The β-Chain of Cell Surface F₀F₁ ATPase Modulates ApoA-I and HDL Transcytosis Through Aortic Endothelial Cells

Clara Cavelier, Pascale M. Ohnsorg, Lucia Rohrer, Arnold von Eckardstein

Objective—Both HDLs and their major protein constituent apolipoprotein A-I (apoA-I) are transported through aortic endothelial cells. The knock-down of the ATP-binding cassette transporters A1 (ABCA1), G1 (ABCG1), and of the scavenger receptor-BI (SR-BI) diminishes but does not completely block the transport of apoA-I or HDL, so that other receptors appear to be involved. The ectopic β-chain of F₀F₁ ATPase has been previously characterized as an apoA-I receptor, triggering HDL internalization in hepatocytes.

Methods and Results—The ectopic presence of the β-chain of F₀F₁ ATPase on the surface of endothelial cells was confirmed by cell surface biotinylation. RNA-interference and the F₀F₁ ATPase inhibitory peptide IF1 reduced cell binding of apoA-I but not HDL, as well as association and transendothelial transport of both apoA-I and HDL. Furthermore, apoA-I stimulated F₀F₁ ATPase catalyzed ATP hydrolysis. The generated ADP as well as apoA-I stimulated the binding, cell association, and internalization of HDL. Both in the presence and absence of ADP inhibition of the purinergic receptor P₂Y₁₂ but not P₂Y₁ decreased the cell association of apoA-I and HDL. Coinhibition of β-ATPase and ABCA1 had no additive effects on the cell association and transport of apoA-I. Reduced cell association of HDL by β-ATPase inhibition was not further decreased by additional knock-down of ABCG1 or SR-BI.

Conclusion—Binding of apoA-I to ectopic F₀F₁ ATPase triggers the generation of ADP, which via activation of the purinergic receptor. P₂Y₁₂ stimulates the uptake and transport of HDL and initially lipid-free apoA-I by endothelial cells. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: apolipoproteins ■ endothelium ■ lipoproteins ■ F₀F₁ ATPase ■ transcytosis

Plasma levels of HDL cholesterol as well as apolipoprotein A-I (apoA-I) are inversely correlated with the risk of atherosclerosis. In addition, both apoA-I and HDL exert several atheroprotective properties within the arterial wall rather than in the blood stream, including cholesterol efflux from macrophage foam cells. HDLs are indeed the most abundant lipoproteins in the extravascular space. Recently, we provided evidence that endothelial cells bind, internalize, and transcytose apoA-I and HDL in a saturable and temperature-dependent manner. By siRNA interference we also showed that the ATP-binding cassette transporter (ABC) A1 modulates endothelial transport of apoA-I, whereas ABCG1 and the scavenger receptor BI (SR-BI) modulate the transport of HDL. In addition, we showed that the transendothelial apoA-I transport is a 2-step process in which apoA-I is initially lipidated by ABCA1 and then further processed by mechanisms that are independent of ABCA1 but involve ABCG1 and SR-BI.

Previously, the ectopically expressed β-chain of F₀F₁ ATPase (β-ATPase) has been identified as a hepatic receptor for apoA-I. F₀F₁ ATPase is an enzymatic complex responsible for the synthesis of ATP in mitochondria, prokaryote membranes, and chloroplasts. The mitochondrial F₀F₁ ATPase (about 600 kDa) is composed of 2 domains: an extramembranous catalytic domain (F₁) and a transmembrane domain (F₀) which functions as a proton channel. The mammalian F₀F₁ ATPase is composed of at least 16 different subunits—F₁: α, β, γ, δ, ε, + IF1, F0; a–g, F6, A6L, and oligomycin-sensitivity conferring protein. Unexpectedly, the F₀F₁ ATPase has been found on the cell surface of endothelial cells, adipocytes, hepatocytes, and tumor cells by immunofluorescence or after biotinylation of the cell surface; the F₁ domain facing outside. Although the mechanism leading to the ectopic expression is still unknown, F₀F₁ ATPase is not the only mitochondrial-matrix protein that was found at extramitochondrial sites and...
shown to be functional in its unusual location.\textsuperscript{18,19} Components of the F\textsubscript{0}F\textsubscript{1} ATPase, usually the $\beta$-chain, have been characterized as cell surface receptors for different ligands involved in tumor cell recognition and lysis, intracellular pH homeostasis, angiogenesis, and lipid metabolism.\textsuperscript{20–23} The $\beta$-chain belongs to the F\textsubscript{1} domain, which contains the binding sites for ATP and ADP as well as the catalytic site for ATP synthesis and hydrolysis.\textsuperscript{11} In hepatocytes, it has been characterized as an apoA-I receptor, which triggers HDL endocytosis.\textsuperscript{19} Indeed, on binding of apoA-I, F\textsubscript{0}F\textsubscript{1} ATPase hydrolyzes ATP. The ADP thereby produced stimulates hepatic HDL uptake by activating the purinergic receptor P2Y\textsubscript{13} under the control of the small GTPase RhoA and its effector ROCK I.\textsuperscript{24,25} P2Y receptors belong to the family of G-protein coupled receptors and are stimulated by extracellular nucleotides such as ATP, ADP, UTP, as well as UDP. In general they mediate downstream effects of the F\textsubscript{0}F\textsubscript{1} ATPase. In endothelial cells the most prominently expressed P2Y receptors are P2Y\textsubscript{1}, P2Y\textsubscript{2}, P2Y\textsubscript{11}, but also P2Y\textsubscript{4}, P2Y\textsubscript{6}, and P2Y\textsubscript{12}.\textsuperscript{26} The ADP-mediated effects in these cells are likely to be mediated by P2Y\textsubscript{1} and by P2Y\textsubscript{12}.\textsuperscript{27,28} In endothelial cells, angiostatin, an endogenous angiogenesis inhibitor, binds to and inhibits cell surface F\textsubscript{0}F\textsubscript{1} ATPase, thus modulating endothelial cell proliferation.\textsuperscript{14,29} Furthermore, it has been shown that apoA-I binds to the F\textsubscript{0}F\textsubscript{1} ATPase expressed on endothelial cells and stimulates proliferation of

\textbf{Figure 1.} Beta-ATPase is expressed on the cell surface of endothelial cells and modulates endothelial cell binding of apolipoprotein A-I (apoA-I).\textbf{A,} Cell surface expression of $\beta$-ATPase was assessed after biotinylation of cell surface proteins. Endothelial cells were transfected with specific $\beta$-ATPase siRNA. Beta-ATPase expression was assessed by RT-PCR (B) and western blotting (C). Effects of RNA interference with $\beta$-ATPase on intracellular ATP concentrations (D), apoA-I binding (E), and HDL binding (F). G, Effects of treatment with the $\beta$-ATPase inhibitor IF1 on the binding of apoA-I or HDL. Data are from quadruple experiments. *$P<0.05$; **$P<0.01$ (ANOVA).
endothelial cells again by promoting extracellular ADP production.\textsuperscript{30} Here we investigated whether cell surface F$_{1}$F$_{o}$ ATPase functions as an apoa-I and/or HDL binding protein on the surface of endothelial cells and thereby influences internalization and transendothelial transport of apoA-I and HDL. Furthermore, we investigated the involvement of P2Y receptors in these processes.

**Methods**

**Endothelial Binding, Association, Internalization, and Transport of ApoA-I and HDL**

The methods for the isolation and radiolabeling of HDL and apoA-I, the cultivation of bovine aortic endothelial cells as well as the quantification of endothelial binding, association, internalization, and transport of apoA-I and HDL have been described previously\textsuperscript{6–9} as well as in the Supplemental Method section, available online at http://atvb.ahajournals.org.

**Pharmacological Treatments and Inhibitors**

Endothelial cells were incubated with either the ATP hydrolysis inhibitor IF$_{1}$ (Abnova, Taipei, Taiwan) at a final concentration of 100 mmol/L in DMEM Hepes pH 6.4 containing 1% BSA or the pH-independent recombinant IF$_{1}$ variant H49K\textsuperscript{30} at a concentration of 1 μmol/L in DMEM Hepes containing 0.2% BSA, for 30 minutes prior to the assay. To test the involvement of specific P2Y receptors in stimulating endocytosis of HDL, 50 μmol/L of the P2Y$_{1}$ inhibitor MRS 2179 (M3808, Sigma, Switzerland) or 100 μmol/L of the P2Y$_{12}$ inhibitor 2-MeSAMP (M1434, Sigma, Switzerland) were added to the cell culture medium 20 minutes prior to the addition of $^{125}$I-HDL. Likewise 1 μmol/L ADP (Sigma, Switzerland) or 50 μg/ml apo-A-I were added to the cells 10 minutes before the addition of $^{125}$I-HDL. All modulators were kept in the medium during the assay.

**siRNA Transfection**

Endothelial cells were transfected when 80% to 90% confluent. Sixty-seven nmol/L BLOCK-iTTM fluorescent oligo and 100 nmol/L Stealth siRNA (Invitrogen) against β-ATPase (GCAGAATCTCCTCCGCTCTGGGTTA), SR-BI (TCTGATCAGCACCACATCTCTGGTCTT), ABCG1 (CGTCCTGCTCTTCTCCGGCTCTTGCTGTGGGTTA), ABCA1 (GGGACTTAGTGGGACGTGTCGAGAATCTCTT), SR-BI (TCTGATCAGCACCACATCTCTGGTCTT) or not coding siRNA were transfected with Lipofectamine 2000 in OPTIMEM according to the manufacturer’s protocol. Six hours after transfection, the medium was replaced by DMEM 5% FCS. Binding and cell association 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 manufacturer’s protocol. Reverse transcription was performed with Superscript II RT (Invitrogen) following the standard procedure. Quantitative PCR was done with LightCycler FastStart DNA Master SYBR Green I (Roche). The expression of P2Y$_{1}$ receptors was analyzed using the anti-P2Y$_{1}$ antibody (A-15) sc-15204 from Santa Cruz Biotechnology and the anti-P2Y$_{12}$ antibody (A-15) sc-15204 from Santa Cruz Biotechnology.

**Cell Surface Biotinylation**

Cell monolayers were biotinylated with 250 μg/mL EZ-link-sulfo-NHS-LC-biotin (Pierce) in PBS containing 0.1 mmol/L NaCl and 1 mmol/L MgCl$_{2}$ at 4°C for 1 hour. The reaction was terminated by 5 minutes incubation in DMEM. Cells were lysed in RIPA buffer (10 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, proteases inhibitor complete EDTA (Roche)). The lysates were incubated with 25 μL streptavidin-conjugated sepharose beads (Amersham Biosciences) at 4°C overnight. The beads were washed 3 times with the lysis buffer and the pulled-down proteins were resolved on a SDS-PAGE. Ten micrograms total proteins from the supernatant and 60 μg total proteins of the beads were loaded on the SDS-PAGE.

**Western Blotting**

Endothelial cells were lysed in RIPA buffer. Proteins were separated on a SDS-PAGE and transblotted onto a PVDF membrane (GE Healthcare). The expression of β-ATPase was analyzed by Western blotting using the anti-β-ATPase antibody MSS03 from MitoSciences (Eugene, OR) in comparison to the expression of actin (antiactin AC-15, Sigma) or GAPDH (anti-GAPDH ab9484, Abcam, Cambridge, MA). The expression of P2Y$_{1}$ receptors was evaluated by Western blotting using the anti-P2Y$_{1}$ antibody (A-15) sc-15204 from Santa Cruz Biotechnology and the anti-P2Y$_{12}$ antibody ab82725 from Abcam (Cambridge, MA).

**Determination of Extracellular and Intracellular ATP Concentrations**

Extracellular and intracellular ATP concentrations were measured by luciferase driven bioluminescence (ATP bioluminescence assay kit

![Figure 2. Inhibition of F$_{1}$F$_{o}$ ATPase decreases cell association (A, B) and transport (C, D) of apolipoprotein A-I (apoA-I) (A, C) and HDL (B, D). Cells were treated with IF1. Data show specific cell association or transport of apoA-I or HDL. Data are means and standard deviations from quadruple experiments. *P<0.05; **P<0.01 (ANOVA).](image-url)
Cells were rinsed twice in DMEM prior incubation in DMEM Hepes 1% BSA with or without 5 μg/mL apoA-I. After 30 minutes incubation, the assay medium was collected to measure the extracellular ATP concentration and the cells were lysed in the provided lysis buffer to determine intracellular ATP concentration. As positive control, intracellular ATP concentrations were measured after inhibition of the mitochondrial ATP synthesis with 5 μmol/L rotenone for 60 minutes.

**Statistical Analyses**

The data for all experiments were analyzed using GraphPad Prism 5 software program. Comparisons between groups were performed using t-test methods. Experiments were routinely performed in triplicates or quadruplets. Each experiment shown is a representative of at least 3 similar experiments. If not indicated otherwise the data are graphically represented as means ±SD, *P<0.05, **P<0.01, ***P<0.001, ns, not significantly different compared to control cells (ANOVA).

**Results**

**Cell Surface Expression of β-ATPase and Role of β-ATPase in ApoA-I Binding**

At first, the presence of the β-chain of the F₀F₁ ATPase (β-ATPase) on the surface of endothelial cells was verified by cell surface biotinylation and streptavidin pull-down (Figure 1A). Beta-ATPase could be pulled down using streptavidin beads, only if the cell surface was biotinylated. By contrast, the intracellular protein GAPDH was not captured by streptavidin, which indicates that the mitochondrial complex was not unspecifically biotinylated. Thus, we confirmed that the β-ATPase is expressed on the surface of endothelial cells. The role of β-ATPase as a binding partner for apoA-I on the surface of endothelial cells was assessed after reducing its expression by RNA interference. Thereby, β-ATPase expression was diminished by 90% on the RNA level and by about 50% on the protein level (Figure 1B and 1C). The energetic status of the cells after reducing the expression of β-ATPase was evaluated by measuring intracellular ATP concentrations. Extracellular ATP concentrations were measured after incubation with or without 5 μg/mL apoA-I. After stimulation with apoA-I or ADP, specific binding (B), cell association (C), and internalization (D) of HDL were analyzed. Data are means and standard deviations from triple experiments. *P<0.05; **P<0.01 (ANOVA).
β-ATPase and inhibits ATP hydrolysis. IF1 inhibited apoA-I binding by about 55% but had no effect on the binding of HDL (Figure 1G). These results indicate that cell surface β-ATPase modulates the binding of apoA-I but not HDL to endothelial cells.

Role of F0F1 ATPase in ApoA-I and HDL Cell Association and Transendothelial Transport
The ATPase inhibitor IF1 was used to evaluate the implication of F0F1 ATPase in cell association, internalization and transport of initially lipid-free apoA-I or HDL. In the presence of IF1, the cellular association (Figure 2A), internalization (data not shown), and transcellular transport (Figure 2B) of initially lipid-free apoA-I were diminished by about 30%, 60%, and 50%, respectively. Similar results were obtained by siRNA interference with β-ATPase, namely a 50% decrease in apoA-I internalization and an 80% decrease in apoA-I transport (Supplemental Figure 1, available online at http://atvb.ahajournals.org). Both the specific association and the transport of HDL transport were diminished by about 50% in the presence of IF1 (Figure 2B and 2D). Taken together, these results suggest that cell surface F0F1 ATPase modulates the cell association and transendothelial transport of both initially lipid-free apoA-I and HDL.

Activity of Cell Surface F0F1 ATPase
To assess the impact of apoA-I on the activity of cell surface F0F1 ATPase, extracellular ATP concentrations were measured in the presence and in the absence of apoA-I and after β-ATPase knock-down (Figure 3A). First, preincubation of nontransfected cells with apoA-I for 30 minutes reduced the extracellular ATP concentration by 50% indicating that apoA-I stimulated the extracellular hydrolysis of ATP. Moreover, in the absence of apoA-I, ATP accumulated extracellularly when β-ATPase was silenced, suggesting that F0F1 ATPase hydrolyzes ATP on the surface of endothelial cells. Finally, the extracellular ATP concentration was reduced only by about 10% in the presence of apoA-I after β-ATPase silencing. These data reveal that on the surface of endothelial cells, F0F1 ATPase hydrolyzes ATP in an apoA-I induced manner.

Effect of ApoA-I and Extracellular ADP on HDL Internalization
We next investigated whether the endocytosis of HDL depends on the ADP produced by F0F1 ATPase or on apoA-I itself. HDL binding was increased by about 50% in the presence of either 1 μmol/L ADP or 50 μg/mL apoA-I (Figure 3B). The cellular association of HDL was enhanced by about 30% and 50% in the presence of ADP and apoA-I, respectively (Figure 3C). The internalization of HDL was augmented by 35% and 25% by stimulation with ADP and apoA-I, respectively (Figure 3D). Also the internalization of initially lipid-free apoA-I was stimulated by ATP and ADP, namely by 20% and 50%, respectively (Supplemental Figure 2A, available online at http://atvb.ahajournals.org). In addition, 2MeS-ADP (ie, a nonhydrolysable form of ADP) but not ATP-S (ie, a nonhydrolysable form of ATP) stimulated apoA-I uptake by 60% (Supplemental Figure 2B and 2C). These data hence indicate that ADP generated by ATPase on stimulation through apoA-I stimulates the internalization of both initially lipid-free apoA-I and HDL by endothelial cells.

Role of P2Y Receptors in HDL Endocytosis
We next tried to identify the P2Y receptor through which ADP stimulates the endocytosis of HDL. First we confirmed the expression of the purinergic receptors P2Y1 and P2Y12 in bovine aortic endothelial cells both on the mRNA (Figure 4A) and protein level (Figure 4B). The cell association of HDL was reduced by about 40% in the presence of either P2Y12 inhibitor 2-MeSAMP and not at all in the presence of the P2Y1 inhibitor MRS 2179 (Figure 4C). Furthermore, the ADP-induced increase in HDL cell association was prevented by the P2Y12 inhibitor 2-MeSAMP, but not by the P2Y1 inhibitor MRS 2179 (Figure 4D). These data indicate that
ADP stimulates the endocytosis of HDL into endothelial cells by activation of the P2Y<sub>12</sub> receptor.

Interaction of β-ATPase With Other ApoA-I and HDL Endocytosis Modulators

Finally, we investigated possible interactions of the β-ATPase with ABCA1, ABCG1, or SR-BI, which were previously shown by us to modulate the transendothelial transport of apoA-I or HDL. By siRNA interference we also showed that ABCA1 modulates the transendothelial transport of apoA-I, whereas ABCG1 and SR-BI modulate the transport of HDL.

In addition, we showed that the transendothelial apoA-I transport is a 2-step process in which apoA-I is initially lipidated by ABCA1 and then further processed by ABCA1-independent mechanisms, most likely involving ABCG1 and SR-BI. In the present study, we provide strong biochemical evidence that apoA-I stimulates the transport of initially lipid-free apoA-I and HDL transport across endothelial cells by inducing ectopic cell surface F<sub>0</sub>F<sub>1</sub> ATPase to generate extracellular ADP, which then stimulates the endocytosis of HDL or lipidated apoA-I by activating the purinergic receptor P2Y<sub>12</sub> (Figure 6).

F<sub>0</sub>F<sub>1</sub> ATPase is the principal ATP synthesis complex in mitochondria. It consists of a catalytic domain F<sub>1</sub> and a transmembrane domain F<sub>0</sub>. The β-chain belongs to the F<sub>1</sub> domain and was shown by various laboratories to be ectopically expressed in the plasma membrane of various cell types including endothelial cells and hepatocytes. In hepatocytes, adipocytes, and endothelial cells, F<sub>0</sub>F<sub>1</sub> ATPase has been characterized as an apoA-I receptor that triggers the internalization of HDL, recycling of apoA-I, and antia apoptotic and proproliferative effects of HDL. We here confirmed that β-ATPase is expressed in the plasma membrane of endothelial cells by biotinylation of the cell surface and streptavidin pull-down (Figure 1A). Reducing the endothelial expression of β-ATPase by siRNA interference or inhibiting ATP hydrolysis by IF<sub>1</sub>, which binds the F<sub>1</sub> domain of F<sub>0</sub>F<sub>1</sub> ATPase, significantly diminished the 4°C binding of apoA-I (Figure 1E and 1G) but not HDL to endothelial cells (Figure 1F and 1G). These results suggest that F<sub>0</sub>F<sub>1</sub> ATPase binds lipid-free apoA-I but not mature HDL particles. In contrast, at 37°C the cell association and the transport of both apoA-I and HDL were diminished by the F<sub>0</sub>F<sub>1</sub> ATPase inhibitor IF<sub>1</sub> (Figure 2). These at first sight controversial findings between the binding and association experiments at 4°C and 37°C are

Discussion

We have previously demonstrated that endothelial cells bind, internalize, and transcytose apoA-I and HDL in a saturable and temperature-dependent manner. By siRNA interference we also showed that ABCA1 modulates the transendothelial transport of apoA-I, whereas ABCG1 and SR-BI modulate the transport of HDL. In addition, we showed that the transendothelial apoA-I transport is a 2-step process in which apoA-I is initially lipidated by ABCA1 and then further processed by ABCA1-independent mechanisms, most likely involving ABCG1 and SR-BI. In the present study, we provide strong biochemical evidence that apoA-I stimulates the transport of initially lipid-free apoA-I and HDL transport across endothelial cells by inducing ectopic cell surface F<sub>0</sub>F<sub>1</sub> ATPase to generate extracellular ADP, which then stimulates the endocytosis of HDL or lipidated apoA-I by activating the purinergic receptor P2Y<sub>12</sub> (Figure 6).
compatible with a model proposed by Martinez and colleagues who suggested that apoA-I binding to ectopic F0F1-ATPase triggers the internalization of HDL by a downstream receptor. In line with observations and conclusions of Howard and colleagues made in experiments with adipocytes, our findings suggest that in addition to mature HDL also lipid-free apoA-I can be internalized by this pathway. However, based on our previously reported findings that initially lipid-free apoA-I is lipidated by ABCA1 for subsequent ABCA1-independent internalization and transport, we assume that a nascent lipidated apoA-I particle rather than lipid-free apoA-I is the substrate for F0F1-ATPase triggered endocytosis. Also the nonadditive effects of ABCA1 interference and F0F1-ATPase inhibition on the endothelial association and transport of apoA-I (Figure 5A and 5B) are in agreement with this model.

Similarly to mitochondrial F0F1 ATPase, cell surface F0F1 ATPase has been shown to catalyze both ATP synthesis and ATP hydrolysis. In the present study, the activity of cell surface F0F1 ATPase was evaluated after F0F1-ATPase silencing, in the presence and in the absence of apoA-I (Figure 3A). Thereby we confirmed previous findings of Martinez and colleagues that binding of apoA-I to ectopic F0F1 ATPase of either hepatocytes or endothelial cells triggers the hydrolysis of ATP. This suggests that like in hepatocytes, the apoA-I induced hydrolysis of ATP triggers the uptake of HDL into endothelial cells. In agreement with this interpretation binding, cell association and internalization of HDL were increased in the presence of extracellular ADP and apoA-I (Figure 3B–3D). The internalization of initially lipid-free apoA-I was also increased in the presence of extracellular ATP and, even more, ADP (Supplemental Figure 2A). In addition, 2MeS-ADP (a nonhydrolysable form of ADP) but not ATP-γS (a nonhydrolysable form of ATP) were found to stimulate the uptake of initially lipid-free apoA-I into endothelial cells (Supplemental Figure 2B and 2C). However, as discussed before, we assume that during the incubation with cells, the initially lipid-free apoA-I was lipidated by ABCA1 so that we likely recorded the stimulated uptake of a nascent HDL particle rather than lipid-free apoA-I.

In hepatocytes, the G-protein coupled purinergic receptor P2Y13 has been identified as the immediate downstream target of dinucleotides that are generated by F0F1 ATPase on binding of apoA-I. By using specific agonists, Martinez and colleagues generated evidence that P2Y13 then signals to a further downstream endocytic HDL receptor that has not yet been identified. Furthermore and pointing to the physiological relevance of Martinez et al’s in vitro findings, P2Y13-deficient mice show a decrease in hepatic HDL cholesterol uptake, hepatic cholesterol content, and biliary cholesterol output, although their plasma HDL and other lipid levels were normal. Endothelial cells express at least 6 P2Y receptors that modulate vascular permeability. However neither is P2Y13 among them nor is any of them stimulated by the specific P2Y13 receptor agonist. Among the known endothelial P2Y receptors, only P2Y1 and P2Y12 are, like P2Y13, stimulated by ADP. Therefore, we tested the effects of the P2Y1 inhibitor MRS 2179 and the P2Y12 inhibitor 2-MeSAMP on the cell association of HDL. Two-MeSAMP but not MRS 2179 inhibited both unstimulated (Figure 4C) and ADP-stimulated association of HDL with endothelial cells (Figure 4D) indicating the involvement of P2Y12 rather than P2Y1 in the endothelial uptake of HDL. Interestingly, P2Y12 and P2Y13, which stimulate endothelial and hepatic endocytosis of HDL, respectively, share a high degree of sequence homology and couple both to members of the Gα

Figure 6. Model of transendothelial transport of apoA-I and HDL. By lipidating apolipoprotein A-I (apoA-I) ABCA1 generates a particle that is processed by a mechanism which, like the transport of mature HDL, involves ABCG1 and SR-BI. The F0F1 ATPase hydrolyzes ATP on binding of apoA-I. The produced ADP binds to P2Y12 and stimulates the internalization and transendothelial transport of lipidated apoA-I and HDL via the activation of G proteins.

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family of G proteins, whereas P2Y1 principally uses the G_{q/11} G protein subunit. In addition to the activation of the phosphatidylinositol 3-kinase via G_{i}, P2Y_{12} has also been shown to activate RhoA and Rho kinase leading to actin cytoskeleton reorganization. Future work will have to unravel the downstream signaling cascade of the endothelial P2Y_{12} receptor, which ultimately leads to endothelial uptake of HDL.

Previously we found that the endothelial binding, uptake, and transport of apoA-I and HDL are modulated by ABCA1, ABCG1, and SR-BI. Our novel findings raise the question whether they interact with ectopic β-ATPase. Our coinhibition experiments did not find any additive effects suggesting that ABCA1, ABCG1, SR-BI, and F_{0}F_{1} ATPase interact in a series of events rather than on independent parallel processes. For example, in the context of our previous findings, we suggest that ABCA1 generates a lipidated apoA-I particle as the substrate for an endocytic pathway, which is triggered via the β-ATPase-ADP-P2Y_{12}-axis. In this regard it is interesting to note that β-ATPase was previously shown to modulate the internalization and resecretion of apoA-I by adipocytes without affecting phospholipid and cholesterol efflux. The interaction of ABCG1, SR-BI, and the β-ATPase-ADP-P2Y_{12}-axis is even more elusive. Figure 6 summarizes our current model of transendothelial transport of apoA-I and HDL: By lipidating apoA-I, ABCA1 generates a particle that is then processed by an ABCA1-independent mechanism for transendothelial transport. Like the transport of mature HDL, the processing of these nascent HDL particles appears to involve ABCG1 and SR-BI. In parallel binding of apoA-I to cell surface F_{0}F_{1} ATPase stimulates the hydrolysis of ATP. The ADP thereby produced binds to the P2Y_{12} receptor of endothelial cells and stimulates the internalization of and hence transendothelial transport of nascent and mature HDL via activation of G proteins.

To conclude, the present study demonstrates that on the surface of endothelial cells the F_{0}F_{1} ATPase hydrolyzes ATP on binding of apoA-I. The ADP thereby produced stimulates apoA-I and HDL internalization and transendothelial transport of initially lipid-free apoA-I and HDL via activation of the P2Y_{12} receptor. Future research will have to characterize the thereby elicited signaling events as well as their downstream targets mediating the endothelial endocytosis of HDL.

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Disclosures
None.

References


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Supplement Material

Figures

**Supplemental figure I.** Role of β-ATPase in apoA-I internalisation and transport. ApoA-I internalisation (A) and apoA-I transport (B) through a monolayer of endothelial cells was evaluated after silencing β-ATPase with specific siRNA. Data are from triple experiments. *P < 0.05; ***P < 0.001 compared to control cells (ANOVA).
Supplemental figure II. Effect of extracellular nucleotides on apoA-I internalisation. ApoA-I internalisation was measured after preincubating endothelial cells for 10 minutes with 100 nM ADP and 100 nM ATP (A), 100 nM ATP-γS (B), and 100 nM 2MeS-ADP (C). The modulators were still present during the assay. Data are from triple experiments. **P < 0.01; ***P < 0.001; ns not significantly different compared to control cells (ANOVA).
Methods

Cell culture – Bovine aortic endothelial cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS) at 37 °C in a humidified 5% CO₂, 95% air incubator.

Isolation and labelling of apoA-I and HDL - Human HDL (1.063<d<1.21 kg/l) was isolated from fresh normolipidemic plasmas of blood donors by sequential ultracentrifugation. The purity of the lipoprotein preparation was verified by SDS polyacrylamide gel electrophoresis (SDS-PAGE) to exclude any contamination with low-density lipoproteins (LDL) or albumin. Lipid-free apoA-I was further purified from delipidated HDL as described previously. ApoA-I and HDL were labelled with ¹²⁵I using the Iodination Beads iodination reagent (Pierce) also as described.

¹²⁵I-apoA-I and ¹²⁵I-HDL binding and cell association – Binding and cell association assays with ¹²⁵I-apoA-I or ¹²⁵I-HDL were performed as described previously. Endothelial cells were incubated with 5 µg/ml of ¹²⁵I-apoA-I or with 10 µg/ml of ¹²⁵I-HDL without (total) or with (non-specific) a 40-fold excess of apoA-I or HDL, respectively, for 2 h at 4 °C (binding) or 30 min at 37 °C (cell association). Specific binding/cell association was calculated by subtracting the values of non-specific binding/cell association from those of total binding/cell association. All experiments were performed at least as triplicates.

¹²⁵I-HDL internalisation – The assay was performed as described for the cell association studies. After 30 min incubation with 10 µg/ml ¹²⁵I-HDL at 37°C, the cells were chilled on ice. The cell layer was washed twice with PBS. Then, the cell surface proteins were biotinylated on ice at 4 °C for 45 min using NHS-biotin (500 µg/ml) (Pierce) in PBS. Then, the cells were lysed in with RIPA buffer. A portion of the supernatant was separated for total protein and radioactivity determination, respectively, and the remaining incubated overnight with streptavidin-sepharose (Amersham Biosciences). The streptavidin beads with the bound cell surface proteins were removed from the supernatant that contained the not biotinylated and consequently internalized proteins. The radioactivity in the supernatant was measured in a Perkin Elmer γ-counter and normalized to the protein contents.
125I-apoA-I and 125I-HDL transport – Transport assays were performed as previously described. In brief, endothelial cells were seeded 2 days in advance on the upper side of porous filter inserts (0.4 µm, BD Biosciences) precoated with collagen type I (BD Biosciences). Medium containing either 5 µg/ml of 125I-apoA-I or 20 µg/ml of 125I-HDL was added to the apical compartment together with (non-specific transport) or in the absence (total transport) of a 40-fold excess of unlabeled apoA-I or HDL, respectively. After incubation for 30 min at 37 °C the media of the basolateral compartment were collected to measure radioactivity. Specific transport was calculated by subtracting the values of non-specific transport from those of total transport.

References