Native LDL Uptigation of ATP-Binding Cassette Transporter-1 in Human Vascular Endothelial Cells

Hailing Liao, Thomas Langmann, Gerd Schmitz, Yi Zhu

Abstract—ATP-binding cassette transporter-1 (ABCA1) mediates the lipid efflux from cells to apolipoproteins. In studying the gene expression and regulation of ABCA1 in human vascular endothelial cells (ECs), we found that native low density lipoprotein (LDL) elevates ABCA1 in both protein and mRNA levels in a time- and dose-dependent fashion. Transfection of full-length human ABCA1 in ECs lowers cellular cholesterol content and increases apolipoprotein (apo) A-I–mediated cholesterol efflux. Transfection of the ABCA1 promoter–luciferase reporter results in a 2-fold induction after LDL exposure. The responsive element was mapped within −116 to −54 of the promoter region with use of promoter deletion constructs, as reported in other cells. A mutation of the DR4 site greatly diminished the LDL effect. Results showing that LDL increases the liver X receptor responsive element (LXRE)–driven luciferase activity demonstrate the effect of LDL on LXR activation. Furthermore, ligands of the retinoid X receptor and LXR activate ABCA1 in ECs at levels of both promoter activation and mRNA induction. Therefore, ABCA1 is expressed in vascular ECs and is transcriptionally upregulated by LDL. Overexpression of ABCA1 in these cells prevents overloading of cholesterol by increasing the efflux of cholesterol. Thus, ABCA1 plays an important role in the homeostasis of cholesterol in the vascular endothelium. (Arterioscler Thromb Vasc Biol. 2002;22:127-132.)

Key Words: ABCA1 ■ cholesterol ■ LDL ■ liver X receptor ■ endothelial cells

High plasma levels of LDL cholesterol and/or low levels of HDL cholesterol are important risk factors for atherosclerotic cardiovascular disease. Epidemiological studies have shown an inverse relationship between levels of HDL cholesterol and the risk of coronary artery disease. It has been proposed that HDL promotes reverse cholesterol transport by facilitating the transfer of cholesterol from peripheral tissues to the liver for disposal. However, the molecular mechanisms for transfer of cholesterol from peripheral cells to HDL were not clear until the recent discovery of mutations in the gene for transfer of cholesterol from peripheral cells to HDL were found in familial HDL deficiency. These findings outline a molecular mechanism for the cellular defect in these disorders and the role of HDL in reverse cholesterol transfer, because ABCA1 stimulates cholesterol and phospholipid efflux to apoA-I and most likely acts as a lipid flippase at the plasma membrane.4

In cells, intracellular cholesterol homeostasis is exquisitely regulated and depends on the balance between cholesterol synthesis, influx, and degradation; cholesterol ester formation; and translocation of cholesterol to the plasma membrane for efflux.3 The free cholesterol (FC) content of cells cultured in normal media containing lipoprotein, when cellular LDL receptors are downregulated, appears to be determined mainly by a balance between the uptake of LDL FC and the efflux of FC to HDL, particularly the apoA-I–enriched, lipid-poor fraction of HDL.6 This response is directly linked to the function of ABCA1, a 240-kDa protein. Plasma HDL cholesterol and phospholipids are nearly absent in patients with Tangier disease.1–3 Furthermore, study of ABCA1 heterozygotes provides direct evidence that the impaired cholesterol efflux is associated with reduced plasma HDL cholesterol levels and an increased risk of coronary artery disease.7 Cultured skin fibroblasts isolated from patients with Tangier disease lack the feature of cholesterol and phospholipid efflux to apoA-I or lipid-poor HDL (see reviews5,8). ABCA1 overexpression in 293 cells markedly increased cellular cholesterol and phospholipid efflux to apoA-I but not to HDL.9

Vascular endothelial cells (ECs), which form a barrier between the vessel wall and lipoproteins and lipids in the circulation, play an important role in maintaining vascular integrity and, when disturbed, can lead to the development of atherosclerotic plaques. To date, study of the regulation of ABCA1 and its role in lipid trafficking in ECs has not been addressed. Because of the significance of lipid efflux in regulating cell surface properties in vascular cells, particularly ECs, we investigated the expression, regulation, and role of ABCA1 in the efflux of cholesterol in ECs. Results demonstrate that ABCA1 is expressed in vascular ECs and transcriptionally upregulated by LDL. The LDL-activated liver X receptor (LXR) apparently plays an important role in ABCA1 induction in ECs. Overexpression of ABCA1 in
these cells could prevent cholesterol overload through enhanced cholesterol efflux.

**Methods**

**Cell Culture and LDL Isolation**

Human umbilical vein endothelial cells (HUVECs) were isolated and maintained as described.10 Human aortic endothelial cells (HAECs) from 2 different patients were a generous gift from Dr S. Allen of the Imperial College of Science, Harefield, UK. The HAECs were cultured in medium 199 containing 15% fetal bovine serum (FBS), 10% AB serum, and 5 mg/mL fibroblast growth factor, as previously reported.11 All experiments were performed with HUVECs up to passage 3 and HAECs up to passage 4, and all cells were used to confluence before LDL treatment. The human hepatoma cell line HepG2 was purchased from the American Type Culture Collection (Manassas, Va.) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% FBS. LDL was isolated from nonfrozen human plasma as described.10,12 During LDL isolation and dialysis, 0.01% EDTA and 20 μM H9262 were added to each sample to prevent LDL oxidation. The oxidation state of LDL was measured by the thio-barbituric acid–reactive substances assay. Malonyldialdehyde equivalents were quantified on a spectrophotometer (at 355 nm). The LDL used in this study contained <0.1 malonyldialdehyde mmol equivalent per milligram of cholesterol. Total cholesterol concentration of LDL was determined by enzymatic assay.13 For all studies, LDL had a final cholesterol concentration of 180 mg/dL (4.68 mmol/L).

**Northern Blot Hybridization**

Total RNA isolation and Northern blotting for human (h) ABCA1 and von Willebrand factor (vWF) expression were performed as described.10 The hABCA1 cDNA probe was generated by reverse transcription–polymerase chain reaction product was used for Northern blot hybridization. hABCA1 and vWF probes were labeled with [32P]dCTP by DECApriming (Ambion).

**ABCA1 Immunoprecipitation and Western Blotting**

After LDL exposure, HUVECs were incubated with 1.0 mg/mL sulfo-NHS-biotin (Pierce) in phosphate-buffered saline (pH 8.0) for 30 minutes to biotinylate the cell surface proteins.15 Cells were solubilized in detergent containing buffer. The rabbit polyclonal antiserum against hABCA1 (R1/61) was generated by the Pineda AK-Service (Berlin, Germany). A peptide derived from the last 20 amino acids of ABCA1, VVDVAVLTSFQDEKVEKSYV, was used to immunize the rabbits, and the antiserum was harvested 6 days after immunization. A total of 500 μg protein in 1.0 mL of cell lysates was incubated with antisera R1/61 (1:200) overnight at 4°C, followed by a 1-hour incubation with protein A/G plus agarose beads. Bound immune complexes were washed 3 times with the lysis buffer. Proteins were resolved by 6% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and visualized with use of a streptavidin–horseradish peroxidase ECL assay as described.12,13

**Plasmids and Transfection**

We used the Targeten reagent (Targeting Systems) for transient transfection.13 For the promoter activation study, the reporter plasmid of the hABCA1 promoter and its mutation and deletion constructs pABCA1(−928)-Luciferase (Luc), pABCA1(156)-Luc, pABCA1(−116)-Luc, pABCA1(−54)-Luc, and pABCA1(−928 DR4 mut)-Luc, as described,16 were generously provided by Dr A. Tall (Columbia University, New York, NY). LKREx3 TK-Luc, a reporter construct containing 3 copies of LXRE from the mouse mammary tumor virus long tandem repeat,17 and cytomegalovirus IE promoter/enhancer (CMX)-hLXRα, the expression plasmid of human LXRα,18 were generously provided by Dr R. Evans (Salk Institute, La Jolla, Calif) and Dr B.M. Forman (City of Hope, Duarte, Calif). Plasmid Rous sarcoma virus (pRSV)–β-galactosidase (β-gal) was cotransfected as a transfection control. After 24 hours of LDL exposure, samples were collected and assayed for luc activity. The results were normalized against β-gal.12 To assess the effect of ABCA1 on cholesterol content in ECs, we obtained the expression plasmid of the full-length coding region of hABCA1 cDNA (nucleotides 118 to 6751; accession number A3012376) in the pcDNA3.1 vector (pcDNA3.1-ABCA1) as described.19 Cells were transfected with pcDNA3.1-ABCA1 or vector for 48 hours and then incubated with 180 mg/dL LDL for different times. After 3 washes with HEPES buffer containing 10 mg/mL heparin, cells were lysed, and the cellular cholesterol level was determined by enzymatic assay and normalized by protein concentration.13

**Assessment of Cholesterol Efflux**

We assessed the cholesterol efflux as described.20 In brief, cells in 6-well plates were transfected with pcDNA3.1-ABCA1 or vector for 24 hours and then labeled with [3H]cholesterol (0.2 μCi/mL) in medium for 24 hours. Cells were washed 3 times with phosphate-buffered saline containing 0.1% bovine serum albumin. Then, medium containing 2% FBS with or without apoA-I (10 μg/mL) was added, and the cells were incubated for various times as indicated. After incubation, the medium was centrifuged to remove any dissociated cells. The cells were washed and lysed in 1N NaOH. Aliquots of medium and cell lysates were assayed by liquid scintillation counting. The results represent radioactivity in the medium as a percentage of the total radioactivity (medium plus cell lysate).13

**Statistics**

Quantitative data were expressed as mean±SEM. Statistical significance of the data was evaluated by Student’s t test. Probability values <0.05 were considered significant. For nonquantitative data, results represent at least 3 independent experiments.

**Results**

**ABCA1 Expression and Regulation by LDL in ECs**

To study the expression and regulation of ABCA1 in ECs, we first detected the mRNA level of ABCA1 in early-passaged HUVECs. Confluent cells were exposed to LDL at a concentration of 180 mg/dL for varying times. Total RNA was collected for Northern blotting. As shown in Figure 1, HUVECs exposed to LDL for 6 hours showed increased ABCA1 mRNA levels, which reached a peak at 12 to 24 hours. To confirm this finding, we cultured HAECs to confluence, followed by LDL exposure for 24 hours. Results demonstrated that LDL at 180 mg/dL upregulated ABCA1 mRNA in HAECs as well. In HepG2 cells, the control, ABCA1 was expressed at a much higher level in the same amount of total RNA (15 μg per lane) but was not upregulated by a 24-hour exposure to LDL.
Furthermore, exposure of ECs to different concentrations of LDL caused an increase of ABCA1 mRNA in a dose-dependent fashion (Figure 2A). To ascertain the expression of ABCA1 at the protein level, we measured ABCA1 protein in HUVECs. After exposure to different concentrations of LDL, cellular membrane proteins were biotinylated, and ABCA1 was immunoprecipitated with its antiserum. Then the ABCA1 protein was visualized on a streptavidin–horseradish peroxidase ECL assay. As shown in Figure 2A, biotinylated ABCA1 in HUVECs was clearly detected and had been increased by exposure to LDL for 24 hours in a dose-dependent fashion, with incremental increases over the concentration range 120 to 180 mg/dL. Therefore, LDL at 180 mg/dL was chosen for the rest of the experiments. Thus, the data above demonstrate that ABCA1 was not only expressed but also upregulated by native LDL in ECs.

**Cholesterol Content in ABCA1-Transfected ECs**

We previously reported that incubation of ECs with LDL increased the cellular cholesterol content of the EC membrane. To assess the effect of ABCA1 on cholesterol content in ECs, we transfected plasmid of hABCA1 or vectors into HUVECs, and [3 H]cholesterol was loaded for 24 hours. Then, fresh medium with or without apoA-I was added, and cholesterol efflux was determined as described in Methods. As shown in Figure 4, the apoA-I did not significantly increase cholesterol efflux in vector-transfected cells at any detected time point, whereas ABCA1-transfected cells promoted 22% more cholesterol efflux than did control cells at 24 hours (P<0.05). The addition of apoA-I increased cholesterol efflux by less than 8% only in controls but increased it 30% to 45% in ABCA1-transfected cells (P<0.05).

**LDL Upregulates the ABCA1 Promoter in ECs**

To characterize further the effect of LDL on ABCA1 expression, we examined the upstream regulatory regions by transfecting HUVECs with ABCA1 promoter–driven reporter constructs. Figure 5A shows that a 24-hour LDL exposure increased the cholesterol content normalized to protein. All the data from treated samples are shown as multiples of basal control (vector, 0 h). Results are mean ± SD of 3 independent experiments, each performed in triplicate.
promoter activities of constructs pABCA1(–928)-Luc, pABCA1(156)-Luc, and pABCA1(–116)-Luc by 170% to 200% compared with controls. Further deletion of a segment located between −200% compared with controls. Further deletion of a segment located between −116 and −54 not only decreased the basal activity but also lacked LDL-responsive activities. Our results point to an LDL/cholesterol-responsive element in the ABCA1 promoter located between −116 and −54. To investigate further the role of cholesterol in LDL activation of the ABCA1 promoter, we loaded FC (20 μg/mL) into the cells in the promoter studies. Similar results were obtained from LDL and cholesterol loading (data not shown). The region between −116 and −54 bp contains a DR4 element located at the noncoding strand between −70 and −55 bp. Several groups reported that the DR4 site was the sterol-responsive element and that LXR and retinoid X receptor (RXR) heterodimers bound to this site. As shown in Figure 5A, the DR4 mutation pABCA1(–928 DR4 mut)-Luc greatly diminished LDL-induced ABCA1 promoter activities but did not affect the basal activity of the promoter.

**LDL Activates the LXRE-Driven Reporter in ECs**

To confirm further the effect of LDL on LXR activation, we cotransfected LXRE3 TK-Luc with CMX-hLXRα in HUVECs and determined the effect of LDL after exposure under low-serum conditions (2% fetal bovine serum) for 24 hours. Figure 5B shows that LXRE activity was slightly increased by LDL in the basic condition by ∼30%. However, when CMX-hLXRα was cotransfected, Luc activity was increased ∼4.5 times, and LDL further increased LXRE activity by 8 times. Thus, LDL upregulated ABCA1 via LXR activation in ECs.

**Ligands for LXR and RXR Activate ABCA1 in ECs**

LXR/RXR heterodimers have been reported to bind to the DR4 site to activate the ABCA1 promoter in macrophages. To study whether LXR/RXR heterodimers are involved in ABCA1 induction in ECs, we used 9-cis-retinoic acid, an RXR ligand, and 22(R)-hydroxycholesterol, an oxysterol LXR ligand. As shown in Figure 6A, 9-cis-retinoic acid or 22(R)-hydroxycholesterol treatment significantly increased ABCA1 mRNA by 24 hours. An additive effect was obtained with combined treatment with both 9-cis-retinoic acid and 22(R)-hydroxycholesterol. The concentration of 9-cis-retinoic acid was 10 μmol/L, as was used in macrophages. However, HUVECs were more sensitive to 22(R)-hydroxycholesterol than were RAW macrophages, because 22(R)-hydroxycholesterol at 10 μmol/L caused significant cytotoxicity under low-serum conditions (2% FBS). We reduced the 22(R)-hydroxycholesterol concentration to 5 μmol/L in all experiments.

To explore further the effect of these LXR/RXR ligands on ABCA1 promoter activation, we transfected pABCA1(–928)-Luc into ECs and then treated them with 9-cis-retinoic acid, 22(R)-hydroxycholesterol, or both for 24 hours. Figure 6B shows that 9-cis-retinoic acid or 22(R)-hydroxycholesterol treatment more than tripled ABCA1 promoter activity. Consistent with the results from Northern blotting, an additive effect was observed on ABCA1 promoter activation with combined treatment.

**Discussion**

Hypercholesterolemia is a well-known risk factor for the development of atherosclerosis. Both lipoproteins and the vascular endothelium play critical roles in the initiation and progression of atherosclerosis. ABCA1, a pivotal regulator of cholesterol efflux from cells to apolipoproteins, is expressed in many organs and cultured cell lines, as well as within atherosclerotic lesions. ABCA1 is upregulated by LDL and
The vascular endothelium, the lining and barrier of the vessel wall, plays an important role in maintaining cholesterol homeostasis. In the present study, we investigated ABCA1 expression, regulation, and role in cholesterol efflux in vascular ECs. Our findings demonstrate that (1) ABCA1 is expressed in human vascular ECs and elevated by native LDL; (2) the overexpression of full-length hABCA1 can lower cellular cholesterol content and increase apoA-I–mediated cholesterol efflux in ECs; (3) LDL increases ABCA1 promoter activity, and the RE can be mapped at the DR4 site within the promoter; and (4) LDL activates LXRE-driven Luc reporter activity. We conclude that ABCA1 is upregulated by LDL in ECs and plays an important role in cholesterol trafficking in ECs. We also confirmed earlier studies involving macrophages16 that the ligands for RXR and LXR activate ABCA1 in ECs. Such a finding may have broad implications for vessel wall homeostasis.

The current understanding is that ABCA1 functions as a cell membrane transporter that facilitates the transfer of phospholipids and cholesterol to poorly lipidated apolipoproteins at the exofacial leaflet of the bilayer. The defect in Tangier disease is that apolipoproteins or nascent, lipid-poor HDL particles fail to acquire a “mature” lipid content and are cleared from the circulation relatively quickly. Cleo and coworkers17 reported that ABCA1 heterozygotes show impaired cholesterol efflux and consequently, impaired reverse cholesterol transport, which is associated with reduced plasma HDL cholesterol levels and an increased risk of coronary artery disease. By in situ hybridization, ABCA1 RNA has been detected in fatty macrophages in early atherosclerotic lesions but not in normal nonatherosclerotic aortas.25 Results from the current study revealed that ABCA1 was upregulated by LDL and cholesterol in cultured vascular ECs. The efflux of lipids to the medium responded to cellular FC levels, ABCA1 expression, and the presence of apoA-I. Thus, ABCA1 enhances the net efflux of lipids not only from nonpolarized cells but also from polarized, distributed ECs on the vessel wall. ABCA1 may be involved in the physiological redistribution of lipids via transcytosis, delivering its lipid cargo from a luminal or basolateral surface.

ABCA1 expression and regulation by LDL in human vascular ECs has not previously been described. Our data demonstrate the presence of ABCA1 mRNA in HUVECs and HAECs and its protein in HUVECs. LDL upregulates ABCA1 in both cell types. Because cells have few LDL receptors, such as quiescent ECs cultured in the presence of serum, cholesterol trafficking appears to be controlled by the mechanism of its efflux.6 Cholesterol efflux responded to FC levels in cells. Thus, FC loading by LDL or other means, which increases cholesterol content in the cells, may cause an adaptive response to maintain cholesterol homeostasis. We have previously reported that elevated extracellular levels of LDL increase intracellular cholesterol content.13,21 Elevated levels of cellular cholesterol content may consequently upregulate ABCA1 expression, which in turn increases FC efflux in ECs. Furthermore, data showing that overexpression of ABCA1 lowered cholesterol content and FC efflux in HUVECs support this concept. Fielding’s group26 reported little ABCA1 mRNA in human ECs and no upregulation, by 30 μg/mL, of FC in these cells. Consequently, these cells had no efflux of phospholipid or FC in the presence of apoA-I. These disparities are likely due to culture conditions and the different cholesterol-loading methods, which may affect the abundance of ABCA1 and its response to extracellular cholesterol loading. We used HUVECs in early passages (passages 2 to 3), and ABCA1 mRNA was detected in the presence of 20% FBS. However, serum deprivation lowered cholesterol content and ABCA1 mRNA levels. These decreases were reversed by addition of 180 mg/dL LDL. When we performed cholesterol efflux assays under low-serum conditions (2% FBS), LDL preexposure increased FC efflux, but the increase in FC efflux to HDL was more than that to apoA-I (data not shown). Thus, other ABCA1-independent mechanisms, such as caveolae and caveolin-1, may be also involved in LDL-mediated intracellular cholesterol regulation. It is postulated that ABCA1 mediates cellular lipid efflux to lipid-poor apoA-I by increasing the direct binding of apoA-I to the cells,5,27 whereas the caveolae and caveolin seem to be involved in cholesterol efflux to lipidated HDL, but not to lipid-free apoA-I.28,29

Different regulatory mechanisms for the expression of ABCA1 have been described (see details in a recent review30). ABCA1 is transcriptionally regulated by LXRxR heterodimers via a DR4 site in the ABCA1 promoter region in macrophages.16,23 In ECs, the DR4 mutation, pABCA1(-928 DR4 mut)-Luc, greatly diminishes both LDL and cholesterol-induced ABCA1 promoter activities. LDL also activates the LXRE-driven reporter. Thus, LDL most likely regulates the ABCA1 promoter through the DR4 site. Results showing that LDL further increases LXRE activities in the cotransfection of hLXRα suggest that LDL provides ligands to LXR for activation.21 The oxysterols might be metabolized from cholesterol...
ABCA1 agonists that modulate cholesterol-mediated gene expression will facilitate the development of new pharmacological agents for treating low HDL and atherosclerosis.

Acknowledgments

This study was supported in part by National Institutes of Health grant HL43023 and the American Heart Association, Western States Affiliate, grant 98–252. We thank Drs A. Tall and S. Allen for providing the constructs and the HAECs in this study. We also thank Dr J. Shy for critically reading this manuscript and his valuable comments.

References


Native LDL Upregulation of ATP-Binding Cassette Transporter-1 in Human Vascular Endothelial Cells
Hailing Liao, Thomas Langmann, Gerd Schmitz and Yi Zhu

doi: 10.1161/hq1201.101772
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/22/1/127