LXR/RXR activation enhances basolateral efflux of cholesterol in CaCo-2 cells

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Abstract Regulation of gene expression of ATP-binding cassette transporter (ABC)A1 and ABCG1 by liver X receptor/retinoid X receptor (LXR/RXR) ligands was investigated in the human intestinal cell line CaCo-2. Neither the RXR ligand, 9-*cis* retinoic acid, nor the natural LXR ligand 22-hydroxycholesterol alone altered ABCA1 mRNA levels. When added together, ABCA1 and ABCG1 mRNA levels were increased 3- and 7-fold, respectively. T0901317, a synthetic non-sterol LXR agonist, increased ABCA1 and ABCG1 gene expression 11- and 6-fold, respectively. ABCA1 mass was increased by LXR/RXR activation. T0901317 or 9-*cis* retinoic acid and 22-hydroxycholesterol increased cholesterol efflux from basolateral but not apical membranes. Cholesterol efflux was increased by the LXR/RXR ligands to apolipoprotein (apo)A-I or HDL but not to taurocholate/phosphatidylcholine micelles. Actinomycin D prevented the increase in ABCA1 and ABCG1 mRNA levels and the increase in cholesterol efflux induced by the ligands. Glyburide, an inhibitor of ABCA1 activity, attenuated the increase in basolateral cholesterol efflux induced by T0901317. LXR/RXR activation decreased the esterification and secretion of cholesterol esters derived from plasma membranes. Thus, in CaCo-2 cells, LXR/RXR activation increases gene expression of ABCA1 and ABCG1 and the basolateral efflux of cholesterol, suggesting that ABCA1 plays an important role in intestinal HDL production and cholesterol absorption.—Murthy, S., E. Born, S. N. Mathur, and F. J. Field. **LXR/RXR activation enhances basolateral efflux of cholesterol in CaCo-2 cells.** ***J. Lipid Res.*** **2002.** 43: **1054–1064.**

Supplementary key words liver X receptor • retinoid X receptor • 9-*cis* retinoic acid • 22-hydroxycholesterol • T0901317 • ABCA1 • ABCG1 • apolipoprotein A-I • high density lipoprotein • cholesterol absorption

Liver X receptors (LXR) of the nuclear receptor superfamily are transcription factors that regulate the transcription of several genes involved in cholesterol metabolism (1–7). It has been proposed that the LXR might act as a sterol sensor. By responding to an influx of excess cellular cholesterol, LXR activates genes that transcribe proteins that enhance the elimination of, or limit the accumulation of, cellular cholesterol (6). Recognized gene targets of LXR include cholesterol 7α-hydroxylase (1, 8), cholesterol ester transfer protein (3), ATP-binding cassette (ABC) membrane transporters (5, 6, 9), and sterol regulatory element-binding protein-1c (SREBP-1c) (7). LXRs bind to DNA of genes as obligate heterodimers complexed with retinoid X receptors (RXRs) (10). This LXR/RXR dimer can be activated by oxysterols (ligands for LXR) or by retinoids (ligands for RXR) (1, 2, 11, 12). LXRs are highly expressed in the intestine, suggesting that these transcription factors might play a role in intestinal cholesterol metabolism (13, 14). Indeed, recent evidence suggests a role of LXR/RXR in the absorption of cholesterol by activating gene expression for the intestinal ABC transporter ABCA1 (6). Moreover, LXR activation was recently used as a tool to identify two ABC transporters, ABCG5 and ABCG8, as gene candidates responsible for abnormal sterol absorption in sitosterolemia (5).

Unlike other dietary fats that are almost completely absorbed, the absorption of cholesterol by the small intestine is inefficient and variable. Humans absorb only 30–65% of the cholesterol they ingest (15). Although several postulates have been offered, the explanation(s) as to why so little of dietary cholesterol is absorbed remains unclear. Since there is a recognized relationship between the efficiency of cholesterol absorbed by the small intestine and serum LDL cholesterol concentrations, and thus a risk of atherosclerosis, a better understanding of this process

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Abbreviations: ABC, ATP-binding cassette transporter; ACAT, acyl-CoA:cholesterol acyltransferase; ActD, actinomycin D; apo, apolipoprotein; FC, free cholesterol; LXR, liver X receptor; MTP, microsomal triglyceride transfer protein; RXR, retinoid X receptor; TC/PC, taurocholate micelles containing phosphatidylcholine; 9cRA, 9-*cis* retinoic acid; 22OHC, 22-hydroxycholesterol.

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could lead to improved therapies for the treatment of hypercholesterolemia (16, 17).

The present study was performed in the human intestinal cell line CaCo-2 to investigate the role of LXR/RXR and its ABC gene targets in cholesterol efflux and the absorption of cholesterol. Because oxysterols also activate the sterol-regulatory element binding pathway and, hence, cholesterol synthesis (18), the effects of oxysterols on cholesterol absorption and metabolism may not be solely related to their activation of the LXR/RXR pathway. Therefore, a synthetic non-steroidal LXR-selective agonist, T0901317, was used in addition to the naturally-occurring physiologic ligands for nuclear receptors 9-*cis* retinoic acid and 22-hydroxycholesterol (19). The results show that 9-*cis* retinoic acid together with 22-hydroxycholesterol enhance gene expression for ABCA1 and ABCG1, as does the non-sterol LXR agonist T0901317. LXR/RXR activation also increases ABCA1 mass. Increased expression of ABCA1 or ABCG1 is associated with increased efflux of cholesterol to apolipoprotein (apo)A-I or HDL from basolateral membranes, suggesting that ABC transporters play an important role in the formation of intestinally derived HDL. In addition, LXR/RXR activation decreases the amount of cholesterol derived from the plasma membrane that is esterified and secreted. Thus, enhancing ABCA1 or ABCG1 expression in CaCo-2 cells depletes the plasma membrane pool of cholesterol that is destined for lipoprotein assembly and secretion.

MATERIALS AND METHODS

Materials

[^{3}H]cholesterol (48.3 Ci/mmol) and [α-^{32}P]UTP (3,000 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Delipidated FBS was from Intracel (Issaquah, WA). Polyclonal horseradish peroxidase (HRP)-conjugated and monoclonal antibodies to human apolipoprotein B (apoB) were from Biodesign International (Saco, ME). TMB Microwell Peroxidase Substrate System including 3,3′,5,5′-tetramethyl benzidine and hydrogen peroxide for measuring HRP was from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Nunc 96-well immunoplates were obtained from PGC Scientific (Gaithersburg, MD). Protease inhibitor mix, 3-[4,5-Dimethylthiazol-2-yl]-2,5-tetrazolium bromide (MTT), Tri Reagent, 9-*cis* retinoic acid, human apoA-I, cholesterol, taurocholate, phoshphatidylcholine from egg yolk, glyburide, goat anti-rabbit polyclonal antibody, GPO Trinder kit, actinomycin D, oleic acid, and fatty acid-free BSA were from Sigma Chemicals (St. Louis, MO). 22-Hydroxycholesterol was purchased from Steraloids (Newport, RI). HDL was isolated from human plasma by differential ultracentrifugation as described previously (20, 21). The microsomal triglyceride transfer protein (MTP) inhibitor, BMS-201038, was a gift from Bristol Myers Squibb (New Brunswick, NJ). T0901317 was a gift from Tularik Inc. (San Francisco, CA). Anti-human ABCA1 rabbit polyclonal antibody was purchased from Novus Biochemicals (Littleton, CO).

Cell culture

CaCo-2 cells were cultured in T-75 flasks (Corning Glassworks, Corning, NY) in DMEM (GIBCO, Grand Island, NY) with 4.5 g/l glucose, and supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. Once the flasks reached 80% confluency, the cells were split and seeded at a density of 0.2 × 10^5 cells/well onto polycarbonate micropore membranes (0.4 μm pore size, 24 mm diameter) inserted into transwells (Costar, Cambridge, MA). For experiments in which apoB mass and cell viability were estimated, cells were sub-cultured in 6.5 mm diameter transwells. For some experiments in which RNA was isolated, cells were grown in 6-well plates. Cells were fed every other day and were used 14 days after seeding.

Stock solutions of taurocholate, phosphatidylcholine, and cholesterol were prepared in 95% ethanol. Oleic acid was dissolved in chloroform. To prepare micelles, appropriate volumes of taurocholate, cholesterol, and oleic acid or taurocholate and phosphatidylcholine were dried under nitrogen and taken up in enough M199 containing 10 mM HEPES to obtain the desired final concentration of lipids. The resulting micellar solution was stirred vigorously at 37°C until clear and then added to cells.

Cell viability/proliferation

Cell viability and proliferation were assessed by measuring the activity of mitochondrial dehydrogenase as described previously (22). This assay is based on the mitochondrial conversion of a tetrazolium salt into a blue formazan product that is released into the medium. Following an overnight incubation with the treatments, the release of the colored formazan dye into the medium was measured spectrophotometrically. Compared with cells incubated for 24 h with 0.2% ethanol, the relative absorbance of medium from cells incubated with 1 μM 9-*cis* retinoic acid, 24.83 μM 22-hydroxycholesterol, or both were 1.15 ± 0.18, 1 ± 0.09, and 1.04 ± 0.07, respectively. Compared with cells incubated with 0.02% DMSO, the relative absorbance of the dye released from cells incubated with 1 μM T0901317 was 1.05 ± 0.06. Similar results were obtained after 48 h of incubation with the treatments.

RNase protection assays

Gene	Primer sequence	Product
Human ABCA1-5164	F: CTT TTG CTG TAT GGG TGG TC	420 bp
Human ABCA1-5583	R: TAC AGG TCT GGG CCT GAT GAA	
Human ABCG1-71	F: GTG GTC TCG CTG ATG AAA GG	311 bp
Human ABCG1-381	R: CCG AGG TCT CTC TTG TGG TC	

DNA-free RNA was extracted from CaCo-2 cells using the High Pure RNA Isolation kit (Boehringer Mannheim, Indianapolis, IN). cDNA was synthesized and amplified using gene-specific forward (F) and reverse (R) primers with Thermoscript RT-PCR System (GibcoBRL Life Technologies, Grand Island, NY). T7 promoters were added to the PCR products using the Lig'nScribe kit (Ambion, Inc., Austin, TX). The products were then amplified by PCR using gene-specific forward primers and T7 adapter primers. Anti-sense [^{32}P]UTP labeled RNA probes were synthesized using T7 promoter-containing PCR products or linearized plasmid as template with the Maxiscript In Vitro Transcription kit (Ambion). The labeled anti-sense RNA probes were purified on denaturing 6% acrylamide/8 M urea gels. Total RNA from CaCo-2 cells was isolated using Tri Reagent (Sigma, St. Louis, MO). mRNA transcripts were estimated by RNase protection assays using RPA III kit from Ambion as described previously (23). Protected fragments were resolved on denaturing 6% acrylamide/8 M urea gels in TBE buffer. Kodak Biomax MS film with TranScreen HE was used to visualize the bands on the gel. In Figs. 1 and 6, densities of the bands were quantitated by the NIH Image J software program and mRNA levels were expressed in relation to control values.

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Validity of the RNase protection assay was established by documenting RNA concentration dependence and running triplicates of each sample on a gel (24–28) (Ambion Technical Bulletin 151). The abundance of 18S rRNA was estimated using an 18S rRNA probe (pTRI RNA 18S, Ambion) that yielded a protected fragment of 80 bp.

ABCA1 mass

Cells were incubated for 18 h with 1 μM T0901317 or 1 μM 9-*cis* retinoic acid and/or 24.83 μM 22-hydroxycholesterol. Control cells received 0.2% ethanol and 0.02% DMSO. After the incubation, cells were rinsed with PBS, scraped and lysed with Buffer C (10 mM HEPES, 1.5 mM magnesium chloride, 10 mM potassium chloride, 1 mM EDTA, 1 mM EGTA, pH 7.4) containing 866.7 μM AEBSF, 0.7 μM Aprotinin, 17.5 μM leupeptin, 30 μM bestatin, 12.5 μM pepstatin A, 11.7 μM E-64, 22.7 μM ALLN, and 1.1 mM dithiothreitol. Cell homogenates were centrifuged for 30 min at 100,000 *g* and the pellet containing total membranes was resuspended in 80 μl Buffer C containing protease inhibitors and 20 μl Laemmli Sample Buffer. Approximately 100 μg membrane protein were separated by SDS-PAGE on an 8% porous gel and transferred to an Immobilon-P PVDF membrane (Millipore, Bedford, MA). After rinsing in TBS (25 mM Tris-HCl, pH 7.5, 150 mM sodium chloride), the membrane was air-dried for 15 min, washed with water-methanol (1:1, v/v) followed by methanol alone. After drying for 10 min under vacuum at room temperature, the membrane was incubated for 1 h with anti-human ABCA1 rabbit polyclonal antibody. The antibody was diluted 300-fold in TBS containing 0.05% Tween-20, 2% non-fat dry milk, and 1% goat serum (Blotto). After washing in TBS containing Tween-20, the membrane was then incubated for 1 h with goat anti-rabbit antibody cross-linked to horseradish peroxidase and diluted 200,000-fold in Blotto. The membrane was washed thoroughly in TBS containing Tween-20 and horseradish peroxidase detected using SuperSignal West Femto Sensitivity Substrate chemiluminescent detection system (Pierce Endogen, Rockford, IL).

Cholesterol efflux

Cells were labeled for 24 h with 5 μCi/well of [^{3}H]cholesterol in the presence of 1% delipidated FBS. After extensive washing to remove unincorporated labeled cholesterol, cells were incubated with 1 μM 9-*cis* retinoic acid and/or 24.83 μM 22-hydroxycholesterol. Control cells received 0.2% ethanol alone. Another set of cells was incubated with 0.02% DMSO in the presence or absence of 1 μM T0901317. Treatments were added to the upper chambers in M199 whereas the lower chambers received M199 alone. After 24 h, media in both chambers were replaced with fresh media containing the respective treatments in the presence or absence of 35 μg/ml HDL protein or 20 μg/ml apoA-I. The acceptors were added to the lower chambers as well. In some experiments 5.5 mM taurocholate micelles containing 250 μg/ml phosphatidylcholine were used as non-specific acceptors and were added to the upper or lower chambers. Following another 24 h of incubation, media from both chambers were collected and centrifuged at 13,500 rpm for 2 min. Aliquots from the supernatants were extracted for lipids according to the method of Bligh and Dyer (29). After rinsing with PBS, cell lipids were extracted with hexanes-isopropylalcohol-water (3:2:0.1, v/v/v). Organic extracts from both cells and media were dried under nitrogen, taken up in 120 μl of chloroform and separated by TLC in hexanes-ethyl ether-methanol-acetic acid (85:15:1:1, v/v/v/v) as previously described (30). Bands corresponding to free cholesterol and cholesteryl esters were visualized under iodine vapor, scraped, and counted by liquid scintillation counting.

Esterification and secretion of plasma membrane-derived cholesterol

The esterification of cholesterol derived from plasma membranes and the secretion of cholesteryl esters were estimated by incubating cells for 18 h with 0.2% ethanol in the presence or absence of 1 μM 9-*cis* retinoic acid and 24.83 μM 22-hydroxycholesterol. Another set of cells were incubated with 0.02% DMSO with or without 1 μM T0901317. Cells were washed and incubated for 90 min at 4°C with 5 μCi/well of [^{3}H]cholesterol in the presence of 1% delipidated FBS. After extensive washing to remove unincorporated labeled cholesterol, cells were incubated for 4 h with 5 mM taurocholate, 200 μM cholesterol, and 500 μM oleic acid. Lipids in cells and basal media were extracted and radioactivity in unesterified, and esterified cholesterol was estimated as described above.

Other analyses

Protein content was estimated using the BCA kit (Pierce Endogen, Rockford, IL). Triglyceride and apoB mass were estimated using the GPO Trinder kit and sandwich ELISA, respectively (22, 31). ACAT activity was estimated in the presence or absence of T0901317, 9-*cis* retinoic acid, and/or 22-hydroxycholesterol as previously described (30). Statistical analysis was performed by Tukey's test.

RESULTS

Effect of RXR/LXR ligands on ABC gene expression and ABCA1 mass

To investigate the regulation of ABCA1 and ABCG1 gene expression by ligands for the LXR/RXR heterodimer, CaCo-2 cells were incubated with the naturally occurring RXR ligand, 9-*cis* retinoic acid, and/or the LXR ligand 22-hydroxycholesterol. In addition, other cells were incubated with the synthetic non-sterol LXR agonist T0901317. Following an 18-h incubation, the abundance of ABCA1 and ABCG1 mRNA was estimated. The results are shown in **Fig. 1**. Neither 9-*cis* retinoic acid nor 22-hydroxycholesterol alone altered gene expression of ABCA1. When the two ligands were added together, however, mRNA levels for ABCA1 were increased 3-fold. The synthetic LXR agonist, T0901317, was more potent than the naturally occurring ligands in enhancing ABCA1 gene expression. ABCA1 gene expression increased with increasing concentrations of T0901317, with 1 μM causing a maximal increase of 11-fold. At 10 μM of T0901317, mRNA levels for ABCA1 were less than what was observed at 1 μM but was still greater than control values.

In contrast to ABCA1, gene expression of ABCG1 was more sensitive to regulation by 9-*cis* retinoic acid and 22-hydroxycholesterol. When either of these ligands was added alone, mRNA levels for ABCG1 increased 2-fold. When the two ligands were added together, ABCG1 mRNA levels were increased 7-fold. In response to 1 μM T0901317, mRNA levels for ABCG1 increased by 6-fold.

In data not shown, estimation of ABCG5 and ABCG8 transcripts by RNase protection was attempted. Both transcripts were in low abundance and difficult to detect. Because of this, a consistent effect of LXR agonists on these two transcripts was not appreciated.

To address whether the induction of gene expression for both ABCA1 and ABCG1 by the ligands was secondary

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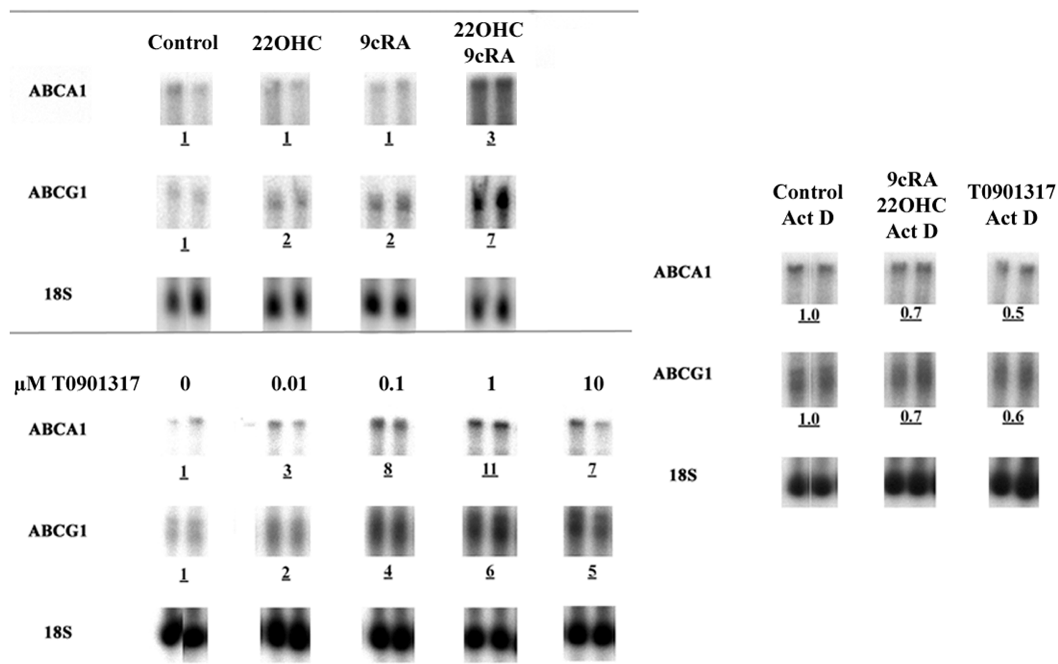

Fig. 1. The effect of liver X receptor/retinoid X receptor (LXR/RXR) ligands on ABCA1 and ABCG1 gene expression. Cells were incubated for 18 h with 0.2% ethanol alone or together with 1 μM 9-*cis* retinoic acid (9cRA) and/or 24.83 μM 22-hydroxycholesterol (22OHC). Alternatively, cells were incubated with 0.02% DMSO with or without increasing concentrations of T0901317. Another set of cells was incubated with 9cRA together with 22OHC or 1 μM T0901317 in the presence of 5 μg/ml actinomycin D (ActD). Following incubation, total RNA was extracted from cells and the abundance of mRNA for ABCA1 and ABCG1 and the levels of 18S rRNA were estimated by RNase protection assays as described in Materials and Methods. A representative autoradiogram from one of three separate experiments is shown together with the relative densities of each of the bands (underlined). N = 2 per treatment.

to enhanced gene transcription, cells were incubated with T0901317 or 9-*cis* retinoic acid and 22-hydroxycholesterol in the presence of a general inhibitor of gene transcription actinomycin D (right panel, Fig. 1). The addition of actinomycin D prevented the increase in mRNA levels for both ABCA1 and ABCG1 in response to 9-*cis* retinoic acid and 22-hydroxycholesterol or T0901317.

Figure 2 shows results from two experiments investigating the effect of LXR/RXR activation on ABCA1 mass. Cells were incubated for 18 h with 9-*cis* retinoic acid and/or 22-hydroxycholesterol or T0901317. Following the incubation, ABCA1 in membranes separated by SDS-PAGE was estimated by immunoblotting. The antibody detected two distinct bands characteristic of ABCA1 at about 220 kDa. In some, but not all, experiments, the density of both bands increased when incubated with 9-*cis* retinoic acid together with 22-hydroxycholesterol or T0901317. It was the density of the lower band, however, that consistently increased following LXR/RXR-ligand activation. It is clear from the results of both experiments that the amount of ABCA1 mass increased in cells incubated with the LXR agonist T0901317, or the combination of 9-*cis* retinoic acid and 22-hydroxycholesterol. In the one experiment, 9-*cis* retinoic acid alone modestly increased ABCA1 mass.

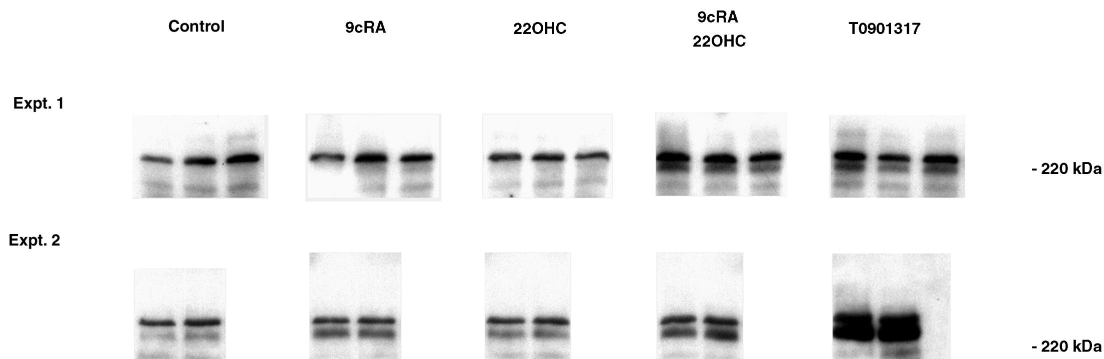

Fig. 2. Effect of LXR ligands on ABCA1 mass. Cells were incubated with 1 μM 9cRA and/or 24.83 μM 22OHC or 1 μM T0901317 in 0.2% ethanol and 0.02% DMSO. After 18 h, cells were rinsed with PBS, lysed, and total membranes isolated as described in Materials and Methods. Proteins were separated by SDS-PAGE and immunoblotted using a polyclonal anti-ABCA1 antibody. Results from two separate experiments are shown. N = 3 in experiment 1 and N = 2 in experiment 2.

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Effect of RXR/LXR ligands on cholesterol efflux

Enhancing gene expression of ABCA1 or ABCG1 has been associated with increased cholesterol efflux from peripheral cells (6). To investigate whether LXR/RXR activation would cause increased cholesterol efflux from intestinal cells, CaCo-2 cells were pre-labeled with cholesterol and then incubated for 24 h with 9-*cis* retinoic acid, 22-hydroxycholesterol, T0901317, or a combination of the ligands. Following the incubation, either apoA-I or HDL was added to both the upper and lower chambers of some of the transwells and the incubations were continued for another 24 h. The percent of total cholesterol released into the upper and lower chambers was then determined. The results are shown in **Fig. 3**. Compared with control cells, in the presence or absence of either apoA-I or HDL, 9-*cis* retinoic acid and 22-hydroxycholesterol did not alter the amount of cholesterol released into the upper chamber (Fig. 3A, top panel). In contrast to HDL, which dramatically increased the amount of cholesterol released into the apical medium, apoA-I was ineffective in increasing cholesterol efflux from the apical membranes. In contrast to what was observed in the upper chamber, compared with control cells, retinoic acid together with 22-hydroxycholesterol significantly increased the amount of cholesterol recovered in the lower chamber (Fig. 3A, bottom panel). This occurred either in the presence or absence of HDL or apoA-I. In the presence of cholesterol acceptors, however, the amount of cholesterol released into the lower chamber by both control cells and cells incubated with the RXR/LXR ligands was enhanced. In data not shown, cholesterol efflux was not altered by either 9-*cis* retinoic acid or 22-hydroxycholesterol when added alone.

Similar to the natural ligands, the synthetic non-sterol LXR ligand T0901317 did not alter the amount of cholesterol released into the upper chamber (Fig. 3B, top panel). In contrast, T0901317 caused a 3-fold increase in cholesterol released into the lower chamber. This was observed in the presence of either apoA-I or HDL (Fig. 3B, bottom panel).

Since apoA-I and HDL are not found in the lumen of the intestine and would not be considered physiologic acceptors of cholesterol released apically, taurocholate micelles containing phosphatidylcholine (TC/PC) were used instead of apoA-I or HDL. When the micelles were added to either the upper or lower chamber, the amount of cholesterol released was not altered by T0901317 (Fig. 3B, top and bottom panels). Since cholesterol efflux into the lower chamber was similar whether HDL or apoA-I was used as an acceptor, HDL was used in the remainder of the experiments.

To address whether preventing the LXR/RXR ligand-induced increase in ABCA1 or ABCG1 gene expression would attenuate cholesterol efflux, the above experiments were performed in the presence of actinomycin D (Act D) (Figs. 3A, B). The increase in cholesterol recovered from the lower chamber of cells incubated with 9-*cis* retinoic acid and 22-hydroxycholesterol or T0901317 was completely prevented by the addition of actinomycin D (lower panels). In contrast, actinomycin D did not alter the amount of cholesterol recovered in the upper chamber (upper panels).

To further investigate the role of ABC transporters in cholesterol efflux, glyburide, a known inhibitor of ABC transporters (32–34), was added to the lower chamber of cells incubated with T0901317. Glyburide significantly attenuated the effect of T0901317 on the basolateral efflux of cholesterol (Fig. 3B, bottom panel). The inhibitor had no effect on the amount of cholesterol released into the upper chamber of either control cells or cells incubated with the LXR agonist (Fig. 3B, upper panel). In data not shown, when glyburide was added to the upper chamber, it had no effect on cholesterol efflux into either the upper or lower chamber of cells incubated with T0901317.

In Fig. 3C, the amount of cholesterol recovered in the lower well after 3, 6, and 24 h of incubation with HDL in the presence or absence of 9-*cis* retinoic acid and 22-hydroxycholesterol is shown. At each of the time points examined, compared with control cells, significantly more cholesterol was recovered in the lower well of cells incubated with the two ligands.

The results of these experiments strongly suggest that activation of LXR/RXR enhances efflux of cholesterol from basolateral membranes of CaCo-2 cells. Because CaCo-2 cells also secrete lipoproteins basolaterally, it was considered that perhaps the ligands were promoting lipoprotein secretion, and hence, increased the secretion of lipoprotein cholesterol. To address this possibility, the above experiment was repeated in the presence of increasing concentrations of the MTP inhibitor BMS 201038. At a concentration of 0.1 μM, BMS 201038 decreased the basolateral secretion of apoB and triacylglycerol by 95% and 98%, respectively (**Fig. 4A** and **B**). Despite preventing triacylglycerol-rich lipoprotein secretion, the MTP inhibitor did not prevent the increased amount of cholesterol recovered in the lower chamber of cells incubated with T0901317 or 9-*cis* retinoic acid and 22-hydroxycholesterol (Fig. 4C). In addition, neither 9-*cis* retinoic acid and 22-hydroxycholesterol nor T0901317 significantly altered the basolateral secretion of apoB or triacylglycerol (data not shown), suggesting that the LXR/RXR ligands were not promoting lipoprotein secretion. Thus, increased amounts of cholesterol recovered in the lower chamber of cells incubated with LXR/RXR ligands were not due to increased secretion of triacylglycerol-rich lipoproteins.

Effect of T0901317 on plasma membrane cholesterol esterification and secretion

We have previously demonstrated that when CaCo-2 cells are driven to synthesize and secrete triacylglycerol-rich lipoproteins, it is cholesterol derived from the plasma membrane that is utilized for lipoprotein assembly, rather than cholesterol derived from bile-salt micelles or cholesterol that is newly-synthesized (35). We also postulated that the plasma membrane cholesterol utilized for lipoprotein assembly is derived from specialized domains of the membrane, such as rafts or sphingomyelin-rich, caveolin-rich microdomains. Although recently challenged, it has been postulated from results in other cells that cholesterol efflux might occur from these specialized domains (33, 36, 37). We speculated, therefore, that by enhancing

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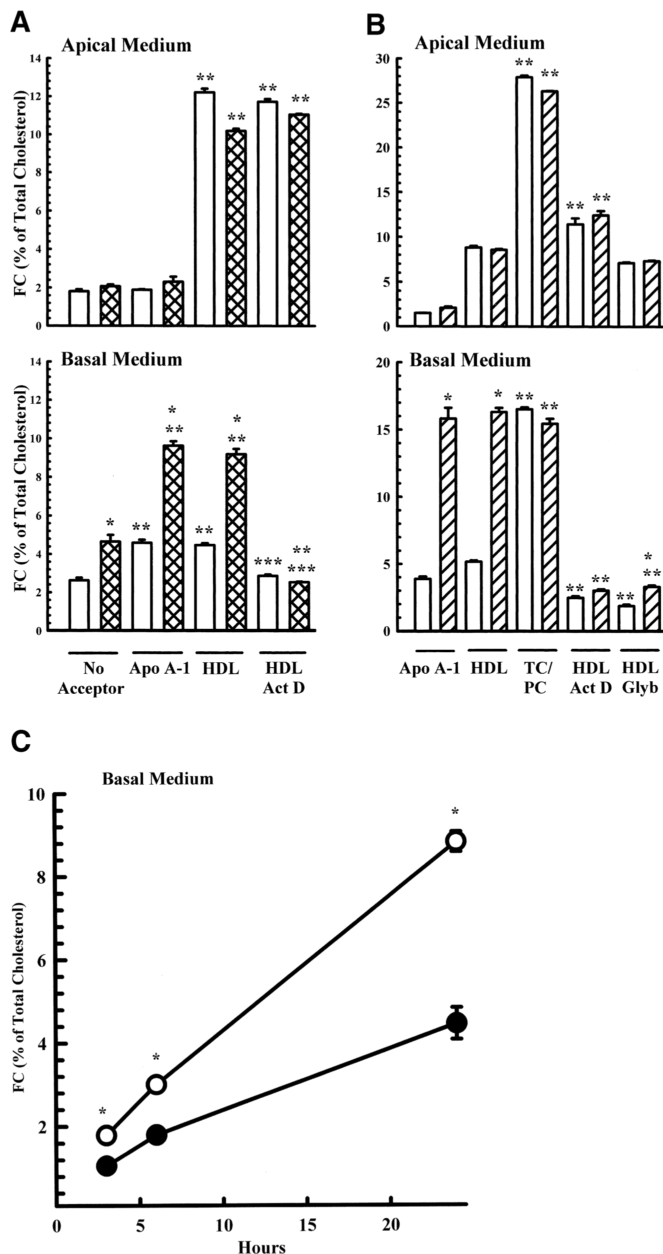

Fig. 3. The effect of LXR/RXR ligands on cholesterol efflux. A: Cells were pre-labeled for 24 h with 5 μCi/well of [^{3}H]cholesterol in the presence of 1% delipidated FBS. After thorough washing to remove unincorporated label, 0.2% ethanol alone or together with 1 μM 9cRA and 24.83 μM 22OHC were added to the upper chambers. The lower chambers contained M199 alone. In addition, some cells received 5 μg/ml Act D. After 24 h of incubation, the medium in the upper chambers was replaced with fresh medium containing the respective treatments with or without 35 μg/ml HDL or 20 μg/ml apolipoprotein (apo)A-I. The lower chambers contained the respective cholesterol acceptors alone. Following incubation for 24 h, the medium in the upper and lower chambers was collected. Lipids in cells and media were extracted and amounts of free and esterified cholesterol were estimated as described in Materials and Methods. Results from one of three representative experiments are shown. Values represent mean ± SEM of the media free cholesterol (FC) expressed as a percent of free and esterified cholesterol in cells and media recovered from both upper and lower chambers. N = 4. Open bar, control; cross-hatched bar, 9cRA and 22OHC. *$P < 0.05$ versus respective control, **$P < 0.05$ versus respective treatment without acceptor, ***$P < 0.05$ versus respective treatment with HDL alone. B: Cells were labeled as described above, and incubated with 0.02% DMSO alone or together with T0901317 in the presence or absence of 5 μg/ml ActD or 1 mM glyburide (Glyb). Glyburide was added only to the lower chamber whereas all other treatments were added to the upper chamber. Cholesterol released from apical and basolateral membranes was estimated in the presence of 20 μg/ml apoA-I, 35 μg/ml HDL, or 5.5 mM taurocholate micelles containing 250 μg/ml phosphatidylcholine (TC/PC). Results from one of three representative experiments are shown as mean ± SEM of FC in media expressed as a percent of free and esterified cholesterol in cells and media. N = 4. Open bar, control; diagonal bar, T0901317. *$P < 0.05$ versus respective control, **$P < 0.05$ versus respective treatment with HDL alone. C: Cells were pre-labeled with cholesterol as described above and then incubated for 24 h with 1 μM 9cRA and 24.83 μM 22HOC. Control cells received 0.2% ethanol alone. Thirty-five micrograms per milliliter of HDL was added to the lower wells and the release of cholesterol into the lower well was estimated at each of the time points examined. Results from three wells are shown as mean ± SEM of FC in media expressed as a percent of free and esterified cholesterol in cells and media. Closed circle, control; open circle, 9cRA and 22OHC. *$P < 0.05$ versus control.

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ABC expression and cholesterol efflux by LXR/RXR activation, the amount of cholesterol contained in these specialized domains would be decreased and less cholesterol would be available for lipoprotein assembly. To address this possibility, cells were incubated for 18 h in the presence or absence of 9-*cis* retinoic acid and 22-hydroxycholesterol or T0901317. Plasma membranes were first labeled with [^{3}H]cholesterol and cells were then incubated with micelles containing 200 μM unlabeled cholesterol. The amount of plasma membrane cholesterol that was transported to the endoplasmic reticulum (cellular cholesteryl esters) and the amount of plasma membrane-derived cholesteryl esters recovered in the lower well were estimated. The results are shown in **Fig. 5A** and **B**. Compared with control cells, in cells incubated with 9-*cis* retinoic acid and 22-hydroxycholesterol, significantly less cholesterol derived from the plasma membrane arrived at the endoplasmic reticulum and was esterified (Fig. 5A, left panel). Moreover, less plasma membrane-derived cholesteryl esters were recovered in the lower well in cells incubated with the ligands (right panel). Similar results were obtained in cells incubated with T0901317 (Fig. 5B). At the concentrations used for these experiments, neither 9-*cis* retinoic acid and 22-hydroxycholesterol nor T0901317 altered ACAT activity in CaCo-2 cells (data not shown). The results suggest that enhancing ABCA1 or ABCG1 expression and cholesterol efflux by LXR/RXR activation depletes a pool of plasma membrane cholesterol that is destined for lipoprotein assembly and secretion.

Effect of micellar cholesterol on ABCA1 and ABCG1 gene expression

The results so far have shown that ligand activation of the LXR/RXR enhances gene expression of ABCA1 and

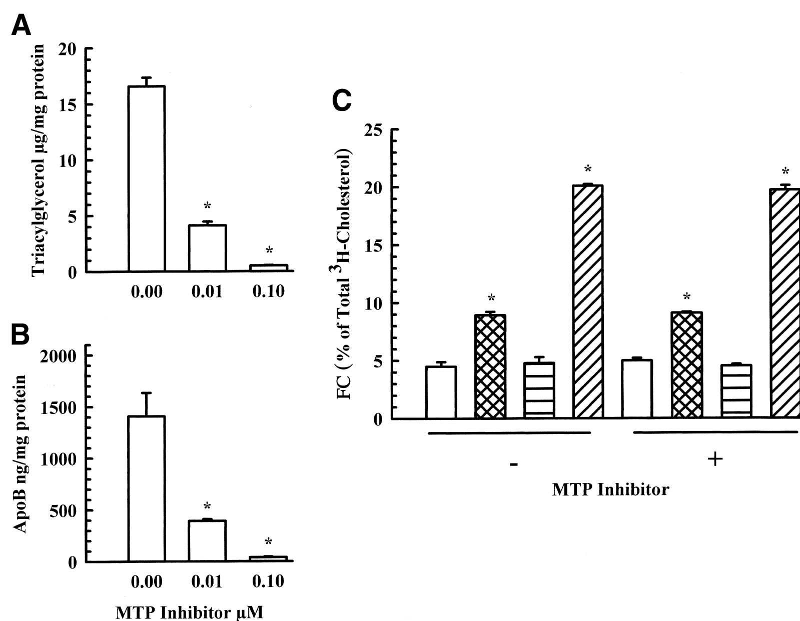

Fig. 4. Effect of MTP inhibition on cholesterol efflux and the secretion of triacylglycerol and apoB. A: Cells were incubated for 18 h with 250 μM oleic acid, 62.5 μM BSA, and increasing concentrations of the MTP inhibitor in 0.1% ethanol. The treatments were added to the upper chamber. Control cells received oleic acid, BSA, and ethanol alone. Following incubation, lipids from medium in the lower chamber were extracted, and the amount of triacylglycerol mass estimated as described in Materials and Methods. Results from four wells are expressed as mean ± SEM. $^*P < 0.05$ versus control. B: Cells were incubated as in A. ApoB mass in the medium from the lower well was analyzed by sandwich ELISA as described in Materials and Methods. $^*P < 0.05$ versus control. C: Cells were pre-labeled with cholesterol and then incubated with 1 μM 9cRA and 24.83 μM 22OHC in ethanol, or 1 μM T0901317 in DMSO as described in Fig. 3. Control cells received 0.2% ethanol or 0.02% DMSO. After 24 h of incubation, medium in the upper chamber was replaced with fresh medium containing the respective treatments with or without 0.1 μM of the MTP inhibitor. 35 μg/ml of HDL was added to both upper and lower chambers and the incubation continued for an additional 24 h. Cholesterol release into the lower chambers was then estimated. Results from 4 wells are shown as mean ± SEM of FC in the basal media expressed as a percent of free and esterified cholesterol in cells and media from both chambers. Open bar, ethanol alone; cross-hatched bar, 9cRA and 22OHC; horizontal bar, DMSO; diagonal bar, T0901317. $^*P < 0.05$ versus respective control.

ABCG1. This is associated with increased cholesterol efflux and decreased triacylglycerol-rich lipoprotein cholesterol secretion. In vivo, however, if intestinal LXR were acting as a cholesterol sensor, influx of cholesterol (or an oxy-derivative) from bile salt micelles would be the natural agonist for LXR. To address this, CaCo-2 cells were incubated with taurocholate micelles containing oleic acid and cholesterol. To enhance the amount of unesterified cholesterol within cells, an ACAT inhibitor was added to some of the dishes. Following incubation, mRNA levels of ABCA1 and ABCG1 were estimated. The results are shown in **Fig. 6**. In cells incubated with micelles containing cholesterol, gene expressions of ABCA1 and ABCG1 were increased 3- and 2-fold, respectively. In the presence of the ACAT inhibitor, mRNA levels for both genes were increased further. Thus, in CaCo-2 cells, the influx of micellar cholesterol, likely acting through LXR, increases the expression of both ABCA1 and ABCG1.

DISCUSSION

Except for the absorption of sterols and certain metallic cations, the small intestine does not usually regulate the absorption of nutrients. It is clear, however, that as the amount of cholesterol in the diet increases, the percentage of cholesterol that is absorbed decreases (15, 38). Moreover, the intestine discriminates between ingested plant sterols and cholesterol by markedly limiting the absorption of plant sterols (15, 38, 39). This suggests that the intestine acts as a barrier to the absorption of sterols and contains a mechanism to protect the organism from excess incoming cholesterol and other sterols.

ABC transporters have recently been implicated in this mechanism. It has been postulated that these proteins regulate the absorption of cholesterol by acting at the apical membrane of intestinal absorptive cells facilitating the efflux of cholesterol back into the lumen. For example, mutations in the newly described ABC half transporter genes ABCG5 and ABCG8 have been found in patients with the disorder β-sitosterolemia suggesting that these transporters regulate sterol absorption in man (5). In a study of mice in which the ABCA1 gene had been deleted, the percent of cholesterol absorbed was found to be increased compared with the amount of cholesterol absorbed by wild-type mice (40). In addition, Repa et al. (6) showed that in mice fed RXR- or LXR-selective agonists intestinal ABCA1 expression was increased, and with this was found an associated decrease in cholesterol absorption. Recent observations, however, have challenged the role of ABCA1 in cholesterol absorption. Groen et al. (41) found that in ABCA1-knock out mice, fecal excretion of

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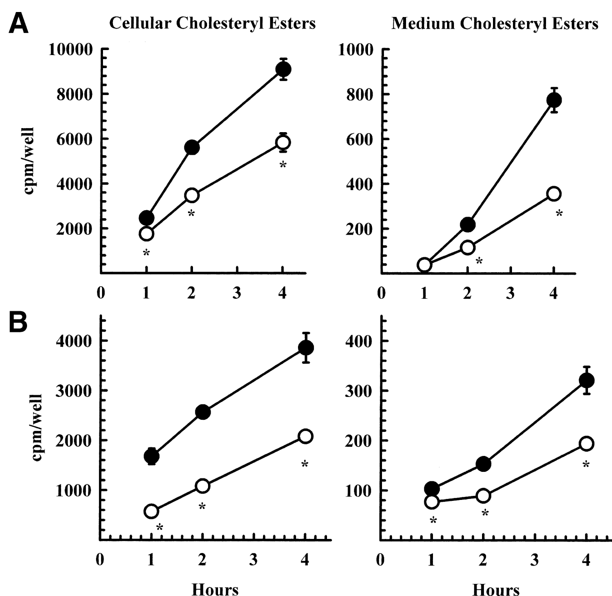

Fig. 5. Effect of T0901317 or 9cRA and 22OHC on the esterification and secretion of plasma membrane cholesterol. Cells were incubated for 18 h with 1 μM 9cRA together with 24.83 μM 22OHC (A) or 1 μM T0901317 (B). The respective controls were 0.2% ethanol and 0.02% DMSO. Cells were then washed and incubated for 90 min at 4°C with 5 μCi/well of [^{3}H]cholesterol and 1% delipidated FCS. After thorough washing, cells were incubated for up to 4 h at 37°C with 5 mM taurocholate, 200 μM cholesterol, and 500 μM oleic acid in the continued presence of the respective treatments. At the indicated times, lipids in cells and media from the lower wells were extracted and the amount of radioactivity in free and esterified cholesterol in cells and media were estimated. Results from one of two representative experiments are expressed as mean ± SEM of cpm/well. N = 6. Closed circle, control; open circle, T0901317 or 9cRA and 22OHC. $*P < 0.05$ versus control.

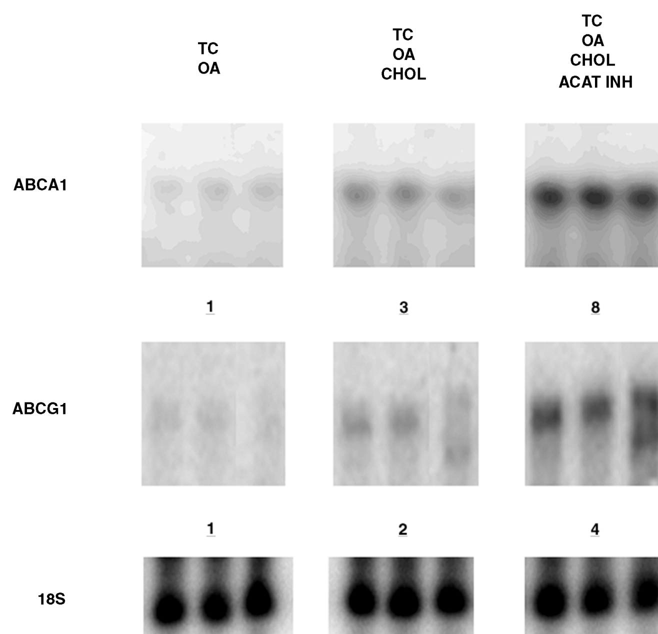

Fig. 6. Effect of cholesterol on the gene expression of ABCA1 and ABCG1. Cells were incubated for 18 h with 5 mM taurocholate, 500 μM oleic acid, and 200 μM cholesterol in the presence or absence of 2 μg/ml of an ACAT inhibitor, PD 128042. Total RNA from cells was extracted and the mRNA abundance of ABCA1, ABCG1, and 18S rRNA was estimated. Results from one of three separate experiments are shown together with the relative optical densities of each of the bands (underlined). N = 3 per treatment.

bile salts and neutral sterols was similar to that observed in wild-type mice. In another study using ABCA1 knock out mice, cholesterol absorption was found to be decreased (42). Thus, the role of ABCA1 in cholesterol absorption is being debated.

Our results provide some insight on the role of ABC transporters on cholesterol flux in an intestinal absorptive cell. Contrary to the prevailing hypothesis of ABC-mediated luminal cholesterol efflux, the present study shows that LXR/RXR activation and enhanced gene expression of ABCA1 or ABCG1 is associated with an increase in cholesterol efflux from basolateral membranes of CaCo-2 cells. Although we have no data that directly address the localization of ABCA1 or ABCG1 in CaCo-2 cells, the combined results strongly support the notion that one or both of the ABC transporters functions at or near the basolateral membrane to facilitate cholesterol efflux. Perhaps, however, this paradigm of ABCA1 functioning at the basolateral membrane of intestinal cells does make sense. The function of ABCA1 in facilitating cholesterol efflux from peripheral cells is thought to be dependent upon the binding of apoA-I to the cell membrane and, in a more recent report, perhaps to ABCA1 itself (34, 43, 44). In Tangier's disease for example, a disease caused by mutation of ABCA1, cholesterol efflux to general cholesterol acceptors remains intact, whereas efflux to apoA-I or HDL is defective (45, 46). Thus, efflux of cholesterol mediated by ABCA1 is dependent upon binding of a specific cholesterol acceptor to the cell. Intestinal cells bind apoA-I and HDL via a receptor-mediated process at the basolateral membrane (47–49). Moreover, apoA-I is normally secreted basolaterally by intestinal absorptive and CaCo-2 cells, thus providing a continuous acceptor for ABCA1-facilitated efflux (50). This would therefore explain why, with enhanced expression of ABCA1 or ABCG1, cholesterol effluxes basolaterally rather than apically. In addition, this also accounts for why we observed regulated cholesterol efflux into the basolateral medium following LXR/RXR activation in the absence of an added acceptor. In support of this hypothesis, we found no evidence for regulated cholesterol efflux when taurocholate micelles containing phosphatidylcholine were used as a cholesterol acceptor.

ABCG1 is thought to play a central role in macrophage cholesterol transport (4, 51). Although found in intestine (51), its role in the intestine is unknown. Unlike ABCA1, which is a full-transporter, ABCG1 is a half-transporter (52, 53). It is believed that, in contrast to full-transporters which are usually localized to plasma membranes, half-transporters are contained in membranes of intracellular organelles such as peroxisomes, mitochondria, and endoplasmic reticulum (52, 54). While this may be true, with the recent observation that genes of half transporters ABCG5 and ABCG8 encode proteins that likely unite to

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form an active transporter, it is conceivable that these functionally combined proteins would do so at the plasma membrane (5). If true, this would imply that there is another gene product that is required for the function of the ABCG1 product in the intestine. The present results cannot separate the possible functions of ABCA1 or ABCG1. Although we suspect that it is ABCA1 that has a major role in regulating cholesterol flux in intestine, all the physiologic data shown in this study are associated with parallel changes in gene expression of ABCA1 or ABCG1.

It is clear that in CaCo-2 cells, the genes for ABCG5 and ABCG8 are in very low abundance when compared with the abundance of ABCA1 and ABCG1. This is consistent with the findings of Lu et al., who had difficulty detecting ABCG8 transcripts in human small intestine (55). Because of their low expression, it was not possible to show a consistent upregulation of ABCG5 or ABCG8 gene expression by LXR/RXR activation. Thus, in the CaCo-2-cell model, little can be said of their possible role in facilitating cholesterol efflux at the apical membrane. In all of the experiments performed, however, we did not see evidence for regulated cholesterol efflux from apical membranes of CaCo-2 cells.

There is a growing body of evidence to suggest that the liver and intestine are the major sources of HDL (56, 57). Although it was recognized several years ago that HDL is produced by the intestine (58), it was never made clear as to how these lipoprotein particles were formed. In fact, it remains unknown whether HDL is assembled within the intestinal cell and then secreted or whether the particle is formed outside the cell. Our data would suggest that intestinal ABCA1 (or ABCG1) plays a pivotal role in HDL production by the intestine. Furthermore, our results would suggest that HDL is assembled extracellularly by an ABCA1-mediated process that enhances the efflux of cholesterol to apoA-I at the basolateral membrane to produce a nascent HDL particle. These nascent particles likely serve as acceptors for ABCA1-mediated cholesterol efflux by peripheral cells, particularly macrophages, and, following their modification by LCAT, these mature particles bind to hepatic scavenger receptors (SR-BI) to eliminate cholesterol through biliary secretion (59). Our data would support similar views of Attie's group from their studies performed in the ABCA1-dysfunctional WHAM chickens (56).

In a previous study performed in CaCo-2 cells, we implicated caveolae, or similar functional cholesterol-, sphingolipid-rich specialized plasma membrane microdomains, as the likely origin for plasma membrane cholesterol that is used for lipoprotein assembly and secretion (60). In the present study, we demonstrated that the cholesterol that is mobilized by an ABCA1-mediated process originates from these same specialized domains. We would postulate, therefore, that the LXR acts as a cholesterol sensor. With an increase in cholesterol influx, ABCA1 expression is increased causing more cholesterol to efflux basolaterally, limiting the amount of cholesterol available for lipoprotein assembly and secretion. Thus, we would surmise, if our data in CaCo-2 cells can be applied to the in vivo situation, enhanced ABCA1 function would decrease cholesterol transported in triacylglycerol-rich lipoproteins whereas ABCA1 deficiency would do the opposite. Although this is in agreement with the results of Repa et al. (6) and McNeish et al. (40), cholesterol absorption in these studies and in the study by Drobnik et al. (42) was based on measurements of fecal cholesterol excretion. Because these estimates do not account for possible changes in basolateral cholesterol efflux, they likely are not true indicators of the amount of cholesterol that is secreted by the intestine in triacylglycerol-rich lipoproteins. Moreover, we would argue that a decrease in basolateral cholesterol efflux in ABCA1 knock out mice and an accompanying increase in triacylglycerol-rich lipoprotein cholesterol would result in little or no change in fecal cholesterol excretion (41, 42). Our postulate is consistent with the observation by McNeish et al. (40) that, in their ABCA1 knockout mice, there was an accumulation of intestinally derived cholesterol in plasma VLDL. Estimating intestinal lymphatic chylomicron cholesterol is, therefore, the only direct way to address the effect of eliminating ABCA1 function on cholesterol absorption.

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