LXR/RXR ligand activation enhances basolateral efflux of β-sitosterol in CaCo-2 cells

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Abstract To examine whether intestinal ABCA1 was responsible for the differences observed between cholesterol and β-sitosterol absorption, ABCA1-facilitated β-sitosterol efflux was investigated in CaCo-2 cells following liver X receptor/retinoid X receptor (LXR/RXR) activation. Both the LXR agonist T0901317 and the natural RXR/LXR agonists 22-hydroxycholesterol and 9-*cis* retinoic acid enhanced the basolateral efflux of β-sitosterol without altering apical efflux. LXR-mediated enhanced β-sitosterol efflux occurred between 6 h and 12 h after activation, suggesting that transcription, protein synthesis, and trafficking was likely necessary prior to facilitating efflux. The transcription inhibitor actinomycin D prevented the increase in β-sitosterol efflux by T0901317. Glybenclamide, an inhibitor of ABCA1 activity, and arachidonic acid, a fatty acid that interferes with LXR activation, also prevented β-sitosterol efflux in response to the LXR ligand activation. Influx of β-sitosterol mass did not alter the basolateral or apical efflux of the plant sterol, nor did it alter ABCA1, ABCG1, ABCG5, or ABCG8 gene expression or ABCA1 mass. Similar to results observed with intestinal ABCA1-facilitated cholesterol efflux, LXR/RXR ligand activation enhanced the basolateral efflux of β-sitosterol without affecting apical efflux. The results suggest that ABCA1 does not differentiate between cholesterol and β-sitosterol and thus is not responsible for the selectivity of sterol absorption by the intestine. ABCA1, however, may play a role in β-sitosterol absorption.—Field, F. J., E. Born, and S. N. Mathur. **LXR/RXR ligand activation enhances basolateral efflux of β-sitosterol in CaCo-2 cells. *J. Lipid Res.* 2004.** 45: **905–913.**

Supplementary key words adenosine 5′-triphosphate binding cassette transporter A1 • absorption • cholesterol • intestine • fatty acid • liver X receptor • retinoid X receptor • sterol

The plant sterols campesterol and β-sitosterol differ from cholesterol only in the addition to the C-24 side chain of a methyl or ethyl group, respectively. Despite these minor modifications to the chemical structure of cholesterol, the intestinal cell discriminates between ingested cholesterol and these and other plant sterols. Compared with cholesterol, plant sterols are poorly absorbed (1–4). Moreover, specificity for the absorption of sterols exists, in that cholesterol is better absorbed than campesterol and campesterol is better absorbed than β-sitosterol (4). How the intestine discriminates among sterols and the reasons for sterol specificity of absorption are unknown. Recent seminal observations, however, have shed light on the mechanisms of sterol absorption by the intestine. In individuals with phytosterolemia, a rare disorder that causes retention of plant sterols in plasma and tissues, mutations in two ABC transporters, ABCG5 and ABCG8, have been identified (5, 6). In these individuals, cholesterol and plant sterols are "hyperabsorbed" (3, 7, 8). It is postulated that the gene products of ABCG5/ABCG8 function together at the apical membrane of absorptive cells and selectively "pump" plant sterols back out into the lumen for excretion (9–11). Although an attractive hypothesis, this has yet to be demonstrated at the cellular level.

ABCA1 is another ABC sterol transporter that is expressed in intestine. It was originally thought to have a role in cholesterol absorption by facilitating cholesterol efflux at the apical membrane of intestinal cells (12). This idea, however, has been challenged (13, 14). ABCA1 has been shown to function not at the apical membrane but at the basolateral membrane to facilitate cholesterol efflux basally (13, 15, 16). Enhanced expression of ABCA1 is thought to be critical for HDL formation by the intestine (13, 16). Its role in sterol absorption, if any, is not clear.

These intestinal ABC transporters are gene targets of liver X receptors (LXRs), transcription factors that together with their obligate partner, retinoid X receptors (RXRs), bind to DNA of their target genes (17). LXRs are expressed in intestine and are thought to play a role in regulating cholesterol absorption (18, 19). For example, animals fed an LXR or RXR agonist have increased fecal excretion of neutral sterols, suggesting that activation of the LXR pathway interferes in some way with cholesterol

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absorption (12). Suppression of cholesterol absorption by LXR activation is independent of intestinal ABCA1 (14). Thus, current thinking suggests that enhanced expression of ABCG5/ABCG8 by LXR causes apical efflux of the sterol back into the intestinal lumen (9–11).

We have previously shown in intestinal cells that LXR activation does not promote apical cholesterol efflux (13). There are no data, however, on the efflux of plant sterols by intestinal cells. In this study, therefore, we examined the role of LXR/RXR activation on β-sitosterol efflux. The results show that LXR/RXR activation enhances the basolateral efflux of β-sitosterol without affecting efflux of the plant sterol apically. Moreover, influx of β-sitosterol mass does not alter gene expression of ABCA1, ABCG5, or ABCG8, or the efflux of β-sitosterol, either apically or basolaterally. ABCA1, functioning at the basolateral membrane of the intestinal cell, does not discriminate between cholesterol and the plant sterol, β-sitosterol. Thus, ABCA1 cannot account for the intestine's ability to discriminate between these two sterols. Moreover, similar to our previous findings with cholesterol efflux (13), we found no evidence for selective apical efflux of the plant sterol to explain the in vivo observations. The results do suggest, however, that ABCA1 may have a role in plant sterol absorption by the small intestine.

MATERIALS AND METHODS

Materials

[4-^{14}C]β-sitosterol (58 mCi/mmol) was purchased from Amersham. [^{3}H]β-sitosterol (42 Ci/mmol) was a generous gift from Dr. Lawrence Rudel, Wake Forest University School of Medicine, Winston-Salem, NC. The specific activity of [^{3}H]β-sitosterol was adjusted to 58 mCi/mmol by the addition of unlabeled β-sitosterol. Both labeled β-sitosterol preparations eluted as a single peak on reverse phase thin-layer chromatography. In all experiments in CaCo-2 cells, the initial experiment was performed using [^{14}C]β-sitosterol. Subsequent experiments were performed using [^{3}H]β-sitosterol. With either ^{14}C- or ^{3}H-labeled β-sitosterol, the results were similar. Experiments in T84 or HT-29 cells were performed using [^{3}H]β-sitosterol. [^{3}H]cholesterol (48.3 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Delipidated fetal calf serum was from Intracel (Issaquah, WA). Protease inhibitor cocktail, Tri Reagent, human apolipoprotein A-I (apoA-I), goat anti-rabbit polyclonal antibody-HRP, fatty acid-free BSA, retinoic acid, glybenclamide, and actinomycin D were from Sigma Chemicals (St. Louis, MO). Arachidonic acid was purchased from Cayman Chemicals (Ann Arbor, MI). T0901317 was a gift from Tularik, Inc. (South San Francisco, CA). Anti-human ABCA1 rabbit polyclonal antibody was purchased from Novus Biochemicals (Littleton, CO). 22-Hydroxycholesterol was obtained from Steraloids (Newport, RI).

Cell culture

CaCo-2 cells were cultured in T-75 flasks (Corning Glassworks, Corning, NY) in Dulbecco's minimum essential medium (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 gentamycin. When 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). Cells were fed every other day and were used 14 days after seeding. T84 and HT-29 cells were gracious gifts from Dr. Michael Welsh, University of Iowa. These cell lines were cultured as described for CaCo-2 cells.

To prepare fatty acid-BSA solutions, appropriate volumes of the fatty acid stock solution were dried under nitrogen with equimolar amounts of sodium hydroxide and taken up in enough M199 containing BSA to obtain the desired final concentration of the fatty acid. The molar ratio of fatty acid to BSA was maintained at 4 to 1. The resulting solution was stirred vigorously at 37°C until clear and was then added to CaCo-2 cells.

Real-time RT-PCR

RNA was extracted from cells and treated with DNase followed by reverse transcription for 4 h at 50°C with SuperScript III (Invitrogen, Carlsbad, CA). cDNA was mixed with the appropriate primers and 2× Sybr Green PCR master mix (Applied Biosystems) and subjected to real-time RT-PCR using an Applied Biosystems model 7000 sequence detection system. Results are shown in **Table 1**.

ABCA1 mass

After 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 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride, 0.8 μM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A, 14 μM E-64, 22.7 μM *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal, 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 100 μl buffer C containing protease inhibitors. After sonication for 20 s, 100 μg membrane proteins in 1× Laemmli sample buffer were separated by SDS-

TABLE 1. Primers for RT-PCR

Gene	Primer Sequence	bp	Accession No.
ABCA1	5′-ATGTCCAGTCCAGTAATGGTTCTGT-3′ 5′- CGAGATATGGTCCGGATTGC-3′	82	NM_005502.2
ABCG1	5′-CGACCGACGACACAGAGACTC-3′ 5′-GAGCACGAGACACCCACAAAC-3′	71	XM_032950.3
ABCG5	5′-GCATGCTGAACGCTGTGAA-3′ 5′-TGGTAGAGGCCGTCCTGACT-3′	76	NM_022436.2
ABCG8	5′-AGCCGCCCTCTTGTTCATG-3′ 5′-GAGTAACATTTGGAGATGACATCCA-3′	75	XM_055525.1
18S	5′-TAAGTCCCTGCCCTTTGTACACA-3′ 5′-GATCCGAGGGCCTCACTAAAC-3′	71	K03432

PAGE on an 8% porous gel (20) and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). After rinsing in Tris-buffered saline (TBS) (25 mM Tris-HCl, pH 7.5, 150 mM sodium chloride), the membrane was air dried for 15 min and 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 600-fold in TBS containing 0.05% Tween-20, 2% nonfat dry milk, and 1% goat serum (Blotto). After washing in TBS containing Tween-20, the membrane was incubated for 1 h with goat anti-rabbit antibody cross-linked to horseradish peroxidase and diluted 100,000-fold in Blotto. The membrane was washed thoroughly in TBS containing Tween-20, and horseradish peroxidase was detected using the SuperSignal West Femto Sensitivity Substrate chemiluminescent detection system (Pierce Endogen, Rockford, IL).

β-Sitosterol efflux

Cells were labeled for 22 h with 0.23 μCi/filter (see figure legends for the amount used for each experiment) of [^{14}C]- or [^{3}H]β-sitosterol in the presence of 1% delipidated fetal calf serum and M199. After extensive washing to remove unincorporated labeled sterol, treatments were added to the upper chambers while the lower chambers received M199 alone. In some experiments, human apoA-I was added to both upper and lower wells at the time of the added treatments. Following 22 h of incubation with the treatments, media from both chambers were collected and extracted for total lipids using chloroform-methanol (1:1; v/v). After the cells were rinsed with PBS, cell lipids were extracted with hexane-isopropyl alcohol-water (3:2:0.1; v/v/v). Organic extracts from media and cells were dried under nitrogen and used for counting in a scintillation counter to estimate labeled β-sitosterol. To verify that the labeled sterol within cells and basolateral media was β-sitosterol, the organic extracts were

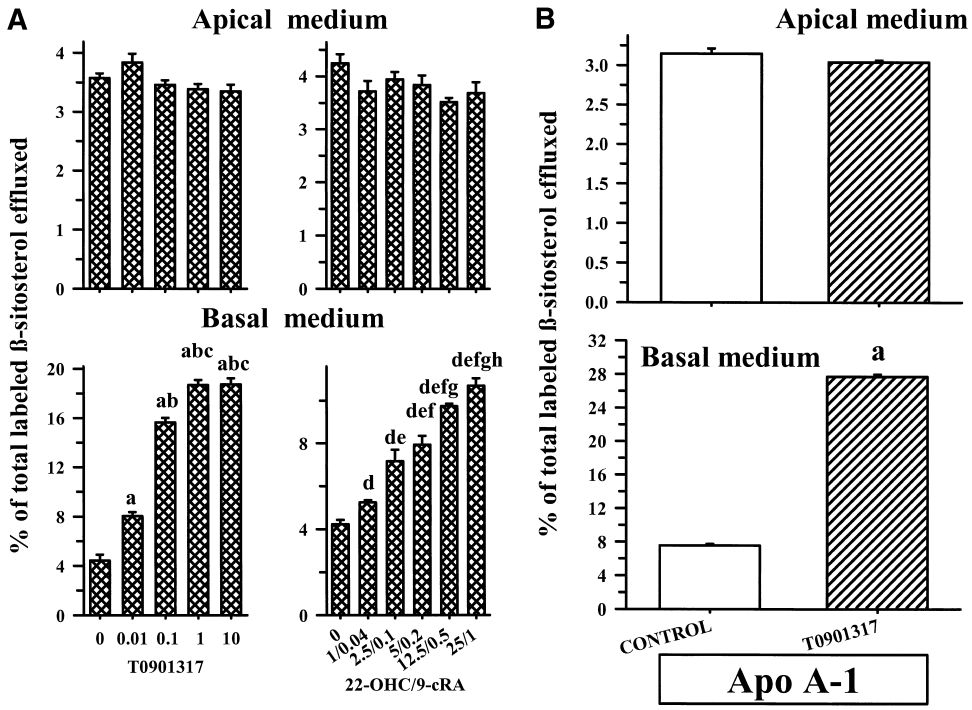

Fig. 1. LXR/RXR activation enhances β-sitosterol efflux. A: CaCo-2 cells were prelabeled for 22 h with 0.23 μCi/well of [^{3}H]- or [^{14}C]β-sitosterol in the presence of 1% delipidated FBS. After thorough washing to remove unincorporated label, increasing amounts of T0901317 in 0.02% DMSO or increasing amounts of 22-hydroxycholesterol/9-*cis* retinoic acid (22-OHC/9-cRA) in 0.2% ethanol were added to the upper chambers. The lower chambers contained M199 alone. After a 22 h incubation, the media in the upper and lower chambers were collected. Lipids in cells and media were extracted. The percent of β-sitosterol efflux into the upper and lower wells was calculated by dividing the amount of radioactivity recovered in the media by the total radioactivity in cells and apical and basal media (~1.74×10^5 dpm/dish). The data represent the mean ± SE from three separate experiments with a total of eight dishes for each treatment. a: $P < 0.05$ versus 0 μM T0901317 (control); b: $P < 0.05$ versus 0.01 μM T0901317; c: $P < 0.05$ versus 0.1 μM T0901317; d: $P < 0.05$ versus 0 μM 22-OHC/0 μM 9-cRA (control); e: $P < 0.05$ versus 1 μM 22-OHC/0.04 μM 9-cRA; f: $P < 0.05$ versus 2.5 μM 22-OHC/0.1 μM 9-cRA; g: $P < 0.05$ versus 5 μM 22-OHC/0.2 μM 9-cRA; h: $P < 0.05$ versus 12.5 μM 22-OHC/0.5 μM 9-cRA. B: CaCo-2 cells were prelabeled for 22 h with 0.13 μCi/well of [^{3}H]β-sitosterol in the presence of 1% delipidated FBS. After thorough washing to remove unincorporated label, apoA-I (6 μg/ml) was added to both the upper and lower wells. T0901317 (in 0.02% DMSO), 1 μM, was added to the upper well. Control cells received the DMSO vehicle alone. After a 22 h incubation, the media in the upper and lower chambers were collected. Lipids in cells and media were extracted. The percent of β-sitosterol efflux into the upper and lower wells was calculated by dividing the amount of radioactivity recovered in the media by the total radioactivity in cells and apical and basal media (~1.2×10^5 dpm/dish). The data represent the mean ± SE from four dishes. a: $P < 0.001$ versus 0 μM T0901317 (control) by Student *t*-test.

applied to a Whatman LKC 18 Linear K reverse phase thin-layer chromatography plate and eluted eight times with a solvent containing methanol-water-chloroform (100:10:7.5; v/v/v) (21). In this system, cholesterol and β-sitosterol eluted with R_f values of 0.63 and 0.39, respectively. All of the label recovered from cells and basolateral media eluted with an R_f value of 0.39, corresponding to β-sitosterol.

Other analyses

Protein content was estimated using the BCA kit (Pierce Endogen). β-Sitosterol mass was estimated by gas-liquid chromatography as described (22). Statistical analysis was performed by the general linear model univariate procedure and Tukey's *t*-test for comparison of the treatments using SPSS software.

RESULTS

LXR/RXR activation enhances β-sitosterol efflux

In a recent study performed in CaCo-2 cells, we demonstrated that LXR/RXR activation enhanced the expression of ABCA1 and ABCG1 and caused cholesterol to efflux into the basolateral medium (13). In the present study, we addressed the efflux of the plant sterol β-sitosterol following LXR/RXR activation. CaCo-2 cells were prelabeled with either [^{3}H]- or [^{14}C]β-sitosterol. They were then incubated with increasing concentrations of either T0901317, a nonsterol ligand for LXR, or 22-hydroxycholesterol and 9-*cis* retinoic acid, natural ligands for LXR and RXR, respectively. Following the incubation, efflux of the labeled sterol into the apical or basolateral medium was estimated. The results with either [^{3}H]- or [^{14}C]-labeled β-sitosterol were the same. The results of this experiment are shown in **Fig. 1A**. With a maximal effect at 1 μM, T0901317 increased the amount of β-sitosterol recovered in the lower well in a concentration-dependent manner. In contrast, the amount of labeled β-sitosterol recovered in the upper well was unchanged. The natural ligands of LXR/RXR, 22-hydroxycholesterol and 9-*cis* retinoic acid, were not as potent as was the synthetic LXR agonist, but they too increased β-sitosterol efflux only into the lower well. This experiment was performed in the absence of the cholesterol acceptor apoA-I. We had previously shown in CaCo-2 cells that the addition of exogenous apoA-I was not required to demonstrate basolateral cholesterol efflux in response to LXR/RXR activation (13). We assumed it was because CaCo-2 cells synthesize and secrete sufficient amounts of apoA-I to promote sterol efflux following LXR/RXR activation. To ensure that the unidirectional basolateral efflux of β-sitosterol was not secondary to the absence of a cholesterol acceptor in the apical medium, cells were again prelabeled with β-sitosterol. Human apoA-I was added to both the upper and lower wells, and T0901317 was added to the upper well. Following the incubation, efflux of labeled β-sitosterol into the apical or basolateral medium was estimated (Fig. 1B). Even in the presence of exogenous apoA-I in the upper and lower well, LXR ligand-induced β-sitosterol efflux occurred only into the lower well. Thus, in CaCo-2 cells, exogenously added apoA-I is not required to promote sterol efflux in response to LXR/RXR activation. Moreover, stimulation of sterol efflux occurs only into the basolateral medium.

To determine how rapidly LXR/RXR activation led to the efflux of β-sitosterol and to ensure that early efflux of the plant sterol into the apical medium was not being overlooked, β-sitosterol efflux was examined over time. Cells prelabeled with β-sitosterol were incubated with or without T0901317 for 3, 6, 12, and 24 h. The amount of labeled sterol recovered in the upper and lower chambers was then estimated. **Figure 2** shows these data. The amount of β-sitosterol recovered in the upper chamber increased modestly over time. Compared with controls, however, addition of the LXR ligand did not alter the amount of labeled sterol found in the apical medium at any time. In control cells without T0901317, the amount of β-sitosterol recovered in the basolateral medium also increased modestly over time. At 3 and 6 h after the addition of the LXR ligand, efflux of β-sitosterol into the lower well was similar to controls. By 12 and 24 h, however, LXR activation caused a 2- and 3-fold increase in sterol efflux, respectively. Thus, the enhancement of β-sitosterol efflux following LXR ligand activation is not immediate and requires between 6 and 12 h before an effect is observed. Moreover, no evidence for LXR-mediated apical efflux of the sterol was observed at any time.

It could be argued that the basolaterol efflux of sterols following LXR/RXR activation is unique to CaCo-2 cells.

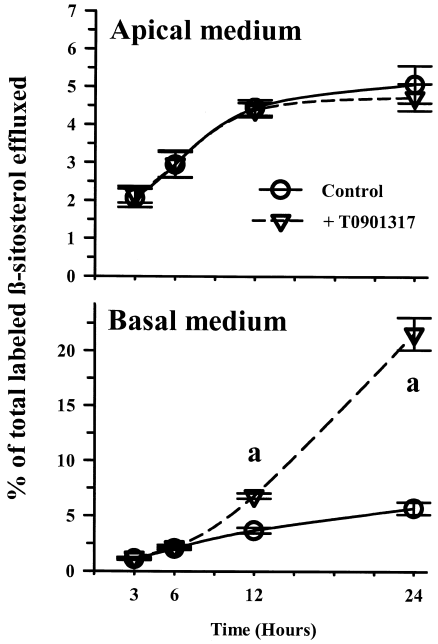

Fig. 2. Effect of LXR activation on β-sitosterol efflux. Cells were prelabeled with β-sitosterol as described in the legend to Fig. 1. After thorough washing to remove unincorporated label, 0 (control) or 1 μM T0901317 in 0.02% DMSO was added to the upper well. After 3, 6, 12 and 24 h, the percent efflux of β-sitosterol into the upper and lower wells was estimated as described in the legend to Fig. 1 and Materials and Methods. The data represent the mean ± SE from three separate experiments, with a total of seven dishes for each time point. a: $P < 0.05$ versus 0 μM T0901317 (control) at 12 h or 24 h.

To address this argument, we performed sterol efflux experiments in two other polarized intestinal epithelial cell lines, T84 and HT-29. After prelabeling with cholesterol, T0901317 was added apically in the presence or absence of apoA-I added to both the lower and upper wells. Following the incubation, efflux of cholesterol into the apical or basolateral medium was estimated. The results are shown in **Fig. 3A**. In contrast to CaCo-2 cells, both T84 and HT-29 cells required the presence of apoA-I to promote cholesterol efflux in response to LXR activation. In the absence of apoA-I, T0901317 did not alter cholesterol efflux either apically or basolaterally. This would suggest that these cell lines do not secrete sufficient amounts of apoA-I to facilitate cholesterol efflux. Moreover, the percent of cellular cholesterol that effluxed into the basolateral compartment by T84 (Fig. 3A, 0.7%) and HT-29 (Fig. 3A, 0.4%) cells in the presence of T0901317 and apoA-I was substantially less than that observed in CaCo-2 cells (10–15%) (13). Similar to that observed in CaCo-2 cells, however, efflux of cholesterol in response to LXR activation was only into the basolateral medium. No apical efflux was observed. In Fig. 3B, results of β-sitosterol efflux following LXR ligand activation in T84 and HT-29 cells are shown. In this experiment, apoA-I was present in both upper and lower chambers. Compared with CaCo-2 cells (Fig. 1B), the percent of cellular β-sitosterol that effluxed into the basolateral compartment was substantially less in T84 and HT-29 cells. Like CaCo-2 cells, however, β-sitosterol efflux occurred only into the basolateral medium in response to LXR activation. Thus, these experiments substantiate that basolateral sterol efflux in response to LXR/RXR activation is not unique to CaCo-2 cells. What is unique to CaCo-2 cells, however, is that these cells synthesize and secrete sufficient amounts of endogenous apoA-I to facilitate sterol efflux.

To determine whether enhanced β-sitosterol efflux following LXR activation requires an increase in transcription, cells were incubated with T0901317 together with actinomycin D, a general inhibitor of gene transcription (**Fig. 4A**). The addition of actinomycin D completely prevented the increase of β-sitosterol efflux into the lower well by the LXR agonist. Glybenclamide is an agent that inhibits the function of ABCA1 (23). When glybenclamide was added to the lower well of cells incubated with

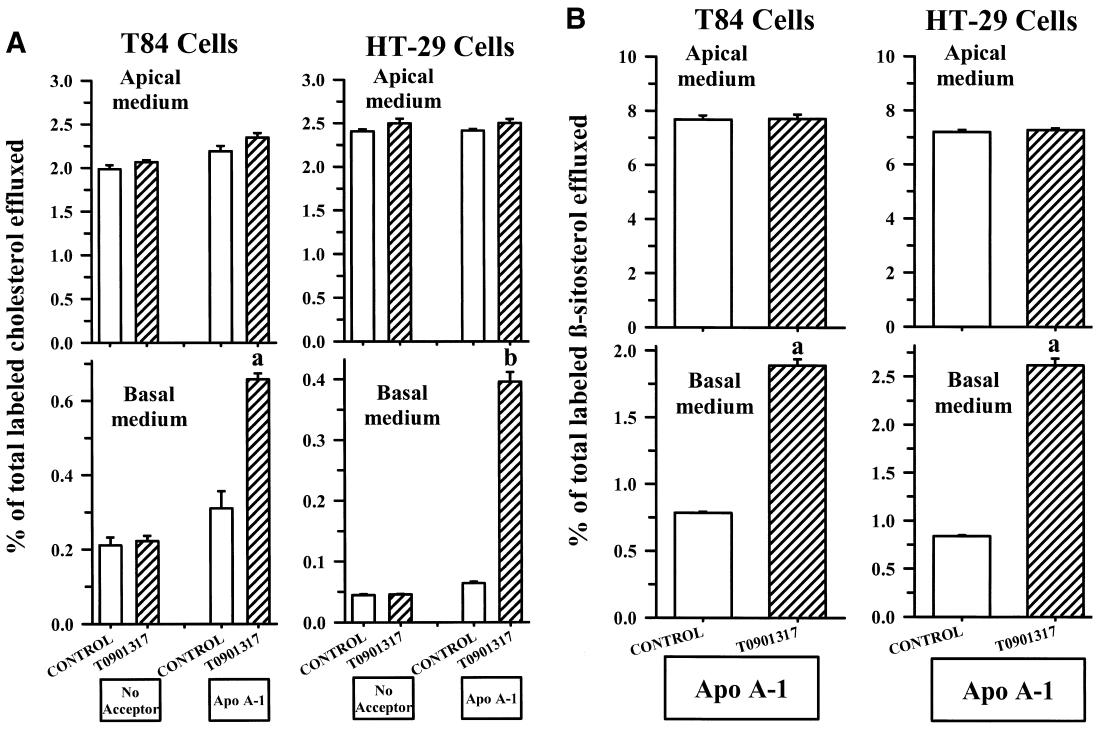

Fig. 3. Sterol efflux in T84 and HT-29 cells by LXR activation. A: T84 and HT-29 cells were prelabeled for 22 h with 3.34 μCi/well of [^{3}H]cholesterol in the presence of 1% delipidated FBS. After thorough washing to remove unincorporated label, apoA-I (6 μg/ml) was added to both the upper and lower wells of half of the dishes. T0901317, 1 μM, was added to the upper well. Control cells received the DMSO vehicle alone. After a 22 h incubation, the media in the upper and lower chambers were collected. Lipids in cells and media were extracted. The percent of cholesterol efflux into the upper and lower wells was calculated by dividing the amount of radioactivity recovered in the media by the total radioactivity in cells and apical and basal media (~1.8×10^6 dpm/dish). The data represent the mean ± SE from three dishes for each treatment. a: $P < 0.002$ versus 0 μM T0901317 with apoA-I (control) by Student *t*-test; b: $P < 0.001$ versus 0 μM T0901317 with apoA-I (control) by Student *t*-test. B: T84 and HT-29 cells were prelabeled for 22 h with 0.28 μCi/well of [^{3}H]β-sitosterol in the presence of 1% delipidated FBS. After thorough washing to remove unincorporated label, apoA-I (6 μg/ml) was added to both the upper and lower wells. T0901317, 1 μM, was added to the upper well. Control cells received the DMSO vehicle alone. After a 22 h incubation, the media in the upper and lower chambers were collected. Lipids in cells and media were extracted. The percent of β-sitosterol efflux was estimated as described in Fig. 1 (total radioactivity recovered in T84 and HT-29 cells, 1.5×10^5 and 1.3×10^5 dpm/dish, respectively). The data represent the mean ± SE from four dishes. a: $P < 0.001$ versus 0 μM T0901317 (control) by Student *t*-test.

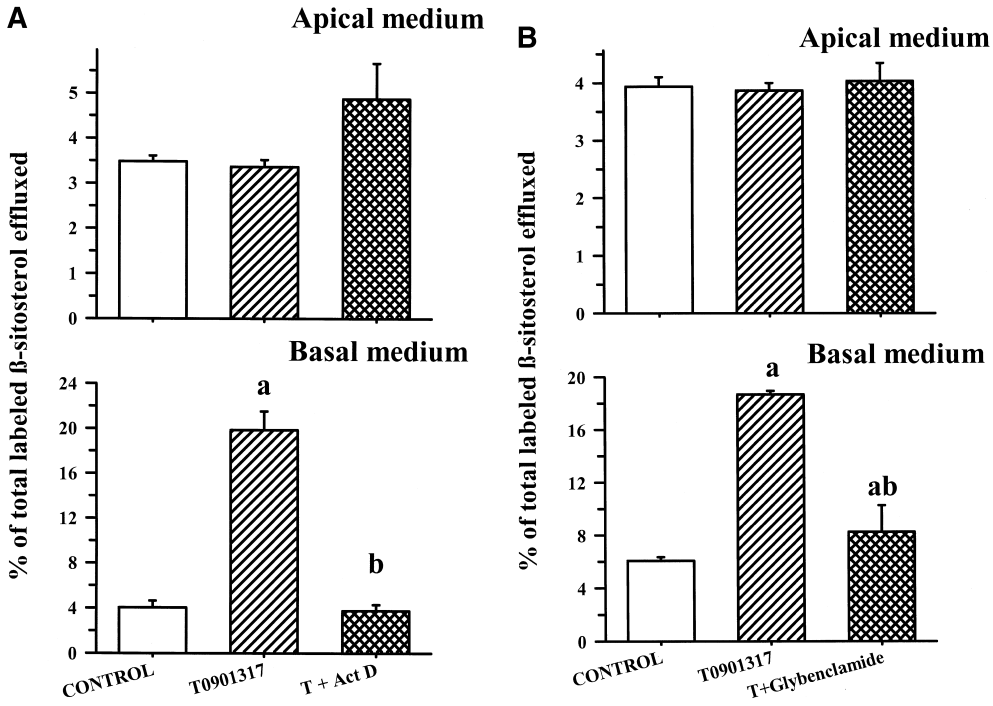

Fig. 4. Actinomycin D and glybenclamide prevent β-sitosterol efflux in response to LXR activation. Cells were prelabeled with β-sitosterol as described in Fig. 1. After thorough washing to remove unincorporated label, cells were incubated with 0 or 1 μM T0901317 ± 5 μg/ml actinomycin D added to the upper well (A) or 0 or 1 μM T0901317 added to the upper well and ± 1 mM glybenclamide added to the lower well (B). After 22 h, the percent of β-sitosterol efflux was estimated as described in Fig. 1. The data represent the mean ± SE from two separate experiments, with a total of four (A) or seven (B) dishes. a: $P < 0.05$ versus 0 μM T0901317 (control). b: $P < 0.05$ versus 1 μM T0901317.

T0901317, it, too, markedly attenuated the increase in β-sitosterol efflux into the lower well that was caused by the LXR agonist (Fig. 4B). Glybenclamide did not alter the amount of sterol label recovered in the apical medium. This finding strongly suggests that an ABC transporter, most likely ABCA1, is responsible for facilitating efflux of the plant sterol basally.

Arachidonic acid interferes with β-sitosterol efflux in response to LXR/RXR activation

It has been demonstrated that polyunsaturated fatty acids interfere with LXR-mediated activation of their gene targets (24–28). Indeed, in a recent study (29), we found that arachidonic acid, by decreasing ABCA1 mass, decreased the efflux of cholesterol in response to LXR activation. We postulated, therefore, that if ABCA1 facilitated β-sitosterol efflux, then arachidonic acid would also attenuate β-sitosterol efflux in response to LXR activation. To address this, cells were prelabeled with β-sitosterol and incubated with T0901317 and increasing concentrations of arachidonic acid. Following the incubation, the amount of labeled sterol found in the upper and lower chambers was estimated. The results are shown in **Fig. 5**. Similar to the effect of arachidonic acid on cholesterol efflux that we had observed previously (29), in a concentration-dependent manner, arachidonic acid caused a significant decrease in β-sitosterol efflux into the basolateral well in response to LXR activation. These results lend further support to the notion that an ABC transporter mediates β-sitosterol efflux.

Influx of β-sitosterol mass does not enhance β-sitosterol efflux

Because LXR activation did not increase efflux of labeled β-sitosterol at the apical membrane, we wanted to exclude the possibility that insufficient cellular plant sterol mass was present to trigger a regulatory response that might act through an ABC transporter located in the apical membrane. To address this possibility, cells were again prelabeled with β-sitosterol. They were then incubated with taurocholate or taurocholate containing 0.2 mM of β-sitosterol in the presence or absence of T0901317. Following the incubation, the amount of labeled β-sitosterol recovered in the upper and lower wells was determined (**Fig. 6**). As expected, the addition of T0901317 to cells incubated with taurocholate alone increased the efflux of β-sitosterol into the lower well. Adding β-sitosterol mass did not alter the amount of labeled sterol found in the basolateral medium in control cells or in response to LXR activation. Moreover, the influx of β-sitosterol mass did not trigger any response for apical sterol efflux in cells incubated with or without the LXR agonist.

β-Sitosterol influx does not alter ABC expression

In intestinal cells, it has been suggested that the influx of a saturated derivative of β-sitosterol, sitostanol, leads to

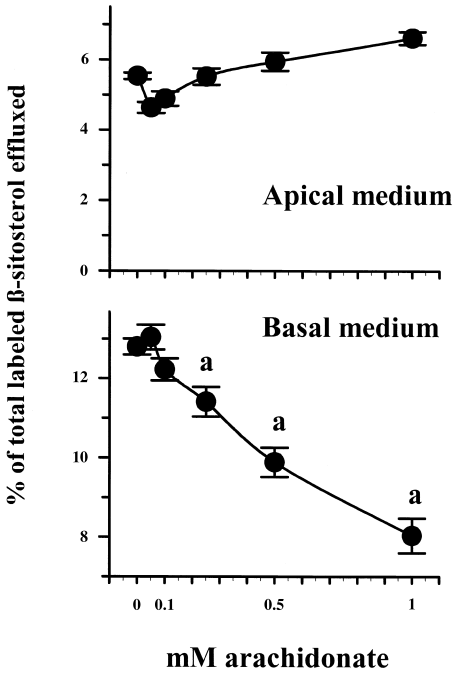

Fig. 5. Arachidonic acid decreases β-sitosterol efflux in response to LXR activation. Cells were prelabeled with β-sitosterol as described in Fig. 1. After thorough washing to remove unincorporated label, increasing concentrations of arachidonic acid complexed with BSA (arachidonic acid-BSA, 4 mol/1 mol) and 1 μM T0901317 in 0.02% DMSO were added to the upper well. After 22 h, the percent of β-sitosterol efflux was estimated as described in Fig. 1. The data represent the mean ± SE from two separate experiments, with a total of six dishes at each concentration of the fatty acid. a: $P < 0.05$ versus 0 mM arachidonic acid (control).

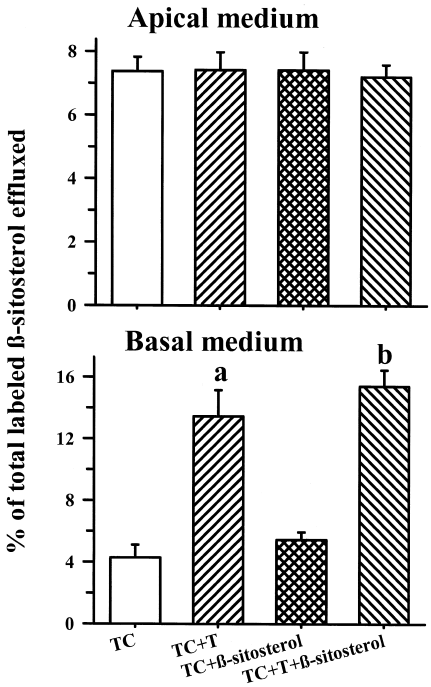

Fig. 6. Effect of β-sitosterol mass on β-sitosterol efflux in response to LXR activation. Cells were prelabeled with β-sitosterol as described in Fig. 1. After thorough washing to remove unincorporated label, 2.5 mM taurocholate (TC), ± 1 μM T0901317 (T) and ± 0.2 mM unlabeled β-sitosterol were added to the upper chamber. After 22 h, the percent of β-sitosterol efflux was estimated as described in Fig. 1. The data represent the mean ± SE from two separate experiments, with a total of five dishes for each treatment. a: $P < 0.05$ versus TC; b: $P < 0.05$ versus TC + β-sitosterol.

enhanced expression of ABCA1 (30). To address whether influx of β-sitosterol enhances gene expression of ABCA1 or other ABC transporters, CaCo-2 cells were incubated for 22 h with taurocholate or taurocholate containing 0.2 mM β-sitosterol. Following the incubation, RNA was extracted and mRNA levels for ABCA1, ABCG1, ABCG5, and ABCG8 were estimated by quantitative RT-PCR. In other experiments, ABCA1 mass was also estimated following incubation with micellar β-sitosterol. Influx of β-sitosterol, which caused the cellular accumulation of 2.4 μg/mg protein of β-sitosterol mass (no β-sitosterol mass was detected in cells incubated with taurocholate alone), did not alter gene expression of any of the ABC transporters (three individual experiments, total of eight dishes per treatment); nor did it alter ABCA1 mass (three individual experiments, total of nine dishes per treatment) (data not shown).

DISCUSSION

The results of this study clearly show that in response to LXR/RXR activation, efflux of the plant sterol β-sitosterol occurs at the basolateral, not apical, membrane of intestinal cells. Because this is similar to that observed previously for cholesterol efflux, the results strongly suggest that LXR/RXR activation does not enhance apical efflux of either this plant sterol or cholesterol (13). Do these results exclude the possibility that in vivo, as postulated, sterols are being "pumped out" into the lumen of the gut by ABC transporters such as ABCG5 or ABCG8 (9–11)? We feel confident that our combined results and the results of others exclude the possibility that ABCA1 plays a role in apical sterol efflux in the intestine (13, 15, 16). We cannot completely exclude the possibility that ABCG5 and ABCG8 facilitate apical sterol efflux in the gut, but we would argue that, if these proteins do facilitate efflux, LXR/RXR ligand activation does not enhance this facilitated sterol transport process. Caution is always required in applying results from cell culture to the in vivo situation, but in all the experiments we have performed in CaCo-2 cells to date, and now in T84 and HT-29 cells, we have yet to demonstrate LXR/RXR-mediated facilitation of apical sterol efflux.

To some degree, the results suggest that ABCA1 is not responsible for the intestine's ability to differentiate among luminal sterols. It is clear that the intestinal absorptive cell can distinguish between plant and animal sterols and that there is selectivity to this process (4). Despite only minor differences in their chemical structures, the intestine absorbs cholesterol to a much greater extent than it does plant sterols (1–4). The observation that

ABCA1 transports both β-sitosterol and cholesterol suggests that this transporter, functioning at the basolateral membrane, does not distinguish between the two sterols. What cannot be determined from our studies, however, is whether the two sterols have a similar affinity for the transporter or similar rates of mass transport. Addressing this question would likely require a cell-free system. If the ABC transporter is nonselective, then another mechanism for intestinal sterol selectivity will need to be considered. Obviously, the "putative" apical sterol transporter could have sterol specificity, but this speculated transport protein has yet to be identified [as reviewed in ref. (31)]. It is unlikely that ABCG5 and ABCG8 play a role in sterol selectivity. Results from a recent study in mice in which abcg5/abcg8 genes were disrupted demonstrate that selectivity of sterol absorption remained and was similar to that found in wild-type mice (11). Thus, intestinal sterol discrimination appears independent of ABCG5/ABCG8. We have previously postulated, as have others, that intestinal ACAT could be contributing to the poor absorption of plant sterols (22, 32–34). Compared with cholesterol, plant sterols are very poor substrates for ACAT, and hence, esterification (22, 34, 35). Because the lack of esterification would preclude assembly of the plant sterol into the core of a lipoprotein particle, subsequent transport into lymph in a triacylglycerol-rich lipoprotein would be inefficient.

In transgenic animals that overexpress hepatic and intestinal ABCG5/ABCG8, fecal excretion of neutral sterols is increased 2-fold and the fractional absorption of dietary cholesterol is decreased (10). This could imply that ABCG5 and ABCG8 function at the level of the intestine, causing increased excretion of cholesterol by potentially facilitating efflux of cholesterol back out into the lumen. Although this explanation seems reasonable, it would then follow that in animals lacking abcg5/abcg8, fractional absorption of dietary cholesterol would be increased. This turns out not to be the case. In abcg5/abcg8 knockout mice, the fractional absorption of cholesterol was found to be similar to that in wild-type animals (11). It remains possible that ABCG5 and ABCG8 do not regulate cholesterol absorption at the level of the intestine. Transgenic mice overexpressing ABCG5/ABCG8 have 6- to 8-fold more biliary cholesterol than their wild-type counterparts, whereas mice lacking abcg5/abcg8 have markedly diminished biliary cholesterol concentrations (10, 11). Results from a previous report demonstrating that the amount of cholesterol secreted into bile plays an important role in regulating the percent of dietary cholesterol absorbed suggest that ABCG5 and ABCG8 may indirectly alter the intestinal excretion of dietary cholesterol by altering biliary cholesterol concentration (36).

It has been suggested that a related plant sterol, the saturated derivative of β-sitosterol, sitostanol, or perhaps a derivative of sitostanol, functions as an LXR ligand. In CaCo-2 cells, sitostanol added in a micellar solution was found to enhance ABCA1 gene expression (30). No data on the efflux of cholesterol or plant sterol were provided. From these results, however, it was postulated that dietary sitostanol would interfere with cholesterol absorption by increasing intestinal ABCA1 expression, which, in turn, would inhibit cholesterol absorption. Although sitostanol, per se, was not investigated in the present study, we found that the influx of β-sitosterol, a sterol that is better absorbed than sitostanol, did not alter ABCA1, ABCG1, ABCG8, or ABCG5 gene expression or ABCA1 mass. Thus, we would argue that naturally occurring plant sterols are unlikely to activate LXR in intestine and therefore would not enhance ABC gene expression. In support of this, Kaneko et al. (37), using an LXR-luciferase reporter assay, showed that the naturally occurring phytosterols β-sitosterol, campesterol, ergosterol, and brassicasterol did not activate LXRα. These investigators, however, did demonstrate that a synthesized derivative of the plant sterol ergosterol, 22E-ergost-22-ene-1α,3 β-diol, was a potent ligand for LXRα, leaving open the possibility that a hydroxy derivative of a phytosterol may act as an LXR agonist in the gut. Whether these plant sterol products are actually synthesized in the gut remains speculative.

During revision of this mansuscript, Plosch et al. (38) observed that plant sterol concentrations were increased in plasma of Abcg5 null mice following the ingestion of T0901317, a treatment that also causes significant induction of ABCA1 expression. Moreover, they found that levels of campesterol and β-sitosterol were reduced in plasma of Abca1 null mice, suggesting that ABCA1 may play a role in plant sterol absorption. At the cellular level, our results would agree with the results observed in mice. Because cholesterol is an excellent substrate for ACAT, most cholesterol will be transported by the intestine in a triacylglycerol-rich lipoprotein particle. Thus, disrupting Abca1 will have little effect on cholesterol absorption [as reviewed in ref. (39)]. In contrast, because β-sitosterol is a poor substrate for ACAT, it will not be transported in a triacylglycerol-rich particle. Instead, β-sitosterol will remain in a cellular compartment that can be utilized by ABCA1 for transport. Thus, disrupting Abca1 will result in a decrease in β-sitosterol absorption. As suggested by Wang and Tall (40), ABCA1 may not be selective in the lipid that it transfers; that is, lipid transport may be related to the availability of the lipid in the vicinity of the ABCA1 transporter. Thus, although intestinal ABCA1 is likely not responsible for distinguishing between cholesterol and β-sitosterol, it is clear that ABCA1 has a role in plant sterol absorption, perhaps by transporting the plant sterol to a nascent HDL particle.

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