophage foam cells (Figure 4M). Taken together, cleavage of apoA-I with chymase impaired both its anti-inflammatory and cholesterol efflux-inducing activities.

Chymase Treatment of ApoA-I Reduces Its Anti-Inflammatory Properties In Vivo

To learn whether chymase would also affect the anti-inflammatory properties of lipid-free apoA-I in vivo, we used the well-established mouse model of LPS-induced inflammation, in which apoA-I has been shown to exert its anti-inflammatory properties.^{7,35,36} We found that LPS administration remarkably increased the levels of 2 circulating proinflammatory mediators (TNF- α and IL-1 β). Concurrent treatment of mice with both LPS and lipid-free apoA-I significantly reduced the level of TNF- α and IL-1 β (Figure 5A and 5B), consistent with the well-known anti-inflammatory effect of apoA-I on LPS-induced inflammation in vivo.³⁵ Importantly, concurrent treatment of mice with LPS and chymase-treated apoA-I failed to significantly diminish the levels of either TNF- α or IL-1 β , indicating that the proteolytic degradation of apoA-I by chymase impaired its anti-inflammatory properties (Figure 5A and 5B). To obtain further insight into the mechanisms by which chymase impaired anti-inflammatory properties of apoA-I in vivo, we determined endotoxin activity using the limulus amoebocyte lysate assay. In line with previous studies demonstrating that both HDL and apoA-I exert their anti-inflammatory properties in vivo via their ability to bind to LPS and neutralize endotoxin,^{35–37} we found that apoA-I strongly neutralized LPS endotoxin activity in vitro (Figure 5C). In sharp contrast, chymase-treated apoA-I had totally lost the ability to neutralize endotoxin activity.

Chymase Treatment Does Not Blunt the Anti-Inflammatory Effects of D-4F on HCAECs, Neutrophils, and Macrophages

The apoA-I mimetic peptide L-4F exerts potent anti-inflammatory effects by reducing the chemotactic activity of human

aortic ECs for monocytes.³⁸ Given the cleavage specificity of chymase, a chymotryptic protease, and the arrangement of aromatic amino acid residues in the 4F structure that determines its functional properties,³⁹ we considered that chymase could cleave L-4F, so resulting in its functional inactivation. As a control, we used D-4F, which is resistant to cleavage by mammalian proteases.⁴⁰ Both untreated L-4F and D-4F decreased VCAM-1 protein expression in TNF- α -activated HCAECs to a similar extent (Figure 6A). However, when chymase-treated L-4F and D-4F were compared, we found that the former had lost, but the latter had maintained its anti-inflammatory effect. Because the remarkable anti-inflammatory properties of both 4F species appear to be accounted for their ability to bind proinflammatory oxidized lipids,³⁸ we also evaluated the effect of chymase on their antioxidant effects in PMA (phorbol 12-myristate 13-acetate)-activated neutrophils. We found that, although apoA-I and both mimetic peptides significantly diminished superoxide production by the activated neutrophils in a dose-dependent manner, chymase treatment fully abolished the antioxidant effect of apoA-I and L-4F, but not that of D-4F (Figure 6B). Similar to findings observed with HCAECs and neutrophils, both untreated and chymase-treated D-4F significantly decreased to a similar extent the mRNA levels of the proinflammatory cytokines (IL-1 β , IL-6, and IL-8) in LPS-activated macrophage foam cells of the 2 phenotypes (GM-Mac and M-Mac) (Figure 6C–CJ). Interestingly, unlike apoA-I, D-4F failed to decrease mRNA levels of TNF- α in either macrophage phenotype.

Discussion

The present study demonstrates that protease-dependent loss of the C-terminal region of apoA-I is sufficient to fully inhibit its anti-inflammatory properties both in vitro and in vivo. The enzymes capable of such cleavage comprised chymase and plasmin, the former known to be secreted by vascular mast cells⁴¹ and the latter by vascular smooth muscle cells and ECs.¹⁸ The repertoire of cell-derived extracellular proteases present in the human atherosclerotic arterial intima and

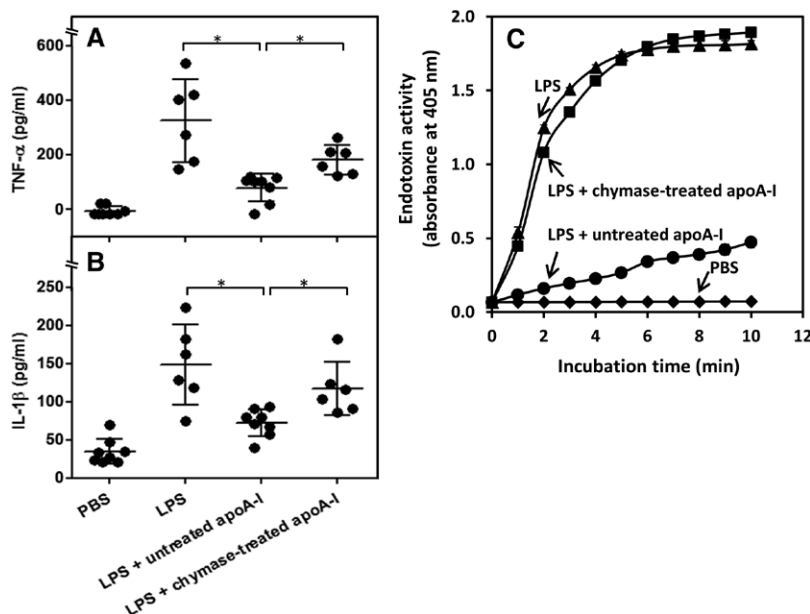


Figure 5. Proteolysis of apolipoprotein A-I (apoA-I) impairs its ability to inhibit lipopolysaccharide (LPS)-induced inflammation in vivo and to neutralize endotoxin activity of LPS in vitro. Mice (6–8 per group) were randomized to receive vehicle (PBS), LPS (1 mg/kg), LPS (1 mg/kg) plus apoA-I (10 mg/kg), or LPS (1 mg/kg) plus chymase-treated apoA-I (10 mg/kg). After 3 h, serum was collected, and TNF- α (A) and IL-1 β (B) concentrations were measured. Data are means \pm SD, * P <0.05. C, LPS (1 μ g/mL) was mixed with untreated apoA-I and chymase-treated apoA-I (10 μ g/mL) and endotoxin activity was measured with kinetic colorimetric limulus amoebocyte lysate (LAL) assay. Data represent the means \pm SD from triplicate wells and are representative of 2 independent experiments.

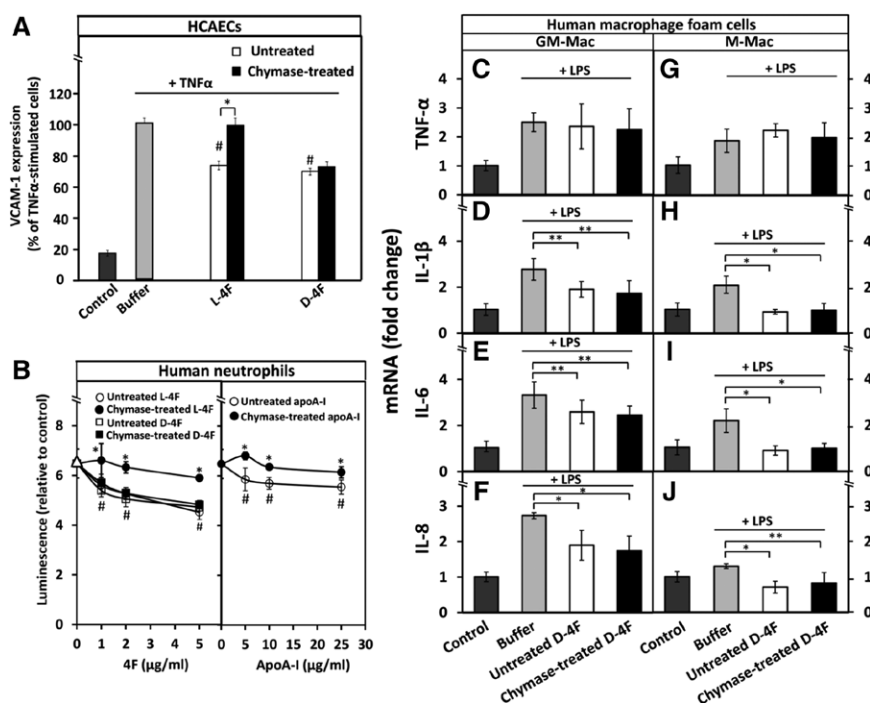


Figure 6. Chymase treatment does not affect the anti-inflammatory effects of D-4F on human coronary artery endothelial cells (HCAECs), neutrophils, and macrophages. **A**, HCAECs were preincubated with untreated or chymase-treated L-4F and D-4F (50 μ g/mL, each) after which the cells were then activated with tumor necrosis factor- α (TNF- α), as described in Figure 1. Nonactivated cells (control) and TNF- α -activated cells preincubated in only medium (buffer) were used as references. Cell-surface expression of vascular cell adhesion molecule-1 (VCAM-1) protein was determined by flow cytometry. Data represent the means \pm SD from 3 independent experiments performed in duplicate. * P <0.01; # P <0.01 (untreated L-4F or untreated D-4F vs buffer). **B**, Superoxide radical production by PMA (phorbol 12-myristate 13-acetate)-activated neutrophils was determined after the cells had been preincubated for 5 min with the indicated concentrations of untreated or chymase-treated L-4F, D-4F, or apolipoprotein A-I (apoA-I). PMA-activated neutrophils preincubated in only medium (buffer) were used as a reference (open triangles). Luminescence emitted by nonactivated neutrophils (control) was set as 1. Data are derived from 2 donors and represent the means \pm SD from 2 independent experiments, each performed in 3 to 6 wells. * P <0.01 (untreated vs chymase-treated L-4F, and untreated vs chymase-treated apoA-I); # P <0.01 (untreated L-4F, D-4F, or apoA-I vs buffer). **C–J**, Human monocyte-derived macrophages were differentiated into GM-Mac and M-Mac subtypes and converted into foam cells by incubation with acetyl low-density lipoprotein (LDL). The foam cells were then incubated for 3 h with untreated or chymase-treated D-4F (50 μ g/mL, each), washed, and activated for 3 h with lipopolysaccharide (LPS). Nonactivated foam cells (control) and LPS-activated foam cells preincubated in only medium (buffer) served as references. LPS-induced mRNA levels of TNF- α , interleukin (IL)-1 β , IL-6, and IL-8 in GM-Mac and M-Mac foam cells were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) and expressed as fold changes relative to the control cells. Data represent the means \pm SD from triplicate wells and are representative of 2 independent experiments. * P <0.01; ** P <0.05.

capable of cleaving the C-terminal domain of apoA-I extends to the members of the matrix metalloproteinase family-3, -7, and -12.¹⁸ Altogether, these findings suggest that an inflamed atherosclerotic lesion is a compartment in which protease-dependent attenuation of the anti-inflammatory functions of HDL could take place.

The structure–function relationships involved in the mechanisms determining the anti-inflammatory activities of apoA-I and HDL are manifold. According to current notions, apoA-I suppresses the expression of surface adhesion molecules in ECs in vitro via interaction with ABCA1, which initiates a cascade of intracellular signaling events that are thought to involve lipid efflux–dependent disruption of lipid rafts.^{5,42} Moreover, apoA-I has been shown to induce COX-2 expression and PGI-2 (prostacyclin) release in unstimulated HUVECs via interaction with ABCA1,⁴³ revealing functional interactions between apoA-I and ABCA1. Furthermore, it was recently shown that the anti-inflammatory and antioxidant effects of the apoA-I mimetic peptide 5A are mediated via the ABCA1 transporter and NF- κ B signaling pathways in HCAECs.¹⁴ Thus, it is likely that, besides being involved

in the transcytotic pathway of lipid-free apoA-I through ECs,^{32,44} binding of apoA-I to ABCA1 also essentially contributes to the anti-inflammatory actions of lipid-free apoA-I in HCAECs. Interestingly, the C-terminal domain of apoA-I has been shown to be required for endothelial binding and transendothelial transport of lipid-free apoA-I.³¹ Here, we have demonstrated that cleavage of the C-terminal domain of apoA-I by chymase prevented binding of apoA-I to HCAECs with high affinity and caused its failure to suppress the initiation of the NF- κ B-mediated downstream inflammatory signaling in TNF- α -activated HCAECs. Similarly, glycation of apoA-I which modifies the conformation of epitopes present in C-terminal and central regions of apoA-I has been shown to impair its anti-inflammatory effects on HCAECs.^{13,45}

Regarding the effect of chymase treatment of apoA-I on its ability to interact with macrophages, we found that chymase-treated apoA-I had lost its anti-inflammatory effects and its ability to promote macrophage cholesterol efflux from human macrophages. It has been found earlier that loss of apoA-I/ABCA1 interaction not only prevents initiation of cholesterol efflux, but also initiation of the anti-inflammatory

effects of apoA-I in these cells,^{46,47} and moreover that silencing of ABCA1 abolishes the inhibitory effect of apoA-I on the production of TNF- α and other inflammatory cytokines in LPS-activated human macrophages.⁴⁸ Moreover, apoA-I has been recently found to exert anti-inflammatory effects on peptidoglycan polysaccharide-activated human macrophages by decreasing NF- κ B activation in an ABCA1-dependent manner.⁴⁹ Thus, we infer that the present observation of the loss of the ability of chymase-treated apoA-I to abolish a proinflammatory response of macrophages to LPS treatment actually reflected lost interaction with macrophage ABCA1, similar to the loss of the apoA-I-dependent induction of cholesterol efflux from macrophage foam cells after chymase treatment of apoA-I.¹⁹ Thus, a failure to bind to macrophage ABCA1 appears to be the molecular link between the losses of the anti-inflammatory and the cholesterol efflux-inducing capacities of the C-terminally truncated apoA-I because of chymase treatment. Similarly, myeloperoxidase-derived hypochlorous acid, which oxidizes apoA-I, impairs the ability of HDL particles to promote cholesterol efflux and also compromises their anti-inflammatory properties.⁵⁰ More recently, non-enzymatic glycation, which induces changes in structure and in lipid-binding activity of apoA-I,⁵¹ was also observed to impair both the cholesterol efflux capacity and anti-inflammatory properties of apoA-I.^{13,45} Thus, several apoA-I-modifying factors alter the structure of apoA-I in such a way that, besides affecting adversely its anti-inflammatory properties, they also impair its cholesterol efflux-inducing capacity.

In vivo studies have demonstrated that administration of apoA-I inhibits inflammatory responses in experimental animals, like in rabbits,^{16,17} mice,³⁵ and rats⁴⁹ subjected to acute or chronic inflammation. Actually, overexpression of human apoA-I in mice attenuates LPS- or high fat-induced inflammation,^{7,8} whereas apoA-I deletion results in loss of its anti-inflammatory actions.⁵² Our in vivo data in mice showed that proteolytic modification of apoA-I by chymase failed to neutralize endotoxin activity, thereby impairing its anti-inflammatory properties in acute inflammation induced by LPS. Similarly, in rabbits with acute vascular inflammation, the anti-inflammatory properties of apoA-I in vivo have been found to be markedly reduced when the animals were infused with glycated apoA-I derived from in vitro modification or from patients with persistent hyperglycemia,¹³ so providing a relevant link between experimental and clinical studies. Of particular relevance to the present work are some recent findings from other laboratories, which attribute the origins of the C-terminally truncated apoA-I in human plasma to mast cell-secreted chymase or to plasmin generated during thrombolytic treatment of coronary patients with tissue plasminogen activator,^{53–55} that is, the 2 proteases also studied here. Obviously, the chymase-generated C-terminally truncated apoA-I must result from extracellular activity of chymase, that is, it requires mast cell degranulation with ensuing release of granule-bound chymase. Regarding plasmin-generated C-terminally truncated apoA-I, also endogenously generated plasmin in atherosclerotic lesions may be involved. Thus, given that apoA-I fragments have been detected in human plasma, albeit at low concentrations, as a result of rapid catabolism of C-terminally truncated apoA-I in vivo,⁵⁶ a small fraction of the lipid-free

or lipid-poor apoA-I appears to be specifically proteolyzed in the extracellular compartment of human body. Regarding the generation of chymase-specific apoA-I degradation products in humans, the cardiovascular system could be one source. Thus, mast cells are particularly abundant in human coronary intima, and the number of activated mast cells in atherosclerotic plaques increase with disease progression.⁵⁷ Activated mast cells have been also demonstrated to accumulate in canine myocardium after ischemia and reperfusion, and moreover, myocardial chymase activity is significantly increased in the acute phase of myocardial infarction in hamsters.^{58–60} Importantly, the substrates for chymase, that is, the chymase-sensitive small lipid-poor pre β -HDL particles, are enriched in the arterial intimal fluid and also in the peripheral lymphatic fluid, which among other tissues and organs, also drains the coronary arterial wall and the myocardium.^{3,4} The challenge is to find a suitable animal model in which chymase-specific apoA-I degradation products would reside in the lymphatic fluid draining an ischemic myocardium.

Finally, considering the demonstrated therapeutic potential of parenterally infused apoA-I in atherosclerotic coronary artery disease,⁶¹ the therapeutic potential of an intravenously infused apoA-I-containing rHDL preparation (including the protease-sensitive apoA-I Milano)^{62,63} or of an apoA-I-mimetic peptide could be locally compromised by extracellular proteolytic activities in the target tissue, notably in an inflamed advanced coronary lesion. This challenge could be overcome by parenteral administration of an apoA-I mimetic peptide, such as the D-4F peptide, in which high anti-atherosclerotic activity is combined with an ability to resist proteolytic attack.

Acknowledgments

We thank Maija Atuegwu, Mari Jokinen, María Arraño de Kivikko, Reija Silvennoinen, and Ilona Kareinen for excellent technical assistance.

Sources of Funding

Wihuri Research Institute is maintained by the Jenny and Antti Wihuri Foundation. This study was also supported by the Academy of Finland (265940 to S.D. Nguyen and K. Öörni and 257545 to M. Jauhainen), the Finnish Foundation for Cardiovascular Research (K. Maaninka and K. Nurmi), and the Ida Montin Foundation (K. Maaninka).

Disclosures

None.

References

- Asztalos BF, Tani M, Schaefer EJ. Metabolic and functional relevance of HDL subspecies. *Curr Opin Lipidol*. 2011;22:176–185. doi: 10.1097/MOL.0b013e3283468061.
- Kane JP, Malloy MJ. Prebeta-1 HDL and coronary heart disease. *Curr Opin Lipidol*. 2012;23:367–371. doi: 10.1097/MOL.0b013e328353eef1.
- Miller NE, Olszewski WL, Hattori H, Miller IP, Kujiraoka T, Oka T, Iwasaki T, Nanjee MN. Lipoprotein remodeling generates lipid-poor apolipoprotein A-I particles in human interstitial fluid. *Am J Physiol Endocrinol Metab*. 2013;304:E321–E328. doi: 10.1152/ajpendo.00324.2012.
- Smith EB. Transport, interactions and retention of plasma proteins in the intima: the barrier function of the internal elastic lamina. *Eur Heart J*. 1990;11(suppl E):72–81.
- Mineo C, Shaul PW. Regulation of signal transduction by HDL. *J Lipid Res*. 2013;54:2315–2324. doi: 10.1194/jlr.R039479.

6. Rao RM, Yang L, Garcia-Cardena G, Lusinskas FW. Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. *Circ Res*. 2007;101:234–247. doi: 10.1161/CIRCRESAHA.107.151860b.
7. Van Linthout S, Spillmann F, Graiani G, Miteva K, Peng J, Van Craeyveld E, Meloni M, Tölle M, Escher F, Subasinghiller A, Doehner W, Quaini F, De Geest B, Schultheiss HP, Tschöpe C. Down-regulation of endothelial TLR4 signalling after apo A-I gene transfer contributes to improved survival in an experimental model of lipopolysaccharide-induced inflammation. *J Mol Med (Berl)*. 2011;89:151–160. doi: 10.1007/s00109-010-0690-6.
8. Cheng AM, Handa P, Tateya S, Schwartz J, Tang C, Mitra P, Oram JF, Chait A, Kim F. Apolipoprotein A-I attenuates palmitate-mediated NF- κ B activation by reducing Toll-like receptor-4 recruitment into lipid rafts. *PLoS One*. 2012;7:e33917. doi: 10.1371/journal.pone.0033917.
9. D'Souza W, Stonik JA, Murphy A, Demosky SJ, Sethi AA, Moore XL, Chin-Dusting J, Remaley AT, Sviridov D. Structure/function relationships of apolipoprotein a-I mimetic peptides: implications for antiatherogenic activities of high-density lipoprotein. *Circ Res*. 2010;107:217–227. doi: 10.1161/CIRCRESAHA.110.216507.
10. Murphy AJ, Woollard KJ, Hoang A, Mukhamedova N, Storzaker RA, McCormick SP, Remaley AT, Sviridov D, Chin-Dusting J. High-density lipoprotein reduces the human monocyte inflammatory response. *Arterioscler Thromb Vasc Biol*. 2008;28:2071–2077. doi: 10.1161/ATVBAHA.108.168690.
11. Smythies LE, White CR, Maheshwari A, Palgunachari MN, Anantharamaiah GM, Chaddha M, Kurundkar AR, Datta G. Apolipoprotein A-I mimetic 4F alters the function of human monocyte-derived macrophages. *Am J Physiol Cell Physiol*. 2010;298:C1538–C1548. doi: 10.1152/ajpcell.00467.2009.
12. Yvan-Charvet L, Kling J, Pagler T, Li H, Hubbard B, Fisher T, Sparrow CP, Taggart AK, Tall AR. Cholesterol efflux potential and antiinflammatory properties of high-density lipoprotein after treatment with niacin or anacetrapib. *Arterioscler Thromb Vasc Biol*. 2010;30:1430–1438. doi: 10.1161/ATVBAHA.110.207142.
13. Nobécourt E, Tabet F, Lambert G, Puranik R, Bao S, Yan L, Davies MJ, Brown BE, Jenkins AJ, Dusting GJ, Bonnet DJ, Curtiss LK, Barter PJ, Rye KA. Nongenzytic glycation impairs the antiinflammatory properties of apolipoprotein A-I. *Arterioscler Thromb Vasc Biol*. 2010;30:766–772. doi: 10.1161/ATVBAHA.109.201715.
14. Tabet F, Remaley AT, Segaliny AI, Millet J, Yan L, Nakhla S, Barter PJ, Rye KA, Lambert G. The 5A apolipoprotein A-I mimetic peptide displays antiinflammatory and antioxidant properties *in vivo* and *in vitro*. *Arterioscler Thromb Vasc Biol*. 2010;30:246–252. doi: 10.1161/ATVBAHA.109.200196.
15. Park SH, Park JH, Kang JS, Kang YH. Involvement of transcription factors in plasma HDL protection against TNF- α -induced vascular cell adhesion molecule-1 expression. *Int J Biochem Cell Biol*. 2003;35:168–182.
16. Patel S, Di Bartolo BA, Nakhla S, Heather AK, Mitchell TW, Jessup W, Celermajer DS, Barter PJ, Rye KA. Anti-inflammatory effects of apolipoprotein A-I in the rabbit. *Atherosclerosis*. 2010;212:392–397. doi: 10.1016/j.atherosclerosis.2010.05.035.
17. Nicholls SJ, Dusting GJ, Cutri B, Bao S, Drummond GR, Rye KA, Barter PJ. Reconstituted high-density lipoproteins inhibit the acute pro-oxidant and proinflammatory vascular changes induced by a periarterial collar in normocholesterolemic rabbits. *Circulation*. 2005;111:1543–1550. doi: 10.1161/01.CIR.0000159351.95399.50.
18. Lee-Rueckert M, Kovanen PT. Extracellular modifications of HDL *in vivo* and the emerging concept of proteolytic inactivation of pre β -HDL. *Curr Opin Lipidol*. 2011;22:394–402. doi: 10.1097/MOL.0b013e32834a3d24.
19. Favari E, Lee M, Calabresi L, Franceschini G, Zimetti F, Bernini F, Kovanen PT. Depletion of pre-beta-high density lipoprotein by human chymase impairs ATP-binding cassette transporter A1- but not scavenger receptor class B type I-mediated lipid efflux to high density lipoprotein. *J Biol Chem*. 2004;279:9930–9936. doi: 10.1074/jbc.M312476200.
20. Lee-Rueckert M, Vikstedt R, Metso J, Jauhiainen M, Kovanen PT. Association of cholesterly ester transfer protein with HDL particles reduces its proteolytic inactivation by mast cell chymase. *J Lipid Res*. 2008;49:358–368. doi: 10.1194/jlr.M700392-JLR200.
21. Lee-Rueckert M, Silvennoinen R, Rotllan N, Judström I, Blanco-Vaca F, Metso J, Jauhiainen M, Kovanen PT, Escola-Gil JC. Mast cell activation *in vivo* impairs the macrophage reverse cholesterol transport pathway in the mouse. *Arterioscler Thromb Vasc Biol*. 2011;31:520–527. doi: 10.1161/ATVBAHA.110.221069.
22. Lee-Rueckert M, Kovanen PT. The mast cell as a pluripotent HDL-modifying effector in atherogenesis: from *in vitro* to *in vivo* significance. *Curr Opin Lipidol*. 2015;26:362–368. doi: 10.1097/MOL.0000000000000224.
23. Cook-Mills JM, Marchese ME, Abdala-Valencia H. Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. *Antioxid Redox Signal*. 2011;15:1607–1638. doi: 10.1089/ars.2010.3522.
24. Maaninka K, Lappalainen J, Kovanen PT. Human mast cells arise from a common circulating progenitor. *J Allergy Clin Immunol*. 2013;132:463–469.e3. doi: 10.1016/j.jaci.2013.02.011.
25. Vucic E, Rosenson RS. Recombinant high-density lipoprotein formulations. *Curr Atheroscler Rep*. 2011;13:81–87. doi: 10.1007/s11883-010-0141-4.
26. Usami Y, Kobayashi Y, Kameda T, Miyazaki A, Matsuda K, Sugano M, Kawasaki K, Kurihara Y, Kasama T, Tozuka M. Identification of sites in apolipoprotein A-I susceptible to chymase and carboxypeptidase A digestion. *Biosci Rep*. 2013;33:49–56. doi: 10.1042/BSR20120094.
27. Vaisar T, Pennathur S, Green PS, et al. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *J Clin Invest*. 2007;117:746–756. doi: 10.1172/JCI26206.
28. Lindstedt L, Kovanen PT. Plasmin and kallikrein reduce HDL-induced cholesterol efflux from foam cells. *Biochem Biophys Res Commun*. 2000;277:552–557. doi: 10.1006/bbrc.2000.3704.
29. Lindstedt L, Lee M, Öörni K, Brömme D, Kovanen PT. Cathepsins F and S block HDL3-induced cholesterol efflux from macrophage foam cells. *Biochem Biophys Res Commun*. 2003;312:1019–1024.
30. Denk A, Goebeler M, Schmid S, Berberich I, Ritz O, Lindemann D, Ludwig S, Wirth T. Activation of NF- κ B via the I κ B kinase complex is both essential and sufficient for proinflammatory gene expression in primary endothelial cells. *J Biol Chem*. 2001;276:28451–28458. doi: 10.1074/jbc.M102698200.
31. Ohnsorg PM, Rohrer L, Perisa D, Katefides A, Chroni A, Kardassis D, Zannis VI, von Eckardstein A. Carboxyl terminus of apolipoprotein A-I (ApoA-I) is necessary for the transport of lipid-free ApoA-I but not prelipidated ApoA-I particles through aortic endothelial cells. *J Biol Chem*. 2011;286:7744–7754. doi: 10.1074/jbc.M110.193524.
32. Rohrer L, Cavalier C, Fuchs S, Schlüter MA, Völker W, von Eckardstein A. Binding, internalization and transport of apolipoprotein A-I by vascular endothelial cells. *Biochim Biophys Acta*. 2006;1761:186–194. doi: 10.1016/j.bbali.2006.01.009.
33. Waldo SW, Li Y, Buono C, Zhao B, Billings EM, Chang J, Kruth HS. Heterogeneity of human macrophages in culture and in atherosclerotic plaques. *Am J Pathol*. 2008;172:1112–1126. doi: 10.2353/ajpath.2008.070513.
34. Henning MF, Herlax V, Bakás L. Contribution of the C-terminal end of apolipoprotein AI to neutralization of lipopolysaccharide endotoxin effect. *Innate Immun*. 2011;17:327–337. doi: 10.1177/1753425910370709.
35. Yan YJ, Li Y, Lou B, Wu MP. Beneficial effects of ApoA-I on LPS-induced acute lung injury and endotoxemia in mice. *Life Sci*. 2006;79:210–215. doi: 10.1016/j.lfs.2006.02.011.
36. Li Y, Dong JB, Wu MP. Human ApoA-I overexpression diminishes LPS-induced systemic inflammation and multiple organ damage in mice. *Eur J Pharmacol*. 2008;590:417–422. doi: 10.1016/j.ejphar.2008.06.047.
37. Levine DM, Parker TS, Donnelly TM, Walsh A, Rubin AL. *In vivo* protection against endotoxin by plasma high density lipoprotein. *Proc Natl Acad Sci U S A*. 1993;90:12040–12044.
38. Van Lenten BJ, Wagner AC, Jung CL, Ruchala P, Waring AJ, Lehrer RI, Watson AD, Hama S, Navab M, Anantharamaiah GM, Fogelman AM. Anti-inflammatory apoA-I-mimetic peptides bind oxidized lipids with much higher affinity than human apoA-I. *J Lipid Res*. 2008;49:2302–2311. doi: 10.1194/jlr.M800075-JLR200.
39. White CR, Datta G, Buck AK, Chaddha M, Reddy G, Wilson L, Palgunachari MN, Abbasi M, Anantharamaiah GM. Preservation of biological function despite oxidative modification of the apolipoprotein A-I mimetic peptide 4F. *J Lipid Res*. 2012;53:1576–1587. doi: 10.1194/jlr.M026278.
40. Navab M, Anantharamaiah GM, Hama S, Garber DW, Chaddha M, Hough G, Lallone R, Fogelman AM. Oral administration of an Apo A-I mimetic peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol. *Circulation*. 2002;105:290–292.
41. Kovanen PT. Chymase-containing mast cells in human arterial intima: implications for atherosclerotic disease. *Heart Vessels*. 1997;(suppl 12):125–127.
42. Prosser HC, Ng MK, Bursill CA. The role of cholesterol efflux in mechanisms of endothelial protection by HDL. *Curr Opin Lipidol*. 2012;23:182–189. doi: 10.1097/MOL.0b013e328352c4dd.

43. Liu D, Ji L, Tong X, Pan B, Han JY, Huang Y, Chen YE, Pennathur S, Zhang Y, Zheng L. Human apolipoprotein A-I induces cyclooxygenase-2 expression and prostaglandin I-2 release in endothelial cells through ATP-binding cassette transporter A1. *Am J Physiol Cell Physiol*. 2011;301:C739–C748. doi: 10.1152/ajpcell.00055.2011.
44. Cavelier C, Rohrer L, von Eckardstein A. ATP-binding cassette transporter A1 modulates apolipoprotein A-I transcytosis through aortic endothelial cells. *Circ Res*. 2006;99:1060–1066. doi: 10.1161/01.RES.0000250567.17569.b3.
45. Hoang A, Murphy AJ, Coughlan MT, Thomas MC, Forbes JM, O'Brien R, Cooper ME, Chin-Dusting JP, Sviridov D. Advanced glycation of apolipoprotein A-I impairs its anti-atherogenic properties. *Diabetologia*. 2007;50:1770–1779. doi: 10.1007/s00125-007-0718-9.
46. Tang C, Liu Y, Kessler PS, Vaughan AM, Oram JF. The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor. *J Biol Chem*. 2009;284:32336–32343. doi: 10.1074/jbc.M109.047472.
47. Yvan-Charvet L, Welch C, Pagler TA, Ranalletta M, Lamkanfi M, Han S, Ishibashi M, Li R, Wang N, Tall AR. Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. *Circulation*. 2008;118:1837–1847. doi: 10.1161/CIRCULATIONAHA.108.793869.
48. Yin K, Deng X, Mo ZC, Zhao GJ, Jiang J, Cui LB, Tan CZ, Wen GB, Fu Y, Tang CK. Tristetraprolin-dependent post-transcriptional regulation of inflammatory cytokine mRNA expression by apolipoprotein A-I: role of ATP-binding membrane cassette transporter A1 and signal transducer and activator of transcription 3. *J Biol Chem*. 2011;286:13834–13845. doi: 10.1074/jbc.M110.202275.
49. Wu BJ, Ong KL, Shrestha S, Chen K, Tabet F, Barter PJ, Rye KA. Inhibition of arthritis in the Lewis rat by apolipoprotein A-I and reconstituted high-density lipoproteins. *Arterioscler Thromb Vasc Biol*. 2014;34:543–551. doi: 10.1161/ATVBAHA.113.302832.
50. Pirillo A, Uboldi P, Catapano AL. Dual effect of hypochlorite in the modification of high density lipoproteins. *Biochem Biophys Res Commun*. 2010;403:447–451. doi: 10.1016/j.bbrc.2010.11.053.
51. Calvo C, Talussot C, Ponsin G, Berthézène F. Non enzymatic glycation of apolipoprotein A-I. Effects on its self-association and lipid binding properties. *Biochem Biophys Res Commun*. 1988;153:1060–1067.
52. Wang W, Xu H, Shi Y, Nandedkar S, Zhang H, Gao H, Feroah T, Weihrauch D, Schulte ML, Jones DW, Jarzembowski J, Sorci-Thomas M, Pritchard KA Jr. Genetic deletion of apolipoprotein A-I increases airway hyperresponsiveness, inflammation, and collagen deposition in the lung. *J Lipid Res*. 2010;51:2560–2570. doi: 10.1194/jlr.M004549.
53. Usami Y, Matsuda K, Sugano M, Ishimine N, Kurihara Y, Sumida T, Yamauchi K, Tozuka M. Detection of chymase-digested C-terminally truncated apolipoprotein A-I in normal human serum. *J Immunol Methods*. 2011;369:51–58. doi: 10.1016/j.jim.2011.04.002.
54. Eberini I, Gianazza E, Breggi L, Klugmann S, Calabresi L, Gomaschi M, Mombelli G, Brusoni B, Wait R, Sirtori CR. Apolipoprotein A-I breakdown is induced by thrombolysis in coronary patients. *Ann Med*. 2007;39:306–311. doi: 10.1080/07853890701288760.
55. Gomaschi M, Ossoli A, Vitali C, Pozzi S, Vitali Serdoz L, Pitzorno C, Sinagra G, Franceschini G, Calabresi L. Off-target effects of thrombolytic drugs: apolipoprotein A-I proteolysis by alteplase and tenecteplase. *Biochem Pharmacol*. 2013;85:525–530. doi: 10.1016/j.bcp.2012.11.023.
56. Schmidt HH, Remaley AT, Stonik JA, Ronan R, Wellmann A, Thomas F, Zech LA, Brewer HB Jr, Hoeg JM. Carboxyl-terminal domain truncation alters apolipoprotein A-I *in vivo* catabolism. *J Biol Chem*. 1995;270:5469–5475.
57. Kaartinen M, Penttilä A, Kovanen PT. Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation*. 1994;90:1669–1678.
58. Frangogiannis NG, Ferrard JL, Mendoza LH, Burns AR, Lindsey ML, Ballantyne CM, Michael LH, Smith CW, Entman ML. Stem cell factor induction is associated with mast cell accumulation after canine myocardial ischemia and reperfusion. *Circulation*. 1998;98:687–698.
59. Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB, Mendoza LH, Spengler RN, Smith CW, Entman ML. Resident cardiac mast cells degranulate and release preformed TNF-alpha, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. *Circulation*. 1998;98:699–710.
60. Jin D, Takai S, Yamada M, Sakaguchi M, Kamoshita K, Ishida K, Sukenaga Y, Miyazaki M. Impact of chymase inhibitor on cardiac function and survival after myocardial infarction. *Cardiovasc Res*. 2003;60:413–420.
61. Nissen SE, Tsunoda T, Tuzcu EM, Schoenhagen P, Cooper CJ, Yasin M, Eaton GM, Lauer MA, Sheldon WS, Grines CL, Halpern S, Crowe T, Blankenship JC, Kerensky R. Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial. *JAMA*. 2003;290:2292–2300. doi: 10.1001/jama.290.17.2292.
62. Calabresi L, Tedeschi G, Treu C, Ronchi S, Galbiati D, Airoidi S, Sirtori CR, Marcel Y, Franceschini G. Limited proteolysis of a disulfide-linked apoA-I dimer in reconstituted HDL. *J Lipid Res*. 2001;42:935–942.
63. Favari E, Gomaschi M, Zanotti I, Bernini F, Lee-Rueckert M, Kovanen PT, Sirtori CR, Franceschini G, Calabresi L. A unique protease-sensitive high density lipoprotein particle containing the apolipoprotein A-I(Milano) dimer effectively promotes ATP-binding cassette A1-mediated cell cholesterol efflux. *J Biol Chem*. 2007;282:5125–5132. doi: 10.1074/jbc.M609336200.

Significance

Activated mast cells are present in human atherosclerotic lesions where they release chymase capable of avidly degrading lipid-free apolipoprotein A-I (apoA-I) and lipid-poor species of high-density lipoprotein. Here, we report that C-terminal cleavage of apoA-I by chymase abolished its anti-inflammatory properties. This is the first demonstration of involvement of a pathophysiologically relevant protease in the regulation of a multitude of anti-inflammatory functions of apoA-I. Because atherosclerotic lesions contain a variety of extracellular proteases capable of C-terminally cleaving apoA-I, local proteolytic inactivation of apoA-I and lipid-poor pre β -high-density lipoprotein particles is likely. Therefore, the use of protease-resistant apoA-I-mimetic peptides may be of special value in the treatment of atherosclerosis.

Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

Carboxyl-Terminal Cleavage of Apolipoprotein A-I by Human Mast Cell Chymase Impairs Its Anti-Inflammatory Properties

Su Duy Nguyen, Katariina Maaninka, Jani Lappalainen, Katariina Nurmi, Jari Metso, Katariina Öörni, Mohamad Navab, Alan M. Fogelman, Matti Jauhiainen, Miriam Lee-Rueckert and Petri T. Kovanen

Arterioscler Thromb Vasc Biol. 2016;36:274-284; originally published online December 17, 2015;

doi: 10.1161/ATVBAHA.115.306827

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2015 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/content/36/2/274>

Free via Open Access

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:

<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:

<http://atvb.ahajournals.org/subscriptions/>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
<http://atvb.ahajournals.org/subscriptions/>

Carboxyl-terminal cleavage of apolipoprotein A-I by human mast cell chymase impairs its anti-inflammatory properties

Su Duy Nguyen¹, Katariina Maaninka¹, Jani Lappalainen¹, Katariina Nurmi¹, Jari Metso², Katariina Öörni¹, Mohamad Navab³, Alan M. Fogelman³, Matti Jauhiainen², Miriam Lee-Rueckert¹, and Petri T. Kovanen^{1*}

¹Wihuri Research Institute, Biomedicum, Helsinki, FI-00290, Helsinki, Finland

²National Institute for Health and Welfare, Genomics and Biomarkers Unit, Biomedicum, Helsinki, Finland

³Division of Cardiology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA, 90095, USA

Running title: Chymase blocks anti-inflammatory effects of apoA-I

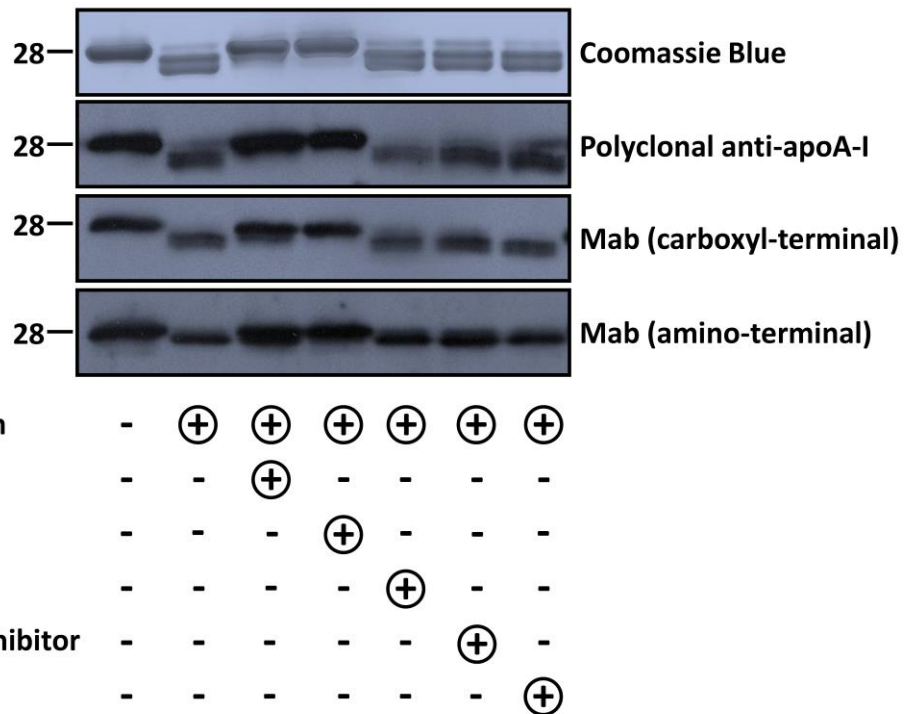
Supplemental Table I: Properties of (A-I)rHDLs

Type	Shape	Mobility	Composition	
			PC: cholesterol: apoA-I (molar ratio)	Lipid: protein (mass ratio)
rHDL-1	Discoidal	Pre β	30:12.5:1	1:1
rHDL-2	Discoidal	Pre β	140:12.5:1	3.9:1
rHDL-3	Discoidal	Pre β	250:12.5:1	6.8:1
HDL ₂	Spherical	α	ND	ND
HDL ₃	Spherical	α	ND	ND

ND: Not determined

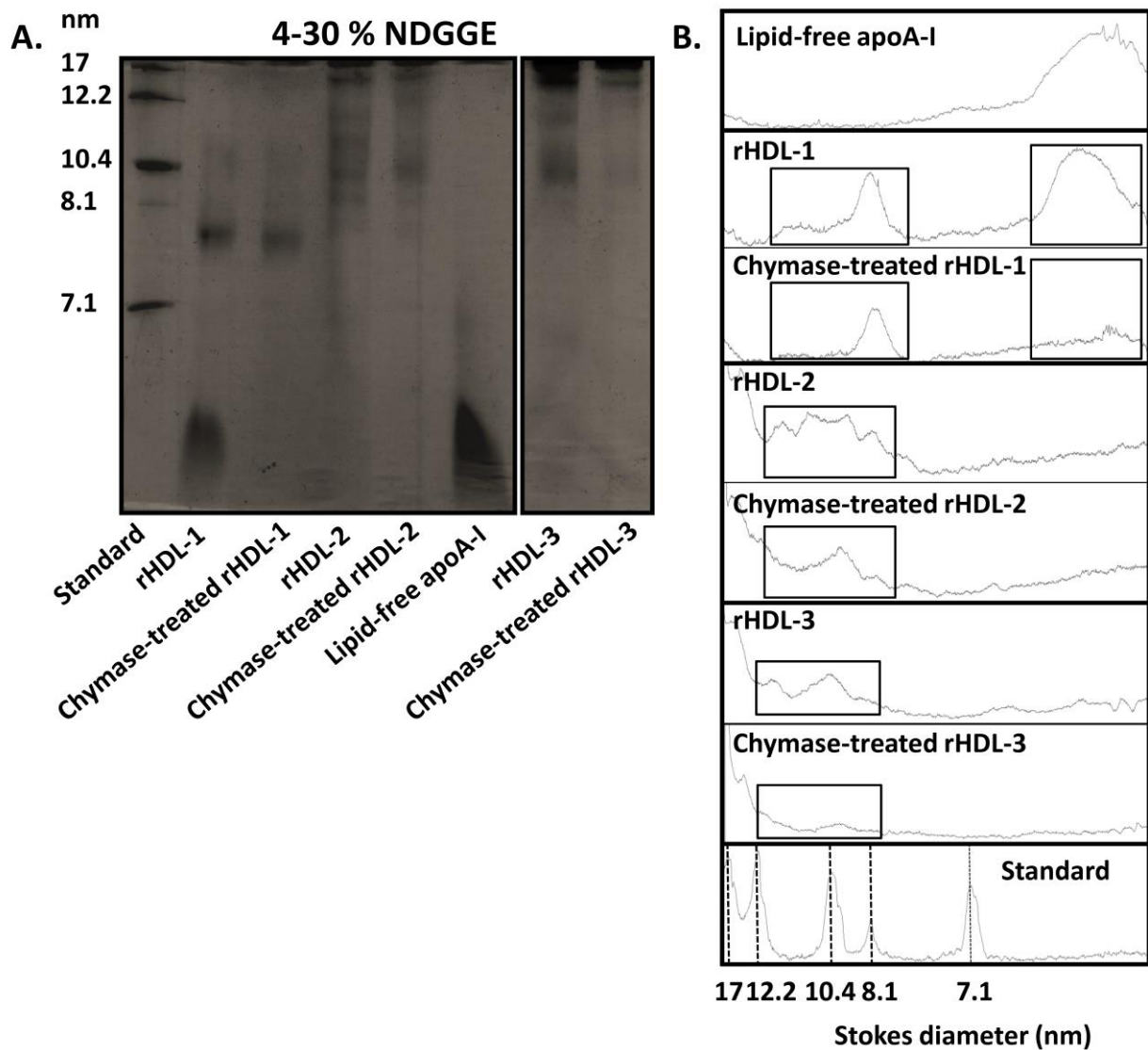
Supplemental Table II: Sequences of primers and fluorogenic probes used in qRT-PCR experiments

Gene	Forward Primers	Reverse Primers	Probes
VCAM-1	5'-CGCAAACACTTTTATGTCAATGTTG-3'	5'-GATTTTCGGAGCAGGAAAGC-3'	5'-CCAGAGATACAACCGTCTTGGTCAGCC--3'
ICAM-1	5'-CCCTGATGGGCAGTCAACA-3'	5'-GCAGCGTAGGGTAAGGTTCTTG-3'	5'-CCTCACCGTGTACTGGACTCCAGAACG-3'
TNF α	5'-GCTGCACTTTGGAGTGATCG-3'	5'-GTTTGCTACAACATGGGCTACAG-3'	5'-CCCAGGCAGTCAGATCATCTTCTCGA-3'
IL-1 β	5'-TTACAGTGGCAATGAGGATGAC -3'	5'-GTCGGAGATTCGTAGCTGGAT -3'	5'-AACAGATGAAGTGCTCCTTCCAGGACC-3'
IL-6	5'-GAACCTTCCAAAGATGGCTGA-3'	5'-CAAACCTCCAAAAGACCAGTGATG-3'	5'-CAGGCAAGTCTCCTCATTGAATCCAGA-3'
IL-8	5'-CTCTTGGCAGCCTTCCTGA-3'	5'-GGTGGAAAGGTTTGGAGTATGTC-3'	5'-TGGCAAAACTGCACCTTCACACAGAG-3'
COX-2	5'-CGAGGGCCAGCTTTCAC-3'	5'-GGCGCAGTTTACGCTGTCTAG-3'	5'-TGATTTAAGTCCACCCCATGGCCC-3'
GAPDH	5'-CCACATCGCTCAGACACCAT-3'	5'-GGCAACAATATCCACTTTACCAGAG-3'	5'-CCAATACGACCAAATCCGTTGACTCC-3'



Supplemental Figure I. SDS-PAGE analysis of apoA-I after treatment with mast cell- conditioned medium.

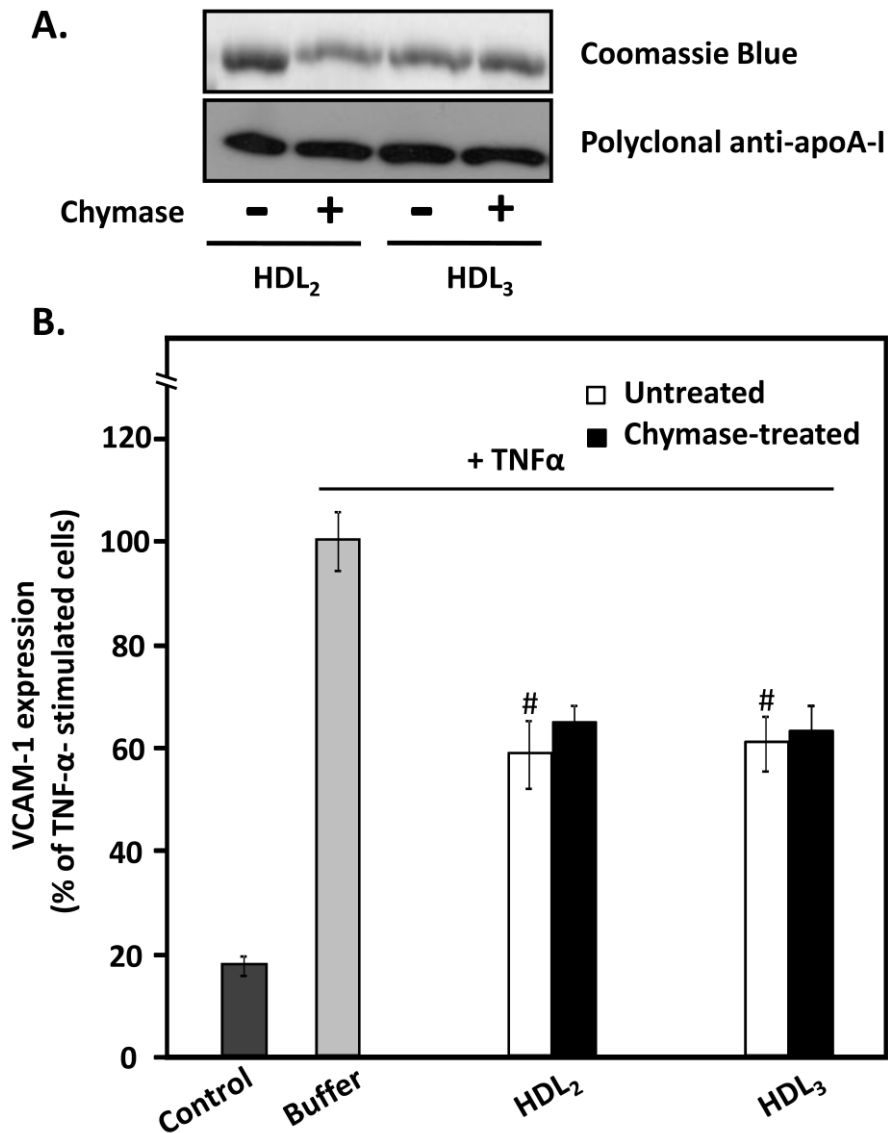
ApoA-I (1 mg/ml) and conditioned medium, in which mature human mast cells generated from circulating progenitors had been activated, were incubated at 37°C for 6 h. Incubations were performed in the absence or presence of the indicated proteinase inhibitors, which were added at concentrations which fully inhibited the respective proteases. The conditioned medium contained 0.15 µg (36 BTEE units)/ml chymase and co-secreted mast cell granule neutral proteases (cathepsin G, tryptase, carboxypeptidase A3, and granzyme B). After incubation, proteins were resolved in NuPAGE Novex 4-12% Bis-Tris gels and detected by Coomassie Blue or resolved in 12.5% SDS-PAGE and immunoblotted with anti-human apoA-I polyclonal antibody or with anti-human apoA-I monoclonal antibodies recognizing either a carboxyl-terminal (amino acids 211-220) or an amino-terminal (amino acids 2-8) region of apoA-I. Similar results were obtained using conditioned medium from mature mast cells derived from progenitors of another human donor.



Supplemental Figure II. Non-denaturing polyacrylamide gradient gel electrophoresis (NDGGE) analysis of rHDLs upon chymase treatment

A, Aliquots of untreated and chymase-treated (A-I)rHDL were electrophoresed by 4-30% NDGGE. The gels were stained with Coomassie blue.

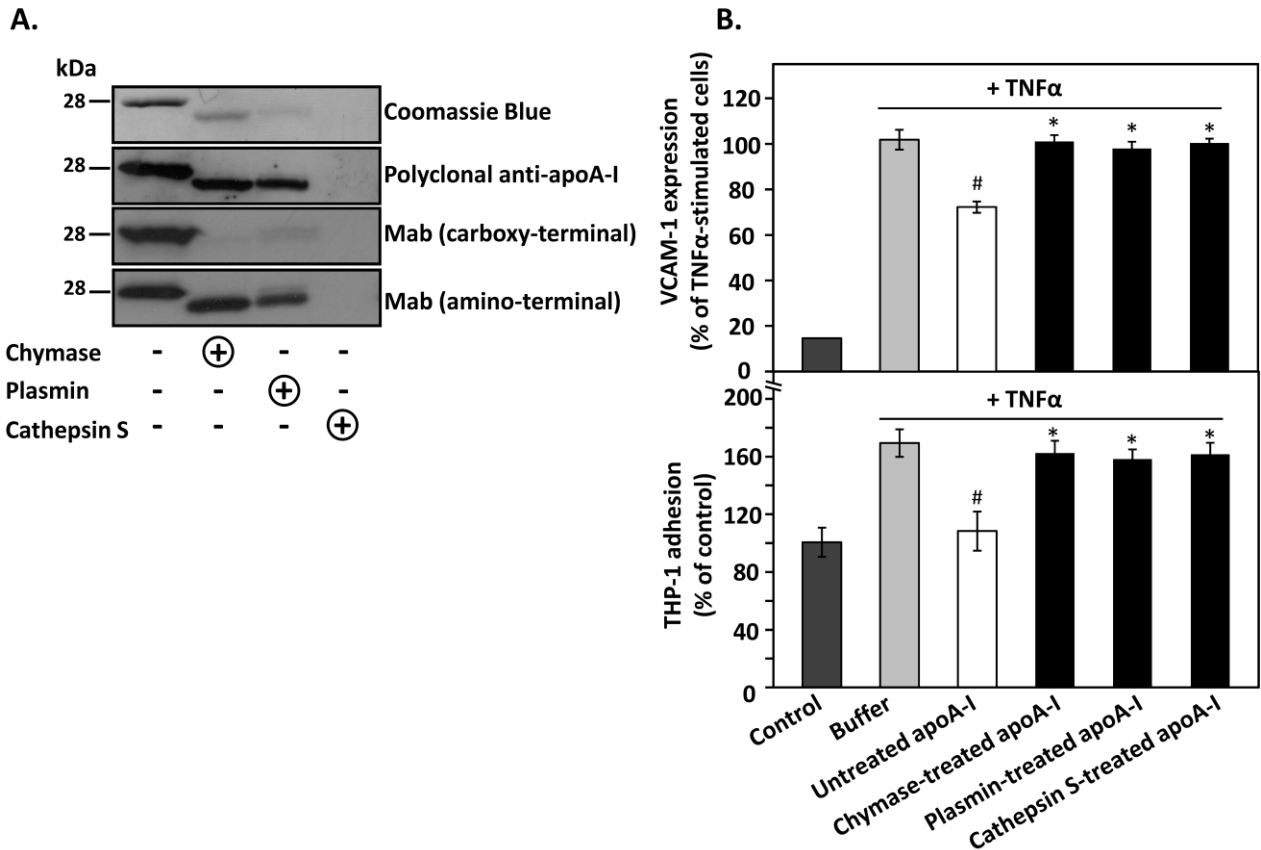
B, The profiles of particle size distribution were analyzed by NIH ImageJ software. The boxes show the areas in which the differences between each untreated and corresponding chymase-treated (A-I)rHDL are present.



Supplemental Figure III. Chymase treatment of mature HDL does not affect its ability to inhibit VCAM-1 expression on activated HCAECs.

A, HDL₂ or HDL₃ (1 mg/ml) were incubated in the absence (untreated) or presence of recombinant human chymase (40 BTEE units/ml) for 6 h. The proteins in the incubation mixture were resolved in 12.5% SDS-PAGE and detected by Coomassie Blue or immunoblotted with anti-human apoA-I polyclonal antibody.

B, HCAECs were preincubated for 16 h with untreated or chymase-treated HDL₂ or HDL₃ (50 μ g/ml, each), and then stimulated with TNF α (10 ng/ml) for 5 h. Non-activated cells (control) and TNF α -activated cells preincubated in only medium (buffer) acted as references. VCAM-1 surface protein expression levels were analyzed by flow cytometry (% of buffer). Data represent the means \pm SD from 3 independent experiments performed in duplicate. # p < 0.01 (untreated vs. buffer).



Supplemental Figure IV. Treatment of apoA-I with plasmin or cathepsin S impairs its ability to attenuate proinflammatory responses in activated HCAECs.

A, ApoA-I (1 mg/ml) was incubated for 1 h in the absence or presence of chymase (40 BTEE U/ml = 0.5 μ g/ml), plasmin (0.1 U/ml = 50 μ g/ml), or cathepsin S (0.15 U/ml = 5 μ g/ml) in TNE buffer. The untreated and protease-treated apoA-I were resolved in 12.5% SDS-PAGE and detected by Coomassie Blue or immunoblotted with anti-human apoA-I polyclonal antibody or with anti-human apoA-I monoclonal antibody recognizing either a carboxyl-terminal (amino acids 211-220) or an amino-terminal (amino acids 2-8) region of apoA-I.

B, HCAECs were preincubated for 16 h with untreated or protease-treated apoA-I (50 μ g/ml, each), and then stimulated with TNF α (10 ng/ml) for 5 h. Non-activated cells (control) and TNF α -activated cells preincubated in only medium (buffer) acted as references. Cell surface expression of VCAM-1 was determined by flow cytometry (top panel). Data represent the mean \pm SD from three to four independent experiments performed in duplicate. In a separate experiment, HCAECs that had been treated as described above were incubated for 1 h with fluorescently labeled THP-1 macrophages. Nonadherent THP-1 cells were removed by gentle washing and the fluorescence of HCAECs-bound THP-1 cells was measured. The fluorescence signals of TNF α -stimulated HCAECs were expressed as percentages of the basal signal from the control cells. Data shown in the panels represent the means \pm SEM from 3 independent experiments performed in triplicate wells. * $p < 0.01$ (untreated vs. protease-treated); # $p < 0.01$ (untreated vs. buffer).

Carboxyl-terminal cleavage of apolipoprotein A-I by human mast cell chymase impairs its anti-inflammatory properties

Su Duy Nguyen¹, Katariina Maaninka¹, Jani Lappalainen¹, Katariina Nurmi¹, Jari Metso², Katariina Öörni¹, Mohamad Navab³, Alan M. Fogelman³, Matti Jauhiainen², Miriam Lee-Rueckert¹, and Petri T. Kovanen^{1*}

¹Wihuri Research Institute, Biomedicum, Helsinki, FI-00290, Helsinki, Finland

²National Institute for Health and Welfare, Genomics and Biomarkers Unit, Biomedicum, Helsinki, Finland

³Division of Cardiology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA, 90095, USA

Running title: Chymase blocks anti-inflammatory effects of apoA-I

Materials and Methods

Preparation of apoA-I-containing reconstituted HDL [(A-I)rHDL]

Lipid-free apoA-I was purified according to a previously published procedure.¹ Three types of (A-I)rHDL particles with varying degrees of lipidation were prepared by the cholate dialysis method.² The molar ratio of egg yolk phosphatidyl choline (PC): cholesterol: apoA-I was 30:12.5:1, 140:12.5:1, and 250:12.5:1 for rHDL-1, rHDL-2, and rHDL-3, respectively. The proteoliposome preparations were dialyzed extensively against endotoxin-free Tris-buffered saline (pH 7.4) at 4°C, stored at 4°C, and used within a week. All (A-I)rHDLs were found to be heterogeneous in size and they exhibited pre β -mobility (Supplemental Table I).

Isolation of high-density lipoproteins

HDL₂ (d = 1.063-1.125 g/ml) and HDL₃ (d = 1.125-1.210 g/ml) were prepared from freshly isolated plasma of healthy volunteers obtained from the Finnish Red Cross by a rapid sequential flotation ultracentrifugation (UC) using KBr for density adjustment.³ This short UC method carried out in a Beckman Optima™ TLX system Table Top Ultracentrifuge with a Beckman fixed-angle rotor (TLA100.3) at 541,000 g yields HDL preparations devoid of pre β -migrating particles.⁴ The amounts of HDL are expressed in terms of their protein concentrations, which were determined by BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as standard.

Treatment of lipid-free apoA-I, (A-I)rHDLs, HDL₂, HDL₃, and of apoA-I mimetic peptides L-4F and D-4F by purified proteases

Lipid-free apoA-I, (A-I)rHDL, HDL₂, HDL₃, or the apoA-I mimetic peptides L-4F and D-4F (1 mg/ml, each) were incubated for 6 h in the absence (untreated) or the presence of 40 BTEE units/ml (units of activity as described by Woodbury⁵) of recombinant human chymase (kindly provided by Teijin Ltd. Hino, Tokyo, Japan) in 5 mM Tris buffer containing 150 mM NaCl and 1 mM EDTA (TNE buffer, pH 7.4) for 6 h at 37°C. Lipid-free apoA-I (1 mg/ml) was also incubated for 1 h with 40 BTEE units/ml of chymase, 0.1 U/ml of plasmin (Sigma: P1867) or 0.15 U/ml of cathepsin S (Calbiochem: 219343) in TNE buffer. After the incubations, chymase and plasmin were fully inhibited by adding soybean trypsin inhibitor (SBTI, final concentration, 100 μ g/ml),^{6,7} whereas cathepsin S was inhibited by adding E-64 (Sigma: E3132, final concentration, 10 μ M).⁸ Aliquots of the incubation mixtures containing the various untreated and protease-treated apoA-I species were subjected to 12.5% SDS-PAGE analysis under reducing conditions. The protein bands were stained with Coomassie Blue, or were transferred to nitrocellulose and immunoblotted with anti-human apoA-I polyclonal antibody, or with anti-human apoA-I monoclonal antibodies recognizing either a C-terminal region (residues 211–220) (MAb 4.1; kindly provided by Drs. Noel Fidge and Dmitri Sviridov, Prahran, Australia) or the N-terminus (residues 2–8) (MAb 4H1; kindly provided by Dr. Yves Marcel, Ottawa, Canada) of apoA-I. Both untreated and protease-treated (A-I)rHDL were further characterized by non-denaturing gradient gel electrophoresis (NDGGE) to assess HDL particle size distribution upon chymase modification. The aliquots were loaded onto self-prepared 4–30% polyacrylamide gradient gels (8.0 cm x 8.0 cm) and run at 125 V under nondenaturing conditions overnight at 4°C to reach equilibrium and then stained with Coomassie blue.⁴ HDL particle size was determined based on the use of high-molecular-weight electrophoresis calibration standards as molecular size markers. The intensities of the bands and particle size distribution profiles were analyzed by NIH ImageJ software.

Proteolysis of lipid-free apoA-I by human mast cell-conditioned medium

Mature human mast cells were isolated and cultured as described previously.⁹ At week 9 of culture, 2×10^6 mast cells were stimulated with $1 \mu\text{M}$ calcium ionophore (A23187; Sigma-Aldrich, St. Louis, MO, USA) in DPBS containing 100 ng/ml kit ligand (KITLG). Mast cell stimulation induces secretion of a mixture of heparin proteoglycans, neutral proteases, and other active compounds into cell culture medium. After 30 min of stimulation, the mast cells were sedimented, the conditioned medium was collected and stored at $-80 \text{ }^\circ\text{C}$ until used. The activity and concentration in the conditioned medium of chymase⁵ and other neutral proteases¹⁰ were determined as reported previously. Lipid-free apoA-I (1 mg/ml) was incubated for 6 h at 37°C in PBS buffer (pH 7.4) in the absence or presence of conditioned medium (containing 40 or 120 BTEE units/ml chymase activity). After the incubations, chymase activity was fully inhibited by addition of SBTI, and aliquots of the incubation mixtures were subjected to 12.5% SDS-PAGE analysis under reducing conditions as described above.

To determine individual contributions of the various proteases present in the mast cell-conditioned medium to apoA-I degradation, each protease was fully inhibited by pre-incubating the conditioned medium for 30 min on ice in the presence of specific protease inhibitors at the following final concentrations: diphenyl $\text{N}\alpha$ -benzoxycarbonyl-L-Arg-Glu-Thr-Phe^P-phosphonate (RETF-(OPh)) (chymase inhibitor; kindly provided by Dr. Gunnar Pejler, Uppsala, Sweden, $2.3 \mu\text{M}$); cathepsin G inhibitor I, (cathepsin inhibitor, Calbiochem, 500 nM); leupeptin (trypsin inhibitor, Sigma, $50 \mu\text{g/ml}$); carboxypeptidase inhibitor from potato tuber (carboxypeptidase A3 inhibitor, Sigma, $25 \mu\text{g/ml}$); and benzyloxycarbonyl-Ala-Ala-Asp-chloromethylketone (granzyme B inhibitor, Enzo Life Science, $20 \mu\text{M}$). Next, aliquots of various inhibitor-pretreated conditioned media which contained chymase (final concentration, 36 BTEE unit/ml corresponding to $0.15 \mu\text{g/ml}$) and co-secreted mast cell granule neutral proteases (cathepsin G, trypsin, carboxypeptidase A3, and granzyme B) were added to apoA-I (final concentration, 1 mg/ml) in PBS, and incubated for 6 h at 37°C . Finally, the aliquots were subjected to high-resolution electrophoresis gel analysis using NuPAGE Novex 4-12% Bis-Tris gels.

Culture of human coronary artery endothelial cells

HCAECs (PromoCell) were cultured in Endothelial Cell Growth Medium MV (Basal Medium; Catalog Number C-22220, PromoCell) supplemented with 5% fetal calf serum, 0.4% endothelial cell growth supplement, 10 ng/ml epidermal growth factor, $90 \mu\text{g/ml}$ heparin, $1 \mu\text{g/ml}$ hydrocortisone (Supplement pack, Catalog Number C-39220, PromoCell), and 100 U/mL penicillin streptomycin solution, and 50 ng/ml amphotericin B to yield Complete Medium in T-75 flask according to the manufacturer's instructions. Confluent HCAECs were washed with 15 ml of PBS, trypsinized, and replated in Complete Medium, as described below. Experiments were performed with cells of 5-8th passage from two donors. HCAECs were seeded at a density of $0.5\text{-}1 \times 10^5$ cells/well in 12 well plates (1.5 ml medium/well) and cultured for 2-3 days until the cells reached 80-85% confluency. Then the HCAECs were washed with Complete Medium (1 ml) and replaced with Basal Medium supplemented with protease inhibitors (SBTI, $100 \mu\text{g/ml}$ or E-64, $10 \mu\text{M}$) and pre-incubated with the untreated or protease-treated apoA-I species or the mimetic peptides L-4F or D-4F ($50 \mu\text{g/ml}$, each) for 16 h, followed by $\text{TNF}\alpha$ (10 ng/ml) stimulation for 5 h. The expression of VCAM-1 and other proinflammatory genes was evaluated by quantitative RT-PCR and the cell surface expression of VCAM-1 protein was measured by flow cytometry.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured HCAECs or human macrophage foam cells (RNeasy kit, QIAGEN). Nucleic acid concentrations were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Purified RNA (0.5 µg) was used to generate cDNA using RT-PCR (M-MLV reverse transcriptase, Promega). Oligonucleotide sequences of the primers for detection of GAPDH (internal control), VCAM-1, ICAM-1, TNF α , IL-1 β , IL-6, IL-8, and COX-2 are shown in supplemental Table II. cDNA was diluted 1:5 and analyzed in duplicates on 96-well optical plates using the TaqMan Gene Expression Master Mix (Applied Biosystems). The thermal cycling parameters were as follows: thermal activation for 10 min at 95°C, and 40 cycles of PCR (melting for 15s at 95°C and annealing/extension for 1 min at 60°C). Relative quantification was calculated with the $2^{-\Delta\Delta CT}$ method¹¹ and normalized to GAPDH.

Flow cytometric analysis of VCAM-1

HCAECs were washed with 1 ml cold PBS, detached from culture plates according to the manufacturer's instructions, and centrifuged at 300 x g for 5 min at 4°C. Cells were then washed two times with FACS buffer (PBS buffer containing 0.02% sodium azide and 0.5% bovine serum albumin), resuspended in 100 µl of FACS buffer containing PE-labeled mouse anti-human VCAM-1 (BD Pharmingen™), and incubated at 4°C in the dark for 30 min. At the end of incubation, the cells were washed 3 times with FACS buffer, and then fixed in FACS buffer containing 2% formaldehyde for 20 min at 4°C. The expression of cell surface VCAM-1 was analyzed by using a LSR II flow cytometer (BD Pharmingen). The PE-labeled mouse IgG1 isotype non-specific antibody was used as a negative control (BD Pharmingen). Cells were gated, and data were obtained from fluorescence channels in a logarithmic mode. Data from 10,000 cells were collected, and processed using the CellQuest program (BD Biosciences).

Adhesion of THP-1 monocytes to HCAECs

The ability of THP-1 monocytes to adhere to TNF α -activated HCAECs was determined using Vybrant cell adhesion assay kit (Molecular Probes). Typically, HCAECs (80-85% confluency) were washed with Complete Medium and replaced with Basal Medium supplemented with protease inhibitors (SBTI, 100 µg/ml or E-64, 10 µM) and incubated for 16 h with untreated or the various protease-treated apoA-I and (A-I)rHDL (50 µg/ml, each), followed incubation for 5 h with TNF α (10 ng/mL). After stimulation, the HCAECs were washed two times with HBSS culture medium (BioWhittaker, Lonza, Switzerland). Concurrently, THP-1 cell suspensions were adjusted to 5×10^6 cells/ml and fluorescently labeled with Calcein AM (final concentration, 5 µM; 30 min,) in PBS. THP-1 cells were washed two times with PBS to remove nonincorporated calcein. The fluorescently labeled-THP-1 cells were resuspended in HBSS culture medium and added (50,000 cells/well) to HCAECs. After incubation for 60 min at 37°C, non-adherent cells were removed by gently washing four times with HBSS culture medium and fluorescence of HCAEC-bound THP-1 cells was measured using VICTOR3 multilabel plate reader (Perkin Elmer, Finland) at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. The fluorescence signal of basal untreated HCAECs (control) was designated as 100%.

Transendothelial migration of monocytes

Monocyte transmigration across an endothelial cell monolayer was determined using the CytoSelect™ Leukocyte Transendothelial Migration Assay kit (Cell Biolabs, Inc., San Diego, USA) according to manufacturer's recommendations with some modifications. Briefly, HCAECs (150,000 cells) were added to the upper side of porous filter inserts (6.5 mm insert diameter, 3 µm pore size, Corning Life Sciences, NY, USA) precoated with rat-tail collagen (10 µg/cm²; BD Biosciences) and cultured in Complete Medium for 2-3 days to obtain tight confluent monolayers of HCAECs. Thereafter, the HCAECs were washed, and incubated for 16 h in Basal Medium supplemented with the protease inhibitor SBTI (100 µg/ml), and either untreated or chymase-treated apoA-I (final concentration, 50 µg/ml) added to either the upper (apical) or lower (basolateral) transwell compartment. The HCAECs were activated by adding TNFα (10 ng/ml) to the apical compartment of the transwell system. After a 5 h stimulation, the HCAECs were washed twice with HBSS culture medium (BioWhittaker, Lonza, Switzerland), and fluorescently labeled-THP-1 cells (200,000 cells in HBSS culture medium) were added to the apical compartment and allowed to migrate for 2 hours. Then, media were carefully aspirated from the apical compartment and non-migratory cells were gently removed by using cotton swabs. The cells in the insert and in the basolateral medium (together comprising the "transmigrated cells") were transferred to new wells containing lysis buffer and incubated for 10 min at room temperature. 200 µl aliquots of lysates were transferred to a 96-well plate for fluorescence measurement. The fluorescence signal of transmigrated cells under control condition (control) was designated as 100%. The net yield of transmigrated cells under control condition was within the 6.5% - 23% range of the total number of THP-1 cells added.

NF-κB nuclear translocation assay

HCAECs (80-85% confluency) grown in Complete Medium were washed with Complete Medium and after which the cells received Basal Medium supplemented with SBTI (100 µg/ml) and either untreated or chymase-treated apoA-I (final concentration, 50 µg/ml, each) for 16 h and finally stimulated with TNFα (10 ng/mL) for 15 min. After the incubation, cells were washed with cold PBS, scraped into cold PBS supplemented with phosphatase inhibitors, and nuclear extracts were prepared using the nuclear extract kit (Active Motif, Rixenart, Belgium) according to the manufacturer's instructions. To detect NF-κB activation, nuclear extracts (2.0 µg) were analyzed using the TransAM NF-κB p65 kit (Active Motif, Rixenart, Belgium) according to the manufacturer's instructions.

Radiolabeling and binding of apoA-I to HCAECs

ApoA-I was labeled with ¹²⁵I using Iodination Beads (Pierce, Rockville, IL) and Na¹²⁵I (Thermo Scientific) according to the manufacturer's instructions to yield final specific activities of 700-800 dpm/ng protein. To study the effect of chymase on apoA-I binding, ¹²⁵I-apoA-I was incubated for 6 h at 37°C in the absence or the presence of chymase. After addition of SBTI for full inhibition of chymase activity, the binding assay of ¹²⁵I-apoA-I was conducted as described previously¹² with some modifications. Briefly, HCAECs were seeded at a density of 2.5- 5 x 10⁴ cells/well in 24 well plates (1.0 ml medium/well) and cultured for 2-3 days until the cells reached 90-95% confluency. Then the cells were chilled on ice, and kept at 4°C throughout the entire experiment. The cells were washed once with Complete Medium, and incubated with the untreated or chymase-treated ¹²⁵I-apoA-I at the indicated concentrations in 0.5 ml Basal Medium containing 0.5 % fatty acid-free BSA in the absence or presence of a 40-fold excess of unlabeled apoA-I. After incubation for 2 h, cells were washed once with PBS containing

0.5% fatty acid-free BSA, followed by three washes with PBS. The cells were then solubilized in 0.25 ml of 0.2 M NaOH for 1 h at room temperature, the radioactivity was measured using Wallac Liquid Scintillation Counter (Wallac, Finland), and the protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL). High-affinity binding was calculated by subtracting the values obtained in the presence of 40-fold excess of unlabeled apoA-I (non-specific binding) from those obtained in its absence (total binding).

Culture of primary human monocyte-derived macrophages

Human monocytes were isolated from buffy coats (Finnish Red Cross Blood Transfusion Center, Helsinki, Finland) by centrifugation in Ficoll-Paque gradient as described.¹³ Washed cells were suspended in DMEM supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, counted, and seeded on 24 well-plates (1.5 million cells per well). After 1 h incubation, non-adherent cells were removed and the medium was replaced with macrophage-SFM medium (Gibco) supplemented with 1% penicillin-streptomycin and 10 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF) or 50 ng/ml of macrophage colony-stimulating factor (M-CSF) (Biosite, San Diego, USA). The cells were cultured for 7 days in the presence of GM-CSF or M-CSF to allow them to differentiate into GM-CSF macrophages (GM-Mac) or M-CSF macrophages (M-Mac) as described previously.¹⁴ The medium was then changed every 2 to 3 days throughout the culture period. The differentiated macrophages were incubated for 24 h in DMEM containing 25 µg/ml of acetylated LDL to generate cholesterol-loaded foam cells. The cells were washed with PBS twice, replaced with DMEM supplemented with SBTI (100 µg/ml), and incubated with untreated or chymase-treated apoA-I (50 µg/ml, each) for 3 h. To prevent the binding of LPS to apoA-I,^{15, 16} macrophage foam cells which had been preincubated with the untreated apoA-I or the chymase-treated apoA-I, were extensively washed, and then challenged with 100 ng/ml of LPS from *Escherichia coli* serotype 0111:B4 (Sigma: Finland) for 3 h. Cells were collected and the expression of proinflammatory genes was evaluated by qRT-PCR. In parallel experiments, cholesterol efflux from macrophage foam cells was measured. For this purpose, the cholesterol-loaded foam cells were incubated in DMEM supplemented with SBTI (100 µg/ml) containing either untreated or chymase-treated apoA-I (50 µg/ml, each) as cholesterol acceptors. After incubation for 3 h, cholesterol efflux were determined, as described previously.⁴

Effects of chymase-treated apoA-I, L-4F, and D-4F on PMA-activated neutrophils

Human neutrophils were isolated from freshly prepared buffy coats by a standard method of centrifugation in a Ficoll Hypaque gradient prior to dextran sedimentation.¹⁷ After dextran sedimentation, all steps were carried out at 4°C. Any remaining erythrocytes were lysed in ice-cold de-ionized water and tonicity was restored by the addition of equal volume of 1.8% NaCl. Cells were washed, adjusted to 4 x 10⁶ cells/ml in HBSS buffer (pH 7.4) (BioWhittaker, Lonza, Switzerland), maintained at 4°C, and used within 2 h after the isolation. The isolated neutrophils contained > 95% viable cells, as determined by the NucleoCounter NC-200 (ChemoMetec A/S).

Superoxide anion production was detected by lucigenin-enhanced chemiluminescence assay.¹⁸ The freshly isolated neutrophils (100,000 cells/well) were preincubated with increasing concentrations of the untreated or chymase-treated apoA-I, L-4F, or D-4F for 5 min at room temperature in 96-well plates in 0.1 ml HBSS solution, followed by the addition of lucigenin (final concentration, 10 µM). After 2 min incubation at room temperature for stabilization, the neutrophils were activated by PMA (final

concentration, 100 nM) for 10 min. The plates were protected from light and the luminescence signal (counts per second) was determined using a VICTOR3 multilabel plate reader (Perkin Elmer, Finland). The luminescence signal of basal unstimulated neutrophils (control) was designated as 1.

The effect of proteolysis on anti-inflammatory properties of apoA-I *in vivo*

Untreated and chymase-treated apoA-I (1 mg/ml) were prepared in PBS buffer (BioWhittaker, Lonza, Switzerland), as described above. Thus, apoA-I was incubated in the absence or presence of recombinant human chymase, after which PMSF (final concentration, 1 mM) was added to the incubation mixture to irreversibly inhibit chymase. The unbound PMSF and small fragments of apoA-I were removed by extensive dialysis against PBS buffer (BioWhittaker, Lonza, Switzerland) at 4°C using a dialysis membrane with molecular weight cutoff of 12 000–14 000 Da. The untreated and chymase-treated apoA-I (0.5 mg/ml) were mixed with LPS (50 µg/ml) and incubated at room temperature for 15-30 min before injection into mice.

Female C57BL/6J mice (aged 18-27 weeks) from Harlan Laboratories (Venray, the Netherlands) were housed 3-5 per cage under controlled conditions for the light/dark cycle, temperature, and humidity. The animals were kept in the same animal facility for at least 1 week before the experiments. Mice were fed a standard chow diet (2016 Teklad Global, Harlan Laboratories), and food and water were provided ad libitum. Animal experiments and the protocols were approved by The Finnish National Animal Experiment Board. Mice (6-8 per group) were randomized to receive a 400 µl intraperitoneal injection of saline vehicle (PBS), LPS (1 mg/kg: *Escherichia coli* serotype 0111:B4; Sigma), LPS (1 mg/kg) plus apoA-I (10 mg/kg), or LPS (1 mg/kg) plus chymase-treated apoA-I (10 mg/ml). Three hours after the injection, the animals were terminally anesthetized with isoflurane and blood was collected by cardiac puncture for determination of proinflammatory mediators. The levels of TNFα and IL-1β in the serum were quantified by using ELISA kits according to the manufacturer's instructions (R&D Systems). Endotoxin activity of LPS and of mixtures of LPS with apoA-I or chymase-treated apoA-I were measured by the limulus amoebocyte lysate (LAL) assay (Lonza group Ltd., Switzerland), as described in a previous study.¹⁹

Statistical analysis

Data are reported as means ± SD. Groups were compared using one-way ANOVA or two-tailed Student's *t*-test, as appropriate. A value of $p < 0.05$ was considered statistically significant.

References

1. Pussinen PJ, Jauhiainen M, Metso J, Pyle LE, Marcel YL, Fidge NH, Ehnholm C. Binding of phospholipid transfer protein (PLTP) to apolipoproteins A-I and A-II: location of a PLTP binding domain in the amino terminal region of apoA-I. *J Lipid Res.* 1998;39:152-161.
2. Jauhiainen M, Dolphin PJ. Human plasma lecithin-cholesterol acyltransferase. An elucidation of the catalytic mechanism. *J Biol Chem.* 1986;261:7032-7043.
3. McPherson PA, Young IS, McKibben B, McEneny J. High density lipoprotein subfractions: isolation, composition, and their duplicitous role in oxidation. *J Lipid Res.* 2007;48:86-95.
4. Nguyen SD, Oorni K, Lee-Rueckert M, Pihlajamaa T, Metso J, Jauhiainen M, Kovanen PT. Spontaneous remodeling of HDL particles at acidic pH enhances their capacity to induce cholesterol efflux from human macrophage foam cells. *J Lipid Res.* 2012;53:2115-2125.
5. Woodbury RG, Everitt MT, Neurath H. Mast cell proteases. *Methods Mol Biol.* 1981;80 Pt C:588-609.
6. Lindstedt L, Kovanen PT. Plasmin and kallikrein reduce HDL-induced cholesterol efflux from foam cells. *Biochem Biophys Res Commun.* 2000;277:552-557.
7. Lee M, Kovanen PT, Tedeschi G, Oungre E, Franceschini G, Calabresi L. Apolipoprotein composition and particle size affect HDL degradation by chymase: effect on cellular cholesterol efflux. *J Lipid Res.* 2003;44:539-546.
8. Lindstedt L, Lee M, Oorni K, Bromme D, Kovanen PT. Cathepsins F and S block HDL3-induced cholesterol efflux from macrophage foam cells. *Biochem Biophys Res Commun.* 2003;312:1019-1024.
9. Lappalainen J, Lindstedt KA, Kovanen PT. A protocol for generating high numbers of mature and functional human mast cells from peripheral blood. *Clin Exp Allergy.* 2007;37:1404-1414.
10. Maaninka K, Lappalainen J, Kovanen PT. Human mast cells arise from a common circulating progenitor. *J Allergy Clin Immunol.* 2013;132:463-469.
11. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods.* 2001;25:402-408.
12. Rohrer L, Cavelier C, Fuchs S, Schluter MA, Volker W, von Eckardstein A. Binding, internalization and transport of apolipoprotein A-I by vascular endothelial cells. *Biochim Biophys Acta.* 2006;1761:186-194.
13. Saren P, Welgus HG, Kovanen PT. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. *J Immunol.* 1996;157:4159-4165.
14. Waldo SW, Li Y, Buono C, Zhao B, Billings EM, Chang J, Kruth HS. Heterogeneity of human macrophages in culture and in atherosclerotic plaques. *Am J Pathol.* 2008;172:1112-1126.
15. Biedzka-Sarek M, Metso J, Kateifides A, Meri T, Jokiranta TS, Muszynski A, Radziejewska-Lebrecht J, Zannis V, Skurnik M, Jauhiainen M. Apolipoprotein A-I exerts bactericidal activity against *Yersinia enterocolitica* serotype O:3. *J Biol Chem.* 2011;286:38211-38219.
16. Henning MF, Herlax V, Bakas L. Contribution of the C-terminal end of apolipoprotein AI to neutralization of lipopolysaccharide endotoxic effect. *Innate Immun.* 2011;17:327-337.

17. Nauseef WM. Isolation of human neutrophils from venous blood. *Methods Mol Biol.* 2007;412:15-20.
18. Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA. Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem.* 1998;273:2015-2023.
19. David SA, Awasthi SK, Balaram P. The role of polar and facial amphipathic character in determining lipopolysaccharide-binding properties in synthetic cationic peptides. *J Endotoxin Res.* 2000;6:249-256.