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Human Endothelial Cells of the Placental Barrier Efficiently Deliver Cholesterol to the Fetal Circulation via ABCA1 and ABCG1

Jasminka Stefulj,* Ute Panzenboeck,* Tatjana Becker, Birgit Hirschmugl, Cornelia Schweinzer, Ingrid Lang, Gunther Marsche, Anton Sadjak, Uwe Lang, Gernot Desoye,† Christian Wadsack†

Abstract—Although maternal–fetal cholesterol transfer may serve to compensate for insufficient fetal cholesterol biosynthesis under pathological conditions, it may have detrimental consequences under conditions of maternal hypercholesterolemia leading to preatherosclerotic lesion development in fetal aortas. Maternal cholesterol may enter fetal circulation by traversing syncytiotrophoblast and endothelial layers of the placenta. We hypothesized that endothelial cells (ECs) of the fetoplacental vasculature display a high and tightly regulated capacity for cholesterol release. Using ECs isolated from human term placenta (HPECs), we investigated cholesterol release capacity and examined transporters involved in cholesterol efflux pathways controlled by liver-X-receptors (LXRs). HPECs demonstrated 2.5-fold higher cholesterol release to lipid-free apolipoprotein (apo)A-I than human umbilical vein ECs (HUVECs), whereas both cell types showed similar cholesterol efflux to high-density lipoproteins (HDLs). Interestingly, treatment of HPECs with LXR activators increased cholesterol efflux to both types of acceptors, whereas no such response could be observed for HUVECs. In line with enhanced cholesterol efflux, LXR activation in HPECs increased expression of ATP-binding cassette transporters ABCA1 and ABCG1, while not altering expression of ABCG4 and scavenger receptor class B type I (SR-BI). Inhibition of ABCA1 or silencing of ABCG1 decreased cholesterol efflux to apoA-I (−70%) and HDL_3 (−57%), respectively. Immunohistochemistry localized both transporters predominantly to the apical membranes of placental ECs in situ. Thus, ECs of human term placenta exhibit unique, efficient and LXR-regulated cholesterol efflux mechanisms. We propose a sequential pathway mediated by ABCA1 and ABCG1, respectively, by which HPECs participate in forming mature HDL in the fetal blood. **(*Circ Res*. 2009;104:600-608.)**

Key Words: maternal–fetal cholesterol transfer ■ endothelial cells ■ HDL ■ liver X receptors

Cholesterol is indispensable during fetal development.[1] It has been long assumed that most, if not all, cholesterol required for fetal growth is synthesized de novo by the fetus itself, thus making it autonomous from maternal or placental cholesterol supply. However, several lines of evidence have cast doubt on this notion.[2,3] Fetuses that lack the ability to synthesize cholesterol, such as those with the Smith–Lemli–Opitz syndrome, are, nevertheless, born with low levels of tissue and plasma cholesterol, indicating that they have acquired maternal cholesterol in utero.[4] Recent exciting studies demonstrated a strong correlation between the size and number of atherosclerotic lesions in human fetal arteries with maternal cholesterol levels.[5,6] Moreover, maternal hypercholesterolemia also modified early predictors of cardiovascular disease in the offspring, thus corroborating the concept of developmental programming of adult disease in human.[7] Considering that progression of atherosclerosis in adults takes ages, these striking results support the assumption of a strong maternal impact on the comparatively short period of fetal development.

These results have sparked strong interest in delineating the mechanism of maternal-to-fetal cholesterol transfer, because identifying molecular targets might offer options for therapeutic intervention. Maternal cholesterol destined to enter the fetal circulation has to traverse the syncytiotrophoblast layer and the endothelium of the fetoplacental circulation. In vitro studies on human syncytiotrophoblast membranes and on human primary trophoblasts[8–10] demonstrated the presence and functional role of low-density lipoprotein (LDL) receptor and the high-density lipoprotein (HDL) receptor scavenger receptor class B type 1 (SR-BI) in cholesterol uptake and subsequent transport across the syncytiotro-

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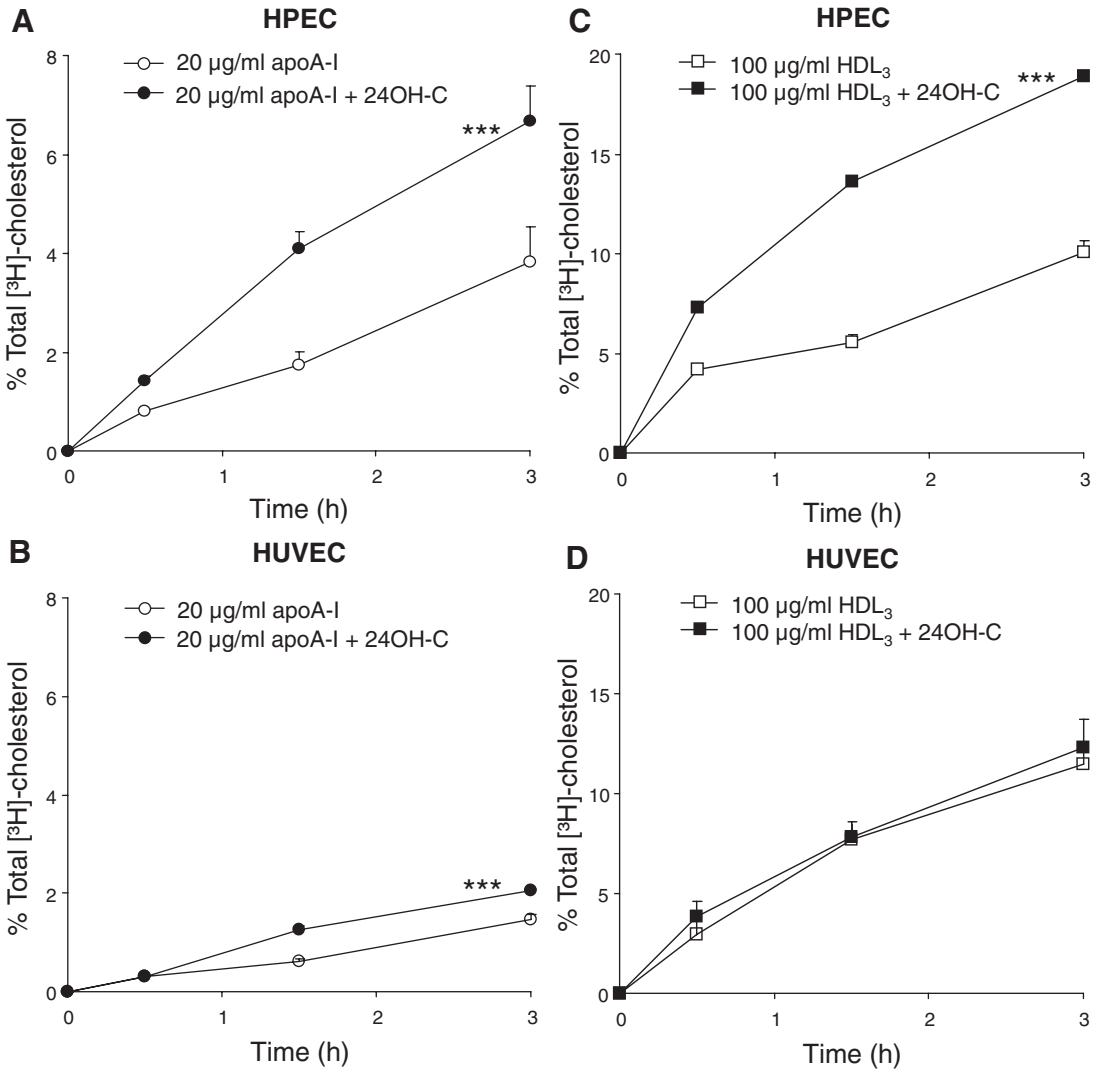

Figure 1. HDL_3- and apoA-I–mediated cholesterol efflux pathways are operative in HPECs and are induced by LXR activation. Basal and 24(S)OH-cholesterol–induced apoA-I–dependent (A and B) and HDL_3-dependent (C and D) cholesterol efflux was determined from [^{3}H]-cholesterol–labeled HPECs (A and C) and HUVECs (B and D) following a 16-hour equilibration period in medium containing 2% FBS in the absence or presence of 10 μmol/L 24(S)OH-cholesterol. Cholesterol efflux to the indicated concentrations of acceptors was measured in aliquots of media taken at the indicated time points and expressed as the radioactivity in the medium relative to total radioactivity in medium and cells (=100%). Cholesterol efflux to serum-free medium was subtracted. Data shown represent means±SEM from 2 experiments performed in triplicates and are representative of 4 independent experiments (different cell preparations). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs corresponding control.

phoblast layer. To date, no reports are available on the mechanisms of cholesterol transfer across the second layer, ie, ECs of fetal vessels in the placenta.

In general, ECs are resistant to excess cholesterol/lipid accumulation. In fact, they are the only cell type in the vascular wall not becoming foam cells during atherosclerosis. The biochemical pathways for sterol synthesis and endocytosis of lipoproteins, as well as for cholesterol efflux, all appear to be active in ECs.[11] However, unlike for macrophages,[12] the mechanisms responsible for the efflux of cellular cholesterol from ECs are still poorly characterized.

The majority of cholesterol in fetal blood is transported by HDL, which makes up to 44% of total lipoproteins in cord blood.[13] A major function of HDL is its role during reverse cholesterol transport, the protective pathway that prevents the excess accumulation of cholesterol.[14] This process involves different pathways of cholesterol efflux from peripheral cells, including transport mediated by specific membrane proteins. The ATP-binding cassette transporters ABCA1 and ABCG1 have been identified as primary gatekeepers for eliminating tissue cholesterol and for the biogenesis of HDL. ABCA1 stimulates cholesterol efflux to lipid-poor apolipoproteins, predominantly to apoA-I,[15,16] whereas ABCG1 promotes efflux of cholesterol and oxysterols to HDL.[17] SR-BI also promotes cholesterol efflux from peripheral cells to HDL but not to lipid-free apoA-I.[18]

Liver-X-receptors (LXRs) are key transcriptional regulators of cholesterol storage, transport, and catabolism. On activation by oxysterols, LXRs induce transcription of specific target genes that in concert regulate feed-forward pathways in HDL-mediated reverse cholesterol transport.[19] These

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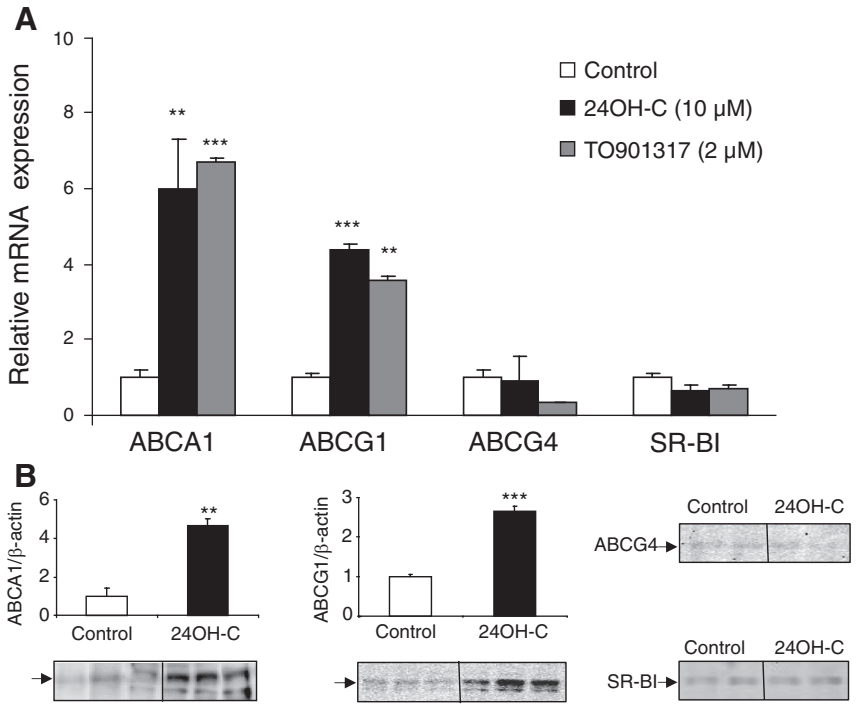

Figure 2. Effects of endogenous and synthetic LXR agonists on mRNA (A) and protein (B) expression levels of cellular cholesterol transporters. HPECs were incubated for 24 hours in the presence of 24(*S*)OH-cholesterol (10 μmol/L), TO901317 (2 μmol/L), or vehicle (EtOH, 0.01%). A, Total RNA was isolated and mRNA of ABCA1, ABCG1, ABCG4, and SR-BI was quantified as described in the expanded Materials and Methods section. Control values were set to 1. B, Immunoblotting and densitometric evaluation of ABCA1 and ABCG1 protein expression levels (normalized to levels of β-actin) was performed as described in the expanded Materials and Methods section. Bars represent means±SD (n=3) from 1 representative of 4 (A) or 2 (B) independent experiments (different cell preparations). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control.

include cellular cholesterol efflux pathways mediated by ABCA1,[20,21] ABCG1,[17] and ABCG4.[22]

The principal objective of our study was to identify cholesterol efflux mechanisms and the transporters involved in fetal ECs of human placenta (HPECs) at term of gestation to test the hypothesis that these cells have a strong and carefully regulated ability to efflux cellular cholesterol to different acceptors.

Materials and Methods

The procedures for isolation and culture of HPECs and HUVECs, cellular cholesterol efflux assays, protein extraction, Western blot analyses, RNA isolation, gene-specific mRNA quantification, small interfering (si)RNA-mediated cellular RNA interference (RNAi), immunohistochemistry, light microscopy, and statistical analyses are described in detail in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.

Results

High Capacity of HPECs to Efflux Cholesterol

Despite the existence of inconsistent reports on the prevailing mechanism(s) for cellular cholesterol export,[23] HDL-dependent reverse cholesterol transport appears to be the common major mechanism for the removal of cholesterol from ECs. Both, lipid-free and lipid-associated apoA-I promote different cholesterol efflux pathways in this process.

The first series of experiments was conducted to analyze the efficiency of cholesterol release from cultured HPECs and HUVECs to lipid-free apoA-I and to HDL_3 (Figure 1). To analyze effects of LXR activation, cholesterol efflux was studied using [^{3}H]-cholesterol–labeled ECs, preincubated in the absence or presence of the endogenous LXR agonist 24(*S*)OH-cholesterol for 16 hours before the addition of cholesterol acceptors to the culture media.

ApoA-I at saturating concentrations of 20 μg/mL[24] (Figure 1A) induced a time-dependent release of cellular [^{3}H]-cholesterol from HPECs, reaching 4±0.7% of total radiolabel detected in medium at 3 hours. 24(*S*)OH-Cholesterol treatment significantly enhanced apoA-I–dependent cholesterol efflux at all time points investigated by 1.7- up to 2.3-fold (1.5 hour). These studies suggest that the LXR-regulated lipid transporter ABCA1, which promotes lipid efflux to apoA-I (and other lipid-free apolipoproteins), is functional in HPECs. In contrast to HPECs, HUVECs displayed negligible (1.5±0.1% at 3 hours) apoA-I–dependent cholesterol release, which was only marginally (2.0±0.04% at 3 hours) inducible by 24(*S*)OH-cholesterol (Figure 1B).

HDL_3 (100 μg/mL), the main cholesterol acceptor in fetal blood, strongly induced [^{3}H]-cholesterol release from HPECs, reaching 10.0±0.6% of [^{3}H]-cholesterol in medium after 3 hours (Figure 1C). LXR activation by 24(*S*)OH-cholesterol significantly enhanced cholesterol efflux to HDL_3 by 1.9-fold (3 hours). Notably, the capacity of HUVECs for HDL_3-dependent cholesterol release in the absence of LXR agonist was almost identical to that observed for HPECs (11.5±1.0% and 10.0±0.6% of [^{3}H]-cholesterol was released to HDL_3 during 3 hours by HUVECs and HPECs, respectively), but in contrast to HPECs, this pathway in HUVECs was not responsive to 24(*S*)OH-cholesterol treatment (Figure 1D). This clearly indicates that LXR-regulated pathways are less important for cholesterol transport/homeostasis in HUVECs.

Similar results for HDL_3 mediated cholesterol efflux were obtained when a synthetic, nonsteroidal LXR agonist (TO901317, 2 μmol/L) was used instead of 24(S)OH-cholesterol (data not shown). These studies indicate that HPECs possess an efficient, LXR-regulated mechanism for efflux of cholesterol to HDL.

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LXR Activation Increases Expression of ABCA1 and ABCG1 in HPECs

Having demonstrated efficient and LXR-regulated cholesterol efflux from HPECs to both lipid-free apoA-I and lipid-associated HDL, we next analyzed expression of known cellular lipid transporters. Using real-time quantitative RT-PCR and immunoblotting, we examined effects of LXR activation on the expression levels of ABCA1, ABCG1, ABCG4, and SR-BI.

Exposure of HPECs to the endogenous agonist of LXRs [24(*S*)OH-cholesterol, 10 μmol/L] for 24 hours induced upregulation of ABCA1 and ABCG1 mRNA (6- and 4-fold, respectively; Figure 2A) and protein (5- and 3-fold, respectively; Figure 2B) levels. Similar results were obtained also with a synthetic, nonsteroidal LXR activator (TO901317, 2 μmol/L; Figure 2A). ABCA1 mRNA levels in HPECs were found to be approximately 3-fold higher than in HUVECs (Figure I, A, in the online data supplement), whereas, under basal conditions, ABCG1 mRNA levels in HPECs were approximately the same as in HUVECs (supplemental Figure I, B). As expected, expression of SR-BI, which is not a direct target gene of LXR, was not affected by LXR activation (Figure 2). Interestingly, LXR agonists did not influence expression of the proposed LXR target gene ABCG4, which was found to be present at negligible levels (Figure 2).

Inhibitors of ABCA1, but Not SR-BI, Decrease Cellular Cholesterol Release

To obtain more direct evidence for the functional relevance of ABCA1 in HPECs, we next analyzed apoA-I–dependent cholesterol efflux in the presence of 2 pharmacological inhibitors of ABCA1, glyburide[25] and probucol.[26]

Probucol and glyburide significantly diminished basal (by 72% and 75%, respectively) and LXR-activated (by 69% and 64%, respectively) apoA-I–dependent [^{3}H]-cholesterol release from HPECs (Figure 3A), demonstrating a major involvement of ABCA1 in this process. Neither of the 2 inhibitors showed any effect on HDL_3-mediated cholesterol efflux (data not shown).

Another chemical inhibitor, BLT-1,[27] was used to examine the role of SR-BI in HDL-dependent efflux of cholesterol from HPECs. HEK cells overexpressing SR-BI (HEK-SR-BI) served as a positive control in these experiments. In contrast to HEK-SR-BI cells, which displayed diminished (50%) cholesterol release to HDL_3 (50 μg/mL) in the presence of BLT-1 (10 μmol/L), HPECs did not respond to BLT-1 treatment (Figure 3B). These data clearly show that SR-BI does not contribute to HDL-mediated cholesterol release from HPECs.

Reduction of ABCG1 Expression by RNAi Decreases Cholesterol Release to HDL_3

Having ruled out a role for SR-BI, the most plausible candidate responsible for HDL-mediated cholesterol release from HPECs that remained was ABCG1. No specific inhibitor for ABCG1 is presently available. Therefore, to confirm our hypothesis, we aimed at reducing ABCG1 expression by applying RNAi before conducting cholesterol efflux assays. A strong significant reduction (76±5.4%) of ABCG1 mRNA

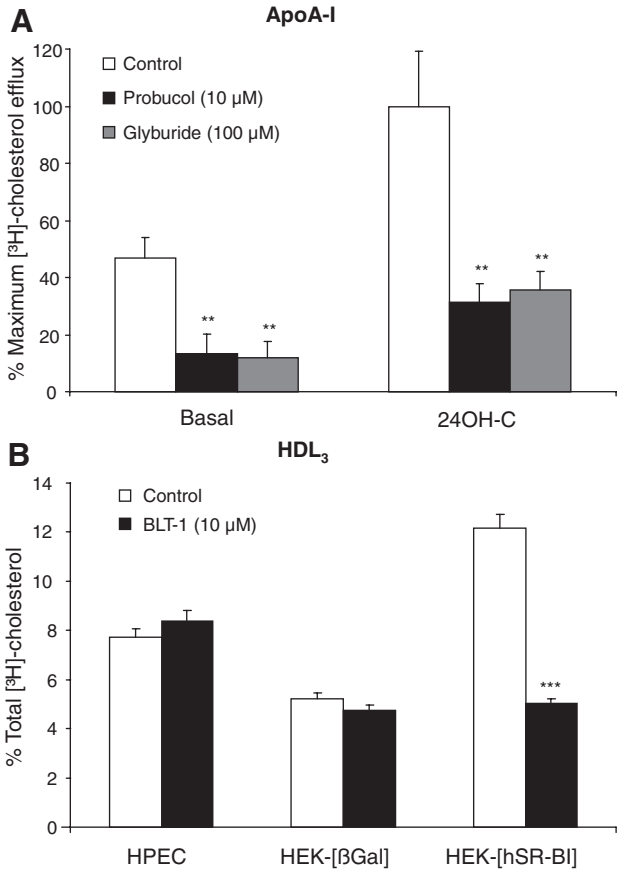

Figure 3. Inhibitors of ABCA1 (probucol, glyburide), but not of SR-BI (BLT-1), reduce cholesterol efflux from HPECs. A, [^{3}H]-Cholesterol–labeled HPECs were incubated for 16 hours in the absence or presence of 24(*S*)OH-cholesterol and/or probucol (10 μmol/L) in medium containing 2% FBS. Cells were then incubated with 20 μg/mL apoA-I in the absence or presence of glyburide (100 μmol/L). Cholesterol efflux was determined after 3 hours. Acceptor-independent cholesterol release was subtracted and data expressed as percentage of maximal cholesterol release [ie, in the presence of 24(*S*)OH-cholesterol, no inhibitor]. B, [^{3}H]-Cholesterol–labeled HPECs, HEK-[β-gal], and HEK-[hSR-BI] were incubated in the presence of HDL_3 (50 μg/mL) for 3 hours in the absence or presence of 10 μmol/L BLT-1. Means±SD (n=3) from 1 representative of 2 independent experiments (different isolations) are shown. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control.

by ABCG1-siRNA, as compared to scrambled siRNA (Figure 4A), resulted in a 49±2.0% to 56±2.2% reduction in time-dependent cholesterol release to 50 μg/ ml of HDL_3 (Figure 4B), in line with (≈45%) diminished ABCG1 protein levels (Figure 4B, inset). Neither SR-BI nor ABCA1 mRNA levels were significantly influenced by the RNA-silencing procedure (data not shown). We, therefore, conclude that HDL_3-mediated cholesterol efflux from HPECs is mainly mediated by ABCG1.

Cholesterol Enrichment of HPECs Increases Acceptor-Mediated Cholesterol Efflux

Cellular cholesterol enrichment with increasing concentrations of LDL or cholesterol (Figure 5C) enhanced apoA-I–mediated cholesterol efflux (Figure 5A) by up to 5.8±0.8-fold (100 μg/mL LDL, 3 hours) as compared to BSA-treated

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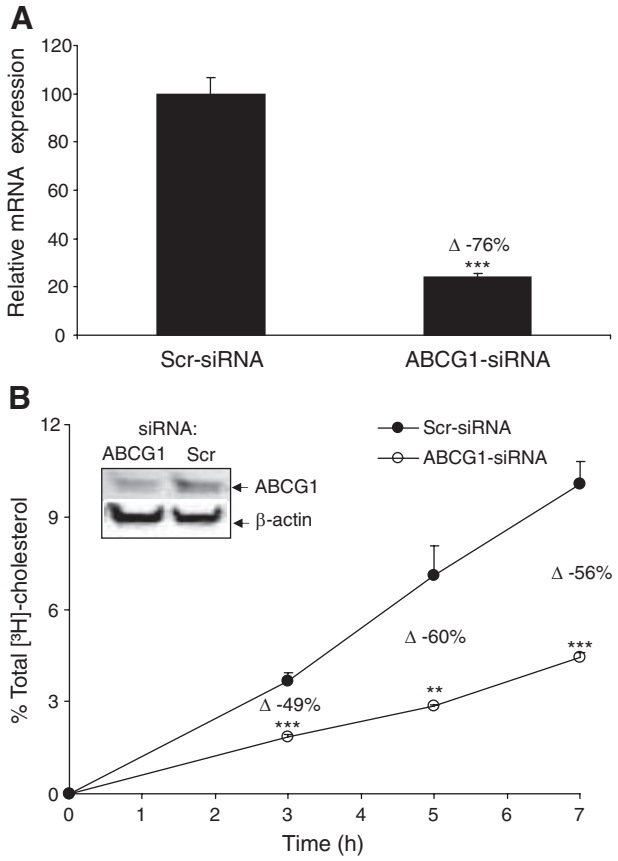

Figure 4. RNAi confirms the contribution of ABCG1 in HDL_3-mediated cholesterol efflux from HPECs. RNAi-mediated silencing of ABCG1 was performed as described in the expanded Materials and Methods section. A, mRNA expression of ABCG1 in HPECs transfected with scrambled (scr-siRNA) or ABCG1-siRNA was determined 30 hours posttransfection. B, Time-dependent HDL_3-mediated (50 μg/mL) cholesterol efflux was determined from scr-siRNA– and ABCG1-siRNA–transfected HPECs 40 hours posttransfection following a 2-hour equilibration period in serum-free medium. Release of [^{3}H]-cholesterol to serum-free medium was subtracted. The insert shows immunoblots of ABCG1 and β-actin, performed in parallel (43 hours posttransfection) on cell proteins isolated from separate wells. Means±SD from 1 typical experiment performed in triplicates are shown. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

(1 mg/mL) HPECs independent of external and cellular cholesterol concentration. In contrast to apoA-I, HDL_3 (Figure 5B) elicited a dose-dependent increase in cholesterol release from HPECs pretreated with LDL (up to 7.5±1.3-fold) or cholesterol (up to 9.9±0.3-fold). Despite the pronounced induction of cholesterol efflux, protein levels of ABCA1 remained unchanged on treatment with either LDL or cholesterol (Figure 5A). Similarly, ABCG1 expression (Figure 5B) remained unchanged on LDL treatment but was induced by cholesterol. These data clearly support a physiological role for acceptor mediated cholesterol release from HPECs. In addition, the induction of the cholesterol efflux activity of HPECs appears not to rely on increasing cellular protein levels of ABCA1 and/or ABCG1 but may involve other features of the mechanism(s) that are changed on cholesterol enrichment, possibly membrane translocation of the transporter(s) and/or increased accessibility of the cholesterol pool determined for efflux to apoA-I and/or HDL_3.

Enrichment of HDL_3 With ApoE Enhances Its Acceptor Capacity

ApoE is prominent in cord blood, and, in contrast to adults, more than 80% of it is associated with HDL.[13] ABCA1 is known to efflux cholesterol to different lipid-free apolipoproteins, including apoE,[28] whereas ABCG1 promotes cholesterol efflux only to lipid-associated particles, ie, HDL.[17] In view of these findings, we aimed to investigate the influence of apoE on cholesterol efflux from HPECs, using lipid-free apoE, HDL_3, and apoE-enriched HDL_3 as acceptor particles (Figure 6). ApoE (20 μg/mL) was similarly efficient as apoA-I in promoting [^{3}H]-cholesterol release from HPECs (Figures 6 and 1A, respectively). Interestingly, apoE-enriched HDL_3 elicited a moderate, but significant, increase in cholesterol release as compared to apoE-free HDL_3, to almost the same extent (1.3-fold) with (Figure 6) or without (data not shown) LXR activation. For all acceptors tested, LXR activation significantly enhanced cellular cholesterol efflux as compared to basal conditions (data not shown).

ABCA1 and ABCG1 Are Located at the Apical Surface of HPECs In Vivo

To define the location of ABCA1 and ABCG1 in HPECs in situ, immunohistochemical analyses of term placental cryosections were performed (Figure 7). Both ABCG1 (Figure 7A) and ABCA1 (Figure 7B) were detected predominantly at the apical surface of the fetal endothelium facing the fetal circulation, thus supporting our hypothesis of cholesterol efflux in the maternal-to-fetal direction.

Discussion

The significance of maternal cholesterol in developmental programming of atherosclerosis and the presence of severe developmental defects in offspring with inborn errors of cholesterol synthesis have prompted interest in delineating the distinct processes involved in cholesterol transfer across the placenta. Although the major mechanisms accounting for the uptake of maternal cholesterol into the placental syncytiotrophoblast have been identified, its subsequent release from the ECs has awaited investigation.

We have recently succeeded in culturing highly purified primary ECs isolated from the human placental vasculature at the end of gestation.[29] This enabled us to demonstrate differences in cholesterol efflux from ECs obtained from different sites within one organ, ie, ECs from the placental "barrier" (HPECs) and from the umbilical cord (HUVECs). The present study provides the first evidence for efficient cholesterol efflux at the end of gestation from human ECs of the placenta to lipid-free apolipoproteins and to HDL and identifies contributing mechanisms. Our key findings demonstrate that: (1) ABCA1 and ABCG1, respectively, accomplish these 2 pathways of acceptor-mediated cholesterol efflux; and (2) both pathways can be induced in HPECs by LXR activation using endogenous or synthetic agonists. In addition, our results exclude a major contribution of SR-BI and ABCG4 to HDL-mediated cholesterol efflux from HPECs. However, a contribution of additional, but still unknown, cholesterol efflux transporters cannot be ruled out. The absence of a significant contribution of ABCG4 concurs

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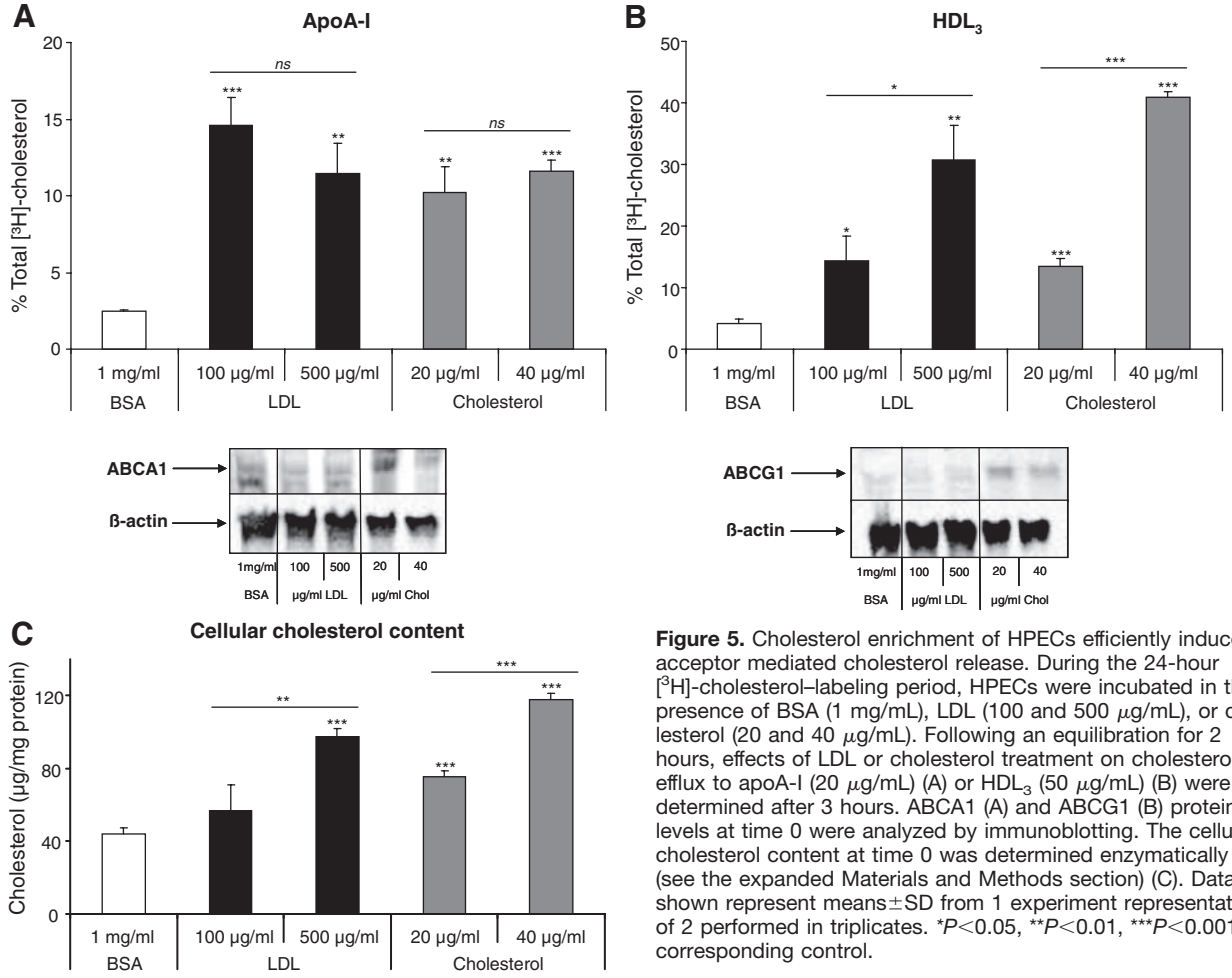

Figure 5. Cholesterol enrichment of HPECs efficiently induces acceptor mediated cholesterol release. During the 24-hour [^{3}H]-cholesterol–labeling period, HPECs were incubated in the presence of BSA (1 mg/mL), LDL (100 and 500 μg/mL), or cholesterol (20 and 40 μg/mL). Following an equilibration for 2 hours, effects of LDL or cholesterol treatment on cholesterol efflux to apoA-I (20 μg/mL) (A) or HDL_3 (50 μg/mL) (B) were determined after 3 hours. ABCA1 (A) and ABCG1 (B) protein levels at time 0 were analyzed by immunoblotting. The cellular cholesterol content at time 0 was determined enzymatically (see the expanded Materials and Methods section) (C). Data shown represent means±SD from 1 experiment representative of 2 performed in triplicates. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs corresponding control.

with the present notion that this transporter is predominantly active in the brain.[30]

Whereas efflux from HPECs to apo-AI and HDL_3 was stimulated by LXR agonists, the same effect was only moderately present or virtually absent in HUVECs. It may be hypothesized that the function of HPECs as a cellular constituent of the maternal–fetal cholesterol transport system requires careful regulation different from the less elaborate function of HUVECs as vascular lining. Stimulation of cholesterol efflux from HPECs via LXR activation may have important physiological, and potentially pharmacotherapeutic, implications. Both major natural LXR activators, 24S- and 27-OH-cholesterol, are present in the fetal circulation,[31] and their concentrations are elevated in inborn errors of cholesterol biosynthesis, such as Smith–Lemli–Opitz.[32,33] It is tempting to speculate that oxysterols are taken up by HPECs from the fetal and/or maternal circulation and stimulate transport of maternal cholesterol into the fetal circulation to compensate, at least in part, for the absence of endogenous fetal cholesterol biosynthesis.

Observations that placental ABCA1 expression is reduced in primary antiphospholipid syndrome, a pregnancy pathology characterized by recurrent pregnancy loss,[34] and that functional loss of ABCA1 in mice causes severe placental malformation[35] highlight the functional importance of ABCA1 in the placenta. Therefore, the expression of functional ABCA1 on HPECs at term of gestation, as observed in our study, might be fundamental for fetal development.

ABCA1 is the best-characterized transporter of active, ATP-dependent cellular cholesterol efflux to lipid-free or lipid-poor apolipoproteins.[15,16] We have demonstrated a major contribution of ABCA1 to cholesterol release from HPECs by: (1) basal expression of ABCA1 mRNA and protein, (2) induction of ABCA1 expression levels along with an increase of cholesterol efflux to apoA-I and apoE, and (3) decreasing effects of ABCA1 inhibitors on cholesterol efflux. Interestingly, our previous studies demonstrated that the apoA-I/ABCA1 pathway is also operative and inducible by LXR activation in porcine brain capillary ECs (pBCECs), which constitute the blood–brain barrier.[36,37] In contrast ECs from other vascular beds such as HUVECs (as confirmed the present study; Figure 1B) and/or human aortic ECs (HAECs) show only a poor ability to efflux cholesterol to lipid-free apoA-I.[23,38,39] Therefore, the apoA-I/ABCA1 pathway appears to be prominently active and regulated by LXR in ECs constituting blood–tissue and blood–blood barriers, ie, blood–brain and placental "barrier." These findings may reflect different functions of HUVECs/HAECs as compared to ECs from barriers. Despite differences in junctional connections, both fetal placental ECs and brain capillary ECs are

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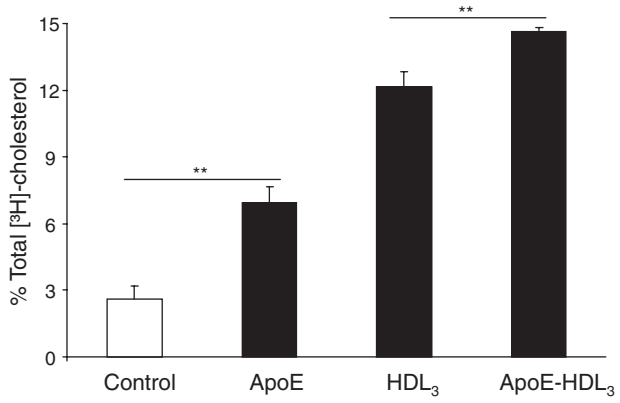

Figure 6. Cholesterol efflux to lipid-free apoE and apoE-enriched HDL_3. LXR-induced cholesterol efflux was determined from [^{3}H]-cholesterol–labeled HPECs following a 16-hour equilibration period in medium containing 2% FBS in the presence of 10 μmol/L 24(*S*)OH-cholesterol. Cholesterol release to medium containing 20 μg/mL apoE or 50 μg/mL protein of HDL_3 or apoE-enriched HDL_3 (apoE-HDL_3) was determined after 3 hours. Data shown represent means±SD (n=3) from 1 representative of 2 independent experiments (different cell preparations). $^{**}P<0.01$ vs corresponding apoE-free condition.

specialized ECs that, in addition to maintaining the integrity of blood vessels, restrict the passage of toxic and immunogenic factors while ensuring efficient solute and nutrient exchange.

In macrophages, ABCG1 promotes efflux of cholesterol and phospholipids onto a variety of lipoprotein particles, including HDL and phospholipids vesicles, presumably also onto nascent HDL particles previously formed via ABCA1 activity.[40] The molecular mechanisms of potential interactions of the respective acceptor particles with these transporters or with specific plasma membrane domains are still not clarified.[41,42] The present study is the first to demonstrate a prominent role of ABCG1 in HDL-mediated cholesterol release from ECs. This conclusion is based on (1) basal expression of ABCG1 mRNA and protein, (2) induction of ABCG1 expression levels along with an increase of cholesterol efflux to HDL, and (3) decreasing effect of ABCG1 silencing on cholesterol efflux to HDL. Interestingly, although expressed in HUVECs (see supplemental Figure I, B, and II, B) and HAECs,[39] ABCG1 does not seem to contribute to cholesterol efflux from these cells. Similarly, we did not find evidence for its role in cholesterol release from pBCECs.[36] The apparent different functions of ABCG1 in various types of ECs might be related to its different cellular localization or posttranscriptional activation and should be addressed in future studies.

The in vivo environment of HPECs is different from other ECs in the human adult and must be considered in any interpretation of our in vitro findings. At term of gestation, total and LDL cholesterol levels in cord blood are approximately one-third, whereas HDL cholesterol level is approximately half that of adult levels, resulting in a lower LDL to HDL ratio in fetal blood. ApoE is prominent in cord blood and more than 80% of it is associated with HDL.[13] It can be hypothesized that the unique lipoprotein composition of cord blood facilitates cholesterol transfer from the maternal to the fetal circulation. Lower levels or even loss of the key apolipoproteins in this process can have detrimental effects on fetal development.[43] We found that lipid-free apoE and apoA-I are comparably efficient in accepting cholesterol from HPECs, whereas, interestingly, apoE-enriched HDL_3 bound cholesterol more efficiently than HDL_3. This may reflect an increased capacity of ABCG1 to efflux cholesterol to apoE-containing HDL versus apoE-free HDL. The source of cord blood apolipoproteins is not clear at present. Although the fetal liver is a likely candidate, HPECs, unlike pBCECs,[36] do not express and secrete endogenous apoA-I or apoE (C Wadsack, C Schweinzer, U Panzenboeck, unpublished data, 2008).[44] It remains to be established whether apolipoproteins pass across HPECs by transcytosis similar to aortic ECs.[45]

In conclusion, ABCA1 and ABCG1 have a major role in mediating net cholesterol efflux from HPECs to apoA-I, apoE, and HDL (Figure 8). This facilitates maternal–fetal cholesterol transfer at the end of gestation and implies LXR activators/antagonists as potential drugs that could provide a future strategy to influence this process. In vivo the functional

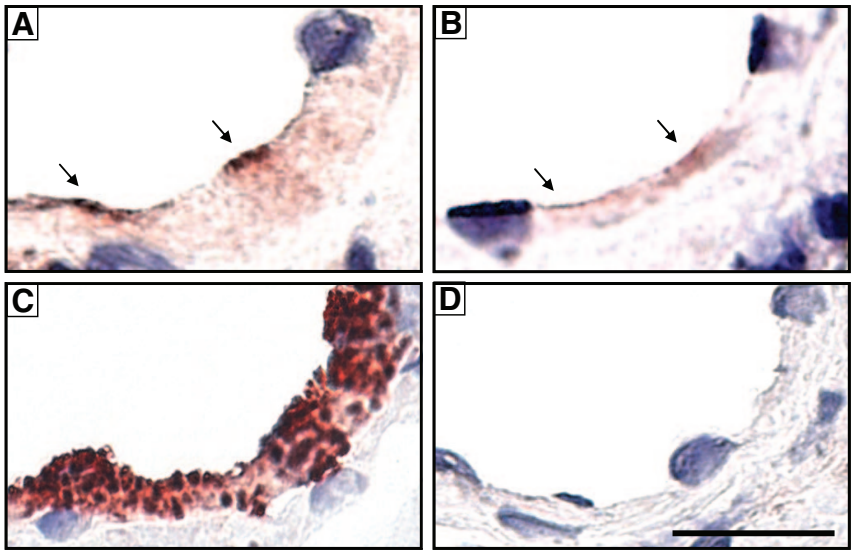

Figure 7. ABCA1 and ABCG1 are predominantly distributed to the apical face of HPECs. ABCG1 (A) and ABCA1 (B) were visualized (arrows) by immunohistochemistry in term placental cryosections as described in the expanded Materials and Methods section. The endothelial marker von Willebrand factor (vWf) showed the typical granular staining on placental ECs (C). D, IgG control. Bar=20 μm.

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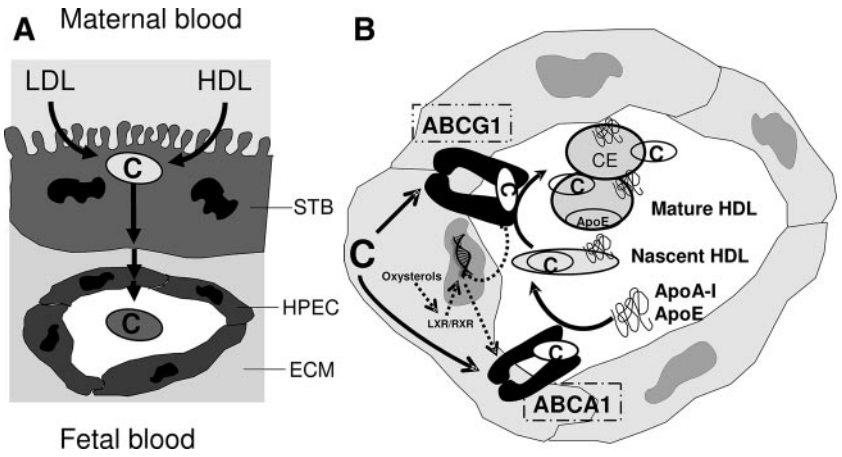

Figure 8. A, Sequential steps in transplacental transfer of lipoprotein-derived cholesterol at the end of gestation. Overall maternal–fetal transfer likely encompasses an "uptake/influx" component for maternal lipoproteins and/or their cholesterol into the syncytiotrophoblast (STB), transport of lipoprotein-derived cholesterol (C) to the basal side of the STB, subsequent release into the villous core for passage through the extracellular matrix (ECM), uptake into HPECs, and, finally, an efflux component by which cholesterol is released from HPECs into the fetal circulation. To date, only the uptake and degradation of LDL and HDL in cultured trophoblasts have been described.[8–10] However, other lipoproteins and their respective receptors have not been analyzed, and the subsequent intraplacental transport steps are still uncharacterized. B, Proposed pathway for cholesterol efflux from placental ECs into the fetal circulation at the end of gestation. The efflux of cholesterol (C) from HPECs into the fetal circulation involves an interactive role of ABCA1, ABCG1, and fetal apolipoproteins. ABCA1 promotes cholesterol efflux to lipid-free/poor apoA-I or apoE in the fetal circulation, thus initiating the formation of nascent, discoidal HDL, which will be further enriched with cholesterol by the cooperation of ABCA1 and ABCG1. ApoE-enriched HDL particles may facilitate cholesterol efflux via the ABCG1 pathway. Esterification of cholesterol by lecithin cholesterol acyl transferase will eventually result in the formation of mature spherical HDL. Oxysterols activate LXRs, which induce expression of ABCA1 and ABCG1, and, hence, will represent an effective mechanism regulating cholesterol efflux (dotted lines).

cooperation of ABCA1 and ABCG1 may allow a sequential loading of cholesterol first onto HDL apolipoproteins derived from fetal liver and, subsequently, onto nascent HDL.

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Disclosures

None.

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Supplement Material

Materials and Methods

Cell culture

Primary human placental endothelial cells (HPEC) were isolated from fetal vessels of human term placenta, as described elsewhere.[1] In brief, HPEC used in experiments were between passage 3 and 7. Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex (New Jersey). HPEC and HUVEC were maintained in Endothelial Cell Basal Medium (EBM; Lonza, Verviers, Belgium) supplemented with fetal bovine serum (FBS, 10%) and antibiotics (1%), and were passaged upon reaching confluency, typically every 5-6 days. Transient transfection of HEK-293 cells with human SR-BI (HEK-293[hSR-BI]) or β-gal (β-galactosidase; HEK-293[β-gal]) was achieved by adenoviral infection as previously described.[2]

Isolation of HDL, LDL and apoA-I

Human apoE-free HDL_3 and LDL was prepared by density gradient ultracentrifugation of plasma obtained from normolipidemic human volunteers (350,000 g; Beckman Instruments, Vienna, Austria).[3] PD10 size exclusion columns were from Amersham Biosciences. ApoA-I was isolated as described.[4] Enrichment of HDL_3 with recombinant human apoE was accomplished by incubating HDL_3 at a protein concentration of 7.5 mg/ml with 2.4 mg/ml apoE in phosphate buffer at 37°C for 1 h.[5]

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Cholesterol efflux

Efflux of cellular cholesterol was measured as described previously.[6] In brief, cells plated on 12-well plates were cholesterol loaded by incubation with 1 μCi/mL of [^{3}H]cholesterol (1.48–2.22 TBq/mmol; New England Nuclear) for 24 hours. Subsequently, cells were washed and cellular cholesterol pools were allowed to equilibrate during 16 hours of incubation in medium containing FBS (2%). Where indicated, 24*(S)*OH-cholesterol (Steraloids) and TO901317 (Cayman Chemical) were added during equilibration period, at 10 μM and 2 μM concentrations, respectively. Before starting efflux incubations, cells were washed twice with serum-free medium. Sterol acceptors were added in serum-free medium at the indicated concentrations. Aliquots of efflux media were collected at indicated time points and centrifuged (1000 g, 7 min) to remove cell debris. At the end of the experiments, cells were lysed in NaOH (0.3 M) and [^{3}H]-cholesterol radioactivity in efflux medium and cell lysates was measured by liquid scintillation counting. [^{3}H]-cholesterol efflux was expressed as the percentage of the radioactivity released from cells into the efflux media relative to the sum of total radioactivity in media plus cells. For cholesterol enrichment experiments, HPEC were incubated in the presence of 100 to 500 μg/ml of LDL or 20 to 40 μg/ml of cholesterol for 24 h. Cellular cholesterol content was determined by an enzymatic kit (Greiner Diagnostics AG, Langenthal, CH). For ABCA1 and SR-BI inhibition studies, probucol (10 μM; Sigma) was added at the beginning of the equilibration period,[7] while glyburide (100 μM; Sigma)[8] and BLT-1 (10 μM, ChemBridge) were added simultaneously with cholesterol acceptors.

Protein Extraction and Western Blot Analysis

Cells were harvested in lysis buffer containing detergents and protease inhibitors (1% Triton X-100, 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM Na_3VO_4, 40 mM NaF, 5 mM EGTA, 0.2% SDS, 0.5% deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride). Protein concentrations in

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cell lysates were determined using Bradford Reagent (Sigma). Proteins (15 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (150 V, 90 min) and electrophoretically transferred to Hybond-P PVDF membranes (Amersham Biosciences). After blocking with 5 % skim milk in PBST (PBS with 0.1 % Tween-20) for one hour at room temperature, membranes were incubated overnight at 4°C with rabbit polyclonal antibodies against human ABCA1 (1:500), ABCG1 (1:2000), ABCG4 (1:1000), SR-BI (1:1500) or β-actin (1:1000). Subsequent incubation with horseradish peroxidase (HRP)-labeled goat polyclonal antibody against rabbit IgG (1:4000) was carried out for 2 hours at room temperature. Antibody against ABCA1 was from Genosphere Biotechnologies, ABCG4 antibody was from Santa Cruz Biotechnology, and all other antibodies were from Abcam (Cambridge, UK). Immunocomplexes were visualized using enhanced chemiluminescence development (ECL Plus Western Blotting detection reagents, Amersham Biosciences). Images were captured and intensity of bands quantified using molecular imager (ChemiDoc XRS) and software (Quantity One 1-D) from Bio-Rad Laboratories.

RNA Isolation and Gene-Specific mRNA Quantification

Total cellular RNA was extracted from HPEC using RNeasy Mini Plus Kit (Qiagen). RNA integrity was checked by agarose gel electrophoresis and total RNA concentrations were measured spectrophotometrically (NanoDrop, Witec AG, Littau, Germany). cDNA was generated from uniform amounts of RNA using iScript cDNA Synthesis Kit (Bio-Rad). Samples prepared without reverse transcriptase served as negative controls in the subsequent quantitative real-time PCR (qPCR) performed on an iCycleriQ Multi-color Real-time PCR Detection System (Bio-Rad), using iQ SYBR Green Supermix (Bio-Rad) according to the manufacture's recommendations. Gene-specific primers were synthesised by Invitrogen; sequences are presented in Online Table I. PCRs were run in triplicates and relative mRNA levels

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were calculated using a standard curve method,[9] following normalization to TATA box binding protein (TBP) as internal control. Representative PCR products were subjected to agarose gel electrophoresis and sequence analysis to confirm primer specificity; melting curve analyses were routinely performed.

Small Interfering RNA (siRNA) Mediated Cellular RNA Interference (RNAi)

Complementary RNA (cRNA) oligonucleotides derived from human ABCG1 sequence (ON-TARGET*plus* SMARTpool targeting human ABCG1) were obtained from Dharmacon (Chicago, USA) and used to downregulate ABCG1 expression in primary HPEC. Scrambled control oligonucleotides (ON-TARGET*plus* si*CONTROL* Non-Targeting Pool) were also from Dharmacon. For cholesterol efflux assay, HPEC were transfected using PrimerFect siRNA Transfection Reagent (Lonza), according to the manufacture's recommendations. 14 hours after transfection, cellular cholesterol pools were radiolabeled by incubation with [^{3}H]-cholesterol (1 µCi/mL) for 24 hours. Subsequently, cholesterol pools were allowed to equilibrate during 2 hours and cholesterol efflux was measured essentially as described above. Expression levels of ABCG1 in transfected HPEC were determined by real-time PCR and immunobloting as described above.

Immunohistochemistry and light microscopy: indirect peroxidase method

Cryostat sections (5 µm) of term placental tissue areas were prepared, air dried for 2 h and stored frozen. Prior to each experiment, staining with antiserum was performed on acetone-fixed (5 min at room temperature) specimens. Tissue sections were rehydrated in PBS and immunolabelled for 30 min at room temperature, using polyclonal rabbit anti human antisera against ABCG1 (1:100, Novus Biologicals, Littleton), ABCA1 (1:100, Abcam, Cambridge,

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England), and von Willebrand factor antibody (vWf, 1:8000, Dako, Glostrup, Denmark) diluted in antibody diluent (Dako). After three washes in PBS the cells were incubated with a biotinylated polyvalent (mouse, rabbit) goat secondary antibody, followed by streptavidin-HRP (Ultravision kit, Labvision, Freemont, USA). The slides were washed again in PBS, and immunlabelling was visualized by a 5 min exposure to 3-amino 9-ethyl-carbazole. In control slides the primary antiserum was replaced by antibody diluent only or rabbit IgG (Dako) respectively. The slides were counterstained with Mayers-hemalum (Merck, Darmstadt, Germany). After washing in distilled water the slides were rinsed in tap water and mounted with Kaiser's glycerol gelatin (Merck).

Statistical analysis

Experiments were routinely performed in triplicates and data are presented as means ± S.D. One representative out of 2-4 independent experiments is shown, if not indicated otherwise. Two-tailed unpaired Student's *t* tests or two-way analysis of variance (ANOVA) were performed using Prism software (GraphPad). Significances were accepted at * $p<0.05$, ** $p<0.01$, *** $p<0.0001$

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Online Table I. Sequences of gene-specific primers (f. - forward, r. - reverse) used in real time PCR.[10,11]

Gene	Primers Sequence (5´-3´)	Source (ID)
ABCA1	f. GCACTGAGGAAGATGCTGAAA r. AGTTCCTGGAAGGTCTTGTTCAC	RTPrimerDB[1] (1139)
ABCG1	f. CAGGAAGATTAGACACTGTGG r. GAAAGGGGAATGGAGAGAAGA	RTPrimerDB[1] (1181)
ABCG4	f. ATCCTGGTTAATGGAAGGCCA r. TCAGGTTAGCAGAGACCATCAT	PrimerBank[2] (20143975a2)
SR-BI	f. TCTTCACACATTCTCACTCAGC r. CCTCCTTATCCTTTGAGCCCT	PrimerBank[2] (33620767a3)
TBP	f. TGCACAGGAGCCAAGAGTGAA r. CACATCACAGCTCCCCACCA	RTPrimerDB[1] (2630)

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Supplemental Figure legends

Online Figure I. Relative expression of mRNAs encoding ABCA1 (A) and ABCG1 (B) in human placental EC (HPEC) and human umbilical vein EC (HUVEC). Cells were incubated for 24 hours in the presence of vehicle (EtOH, 0.01 %), 24(S)OH-cholesterol (10 µM) or TO901317 (2 µM). Total RNA was isolated and mRNA expression of ABCA1 and ABCG1 (normalized to TATA box binding protein) quantified as described in Materials and Methods. Control values in HUVEC were set to 1. Bars represent means ± SD (n=4) from one representative out of 2 independent experiments (different cell preparations). * $p<0.05$, ** $p<0.01$ for HUVEC versus HPEC.

Online Figure II. Protein expression levels of ABCA1 (A) and ABCG1 (B) in human umbilical vein EC (HUVEC). Cells were incubated for 24 hours in the presence of vehicle (EtOH, 0.01 %; control), 24(S)OH-cholesterol (10 µM) or TO901317 (2 µM). Immunoblotting and densitometric evaluation of ABCA1 and ABCG1 protein expression levels (normalized to levels of ß-actin) was performed as described in the Materials and Methods section. Blots shown are representative of 2 separate experiments.

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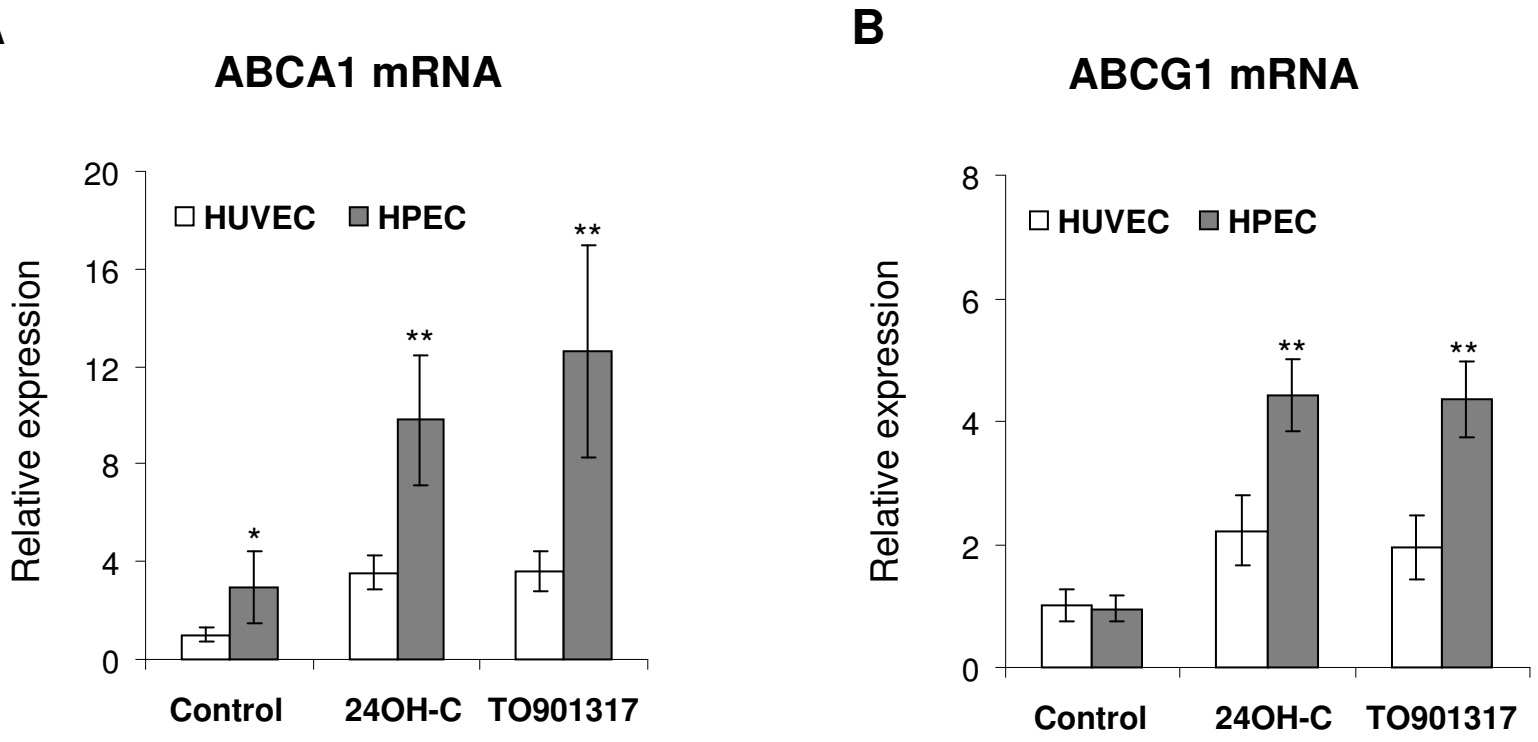
Online Figure I



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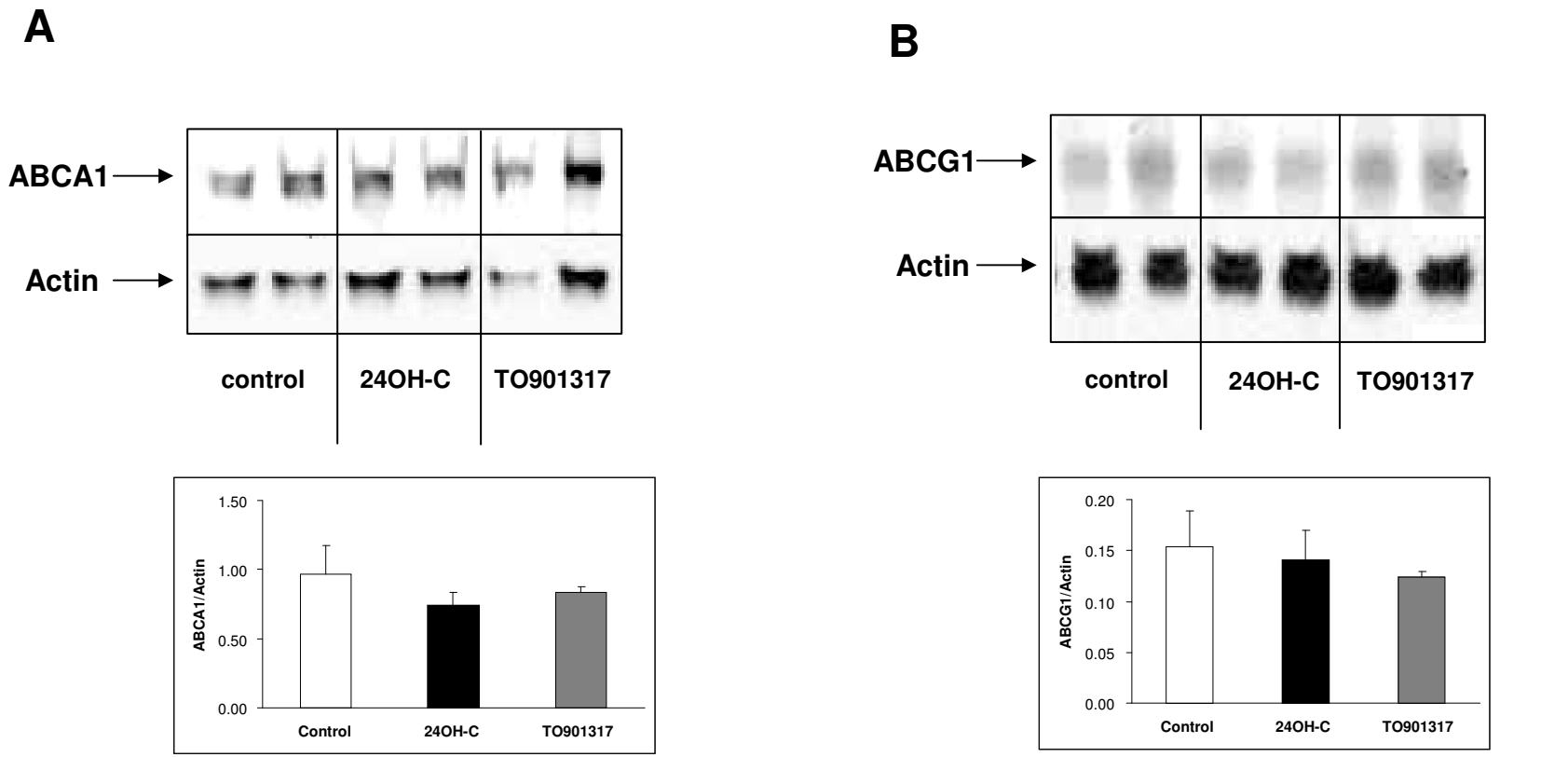
Online Figure II



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