Cultured gallbladder epithelial cells synthesize apolipoproteins A-I and E

Jin Lee,1,3 Aimee Tauscher,1,3 Dong Wan Seo,1,3 John F. Oram,2 and Rahul Kuver1,3

1Division of Gastroenterology and 2Division of Endocrinology and Metabolism, University of Washington School of Medicine, Seattle 98195; and 3Puget Sound Veterans Affairs Health Care System, Seattle Division, Seattle, Washington 98108

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Lee, J., A. Tauscher, D. W. Seo, J. F. Oram, and R. Kuver. Cultured gallbladder epithelial cells synthesize apolipoproteins A-I and E. Am J Physiol Gastrointest Liver Physiol 285: G630–G641, 2003. First published May 28, 2003; 10.1152/ajpgi.00101.2003.—Gallbladder epithelial cells (GBEC) are exposed to high and fluctuating concentrations of biliary cholesterol on their apical (AP) surface. GBEC absorb and efflux cholesterol, but the mechanisms of cholesterol uptake, intracellular trafficking, and efflux in these cells are not known. We previously reported that ATP binding cassette (ABC)A1 mediates basolateral (BL) cholesterol efflux in cultured polarized GBEC. In addition, the nuclear hormone receptors liver X receptor (LXRα) and retinoid X receptor (RXR) mediate both AP and BL cholesterol efflux. An interesting finding from our previous study was that apolipoprotein (apo)A-I applied to the AP surfaces of cells elicited BL ABCA1-mediated cholesterol efflux. Because ABCA1-mediated cholesterol efflux requires the presence of a cholesterol acceptor, we hypothesized that GBEC synthesize and secrete endogenous apo into the BL compartment. Here, we demonstrate that cholesterol loading of cells with model bile and AP apoA-I treatment is associated with an increase in the synthesis of apoE mRNA and protein. Furthermore, apoE is secreted into the BL compartment. LXRα/RXR ligands stimulate the synthesis of endogenous apoA-I mRNA and protein, as well as apoE mRNA. BL secretion of apoA-I is elicited by LXRα/RXR ligands. Therefore, GBEC synthesize apoA-I and -E and efflux cholesterol using ABCA1- and non-ABCA1-mediated pathways. These processes may alter gallbladder biliary cholesterol concentrations and thereby influence gallstone formation.

ATP binding cassette A1; cholesterol; liver X receptor; retinoid X receptor; oxysterol

A major function of the gallbladder (GB) is to store and concentrate bile (53). This process, in conjunction with secretion of cholesterol into bile by the canalicular membrane of the hepatocyte, leads to exceedingly high concentrations of cholesterol in GB bile, which is a prerequisite for cholesterol gallstone formation (24). Consequently, GB epithelial cells (GBEC) coexist with extremely high and variable concentrations of biliary cholesterol. GBs also absorb cholesterol (18, 37), but the fate of the absorbed cholesterol is not known. Absorbed cholesterol from bile appears in the subepithelial GB wall (8). Cholesterol in the subepithelial layer may contribute to cholelithiasis, and this may impair GB motility (5). The latter is a risk factor for gallstone formation. On the other hand, studies in ex vivo arterially-perfused human GBs have shown that cholesterol absorption by the GB, and hence the ability to modify cholesterol concentrations in GB bile to a more anti-lithogenic profile, is disrupted in patients with cholelithiasis (7). These findings, performed in a variety of model systems from various species, point not only to the potentially critical role played by the GB in modifying biliary cholesterol concentrations, but also to the gaps in our knowledge with respect to cholesterol uptake, intracellular trafficking and efflux mechanisms in the GB. Significant advances have been made in understanding cholesterol uptake, intracellular trafficking and efflux mechanisms in macrophages, hepatocytes and enterocytes. Specific cholesterol transport proteins have been implicated, including ATP binding cassette (ABC)A1 in macrophages (42) and the half-transporters ABCG5/ABCG8 in hepatocytes and enterocytes (2, 55, 56). In addition, the HDL receptor SR-BI has been implicated in cholesterol uptake and efflux in numerous cell types and has been detected in the GB (1, 20, 36). Therefore, the opportunity now exists to determine the cellular and molecular mechanisms of cholesterol uptake, intracellular trafficking, and efflux in GBEC.

We recently showed that ABCA1, a member of the ABC super family of transport proteins, mediates cholesterol efflux from the basolateral (BL) membrane of polarized GBEC (32). We now extend those studies in an effort to provide a clearer understanding of the pathways involved in cholesterol uptake, intracellular trafficking, and efflux in these cells. Several questions were raised by our previous work. First, the cholesterol acceptor apolipoprotein (apo)A-I elicited BL cholesterol efflux via the ABCA1-mediated pathway even when supplied to the apical (AP) surfaces of cells. Because we had eliminated the presence of paracellular or transcellular passage of apoA-I, we speculated that the cells synthesized apolipoproteins that could serve as cholesterol acceptors. Second, because bile contains a diverse...
array of oxysterols (14), activation of nuclear hormone receptors such as liver X receptor (LXR)α could be a means of regulating cholesterol transport pathways at the transcriptional level. Indeed, incubation with ligands for the nuclear hormone receptors LXRα/retinoid X receptor (RXR) leads to cholesterol efflux from both the AP and BL surfaces of the cells (32), implying that both ABCA1 and non-ABCA1-mediated pathways for cholesterol efflux are activated. We have explored these observations further to better define the mechanisms whereby LXRα/RXR exerted these effects. These studies lay the foundation for an understanding at the cellular and molecular level of cholesterol uptake, intracellular trafficking, and efflux in GBEC.

MATERIALS AND METHODS

Materials. MEM, FBS, trypsin/EDTA, penicillin/streptomycin, 1-α-phosphatidylycerine, 8-bromo-cyclic AMP (8-BrcAMP), 9-cis-retinoic acid, 22-(R)-hydroxycholesterol, and peroxidase-conjugated anti-rabbit IgG were from Sigma (St. Louis, MO). Vitrogen was from Celtrix (Palo Alto, CA). Transwell cell culture plates (diameter 12 or 24 mm; pore size 3.0 µm) were from Costar (Cambridge, MA). Taurocholic acid, fatty acid-free BSA and cholesterol were from Calbiochem (La Jolla, CA). [14C]cholesterol was from New England Nuclear (Boston, MA). [35S]methionine and enhanced chemiluminescence detection reagents (ECL-Plus) were from Amersham Pharmacia (Piscataway, NJ). Fluorescein-conjugated goat anti-rabbit IgG was from Molecular Probes (Eugene, OR). The goat anti-human polyclonal apoE antibody, the peroxidase-conjugated anti-goat IgG antibody, and the goat polyclonal anti-human apoA-I antibody were from Rockland (Gilbertsville, PA). Protein G-agarose conjugate was from ECL-Plus M III mass detector (Deerfield, IL).

RT-PCR-GBEC were cultured to confluence on six-well Transwells, then treated as outlined below. Each treatment group consisted of three wells. Treatment groups were as follows: 1) AP apoA-I treatment: 10 µg/ml delipidated apoA-I was added to the AP compartment in SFM, and SFM was added to the BL compartment. Treatment continued for 8 h before harvesting. 2) Model bile treatment: model bile was applied to the AP compartment in SFM, and SFM alone was added to the BL compartment. Treatment continued for 48 h before harvesting. 3) AP apoA-I + model bile treatment: model bile was added to AP wells in SFM, and SFM alone was added to BL wells. At the same time, 10 µg/ml apoA-I was added to the AP compartment. The total time of incubation was 48 h, with apoA-I added 8 h before harvesting. 4) No-treatment group: cells were cultured in SFM and then harvested. 5) LXRα/RXR treatment: cells were incubated in SFM with 10 µM each of 22-(R)-hydroxycholesterol and 9-cis-retinoic acid, added both apically and basolaterally, before harvesting. Treatment time was 24 h. Cells were harvested, and RNA was extracted using the RNeasy Minikit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed first followed by PCR with the following primer sequences: ApoE forward: 5’-CAG GTC ACC CAG GAA CTG AC-3’; apoE reverse: 5’-GCG TGC TCA TCT CCT CCA-3’; GAPDH forward: 5’-ATC ACT GCC ACC CAG AAC-3’; GAPDH reverse: 5’-GCC AGG TCA GAT CCA CAA-3’. Primers were based on the canine apoE sequence (33). PCR was performed under the following conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 55°C for 30 s, 68°C for 90 s, then followed by 72°C for 7 min.

Western blot analysis. GBEC were cultured to confluence on Transwell inserts. For Western blot analysis with the apoE antibody, model bile was added to the AP compartment before harvesting of cells. Certain cells were treated with model bile as well as 10 µg/ml apoA-I added to the AP compartment (model bile + apoA-I group). For Western blot analysis with the ABCA1 antibody or the SR-BI antibody, model bile was added to the AP compartment with or without 10 µM each of 22-(R)-hydroxycholesterol and 9-cis-retinoic acid added to both sides of certain Transwells. Incubation with preimmune serum was used as a negative control for the ABCA1 Western blots. For the apoE and SR-BI Western blots, we used cell lysates from human GB myofibroblasts, which do not express these proteins, as negative controls.
The AP and BL media were collected and concentrated 10-fold with a Centricron 10 microconcentrator (Amicon, Beverly, MA). Cells were harvested with SDS loading buffer [250 mM Tris (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromphenol blue, and 2% β-mercaptoethanol]. Protein content of media and cell extracts was measured by the Lowry method. SDS-PAGE with a 4% stacking gel and a 6% (for ABCA1 Western) or 12% (for apoE Western) resolving gel was performed, followed by transfer to a polyvinylidene difluoride membrane. For the SR-BI Western blots, a non-denaturing gel was used. The membranes were blocked with 1% BSA in PBS/Tween-20 (0.05%; vol/vol) at 4°C for 16 h, and then incubated with a rabbit polyclonal anti-human ABCA1 antibody or goat polyclonal anti-human apoE antibody or rabbit polyclonal anti-human SR-BI antibody for 1 h at room temperature. The membrane was then washed with 0.05% Tween-20 in PBS and incubated with peroxidase-conjugated anti-rabbit or anti-goat IgG for 1 h at room temperature. The membrane was washed and incubated with ECL-Plus detection system (Amersham, Piscataway, NJ) for 2 min, and autoradiography was performed. The signal intensities for specific bands on the Western blots were quantified using National Institutes of Health Image J density analysis software (version 1.20).

Immunofluorescence studies. GBEC were cultured on 12-mm diameter Transwell inserts until confluent. Cells were treated with 10 μM each of 22-(R)-hydroxycholesterol and 9-cis-retinoic acid as described above. Cells were washed twice with ice-cold PBS, and the Transwell membranes were cut out and placed on a culture plate. Cells were fixed in methanol/acetic acid (1:1; vol/vol), incubated at −20°C for 15 min, and washed with ice-cold PBS. Blocking buffer (PBS with 1% BSA) was added for 15 min. The rabbit polyclonal anti-human ABCA1 antibody was applied for 60 min. After being washed with PBS, the Alexa 568 goat anti-rabbit IgG secondary antibody was added for 60 min. Cells were washed three times with PBS and sealed in mounting media. A Zeiss Axioplan fluorescence microscope fitted with a Hamamatsu C4880 integrating digital camera and MCID software were used for image acquisition and analysis at ×400 magnification.

Confocal laser-scanning immunofluorescence microscopy. Slides were prepared as described for immunofluorescence microscopy studies. Scanned images were acquired with ×400 optical and ×3 digital magnification using a laser-scanning spectral confocal microscope system (Leica DM-R upright fluorescence microscope and Leica TCS-SP confocal scanner). For the colocalization studies, the same slides were treated with both the goat anti-human apoE antibody and the chicken anti-canine Na+/K+ ATPase antibody, and scanning confocal images were obtained. In these studies, the secondary antibodies were Alexa 488 donkey anti-goat IgG and rhodamine-conjugated donkey anti-chicken, respectively. For the SR-BI confocal studies, the secondary antibody used was rhodamine goat anti-rabbit IgG.

Metabolic labeling with [35S]methionine and apoA-I immunoprecipitation. GBEC were cultured on 12-mm diameter Transwell inserts without Vitrogen until confluent. The protocol for metabolic labeling and apoA-I immunoprecipitation was followed as described (13). Cells were washed three times with methionine-free DMEM. Cells were then labeled with 20 μCi/ml [35S]methionine for 24 h in methionine-free DMEM in the presence or absence of 10 μg/ml apoA-I added to the AP compartment. Aliquots (250 μl) of AP and BL media and cellular protein extracted with RIPA buffer [50 mM Tris, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS], diluted with 250 μl NET buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 0.02% azide), and incubated with 25 μl of protein G-agarose for 1 h at 4°C to remove nonspecifically bound proteins. Supernatants were removed after centrifugation (12,000 g for 5 min) and incubated overnight with goat anti-human apoA-I antibody (or albumin as a control) at 4°C. Protein G-agarose (25 μl) was added for 1 h at 4°C. Subsequently, the tubes were centrifuged for 5 min at 12,000 g, and the pellet was washed three times with RIPA buffer. Immunoprecipitated proteins were analyzed by SDS-PAGE with a 12% polyacrylamide gel followed by autoradiography. Alternatively, the cell pellet was dissolved in scintillation fluid, and scintillation counts were obtained. These experiments were performed twice with equivalent results.

ApoA-I ELISA assay. Cells were cultured to confluency on 24-mm Transwell inserts, then treated on the AP and BL compartments with 10 μM each of 22-(R)-hydroxycholesterol and 9-cis-retinoic acid. After incubation for 48 h, media from the AP and BL compartments were collected and concentrated using Centricron 10. A separate aliquot of media from each compartment was used for protein quantification using the Lowry method. Six samples per each treatment condition were assayed for apoA-I by an ELISA kit per the manufacturer’s instructions (AlerChek, Portland, ME). This experiment was repeated with equivalent results.

Thin-layer chromatography. The cholesterol efflux assay was performed as described (32). Cells were treated with a combination of model bile containing [14C]cholesterol, 1 mM 8-Br-cAMP, 10 μg/ml apo A-I, and/or the combination of 10 μM 22-(R)-hydroxycholesterol and 10 μM 9-cis-retinoic acid. The LXRs/RXR ligands were added apically and basolaterally 24 h before model bile treatment, again at the time of model bile treatment, and also at the time of addition of the efflux media. Media were collected as described (32), but triplicate wells were pooled into a single glass test tube. Each pooled sample was mixed with chloroform/methanol (2:1) and shaken. Tubes were centrifuged at 1,760 g, and the chloroform layer was extracted, transferred to a clean test tube, and evaporated to dryness. The residue was resuspended in 50 μl chloroform, and concentrated samples were applied to channels on a prescribed silica thin-layer chromatography (TLC) plate (Whatman K6D) and developed in hexane-diethyl ether-glacial acetic acid (85:15:1) until the solvent front was 1/4 inch from the top of the plate. Each channel was divided into eight sections of 2 cm each. Each section was scraped into a 20-ml scintillation vial. Scintillation fluid (10 ml; Opti-Fluor, Packard Biosciences, Meriden, CT) was added and mixed well. Samples were counted using the same program and instrument as the cholesterol efflux assay. Cholesterol and cholesterol ester standards were run with each plate.

Cholesterol efflux assay with recombinant helix 10 apoA-I truncation mutant. Recombinant wild-type apoA-I and the helix 10 apoA-I truncation mutant were prepared as described (44). The cholesterol efflux assay was performed as described (32), except that recombinant helix 10 truncated apoA-I was added to either the AP or the BL compartment instead of wild-type human apoA-I. As controls, wild-type human apoA-I or recombinant apoA-I was added to either the AP or BL compartments. BL cholesterol efflux was measured as described (32).

Cross-linking. Confluent monolayers of GBEC in six-well Transwells were placed on ice and washed three times with SFM with 1% BSA. ApoA-I (20 μg) was added in 2 ml SFM with 1% BSA, and the cells were incubated for 60 min at 37°C under an atmosphere of 95% air-5% CO2. Cells were cooled on ice and washed twice with 2 ml of SFM with 1% BSA and three times with 2 ml PBS. The water-soluble homo-
bifunctional cross-linker 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP; Pierce, Rockford, IL) was dissolved immediately before use in PBS to a concentration of 200 μM, and 1 ml was added per well. Cells were incubated for 45 min at room temperature, after which cells were cooled on ice, and the incubation medium was removed. Cells were washed with PBS with 50 mM glycine. Transwell membranes were cut out, and cells were lysed in 400 μl of 0.02 M sodium phosphate, pH 7.4, 1 mM MgCl₂, 0.5% NP-40, 10 μg/ml aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, and 10 mM glycine and centrifuged for 10 min at 10,000 g at 4°C. The samples were then used for immunoprecipitation.

**Immunoprecipitation.** Cell lysates were incubated with magnetic protein G beads for 60 min at 4°C. Beads were precipitated with a magnet (Dynalabs, Lake Success, NY), and the supernatant was then incubated with 2 μl of anti-SR-B1 antibody for 1 h at 4°C. Protein G beads (2 mg) were added, incubated for 1 h at 4°C, and then beads were precipitated. Beads were washed two times with 1 ml 1% Triton X-100, 40% acetic acid added, vortexed, and incubated for 10 min. Beads were precipitated, and the supernatant was transferred to a tube containing SDS-PAGE loading buffer. SDS-PAGE was run on a 12% gel. Western blot analysis was then performed with the apoA-I antibody.

**Statistical analysis.** Results for each experiment are expressed as the means ± SD of duplicate cultures, and all results described are representative of at least two separate experiments. Student's t-test for unpaired two groups were used, and P < 0.05 was considered significant. The results are expressed as means ± SE, where the results of multiple experiments are pooled. The ANOVA test was used for the ELISA results.

**RESULTS**

**Model bile treatment leads to cholesterol loading in GBEC.** We have previously used an assay to measure cholesterol efflux that involved treatment of confluent GBEC with model bile for 48 h (32). Although the term “cholesterol loading” was used, a quantitative measure of cholesterol uptake by cells using this method was not previously provided. We therefore cultured canine GBEC under conditions identical to that used for the cholesterol efflux assay and used HPLC to measure cellular cholesterol levels without and with a 48-h AP treatment with model bile. The results showed a ~1.6-fold increase in cholesterol content in the model bile-treated cells (Fig. 1).

ApoE is a candidate cholesterol acceptor for ABCA1-mediated cholesterol efflux. AP apoA-I is capable of eliciting BL ABCA1-mediated cholesterol efflux in polarized GBEC (32). Because cholesterol efflux mediated by ABCA1 is dependent on the presence of a cholesterol acceptor, we hypothesized that GBEC are capable of synthesizing apolipoproteins that would serve in this capacity. We considered the possibility that endogenous synthesis of apoE was stimulated by model bile treatment or AP apoA-I treatment. This mechanism has been reported in macrophages, where apoA-I elicits apoE secretion (46). RT-PCR using canine apoE sequence primers showed that apoE mRNA was increased on cholesterol loading of cells with model bile and AP application of apoA-I (Fig. 2A, lane 1) and less prominently with model bile treatment alone (lane 2). There was no detectable signal for apoE mRNA following only AP apoA-I treatment (lane 5).

Biliary sterols include oxysterols (14), which could transcriptionally regulate cholesterol transport processes in GBEC via activation of nuclear hormone receptors. Because activation of the nuclear hormone receptors LXRα/RXR has been linked to upregulation of transcription of apoE (29, 34), we looked for evidence of this in our system. RT-PCR for the apoE message showed an increase following treatment with 22-(R)-hydroxycholesterol and 9-cis-retinoic acid (Fig. 2B). The increase in the signal was comparable with that seen when cells were cholesterol loaded following model bile treatment.

We also looked for evidence of apoE expression in cultured GBEC. Immunoblots showed apoE expression at baseline (Fig. 3). As with the RT-PCR results, cholesterol loading with model bile increased apoE expression within cells (P < 0.05). A significant increase in apoE cellular expression was also seen with the combination of model bile treatment and AP apoA-I treatment (P < 0.05), whereas AP apoA-I treatment alone showed a slight increase in apoE expression that was not statistically significant.

Subcellular localization of apoE in GBEC was examined using confocal immunofluorescence microscopy. These studies were performed on polarized cells under baseline conditions. The apoE signal was predominantly intracellular (Fig. 4). The plasma membrane marker Na⁺-K⁺-ATPase did not show colocalization with the apoE signal.

We also looked for evidence of apoE secretion into the AP and BL media by performing immunoblots on concentrated media following incubation of cells with model bile and/or with AP apoA-I treatment. No apoE was demonstrable in the AP media. In the BL media, a band corresponding to apoE was detected (Fig. 5). ApoA-I added to the AP compartment led to an increase in intensity of the apoE signal in the BL media, suggesting that AP apoA-I signaled the cell to secrete...

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![Figure 1](http://ajpgi.org)
apoE into the BL compartment. An additional increase in the apoE signal was seen when the cells were treated with model bile in addition to AP apoA-I treatment. These changes were subtle, however, and the low levels of apoE detected in the media did not allow a quantitative assessment of these changes.

Lack of effect of the helix 10 apoA-I truncation mutant applied apically on BL cholesterol efflux. As a first step to determine whether AP apoA-I served in a signaling role to elicit BL cholesterol efflux, we examined whether a COOH-terminal apoA-I truncation mutant was effective in eliciting BL cholesterol efflux. The apoA-I helix 10 COOH-terminal truncation mutant has been shown to be critical for eliciting ABCA1-mediated cholesterol efflux (44). Recombinant apoA-I with a helix 10 truncation applied to the AP surface of GBEC did not elicit BL cholesterol efflux; cholesterol efflux rates were identical to control wells (n = 3). In addition, no BL cholesterol efflux was elicited with BL application of the apoA-I helix 10 truncation mutant, consistent with studies in the mouse macrophage RAW264.7 cell line (6, 44). Wild-type apoA-I elicited BL cholesterol efflux when applied to either the AP or the BL compartment, as previously reported (32). These results suggest that the COOH-terminal helix 10 of apoA-I is critical not only for the ABCA1-mediated cholesterol efflux pathway, but for the AP membrane putative signaling role.

Scavenger receptor class B type I expression is not altered by model bile or AP apoA-I treatment. Scavenger receptor class B type I (SR-BI) is a receptor for HDL and has been shown to bind to multiple sites on apoA-I, the major apolipoprotein found in HDL (54). Because apoA-I and not HDL is present in bile, we hypothesized that biliary apoA-I binds to SR-BI on the AP membrane of GBEC, and this may be a mechanism whereby AP apoA-I signaling is initiated. We therefore looked for evidence of SR-BI expression in cultured GBEC. SR-BI expression was confirmed on the AP plasma membrane by confocal immunofluorescence microscopy. Na⁺-K⁺-ATPase showed a predominantly BL membrane signal, whereas the SR-BI signal was predominantly AP, as shown on both xy and xz images (Fig. 6). This finding is consistent with an AP membrane localization of SR-BI as reported in mouse and human GB epithelium (20, 36). Because expression of this HDL receptor on the AP plasma membrane may be a mechanism of both cholesterol uptake and efflux, we asked whether expression levels of SR-BI were altered under conditions of cholesterol loading by treatment with model bile. As shown in Fig. 7A, there was no change in expression of SR-BI following AP model bile treatment. We also noticed no significant change in expression of SR-BI on immunoblotting following treatment of cells with AP apoA-I, LXRα/RXR ligands, or 8-Br-cAMP (Fig. 7B).

To determine whether apo A-I binding to the AP surface of GBEC involves a direct interaction with
SR-BI, we treated confluent monolayers with apoA-I along with the cross-linking agent DTSSP. Cell lysates were then immunoprecipitated with the SR-BI antibody, and the immunoprecipitate was electrophoresed using SDS-PAGE. Western blot analysis was then performed with the apoA-I antibody. Three bands at approximate molecular masses 50, 80, and 110 kDa were noted (Fig. 7C). Whereas the identity of the smaller two bands is unclear, the 110-kDa band is consistent with a complex comprised of SR-BI (82 kDa) and apoA-I (28 kDa). This molecular mass is one-half the size reported by Williams et al. (54) in similar cross-linking experiments with apoA-I and SR-BI.

LXRα/RXR ligands increase ABCA1 expression. Treatment with ligands for the nuclear hormone receptors LXRα/RXR led to AP and BL cholesterol efflux (32). BL cholesterol efflux in GBEC proceeds, in part, via ABCA1, which is transcriptionally regulated by LXRα. We therefore looked for evidence of upregulation of ABCA1 expression in cells treated with LXRα/RXR ligands. Immunoblots performed on cells treated with LXRα/RXR ligands showed increased ABCA1 expression compared with untreated cells (Fig. 7A). These experiments were performed in the absence of cholesterol loading with model bile. When cells were cholesterol loaded with model bile, ABCA1 expression increased to the same extent whether or not LXRα/RXR stimulation was provided.

We also performed immunofluorescence studies to assess ABCA1 expression with or without LXRα/RXR ligand stimulation. The immunofluorescence data corroborated the results from the immunoblots, showing increased expression of ABCA1 following LXRα/RXR ligand treatment compared with no treatment (Fig. 8B). ABCA1 expression levels in GBEC are therefore subject to regulation by several distinct mechanisms, including activation of LXRα/RXR and stimulation with 8-Br-cAMP (32).

LXRα/RXR ligand treatment elicits apoA-I expression. ApoA-I is present in bile; therefore, we looked for endogenous apoA-I synthesis and secretion in response to exogenous AP apoA-I. These studies were performed using 35S metabolic labeling of cells under conditions designed to elicit cholesterol efflux, followed by immunoprecipitation with an apoA-I antibody. These studies were negative (data not shown). On the other hand, confocal immunofluorescence studies showed an intracellular signal with an apoA-I antibody, consistent with a prior report (45). We therefore asked whether LXRα/RXR ligands are capable of eliciting apoA-I expression. 35S metabolic labeling studies in cells treated with 9-cis-retinoic acid and 22-(R)-hydroxycholesterol demonstrated increased apoA-I expression (Fig. 9A). Scintillation counts of the pellet obtained by immunoprecipitation with either the apoA-I antibody or with albumin showed significantly increased counts in the apoA-I immunoprecipitated pellet compared with the albumin control (Fig. 9B). Because apoA-I is a lipid acceptor that acts in concert with ABCA1 to mediate cholesterol efflux (32, 43), the finding that LXRα/RXR receptor activation leads to apoA-I expression suggests an alternative transcriptional level of control of cholesterol efflux and an indirect means to control activation of ABCA1.

LXRα/RXR ligand treatment elicits basolateral apoA-I secretion. BL apoA-I was a stimulus for BL cholesterol efflux mediated by ABCA1 (32). Increased association of 125I-labeled apoA-I was observed under
conditions that elicited BL ABCA1-mediated cholesterol efflux. We therefore asked whether apoA-I secretion into the media could be detected following LXRα/RXR stimulation. With the use of an ELISA, increased BL apoA-I secretion was observed following treatment with LXRα/RXR ligands (Fig. 10). There was no detectable apoA-I in the AP compartment. These results show that BL cholesterol efflux, regulated by LXRα/RXR and proceeding via the ABCA1 pathway, could be coupled to BL apoA-I secretion, a process that would provide a cholesterol acceptor.

**LXRα/RXR ligand treatment decreases esterified cholesterol levels in GBEC.** We previously used a cholesterol efflux assay to measure ABCA1 function in cultured GBEC (32). To define the radiolabeled species measured in that assay, TLC was performed on cell extracts and on AP and BL media. For these studies, the cholesterol efflux assay protocol was followed (32). The results showed that the effluxed radiolabel was present entirely in the form of unesterified cholesterol and was found predominantly in the BL compartment when cells were treated with model bile, cAMP, and the cholesterol acceptor apoA-I provided in the BL compartment. Within the cells, the radiolabel was found either as unesterified cholesterol (60.9 ± 13%) or esterified cholesterol (39.1 ± 13%). These findings were consistent regardless of whether cells were treated with cAMP analog and with AP or BL apoA-I. With LXRα/RXR treatment, however, the percentage of unesterified cholesterol in cells increased to 86.5% ± 3.6% (P < 0.02 compared with the control value) and the percentage of esterified cholesterol in cells decreased to 13.5% ± 3.6% (P < 0.02 compared with the control value). LXRα/RXR treatment therefore allows for a greater percentage of the radiolabeled cholesterol that is supplied in model bile to remain unesterified in GBEC, thereby preserving its availability for subsequent efflux from the cell.
DISCUSSION

Although it has long been appreciated that bile contains various apolipoproteins (51), and whereas apoA-I, -A-II, and -B were previously found in GB epithelium (45), the origin and functional significance of apolipoproteins in the GB and in bile has not been adequately explained. Biliary apoA-I possesses antinucleating properties, although the mechanisms by which apoA-I inhibits cholesterol crystallization and stone formation are not well defined (16, 21). ApoA-I is transported from the AP to the BL side of human and murine GBEC (50), although this was not confirmed in our GBEC system (32). In the current report, we provide evidence not only of endogenous synthesis and secretion of apoA-I and -E by GBEC, but we also endeavor to place these apolipoproteins in a functional context with respect to cholesterol uptake, intracellular trafficking, and efflux by these cells. ApoE mRNA and protein levels are increased following cholesterol loading of cells by treatment with model bile and, to a greater extent, following model bile and AP apoA-I treatment. LXRα/RXR treatment elicits diverse effects: ApoA-I synthesis and secretion via the BL membrane, upregulation of apoE mRNA synthesis, and increased ABCA1 expression. These results suggest that the GB epithelial cell is subjected to signals emanating from bile that influence cellular cholesterol uptake, intracellular trafficking, and efflux. These signals are biliary cholesterol itself, oxysterols acting via the nuclear hormone receptor LXRα, and biliary apoA-I.

Cholesterol loading of cells by treatment with model bile increases expression of ABCA1, which is found on the BL membrane (32). Cholesterol loading also increases the mRNA for apoE and increases apoE protein expression. These results imply that cholesterol absorption by the GB epithelial cell prompts compensatory mechanisms that allow the cell to transport and dispose of excess cholesterol, with apoE playing a role in this regard. Previously, we had found that ABCA1-mediated BL cholesterol efflux could be elicited by the addition of apoA-I to the AP compartment. In the absence of transcytosis of apoA-I in our model system, this finding required a mechanism that would allow ABCA1 to efflux cholesterol to the BL compartment in
functions in a signaling role at the AP plasma membrane of GBEC. Because SR-BI is localized to the AP plasma membrane of GBEC, consistent with its localization in murine and human GB epithelium (20, 36), we hypothesized that biliary apoA-I interacts with SR-BI and thereby initiates a signaling cascade that ultimately leads to BL cholesterol efflux. Such a pathway may also involve synthesis and secretion of apolipoproteins from the BL side of the cell. SR-BI interacts with apoA-I (54) and is postulated to be the mechanism whereby SR-BI interacts with HDL in the hepatocyte. We postulated that a similar phenomenon, albeit with biliary apoA-I and without HDL, occurs at the interface between bile and GBEC. To test this model, several experiments were performed. First, we found that the helix 10 truncation mutant of apoA-I, which has been implicated in the apoA-I/ABCA1 functional interaction (44), did not elicit BL cholesterol efflux when applied to either the AP or BL surface of GBEC. This suggests that the same COOH-terminal helix 10 region of apoA-I is important in both the functional interaction with ABCA1 at the BL surface and in the binding/signalizing role postulated at the AP surface. Clearly, further studies are needed to define the AP binding/signalizing role that biliary apoA-I may play. Second, we looked for an interaction between apically applied apoA-I and SR-BI. Cross-linking experiments following AP application of apoA-I and immunoprecipitation with the SR-BI antibody and subsequent Western blot analysis with an apoA-I antibody revealed three bands, one of which (∼110 kDa) corresponds to an SR-BI-apoA-I complex and is one-half the size of a similar complex reported elsewhere (54). As postulated by these authors, the mass of this complex could be accounted for by apoA-I cross-linking to itself (providing ∼60 kDa of the total, because apoA-I molecular mass is 28 kDa), with the remainder coming from SR-BI multimers or SR-BI interacting with other membrane proteins. Another protein that acts as an apoA-I receptor is the β-chain of ATP synthase, with a molecular mass of 50 kDa (35); this protein could conceivably also

the absence of a cholesterol acceptor. The finding that apoE synthesis is increased with cholesterol loading (and enhanced even further following cholesterol loading and AP apoA-I treatment) and is secreted into the BL compartment suggests a plausible mechanism.

How could endogenously synthesized apoE facilitate ABCA1-mediated BL cholesterol efflux? The simplest explanation is concomitant secretion of apoE into the BL compartment, with this apolipoprotein acting as the cholesterol acceptor for ABCA1-mediated efflux. However, because the relationship between apoE and ABCA1 is complex in other cell types, this complexity may be maintained in GBEC. For example, cholesterol efflux mediated by endogenous apoE is independent of ABCA1 (17), and ABCA1 modulates the secretion of apoE (52). Therefore, the possibility exists that apoE mediates BL cholesterol efflux in GBEC independent of ABCA1. Alternatively, a secretion-capture mechanism for apoE may exist at the BL membrane of the GBEC, as has been proposed for hepatocytes (19). Such a mechanism might explain the low levels of apoE secreted into the BL compartment, relative to the amount of apoE found within the cell.

The finding that apically applied apoA-I elicited BL cholesterol efflux in the absence of transcytosis of this apolipoprotein raised the possibility that apoA-I in bile

Fig. 9. LXR<sub>R</sub>/RXR ligand treatment elicits apoA-I expression. A: immunoprecipitation: [35S]methionine labeling of cells was performed, followed by immunoprecipitation with an apoA-I antibody. Lane 1: no treatment; lane 2: LXR<sub>R</sub>/RXR treatment. Shown are the results of SDS-PAGE following immunoprecipitation. This experiment was repeated with similar results. B: quantitation of radioactivity in the immunoprecipitated pellet. Shown are the results of scintillation counts of pellets precipitated with either the apoA-I antibody or with albumin in untreated cells and in cells treated with ligands for LXR<sub>R</sub>/RXR. *P < 0.01 between the LXR<sub>R</sub>/RXR treated/apoA-I antibody immunoprecipitated group vs. no-treatment group and the LXR<sub>R</sub>/RXR treated/apoA-I antibody immunoprecipitated group. CPM, counts/min.

Fig. 10. LXR<sub>R</sub>/RXR ligand treatment elicits BL apoA-I secretion. ApoA-I in the BL media was measured using an ELISA. Shown are the results of apoA-I secretion into the BL compartment under baseline conditions (no treatment) compared with the LXR<sub>R</sub>/RXR ligand treatment group. No apoA-I was detectable in the AP media. This experiment was repeated 3 times with similar results. *P < 0.01 vs. no-treatment group.

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be part of the immunoprecipitated SR-BI/apoA-I complex. Further studies are needed to elucidate the identity of the three bands identified in these cross-linking experiments.

Oxysterols in bile may be another means to regulate cholesterol uptake, intracellular trafficking, and efflux in GBEC. Numerous oxysterol species have been found in bile, some of which may activate LXRα, although to date, identity has been assigned to only two species, cholesta-4,6-diene-3-one and cholest-4-ene-3-one. Although these two oxysterols are not known to be activators of LXRα, the presence of numerous other as yet unassigned oxysterol species in bile leaves open the possibility that nuclear hormone receptor ligands may originate in bile. Alternatively, oxysterols generated intracellularly and reflecting cellular free cholesterol levels could serve as the ligands for LXRα. LXRα activation upregulates ABCA1 expression in GBEC, consistent with findings in other cell types (9, 49). In addition, LXRα/RXR activation leads to the upregulation of apoE mRNA as well as apoA-I synthesis and secretion. Because BL cholesterol efflux activated by LXRα/RXR ligands likely proceeds via an ABCA1-mediated pathway (32), the finding that GBEC also secrete apoA-I into the BL compartment following LXRα/RXR stimulation suggests a mechanism that allows the presence of a cholesterol acceptor that facilitates ABCA1-mediated cholesterol efflux on the BL membrane. Although we did not demonstrate apoE secretion into the BL membrane following LXRA/RXR treatment, nevertheless the upregulation of synthesis of apoE by LXRA/RXR may be another means to facilitate cholesterol efflux from the BL membrane, as discussed above. Therefore, oxysterols originating in bile or inside the cell, acting via activation of LXRA/RXR, may be master regulators of cholesterol transport in GBEC, as has been shown for the hepatocyte and the enterocyte (4, 48). The finding that LXRA/RXR ligand treatment significantly decreases the esterified cholesterol pool in GBEC is consistent with the concept that activation of these nuclear hormone receptors stimulates diverse pathways to facilitate cellular cholesterol efflux mechanisms, as proposed for hepatocytes and enterocytes (48).

Our laboratory previously showed (32) that AP cholesterol efflux is activated by LXRA/RXR using a non-ABCA1-mediated mechanism. Whereas the mechanism of AP cholesterol efflux in GBEC was not directly investigated, tantalizing clues exist. First, LXRA/RXR activates the half-transporters ABCG5/ABCG8 (47), which have been implicated in biliary cholesterol secretion and intestinal cholesterol absorption (56). Second, ABCG5/ABCG8 is localized on the AP membrane of hepatocytes and enterocytes (12). Third, ABCG5/ABCG8 mRNA transcripts and protein are present in murine GBEC by RT-PCR and immunoblotting (A. Tauscher and R. Kuver, unpublished observations). Therefore, it is tempting to speculate that ABCG5/ABCG8 is present on the AP membrane of GBEC and mediates LXRA/RXR-activated AP cholesterol efflux. Arguing against this model is the finding that no significant difference in absorption rates of β-sitosterol and cholesterol was seen in human GB explants (18). Further studies into the mechanisms of apically directed cholesterol efflux regulated by LXRA/RXR ligands in GBEC are therefore necessary.

The quantity of apoA-I and -E secreted into the BL media was low. This, in concert with the low levels of cholesterol efflux documented in GBEC (32), indicates that in this model system, uptake, trafficking, and efflux pathways do not involve large amounts of cholesterol. From a clinical perspective, this is not surprising, as cholecystectomy does not lead to measurable changes in serum cholesterol levels. Low levels of cholesterol flux, in the setting of GB bile that contains extremely high levels of cholesterol, is likely, over time, to lead to cholesterol accumulation unless counteracting cellular mechanisms exist. Because the GB epithelium is exposed for long periods to the highest concentrations of cholesterol found anywhere in vivo, we speculate that GBEC have evolved mechanisms to defend themselves against the accumulation of excessive amounts of cholesterol. The expressions of apoA-I and -E, along with expression of ABCA1 and SR-BI, are hypothesized to serve the function of properly disposing of excess cholesterol that enters GBEC from bile. The significance of the findings of this work lies not so much in the quantity of cholesterol that is transported but in the fact that such vectorial transport mechanisms exist at all in GBEC. Whether such mechanisms are dysfunctional in vivo in the setting of cholesterol gallstone formation remains to be determined. Perhaps more exciting is the prospect that these mechanisms could be manipulated to therapeutic benefit either by pharmacological or genetic means. The results described in this work are done in a model in vitro system; in vivo, where there is a constant flux of cholesterol into and out of the biliary and plasma compartments, even low levels of perturbations in cholesterol uptake, intracellular trafficking, and efflux could, over time, add up to physiologically relevant phenomena. Therefore, it will be important to follow up the current findings in in vivo model systems.

apoA-I and -E synthesis and secretion have been well studied in hepatocytes (3, 15). For example, in monkey hepatocytes and in HepG2 cells, the majority of the apoA-I that is synthesized is secreted (31). The story with apoE is more complex, with multiple roles postulated for this lipoprotein including serving as a ligand for receptor-mediated uptake of lipoproteins, remnant lipoprotein clearance, and intracellular functions such as modulation of triglyceride incorporation into newly synthesized VLDL and control of cholesterol efflux (10). An interesting phenomenon is that a significant pool of apoE is recycled in the hepatocyte, and apoE has been postulated to act in a secretion-recapture role with respect to remnant lipoprotein metabolism (19). Although the GBEC and the hepatocyte are clearly functionally distinct, whether shared roles exist for these apolipoproteins in the GB are not known. ABCA1 can perhaps provide an instructive analogy: both hepatocytes and GBEC express ABCA1 on the BL plasma
membrane (32, 38), yet the physiological significance of this transporter is clearly different, with hepatocyte ABCA1 playing a role in regulating plasma HDL levels, whereas GBEC ABCA1 does not.

This study is the first to document that GBEC are capable of synthesizing and secreting apoA-I and -E. The source of biliary apoA-I has been presumed to be the hepatocyte, although the intrahepatic biliary tree also expresses apoA-I mRNA and protein (40, 41). Given the complexities of apolipoprotein function, additional studies are needed to define the roles of these apolipoproteins in the GB. Additional studies are needed to further define the functional relevance of these apolipoproteins and to determine how the GB epithelial cell handles the fluctuating biliary cholesterol levels it routinely encounters on its AP surface.

Our studies were performed on cultured canine GBEC, a model system that has provided insights into several areas of GB cell physiology (25–27). Future investigations could be performed in murine GBEC, as investigations could be performed in murine GBEC, as identification of adjacent ABC transporters. Although the intrahepatic biliary tree is capable of synthesizing and secreting apoA-I and -E, whereas GBEC ABCA1 does not. ABCA1 playing a role in regulating plasma HDL levels.

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DISCLOSURES

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