LXR-induced reverse cholesterol transport in human airway smooth muscle is mediated exclusively by ABCA1

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Delvecchio CJ, Bilan P, Nair P, Capone JP. LXR-induced reverse cholesterol transport in human airway smooth muscle is mediated exclusively by ABCA1. Am J Physiol Lung Cell Mol Physiol 295: L949–L957, 2008. First published September 26, 2008; doi:10.1152/ajplung.90394.2008.—The association of hypercholesterolemia and obesity with airway hyperresponsiveness has drawn increasing attention to the potential role of cholesterol and lipid homeostasis in lung physiology and in chronic pulmonary diseases such as asthma. We have recently shown that activation of the nuclear hormone receptor liver X receptor (LXR) stimulates cholesterol efflux in human airway smooth muscle (hASM) cells and induces expression of the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, members of a family of proteins that mediate reverse cholesterol and phospholipid transport. We show here that ABCA1 is responsible for all LXR-mediated cholesterol and phospholipid efflux to both apolipoprotein AI and high-density lipoprotein acceptors. In contrast, ABCG1 does not appear to be required for this process. Moreover, we show that hASM cells respond to increased levels of cholesterol by inducing expression of ABCA1 and ABCG1 transporters, a process that is dependent on LXR expression. These findings establish a critical role for ABCA1 in reverse cholesterol and phospholipid transport in airway smooth muscle cells and suggest that dysregulation of cholesterol homeostasis in these cells may be important in the pathogenesis of diseases such as asthma.

Asthma is a multifactorial chronic inflammatory disease of the lung that is characterized by airway hyperresponsiveness (AHR). Disease progression involves bronchoconstriction, recruitment of leukocytes, airway remodeling, and increased smooth muscle mass (10). Many factors are proposed to contribute to the initiation and progression of asthma including genetic predisposition and environmental factors such as pollutants (9). The increased coincidence of obesity and asthma, as observed with recent longitudinal and cross-sectional studies (16, 42), further suggests that metabolic dysregulation may also contribute to AHR. As well, hypercholesterolemia, which is often observed in obese patients with asthma, has been associated with AHR in animal models of lung disease (1, 33, 47). Consistent with the latter, statin therapy has been shown to reduce pulmonary inflammation and proliferation of airway smooth muscle cells (39, 47). Further evidence to support a crucial role for lipid homeostasis in the lung come from animal studies of the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1. ABCA1 and ABCG1 mediate cholesterol and phospholipid efflux from peripheral cells to extracellular acceptors such as apolipoprotein AI (apoAI) and HDL. This transport back to the liver in a process termed “reverse cholesterol transport” (45). Severe pulmonary abnormalities are observed in both ABCA1 and ABCG1 knockout mouse models. Thus abca1−/− mice display massive lipid accumulation in alveolar macrophages, type II pneumocytes and lung parenchyma, abnormal lung morphology, and shallow breathing (6). Similarly, abcg1−/− mice display progressive lipid accumulation in alveolar macrophages and type II pneumocytes (5). Moreover, patients who suffer from pulmonary alveolar proteinosis (PAP) have reduced ABCG1 expression in alveolar macrophages (40). Interestingly, gm-csf−/− mice, which recapitulate disease phenotypes of PAP patients, show reduced ABCG1 expression in alveolar macrophages, implicating ABCG1 as well as the proinflammatory cytokine granulocyte/macrophage colony-stimulating factor (GM-CSF) as crucial regulatory components of lipid and surfactant metabolism in the lung (40). Taken together, the foregoing indicates that metabolic defects in lipid and cholesterol homeostasis may contribute to AHR and lung disease; however, the physiological relevance and mechanisms involved remain unknown.

Although ABC transport proteins have been highly characterized in the macrophage, their role in other lung cell types, such as human airway smooth muscle (hASM), that are crucial in diseases such as asthma, has not been investigated. hASM cells regulate bronchomotor tone and contribute to exaggerated bronchoconstriction (hyperresponsiveness) during an asthmatic attack and to increased smooth muscle mass in the asthmatic airway, which leads to airway constriction and eventual irreversible tissue remodeling (2). Airway smooth muscle cells also mediate immune modulation and inflammation in the airway by promoting the recruitment, activation, and migration of inflammatory cells through the expression of cytokines and inflammatory mediators (21). We have recently demonstrated that both ABCA1 and ABCG1 are highly expressed in hASM and that their expression is regulated by liver X receptor (LXR; subtypes -α and -β), a ligand-activated nuclear hormone receptor that governs cholesterol and fatty acid homeostasis by controlling the expression of key target genes that control cholesterol catabolism, storage, adsorption, and transport (13). Ligand activation of LXR also results in robust cholesterol efflux in airway smooth muscle cells, presumably through the actions of ABC transporters. Given the emerging importance of cholesterol and lipid metabolism in the lung, we sought to further characterize the function of ABCA1 and ABCG1 in hASM cells using small interfering RNA (siRNA) knockdown approaches and small molecule inhibitors of these transporters.
We show here that LXR ligand-induced reverse cholesterol and phospholipid transport to both apoAI and HDL is mediated exclusively by ABCA1, whereas ABCG1 appears to play no observable role in this process. Moreover, we show that cholesterol loading of hASM cells specifically increases the expression of ABCA1 and ABCG1 and that this induction is dependent on LXRα/β expression. Our findings indicate that cholesterol and lipid homeostasis are of importance in normal hASM function and suggest that dysregulation of these pathways may contribute to the pathogenesis of respiratory diseases such as asthma.

MATERIALS AND METHODS

Reagents. Human apoAI, TO901317 (T1317), GW3965, 9-cis retinoic acid (9-cisRA), IL-1β, and probucol were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). HDL purified by ultracentrifugation (d = 1.063–1.21 g/ml) was purchased from Biomedical Technologies (Stoughton, MA). [3H]cholesterol and [3H]choline were purchased from PerkinElmer (Boston, MA). Gene-specific siRNA oligonucleotides were purchased from QIAGEN (Chatsworth, CA). All other chemicals were purchased from Sigma-Aldrich unless stated otherwise.

hASM. hASM cells were obtained as described previously (29) from human lungs that were resected at St. Joseph’s Healthcare (Hamilton, Ontario, Canada) following approval from the Institutional Review Board and the consent of patients undergoing resection. Smooth muscle tissue was isolated from disease-free areas of the bronchi. Airway smooth muscle cells were grown in RPMI media supplemented with 10% FBS, 1% l-glutamine, and 1% penicillin-streptomycin. All experiments were done with cells at passage 5 or earlier. All experiments described herein include a 24- to 48-h serum deprivation step. As has been shown previously, serum removal results in the expression of contractile proteins and other differentiation markers and increases the number of caveolae, which is thought to represent a more physiological relevant state since cells are not normally exposed to high levels of serum (17). This step also synchronizes hASM cell cultures resulting in decreased heterogeneity and more uniform responses.

siRNA transfections. hASM cells were transfected with siRNA target-specific oligonucleotides using HiPerFect Transfection Reagent and reverse-transfection protocols according to manufacturer’s instructions (QIAGEN). Briefly, siRNA (19–285 ng) was incubated with 1.5 μl of HiPerFect reagent in 100 μl of serum-free media at a final concentration of 5–75 nM and incubated for 10 min at room temperature. During the incubation period, hASM cells were harvested and split into 48-well dishes (5 × 10^5 cells per well in a volume of 250 μl), and 100 μl of siRNA complex was added to each well. Cells were incubated for 24 h or longer (as indicated in the figure legends) and analyzed by real-time PCR as described below.

Real-time PCR. Total RNA was isolated from hASM cells using the RNeasy QIAGEN kit, and cDNA was prepared from 1 μg of RNA by reverse transcription using the QuantiTect Reverse Transcription kit (QIAGEN) according to the manufacturer’s instructions. Real-time PCR was performed using Platinum SYBR Green SuperMix-UDG with ROX PCR mix (Invitrogen, Burlington, Ontario, Canada). Briefly, 5 μl of SYBR Green SuperMix, 2.55 μl of H2O, 1.25 μl of primer sets, and 1.25 μl of cDNA was mixed with a final reaction volume of 10 μl, and PCR amplification was carried out in 384-well plates in an Applied Biosystems 7900HT real-time PCR machine (Applied Biosystems, Foster City, CA). Relative expression was determined using the 2^(-ΔΔCt) method (7) and by normalizing to β-actin expression levels. Primer sequences used were: hABC1A, forward 5’-CAGGTGAAAGCCTGGAACCT-3’; hABC1B, reverse 5’-GGCAAGAACAATCTGACA-3’; hABCG1, forward 5’-GATTACATTCCACACGACTTGC-3’; hABCG1, reverse 5’-TCTGCGCTATCTTCTC-3’; hβ-actin, forward 5’-CCTCTTCCAGCCT-3’; hβ-actin, reverse 5’-GGATGTCCAGTCACCCTTCTC-3’; hSR-BI, forward 5’-GTACAGGGAGTCTAGCACA-3’; hSR-BI, reverse 5’-GAAGCTGGAGTGGGTACT-3’.

Cholesterol efflux. Cholesterol efflux assays were carried out as previously described (13) with minor modifications. Briefly, hASM cells were split into 48-well dishes at a concentration of 5 × 10^5 cells per well and allowed to adhere overnight followed by incubation for 48 h in DMEM plus 10% FBS plus [3H]cholesterol (5 μCi/ml). Cells were then washed and incubated for an additional 18 h with equilibration medium (DMEM + 2% BSA) supplemented with T1317 (1 μM), 9-cisRA (1 μM), and probucol (10 μM) where indicated. Efflux was initiated by the addition of efflux medium (DMEM + 0.2% BSA) plus either vehicle, apoAI (50 μg/ml), or HDL (50 μg/ml) where indicated. Radioactivity was measured by scintillation counting, and cholesterol efflux was calculated by dividing the amount of [3H]cholesterol in the media by the total [3H]cholesterol associated with the cells plus media. For efflux experiments using cells that were transfected with siRNA, cell plating and transfections were done on the same day and the siRNA complex was incubated with the cells overnight. The following day, the media was removed and replaced with labeling media and incubated for 48 h as described above.

Western blot analysis. hASM cells were transfected with siRNA (50 nM) as indicated in the figure legends, and cholesterol efflux assays were carried out as described above. Total protein was then isolated using 1% Triton X-100 detergent, and Western blot analysis was carried out (25 μg of total protein for each sample) using a commercially available kit (Amersham Biosciences, Baie-d’Urfé, Québec, Canada) according the manufacturer’s instructions. Following transfer to nitrocellulose, blots were incubated with rabbit anti-ABCA1 (1:1,000; Novus Biologicals, Littleton, CO) or rabbit anti-ABCG1 polyclonal antibody (1:2,000; Novus Biologicals) for 1 h followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000; Amersham Biosciences) for an additional hour and visualized by enhanced chemiluminescence. Blots were probed with rabbit anti-β-actin as a loading control.

Cytoxicity assay. hASM cells (2.0 × 10^5 cells per well) were seeded into 96-well dishes and allowed to adhere overnight. Cells were then untreated or treated with probucol (10 μM) in the absence or presence of T1317/9-cisRA (1 μM each) for 18 h in 2% BSA-RPMI. Cells also received equivalent levels of vehicle (DMSO). Cell viability was measured using the Cell Counting Kit-8 according to manufacturer’s instructions (Dojindo, Rockville, MD). Colorimetric absorbance readings were performed at 450 nm on a SpectraMax Plus plate reader (Molecular Devices-Amersham Biosciences).

Phospholipid efflux. Phospholipid efflux assays were carried out as previously described (43). Briefly, hASM cells were split into 48-well dishes at a concentration of 5 × 10^5 cells per well and allowed to adhere overnight. The media was then replaced by DMEM plus 2% BSA plus [3H]choline (5 μCi/ml), and cells were incubated for 48 h. Cells were washed and incubated for an additional 18 h in DMEM plus 2% BSA supplemented with T1317 (1 μM) and 9-cisRA (1 μM) where indicated. Phospholipid efflux was initiated by the addition of efflux medium (DMEM + 0.2% BSA) plus either vehicle, apoAI (50 μg/ml), or HDL (50 μg/ml) where indicated in a total volume of 250 μl. Supernatants (200 μl) were collected after 5 h, and lipid was extracted using Folch mixture (2:1 chloroform-to-methanol ratio) and measured by scintillation counting. Lipids were extracted from cell monolayers using the hexane-to-isopropanol method as described (43). Phospholipid efflux was calculated as the amount of extracellular [3H]choline/total [3H]choline associated with the lipid fraction of the cells plus media.

Cholesterol loading and unloading with methyl-β-cyclodextrin. hASM cells were plated in six-well dishes and allowed to adhere overnight, transfected with the indicated siRNA as described above, and serum starved for 24 h in 0.2% BSA-RPMI. Cells were then incubated with methyl-β-cyclodextrin (MβCD; 5 μg/ml) or MβCD in...
complex with cholesterol (5 μg/ml; Sigma-Aldrich) in 0.2% BSA-RPMI for an additional 24 h where indicated in the figure legends. RNA was isolated and quantified by RT-PCR as described above.

Analysis of HDL and apoAI preparations by fast-performance liquid chromatography. Purified HDL and pure apoAI described above were separated by gel filtration chromatography using an AKTA FPLC with a Superose 6 HR 10/30 column and eluted into 80-μl fractions (35). Fractions were analyzed for total cholesterol using the Infinity Cholesterol Liquid Stable Reagent Kit according to manufacturer’s instructions (Thermo Electron, Pittsburgh, PA). HDL and free apoAI fractions were also analyzed for apoAI content by Western blot as described above. Briefly, 10 μl of each fraction was separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with goat anti-apoAI (1:10,000; Midland Bioproducts, Boone, IA) for 1 h followed by rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (1:10,000, Amersham Biosciences) for an additional hour and visualized by enhanced chemiluminescence.

Statistical analysis. Unpaired t-tests were used for comparison of groups. P < 0.05 was considered significant.

RESULTS

ABCA1, but not ABCG1, mediates LXR-stimulated cholesterol efflux to apoAI and HDL. We (13) have previously demonstrated that activation of endogenous LXR in hASM cells by various receptor agonists induces expression ABCA1 and ABCG1 at the mRNA and protein levels and stimulates cholesterol efflux. To determine whether reverse cholesterol transport in hASM cells requires ABCA1 and/or ABCG1 and, if so, to assess the contribution of each receptor in this process, we employed knockdown strategies using siRNAs to specifically reduce the expression of ABCA1 or ABCG1 and tested the effects on cholesterol efflux. As previously demonstrated, cholesterol efflux in airway smooth muscle cells, using apoAI as an acceptor, was increased twofold over control levels in the presence of the LXR ligand T1317 and 9-cisRA, a ligand for the LXR obligate heterodimerization partner retinoic X receptor (RXR) (Fig. 1A). Knockdown of ABCA1, but not ABCG1, blocked all LXR ligand-induced cholesterol efflux to apoAI acceptor. To determine the efficiency and specificity of knockdown of ABC transporters, we isolated RNA and protein from cells treated in parallel during the cholesterol efflux assays and analyzed expression levels by RT-PCR and Western blot analysis. As shown in Fig. 1, siRNAs targeted to ABCA1 or ABCG1 showed specific and efficient knockdown of each respective transporter at both the RNA (Fig. 1B) and protein levels (Fig. 1C). In each case, LXR-dependent induction over control levels was reduced 85–95%. These above findings establish that ABCA1 is necessary and sufficient for LXR-mediated cholesterol efflux to apoAI.

Previous reports have suggested that, in macrophages, ABCG1 is responsible for preferential cholesterol efflux to the lipid-rich acceptor HDL. To determine whether this is also the case in hASM cells, we repeated the above experiment with HDL in place of apoAI. As shown in Fig. 2A, LXR activation increased cholesterol efflux to HDL by twofold over control levels. Surprisingly, knockdown of ABCG1 did not alter the ability of hASM cells to efflux cholesterol to HDL (Fig. 2A). Thus, in contrast to what has been reported in the macrophages (18, 27, 41, 44), HDL does not serve as a cholesterol acceptor for ABCG1-mediated cholesterol efflux in hASM cells. To identify which transporters were responsible for cholesterol efflux to HDL, we investigated the role of the scavenger receptor B type I (SR-BI) and ABCA1, both of which have also been reported to efflux cholesterol to HDL in macrophages (13, 14, 28). Knockdown of SR-BI (>80%) had no effect on efflux to HDL acceptors (Fig. 2, B and C), whereas knockdown of ABCA1 blocked all LXR ligand-induced efflux to HDL (Fig.
To ensure that the observed efflux was not due to contaminating free apoAI in the HDL preparations, we fractionated HDL and pure apoAI protein preparations by fast-performance liquid chromatography (FPLC). ApoAI in the HDL preparations eluted in fractions rich in cholesterol and as a distinct and separate peak, with only minor overlap, compared with pure apoAI (data not shown). These findings indicate that the majority of the apoAI in the HDL preparations was associated with HDL particles and not in free form. Furthermore, the observation that the raw percentage of cholesterol efflux observed with HDL preparations was greater compared with free apoAI (compare Fig. 1A vs. Fig. 2A), despite using equivalent protein amounts (50 μg/ml), indicates that ABCA1-dependent cholesterol efflux to HDL was not a result of contaminating free apoAI in the HDL preparations. The finding that ABCA1 mediated efflux to HDL was further confirmed by using an independent siRNA construct to reduce expression of ABCA1 (Fig. 2D) and by undertaking dose-response and time course experiments. As shown in Fig. 3, increasing amounts of ABCA1 siRNA blocked LXR-stimulated efflux in a dose-dependent manner, an effect that was evident within 1–2 h (Fig. 4, A and B). To independently confirm that ABCA1 is involved in cholesterol efflux to both HDL and apoAI, cells were incubated with probucol, a small molecule that has been shown to specifically block ABCA1-dependent cholesterol efflux while having no effect on ABCG1 (15). As shown in Fig. 5A, probucol inhibited all LXR-mediated cholesterol efflux to both HDL and apoAI while having no effect on cell viability (Fig. 5B). The foregoing establishes that ABCA1 mediates all LXR ligand-induced cholesterol efflux in hASM cells to both apoAI and HDL acceptors and, moreover, that ABCG1 is not involved in this process.

Phospholipid efflux in hASM is mediated by ABCA1. Phospholipids constitute approximately 80–90% of the lipid found

Fig. 2. ABCA1 expression, but not ABCG1, is required for LXR-induced cholesterol efflux to HDL. A and B: hASM cells were transfected with the siRNA (50 nM) targeting ABCA1, ABCG1, or scavenger receptor B type I (SR-BI), as indicated, and subsequently labeled with [3H]cholesterol in the presence or absence of LXR/RXR ligands T1317 (1 μM) and 9-cis-RA (1 μM). Cells were then incubated in the presence of HDL (50 μg/ml) for 5 h, after which supernatants were collected, and extracellular cholesterol was measured by scintillation counting as described in MATERIALS AND METHODS. C: cells were transfected with the indicated siRNA and treated as in B, and total RNA was isolated, and SR-BI levels were quantified by real-time PCR. The data represent the average of triplicates (± SD) normalized using β-actin as an internal standard and taking untreated control luciferase siRNA as 1. D: cells were transfected with either luciferase siRNA (negative control) or 1 of 2 siRNA constructs targeting nonoverlapping sequences in ABCA1 mRNA as indicated, and cholesterol efflux was measured as above. The values are expressed as the percentage of cholesterol in the supernatants relative to total cholesterol associated with the cells plus supernatants and are the average of 3 experiments done in triplicate (± SD).
in surfactant and, moreover, play a crucial role in lipid-mediated signal transduction pathways in airway smooth muscle cells (37, 40). Since ABCA1 and ABCG1 knockout mouse models each display significant accumulation in phospholipid levels in the lung (5, 6), we sought next to determine which transporter regulated phospholipid efflux in airway smooth muscle cells. ABCG1 has been previously reported to promote phospholipid efflux, specifically sphingomyelin and phosphatidylcholine, to apoAI when overexpressed in human embryonic kidney (HEK-293) cells, although others have shown no change in type II pneumocytes (5, 20). Additionally, ABCG1 is proposed to be the major phospholipid transporter in alveolar macrophages since elevated levels of ABCA1 cannot compensate for the loss of ABCG1 in alveolar macrophages isolated from abcg1−/− mice (40). To determine whether these pathways are conserved and to possibly assign a function for ABCG1 in hASM cells, we knocked down ABCA1 or ABCG1 and measured phospholipid efflux following incubation of cells with [3H]choline. As shown in Fig. 6, A and B, LXR agonists increased phospholipid efflux to apoAI and HDL by twofold. LXR-dependent efflux was inhibited in the absence of ABCA1 but not in the absence of ABCG1. These findings indicate that ABCA1, but not ABCG1, regulates phospholipid efflux in hASM cells.

**DISCUSSION**

A correlation between high cholesterol levels and AHR has raised many questions in regard to the molecular mechanisms mediating this response and the physiological relevance. Our recent findings that LXR agonists reciprocally regulate the expression of multiple inflammatory mediators in airway

![Fig. 4. Effects of ABCA1 knockdown on kinetics of cholesterol efflux in hASM cells. HASM cells were transfected with the indicated concentration of ABCA1 siRNA and subsequently labeled with [3H]cholesterol for 48 h. Cells were then treated with T1317 (1 μM) and 9-cisRA (1 μM) for an additional 18–24 h in 2% BSA. Cholesterol efflux was initiated by the addition of HDL (50 μg/ml; A) or apoAI (50 μg/ml; B) in 0.2% BSA, and extracellular cholesterol was measured by scintillation counting as described in MATERIALS AND METHODS. The values are expressed as the percentage of cholesterol in supernatants relative to total cholesterol associated with the cells plus supernatants. The data are representative of at least 2 experiments done in triplicate (± SD). *P < 0.05 vs. control indicated by †.

![Fig. 3. LXR-induced cholesterol efflux to HDL correlates with ABCA1 expression in hASM cells. HASM cells were transfected with the indicated concentration of ABCA1 siRNA and subsequently labeled with [3H]cholesterol for 48 h. Cells were then treated with T1317 (1 μM) and 9-cisRA (1 μM) for an additional 18–24 h in 2% BSA. Cholesterol efflux was initiated by the addition of HDL (50 μg/ml) in 0.2% BSA, and, following incubation for 5 h, supernatants were collected, and extracellular cholesterol was measured by scintillation counting as described in MATERIALS AND METHODS. The values are expressed as the percentage of cholesterol in supernatants relative to total cholesterol associated with the cells plus supernatants. The data are representative of at least 2 experiments done in triplicate (± SD).

**ABCA1 and ABCG1 expression is elevated on cholesterol loading.** Cells sense cholesterol and respond in part by inducing expression of ABCA1 and G1 transporters (23). To determine whether this is the case in hASM cells, cells were treated with cholesterol in complex with MβCD to load cells with cholesterol or with MβCD alone to deplete cells of cholesterol. As shown in Table 1, ABCA1 and ABCG1 expression levels were induced 11-fold and 18-fold, respectively, by cholesterol loading, whereas expression levels were decreased by 70–90% by cholesterol depletion relative to controls. As expected, knockdown of LXRα/β isoforms blocked the induction of ABCA1 and ABCG1 following cholesterol loading (Table 1). Thus both ABCA1 and ABCG1 mRNA levels are sensitive to cellular cholesterol levels. These findings imply that ABCG1 does in fact respond to changes in cholesterol levels in hASM cells; however, its precise physiological role remains to be determined.

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smooth muscle cells in vitro as well as promote reverse cholesterol transport through the induction of ABC transporters suggest a possible molecular link between airway inflammation and cholesterol homeostasis. However, the role of lipid and cholesterol homeostasis in airway smooth muscle function is not understood, and further studies are warranted. In contrast, lipid homeostasis plays a well-established role in vascular smooth muscle biology where elevated levels of intracellular cholesterol and oxidized phospholipids, derived from uptake of oxidized (ox) LDL through scavenger receptors such as SR-BI and CD36 (8), induces a proinflammatory reaction by vascular cells that can eventually lead to atherosclerotic lesion development (32, 36). It is interesting to note that asthma is in many ways a disease analogous to atherosclerosis in that both diseases are characterized by chronic inflammation and involve increased smooth muscle hyperplasia and hypertrophy, proinflammatory gene expression, lesion development, extracellular matrix remodeling, and infiltration of inflammatory leukocytes. Analogous to atherosclerotic lesions, the airways of asthmatic patients display endothelial and microvascular damage, which is thought to expose the lung to blood plasma and its constituents including lipoprotein particles such as LDL (34). Thus the asthmatic airway may be subject to a similar inflammatory microenvironment as vascular cells following endothelial damage.

Using siRNA knockdown approaches, we have shown that ABCA1 is necessary and sufficient for all LXR ligand-induced

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Fig. 5. Probucol treatment inhibits cholesterol efflux to apoAI and HDL in hASM. A: hASM cells were plated in 48-well dishes and labeled with [3H]cholesterol for 48 h. Cells were then treated with vehicle, T13179-cisRA (1 μM each), and/or probucol (10 μM), as indicated, for an additional 18 h. Cholesterol efflux was initiated by the addition of 0.2% BSA, apoAI (50 μg/ml), or HDL (50 μg/ml), as indicated, and extracellular cholesterol was measured as above. The values are expressed as the percentage of cholesterol in supernatants relative to total cholesterol associated with the cells plus supernatants. The data represent the average of 3 experiments done in triplicate (± SD). B: hASM cells were plated into 96-well dishes and treated as described in MATERIALS AND METHODS. Cell viability was measured in the presence of the indicated compounds and compared with untreated cells taken as 100%.

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Fig. 6. ABCA1 expression is required for phospholipid efflux in hASM cells. A and B: hASM cells were plated in 48-well dishes and transfected with the indicated siRNA. Cells were subsequently labeled with [3H]choline in the presence or absence of T1317 (1 μM) and 9-cisRA (1 μM). Phospholipid efflux was initiated by the addition of apoAI (50 μg/ml; A) or HDL (50 μg/ml; B) where indicated. Following incubation for 5 h, lipids were extracted from supernatants and cell monolayers as described in MATERIALS AND METHODS, and radioactivity was measured by scintillation counting. Values are expressed as the percentage of phospholipids in the supernatants relative to total phospholipids associated with the cells plus supernatants. The data are representative of at least 2 trials done in triplicate (± SD).
cholesterol and phospholipid efflux in hASM cells, whereas ABCG1 appears not to be required. We further demonstrate that ABCA1 effluxes cholesterol to both apoA1 and HDL acceptors, in concordance with others who have shown, in endothelial cells, that ABCA1 but not ABCG1 mediates cholesterol efflux to HDL (22, 26). We confirmed these results using probucol, a small molecule inhibitor specific for ABCA1 as demonstrated by the ability of this compound to inhibit cholesterol efflux in normal fibroblasts but not in fibroblasts derived from Tangier’s patients who lack ABCA1 (15). The raw percentages of efflux observed from hASM to both apoA1 (4–14%) and HDL (30–40%) are also highly consistent with previous reports in other cell types including macrophages (15, 22). Recently, Mukhamedova et al. (25) using in vitro labeled cholesterol mass efflux to HDL was reported to be dramatically reduced in peritoneal macrophages isolated from abca1<sup>+/−</sup> mice, however, abcgl<sup>+/−</sup> mice also displayed reduced capacity to efflux cholesterol to HDL, suggesting that, in macrophages, both transporters contribute to efflux to HDL (28). These findings point to a possible cell type-specific role for ABCG1 and cholesterol efflux in macrophages compared with smooth muscle cells. Why airway smooth muscle cells differ from the macrophage in mechanisms of cholesterol efflux to HDL is currently unclear. The macrophage is a highly specialized cell able to phagocytose extracellular materials including apoptotic cells and surfactant (12). Both processes dramatically increase intracellular sterol content, and thus alveolar macrophages have possibly evolved separate mechanisms to handle increased intracellular lipids (12). Additionally, it is possible that macrophage cell types may express additional cofactor proteins that work in conjunction with ABCG1 to mediate efflux of lipids that are not present in hASM. Thus, for hASM cells, it is perhaps sufficient and efficient that ABCA1 can both lipidate apoA1 particles to form nascent HDL and in addition, further lipidate HDL itself.

HDL. Although ABCG1 is robustly induced in hASM cells by activation of LXR, its function in these cells remains unclear. Recent findings indicate that abcgl<sup>+/−</sup> have elevated levels of multiple proinflammatory mediators in the lungs (4), and it was suggested that this was likely due to local elevated cholesterol levels. However, the progressive lipid accumulation observed in abcgl<sup>+/−</sup> mice can be reversed by bone marrow transplantation of wild-type cells suggesting that hematopoietic ABCG1 is required for proper lipid homeostasis in the lung (46). In agreement with these findings, we did not observe an effect on LXR-induced cholesterol transport after ABCG1 knockdown in airway smooth muscle, a non-hematopoietic cell. Whether ABCG1 is linked to roles unrelated to direct lipid efflux in hASM cells (e.g., intracellular lipid trafficking) remains to be tested. Recently, transforming growth factor-β (TGF-β) signaling has been shown to specifically increase the expression of ABCG1 and not ABCA1 (3). Since TGF-β plays a fundamental role in ECM production in hASM cells, it is possible that ABCG1 has a function in this context (24, 30).

Our findings raise several questions as to the role of cholesterol efflux and metabolism to AHR in vivo. Although we (13) and others (38) have shown that LXR agonists reduce the inflammatory response in multiple lung cell types, a direct correlation between reverse cholesterol transport and AHR remains to be determined in vivo. Baldan et al. (4) have proposed that cholesterol itself is proinflammatory, and thus elevated levels may exacerbate an inflammatory milieu thereby accelerating the remodeling process occurring in the asthmatic lung. The beneficial effects of LXR may thus be partially due to efflux of excess cholesterol for transport back to the liver and eventually excretion via an ABCA1-dependent mechanism. In support of this, statin therapy, a cholesterol-lowering drug, is recently reported as a potential therapeutic avenue for the treatment of lung diseases (11, 19, 31).

In summary, we demonstrate that LXR ligand-induced cholesterol and phospholipid efflux in airway smooth muscle cells is mediated exclusively by ABCA1 and that elevated levels of cholesterol affect biological processes that are highly relevant to the pathophysiology of asthma. Our findings may thus have relevance to understanding the molecular mechanisms that link obesity and hypercholesterolemia to airway diseases such as asthma. Future studies investigating the mechanisms of high cholesterol diet on AHR and the effects of LXR activation in vivo will provide evidence to support cholesterol lowering as a potential therapeutic avenue in treating asthma in hypercholesterolemic and obese patients.

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GRANTS

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Table 1. ABCA1 and ABCG1 mRNA levels are increased by cholesterol loading and decreased by cholesterol depletion

<table>
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<tr>
<th>Gene of Interest</th>
<th>0.2% BSA</th>
<th>MβCD</th>
<th>MβCD-Chol</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA</td>
<td>Luciferase</td>
<td>LXRa/β</td>
<td>Luciferase</td>
</tr>
<tr>
<td>ABCA1</td>
<td>1 (± 0.03)†</td>
<td>0.70 (± 0.09)</td>
<td>0.14 (± 0.02)*</td>
</tr>
<tr>
<td>ABCG1</td>
<td>1 (± 0.11)†</td>
<td>0.13 (± 0.02)*</td>
<td>0.07 (± 0.01)*</td>
</tr>
</tbody>
</table>

Human airway smooth muscle (hASM) cells were transfected with the indicated smaller interfering RNA (siRNA) and serum-starved for 24 h followed by treatment with vehicle (0.2% BSA), methyl-β-cyclodextrin (MβCD; 5 μg/ml) alone, or MβCD in complex with cholesterol (MβCD-Chol; 5 μg/ml) for an additional 24 h. RNA was then isolated, and levels of ABCA1 and ABCG1 were quantified by real-time PCR. The data represent relative fold changes and are the average of triplicates (± SD) normalized using β-actin as an internal standard with control vehicle treated taken as 1. *P < 0.05 vs. control indicated by †. ABCA1 and ABCG1, ATP-binding cassette (ABC) transporters; LXR, liver X receptor.
DISCLOSURES
C. J. Delvecchio, P. Bilan, and J. P. Capone have nothing to disclose. P. Nair has received lecture fees from GlaxoSmithKline, Merck, and AstraZeneca.

REFERENCES

