ATP-Binding Cassette Transporter A1 Modulates Apolipoprotein A-I Transcytosis Through Aortic Endothelial Cells

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Abstract—High-density lipoproteins and their major protein constituent apolipoprotein A-I (apoA-I) possess diverse atheroprotective properties. Most of them must be exerted within the arterial wall. Actually, high-density lipoproteins are the most abundant lipoproteins within the arterial intima. We have recently reported that apoA-I is transcytosed through aortic endothelial cells. In the present study, we evaluate the role of ATP-binding cassette transporter A1 (ABCA1) and scavenger receptor BI (SR-BI) in this process. Using pharmacological interventions and RNA interference, we investigated whether ABCA1 and SR-BI modulate apoA-I binding, internalization and transcytosis in endothelial cells. Upregulation of ABCA1 with oxysterols increased apoA-I binding and internalization. Trapping ABCA1 on the cell surface with cyclosporin A enhanced apoA-I binding but decreased its internalization and transcytosis. In addition, apoA-I binding, internalization, and transcytosis were reduced by at least 50% after silencing ABCA1 but not after knocking down SR-BI. The integrity of the endothelial cell monolayer was affected neither by cyclosporin A treatment nor by ABCA1 silencing, as controlled by measuring inulin permeability. Finally, in ABCA1-GFP–expressing cells, fluorescently labeled apoA-I colocalized intracellularly with ABCA1-GFP. However, apoA-I–containing vesicles did not colocalize with the late endosome marker LAMP-1 (lysosome-associated membrane protein-1). In conclusion, ABCA1, but not SR-BI, modulates the transcytosis of apoA-I through endothelial cells. (Circ Res. 2006;99:1060-1066.)

Key Words: high-density lipoproteins ▪ endothelium ▪ apolipoprotein A-I ▪ transcytosis ▪ ABCA1

The plasma levels of both high-density lipoproteins (HDLs) and their major apolipoprotein, apoA-I, are inversely correlated with the risk of cardiovascular diseases. HDLs and apoA-I are exerting diverse functions that may help to protect from atherosclerosis. They reduce oxidative damage, correct endothelial dysfunction, inhibit inflammation, and mediate reverse cholesterol transport. At least some of these antiatherogenic actions of HDLs and apoA-I must be exerted within the arterial wall, rather than in the plasma compartment. Actually, HDLs are the most abundant lipoproteins in the extravascular space, presumably because of their small size and relative high molar plasma concentration.

Two principal mechanisms of transendothelial protein transport have been controversially discussed: porous transport and transcytosis. Porous transport refers to the passive and convective transport of proteins across large pores located either paracellularly or transcellularly. Transcytosis designates the shuttling of proteins in plasmalemmal vesicles across the endothelium. Recently, we have reported that endothelial cells bind, internalize, and transport apoA-I in a specific manner. In addition, within the first 30 minutes, approximately 50% of apoA-I that associates with the cells is transcytosed, which indicates that apoA-I transcytosis is a major process. Finally, apoA-I–specific transport was temperature dependent, and apoA-I was modified during transport. Thus, this phenomenological study provided the first evidence that apoA-I is transcytosed through endothelial cells. However, the receptor or transporter modulating apoA-I transcytosis has not yet been identified.

ATP-binding cassette transporter A1 (ABCA1) and scavenger receptor BI (SR-BI) are known apoA-I– or HDL-binding proteins. ABCA1 belongs to the ATP-binding cassette transporter family and mediates the efflux of phospholipids and cholesterol onto apoA-I. Its activity is rate limiting for HDL biogenesis in the liver and helps to maintain the cholesterol homeostasis of macrophages in the vascular wall. SR-BI belongs to the scavenger receptor family. In the liver, it mediates the selective uptake of chemokine esters from HDLs, via a mechanism that is still unclear. In addition, SR-BI contributes to the activation of endothelial nitric oxide synthase by HDLs.
The present study addresses the question of whether ABCA1 and SR-BI modulate apoA-I binding, internalization, and transport.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells (BAECs) were cultured in DMEM supplemented with 5% FCS at 37°C in a humidified 5% CO2, 95% air incubator.

Isolation and Labeling of ApoA-I

Lipid-free human apoA-I was isolated from plasma HDL and labeled with 125I using the Iodo-Beads iodination reagent (Pierce) as described previously.

Pharmacological Treatments

ABCA1 expression was stimulated by a mixture of 22-R-hydroxycholesterol (HC) and 9-cis-retinoic acid (RA) (Sigma) for 4 hours.

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Small Interfering RNA Transfection

BAECs were transfected when 80% to 90% confluent. BLOCK-it fluorescent oligo (67 nmol/L and 100 nmol/L Stealth small interfering RNA (siRNA) (Invitrogen) against either SR-BI (GTCAAGCAAGTTCAACTTGCCATT) or ABCA1 (GGGAGCTAGTG-GGGAGAACATCTTCTT) were transfected with Lipofectamine 2000 in OPTIMEM according to the protocol of the manufacturer. Six hours after transfection, the medium was replaced by DMEM 5% FCS. Binding, internalization, and transport assays were conducted 2 to 3 days after transfection. The efficiency of the silencing was evaluated by quantitative RT-PCR and Western blotting.

Quantitative RT-PCR

RNA was isolated with RNeasy mini (Qiagen) according to the protocol of the manufacturer. Reverse transcription was performed with Superscript II RT (Invitrogen) following the standard protocol. Quantitative PCR was performed with LightCycler FastStart DNA Master SYBR Green I (Roche). ABCA1 (GTCTTACATCATCTGCTTCC, CCTCACTATCATTCTCATCATT, 60°C, 5 mmol/L MgCl2) transcription levels were normalized to GAPDH (GTCTGATCCACTTGATGTCATC, 58°C, 5 mmol/L MgCl2) and SR-BI (GGAATCCCCATCTTCATCTGCTTCC, CCTCACATCTTCATCTTCATCATC, 60°C, 5 mmol/L MgCl2) transcription levels were normalized to GAPDH (GTCTTACATCATCTGCTTCC, CCTCACTATCATTCTCATCATT, 60°C, 5 mmol/L MgCl2) and SR-BI (GGAATCCCCATCTTCATCTGCTTCC, CCTCACATCTTCATCTTCATCATC, 60°C, 5 mmol/L MgCl2). Late endosomes were stained with LAMP-1 (lysosome-associated membrane protein-1) antibodies (ab19294, Abcam). Confocal microscopy was performed with a ×63 oil-immersion lens in the sequential mode.

Results

The aim of this study was to characterize the role of 2 well-known apoA-I– or HDL-binding proteins (ABCA1 and SR-BI) in apoA-I transcytosis. Their involvement in apoA-I binding, internalization, and transport was assessed.

ABCA1 and SR-BI Are Expressed in Endothelial Cells

The expression of ABCA1 and SR-BI in BAECs was evaluated by RT-PCR and Western blotting (Figure 1). Both apoA-I candidate receptors were found to be expressed in BAECs. Moreover, the effect of known modulators of ABCA1 was tested in these cells. First, we verified that the expression of ABCA1 is stimulated by a mixture of HC and RA (Figure 1A). Second, in cells treated with CsA, a broad-spectrum multidrug-resistance modulator, the cell sur-
face expression of ABCA1 was enhanced (Figure 1C). Third, expression of both ABCA1 and SR-BI was diminished by RNA interference (Figure 1B). Both candidate receptors were knocked down with an efficiency of more than 90% on the RNA level. The remaining protein expression of ABCA1 and SR-BI after silencing was approximately 50%, as assessed by Western blotting.

ABCA1, but Not SR-BI, Is Involved in ApoA-I Binding and Cell Association

The role of ABCA1 and SR-BI in apoA-I binding (4°C) and cell association (37°C) in BAECs was assessed after pharmacological treatments and siRNA-mediated silencing. First, apoA-I binding and cell association were increased by 50% and 35%, respectively, when the cells were stimulated with HC and RA (Figures 2A and 3A). Second, after 4 hours of treatment with CsA, apoA-I binding and cell association to BAECs were also augmented by 50% and 45%, respectively (Figures 2B and 3B), consistent with the previous finding that CsA traps ABCA1 on the cell surface. These 2 results indicated that an ABC transporter modulates apoA-I binding and cell association to endothelial cells. Third, reducing the expression of SR-BI by RNA interference affected neither apoA-I–specific binding (Figure 2C) nor apoA-I–specific cell association (Figure 3C). However, silencing ABCA1 reduced apoA-I binding by 40% (Figure 2C) and apoA-I cell association by 50% (Figure 3C). Therefore, we studied further only the implication of ABCA1 in apoA-I internalization and transcytosis in endothelial cells.

ABCA1 Plays a Role in ApoA-I Internalization

The contribution of ABCA1 to apoA-I internalization was further investigated using the treatments described above. ApoA-I uptake in cells stimulated with HC and RA was twice as high as in unstimulated cells (Figure 4A). In contrast, treating the cells with the ABC transporter inhibitor CsA reduced apoA-I internalization by 45% (Figure 4B). In addition, after reducing ABCA1 expression specifically by RNA interference, apoA-I uptake was lowered by approximately 60% (Figure 4C). Furthermore,
the colocalization of apoA-I with ABCA1 in endothelial cells was assessed after expressing an ABCA1-GFP fusion protein. ABCA1 was observed both on the cell surface and intracellularly. Fluorescently labeled apoA-I was found in intracellular vesicles that seemed to colocalize with ABCA1-GFP (Figure 5A). These results suggest that ABCA1 modulates not only apoA-I binding and cell association but also apoA-I internalization in BAECs. In fibroblasts, ABCA1 colocalizes with apoA-I in late endosomes.\textsuperscript{21} In endothelial cells, however, apoA-I was observed in vesicles that did not colocalize with the late endosome marker LAMP-1 (Figure 5B).

**ABCA1 Modulates ApoA-I Transcytosis**

Finally, we characterized the implication of ABCA1 in apoA-I transport through a monolayer of endothelial cells. In BAECs treated with CsA, apoA-I transport was reduced by a factor 2 as compared with untreated cells (Figure 6A). When ABCA1 expression was decreased by RNA interference, we observed an up to 70\% reduction in apoA-I transport (Figure 6B). In addition, inulin permeability was measured to evaluate the integrity of the endothelial cell monolayer after treatment with CsA and siRNA transfection (Figure 6C and 6D). Both interventions did not affect inulin permeability, demonstrating that the effects previously described are specific for apoA-I. Taken together, these results indicate that apoA-I transport is modulated by ABCA1.

**Discussion**

We have previously reported that vascular endothelial cells transcytose apoA-I in a specific manner. Here, we are providing strong evidence that ABCA1 but not SR-BI is modulating this process.

After confirming the expression of ABCA1 in endothelial cells,\textsuperscript{22–24} we assessed the role of ABCA1 in apoA-I binding and cell association, using pharmacological treatments and RNA interference. Initially, we verified that, as in macrophages,\textsuperscript{25,26} the expression of ABCA1 is upregulated in cells incubated with a mixture of HC and RA (Figure 1A). We also confirmed that ABCA1 is trapped on the cell surface of cells treated with CsA (Figure 1C), as previously reported for macrophages.\textsuperscript{20} After either treatment, apoA-I binding (Figure 2A and 2B) and apoA-I cell association (Figure 3A and 3B) were increased. Although these treatments are not specifically modulating ABCA1, they provided evidence that an ABC transporter is involved in apoA-I binding and cell association to endothelial cells. Furthermore, when the expression of ABCA1 was specifically reduced by RNA interference, both apoA-I binding (Figure 2C) and apoA-I cell association (Figure 3C) were lowered. Thus, our results and the literature agree that ABCA1 modulates apoA-I binding. Indeed, overexpression of ABCA1 in macrophages increases apoA-I binding to the cell surface.\textsuperscript{27} However, it is still controversially discussed whether ABCA1 binds directly apoA-I or whether it modifies the lipid distribution at the...
plasma membrane facilitating apoA-I docking. Hence, it is likewise unclear whether ABCA1 can be considered as a receptor for apoA-I in endothelial cells.

We further evaluated the involvement of ABCA1 in apoA-I internalization (Figure 4). Similarly to apoA-I binding and cell association, apoA-I internalization was increased after stimulating ABCA1 with HC and RA. On the contrary, CsA inhibited apoA-I uptake. When ABCA1 expression was reduced by RNA interference, apoA-I uptake was also diminished. Furthermore, in endothelial cells expressing the fusion protein ABCA1-GFP, apoA-I seemed to colocalize with ABCA1 intracellularly (Figure 5A). Thus, it seems that ABCA1 plays a critical role in apoA-I internalization, which is consistent with data obtained previously in macrophages and fibroblasts. In macrophages, CsA inhibits apoA-I uptake and resecretion by trapping ABCA1 on the cell surface. In fibroblasts, apoA-I colocalizes with ABCA1 intracellularly and was found in late endosomes. In endothelial cells, however, apoA-I–containing vesicles did not colocalize with the late endosomal marker LAMP-1 (Figure 5B). Similar results have been previously reported for the interaction of HDL with endothelial cells. Therefore, it seems that the fate of HDL and apoA-I in endothelial cells differs from their fate in fibroblasts (ie, lipid efflux). Interestingly, although both processes require a functional ABCA1, they do not involve the same intracellular compartment.

It seems that ABCA1 also critically modulates apoA-I transcytosis. Treatment with CsA and knock-down of ABCA1 expression specifically reduced apoA-I transport (Figure 6). ABCA1 might modulate apoA-I transcytosis, only because of its role in apoA-I binding and internalization. Consequently, the transporter would not regulate later events such as apoA-I intracellular trafficking or apoA-I secretion at the basolateral membrane. Nevertheless, a contribution of ABCA1 to apoA-I intracellular trafficking cannot be excluded and is even suggested by the finding that ABCA1 and apoA-I are colocalized intracellularly (Figure 5A). Moreover, when CsA was added into only the basolateral compartment, it inhibited apoA-I transport, although apoA-I cell association and, hence, ABCA1 availability on the apical site remained unchanged (data not shown). Thus, ABCA1 might modulate not only the uptake of apoA-I but also its intracellular trafficking and, possibly, its resecretion. Further research, however, will have to characterize the role of ABCA1 in these processes.

In addition, we found that SR-BI is expressed in endothelial cells, as previously published by others. Its expression could be diminished by RNA interference (Figure 1B). After reduction of SR-BI expression, apoA-I binding (4°C) and cell association (37°C) to endothelial cells were not changed (Figures 2C and 3C). On the contrary, HDL binding was reduced (data not shown). Therefore and because SR-BI has already been reported to bind lipidated apoA-I and HDL, rather than lipid-free apoA-I, we did not investigate further the implication of SR-BI in apoA-I transcytosis.

At first sight, our findings assign a novel function to ABCA1, which is known to mediate efflux of phospholipids and cholesterol onto apoA-I. However, it is important to recall that the mechanism by which ABCA1 mediates lipid efflux is not yet resolved. Most authors assume that ABCA1 acts on the cell surface and translocates phospholipids and cholesterol from the inner leaflet to the outer leaflet of the plasma membrane onto apoA-I. However, lipid efflux from macrophages onto apoA-I has also been suggested to involve retroendocytosis, where apoA-I is internalized, interacts with intracellular lipid pools, and is resecreted as lipidated particle. In human and murine macrophages that lack ABCA1, this process is defective. Furthermore, after transcytosis through endothelial cells, apoA-I seems to be lipidated.
ApoA-I transport

![Graphs showing ApoA-I transport](image)

**Figure 6.** Role of ABCA1 in apoA-I transcytosis. $^{125}$I-apoA-I (5 

Therefore, our finding that ABCA1 modulates apoA-I internalization and transport in endothelial cells may indirectly support the retroendoцитosis hypothesis of cholesterol efflux.

According to the current opinion, ABCA1 helps protecting against the development of atherosclerosis by 2 major mechanisms. It catalyzes a limiting step in HDL biogenesis in the liver, and it mediates cholesterol efflux from macrophages. Many of the antiatherogenic effects of apoA-I and HDL are to be executed within the vascular wall. Therefore, by modulating the transport of apoA-I through the endothelium into the arterial wall, ABCA1 may exert an additional atheroprotective activity. In this context, it is important to note that several mutations in ABCA1 were associated with cardiovascular risk independently of HDL cholesterol.

Porous transport and transcytosis are the 2 major mechanisms controversially discussed for the transendothelial transport of proteins. The identification of ABCA1 as a rate-limiting factor in apoA-I transport through endothelial cells supports the concept that proteins are transcytosed through the endothelium. However, future research will have to show the physiological and pathological relevance of the ABCA1-mediated transendothelial transport of apoA-I.

In summary, ABCA1, but not SR-BI, modulates apoA-I binding, internalization, and transcytosis through aortic endothelial cells. This study is the first molecular characterization of the transendothelial transport of apoA-I. More generally, the identification of ABCA1 as a modulator of apoA-I transcytosis provides evidence that transcytosis is a relevant mechanism for the transendothelial transport of proteins.

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**Disclosures**

None.

**References**


