Apolipoprotein AI and High-Density Lipoprotein Have Anti-Inflammatory Effects on Adipocytes via Cholesterol Transporters

ApoAI and HDL inhibit inflammation in adipocytes and adipose tissue similar to their effects in other cell types. These effects are consistent with disruption and removal of cholesterol from LRs, which are regulated by cholesterol transporters, such as ATP-binding cassette A-1, ATP-binding cassette G-1, and scavenger receptor B-1. (Circ Res. 2013;112:1345-1354.)

Key Words: ABC transporters □ adipocytes □ apolipoprotein AI □ cholesterol □ HDL

Obesity, especially visceral obesity, is accompanied by adipose tissue inflammation, which, in turn, is associated with insulin resistance and an increased risk of cardiovascular disease.1,2 A hallmark of adipose tissue inflammation is the accumulation of macrophages3,4 and other immune cells5–7 that are recruited to adipose tissue by chemotactic factors after exposure of adipocytes to saturated fatty acids, such as palmitate, occurs via translocation of NADPH oxidase 4 into lipid rafts (LRs). The anti-inflammatory effects of apolipoprotein AI (apoAI) and high-density lipoprotein (HDL) on macrophages and endothelial cells seem to occur via cholesterol depletion of LRs. However, little is known concerning anti-inflammatory effects of HDL and apoAI on adipocytes.

Methods and Results: In 3T3-L1 adipocytes, apoAI, HDL, and methyl-β-cyclodextrin inhibited chemotactic factor expression. ApoAI and HDL also disrupted LRs, reduced plasma membrane cholesterol content, inhibited NADPH oxidase 4 translocation into LRs, and reduced palmitate-induced reactive oxygen species generation and monocyte chemotactic factor expression. Silencing ATP-binding cassette A-1 abrogated the effect of apoAI, but not HDL, whereas silencing ATP-binding cassette G-1 or scavenger receptor B-1 abrogated the effect of HDL but not apoAI. In vivo, apoAI transgenic mice fed a high-fat, high-sucrose, cholesterol-containing diet showed reduced chemotactic factor and proinflammatory cytokine expression and reduced macrophage accumulation in adipose tissue.

Conclusions: ApoAI and HDL have anti-inflammatory effects in adipocytes and adipose tissue similar to their effects in other cell types. These effects are consistent with disruption and removal of cholesterol from LRs, which are regulated by cholesterol transporters, such as ATP-binding cassette A-1, ATP-binding cassette G-1, and scavenger receptor B-1. (Circ Res. 2013;112:1345-1354.)

Key Words: ABC transporters □ adipocytes □ apolipoprotein AI □ cholesterol □ HDL

Original received November 19, 2012; revision received February 25, 2013; accepted March 15, 2013. In February 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 11.98 days.


Current address for T.U.: Division of General Medicine I, Saitama Medical Center, Jichi Medical University, Saitama Japan.

*These authors contributed equally to this work.

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.111.300581/-/DC1.
High-density lipoprotein (HDL) and apolipoprotein AI (apoAI) are believed to protect against the development of atherosclerosis, in part, because of their role in promoting cholesterol efflux and reverse cholesterol transport. However, in addition to these properties, HDL and apoAI also have anti-inflammatory properties. The most studied of these is its ability to inhibit adhesion molecule expression by endothelial cells, which reduces monocyte adhesion. At the level of the artery wall, this property of HDL would reduce recruitment of monocyte-macrophages and inhibit atherogenesis. HDL and apoAI also have been shown to have anti-inflammatory effects on monocytes and macrophages. However, little is known concerning their effect on adipocytes.

HDL and apoAI can exert an anti-inflammatory effect on macrophages via ATP-binding cassette A-1 (ABCA-1) and ABCG-1-mediated cholesterol efflux from plasma membrane microdomains known as lipid rafts (LRs), which are enriched in cholesterol, glycosyl-phosphatidylinositol–linked proteins, glycosphingolipids, cholesterol, and caveolin. Free cholesterol accumulation in LRs is associated with increased signaling via proteins concentrated in these microdomains. NOX4 is the source of ROS after exposure of adipocytes to palmitate in vitro, and its activity is increased by its translocation into LR after exposure to palmitate. Cholesterol efflux occurs via 2 main pathways: first, free cholesterol simply diffuses from the plasma membrane through the aqueous medium to HDL, and second, cholesterol efflux is mediated by transporters in the plasma membrane (ABCA-1, ABCG-1, and scavenger receptor [SRB-1]). ABCA-1 transports free cholesterol from plasma membrane to lipid-poor apoAI, and ABCG-1 transports cholesterol to mature HDL particles. These transporters regulate the cholesterol content of LR by efflux to HDL and apoAI. In turn, disruption and depletion of cholesterol in LR block signal transduction. Whether and how HDL and apoAI affect inflammation in adipocytes are unknown.

To test the hypothesis that HDL has anti-inflammatory effects on adipocytes analogous to other cell types, we first exposed fully differentiated 3T3-L1 adipocytes to normal human HDL and apoAI in vitro to test their effect on palmitate-induced inflammation. We found that HDL and apoAI inhibited palmitate-induced ROS generation and chemotactic factor expression by decreasing LR formation and translocation of NOX4 into LR. Moreover, these effects of HDL and apoAI were dependent on cholesterol transporters in the plasma membrane. To determine whether HDL inhibited adipose tissue inflammation in vivo, we evaluated the effect of increased HDL levels that resulted from overexpression of the human apoAI transgene on adipose tissue inflammation induced by a high-fat, high-sucrose, cholesterol-containing diet (HFHSC) in mice. We found that adipose tissue inflammation also was ameliorated in human apoAI transgenic mice. Our finding suggests that HDL and apoAI have anti-inflammatory properties on adipocytes and adipose similar to their effects in other cell types.

### Methods

Reagents and other detailed methods are described in the Online Data Supplement.

**Preparation of Fatty Acid: Albumin Complexes**
Free fatty acids were prepared by conjugation with albumin, as described previously.

**Multiplex Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction**
Reverse transcription-polymerase chain reaction was performed using the TaqMan Master kit (Applied Biosystems) in the ABI prism 7900HT system (Online Data Supplement).

**Quantification of Cholesterol Levels in Cellular Membranes of 3T3L-1 Adipocytes**
Cellular membranes were isolated from 3T3L-1 adipocytes as described previously. Membrane preparations were suspended in 100 μL of ethanolic potassium hydroxide (1 mol/L), and cholesterol-d7 was added as the internal standard. After saponification, the lipid fractions were extracted from the membrane preparations with hexane and dried under nitrogen gas. Total cholesterol levels were determined after derivatization using liquid chromatography electrospray ionization tandem mass spectrometry as described previously.

**In Vitro ABCA-1, ABCG-1, and SRB-1 Gene Silencing**
For experiments in which we tested the roles of ABCA-1, ABCG-1, and SRB-1 in mediating ROS generation and the expression of SAA3 and MCP-1, 2 days after completion of the differentiation protocol 3T3-L1 adipocytes were transiently transfected with small interfering RNA (siRNA) duplexes for ABCA-1, ABCG-1, and SRB-1 or scrambled sequences, which were synthesized and purified by Ambion using the DeliverX system (Panomics), as described previously. Briefly, the cells were incubated with 300 μL of siRNA/siRNA transfection reagent complex for 3 minutes. After that, 300 μL of serum-free media was added and incubated for 24 hours. Four days later, mRNA and protein were analyzed to confirm the efficiency of silencing by reverse transcription-polymerase chain reaction and Western blotting.

**Quantification of ROS**
ROS generation was assessed as CM-H2DCFDA (Molecular Probes) fluorescence, which was monitored by fluorescence-activated cell sorting (FACSCanto, Becton-Dickinson) as described previously.

**Visualization of LR**
To detect LR in plasma membranes, Alexa Fluor 594–conjugated cholera toxin subunit-β (CTB) was used to stain 3T3-L1 adipocytes.
Briefly, cultured 3T3-L1 adipocytes were incubated with 1 μg/mL of Alexa Fluor 594–conjugated CTB for 15 minutes at 4°C. After washing twice with cold PBS, cells were fixed in 4% paraformaldehyde for 20 minutes at 4°C. Fixed cells were photographed by fluorescent microscopy (Nikon Eclipse 80i).

Detergent-Free LR Fractionation
LR and non-LR fractions from adipocytes were obtained by Optiprep gradient centrifugation using a detergent-free protocol (Online Data Supplement).

Animals, Diet, and Tissue Collection
To investigate the role of HDL and apoAI in adipose tissue inflammation, adult (10-week-old) male human apoAI overexpressing transgenic (apoAI<sup>tg/tg</sup>) and control C57BL/6 mice were fed either a HFHSC diet (35.5% calories as fat and 36.6% as carbohydrate, 0.15% added cholesterol, BioServ No. F1850), or chow (control) diets for 24 weeks. In a parallel experiment, 10-week-old male low-density lipoprotein receptor (LDLR)<sup>−/−</sup> apoAI<sup>tg/tg</sup> mice and control LDLR<sup>−/−</sup> mice also were fed either HFHS or chow diets for 16 weeks. ApoAI<sup>tg/tg</sup> and LDLR<sup>−/−</sup> apoAI<sup>tg/tg</sup> mice were generated from the Jackson laboratory, and LDLR<sup>−/−</sup> apoAI<sup>tg/tg</sup> mice were generated from these homozygous mice. At euthanization, adipose tissues were either fixed in 10% formalin for macrophage staining using a Mac2 antibody, or snap-frozen at −70°C for isolation of total RNA. Metabolic variables were measured in blood samples obtained from the retro-orbital sinus after a 4-hour fast. Cholesterol and triglycerides in plasma and fast-phase liquid chromatography fractions were measured using colorimetric assays (2-tailed and <i>P</i> < 0.05 was considered significant).

Results
ApoAI and HDL Disrupt LRs and Inhibit Palmitate-Induced Generation of Chemotactic Factors
We previously showed that exposure of differentiated 3T3-L1 adipocytes to palmitate (250 μmol/L) increased chemotactic factor gene expression. We also showed that chemotactic factor generation was linked to the translocation of NOX4 into LRs, which led to ROS generation. Cholesterol is an essential component of LRs. Depletion of cholesterol in the plasma membrane disrupts LRs and blocks the assembly of proteins, resulting in inhibition of signal transduction. Therefore, we investigated whether modulation of membrane cholesterol using methyl-β-cyclodextrin, a compound that depletes membrane cholesterol and disrupts LRs, affects palmitate-induced chemotactic factor gene expression. Methyl-β-cyclodextrin totally blocked palmitate-induced Saa3 and Mcp-1 gene expression (Figure 1A and 1B). Moreover, adding back cholesterol reversed the effect of methyl-β-cyclodextrin on Saa3 and Mcp-1 gene expression (Figure 1A and 1B).

When cells were pre-exposed to simvastatin (10 μmol/L), an inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase, for 24 hours to reduce cholesterol biosynthesis, we also found that palmitate-induced Saa3 and Mcp-1 gene expression was blocked (data not shown). Because apoAI and HDL have the ability to remove cholesterol from cell membranes, we also investigated whether apoAI and HDL could inhibit this effect of palmitate. We first determined whether apoAI and HDL could decrease the cholesterol level of membranes in 3T3-L1 adipocytes. Indeed, apoAI and HDL decreased membrane cholesterol levels before and after exposure of adipocytes to palmitate (Online Figure I). Palmitate exposure alone led to a significant increase in membrane cholesterol (Online Figure I). Both apoAI and HDL blocked the increase of Saa3 and Mcp-1.

Figure 1. Methyl-β-cyclodextrin (MβCD), apolipoprotein AI (apoAI), and high-density lipoprotein (HDL) inhibit chemotactic factor expression. 3T3-L1 adipocytes were pre-exposed to MβCD (10 μmol/mL; A, B), cholesterol-loaded MβCD (A, B), apoAI and HDL (at the indicated concentrations in microgram protein per milliliter; C–F) for 6 h. After that, adipocytes were incubated with or without 250 μmol/L palmitate for 24 h. Saa3 and Mcp-1 gene expression was analyzed by multiplex real-time reverse transcription polymerase chain reaction, normalized to GAPDH (A–F). *<i>P</i> < 0.001 vs control media, **<i>P</i> < 0.001 vs palmitate, #<i>P</i> < 0.001 vs palmitate plus MβCD.

Statistical Analysis
Statistical significance was determined by Student <i>t</i> tests (2-tailed and paired). All data are shown as mean±SD of 3 independent experiments performed in triplicate. *<i>P</i> < 0.05 was considered significant.
lipoprotein (HDL) disrupt palmitate-induced lipid raft (LR) formation and NADPH oxidase 4 (NOX4) translocation into LRs. 3T3-L1 adipocytes were pre-exposed to apoAI (50 μg protein/mL) or HDL (50 μg protein/mL) for 6 h. After that, adipocytes were incubated with or without 250 μmol/L palmitate for 24 h. A, LRs were stained by Alexa Fluor 594–conjugated cholera toxin subunit β (CTB) and photographed by fluorescent microscopy (Nikon Eclipse 80i, original magnification, ×400). B, LRs were isolated andfractionated by ultracentrifugation using a detergent-free fractionation method. Proteins from OptiPre-gradient fractions were immunoblotted with anti-NOX4 antibody and anticafeolin-1 (CAV1) antibody (B). Fractions 6 to 8 contain LRs andfractions 1 to 4 are non-LR containing fractions.

Mcp-1 gene expression induced by palmitate in a dose-dependentmanner (Figure 1C–1F), although Mcp-1 expression seemed to be more sensitive to the effects HDL and apoAI possibly because the promoter of the Mcp-1 gene might be more sensitive to the anti-inflammatory effects of HDL and apoAI than the Saa3 gene.

Moreover, cotreatment with apoAI and HDL synergistically induced these chemotactic factor expression induced by palmitate (Online Figure II). Because a trace of HDL in the serum used for adipocyte cells culture could potentially affect these results, we also performed experiments using lipoprotein-deficient serum and found no differences between these 2 conditions (data not shown). To determine whether apoAI and HDL disrupt LR formation, we visualized the LRs using Alexa Fluor 594–conjugated CTB, which selectively binds to LRs in the plasma membrane. Exposure of adipocytes to palmitate increased CTB-stained microdomains of LRs compared with controls, whereas pre-exposure to either apoAI or HDL reduced these microdomains (Figure 2A). Finally, we investigated whether apoAI and HDL blocked NOX4 translocation into LRs isolated by ultracentrifugation. Non-LRs sediment in the lower fractions, whereas LRs float toward the top of the centrifuge tube. The presence of LRs was judged by detection of caveolin-1. In the absence of palmitate exposure, all NOX4 protein was present in non-LR fractions. After exposure to palmitate, NOX4 proteins were translocated into LRs (Figure 2B). However, pre-exposure of cells to either apoAI or HDL inhibited palmitate-induced translocation of NOX4 into LRs (Figure 2B). These results imply that apoAI and HDL disrupt the formation of LRs, causing disturbance of palmitate-induced NOX4 translocation, which, in turn, inhibits chemotactic factor expression.

Anti-Inflammatory Effect of ApoAI and HDL on Palmitate-Induced Chemotactic Factor Production Is Dependent on ABC Transporters and Scavenger Receptor B1

Because reverse cholesterol transport by apoAI and HDL is mediated by transporters in the plasma membrane (ABCA-1, ABCG-1, and SRB-1), we first tested which of these transporters is important for apoAI or HDL to exert their effects on palmitate-induced chemotactic factor gene expression. ABCA-1, ABCG-1, and SRB-1 silencing in 3T3-L1 adipocytes by their specific siRNAs was confirmed by demonstrating significant reduction of their respective expression levels by reverse transcription-polymerase chain reaction (data not shown) and Western blotting (Online Figure III). When ABCA-1 was silenced, the effect of apoAI on inhibiting Saa3 and Mcp-1 gene expression induced by palmitate was reversed, whereas HDL still inhibited palmitate-induced expression of these chemotactic factors (Figure 3A and 3B). Conversely, the ability of HDL to inhibit palmitate-induced chemotactic factor gene expression was reversed by silencing ABCG-1 or SRB-1, whereas apoAI still inhibited these events (Figure 3C–3F).

ApoAI and HDL Regulate Palmitate-Induced Formation of LRs, Translocation of NOX4 and ROS Generation via ABCA-1, ABCG-1, and SRB-1

To investigate the roles of ABCA-1, ABCG-1, and SRB-1 in LR formation induced by palmitate, we evaluated CTB-stained microdomains in the plasma membrane of adipocytes in which these transporters had been silenced using siRNA. In control cells, palmitate resulted in increased CTB-stained microdomains. In ABCA-1–silenced cells, apoAI did not decrease the CTB-stained LRs, whereas HDL did (Figure 4). Conversely, in ABCG-1–silenced or SRB-1–silenced cells, HDL failed to reduce the CTB-stained LRs, whereas apoAI did (Figure 4).

Next, we investigated whether ABCA-1, ABCG-1, and SRB-1 regulate NOX4 translocation into LRs because we had previously shown that exposure of adipocytes to palmitate leads to translocation of NOX4 to LRs. Consistent with the findings with CTB staining, in ABCA-1–silenced cells, palmitate-induced NOX4 translocation into LRs was not blocked by apoAI, whereas HDL inhibited NOX4 translocation (Figure 5). Conversely, in ABCG-1–silenced
or SRB-1–silenced cells, palmitate-induced NOX4 translocation was not blocked by HDL, whereas apoAI inhibited NOX4 translocation (Figure 5).

Finally, we evaluated how these transporters affect NOX4-derived ROS generation. Similar to the findings with CTB staining and NOX4 translocation, silencing ABCA-1 reduced the ability of apoAI to suppress palmitate-induced ROS generation, whereas HDL still reduced palmitate-induced ROS (Figure 6). Silencing of ABCG-1 or SRB-1 reduced the ability of HDL to suppress palmitate-induced ROS generation, whereas apoAI still reduced palmitate-induced ROS (Figure 6).

Because silencing these transporters could potentially affect palmitate uptake, thereby changing palmitate concentration in cells, we measured the uptake of 14C-labeled palmitate as apoAI still reduced palmitate-induced ROS (Figure 6). Silencing of ABCG-1 or SRB-1 reduced the ability of HDL to suppress palmitate-induced ROS generation, whereas apoAI still reduced palmitate-induced ROS (Figure 6).

Adipose Tissue Inflammation Is Reduced in Human ApoAI Transgenic Mice
To extend our in vitro findings in 3T3-L1 adipocytes to an in vivo model, we used male apoAI transgenic mice fed a HFHSC diet, which previously has been shown to result in obesity, insulin resistance, and atherosclerosis.31 ApoAI and HDL cholesterol levels are increased 2-fold in mice overexpressing the human apoAI transgene.35 As expected, lipoprotein distribution profiles of apoAI transgenic mice fed chow or HFHSC diet revealed increased HDL particles (Online Figure IVA and IVE). Plasma triglyceride and cholesterol levels were increased in apoAI/tg mice fed the HFHSC diet (Online Figure IVB and IVC). However, there were no changes in plasma triglyceride and cholesterol profiles between LDLR−/− and LDLR−/− apoAI/tg mice fed the HFHSC diet (Online Figure IVF and IVG). Levels of SAA (a circulating inflammatory marker) showed a trend toward reduction in the apoAI transgenic mice fed the HFHSC diet, but the changes did not reach statistical significance (Online Figure IVD and IVH). Next, we investigated inflammation in adipose tissue. First, in response to consumption of the HFHSC diet, expression of chemotactic factor gene, Saa3, was decreased in intra-abdominal adipose tissue of apoAI/tg (Figure 7A) and LDLR−/− apoAI/tg mice (Online Figure IV) versus controls (C57BL/6 or LDLR−/−), whereas another chemotactic factor gene, Mcp-1, was only decreased in intra-abdominal adipose tissue of LDLR−/− apoAI/tg mice (Figure 7A; Online Figure IV). Second, mRNA expression of macrophage markers was decreased in intra-abdominal adipose tissue of apoAI/tg (Figure 7B) and LDLR−/− apoAI/tg mice (Online Figure IV) fed the HFHSC diet. Immunohistochemical staining also showed a decrease of macrophages (detected with Mac2) in intra-abdominal adipose tissue of apoAI/tg mice compared with C57BL/6 mice fed the HFHSC diet (Figure 7C). Third, mRNA expression level of proinflammatory cytokines (Tnfa,
Il1β, and Il6) was decreased in intra-abdominal adipose tissue of apoAI<sup>tg</sup>/tg (Figure 7D) and LDLR<sup>−/−</sup> apoAI<sup>tg</sup>/tg mice (Online Figure V) fed the HFHSC diet. Interestingly, Nox4 mRNA expression level was decreased in apoAI<sup>tg</sup>/tg (Figure 7E) and in LDLR<sup>−/−</sup> apoAI<sup>tg</sup>/tg mice (Online Figure V) fed the HFHSC diet. These findings imply that apoAI and HDL also exert an anti-inflammatory effect on adipose tissue in vivo.

**Discussion**

We previously have shown that both excess glucose<sup>9</sup> and saturated fatty acids, such as palmitate,<sup>10</sup> increase the expression of the monocyte chemotactic factors, SAA3, and MCP-1, which induce monocyte chemotaxis. In this study, we show that apoAI and HDL have anti-inflammatory properties in cultured adipocytes in addition to their well-described effects on vascular cells, such as endothelial cells and macrophages.<sup>16,18–20,36</sup> Addition of either normal human HDL or its major apolipoprotein, apoAI, both reduced palmitate-induced expression of Saa3 and Mcp-1 in a dose-dependent and additive fashion. These effects are likely related to the cholesterol content of adipocyte membranes because they could be mimicked by removal of cholesterol from cells by exposure to methyl-β-cyclodextrin and restored by adding back cyclo-dextrin to which cholesterol had been added. Moreover, the anti-inflammatory effect of apoAI seemed to be ABCA-1 dependent because it could be blocked by silencing ABCA-1. The effects of intact HDL seemed to depend on ABCG-1 and SRB-1 because they could be reversed by silencing either of these genes. All of these proteins are present in 3T3-L1 adipocytes in vitro. We also were able to show that Saa3 and Mcp-1 expression and macrophage accumulation in adipose tissue were reduced in vivo in mice with high HDL levels as a result of apoAI overexpression. Finally, we had previously shown that palmitate induction of SAA3 and MCP-1 in adipocytes was dependent on the generation of ROS by NOX4 after translocation to LRs.<sup>28</sup> In this study, we extend those findings by demonstrating that NOX4 translocation to LRs is inhibited by both HDL and apoAI. Several mechanisms have been proposed by which HDL might be able to exert an anti-inflammatory effect on cells, including induction of scavenger receptor class B<sup>17</sup> and hydrolysis of some HDL components by endothelial lipase,<sup>38</sup> both PPAR-α-dependent processes. In addition, effects of apoAI and intact HDL on ABCA-1–mediated and ABCG-1–mediated cholesterol efflux from macrophages have been...
invoked to play a role in the anti-inflammatory properties of HDL in macrophages. A similar mechanism seems to be operative in adipocytes because palmitate increased the cholesterol content of rafts, as assessed by cholera toxin fluorescence, an effect that was reduced by pretreatment of the cells with either HDL or apoAI. Additional evidence that the cholesterol content of the cell membrane plays an important role in determining the ability of adipocytes to respond to palmitate is the observation that disruption of cell membrane rafts by the addition of cyclodextrin had the same anti-inflammatory effect as exposure of cells to either HDL or apoAI, and that the proinflammatory effect could be restored by exposing the cells to cyclodextrin that had been preloaded with cholesterol.

ABCA-1 transports cholesterol from cell membranes to lipid-poor apoAI, whereas whole HDL accepts cholesterol from both ABCG-1 and SRB-1. Although ABCG-1 is reported to reside in the endoplasmic reticulum, it could mediate transfer of cholesterol to the plasma membrane from where it could be desorbed to exogenously added lipids, such as HDL. Our results indicate that silencing ABCA-1 reversed the anti-inflammatory effect of apoAI but had no influence on the effect of intact HDL. Conversely, silencing ABCG-1 or SRB-1 reversed the inhibitory effect of HDL but not apoAI on palmitate induction of chemotactic factors. Because of the known functions of these proteins in transferring cholesterol to either apoAI or HDL, these findings are consistent with the cholesterol content of plasma membranes playing a major role in the ability of adipocytes to respond to the proinflammatory effects of palmitate.

Although some controversy exists regarding the definition or even the existence of LRs, proteins involved in cell signaling clearly cluster in certain domains of the plasma membrane. Accumulation of free cholesterol of the plasma membrane is associated with increased signaling via TLR4, which reside in these domains—the presence of apoAI or HDL reduces the cholesterol content of these rafts and TLR4 signaling in other cell types. The presence of ABCG-1 and HDL also protect against endothelial cell dysfunction in mice fed a Western-type diet, consistent with an inhibition of activation of TRL4 by saturated fatty acid in endothelial cells. We recently showed that

**Figure 5.** High-density lipoprotein (HDL) and apolipoprotein Al (apoAl) block the palmitate-induced NADPH oxidase 4 (NOX4) translocation via ATP-binding cassette A-1 (ABCA-1), ABCG-1, and scavenger receptor B-1 (SRB-1). 3T3-L1 adipocytes were transfected with a siRNA specific for ABCA-1, ABCG-1, SRB-1, or a scrambled small interfering RNA (siRNA; negative control) as indicated. Twenty-four hours later, siRNA was removed and the cells were cultured for a further 3 days. After that, the cells were pre-exposed to apoAI (50 μg/mL) or HDL (50 μg protein/mL) for 6 h, and then incubated with or without added palmitate (250 μM/L) for 24 h. Lipid rafts (LRs) were isolated and fractionated by ultracentrifugation using a detergent-free fractionation method. Proteins from OptiPre-gradient fractions were immunoblotted with anti-NOX4 and anticaevenol-1 (CAV1) antibodies. PNS indicates postnuclear supernatant fraction.

**Figure 6.** Apolipoprotein AI (ApoAI) and high-density lipoprotein (HDL) affect palmitate-induced NADPH oxidase 4 (NOX4)–derived reactive oxygen species generation via ATP-binding cassette A-1 (ABCA-1), ABCG-1, and scavenger receptor B-1 (SRB-1). 3T3-L1 adipocytes were transfected with a small interfering RNA (siRNA) specific for ABCA-1, ABCG-1, SRB-1, or a scrambled siRNA (negative control) as indicated. Twenty-four hours later, the siRNA was removed and the cells were cultured for a further 3 days. After that, the cells were exposed to apoAI (50 μg/mL) or HDL (50 μg protein/mL) for 6 h, and then incubated with or without added palmitate (250 μM/L) for 24 h. Cells were subjected to fluorescence activated cell sorter analysis using CM-H$_2$DCFDA. Results are plotted as counts (number of cells) on the vertical axis, vs dihydrodichlorofluorescein fluorescence intensity on the horizontal axis. Cells exposed to control media are shown in red, and the peak value for cells exposed to 250 μM/L palmitate is indicated by the dashed lines. Cells exposed to the indicated treatments are shown in blue.
factor expression induced by palmitate. In the present study, we show that pre-exposure of cells to either HDL or apoAI markedly inhibited the translocation of NOX4 to LR fractions of the plasma membrane and ROS generation. Moreover, silencing ABCA-1 reversed the effect of apoAI but not HDL, whereas silencing ABCG-1 or SRB-1 reversed the effect of HDL but not apoAI on palmitate-mediated NOX4 translocation to the plasma membrane. In view of the effects of apoAI and HDL and the silencing of these transporter proteins on chemotactic factor gene expression, these findings are consistent with apoAI and HDL effects on NOX4 translocation playing an important role in their ability to influence gene expression.

To test whether similar changes occur in vivo, we chose to emulate saturated fatty acid–induced adipose tissue inflammation in control mice and in mice expressing the human apoAI transgene. We previously have shown that a diet rich in saturated fat and sucrose with a moderate amount of added cholesterol, the so-called HFHSC diet, led to profound proinflammatory effects on intra-abdominal adipose tissue. To test the effect of apoAI and HDL in vivo, we chose to study apoAI transgenic mice, which have increased levels of human HDL. These mice show a reduction of atherosclerosis when present on an atherosclerosis prone background, suggesting that vascular inflammation is reduced in these
mice. Our findings indicate that high levels of apoAI that result from expression of the human apoAI transgene also inhibit the expression of monocyte chemotactic factors and the accumulation of macrophages in adipose tissue in mice. Moreover, expression of inflammatory genes, such as Tnfα and Il6, were reduced in adipose tissue from these mice, consistent with our in vitro findings. The reason why MCP-1 expression was not reduced in adipose tissue from the apoAI transgenic mice on the C57BL6 background whereas it was in the transgenic mice on the LDLR-deficient background is not clear. However, macrophage and inflammatory genes were reduced in both apoAI transgenic strains of mice.

Potential shortcomings of our study include lack of comparison of gene expression in the adipocyte versus stromal vascular fractions and of knowledge of the cellular distribution of NOX4 in adipose tissue. These should be determined in future studies of this nature.

Accumulation of macrophages in adipose tissue of both mice and humans is a hallmark of obesity.3,4 Adipose tissue inflammation and obesity are features of the metabolic syndrome in humans49 and in a mouse model that has many of the features of the metabolic syndrome.26 Moreover, obesity-associated inflammation predisposes to the development of both type 2 diabetes mellitus and cardiovascular disease.49 Therefore, strategies to reduce adipose tissue inflammation have potentially important therapeutic implications. Although simply raising HDL cholesterol levels has not been shown to reduce atherosclerosis50 nor do some genetic polymorphs that raise HDL levels protect against cardiovascular disease, it is clear from human epidemiological studies that HDL has a strong inverse relationship with cardiovascular disease.51 In addition, experiments in mice that either overexpress or are lacking in apoAI clearly show a very strong relationship with atherosclerosis.48,52 HDL also clearly has anti-inflammatory12–14 and other14 potentially atheroprotective properties on cells of the artery wall. The findings presented in this study, which demonstrate anti-inflammatory effects of HDL and apoAI on adipose tissue inflammation, offer additional possibilities for the prevention of diabetes mellitus and atherosclerosis associated with adipose tissue inflammation.

Sources of Funding
This work was supported by National Institutes of Health grants HL094352, HL092969, and DK-035816.

Disclosures
None.

References
What Is Known?

- High-density lipoproteins (HDL) and apolipoprotein A-I (apoA-I) have atheroprotective effects, which may be, in part, because of their anti-inflammatory properties.
- The mechanism by which HDL and apoA-I exert their anti-inflammatory effects in macrophages and endothelial cells seems to be via the depletion of lipid rafts on cell membranes.

What New Information Does This Article Contribute?

- Cholesterol depletion of lipid rafts in macrophages by HDL, apoA-I, or methyl-β-cyclodextrin inhibited palmitate-mediated chemotactic factor expression.
- HDL and apoA-I have anti-inflammatory effects on macrophages similar to other cell types, such as macrophages and endothelial cells.
- Similar findings were observed in adipose tissue in vivo as a result of apoA-I overexpression.

HDL and apoA-I exert anti-inflammatory effects on macrophages and endothelial cells by depleting cholesterol in lipid rafts. However, it is unclear whether HDL and apoA-I have similar effects on adipocytes. We found that both HDL and apoA-I inhibit palmitate-mediated induction of monocyte chemoattractant factor gene expression in cultured adipocytes by a mechanism consistent with the disruption and the removal of cholesterol from lipid rafts. Moreover, overexpression of apoA-I in vivo reduced chemotactic factor expression and macrophage accumulation in adipose tissue in mice fed a high-fat diet. These findings indicate that HDL and apoA-I have anti-inflammatory effects on adipocytes and adipose tissue, similar to their better-known effects on vascular cells, such as macrophages and endothelial cells. Because macrophage accumulation in adipose tissue is an important harbinger of insulin resistance and cardiovascular disease, these results have important translational implications for the prevention and the management of insulin resistance and obesity-associated cardiovascular disease by controlling adipose tissue inflammation and its downstream consequences.
Apolipoprotein AI and High-Density Lipoprotein Have Anti-Inflammatory Effects on Adipocytes via Cholesterol Transporters: ATP-Binding Cassette A-1, ATP-Binding Cassette G-1, and Scavenger Receptor B-1

Tomio Umemoto, Chang Yeop Han, Poulami Mitra, Michelle M. Averill, Chongren Tang, Leela Goodspeed, Mohamed Omer, Savitha Subramanian, Shari Wang, Laura J. Den Hartigh, Hao Wei, Eung Ju Kim, Jinkyu Kim, Kevin D. O'Brien and Alan Chait

Circ Res. 2013;112:1345-1354; originally published online March 15, 2013; doi: 10.1161/CIRCRESAHA.111.300581

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/112/10/1345

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/03/15/CIRCRESAHA.111.300581.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental methods

Reagents and cell culture

Palmitate (16:0), and methyl-β-cyclodextrin (MβCD) were purchased from Sigma. Alexa Fluor 594 conjugated cholera toxin subunit β (CTB) was purchased from Invitrogen. HDL (d=1.063 to 1.210 g/mL) was isolated from plasma of healthy human volunteers by ultracentrifugation. Human apoA-I was purchased from Academy Bio-Medical Co. 3T3-L1 murine pre-adipocytes, obtained from American Type Tissue Culture Collection, were propagated and differentiated according to standard procedures. Fully differentiated 3T3-L1 adipocytes were pre-treated with 50μg/ml HDL or apoA-I for 6h in DMEM containing 5mmol/L glucose and 10% fetal bovine serum, and then washed three times with PBS. After that, adipocytes were incubated with 250μmol/ml palmitate for 24h in DMEM containing 5mmol/L glucose and 10% fetal bovine serum.

Multiplex real-time quantitative reverse-transcription polymerase chain reaction

(RT-PCR) was performed using the TaqMan Master kit (Applied Biosystems) in the ABI prism 7900HT system. Saa3, Mcp-1, Tnfα, Il1β, Il6, Cdl1b, Cdl1c, F4/80, Mac2, NOX4, Srb-1, Abca-1 and Abcg-1 primers and a FAM probe were obtained from Applied Biosystems (Assay-on-Demand). Primers and TaqMan probes specific for GAPDH were as follows: for GAPDH, forward primer, 5'AGCCTCGTCCCGTAGACAAA3'; reverse primer, 5'ACCAGGCGCCCAATACG3'; probe, HEX-5'AAATCCGTTCACACCGACCTTCACCA3'-BHQ1, Each sample was analyzed in triplicate and normalized in multiplex reactions using GAPDH as control.

Detergent-free lipid raft fractionation

Lipid raft (LR) and non-LR fractions from adipocytes were obtained by Optiprep gradient centrifugation using a detergent-free protocol. Briefly, the cell pellet was homogenized in buffer (250mmol/L sucrose, 1mmol/L EDTA, 500mmol/L sodium bicarbonate, pH 11), with 15 strokes of a loose fitting Dounce homogenizer and sonicated (20 times for 30s). After centrifugation (1,000 × g, 10 min), the post-nuclear supernatant fraction was added to 60% Optiprep to make 35% Optiprep (final concentration). The 35% Optiprep samples were overlaid with 5–35% Optiprep as a discontinuous gradient and centrifuged at 60,000 rpm in a Beckman NVT 65.2 rotor for 90 min at 4 °C. After centrifugation, nine 0.5-ml fractions were collected using a fraction collector. The fractionated proteins were subjected to immunoblotting using antibodies against NOX4 (ABCAM) and caveolin-1 (Cell Signaling).

Figure Legends

Supplemental figure I. ApoA-I and HDL decrease cholesterol levels.

3T3-L1 adipocytes were pre-exposed to apoA-I (50mg/ml) and HDL (50μg protein/ml) for 6h. Thereafter, adipocytes were incubated with or without 250 mol/ml palmitate for 24 h. Membrane fractions were collected from cells pre-exposed to apoA-I and HDL for 6h, and from cells exposed to palmitate for 24h after the pre-exposure to apoA-I and HDL. Membrane cholesterol levels were measured by LC-MS/MS and normalized to membrane protein concentration. *P < 0.001 vs. control media, **P < 0.001 vs. palmitate.

Supplemental figure II. ApoA-I and HDL inhibits chemotactic factor expression.

3T3-L1 adipocytes were pre-exposed to HDL and apoA-I (50μg/ml) for 6h. After that, adipocytes were incubated with or without 250 mol/ml palmitate for 24h. Saa3 and Mcp-1 gene expression was analyzed
by multiplex real-time RT-PCR, normalized to GAPDH (A-B).

**Supplemental figure III. The efficiency of silencing ABCA-1, ABCG-1 and SRB-1.**
3T3-L1 adipocytes were transfected with siRNAs specific for ABCA-1, ABCG-1, SRB-1 or a scrambled siRNA (negative control) as indicated. 24h later the siRNA was removed and the cells were cultured for a further 4 days. After that, cell lysates were analyzed by immunoblot using ABCA-1, ABCG-1 and SRB-1 antibodies, and were normalized to GAPDH.

**Supplemental figure IV. Lipids, lipoproteins, SAA and insulin levels in human apoA-I transgenic mice.**
ApoA-I+/+ and C57BL/6 control mice were fed a chow or a HFHSC diet for 24 weeks (A-D; n=9). LDLR−/− apoA-I+/+ mice and LDLR−/− control mice were fed a chow diet or HFHSC diet for 16 weeks (E-H; n=7). Plasma lipoprotein distribution (A and E), plasma triglycerides (B and F), plasma cholesterol (C and G), and plasma SAA (D and H) levels were measured. *P < 0.05 vs. non-transgenic mice.

**Supplemental figure V. Overexpression of human apoA-I inhibits chemotactic factor expression and macrophage accumulation in adipose tissue of LDL receptor deficient (LDLR−/−) mice.**
LDLR−/− apoA-I+/+ mice and LDLR−/− control mice were fed a chow diet or HFHSC diet for 16 weeks (A-F; n=7). Epididymal fat was isolated and analyzed by real-time RT-PCR using Saa3, Mcp-1 (A), Mac2, Cd11b, Cd11c, F4/80 (B), Tnfα, Il6 (C) and Nox4 (D)-specific primers and probes and normalized to Gapdh. *P < 0.005 vs. chow, **P < 0.005 vs. C57BL/6 or LDLR−/− in HFHSC
Cholesterol (mg/mg protein)

Palmitate
ApoA-I (50μg/ml)
HDL (50μg/ml)

Supplemental figure I
Supplemental figure II
Supplemental figure III
Supplemental figure IV
Supplemental figure IV
Supplemental figure IV
Supplemental figure IV
Supplemental figure V

**Figure A**

- **Saa3**
  - **Chow**
  - **HFHSC**

- **Mcp-1**
  - **Chow**
  - **HFHSC**

*Note: The figure illustrates the copy number of Saa3 and Mcp-1 genes in different dietary conditions (Chow vs. HFHSC) and genetic backgrounds (LDLR⁻/⁻ vs. LDLR⁻/⁻ & ApoA⁻/⁻/⁻). The bars represent the mean ± standard error of the mean (SEM). The asterisks indicate statistical significance: *p < 0.05, **p < 0.01.*
**B**

**Mac2**

- **LDLR**/−
- **LDLR**/− & ApoA-I^tg/tg

**Cd11b**

**Cd11c**

**F4/80**

*Supplemental figure V*
Supplemental figure V
Supplemental figure V

**F**

**Nox4**

- LDLR<sup>−/−</sup>
- LDLR<sup>−/−</sup> & ApoA<sup>−/−</sup>

Chow  |  HFHSC

- Nox4/Gapdh copy number

<table>
<thead>
<tr>
<th></th>
<th>Chow</th>
<th>HFHSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>LDLR&lt;sup&gt;−/−&lt;/sup&gt; &amp; ApoA&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.01