ATP-Binding Cassette Transporter 1 Participates in LDL Oxidation by Artery Wall Cells

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**Objective**—We have previously reported that products of the lipoxygenase pathway, hydroperoxyoctadecadienoic acid and hydroperoxyeicosatetraenoic acid, as well as cholesterol linoleate hydroperoxides, collectively termed seeding molecules, are removed by apolipoprotein A-I (apoA-I) from the artery wall cells and render low density lipoprotein (LDL) resistant to oxidation by human artery wall cells. The mechanisms by which oxidized lipids are transported and/or transferred to lipoproteins and the pathways by which apoA-I facilitates their removal remain unclear. ATP-binding cassette transporter 1 (ABCA1) is known to facilitate the release of cellular phospholipids and cholesterol from the plasma membrane to apoA-I and high density lipoprotein. Therefore, we evaluated whether ABCA1 participates in LDL oxidation.

**Methods and Results**—In this report, we show that (1) chemical inhibitors of ABCA1 function, glyburide and DIDS, block artery wall cell–mediated oxidative modification of LDL, (2) inhibition of ABCA1 with the use of antisense (but not sense) oligonucleotides prevents LDL-induced lipid hydroperoxide formation and LDL-induced monocyte chemotactic activity by the artery wall cells, and (3) oxysterols that induce ABCA1 expression, such as 22(Δ6)hydroxycholesterol, enhance cell-mediated LDL oxidation. Furthermore, we also show that 22(Δ6)hydroxycholesterol induces the production of reactive oxygen species in the artery wall cells, which can be removed by incubating the artery wall cells with apoA-I.

**Conclusions**—Our data suggest that ABCA1 plays an important role in artery wall cell–mediated modification/oxidation of LDL by modulating the release of reactive oxygen species from artery wall cells that are necessary for LDL oxidation. (Arterioscler Thromb Vasc Biol. 2002;22:1877-1883.)

Key Words: ATP-binding cassette transporter 1 | LDL oxidation | atherosclerosis | artery wall cells | oxysterols

Low density lipoprotein is the major source of extracellular cholesterol and phospholipid, and some of these phospholipids can yield oxidized phospholipids that induce an inflammatory response. An important step in the oxidation of LDL by artery wall cells involves the seeding of LDL with products of the lipoxygenase pathway: hydroperoxyoctadecadienoic acid (HPODE) and hydroperoxyeicosatetraenoic acid (HPETE), as well as cholesterol linoleate hydroperoxides, collectively termed seeding molecules. ApoA-I, a major protein component of HDL particles, is known to remove cholesterol and phospholipids from artery wall cells. It is widely accepted that the atheroprotective actions of apoA-I or HDL arise from their participation in a normal physiological process known as reverse cholesterol transport. Recently, our laboratory has shown that apoA-I and an apoA-I mimetic peptide remove “seeding molecules” from LDL and render LDL resistant to oxidation by human artery wall cells. These findings project a new function for apoA-I in removing oxidized lipids from lipoproteins and cells and further solidify its role as an antiatherogenic apolipoprotein. However, the mechanisms by which seeding molecules are transported and/or transferred to lipoproteins and the pathways by which apoA-I facilitates the removal of seeding molecules remain unclear.

ATP-binding cassette transporter A1 (ABCA1) mediates cholesterol secretion from cells and functions as a rate-controlling protein in the apoA-I–dependent active transport of cholesterol and phospholipids. ApoA-I–mediated lipid transport requires a functional ABCA1 protein and specific binding and/or docking interactions of apoA-I with the plasma membrane. Mutations in the ABCA1 gene result in the loss of apoA-I–mediated removal of cellular lipids and cause Tangier disease and Familial HDL deficiency. Disruption of the ABCA1 gene in mice results in HDL deficiency and impaired cholesterol and lipid transport, and overexpression of ABCA1 leads to increased cholesterol and phospholipid efflux to apoA-I in transgenic mice. Moreover, fibroblasts from patients with Tangier disease are defective in transferring phospholipids onto apoA-I. Although ABCA1 was shown to generate a regulated anion flux when expressed in Xenopus laevis oocytes, to date, ABCA1 has not been implicated in the transport of anionic biomol-
cules, such as oxidized lipids or reactive oxygen species (ROS). Because apoA-I and ABCA1 are functionally linked in cholesterol and phospholipid efflux from cells and because apoA-I also facilitates the removal of seeding molecules from cells,7,8 we hypothesized that ABCA1 may play a role in cell-mediated oxidation of LDL by regulating the transfer or transport of seeding molecules.

In the present study, we investigated whether ABCA1 participates in the oxidative modification of LDL by human artery wall cells. We have used chemical inhibitors of ABCA1 function, inducers of ABCA1 expression (oxysterols), and specific inhibition of ABCA1 protein expression (antisense oligonucleotides), and we have shown that ABCA1 plays a key role in cell-mediated oxidative modification of LDL by human artery wall cells.

Methods

Materials
All cell culture media and reagents were supplied by GIBCO-BRL. Human apoA-I, glyburide, DIDS, and 22(R)-hydroxycholesterol (22-R-OHC) were obtained from Sigma Chemical Co. Oligonucleotides were purchased from Operon Technologies. LDL (density 1.019 to 1.063 g/mL) and HDL (density 1.063 to 1.21 g/mL) were isolated on the basis of the protocol described by Havel et al.26

Cell Cultures
Human aortic endothelial cells (HAECs) and human aortic smooth muscle cells (HASMCs) were isolated and cultured as described previously.7,8 Monocytes were prepared by a modification of the method reported by Colotta et al.27 from the blood of normal volunteers after obtaining written consent under a protocol approved by the Human Research Subject Protection Committee of the University of California, Los Angeles. Cocultures were set up in 6-well culture dishes treated with 0.1% gelatin at 37°C overnight. HASMCs were added at a density of 1×10^5 cells/cm². Cells were cultured for 2 days, and HAECs were subsequently added at 2×10^5 cells/cm² and were allowed to grow, forming a complete monolayer of confluent HAECs in 2 days. In all experiments, HAECs and autologous HASMCs (from the same donor) were used at passages 4 to 6.

Northern and Western Analyses
Northern analyses were performed as described previously.26 Human ABCA1 cDNA was provided by Dr Peter Edwards (University of California, Los Angeles). Western analyses were performed as described previously.8 Briefly, protein extracts from human artery wall cocultures (60 °C) were subjected to SDS-PAGE on 5% Tris-HCl gels. Proteins were electroblotted onto Hybond ECL Nitrocellulose membranes (Amersham) and incubated for 3 hours with rabbit anti-human ABCA1 antisera (Novus Biologicals) at 1:100 dilution. Proteins were detected by using an ECL Western blotting kit (Amersham) according to the manufacturer’s suggested protocol.

Antisense Oligonucleotide Experiments
Antisense oligonucleotide experiments were performed as described previously.26 Briefly, HAEC/HASMC cocultures were set up in 6-well plates. Phosphorothioate oligonucleotides (Operon Technologies) were used at a final concentration of 100 nmol/L for all antisense transfection experiments. Appropriate amounts of the oligonucleotides were diluted in 200 µL serum-free medium 199 in 0.5-mL Eppendorf tubes. Three microliters of SuperFect reagent (Qiagen, Inc) was added to each tube and incubated at room temperature for 15 minutes to allow SuperFect reagent–DNA complex formation. During the incubation, the HAECs were washed with PBS and supplemented with 0.8 mL complete medium 199. The transfection complexes were added to the wells and incubated for 2 hours. The cultures were washed in PBS and supplemented with complete medium 199. Eighteen hours later, the transfection protocol was repeated, and cultures received 250 µg/mL of LDL. Six hours later, supernatants were removed and assayed for Auerbach lipid hydroperoxide equivalents30 (left) and monocyte chemotactic activity (right). Similar results were obtained in 3 independent experiments. *P<0.05.

Other Methods
Monocyte chemotactic activity assays were performed as described previously.7,8 Protein concentrations were determined by using the Bradford reagent (Bio-Rad). Lipid hydroperoxides were determined by the method reported by Auerbach et al.30 Statistical significance was determined by ANOVA. The analyses were first carried out with the Excel application program (Microsoft), followed by a paired Student t test to identify significantly different means. Significance is defined as P<0.05.

Results

Inhibitors of ABCA1 Function Impair Artery Wall Coculture–Mediated Modification of LDL
To determine whether ABCA1 plays a role in cell-mediated oxidation of LDL, we first examined the effect of inhibitors of ABCA1 function, glyburide and DIDS, on artery wall coculture–mediated oxidation of LDL. As expected, on the basis of our previous studies,7,8 when cocultures were incubated with LDL (250 µg/mL, 18 hours), LDL-induced lipid hydroperoxides and LDL-induced monocyte chemotactic activity were elevated in the coculture supernatants (Figure 1). However, pretreatment of the cocultures with either glyburide (100 µmol/L) or DIDS (100 µmol/L) in medium 199 containing 10% lipoprotein-deficient serum (LPDS), LDL (250 µg/mL) was added to the cocultures, and 18 hours later, the supernatants were removed and assayed for Auerbach lipid hydroperoxide equivalents30 (left) and monocyte chemotactic activity (right). Similar results were obtained in 3 independent experiments. *P<0.05.
DIDS at the concentrations used in coculture experiments showed no cytotoxicity and completely blocked HDL-mediated cholesterol efflux (data not shown) in human monocyte macrophages. These results suggest that cell-mediated LDL oxidation and cholesterol efflux share a common mechanism(s).

**Inducers of ABCA1 Expression Enhance Coculture-Mediated Modification of LDL**

Because inhibitors of ABCA1 function impaired the ability of artery wall cells to modify/oxidize LDL, we next examined the effect of inducers of ABCA1 expression on coculture-mediated modification of LDL. 22-R-OHC is a potent inducer of ABCA1 transcription in macrophages. ABCA1 mRNA (Figure 2A) and protein (Figure 2B) were induced after 22-R-OHC treatment in artery wall cocultures. 22-R-OHC that was incubated directly with LDL did not affect either the lipid hydroperoxide content of LDL or LDL-induced monocyte chemotactic activity. However, cocultures pretreated with 22-R-OHC oxidized LDL significantly more than did the untreated cocultures (Figure 2C, left), and cocultures pretreated with 22-R-OHC (25 μmol/L) had significantly higher monocyte chemotactic activity (Figure 2C, right). The results were significant and consistent in dose-response experiments in which 22-R-OHC concentrations between 5 and 50 μmol/L were tested.

**Antisense Oligonucleotides to ABCA1 Disrupt Coculture-Mediated Oxidation of LDL**

We next examined the effect of selective inhibition of ABCA1 on coculture-mediated oxidation of LDL. Antisense oligonucleotides targeted to human ABCA1 mRNA have been previously used to successfully block the expression of ABCA1 protein and ABCA1 function in macrophages. Transfection with oligonucleotides alone, either sense or antisense, did not affect the accumulation of lipid hydroperoxides or monocyte chemotactic activity in untreated artery wall coculture supernatants (Figure 3). Pretreatment of cocultures with antisense oligonucleotides significantly impaired cell-mediated oxidative modification of LDL, as was evident from the decrease in LDL-induced lipid hydroperoxides (Figure 3, left) and LDL-induced monocyte chemotactic activity (Figure 3, right) in the coculture supernatants. These data suggest that ABCA1 modulates the cell-
mediated oxidative modification of LDL by artery wall cells. Furthermore, antisense oligonucleotides to ABCA1 inhibited the accumulation of ABCA1 protein in artery wall cocultures pretreated with 22-R-OHC (Figure 4A), and they also prevented LDL-induced monocyte chemotactic activity in the supernatants of cocultures that had been pretreated with 22-R-OHC (Figure 4B).

22-R-OHC Induces the Accumulation of Intracellular ROS

Incubating cocultures with 22-R-OHC not only induced ABCA1 expression (Figure 2A) but also enhanced coculture-mediated modification of LDL (Figure 2B). Moreover, antisense oligonucleotides to ABCA1 attenuated 22-R-OHC-mediated LDL oxidation (Figure 4B). The increase in LDL oxidation, after exposure to 22-R-OHC, could be due to either an increase in the transport of ROS alone (via increases in ABCA1 expression) or an increase in the synthesis of ROS. To test the latter possibility, we examined the accumulation of intracellular ROS in 22-R-OHC–treated cocultures. We have recently reported the use of 2’,7’-dichlorofluorescin diacetate (DCFH-DA) to measure intracellular oxidative stress.33 This assay quantifies the fluorescence emitted when the nonfluorescent DCFH-DA is oxidized to the highly fluorescent dichlorofluorescein (DCF) by intracellular ROS. Artery wall cocultures were incubated with 100 μmol/L DCFH-DA for 1 hour and subsequently treated with varying concentrations (0 to 50 μmol/L) of 22-R-OHC. No significant difference in emitted fluorescence was observed between cells treated with vehicle alone or untreated cells (data not shown). Emitted fluorescence was significantly higher (at all time points tested) in cocultures treated with 22-R-OHC (50 μmol/L) than in control cells (Figure 5A). 22-R-OHC incubated alone (in the absence of cells) with DCFH-DA did not cause any changes in emitted fluorescence (data not shown), suggesting that 22-R-OHC, by itself, does not oxidize DCFH-DA to the fluorescent DCF. These experiments demonstrate that oxysterols, such as 22-R-OHC, induce the synthesis and/or accumulation of ROS in artery wall cocultures.

ApO-A-I Renders 22-R-OHC–Incubated Cocultures Unable to Oxidize LDL

ApO-A-I removes ROS, including HPETE and HPODE, and renders artery wall cells unable to oxidize LDL.7,8 We next examined whether apoa-I is able to remove 22-R-OHC–induced ROS from the artery wall cocultures and thus prevent 22-R-OHC–induced LDL oxidation by cocultures. Cocultures pretreated with 22-R-OHC oxidized LDL significantly more than did untreated cocultures (Figure 5B, left), and cocultures pretreated with 22-R-OHC (25 μmol/L) had significantly higher monocyte chemotactic activity (Figure 5B, right). However, apoa-I (100 μg/mL) added to the cells for 6 hours after 22-R-OHC treatment and removal before the addition of LDL significantly inhibited the ability of cocultures to modify LDL (Figure 5B).

Discussion

In presenting possible explanations for the decrease in atherosclerosis in 12/15-lipoxygenase–knockout mice, Cyrus et al34 concluded that several mechanisms could explain their findings, but they favored one in which “...lipooxygenase-derived hydroperoxides or secondary reactive lipid species may be transferred across the cell membrane to ‘seed’ the extracellular LDL, which would then be more susceptible to a variety of mechanisms that could promote lipid peroxidation.” Because ABCA-1 mediates the transport of cholesterol and phospholipids from cells by apoa-I,35 we hypothesized that ABCA1 might also participate in the transfer of the seeding molecules from artery wall cells to LDL. In the present study, we demonstrated that ABCA1 modulates artery wall cell–mediated oxidation of LDL. Recent evidence suggests that ABCA1 may have a more indirect role in cholesterol transport and thus functions as a cholesterol efflux regulatory protein.36,37 Several reports also indicate that ABCA1 is not confined to regulating the transport of cholesterol and...
phospholipids alone. ABCA1 modulates the secretion of apoE from human monocyte-derived macrophages, interleukin-1β secretion, and α-tocopherol. Our data suggest that ABCA1 may also modulate the secretion of ROS from artery wall cells that are necessary for LDL oxidation.

We found that 22-R-OHC not only induced accumulation of intracellular ROS (Figure 5) in artery wall cocultures but also significantly increased artery wall cell–mediated LDL oxidation (Figure 2). We previously demonstrated that human artery wall cells contain 12-lipoxygenase protein and mRNA and that chemical inhibitors and antisense oligonucleotides of 12-lipoxygenase inhibited the ability of the cells to oxidize LDL. On the basis of our previous results, we hypothesized that oxysterols may activate 12-lipoxygenase, resulting in increased synthesis of HPETE and HPODE and thus causing an increase in LDL oxidation. However, inhibiting 12-lipoxygenase did not affect the 22-R-OHC–mediated increase in LDL-induced monocyte chemotactic activity (data not shown). Furthermore, we did not find any changes in 15-lipoxygenase and 5-lipoxygenase (message or protein) after treatment of the artery wall cocultures with 22-R-OHC (data not shown). It is possible that oxysterol may regulate LDL oxidation by artery wall cells either via post-translational modifications of lipoxygenases or via a novel previously unidentified pathway(s), such as increasing phospholipase activity in the artery wall cells, resulting in increased synthesis of arachidonic acid and linoleic acid, the substrates for lipoxygenases. We are currently evaluating these possibilities.

Interestingly, Liao et al recently reported that native LDL induces ABCA1 mRNA and protein in vascular endothelial cells. ABCA1 protein induction by native LDL at 24 hours was significant, but it was only moderate in HAECS compared with HUVECs, which were the predominant cells used in their studies. In our experiments, LDL alone did not significantly induce ABCA1 message or protein in HAECS or cocultures after 6 hours of LDL treatment (data not shown). Liao et al also suggest that overexpression of ABCA1 by LDL prevents overloading of cholesterol by increasing the efflux of cholesterol. The data and conclusions from their studies are consistent with our hypotheses that reverse cholesterol transport and LDL oxidation/metabolism may be linked in the artery wall.

In light of our findings, one might expect that the balance between the transport out of the cell of the seeding molecules by ABCA1 to extracellular LDL (favoring LDL oxidation) and the generation of higher levels of HDL (which could prevent LDL oxidation) might be a complex process, and it might be difficult to predict whether ABCA1 would promote or retard atherogenesis. In fact, some of the unexpected results from studies on ABCA1 transgenic and knockout mice do not support a simple reverse cholesterol transport model.

Joyce et al recently reported that transgenic expression of ABCA1 reduced atherosclerosis in C57BL/6j mice fed an atherogenic diet containing cholic acid but actually increased atherosclerosis in apoE-null mice that were also transgenic for human ABCA1. In contrast, Singaraja et al reported that human ABCA1–overexpressing transgenic mice had reduced atherosclerosis. Aiello et al reported that the complete absence of ABCA1 did not affect the development, progression, or composition of atherosclerotic lesions in either LDL receptor–null or apoE-null mice fed either a chow or atherogenic diet. However, Aiello et al and Van Eck et al, using bone marrow transplantation, found that the absence of ABCA1 from macrophages resulted in increased atherosclerosis in apoE-null and LDL receptor–null mice. Thus, the role of ABCA1 in atherosclerosis is clearly complex.

Our data suggest that reverse cholesterol transport and LDL oxidation may share common mechanisms (Figure 6) in at least 2 ways: (1) ABCA1 is required for reverse cholesterol transport and LDL oxidation. (2) Cellular cholesterol levels may determine the cellular levels of 22-R-OHC, which, in part, regulates cell-mediated LDL oxidation by an as-yet-identified pathway.
would be favored, whereas at low levels of LDL relative to HDL formation, LDL oxidation would not be favored. Thus, the rate of formation and clearance of mature HDL relative to LDL levels would determine in part the rate of LDL oxidation and atherogenesis.

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