Acidic Extracellular Environments Strongly Impair ABCA1-Mediated Cholesterol Efflux From Human Macrophage Foam Cells

Miriam Lee-Rueckert, Jani Lappalainen, Hannele Leinonen, Tero Pihlajamaa, Matti Jauhiainen, Petri T. Kovanen

Objective—In the deep microenvironments of advanced human atherosclerotic lesions, the intimal fluid becomes acidic. We examined the effect of an acidic extracellular pH on cholesterol removal (efflux) from primary human macrophages.

Methods and Results—When cholesterol efflux from acetyl-low-density lipoprotein-loaded macrophages to various cholesterol acceptors was evaluated at pH 7.5, 6.5, or 5.5, the lower the pH the more was cholesterol efflux reduced. The reduction of efflux to lipid-free apolipoprotein A-I was stronger than to high-density lipoprotein, or to plasma. Cholesterol efflux to every acceptor was severely compromised also at neutral pH when the macrophages had been loaded with cholesterol at acidic pH, or when both loading and efflux were carried out at acidic pH. Compatible with these observations, the typical upregulation of ABCA1 and ABCG1 mRNA levels in macrophages loaded with cholesterol at neutral pH was rapidly attenuated in acidic medium. The secondary structure of apolipoprotein A-I did not change over the pH range studied, supporting the notion that the inhibitory effect of acidic pH on cholesterol efflux rather impaired the ability of the foam cells to facilitate ABCA1-mediated cholesterol release. Secretion of apolipoprotein E from the foam cells was fully inhibited when the pH was 5.5, which further reduced cholesterol efflux.

Conclusion—An acidic pH reduces cholesterol efflux via different pathways and particularly impairs the function of the ABCA1 transporter. The pH-sensitive function of human macrophage foam cells in releasing cholesterol may accelerate lipid accumulation in deep areas of advanced atherosclerotic plaques where the intimal fluid is acidic. (Arterioscler Thromb Vasc Biol. 2010;30:1766-1772.)

Key Words: acidic environment • macrophage foam cells • cholesterol efflux • ATP binding cassette transporters • apolipoprotein E

A hallmark of atherosclerosis is the conversion of intimal macrophages into cholesterol-laden foam cells. High-density lipoproteins (HDL) are the most efficient acceptors of cellular cholesterol, so initiating the reverse cholesterol transport from the macrophage foam cells in the intima. Specific cellular transporters preferentially promote efflux of cholesterol from macrophages to different subpopulations of HDL particles. The ABCA1 transporter mediates unidirectional efflux of cholesterol to lipid-free apolipoprotein (apo) A-I and to lipid-poor pre-β-migrating HDL, whereas the ABCG1 transporter facilitates cholesterol efflux to mature α-HDL but not to lipid-free apoA-I or small-sized pre-β-HDL particles. Importantly, studies in vitro have evidenced that both transporters become abundantly expressed in cholesterol-loaded macrophages. In addition, diffusion-based efflux of cholesterol to mature HDL particles occurs in all types of cells and is facilitated by the scavenger receptor type I (SR-BI), which moderately contributes to cholesterol release from macrophages. Human macrophages can adapt to acidic environments, such as within the core of solid tumors where the extracellular pH may be as low as 5.2. In advanced atherosclerotic lesions, also, the extracellular fluid gradually becomes acidic, and such low pH values have even been suggested as a novel marker of plaque vulnerability. This heterogeneity of pH values well agrees with the general concept of advanced atherosclerotic lesions being “complex” lesions. Acidification of the intimal fluid in advanced atherosclerotic lesions partially depends on redox potential changes, local hypoxia, and on the activation of macrophages that renders them particularly sensitive to hypoxic conditions. Importantly, due to the increased thickness of atherosclerotic lesions, macrophages are subjected to low diffusion of oxygen that stimulates anaerobic glycolysis for cellular ATP generation. As a consequence, such activated macrophages produce and secrete metabolites, such as lactic acid and CO2, to the extracellular fluid and, thus, acidify their pericellular milieu.
First, we evaluated the effect of acidic pH on cellular cholesterol efflux from macrophage foam cells. To this end, human monocyte-derived macrophages were incubated in custom-made DMEM culture media of pH 5.5 to 7.5, and their responses to cholesterol loading and cholesterol efflux were evaluated. No differences were found in macrophage morphology after incubation under acidic conditions. Cell viability, as evaluated by Trypan blue exclusion test and lactate dehydrogenase released to media, remained similar on incubation in neutral and acidic media. The present work is based on the study of human monocyte-derived macrophages from altogether more than 40 healthy donors. A detailed description of the methods is provided in the supplemental data, available online at http://atvb.ahajournals.org.

Results
Efflux of Cholesterol From Macrophage Foam Cells to apoA-I Is Reduced in Acidic Medium

Interestingly, activated cultured murine macrophages are able to decrease the pH in their peripheral milieu down to 3.6, and this response increases when the cells are exposed to oxidized low-density lipoprotein (LDL).17

Interestingly, it was recently found that macrophages create acidic extracellular surface-connected compartments to digest aggregated LDL particles, in which pH as low as 5.0 was recorded.18 Whether an acidic extracellular fluid is able to influence cholesterol efflux from macrophages in vivo is not known. Here, we studied in vitro the effect of an acidic challenge on the ability of human monocyte-derived macrophage foam cells to release cholesterol to established cholesterol acceptors. We found that various cholesterol efflux pathways, particularly that facilitated by ABCA1 transporter, were inhibited at acidic pH. We conclude that, by reducing cholesterol efflux, an acidic environment favors the formation and maintenance of human cholesterol-laden macrophage foam cells.

Methods

Human monocyte-derived macrophages were incubated in custom-made DMEM culture media of pH 5.5 to 7.5, and their responses to cholesterol loading and cholesterol efflux were evaluated. No differences were found in macrophage morphology after incubation under acidic conditions. Cell viability, as evaluated by Trypan blue exclusion test and lactate dehydrogenase released to media, remained similar on incubation in neutral and acidic media. The present work is based on the study of human monocyte-derived macrophages from altogether more than 40 healthy donors. A detailed description of the methods is provided in the supplemental data, available online at http://atvb.ahajournals.org.

Figure 1. Cholesterol efflux from cholesterol-loaded macrophages to apoA-I in culture medium of acidic pH. Human monocyte-derived macrophages were converted into cholesterol-loaded foam cells by incubating them in the presence of 25 μg/mL of [3H]CE-acetyl-LDL for 48 hours in DMEM of pH 7.5. After cholesterol loading, the cells were washed, fresh DMEM of pH 7.5, 6.5, or 5.5 containing increasing concentrations of apoA-I was added, and cholesterol efflux was allowed to proceed for 16 hours (A). Time course of cholesterol efflux to apoA-I (10 μg/mL) from macrophages preincubated with acetyl-LDL at pH 7.5 was determined for up to 6 hours (B) or 16 hours (inset). Efflux of cholesterol was calculated as dpmmedium/dpmcells×100, and for each data point at each pH, efflux in the absence of apoA-I was subtracted from that in the presence of apoA-I. The pH values in each panel denote pH of medium during cholesterol efflux. Values in each panel are means±SD of triplicate wells. Macrophages derived from 9 individual donors were studied, and the data shown correspond to macrophages derived from representative donors.

Reduced Efflux of Cholesterol to apoA-I in Acidic Conditions Is Not Caused by Changes in apoA-I Protein Structure

To evaluate whether the impaired cholesterol efflux observed in acidic pH was related to a pH-sensitive folding of apoA-I, dilutions of apoA-I in buffers of pH 7.5, 6.5, and 5.5 were kept at 4°C or incubated at 37°C overnight, and their far-UV circular dichroism spectra were recorded at 37°C using Jasco J-715 spectropolarimeter. An overlay of the circular dichroism spectra showed that the secondary structure of apoA-I was not modified within the pH range (supplemental Figure I). Indeed, apoA-I spectra are characteristic for an α-helix structure with 2 negative bands at 222 and 208 nm and a positive one at 193 nm. In further analysis, the spectra were subjected to secondary structure estimation (supplemental Table I). The results showed that the α-helical content of apoA-I remained constant (41±1%) over the pH range 5.5 to 7.5, and the overnight incubation had no effect on the α-helical content. Because the ability of apoA-I to stimulate cellular cholesterol efflux depends on its amphipathic helical segments, our data strongly suggest that its intrinsic activity as cholesterol acceptor was not affected under the pH range studied. The α-helical content at neutral pH agreed with previous studies reporting 44 to 46% helicity, but the lack of acidification effect found here contrasts data reporting a decrease in apoA-I helicity from 45 to 30% when the pH was decreased from 7.0 to 5.0.

Methods

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Acidic pH Reduced Cholesterol Efflux From Macrophage Foam Cells to HDL₂ and to Human Plasma

Next, we evaluated whether the efficient pH-dependent inhibitory effect on cholesterol efflux to purified lipid-free apoA-I might also apply to other efflux pathways stimulated by human plasma. Initially, macrophages were loaded with cholesterol at neutral pH, and a time-course of cholesterol efflux to 2.5% human plasma (which contains very low concentration of lipid-poor apoA-I) was evaluated, up to 16 hours. Cholesterol efflux to plasma was linear, and similarly to efflux to apoA-I, at each time point, efflux declined with lowering of the pH (data not shown).

To further analyze the pH effect on cholesterol efflux via different pathways, the foam cells were incubated with various cholesterol acceptors in acidic medium. We also evaluated the effect on cholesterol efflux of the acidic pH already during cholesterol loading of the cells. For these aims, macrophages were loaded with cholesterol at either neutral pH (Figure 2, upper panels) or at acidic pH (Figure 2, lower panels), after which cholesterol efflux to increasing concentrations of apoA-I, HDL₂, and plasma was determined (supplemental Table III), these data further suggested that a low pH also partially impaired the foam cell competence to release cholesterol via a passive diffusion-based mechanism. This inhibitory effect was more pronounced when macrophages were already loaded in acidic pH; thus, efflux to HSA dropped >50% independently of the pH the efflux had occurred. Altogether, these findings revealed that various cholesterol efflux pathways in macrophage foam cells were affected to varying extent by the pH (Figure 2C) were less marked (∼20%). Interestingly, when plasma was used as an acceptor, the high-affinity component of cholesterol efflux occurring at low plasma concentrations was lost at acidic pH, evidenced by the increase of the apparent Km value (0.6% at neutral pH versus 11% at acidic pH, vol/vol) (Figure 2C). This indicated that diffusional efflux processes stimulated by plasma components, such as mature HDL particles, were more resistant to acidification of the pericellular environment. In contrast, when macrophages were challenged by acidic pH already during cholesterol loading (Figure 2D through 2F), cholesterol efflux to every studied acceptor was severely attenuated at both pH values. To gain further insight on the mechanism responsible for the residual efflux stimulated by plasma in acidic pH (Figure 2C) despite a strong inhibition of the ABCA1 pathway, we used human albumin to evaluate apoA-I-independent efflux. Thus, 1 mg/mL of HSA was added to efflux media of macrophage foam cells generated in acidic pH (Figure 2C). This indicated that diffusional efflux processes stimulated by plasma components, such as mature HDL particles, were more resistant to acidification of the pericellular environment. In contrast, when macrophages were challenged by acidic pH already during cholesterol loading (Figure 2D through 2F), cholesterol efflux to every studied acceptor was severely attenuated at both pH values. To gain further insight on the mechanism responsible for the residual efflux stimulated by plasma in acidic pH (Figure 2C) despite a strong inhibition of the ABCA1 pathway, we used human albumin to evaluate apoA-I-independent efflux. Thus, 1 mg/mL of HSA was added to efflux media of macrophage foam cells generated at either neutral or acidic pH, and cholesterol efflux was measured (supplemental Table II). Efflux to HSA in acidic pH from foam cells generated at neutral pH was only partially reduced (by ∼30%), suggesting that, besides ABCG1 partial contribution, such passive cholesterol efflux also sustained efflux to plasma in acidic pH. Because, like apoA-I, the secondary structure of HSA (α-helicity ∼62%) was not modified at acidic pH (supplemental Table III), these data further suggested that a low pH also partially impaired the foam cell competence to release cholesterol via a passive diffusion-based mechanism. This inhibitory effect was more pronounced when macrophages were already loaded in acidic pH; thus, efflux to HSA dropped >50% independently of the pH the efflux had occurred. Altogether, these findings revealed that various cholesterol efflux pathways in macrophage foam cells were affected to varying extent by the pH.
distinct degrees by acidic pH, the ABCA1-mediated pathway being particularly sensitive. Moreover, exposure of macrophages to an acidic environment during cholesterol loading for at least 24 hours rendered the foam cells even less sensitive to cholesterol release induction irrespective of the efflux pH.

Induction of ABCA1 and ABCG1 Gene Expression in Human Macrophages During Cholesterol Loading Is Impaired in Acidic pH

In response to cholesterol accumulation, ABCA1 and ABCG1 gene expression is upregulated in human macrophages, whereas the expression of SR-BI is either increased or decreased. We next investigated whether cholesterol loading of macrophages at acidic pH would influence the expression of such lipid efflux-related genes. It appeared that the 6- to 8-fold induction of ABCA1 and ABCG1 mRNA expression during cholesterol loading at pH 7.5 was progressively attenuated at pH 6.5 or 5.5 (Figure 3). Moreover, at pH 5.5, the cholesterol loading-dependent mRNA induction was fully blocked, because the expression of both ABC transporters remained within basal levels. In contrast to the ABC transporters, cholesterol loading did not induce significant change in SR-BI mRNA expression at any pH value (Figure 3C). Overall, the data revealed that lowering of extracellular pH significantly reduced mRNA expression of the 2 ABC transporters that, otherwise, are strongly upregulated during foam cell formation at neutral pH and are considered as major determinants of cholesterol efflux under the more physiological pH conditions.

Protein Levels of ABC Transporters Are Reduced in Macrophages Loaded With Cholesterol in Acidic Medium

Next, ABCA1 and ABCG1 protein levels were evaluated by semiquantitative Western blot analysis in macrophages incubated with or without acetyl-LDL at neutral or acidic pH. As expected, at neutral pH, both proteins were upregulated in response to increasing concentrations of acetyl-LDL. Of note, incubation of macrophages in acidic pH markedly reduced the levels of both ABC proteins also in nonloaded cells (supplemental Figure IIA). Beyond a natural variation in responses among macrophages derived from different individuals, ABCA1 seemed to be more affected than ABCG1. This notion was compatible with major defects observed in cholesterol efflux to apoA-I than to HDL at acidic pH (see Figure 2). Densitometric analysis from 5 monocyte donors confirmed that incubation in acidic pH significantly reduced ABCA1 protein levels both in basal and cholesterol-loaded macrophages (Figure 4, upper panels), whereas levels of ABCG1 were only reduced in cholesterol-loaded cells (Figure 4, lower panels). This may be related to the predominant intracellular location of ABCG1 in nonloaded macrophages. Despite the inhibitory effect of the acidic pH on
ABC1 and ABCG1 mRNA levels (Figure 3), the protein levels were increased in the foam cells (supplemental Figure III). The moderate increase in ABC1 protein found during loading at acidic pH despite virtually identical ABC1 mRNA levels suggested altered ABC1 protein turnover. ABC1 protein levels were also reduced in foam cells generated at neutral pH further exposed to cholesterol efflux to apoA-1 at acidic pH (supplemental Figure IIB). This finding suggested that exposure to the acidic pH destabilized ABC1 protein in macrophages already overexpressing ABC1 mRNA and protein, even in the presence of apoA-1, which stabilizes and protects ABC1 protein from proteolytic degradation.31 The stronger inhibitory effect of the acidic medium on the ABC1 protein levels seemed, therefore, to involve its posttranscriptional regulation during foam cell formation.31,32

**Secretion of Endogenous apoE by Macrophage Foam Cells Is Attenuated in Acidic pH**

Secretion of apoE is also an important component of the cholesterol efflux machinery in human macrophages.33,34 To evaluate whether apoE secretion by macrophages was sensitive to pH, the cells were incubated at acidic pH with or without acetyl-LDL, and apoE was quantitated in medium (supplemental Table IV). As demonstrated previously,34 loading of macrophages with cholesterol at neutral pH stimulates their basal secretion of apoE (>3-fold). In sharp contrast, apoE secretion by foam cells was strongly inhibited at pH 5.5, and furthermore, the basal secretion of apoE by nonloaded macrophages was fully blocked. A time course of apoE mRNA expression and apoE secretion by the foam cells at pH 7.5, 6.5, and 5.5 showed that, at pH 6.5, neither the mRNA levels nor the secretion rate of apoE were affected when compared with neutral conditions (Figure 5). In contrast, although the mRNA levels remained largely unchanged at pH 5.5, the protein secretion was fully inhibited after 3 hours of incubation, which indicated that failure to secrete apoE at pH 5.5 might involve a posttranscriptional event.

**Discussion**

Here, we demonstrated that lowering of the extracellular pH progressively reduced cholesterol efflux from human monocyte-derived macrophage foam cells, particularly the efflux component facilitated by the ABCA1 transporter, whereas the ABCG1-mediated pathway and the aqueous cholesterol diffusion appeared to be also impaired, although to a lower degree. This inhibitory effect on cholesterol efflux was also exerted on macrophages exposed to acidic pH already during their conversion into foam cells. Because the ABC-facilitated efflux processes have additive effects in stimulating net cholesterol removal from macrophages and they promote macrophage-specific reverse cholesterol transport in vivo,34 the present observations suggest that an acidic microenvironment may significantly attenuate this major physiological antiatherosclerotic mechanism. This experimental model may closely simulate the pathological fate of human macrophages surrounded by acidic intimal fluid in local areas of advanced atherosclerotic lesions.12

A key role of the ABCA1 pathway in macrophages has been related to selective removal of a cholesterol pool from the plasma membrane that can trigger cytotoxic events.36 Moreover, ABCA1 has been recently claimed to be solely responsible for enhanced cholesterol efflux in human foam cells,37 which is in line with the inverse correlation observed between ABCA1-mediated cholesterol efflux and carotid arterial-wall thickness.38 Although cholesterol efflux likely occurs at every stage of the atherosclerotic process, the dynamics of this process appears to be under the influence of the atherosclerotic plaque microenvironment. In fact, a dramatic drop in ABCA1 protein level was reported in whole extracts of human carotid atheromas,39,40 suggesting a decrease in ABCA1 protein levels. Of note, it was demonstrated by in situ hybridization that ABCA1 mRNA expression in macrophages from atherosclerotic lesions was not evenly distributed, being nearly absent specifically in the macrophage-rich core of advanced plaques of human atherosclerotic aorta,41 where, most likely, acidification of the intimal fluid had occurred.

The present study revealed that acidification of the extracellular environment was sufficient to rapidly impair cholesterol efflux from cultured cholesterol-loaded human macrophages even in the presence of abundant amounts of physiological cholesterol acceptors. Among the various efflux pathways, the one mediated by the ABCA1 transporter was rapidly (within 1 hour) (Figure 1B) and most strongly
by impairing macrophage cholesterol efflux and stimulating foam cell generation, an acidic intimal fluid can be considered as a potent proatherogenic factor within advanced human atherosclerotic plaques.

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Disclosures

None.

References


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Supplement material

Online Data Supplement: Methods

Human plasma lipoproteins

LDL (1.019 – 1.050 g/mL) and HDL₂ (1.050 – 1.125 g/mL) were isolated from human plasma by sequential ultracentrifugation using KBr for density adjustments. The concentrations of lipoproteins used in the experiments are given in terms of their protein content. LDL was acetylated with acetic anhydride¹, and the acetyl-LDL was radiolabeled by treatment with [1,2⁻³H]-cholesteryl linoleate (Amersham Biosciences, Piscataway, NJ) dissolved in dimethylsulfoxide². The specific activities of the [³H]CE-acetyl-LDL preparations ranged from 50-90 dpm/ng protein. Lipoprotein preparations were endotoxin-free as assayed by the Limulus Amebocyte Lysate assay (Cambrex Bio Science Walkersville Inc. MD).

Primary monocyte-macrophage cultures

Buffy coats, provided by the Finnish Red Cross, were layered over Ficoll-Paque, centrifuged at 800 x g for 30 min, and then the mononuclear cells were recovered as a single cell layer. The mononuclear cells were sedimented, and the cell pellets were washed three times with PBS to eliminate platelets, and finally were suspended in DMEM. The resuspended mononuclear cells were plated into 24-well plates (1.5 million cells per well) and allowed to adhere for 1 h, and the medium was subsequently removed and replaced with macrophage-medium (Invitrogen, Carlsbad, CA) containing GM-CSF (Biosite Ltd, UK). The medium was then changed every two to three days throughout the
cell culture period. After seven days, when the monocytes were phenotypically converted to macrophages, as confirmed by immunostaining for CD68, the cells were used for the experiments.

**Macrophages cultured in acidic pH**

Human monocyte-derived macrophages were incubated at 37°C for up to 48 h in culture medium of pH 7.5, 6.5 or 5.5. To minimize experimental variation and to avoid high osmolarity derived from acidification of the culture medium by addition of inorganic acids, we purchased custom-made acidic media (DMEM, Hyclone, UT) and compared the results with those using the corresponding medium of neutral pH from the same provider. In some experiments foam cells were harvested, cell protein was solubilized, and ABCA1 and ABCG1 protein levels were detected in the cell lysates by western blotting, as described below. Concentrations of apoE in medium were determined by ELISA analysis. Macrophage morphology was monitored by light microscopy after cholesterol loading, and after cholesterol efflux. Cell viability and cellular integrity were determined by Trypan blue exclusion test and by measuring lactate dehydrogenase activity in the medium (LDH, Cytotoxicity Detection Kit, Roche Diagnostics, IN).

**Generation of macrophage foam cells and measurement of cellular cholesterol efflux**

To generate foam cells, the monocyte-derived macrophages were incubated in DMEM of the various studied pHs containing 25 μg/ml of [³H]CE-acetyl-LDL for 24 or 48 h. To measure cholesterol efflux, macrophages were washed with PBS, and fresh media of the various pHs were added. The efflux medium contained lipid-free apoA-I (kindly provided
by Dr. Peter Lerch, Swiss Red Cross, Bern, Switzerland), ultracentrifugally isolated HDL₂ or a pool of human plasma at the concentrations indicated in the Figure legends. In an additional experiment fatty acid-free human serum albumin (HSA, Sigma) was used as cholesterol acceptor. After incubation for 16 h with the various cholesterol acceptors, the media were collected, centrifuged at 200 g for 10 min to remove cellular debris, and the radioactivity of each supernatant was determined by liquid scintillation counting. Cells were solubilized by treatment with 0.2 M NaOH, and protein content and radioactivity were determined in the cell lysates. Cholesterol efflux was expressed as fractional efflux that was calculated as \( \frac{\text{dpm}_{\text{medium}}}{(\text{dpm}_{\text{cells}} + \text{dpm}_{\text{medium}})} \times 100 \). Cholesterol efflux to incubation medium in the absence of any added cholesterol acceptors was considered as basal efflux, and was subtracted from the efflux values obtained in the presence of acceptors.

**Analysis of the secondary structure of apoA-I at varying pH by far-UV CD spectropolarimetry**

ApoA-I was diluted to a concentration of 0.17 mg/mL in sodium phosphate buffer of pH 7.5 or pH 6.5, or in a buffer containing 5 mM MES and 100 mM NaF, pH 5.5. The samples were either stored at 4°C or incubated at 37°C overnight, and CD spectra for the wavelength region 190-250 nm were recorded in 0.1-cm cells with Jasco J-715 spectropolarimeter at 37°C with a scanning speed of 50 nm/min, band width of 1 nm and response time of 1 s, averaging five accumulations per spectrum. After smoothing correction and background subtraction, the data were converted to mean residue ellipticity \([\theta]\) for secondary structure estimation using the DichroWeb server\(^4\). CONTIN
LL program\textsuperscript{5} was used with reference protein sets 4 (=contin A) and 7 (=contin B)\textsuperscript{6,7}, including data measured at 190-240 nm. For the K2D program\textsuperscript{8}, data measured at 200-250 nm were included. Estimated percentage contents of $\alpha$-helix, $\beta$-strand and other secondary structure elements are listed, along with means and SD values. A similar CD analysis was carried out for dilutions of human serum fatty acid-free albumin (HSA) in the same pH buffers.

**Western blotting of ABCA1 and ABCG1**

Macrophages were washed with PBS and lysed with buffer containing 250 mM Tris-HCl, pH 6.8, 8 % w/v SDS and complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, IN). The cells were scraped off from the wells and sonicated briefly to break DNA strands and reduce sample viscosity. Protein concentrations were determined in the cell lysates and 30 µg of protein from each sample was loaded onto a 5% SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membrane (Trans Blot 0.2 µm, Bio Rad Laboratories) and the proteins of interest were detected by immunoblotting using either a rabbit anti-ABCA1 antibody or a rabbit anti-ABCG1 antibody (Novus Biologicales, CO, 1:1000, 1:2500 dilutions, respectively). After incubation with horseradish peroxidase-conjugated anti-rabbit IgG (BioRad, Hercules, CA) as secondary antibody, immunocomplexes were detected by ECL system (Hyperfilm HPC film, Amersham Biosciences).
Analysis of mRNA expression

To analyze mRNA levels in monocyte-derived macrophages, quantitative real-time RT-PCR was applied. Briefly, total cellular RNA was first isolated using the RNeasy Plus Kit with genomic DNA removal according to manufacturer’s instructions (Qiagen, Valencia, CA). Nucleic acid concentrations were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA from each sample was then converted to cDNA using Moloney-Murine Leukaemia Virus (M-MLV) reverse transcriptase and random hexamers (both from Life Technologies, Gaithensburg, CA) for 55 min at 37°C. For PCR primer design, nucleotide sequences were retrieved from the GenBank database (National Center for Biotechnology Information, Bethesda, MD) and synthesized as shown in online-only Data Supplement, Table V. For PCR, the samples were amplified in duplicate with ABI Prism 7500 Sequence Detector System using TaqMan Universal or Power SYBR Green PCR Master Mix (all from PE Applied Biosystems) with the following program: a 10 min pre-incubation at 95°C followed by 40 amplification cycles consisting of 15 sec denaturation at 94°C and 60 sec primer annealing and polymerase extension at 60°C. For data analysis, the results were normalized relative to an endogenous control glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (a commercial assay from PE Applied Biosystems) by applying the delta-deltaCt algorithm.
Reference List


Online Data Supplement: Additional Figures

Online Data Supplement Figure I. Analysis of the secondary structure of apoA-I at varying pH by far-UV CD spectropolarimetry. ApoA-I stock solution was diluted to a concentration of 0.17 mg/mL either in sodium phosphate buffers of pH 7.5 (solid line) or pH 6.5 (dotted line), or in 5 mM MES, 100 mM NaF, pH 5.5 (dashed line). The samples were incubated at 37°C overnight and CD spectra for the wavelength region 190-250 nm were recorded as described in Methods. The apparent minor changes in the region 197-235 nm are not indicative of changes in secondary structure, but rather reflect minor differences in protein amounts between samples, so evidenced by perfect alignment of spectra after multiplication with a suitable constant (not shown).

Online Data Supplement Figure II. Effect of the acidic culture medium on the levels of ABCA1 and ABCG1 proteins in human macrophages after cholesterol loading and after efflux of cholesterol from the foam cells. Macrophages were loaded with cholesterol by incubating them for 48 h in the presence of increasing concentrations of acetyl-LDL in DMEM of pH 7.5 or 5.5 (panel A), or 25 μg/mL of acetyl-LDL in DMEM of pH 7.5 and then the foam cells were subjected to efflux for 16 h in the presence of apoA-I (10 μg/mL) (panel B). Cells were sonicated and the cellular lysates were electrophoresed, blotted, and the membranes were probed with anti-ABCA1 or anti-ABCG1, as indicated. Western blotting shown for each ABC transporters corresponds to one representative monocyte donor. Similar results were obtained with two other monocyte donors.
Online Data Supplement Figure III. Levels of ABCA1 and ABCG1 proteins in macrophage foam cells after loading with cholesterol in neutral or acidic culture medium. The intensity of ABCA1 and ABCG1 western blot protein bands of cholesterol-loaded macrophages from the experiment described in Figure 4 were compared after loading of the cells at pH 7.5 and 5.5. For universal data comparison the intensity of the basal ABC protein bands before loading acted as control and was set to 100%. Panel bars represent the means ± SD (% difference relative to control) from 5 donors.
ApoA-I was diluted to a concentration of 0.17 mg/mL in buffer of pH 7.5, 6.5 or 5.5. The samples were either stored at 4°C or incubated at 37°C overnight. After centrifugation for 2 min at 16,000 x g, far-UV circular dichroism spectra were recorded as described in Methods. CD spectra of apoA-I measured at 25°C were identical to the respective spectra measured at 37°C (not shown).
Table II online supplement. Efflux of cholesterol to human serum albumin (HSA) at neutral and acidic pH from macrophage foam cells generated in neutral or acidic culture medium.

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<td>7.5</td>
<td>5.5</td>
<td>6.7 ± 0.53</td>
<td>0.01</td>
</tr>
<tr>
<td>5.5</td>
<td>7.5</td>
<td>4.8 ± 0.50</td>
<td>0.01</td>
</tr>
<tr>
<td>5.5</td>
<td>5.5</td>
<td>3.9 ± 0.14</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Monocyte-derived macrophages were incubated for 24 h in DMEM either at pH 7.5 or at pH 5.5 containing 25 μg/mL of [³H]CE-acetyl-LDL. The cells were then washed and efflux of cholesterol to fatty acid-free human albumin (1 mg/mL, which mimics HSA concentration in 2.5% of plasma) in DMEM of pH 7.5 or 5.5 was measured after 16 h of incubation, as described in Methods. Values are means ± SD of triplicate wells derived from 3 individual donors. P-values denote statistical differences vs. the control cells (loading and efflux at pH 7.5) determined by Student's t test.
Table III online supplement. Secondary structure estimation from CD spectra of nonincubated and preincubated HSA at different pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>incub.</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; struct.</th>
<th>contin A (%)</th>
<th>contin B (%)</th>
<th>mean (%)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>no</td>
<td>helix</td>
<td>62</td>
<td>61</td>
<td>61</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>strand</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>other</td>
<td>36</td>
<td>37</td>
<td>36</td>
<td>0.8</td>
</tr>
<tr>
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<td>yes</td>
<td>helix</td>
<td>62</td>
<td>61</td>
<td>62</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>strand</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>other</td>
<td>36</td>
<td>38</td>
<td>37</td>
<td>1.1</td>
</tr>
<tr>
<td>6.5</td>
<td>no</td>
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<td>61</td>
<td>60</td>
<td>61</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td>other</td>
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<td>37</td>
<td>37</td>
<td>0.5</td>
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<td>6.5</td>
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<td>helix</td>
<td>63</td>
<td>63</td>
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<td>0.1</td>
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<tr>
<td></td>
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<td></td>
<td>other</td>
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<td>35</td>
<td>35</td>
<td>0.3</td>
</tr>
<tr>
<td>5.5</td>
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<td>helix</td>
<td>63</td>
<td>62</td>
<td>63</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>strand</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0.2</td>
</tr>
<tr>
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<td></td>
<td>other</td>
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<td>36</td>
<td>35</td>
<td>0.7</td>
</tr>
<tr>
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<td>helix</td>
<td>64</td>
<td>63</td>
<td>64</td>
<td>0.4</td>
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<tr>
<td></td>
<td></td>
<td>strand</td>
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<td>1</td>
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</tr>
<tr>
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<td></td>
<td>other</td>
<td>34</td>
<td>35</td>
<td>35</td>
<td>0.6</td>
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</tbody>
</table>

HSA was diluted to 0.10 mg/ml with buffers of pH 7.5, 6.5 or 5.5, and either stored at 4°C or incubated at 37°C overnight. After centrifugation for 2 min at 16,000 x g, far-UV circular dichroism spectra were recorded at 37°C as described in Methods. Estimated percentage contents of α-helix, β-strand and other secondary structure elements are listed, along with means and SD values.
Table IV online supplement. Effect of acidic pH medium on apoE secretion from human macrophages in the basal state and after cholesterol loading

<table>
<thead>
<tr>
<th></th>
<th>Donor A</th>
<th>Donor B</th>
<th>p-value</th>
<th></th>
<th>Donor A</th>
<th>Donor B</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acetyl-LDL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Acetyl-LDL</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>p-value</td>
<td>+</td>
<td></td>
<td>p-value</td>
<td>+</td>
<td>p-value</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>μg/mg cell protein</th>
<th>μg/mg cell protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.5</td>
<td>pH 5.5</td>
</tr>
<tr>
<td>0.48 ± 0.04</td>
<td>0.00</td>
</tr>
<tr>
<td>1.69 ± 0.03</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Monocyte-derived macrophages were incubated in the absence or presence of 25 μg/ml of acetyl-LDL in DMEM of pH 7.5 or 5.5. After 24 h of incubation media was collected, centrifuged at low speed to eliminate cell debris, and apoE was determined by ELISA. Cells were sonicated and their total protein contents were measured. Macrophages derived from 2 different donors (A and B) were studied. Values are mean ± SD of triplicate wells. P-values denote statistical differences determined by Student's t test.
### Table V online supplement. Real-time PCR oligonucleotide sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>5’-CATCTTTGGGACACCTCAGA-3’</td>
<td>5’-ATGAATTGTGCTGGGCATT-3’</td>
</tr>
<tr>
<td>ABCG1</td>
<td>5’-CCATCCCCACGTACCTACA-3’</td>
<td>5’-GCAGATCTTCCCCGGTCTAAG-3’</td>
</tr>
<tr>
<td>SR-B1</td>
<td>5’-CCTTGTTCCTGGACATCCAC-3’</td>
<td>5’-CTCAATCTTCCCAGTTGTCCA-3’</td>
</tr>
<tr>
<td>ApoE</td>
<td>5’-GAGCAGGCCCAGCAGATAC-3’</td>
<td>5’-CTTCGGCGTTCAGTGATTGT-3’</td>
</tr>
</tbody>
</table>
Figure I online supplement
A. Cholesterol loading

<table>
<thead>
<tr>
<th>Acetyl-LDL (μg/ml)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 5.5</td>
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</tbody>
</table>

B. Cholesterol loading and efflux

<table>
<thead>
<tr>
<th>Loading</th>
<th>pH 7.5</th>
<th>pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efflux</td>
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<td>pH 5.5</td>
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</table>

<table>
<thead>
<tr>
<th>ApoA-I</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
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<tbody>
<tr>
<td>ABCA1</td>
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kDa

-250
Figure III online supplement

<table>
<thead>
<tr>
<th>Acetyl-LDL</th>
<th>pH 7.5</th>
<th>pH 5.5</th>
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</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**ABCA1**
- Densitometry of band (AU%)
- P = 0.002
- P = 0.04

**ABCG1**
- Densitometry of band (AU%)
- P = 0.001
- P = 0.02