Liver-X-receptor-mediated increase in ATP-binding cassette transporter A1 expression is attenuated by fatty acids in CaCo-2 cells: effect on cholesterol efflux to high-density lipoprotein

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The effect of fatty acids on LXR (liver X receptors)-mediated enhancement of ABCA1 (ATP-binding cassette transporter A1) expression and cholesterol efflux was investigated in human intestinal cells CaCo-2. LXR activation by T0901317 increased basolateral cholesterol efflux to lipoprotein particles isolated at a density of 1.21 g/ml or higher. Oleic and arachidonic acids attenuated the amount of cholesterol isolated from these particles. Stearic, linoleic and docosahexaenoic acids also decreased cholesterol efflux from basolateral membranes, with the polyunsaturated fatty acids being the most potent. Although oleic, arachidonic and docosahexaenoic acids modestly decreased ABCA1 mRNA levels in response to LXR activation, stearic and linoleic acids did not. Except for oleic acid, all fatty acids substantially attenuated an increase in ABCA1 mass secondary to LXR activation. Inhibiting acyl-CoA:cholesterol acyltransferase activity prevented the decrease in cholesterol efflux caused by oleic acid. Thus, in response to LXR activation, all fatty acids decreased the efflux of cholesterol from the basolateral membrane of CaCo-2 cells. Although modest suppression of ABCA1 gene expression by oleic, arachidonic and docosahexaenoic acids cannot be completely excluded as a mechanism, the predominant effect of fatty acids on ABCA1 expression and cholesterol efflux is at a post-transcriptional level.

Key words: ATP-binding cassette transporter A1 (ABCA1), CaCo-2 cells, cholesterol, fatty acids, liver X receptor (LXR), retinoid X receptor (RXR).

INTRODUCTION

Results from numerous epidemiological studies have documented a reciprocal relationship between atherosclerotic coronary heart disease and HDL (high-density lipoprotein) levels [1]. It is believed that HDL plays a pivotal role in reverse cholesterol transport, a process that delivers cholesterol from the arterial wall to the liver for disposal [2–4]. By promoting efflux of excess cholesterol from macrophages contained within the arterial wall, HDL prevents the accumulation of cellular cholesteryl esters and foam-cell formation. Cholesterol carried in HDL returns to the liver, whereby the sterol is converted into bile acids and secreted into bile [5,6]. Secreted bile acids enter the enterohepatic circulation or are lost by faecal excretion. Loss of biliary cholesterol or bile acids via the gastrointestinal tract constitutes the major pathway for cholesterol elimination from the body.

Reverse cholesterol transport is facilitated by a cell-surface transporter called ABCA1 (ATP-binding cassette transporter A1) [7]. This transporter facilitates the efflux of cholesterol and phospholipids to lipid-poor apolipoproteins, resulting in the formation of nascent HDL particles [8–10]. Mutations in ABCA1 are responsible for the phenotype found in Tangier disease [11–14]. This rare disorder is characterized by severe HDL deficiency, accumulation of cholesteryl esters in macrophages in various tissues and premature atherosclerosis [15]. Thus it is clear that ABCA1 is critical for the normal production of HDL.

ABCA1 gene is a target for the nuclear hormone transcription factor, LXR (liver X receptor) [16,17]. LXR is believed to act as a sterol sensor in cells up-regulating genes involved in cholesterol elimination when cells are faced with excess sterols. LXR is activated by the binding of specific oxysterols and, after ligand activation, the LXR forms obligate heterodimers with RXRs (retinoid X receptors), which then enhance the transcription of such genes as those for cholesterol 7α-hydroxylase, lipoprotein lipase, cholesteryl ester transfer protein, SREBP-1c (sterol-regulatory-element-binding protein 1c) and other ABC sterol transporters, such as ABCG1, ABCG5 and ABCG8 [18,19].

Besides being expressed in macrophages and parenchymal cells of several organs, ABCA1 and LXR mRNAs are highly expressed in intestine, suggesting that these proteins might play a role in intestinal cholesterol metabolism [20,21]. Indeed, in an earlier study, enhanced expression of ABCA1 was initially thought to inhibit cholesterol absorption by facilitating efflux of enterocyte cholesterol back out into the lumen [16]. More recent results in cell culture and in animal models of ABCA1 gene non-function/dysfuction, however, indicate that ABCA1 functions at the basolateral, not the apical, membrane of the intestinal cell and is not the sterol transporter responsible for inhibiting cholesterol absorption [22–24]. Moreover, these latter studies increase the possibility that ABCA1 is responsible for nascent HDL production by the intestine.

Fatty acid flux through the intestine is substantial. After re-esterification, most of these absorbed fatty acids are transported as triacylglycerols or cholesteryl esters in triacylglycerol-rich lipoproteins. However, results also suggest that certain dietary fatty acids also affect HDL levels. In particular, polyunsaturated fatty acids, which are recommended in lieu of dietary saturated fatty acids, have been shown to decrease HDL levels

Abbreviations used: ABCA1, ATP-binding cassette transporter A1; ACAT, acyl-CoA:cholesterol acyltransferase; apoA1, apolipoprotein A1; HDL, LDL and VLDL, high-density, low-density and very-low-density lipoproteins respectively; LXR, liver X receptor; MTP, microsomal triacylglycerol transfer protein; PPARα, peroxisome-proliferator-activated receptor-α; RT, reverse transcriptase; RXR, retinoid X receptor; SREBP-1c, sterol-regulatory-element-binding protein 1c.

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[25]. Thus, in light of what we know, this could have an adverse effect on reverse cholesterol transport and atherosclerosis. Since the reason why polyunsaturated fatty acids should lower HDL is not clear, and since the intestine is probably a major source of nascent HDL, we set out to investigate whether specific fatty acids regulate intestinal ABCA1 expression, cholesterol efflux and HDL production. Using the cultured human intestinal cell line, CaCo-2, we demonstrate that in response to LXR activation, fatty acids decreased the basolateral efflux of cholesterol to nascent HDL particles. Although some fatty acids modestly decreased ABCA1 gene expression, the major effect of fatty acids on ABCA1 expression was a decrease in its mass. In contrast, oleic acid decreased cholesterol efflux not by interfering with ABCA1 expression, but by decreasing the amount of cholesterol available for efflux.

MATERIALS AND METHODS

Materials

[^{3}H]Cholesterol (48.3 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA, U.S.A.). Delipidated foetal calf serum was from Intracel (Issaquah, WA, U.S.A.). Protease inhibitor cocktail, TRI Reagent, human apolipoprotein A1 (apoA1), HDL from human plasma, goat anti-rabbit polyclonal antibody–horseradish peroxidase, oleic acid, stearic acid, HDL and fatty acid-free BSA were from Sigma Chemicals (St. Louis, MO, U.S.A.). Arachidonic acid and docosahexaenoic acid were from Cayman Chemicals (Ann Arbor, MI, U.S.A.). The MTP (microsomal triacylglycerol transfer protein) inhibitor, BMS-201038, was a gift from Bristol Myers Squibb (New Brunswick, NJ, U.S.A.). T0901317 was a gift from Tularik (South San Francisco, CA, U.S.A.). Anti-human ABCA1 rabbit polyclonal antibody was purchased from Novus Biochemicals (Littleton, CO, U.S.A.).

Cell culture

CaCo-2 cells were cultured in T-75 flasks (Corning Glassworks, Corning, NY, U.S.A.) in Dulbecco's minimum essential medium (Gibco, Grand Island, NY, U.S.A.) with 4.5 g/l glucose, and supplemented with 10 % (v/v) foetal bovine serum (Atlanta Biologicals, Norcross, GA, U.S.A.), 2 mM glutamine, 100 units/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 2×10^5 cells/well on to polycarbonate micropore membranes (0.4 μm pore size, 24 mm diameter) inserted into transwells (Costar, Cambridge, MA, U.S.A.). Cells were fed on alternate days and were used 14 days after seeding.

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 a sufficient amount of M199 containing BSA to obtain the desired final concentration of the fatty acid. The molar ratio of fatty acid to BSA was maintained at 4:1. The resulting solution was stirred vigorously at 37 °C until clear and then added to cells.

Real-time RT (reverse transcriptase)–PCR

DNase-free RNA was extracted from cells and subjected to reverse transcription for 4 h at 50 °C with SuperScript III (Invitrogen, Carlsbad, CA, U.S.A.). After inactivation for 15 min at 70 °C, the reverse-transcribed RNA with the appropriate primers for ABCA1 (forward primer 5′-ATGTCCAGTCCAGTAATGGTTCTGT-3′ and reverse primer 5′-CGAGATATGGTCCGGATTGC-3′, NM_005502.2) or 18 S rRNA (forward primer 5′-TAAGTCCC-TGCCCTTTGTACACA-3′ and reverse primer 5′-GATCCGAG-GGCCTCACTAAAC-3′, K03432) were mixed with the 2 × Sybr Green PCR master mix (Applied Biosystems, Foster City, CA, U.S.A.) and subjected to real-time RT–PCR using an Applied Biosystems model 7000 sequence detection system.

ABCA1 mass

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 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 0.8 μM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A, 14 μM E-64, 22.7 μM *N*-acetyl-leucyl-leucyl-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 of buffer C containing protease inhibitors. After sonication for 20 s, 100 μg of membrane proteins in 1× Laemmli sample buffer was separated by SDS/PAGE on an 8 % porous gel [26] and transferred to an Immobilon-P PVDF membrane (Millipore, Bedford, MA, U.S.A.). 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, washed with water/methanol (1:1, v/v), followed by methanol alone. After drying for 10 min under vacuum at room temperature (25 °C), 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 % non-fat dry milk and 1 % goat serum (Blotto). After washing with TBS containing Tween-20, the membrane was then 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 SuperSignal West Femto Maximum Sensitivity Substrate chemiluminescent detection system (Pierce Endogen, Rockford, IL, U.S.A.).

Cholesterol efflux

Cells were labelled for 24 h with 2.5 μCi/well [^{3}H]cholesterol in the presence of 1 % delipidated foetal calf serum and M199. After extensive washing to remove unincorporated labelled cholesterol, treatments were added to the upper chambers, whereas the lower chambers received M199 alone. In some experiments, human apoA1 (15 μg/2.5 ml) was added to the lower well. In all experiments, 0.1 μM MTP inhibitor, BMS-201038, was added together with the treatments apically. The MTP inhibitor completely prevented the increase in secretion of triacylglycerol and mass of apoB in response to 1 mM oleic or arachidonic acid. After 24 h of incubation with the treatments, media from both chambers were collected. Apical media were centrifuged at 17 320 ***g*** for 2 min to remove cell debris. Aliquots from the media were taken for radioactivity counting to estimate [^{3}H]cholesterol efflux, both apically and basally. After rinsing the cells with PBS, cell lipids were extracted with hexane/isopropyl alcohol/water (30:20:1, by vol.). Organic extracts from cells were dried under nitrogen, taken up in 1 ml of chloroform, and aliquots were taken for counting to estimate cell-associated [^{3}H]cholesterol. In some experiments, cell lipids were separated by TLC to estimate the amount of labelled unesterified and esterified cholesterol [22].

Other analyses

Protein content was estimated using the BCA kit (Pierce Endogen, Rockford, IL, U.S.A.). Using SAS software, statistical analysis was performed by linear mixed model analysis followed by

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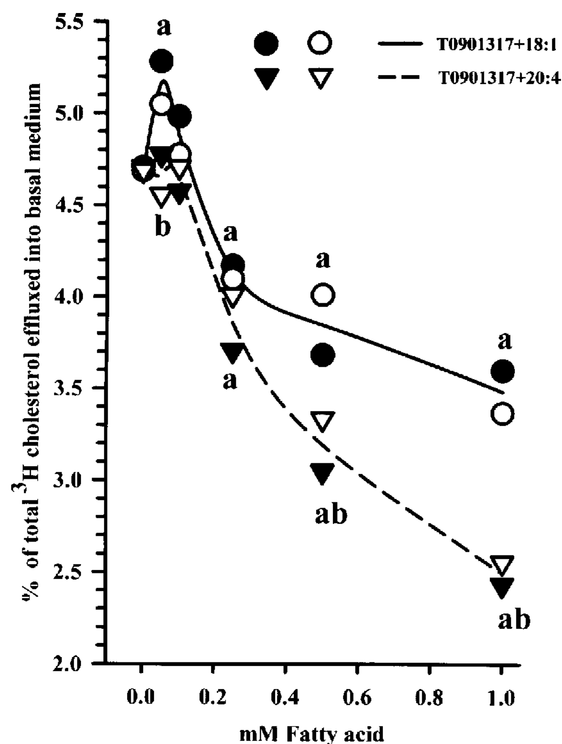

Figure 1 Effect of oleic and arachidonic acids on cholesterol efflux

Cells were prelabelled for 24 h with 2.5 μCi/well [^{3}H]cholesterol in the presence of 1 % delipidated foetal calf serum. After thorough washing to remove unincorporated label, the medium containing 1 μM T0901317 (0.02 % DMSO) and indicated amounts of oleic or arachidonic acid complexed with BSA (4 mol of fatty acid/mol of BSA) were added to the upper chambers. MTP inhibitor (0.1 μM) was included in apical media of all experiments. The lower chambers contained M199 alone. After 24 h, lipids in media collected from both chambers and from cells were extracted and the amounts of unesterified cholesterol were estimated as described in the Materials and methods section. Unesterified cholesterol in basal medium is expressed as a percentage of total labelled cholesterol in cells and media recovered from both upper and lower chambers (2.52×10^6 d.p.m./dish). The closed symbols represent the means from experiment no. 1 with $n = 2$ dishes at each concentration. The open symbols represent the means from experiment no. 2 with $n = 3$ dishes for each concentration. The lines are drawn through the means for the two experiments. The data were subjected to linear mixed model analysis followed by Bonferroni's test to determine significance between treatments at each concentration. a, $P < 0.05$ versus control without fatty acids; b, $P < 0.05$ versus T0901317 + 18:1.

Tukey's T test for comparison of the treatments (Figures 2–6). For results shown in Figure 1, Bonferroni-adjusted P value was used instead for comparison of the treatments. The results shown in Figure 2 (right panel) were analysed by one-way ANOVA using SigmaStat software.

RESULTS

Fatty acids decrease basolateral cholesterol efflux

In CaCo-2 cells, activation of LXR enhances efflux of cellular cholesterol from the basolateral membrane [22]. In these cells and in other cell types, fatty acids have been shown to interfere with activation of the LXR pathway [27–29]. Since intestinal cells are exposed to high concentrations of dietary and biliary fatty acids, we determined whether an increase in fatty acid flux would alter cholesterol efflux in response to LXR activation. To address this initially, two common and representative dietary fatty acids, oleic and arachidonic acid, were studied. CaCo-2 cells were prelabelled with cholesterol and incubated with the LXR agonist, T0901317, in the presence or absence of increasing concentrations of the two fatty acids. The amount of cellular cholesterol that

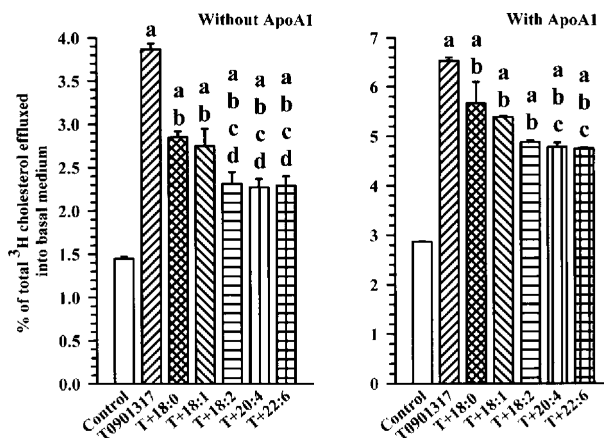

Figure 2 Effect of fatty acids on cholesterol efflux

The experiment was performed as described in Figure 1. After thorough washing to remove unincorporated label, 0.25 mM BSA alone or together with 1 mM stearic (T + 18:0), oleic (T + 18:1), linoleic (T + 18:2), arachidonic (T + 20:4) or docosahexaenoic (T + 22:6) acids was added to the upper chambers. Except for control dishes, 1 μM T0901317 (T) was added to the apical medium together with the fatty acids. In another set of dishes, apoA1 (15 μg/2.5 ml) was added to the lower wells. Unesterified cholesterol in basal medium is expressed as a percentage of total labelled cholesterol in cells and media recovered from both upper and lower chambers (2.14×10^6 d.p.m./dish). Left panel: values represent the means ± S.E.M. for three separate experiments with 3–4 dishes for each treatment per experiment. Right panel: values represent the means ± S.E.M. for three dishes. a, $P < 0.05$ versus BSA control; b, $P < 0.05$ versus T0901317; c, $P < 0.05$ versus T + 18:0; d, $P < 0.05$ versus T + 18:1.

effluxed into the basolateral medium was estimated. To eliminate the contribution of cholesterol associated with triacylglycerol-rich lipoprotein secretion, an MTP inhibitor, BMS 201038, at a concentration of 0.1 μM, was added to the apical medium of this experiment and all subsequent experiments. In the presence of either 1 mM oleic or arachidonic acid, the MTP inhibitor completely prevented the increase in secretion of triacylglycerol or apoB mass (results not shown). Figure 1 shows the results of this experiment. At concentrations of 50 or 100 μM, neither fatty acid interfered with the basolateral efflux of cholesterol in response to T0901317. At concentrations of 250 μM or above, however, both fatty acids decreased cholesterol efflux. Arachidonic acid was more potent than oleic acid in attenuating the effect of the LXR agonist. Cholesterol efflux into the apical medium was not altered by either the LXR agonist or the fatty acids (results not shown). In the absence of T0901317, approximately equal amounts of cholesterol label were recovered in the upper and lower chambers, ranging from 1 to 2 % of total cellular cholesterol label.

To examine the specificity (or non-specificity) of this effect, cells were incubated with T0901317 and fatty acids of various chain lengths and saturation. After the incubation, the amount of labelled cholesterol that effluxed basolaterally was again estimated (Figure 2, left panel). All fatty acids tested attenuated the effect of the LXR agonist on cholesterol efflux. Compared with the saturated and monounsaturated fatty acids, stearic and oleic acid, the polyunsaturated fatty acids, linoleic, arachidonic and docosahexaenoic acids, appeared to be relatively more effective in suppressing cholesterol efflux.

We have previously shown that in response to LXR activation, cholesterol efflux into the basolateral medium is enhanced in the absence of exogenously added apoA1 as a cholesterol acceptor [22]. It was assumed that CaCo-2 cells constitutively secrete endogenous apoA1 that then acts as a sterol acceptor. To ensure that this remained true under conditions of fatty acid flux, apoA1 was added to the lower wells and the experiment was repeated (Figure 2, right panel). Compared with control dishes without

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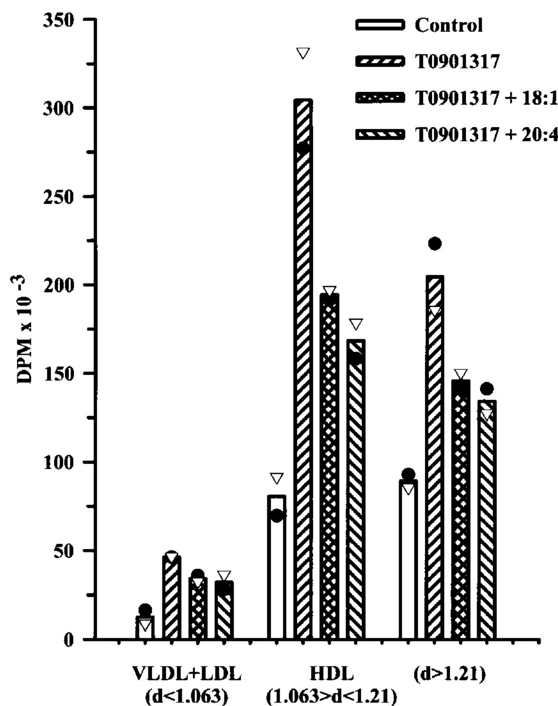

Figure 3 Effect of oleic and arachidonic acids on the efflux of cholesterol to lipoproteins isolated in the HDL density range

The experiment was performed as described in Figure 1. After thorough washing to remove unincorporated label, 0.25 mM BSA alone (open bars) or together with 1 μM T0901317 (rising right bars), 1 μM T0901317 + 1 mM of oleic acid (T0901317 + 18:1) (diagonal cross-hatch bars) or 1 μM T0901317 + arachidonic acid (T0901317 + 20:4) (rising left bars) was added to the upper chambers. The lower chambers contained 35 μg of HDL per ml of M199. At the end of 24 h, the basal medium was collected and the density was adjusted to 1.063 g/ml with solid KBr. The medium was centrifuged at 314 000 ***g*** for 18 h in a TLA-100.2 rotor using a Beckman Optima TL tabletop ultracentrifuge. At the end of the centrifugation, 20 % of the volume at the top was removed (VLDL + LDL fraction). The density of the subnatant was then adjusted to 1.21 g/ml by the addition of solid KBr. After a second centrifugation at 314 000 ***g*** for 18 h, the top 20 % of the volume was collected (HDL fraction). Radioactivity in the isolated fractions and 1.21 g/ml subnatant was determined. The Figure shows a representative experiment of two having similar results. The values represent the average of two dishes and the range is shown by open and closed symbols.

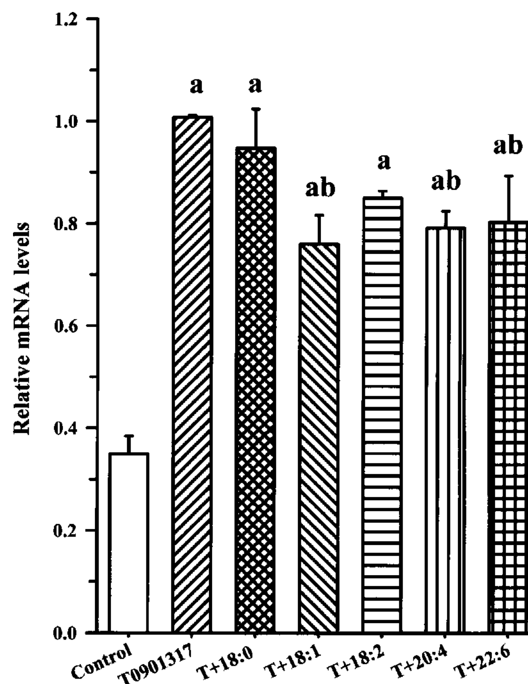

Figure 4 Effect of fatty acids on ABCA1 gene expression

Cells were incubated for 24 h with 0.25 mM BSA alone or together with 1 mM of stearic (T + 18:0), oleic (T + 18:1), linoleic (T + 18:2), arachidonic (T + 20:4) or docosahexaenoic (T + 22:6) acids added to the upper chambers. Except for control dishes, 1 μM T0901317 (T) was added to the apical medium together with the fatty acids. After the incubation, total cellular RNA was extracted and the abundances of ABCA1 mRNA and of 18 S rRNA were estimated by real-time RT–PCR as described in the Materials and methods section. mRNA abundance of ABCA1 was normalized to 18 S rRNA. The values represent the means ± S.E.M. for three experiments. For each treatment 3–4 dishes were used per experiment. a, $P < 0.05$ versus BSA control; b, $P < 0.05$ versus T0901317.

apoA1, basolateral cholesterol efflux was increased in dishes containing apoA1. Similar to what was observed in the absence of added apoA1, however, all fatty acids suppressed cholesterol efflux in response to LXR activation, with polyunsaturated fatty acids being relatively more potent.

Oleic and arachidonic acids decrease the efflux of cholesterol to lipoproteins isolated in the HDL density range

It is believed that the bulk of circulating HDL originates from liver and intestine [30,31]. We have postulated that cellular cholesterol, effluxing from the basolateral membrane of CaCo-2 cells in response to LXR activation, represents cholesterol in nascent HDL particles [22]. To address this, cells were labelled with cholesterol and incubated with T0901317 in the presence or absence of oleic or arachidonic acid. After the incubation, the density of the basolateral medium was adjusted to 1.063 g/ml. After centrifugation, lipoproteins floating at this density were collected and the density of the remaining medium was readjusted to 1.21 g/ml. After another centrifugation, lipoproteins floating at this density and the subnatant were collected. The amount of labelled cholesterol recovered in these fractions was estimated. The results are shown in Figure 3. Most of the label was recovered in lipoproteins isolated at density 1.21 g/ml or greater. In the presence of an MTP inhibitor, very little labelled cholesterol was found in lipoproteins isolated at a density of 1.063 g/ml or less. The LXR agonist significantly increased the efflux of cellular cholesterol to particles isolated at density 1.21 g/ml or greater. Both oleic and arachidonic acid significantly attenuated the efflux of cholesterol to these particles.

Effect of fatty acids on ABCA1 gene expression

ABCA1, a gene target of LXR, facilitates the efflux of cellular cholesterol to apoA1 or lipid-poor HDL particles [8–10]. In CaCo-2 cells, LXR activation with either T0901317 or 22-hydroxycholesterol/9-*cis*-retinoic acid increases ABCA1 expression [22]. Thus, to address a possible mechanism to understand why fatty acids decrease cholesterol efflux, ABCA1 mRNA levels were estimated in cells that were incubated with T0901317 and fatty acids of different chain lengths and saturation. The results are shown in Figure 4. As expected, the LXR agonist significantly increased ABCA1 mRNA levels. None of the fatty acids, however, caused substantial changes to ABCA1 gene expression. Statistically, oleic, arachidonic and docosahexaenoic acids significantly decreased ABCA1 mRNA levels by approx. 20 %. Since the suppression of ABCA1 mRNA abundance by these fatty acids was modest, and since stearic and linoleic acids had no significant effect on gene expression, and yet both caused a decrease in cholesterol efflux, the results suggested that the effects of the fatty acids on cholesterol efflux could not be attributed to changes in ABCA1 gene expression.

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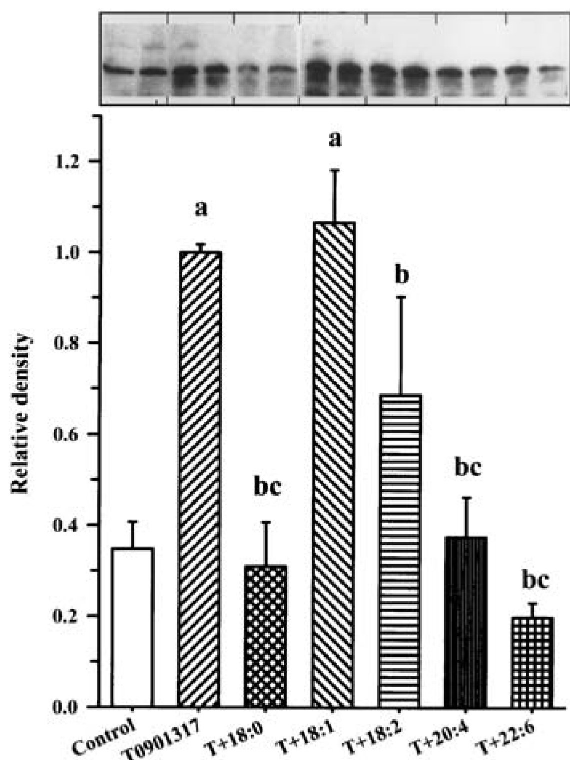

Figure 5 Effect of fatty acids on ABCA1 mass

Cells were incubated with the fatty acids as described in Figure 4. After the incubation, cells were rinsed with PBS, lysed, and total membranes isolated as described in the Materials and methods section. Proteins were separated by SDS/PAGE and immunoblotted using a polyclonal anti-ABCA1 antibody. The top of the Figure shows a representative immunoblot, with each lane depicting a separate dish. For control, T0901317 (T), T + 18:1 and T + 20:4, the data are derived from four separate experiments. For each treatment, 3–4 dishes were used per experiment. Data for T + 18:0, T + 18:2 and T + 22:6 are from one experiment with $n = 3$. a, $P < 0.05$ versus BSA control; b, $P < 0.05$ versus T0901317; c, $P < 0.05$ versus T + 18:1.

Effect of fatty acids on ABCA1 mass

We next addressed whether fatty acids altered ABCA1 mass in response to LXR activation. Cells were incubated with T0901317 or T0901317 and the various fatty acids. After the incubation, ABCA1 mass was estimated by immunoblotting. The results are shown in Figure 5. A representative immunoblot is shown above the bar graph, with each lane representing a separate dish. Similar to what we and others have observed before, the antibody against ABCA1 detects two bands at approx. 220 kDa [32]. LXR activation consistently and reproducibly increased the intensity of the lower band in all experiments. Although in this blot, LXR activation modestly increased the intensity of the upper band, this was not a consistent finding in all experiments. ABCA1 mass was estimated by determining the intensity of the lower band. Stearic, arachidonic and docosahexaenoic acids markedly reduced ABCA1 mass in response to LXR activation. Linoleic acid also significantly suppressed ABCA1 mass, but the effect was more modest. In distinct contrast with the other fatty acids, oleic acid did not alter the mass of ABCA1.

Effect of oleic acid on esterification of plasma membrane cholesterol

Since oleic acid did not alter ABCA1 mass, but did suppress cholesterol efflux in response to LXR activation, another explanation for the effect of oleic acid on cholesterol efflux was pursued. Previous observations have suggested that ACAT (acyl-CoA:cholesterol acyltransferase) and ABCA1 utilize the same pool of membrane cholesterol as substrate [22]. In addition, compared with other fatty acids, oleic acid is the preferred substrate for cholesteryl esterification by ACAT [33,34]. Thus we postulated that oleic acid was promoting the flux of membrane cholesterol to ACAT, directing it away from ABCA1. This would make less cholesterol available for ABCA1 and lead to diminished cholesterol efflux. We addressed this possibility in the following manner. Cells were prelabelled with cholesterol and incubated for 18 h with T0901317 and either 1 mM oleic or arachidonic acid. After the incubation, the percentage of cellular cholesterol that was esterified was estimated. In cells incubated with the LXR agonist alone, between 6 and 8 % of cellular cholesterol was esterified. In cells incubated with oleic acid, approx. 16–20 % was esterified, compared with only 6–7 % in cells incubated with arachidonic acid. Thus, compared with arachidonic acid, oleic acid was directing at least twice the amount of membrane cholesterol to ACAT for esterification. If this were the explanation for the observed decrease in cholesterol efflux by oleic acid, preventing cholesterol esterification should reverse the inhibitory effect of oleic acid on cholesterol efflux. To test this, cells were prelabelled with cholesterol and incubated with T0901317, T0901317 and oleic acid, or T0901317, oleic acid and a potent ACAT inhibitor, PD128042. After the incubation, the percentage of cellular cholesterol esterified and the percentage of cholesterol recovered in the basolateral well were estimated in the presence or absence of apoA1 added to the lower well. Figure 6 shows these results. The results were similar whether apoA1 was added to the lower well (right panel) or not (left panel). The upper panels depict the percentage of cellular cholesterol esterified. Addition of oleic acid caused a 2–3-fold increase in the amount of cholesteryl esters within cells. This increase, however, was completely prevented in cells incubated with the ACAT inhibitor. The lower panels in Figure 6 depict the percentage of cholesterol effluxed. In response to LXR activation, cholesterol efflux was attenuated 25–30 % by oleic acid. In cells incubated with oleic acid and the ACAT inhibitor, however, the percentage of cholesterol efflux was similar to cells incubated with the LXR agonist alone. These results suggest that oleic acid probably decreases cholesterol efflux by diverting cholesterol away from ABCA1 and towards ACAT.

DISCUSSION

The results of the present study clearly demonstrate that an increase in fatty acid flux decreases the efflux of cellular cholesterol from the basolateral membrane of CaCo-2 cells in response to LXR activation. Although all the fatty acids tested suppressed efflux, the effect was more pronounced with polyunsaturated fatty acids. In previous studies performed in CaCo-2 cells and other cell lines, it has been demonstrated that fatty acids, specifically unsaturated fatty acids, interfere with LXR activation [27–29]. In HEK-293 cells and rat hepatoma cells, unsaturated fatty acids decreased gene expression of SREBP-1c, another recognized gene target of LXR [28,29]. In CaCo-2 cells, we also showed evidence for attenuation of SREBP-1c gene expression by an influx of polyunsaturated fatty acids and postulated that the mechanism was probably an interference of LXR activation by the fatty acids [27]. Thus it seemed logical that if these fatty acids interfered with LXR activation, other gene targets of LXR would similarly be affected.

ABCA1, a sterol transporter critical for facilitating cholesterol efflux, is such a gene target. In intestine, there is clear evidence, both *in vitro* and *in vivo*, that LXR activation enhances the

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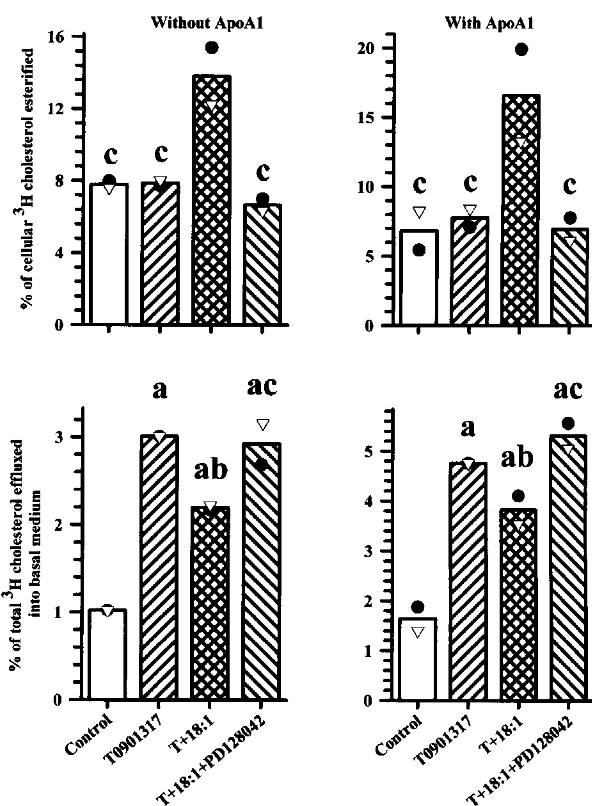

Figure 6 Effect of ACAT inhibition on cholesterol efflux

After labelling the cells with [^{3}H]cholesterol as described in Figure 1, cells were washed and incubated for 18 h with 1 μM T0901317, T0901317 and 1 mM oleic acid, or T0901317, oleic acid, plus 4 μg/ml of the ACAT inhibitor, PD128042. In some of the dishes, apoA1, 15 μg/2.5 ml, was added to the lower well. After the incubation, the amount of label found in cellular cholesteryl ester (upper panels) and the amount of cholesterol effluxed into the lower well (lower panels) were estimated. The total cholesterol radioactivity in cells and media was 2.66×10^{6} d.p.m./dish for experiments without apoA1 and 3.5×10^{6} d.p.m./dish for experiments with apoA1. The closed symbols represent the mean from experiment no. 1 and the open symbols represent the mean from experiment no. 2. For each treatment, three dishes were used per experiment. The bar height represents the mean of the two experiments. a, $P < 0.05$ versus control; b, $P < 0.05$ versus T0901317; c, $P < 0.05$ versus T + 18:1.

expression of ABCA1 [16,22–24]. We previously demonstrated in CaCo-2 cells that the LXR-mediated increase in ABCA1 mass was secondary to an increase in transcription of the ABCA1 gene [22]. Thus, in the present study, we postulated that polyunsaturated fatty acids would predominantly interfere with transcription of ABCA1 in response to LXR activation. Our results demonstrate clearly that this is not the case. Stearic, arachidonic and docosahexaenoic acids essentially prevented the increase in ABCA1 mass in response to LXR activation, and yet the effects of the fatty acids on ABCA1 gene expression were either non-existent or modest at best. Thus these results suggest that these fatty acids exert their suppressive effects on ABCA1 mass at a post-transcriptional step. While we were performing our studies, Wang and Oram [32] showed in macrophages that unsaturated fatty acids enhanced the rate of degradation of ABCA1 mass without altering ABCA1 gene expression. It is clear, therefore, that fatty acids alter LXR-mediated events at several different levels of regulation.

In the present study, we elected to activate LXR to enhance both ABCA1 expression and cholesterol efflux to investigate the regulation of this LXR-mediated process by fatty acids. In the absence of LXR activation, estimation of ABCA1 mass is difficult, and with treatments that could potentially (and did) suppress the mass of ABCA1 further, estimation of ABCA1 protein would have been even more difficult. It is possible, therefore, that by simultaneously activating LXR by T0901317, a fatty acid-induced inhibition of ABCA1 transcription could have been masked. For the LXR target gene, SREBP-1c, this was found to be true [28]. In FTO-2B cells, LXR activation by T0901317 completely prevented the attenuation of SREBP-1c gene expression by fatty acids. Thus, in the present study, LXR activation may have masked the effect of fatty acids on 'basal' ABCA1 gene expression. This, however, cannot explain our present findings. All fatty acids decreased cholesterol efflux in response to LXR activation, without changing or only modestly attenuating ABCA1 gene expression.

Synthesized agonists of peroxisome-proliferator-activated receptor-α (PPARα) enhance the expression of ABCA1, probably through an LXR pathway [35–37]. Since non-esterified fatty acids, particularly polyunsaturated fatty acids, are natural ligands of PPARα [38,39], it could be postulated that fatty acid flux through an intestinal cell might activate PPARα and, thus, enhance ABCA1 expression and basolateral cholesterol efflux. PPARs play a critical role in regulating the oxidation machinery for fatty acids [40,41]. In the small intestine, however, we and others have demonstrated that fatty acid oxidation is a minor pathway of fatty acid metabolism [42–44]. The predominant fate of fatty acids in the upper gut is esterification and transport. Since esterified fatty acids are not ligands for PPARα, it is not probable that under normal conditions PPARs play a significant role in the fatty acid metabolism in this organ. Moreover, our present results are in contrast with what would have been expected with an increase in fatty acid flux, i.e. enhanced ABCA1 expression by activation of a PPARα pathway. In fact, just the opposite was observed. Fatty acids attenuated ABCA1 expression. In our experiments, however, we cannot completely exclude the possibility that fatty acid flux might regulate PPARα and ABCA1 expression, as all of our experiments were performed in the presence of a very potent LXR activator that could potentially obscure any PPARα-enhancing effect on ABCA1 expression. Additional work will be necessary to address the role of PPARs in regulating ABCA1 expression and cholesterol trafficking in small intestine.

Results observed with oleic acid are surprising. They provide yet another mechanism for regulating cholesterol efflux and HDL formation by the intestine, a mechanism that is independent of ABCA1 expression. As we have postulated previously [22], results of the present study suggest that ACAT and ABCA1 utilize the same cholesterol substrate pool within the cell. Oleic acid, by directing a substantial amount of plasma membrane cholesterol to ACAT, clearly decreases the available pool of cholesterol for ABCA1. This results in a decrease in basolateral cholesterol efflux and HDL formation. If this observation can be applied to the *in vivo* situation, it would indicate that diets enriched in oleic acid would preferentially promote intestinal cholesterol secretion in triacylglycerol-rich lipoproteins and less cholesterol secretion in nascent HDL. Because of the major contribution of the liver to plasma VLDL (very-low-density lipoprotein) and HDL cholesterol, this hypothesis would have to be tested in lymph-cannulated animals. To our knowledge, this has not been done.

Whether SREBP-1c or ABCA1 is investigated as gene targets of LXR, it seems clear that all fatty acids are not equal in interfering with LXR-mediated events. From combined results of previous reports, the pattern of inhibition clearly favours unsaturated fatty acids over saturated fatty acids and, in most studies, polyunsaturated fatty acids over monounsaturated fatty acids [27–29,32]. This has been generally true for our studies in CaCo-2 cells. In our previous study, neither stearic nor oleic acid altered mass or gene expression of SREBP-1c, whereas

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polyunsaturated fatty acids profoundly decreased both mass and gene expression. In addition, oleic acid did not interfere with LXR-mediated enhancement of SREBP-1c gene expression [27]. In the present study, in response to LXR activation, oleic acid also had little or no effect on ABCA1 gene or protein expression, whereas the polyunsaturated fatty acids markedly decreased ABCA1 mass. Unlike the previous study, however, in which stearic acid did not alter SREBP-1 mass, the saturated fatty acid unequivocally decreased the mass of ABCA1 in the present study. We do not have a good explanation for this. Although others have observed interference of LXR-mediated activation by stearic acid [28], it was attributed to the conversion of stearic into oleic acid by stearoyl-CoA desaturase-1. Obviously, this cannot be implicated in our study, as the effects of stearic and oleic acid on ABCA1 expression differed significantly. Caution should be used, therefore, in making generalizations about the effects of certain fatty acids on LXR-mediated events, as it seems to be partially dependent on cell type and the gene target of interest.

ABCA1 functions at the basolateral membrane of the intestinal cell facilitating the transfer of cellular cholesterol to apoA1, an apoprotein that is secreted by the intestine [22,23]. It is postulated that intestinal ABCA1 is critical for nascent HDL formation by the intestine [30]. The present findings suggest, therefore, that an increase in fatty acid flux, but particularly polyunsaturated fatty acid flux, will cause a decrease in HDL cholesterol derived from intestine. Indeed, it has been demonstrated in several studies that diets enriched with either monounsaturated or polyunsaturated fat cause a modest decrease in plasma HDL [45]. By attenuating the expression and/or activity of intestinal ABCA1, the results of the present study provide at least a partial explanation as to why an increase in dietary unsaturated fatty acids decreases HDL levels. Although a decrease in intestinal HDL production by a diet enriched in unsaturated fatty acids might be considered detrimental, the decrease in HDL does not translate into increased risk for atherosclerosis. In fact, the opposite occurs. Since LDL (low-density lipoprotein) cholesterol levels decrease even further by diets enriched in unsaturated fat, the HDL/LDL ratio remains favourable. Numerous clinical studies have demonstrated that diets enriched in unsaturated fats significantly reduce cardiovascular events [45–48].

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