Polyunsaturated fatty acids decrease the expression of sterol regulatory element-binding protein-1 in CaCo-2 cells: effect on fatty acid synthesis and triacylglycerol transport

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Regulation of sterol regulatory element-binding proteins (SREBPs) by fatty acid flux was investigated in CaCo-2 cells. Cells were incubated with 1 mM taurocholate with or without 250 μ M 18:0, 18:1, 18:2, 20:4, 20:5 or 22:6 fatty acids. Fatty acid synthase (FAS) and acetyl-CoA carboxylase mRNA levels and gene and protein expression of SREBPs were estimated. 18:2, 20:4, 20:5 and 22:6 fatty acids decreased the amount of mature SREBP-1 and mRNA levels of SREBP-1c, SREBP-1a, FAS and acetyl-CoA carboxylase. SREBP-2 gene or mature protein expression was not altered. Liver X receptor (LXR) activation by T0901317 increased gene expression of SREBP-1c, SREBP-1a, FAS and acetyl-CoA carboxylase without altering SREBP-2. 20:5, but not 18:1, prevented the full expression of SREBP-1c mRNA by T0901317. T0901317 increased SREBP-1 mass without altering the mass of mature SREBP-2.

Although only 18:2, 20:4, 20:5 and 22:6 suppressed SREBP-1, acetyl-CoA carboxylase and FAS expression, all fatty acids decreased the rate of fatty acid synthesis. T0901317 increased endogenous fatty acid synthesis yet did not increase secretion of triacylglycerol-rich lipoproteins. In CaCo-2 cells, polyunsaturated fatty acids decrease gene and protein expression of SREBP-1 and FAS mRNA, probably through interference with LXR activity. Since all fatty acids decreased fatty acid synthesis, mechanisms other than changes in SREBP-1c expression must be entertained. Increased endogenous fatty acid synthesis does not promote triacylglycerol-rich lipoprotein secretion.

Key words: apolipoprotein B, cholesterol, liver X receptor.

INTRODUCTION

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that regulate the transcription of several key genes in the biosynthetic pathways of cholesterol and fatty acids (see [1] for a review). Three SREBPs have been described, designated SREBP-1a, -1c and -2. SREBP-1a and SREBP-1c are products of a single gene that are derived by using alternative transcriptional start sites [2,3]. SREBP-2 is a product of a different gene and shares approx. 50% homology with the SREBP-1 isoforms [4]. SREBPs are bound to membranes of the endoplasmic reticulum. The membrane-bound protein is conventionally called the precursor or membrane form. In times of cellular sterol deficiency, SREBPs are transported to the Golgi where the precursor form of SREBP undergoes a two-step proteolysis that releases the N-terminal 68 kDa domain of the protein [5]. This 'mature or nuclear form' enters the nucleus and binds to a 10 bp sterol regulatory element of target genes that will then enhance transcription [6,7]. Movement of SREBPs to the Golgi and proteolysis of the precursor are prevented by excess cellular cholesterol, leading to suppression of transcription of these genes [8–10]. Although there is some overlap in their gene targets, SREBP-2 preferentially enhances transcription of genes that control cholesterol synthesis, whereas SREBP-1 enhances genes of the fatty acid synthetic pathway [11–14].

It is clear that changes in cellular cholesterol flux regulate SREBP expression [8–10,15–18]. There is now growing evidence to suggest that certain fatty acids alter SREBP expression as well

[19–24]. This observation may be particularly important in absorptive cells that line the small intestine. These cells are exposed to very high levels of dietary and biliary-derived fatty acids. Unlike peripheral cells that tend to store or oxidize incoming fatty acids, absorptive cells rapidly transport luminal fatty acids into lymph as esterified lipids contained within chylomicrons. The regulation of SREBPs by fatty acid flux in intestinal absorptive cells, therefore, may differ from that observed in cells of the periphery or, for that matter, from hepatocytes that procure dietary fatty acids via uptake of remnant lipoprotein particles. Since chylomicrons also contain cholesterol and cholesteryl esters, and endogenously synthesized fatty acids contribute to exported triacylglycerols [25], the interplay that occurs between cholesterol and fatty acid biosynthesis, as regulated by intestinal SREBPs, may be important for normal chylomicron assembly and secretion during influx of fatty acids.

SREBP-1c, the isoform of SREBP-1 that impacts fatty acid synthesis more so than cholesterol synthesis, is a target gene for liver X receptors (LXRs) [26]. LXRs, whose ligands are naturally occurring oxysterols, belong to a class of nuclear hormone receptor transcription factors that are highly expressed in intestine as well as in other cells [27–30]. LXRs bind to DNA as a heterodimer along with retinoid X receptors (RXRs) to enhance the transcription of certain target genes [31–33]. Recent evidence suggests that polyunsaturated fatty acids interfere with LXR activation, which in turn leads to a decrease in expression of SREBP-1c [24].

Abbreviations used: apoB, apolipoprotein B; FAS, fatty acid synthase; LXR, liver X receptor; RT-PCR, reverse transcriptase PCR; RXR, retinoid X receptor; SREBP, sterol regulatory element-binding protein.

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The present study was undertaken in the human intestinal cell line, CaCo-2, to address the regulation of intestinal SREBPs by micellar fatty acid flux and to investigate the role of SREBP-1c in fatty acid synthesis and triacylglycerol transport. The results show that 18- and 20-carbon polyunsaturated fatty acids suppress SREBP-1c mRNA levels and SREBP-1 protein mass resulting in a decrease in fatty acid synthase (FAS) and acetyl-CoA carboxylase gene expression and fatty acid synthesis. Polyunsaturated fatty acids probably suppress SREBP-1c gene expression by interfering with LXR activation. Enhancing SREBP-1c expression by LXR activation increases FAS and acetyl-CoA carboxylase gene expression and fatty acid synthesis but does not augment apolipoprotein B (apoB) or triacylglycerol transport.

MATERIALS AND METHODS

[¹⁴C]Acetate (55 mCi/mmol) and [³²P]UTP (3000 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, U.S.A.). ³H-water (1 Ci/mmol) was purchased from ICN Biochemicals (Irvine, CA, U.S.A.). Protease inhibitors, BSA, cholesterol, taurocholic acid, stearic acid, oleic acid, linoleic acid and Tri Reagent were purchased from Sigma (St. Louis, MO, U.S.A.). Arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Superscript II RNase inhibitor, Lig'nScribe, MAXIScript, MEGAScript, pTRI RNA 18 S and RNase protection assay (RPA) III kits were purchased from Ambion (Austin, TX, U.S.A.). Medium 199 was from Gibco-BRL Life Technologies (Grand Island, NY, U.S.A.). The LXR agonist T0901317 [34] was kindly supplied by Tularik (South San Francisco, CA, U.S.A.). RNase-free DNase was purchased from Promega (Madison, WI, U.S.A.). Superscript II RNase H⁻ reverse transcriptase was obtained from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.). SYBR Green PCR mix was purchased from Applied Biosystems (Foster City, CA, U.S.A.).

Cell culture

CaCo-2 cells were grown in T-75 flasks as described previously [35]. They were subcultured on micropore filters inserted in Transwells (Costar, Cambridge, MA, U.S.A.). Cells were used 14 days after plating and medium was changed every 2 days.

Solution of taurocholate and cholesterol were prepared in 95% ethanol. Fatty acids were dissolved in chloroform. Appropriate amounts of taurocholate and fatty acids were mixed and solvents were evaporated under a stream of nitrogen. Medium 199 with 10 mM Hepes was then added to obtain the

desired concentrations of the lipids. The solution was warmed to 37 °C and stirred vigorously before use. All solutions were clear prior to adding to cells. Following incubation of cells with fatty acids, cell viability was monitored by mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into blue formazan product that is released into the medium [36]. Taurocholate was used at 1 mM to dissolve fatty acids because eicosapentaenoic and docosahexaenoic acids modestly decreased cell viability when dissolved in 5 mM taurocholate.

In experiments where oleic acid was used to stimulate lipoprotein synthesis and secretion, the fatty acid was complexed with albumin as described previously [17].

To study the effect of LXR activation on fatty acid synthesis, FAS and acetyl-CoA carboxylase gene and SREBP gene and protein expression, CaCo-2 cells were incubated with medium 199 containing 1 µM T0901317 in 0.02% DMSO. Control cells were incubated with 0.02% DMSO alone.

RNase protection assays

The plasmid preparations for human SREBP-1a and -1c were a generous gift from Dr M. S. Brown and Dr J. L. Goldstein (University of Texas Southwestern Medical School, Dallas, TX, U.S.A.). Antisense RNA probes for human SREBP-2 and FAS were prepared and RNase protection assays were performed as described in [17].

Estimation of relative expression of mRNA levels by real-time reverse transcriptase PCR (RT-PCR)

RNA was extracted from CaCo-2 cells using Tri reagent. RNA (2 µg) was treated with RNase-free DNase to remove contaminating genomic DNA. After inactivating the DNase by addition of EGTA and heating at 65 °C for 10 min, the RNA was transcribed to cDNA by SuperScript II RNase H⁻ reverse transcriptase using random hexamers at 24 °C for 10 min and at 40 °C for 4 h. After stopping the reaction by incubation at 90 °C for 15 min, one-twenty-fifth of the reverse transcriptase mixture was mixed with 2 × SYBR Green PCR master mix and gene-specific primers in a final volume of 20 µl. The primers (see Table 1) were designed with Primer Express software. The primer pairs were selected to yield a single amplicon based on dissociation curves and analysis by acrylamide gel electrophoresis. Real-time

Table 1 Real time RT-PCR primers for human

	Sequence	Size (bp)	Accession no.
18 S	5'-TAAGTCCCTGCCCTTTGTACACA-3' 5'-GATCCGAGGGCCTCACTAAC-3'	71	K03432
SREBP-1a	5'-TCAGCGAGGCGGCTTTGGAGCAG-3' 5'-CATGTCTTCGATGTCGGTCAG-3'	80	U00968
SREBP-1c	5'-GGAGGGGTAGGGCCAACGGCCT-3' 5'-CATGTCTTCGAAAGTGCAATCC-3'	80	S66167
SREBP-2	5'-CTGAAGCTGGCAATCAAAAG-3' 5'-TCATCAATAGAGGGCTTCCT-3'	200	NM_004599.1
FAS	5'-ACAGGGACAACCTGGAGTTCT-3' 5'-CTGTGGTCCCACTTGATGAGT-3'	151	NM_004104
Acetyl-CoA carboxylase	5'-GAAGGGCTTATATGCCTATGAACCTAAC-3' 5'-GGGCAGCATGAACCTGAATT-3'	92	U19822

Figure 1 Effect of fatty acids on gene expression of SREBPs, FAS and acetyl-CoA carboxylase

CaCo-2 cells were incubated for 18 h in medium containing 1 mM taurocholate alone (TC) or with 250 μ M stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4), eicosapentaenoic (20:5) or docosahexaenoic (22:6) acid. Left-hand panel: mRNA levels of SREBP-1a, SREBP-1c, SREBP-2 and FAS were estimated by RNase protection assays as described in the Materials and methods section. 18 S rRNA was used to ensure equal loading of RNA. The Figure shows a representative autoradiogram of two separate experiments with similar results. Each assay represents RNA from two dishes; $n = 6$ samples for each fatty acid. The number below each autoradiogram reflects the mean fold-change from taurocholate alone. Right-hand panel: mRNA levels of SREBP-1a, SREBP-1c, SREBP-2, FAS and acetyl-CoA carboxylase were determined by real-time quantitative RT-PCR as described in the Materials and methods section. In these experiments 18 S rRNA was used as an endogenous control to normalize the expression of other genes. The results show the means $\pm$ S.E.M. of fold change for the treatment over taurocholate alone. Each assay represents RNA from two dishes; $n = 9$ samples for each fatty acid from three separate experiments. * $P < 0.05$ versus taurocholate alone.

quantitative PCR was performed in an ABI Prism model 7000 sequence-detection system. The thermal cycler parameters were: hold for 2 min at 50 $^{\circ}$ C and 10 min at 95 $^{\circ}$ C for one cycle followed by amplification of cDNA for 40 cycles with melting for 15 s at 95 $^{\circ}$ C and annealing and extension for 1 min at 60 $^{\circ}$ C. The values were normalized using 18 S rRNA as an endogenous internal standard. A serial dilution of a standard was run on each plate for each mRNA and used to calculate the relative levels of the mRNA.

Immunoblot analysis

The methods used for estimation of SREBP mass in CaCo-2 cells by immunoblotting have been described previously [17].

Fatty acid synthesis

CaCo-2 cells were incubated for 18 h with 1 mM taurocholate or taurocholate containing 250 μ M of the respective fatty acid. At

4 and 2 h prior to harvesting the cells (to ensure linearity of the incorporation), [^{14}C]acetate was added at a final concentration of 0.6 mM and specific activity of 11 d.p.m./pmol. In other experiments, [^3H]water was added at a final specific activity of 8 mCi/ml. Lipids were extracted from cells and radioactivity in the fatty acid fraction was determined as described earlier [17].

FAS assay

FAS activity in CaCo-2 cytosol was assayed as described by Kumar and Dodds [37]. Briefly, CaCo-2 cells were homogenized in 100 mM potassium phosphate buffer, pH 7, 0.1 mM Na-EDTA and 1 mM dithiothreitol. The homogenate was centrifuged at 100 000 *g* for 30 min to obtain cytosol. The reaction mixture contained 200 μg of cytosolic protein, 100 mM potassium phosphate buffer, pH 7, 0.1 mM Na-EDTA, 1 mM dithiothreitol, 0.3 mM NADPH, 0.05 mM acetyl-CoA and 0.15 mM malonyl-CoA. The oxidation of NADPH at 340 nm was measured spectrophotometrically in a linear range at 1 min intervals.

Other analyses

An aliquot of the membrane preparation was taken and proteins were precipitated by adding 50 μl of 0.15% deoxycholate and 0.45 ml of 10% trichloroacetic acid to remove substances that interfere with protein estimation. Protein content was then determined using the BCA method (Pierce Endogen, Rockford, IL, U.S.A.). Triacylglycerol and apoB mass were estimated using the GPO Trinder reagent (Sigma) and sandwich ELISA respectively [36,38].

Statistical analysis

To determine whether significant differences existed between fatty acids, the data were analysed by one-way ANOVA and the Student–Newman–Keuls method, using SigmaStat software (SPSS, Chicago, IL, U.S.A.). $P < 0.05$ was considered significant.

RESULTS

Effect of fatty acids on gene expression of SREBPs, FAS and acetyl-CoA carboxylase

To investigate the regulation of gene expression of SREBPs, FAS and acetyl-CoA carboxylase by fatty acid influx, CaCo-2 cells were incubated for 18 h with 1 mM taurocholate or taurocholate containing 250 μM of stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4), eicosapentaenoic (20:5) or docosahexaenoic (22:6) acid. Following the incubation, mRNA levels for SREBP-1a, SREBP-1c, SREBP-2, FAS and acetyl-CoA carboxylase were estimated by RNase protection assays or RT-PCR (Figure 1). 18 S rRNA was used as a control to ensure that equal amounts of RNA were being analysed. The saturated and monoenoic 18-carbon fatty acids, 18:0 and 18:1, did not significantly alter mRNA levels of any of these genes. In contrast, 18:2 and the 20-carbon polyunsaturated fatty acids, 20:4, 20:5 and 22:6, all decreased mRNA levels of SREBP-1a, SREBP-1c, FAS and acetyl-CoA carboxylase. None of the fatty acids significantly altered mRNA levels of SREBP-2. To examine whether the effect of polyunsaturated fatty acids on gene expression was concentration-dependent, cells were incubated with increasing concentrations of either 18:1 or 20:5. Following the incubation, mRNA levels were estimated by RT-PCR (Figure 2). In concentrations up to 750 μM , 18:1 did not decrease mRNA levels of any of the genes. In contrast, 20:5, although

Figure 2 Effect of fatty acid concentration on gene expression of SREBPs, FAS and acetyl-CoA carboxylase

CaCo-2 cells were incubated for 18 h in medium containing 1 mM taurocholate alone (0) or with 50, 100, 250, 500 or 750 μM 18:1 or 20:5. mRNA levels of SREBP-1a, SREBP-1c, SREBP-2, FAS and acetyl-CoA carboxylase were determined by real-time quantitative RT-PCR as described in the Materials and methods section. In these experiments 18 S rRNA was used as an endogenous control to normalize the expression of other genes. The results show the means $\pm$ S.E.M. fold change for the treatment versus taurocholate alone. Each assay represents RNA from two dishes with $n = 7$ samples for each fatty acid from two separate experiments. * $P < 0.05$ versus taurocholate alone.

there was some variability, caused suppression of these genes between 50 μM (in the case of acetyl-CoA carboxylase) and 250 μM . Except for expression of SREBP-1a in this experiment, concentrations of 20:5 higher than 250 μM did not significantly decrease gene expression further. Again, neither 18:1 nor 20:5, at 750 μM , altered the expression of SREBP-2.

Effect of fatty acids on SREBP mass

To address the effect of fatty acid influx on SREBP mass, cells were again incubated for 18 h with taurocholate alone or taurocholate containing the individual fatty acids. After the incubation, proteins were extracted from total cellular membranes and the amount of precursor and mature forms of

Figure 3 Effect of fatty acids on SREBP mass

CaCo-2 cells were incubated as described for Figure 1. Following the incubation, total membranes were prepared and SREBP mass was estimated by standard immunoblotting as described in the Materials and methods section. The Figure shows a representative immunoblot of three separate experiments all with similar results. Each assay represents cells pooled from two dishes with $n = 3$ samples for each fatty acid. The number above each immunoblot represents the mean fold change of precursor protein from taurocholate alone (TC). The number below each immunoblot represents the mean fold change of mature protein from taurocholate alone.

SREBP-1 and SREBP-2 were estimated by standard immunoblotting. A representative blot is shown in Figure 3. Compared with SREBP-1 mass in cells incubated with taurocholate alone, neither the precursor nor the mature form was altered in cells incubated with 18:0 or 18:1. In contrast, the mature form of SREBP-1 was significantly decreased by 18:2, 20:4, 20:5 and 22:6. Precursor mass was decreased in cells incubated with 18:2 and 20:5. None of the fatty acids altered the precursor or mature form of SREBP-2.

Effect of LXR activation on SREBP mRNA levels and mass

In other cells, SREBP-1c is a recognized gene target for LXR [26]. To investigate whether intestinal SREBP-1c expression is regulated by LXR activation, CaCo-2 cells were incubated for 18 h with a non-sterol agonist of the LXR, T0901317. T0901317 is a specific agonist for LXR α and LXR β having little or no activity for other nuclear hormone receptors [34]. Following the incubation, mRNA levels for SREBPs, FAS and acetyl-CoA carboxylase were estimated by RNase protection assay or RT-PCR. The results are shown in Figure 4 (left-hand and middle panels, respectively). In cells incubated with T0901317, mRNA levels of SREBP-1a, SREBP-1c, FAS and acetyl-CoA carboxylase were significantly increased. The stimulation of gene expression by the LXR agonist was concentration-dependent with a maximal effect occurring at 1 μ M (Figure 4, right-hand panel). In contrast, T0901317 had no effect on SREBP-2 mRNA levels (Figure 4).

To investigate whether fatty acids interfere with the action of LXR, cells were incubated with taurocholate alone or taurocholate containing either 18:1 or 20:5 in the presence or absence of T0901317 (Figure 5). As shown above, in the absence of T0901317 (Figure 5, left-hand panel), 18:1 did not significantly alter mRNA levels of either SREBP-1c or FAS, whereas 20:5

significantly decreased them. In the presence of T0901317, mRNA levels of SREBP-1c and FAS were increased approx. 2.5-fold (Figure 5, right panel, TC). 18:1, which by itself did not alter SREBP-1c or FAS gene expression, also did not interfere with the action of the LXR agonist. In contrast, 20:5 prevented the full expression of SREBP-1c and FAS in response to the LXR agonist.

To address whether an increase in gene expression of SREBP-1c by LXR activation would result in an increase in the amount of SREBP-1 mass, CaCo-2 cells were incubated with taurocholate alone or taurocholate and T0901317. The amount of SREBP-1 and SREBP-2 mass was estimated following the incubation. The results are shown in a representative immunoblot in Figure 6. Both the precursor and mature forms of SREBP-1 were increased in cells incubated with T0901317. In contrast, the mature form of SREBP-2 was unaltered by LXR activation whereas the precursor form of SREBP-2 was modestly decreased.

Effect of fatty acids or T0901317 on fatty acid synthesis and triacylglycerol and apoB mass secretion

Because polyunsaturated fatty acids with chain lengths of 18- and 20-carbons suppressed FAS and acetyl-CoA carboxylase mRNA levels and SREBP-1 gene and protein expression, we asked whether these alterations would result in changes in fatty acid synthesis. Cells were incubated for 18 h with taurocholate alone, taurocholate and the respective fatty acid, or taurocholate and the LXR agonist T0901317. During the last 2 and/or 4 h of the incubation, labelled acetate or tritiated water was added and the incorporation of labelled substrate into fatty acids was estimated. The results are shown in Figure 7: the upper panel in Figure 7 shows the results with labelled acetate and the lower panel shows the results with labelled water. Estimates of changes in fatty acid synthesis were quite similar using the two different substrates.

Figure 4 Effect of T0901317 on SREBPs, FAS and acetyl-CoA carboxylase gene expression

Left-hand panel: CaCo-2 cells were incubated for 18 h with 1 mM taurocholate alone or taurocholate and 1 μ M T0901317. All media contained 0.02% DMSO. Following the incubation, mRNA levels were estimated by RNase protection assays. Each assay represents RNA from two dishes with $n = 6$ samples for each treatment. The number below the autoradiogram reflects the mean fold change from the control. Middle panel: the experiment described in the left-hand panel was repeated with the exception that mRNA levels of SREBP-1a, SREBP-1c, SREBP-2, FAS and acetyl-CoA carboxylase were estimated by real-time RT-PCR. The data represent means $\pm$ S.E.M. from three samples from each treatment. * $P < 0.05$ versus taurocholate alone (TC). Right-hand panel: effect of T0901317 concentration on gene expression. mRNA levels were estimated by RNase protection assays. Each assay represents RNA from two dishes with $n = 3$ samples for each treatment. The number below the autoradiogram reflects the mean fold change from control.

Interestingly, all the fatty acids suppressed fatty acid synthesis significantly. The effect of 18:1 was more modest than that of the other fatty acids, but the inhibitory effect was consistent and significant. In cells incubated with taurocholate and T0901317, the rate of fatty acid synthesis was increased by approx. 50%. To further substantiate this inhibitory effect of fatty acids on fatty acid synthesis, cytosolic FAS activity was estimated in cells incubated with the fatty acids. All fatty acids, including 18:0 and 18:1, suppressed FAS activity by 15–26% (results not shown).

It was next asked whether increasing SREBP-1 expression and rates of fatty acid synthesis by LXR activation would enhance the secretion of triacylglycerol-rich lipoproteins. CaCo-2 cells, grown on semi-permeable filters, were incubated for 18 h with increasing concentrations of T0901317 in the presence or absence of oleate to drive lipoprotein secretion. Following the incubation, the amount of triacylglycerol and apoB mass that was secreted into the basolateral medium was estimated. The results are shown in Figure 8. In the presence or absence of oleic acid, T0901317 did not alter either the secretion of triacylglycerol or

apoB. It is clear from these results that enhancing SREBP-1 expression and rates of fatty acid synthesis by LXR activation do not cause an increase in the secretion of triacylglycerol-rich lipoproteins.

DISCUSSION

The intestinal absorptive cell takes up and transports large amounts of dietary-derived fatty acids. Unlike the liver and peripheral cells, an enterocyte does not store fatty acids as neutral lipids; nor does it divert substantial amounts of fatty acids to oxidation pathways [38]. Instead, fatty acids taken up by absorptive cells are rapidly and efficiently esterified to neutral lipids, predominately triacylglycerols and cholesteryl esters, and within hours are secreted within chylomicron particles. We initially postulated, therefore, that fatty acid influx into intestinal cells would not alter the expression of genes that regulate fatty acid synthesis, i.e. SREBP-1, FAS or acetyl-CoA carboxylase. To our knowledge, regulation of fatty acid synthesis by fatty acid flux

Figure 5 Eicosapentaenoic acid, 20:5, interferes with LXR activity

CaCo-2 cells were incubated for 18 h with 1 mM taurocholate (TC) or taurocholate containing 250 μ M 18:1 or 20:5 in the presence or absence of 1 μ M T0901317. Following incubation, mRNA levels of SREBP-1c and FAS were estimated by RNase protection assays. Each assay represents RNA from two dishes with $n = 6$ samples for each treatment. The number below the autoradiogram reflects the mean fold change from taurocholate alone.

Figure 6 Effect of T0901317 on SREBP mass

CaCo-2 cells were incubated for 18 h with 1 mM taurocholate (TC) or taurocholate and 1 μ M T0901317. Following the incubation, total membranes were prepared and SREBP mass was estimated by standard immunoblotting as described in the Materials and methods section. The Figure shows a representative autoradiogram of two individual experiments with the same results. Each assay represents an individual dish with $n = 3$ dishes for each treatment. The number above the immunoblot represents the mean fold change of precursor protein from taurocholate alone. The number below the immunoblot represents the mean fold change of mature protein from taurocholate alone.

has not been previously addressed in the intestine. In addition, although it has been shown that endogenous fatty acids are utilized by intestinal cells to synthesize lipoprotein lipids [25], we postulated that it is luminal fatty acid flux that drives lipoprotein

Figure 7 Effect of fatty acids on fatty acid synthesis

CaCo-2 cells were incubated as described in Figure 1. Upper panel: at 4 and 2 h prior to harvesting the cells, 0.6 mM [¹⁴C]acetate, with specific activity of 11 d.p.m./pmol, was added. Incorporation of labelled acetate into fatty acids was estimated as described in the Materials and methods section. The data are presented as the percentage change from taurocholate alone (TC) and represent the means $\pm$ S.E.M. of 9 dishes from three experiments for each treatment. Lower panel: 4 h prior to harvesting the cells, [³H]water, with a specific activity of 8 mCi/ml of medium, was added. The incorporation of labelled water into fatty acids was estimated. Data are percentage change from taurocholate alone and are means $\pm$ S.E.M. of 7 dishes from two experiments for each treatment. * $P < 0.05$ compared with taurocholate alone.

production and that enhancing endogenous fatty acid synthesis would not cause, or further promote, lipoprotein assembly and secretion.

It is clear from results of this study that the influx of 18- and 20-carbon polyunsaturated fatty acids down-regulates SREBP-1c gene expression and decreases the amount of the nuclear form of the protein. These findings parallel the regulation of FAS and acetyl-CoA carboxylase gene expression, gene targets for SREBP-1c [11,39,40]. Thus down-regulation of gene expression of SREBP-1, FAS and acetyl-CoA carboxylase by fatty acid flux does not support our original hypothesis that intestinal fatty acid influx would bypass 'regulatory pathways' of fatty acid biosynthesis. In human embryonic and monkey kidney cells (HEK-293 and CV-1, respectively), the ability of individual fatty acids to inhibit SREBP-1 expression seemed to be related to chain length and degree of unsaturation [20,23]. The longer the chain length and the higher degree of unsaturation, the more potent the fatty acid was in suppressing SREBP-1 expression. Unlike our results, however, 18:1 did decrease SREBP-1c

Figure 8 Effect of T0901317 on apoB and triacylglycerol secretion

CaCo-2 cells, grown on semi-permeable membranes separating an upper and lower well, were incubated for 18 h with 0, 0.1 and 1.0 μM T0901317 with or without 250 μM oleic acid attached to albumin (4:1, mol/mol). Following incubation, the amounts of apoB and triacylglycerol mass in the lower well were estimated as described in the Materials and methods section. The data represent means $\pm$ S.E.M. from a total of four dishes.

expression. In FTO-2B rat hepatoma cells, fatty acids of 18- (including 18:0 and 18:1) and 20-carbon chain length also decreased mRNA levels of SREBP-1c [24], whereas, in another liver cell line, HepG2, 18:1, at concentrations as high as 200 μM , had no effect on either SREBP-1 or FAS gene expression [21]. Obviously, the effects of fatty acids on SREBP-1 expression differ depending upon the cell line being studied. If the mechanism by which fatty acids regulate SREBP-1c expression is related to interference of LXR activity, then it is clear in CaCo-2 cells that 18:1 (and presumably 18:0) will not alter SREBP-1c expression, as 18:1 was ineffective in suppressing LXR activity.

In CaCo-2 cells, the intracellular fate of individual fatty acids differs. For example, in contrast to 18:1, which is predominately incorporated into triacylglycerols, 20:5 is incorporated into phospholipids [38]. In addition, 18:1 is very potent in promoting the secretion of triacylglycerols and apoB [41–44], whereas 20:5 tends to retard or delay the secretion of apoB-containing triacylglycerol-rich lipoproteins [42]. Thus in an intestinal cell, whether an individual fatty acid alters the expression of genes that control fatty acid synthesis might also be dependent upon the specific fate of that fatty acid within the cell.

In a previous study, following the addition of fatty acids, changes in SREBP-1a gene expression paralleled those observed in SREBP-1c [23]. Our results in CaCo-2 cells agree. Like the suppression observed in SREBP-1c gene expression by fatty acids, only the 18- and 20-carbon polyunsaturated fatty acids

decreased gene expression of SREBP-1a. Possible explanations for why SREBP-1a and SREBP-1c are similarly affected by fatty acids have already been discussed [23]. In addition, because T0901317 also increased SREBP-1a mRNA levels, it is likely that polyunsaturated fatty acids interfere with the action of LXR on SREBP-1a as well as SREBP-1c expression. Indeed, in FTO-2B cells, T0901317 increased gene expression of SREBP-1a and reversed the suppressive effects of arachidonic acid on SREBP-1a gene expression [24]. Although up-regulation of SREBP-1a expression by LXR activation seems to occur in cell culture, there is no evidence that it happens *in vivo*. For example, in mice fed a rexinoid, which enhances LXR/RXR activation, only the SREBP-1c transcript in liver, intestine and white fat was enhanced. The SREBP-1a transcript was unaltered [26]. Recently, in mice fed an LXR agonist, SREBP-1c mRNA levels were increased without altering the expression of SREBP-1a [45]. In hamster small intestine, SREBP-1a is a minor transcript compared with SREBP-1c [16]. Feeding hamsters a cholesterol-rich or cholesterol-depletion diet caused marked changes in intestinal SREBP-1c mRNA levels without altering expression of SREBP-1a [18]. We would surmise, therefore, that *in vivo* intestinal SREBP-1a and SREBP-1c are regulated independently and that SREBP-1a probably does not play a significant role in regulating either cholesterol or fatty acid synthesis in this organ.

None of the fatty acids altered gene or protein expression of SREBP-2. In previous studies performed in cell culture, changes in cholesterol flux caused co-ordinate regulation of both SREBP-1 and SREBP-2 [1]. It is clear from the results of this study and those of others that, in contrast to sterol flux, fatty acid flux does not regulate SREBP-1 and SREBP-2 co-ordinately [23,24]. This is partially explained by the observation that SREBP-1c, but not SREBP-2, is a target gene for LXR. LXR activation by T0901317 increased gene expression of SREBP-1c and protein expression of SREBP-1 without altering either gene or protein expression of SREBP-2. Thus in CaCo-2 cells, SREBP-1 and SREBP-2 are not co-ordinately regulated by fatty acid flux. This makes sense because, as observed previously in other cell types, polyunsaturated fatty acids interfere with ligand-dependent activation of LXR. Therefore SREBP-1c expression will be regulated by this mechanism, whereas SREBP-2 will not [24]. In CaCo-2 cells, the mechanism by which polyunsaturated fatty acids regulate SREBP-1 expression appears similar.

It was unexpected that 18:0 and 18:1 decreased fatty acid synthesis as neither fatty acid altered SREBP-1c, FAS or acetyl-CoA carboxylase expression. Although the modest inhibition of fatty acid synthesis by 18:1 was the least of all the fatty acids (approx. 20%), the effect was consistent and real. Interestingly, 18:0 was as potent as the other fatty acids in suppressing fatty acid synthesis. One could implicate a mechanism of 18:0 modification, such as desaturation, chain shortening or chain elongation. This does not negate the observation, however, that 18:0 and 18:1 inhibited fatty acid synthesis independently of any changes in SREBP-1c or FAS. Perhaps these fatty acids interfere with one or more regulatory enzymes in the fatty acid synthetic pathway not under control of SREBP-1c; or perhaps 18:0 and 18:1 alter post-transcriptional/post-translational activity of regulatory enzymes in fatty acid synthesis. Further studies will be necessary to examine these possibilities.

Although LXR activation by T0901317 increased endogenous fatty acid synthesis by some 50%, we observed no alterations in triacylglycerol-rich lipoprotein secretion. This does not imply that newly synthesized fatty acids are not used for lipoprotein assembly and secretion. In fact, there are data to suggest that some endogenously synthesized fatty acids are transported in chylomicrons [25]. The results do suggest, however, that intestinal

lipoprotein production is driven by luminal fatty acid flux and not by an increase in endogenous production of fatty acids. The data are also consistent with *in vivo* findings that LXR/RXR activation does not alter triacylglycerol absorption or apoB expression in the intestine [29].

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REFERENCES

- Brown, M. S. and Goldstein, J. L. (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**, 331–340
- Shimomura, I., Shimano, H., Horton, J. D., Goldstein, J. L. and Brown, M. S. (1997) Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J. Clin. Invest.* **99**, 838–845
- Hua, X., Wu, J., Goldstein, J. L., Brown, M. S. and Hobbs, H. H. (1995) Structure of the human gene encoding sterol regulatory element binding protein-1 (SREBP1) and localization of SREBP1 and SREBP2 to chromosomes 17p11.2 and 22q13. *Genomics* **25**, 667–673
- Hua, X., Yokoyama, C., Wu, J., Briggs, M. R., Brown, M. S., Goldstein, J. L. and Wang, X. (1993) SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11603–11607
- Brown, M. S. and Goldstein, J. L. (1999) A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11041–11048
- Horton, J. D. and Shimomura, I. (1999) Sterol regulatory element-binding proteins: activators of cholesterol and fatty acid biosynthesis. *Curr. Opin. Lipidol.* **10**, 143–150
- Edwards, P. A., Tabor, D., Kast, H. R. and Venkateswaran, A. (2000) Regulation of gene expression by SREBP and SCAP. *Biochim. Biophys. Acta* **1529**, 103–113
- Sakai, J., Nohturfft, A., Goldstein, J. L. and Brown, M. S. (1998) Cleavage of sterol regulatory element-binding proteins (SREBPs) at site-1 requires interaction with SREBP cleavage-activating protein. Evidence from *in vivo* competition studies. *J. Biol. Chem.* **273**, 5785–5793
- Nohturfft, A., DeBose-Boyd, R. A., Scheek, S., Goldstein, J. L. and Brown, M. S. (1999) Sterols regulate cycling of SREBP cleavage-activating protein (SCAP) between endoplasmic reticulum and Golgi. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11235–11240
- DeBose-Boyd, R. A., Brown, M. S., Li, W. P., Nohturfft, A., Goldstein, J. L. and Espenshade, P. J. (1999) Transport-dependent proteolysis of SREBP: relocation of site-1 protease from Golgi to ER obviates the need for SREBP transport to Golgi. *Cell* **99**, 703–712
- Shimano, H., Horton, J. D., Shimomura, I., Hammer, R. E., Brown, M. S. and Goldstein, J. L. (1997) Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells. *J. Clin. Invest.* **99**, 846–854
- Sheng, Z., Otani, H., Brown, M. S. and Goldstein, J. L. (1995) Independent regulation of sterol regulatory element-binding proteins 1 and 2 in hamster liver. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 935–938
- Shimomura, I., Bashmakov, Y., Shimano, H., Horton, J. D., Goldstein, J. L. and Brown, M. S. (1997) Cholesterol feeding reduces nuclear forms of sterol regulatory element binding proteins in hamster liver. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12354–12359
- Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L. and Shimano, H. (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J. Clin. Invest.* **101**, 2331–2339
- Nohturfft, A., Yabe, D., Goldstein, J. L., Brown, M. S. and Espenshade, P. J. (2000) Regulated step in cholesterol feedback localized to budding of SCAP from membranes. *Cell* **102**, 315–323
- Field, F. J., Born, E., Murthy, S. and Mathur, S. N. (2001) Gene expression of sterol regulatory element-binding proteins in hamster small intestine. *J. Lipid Res.* **42**, 1–8
- Field, F. J., Born, E., Murthy, S. and Mathur, S. N. (2001) Regulation of sterol regulatory element-binding proteins by cholesterol flux in CaCo-2 cells. *J. Lipid Res.* **42**, 1687–1698
- Field, F. J., Born, E., Murthy, S. and Mathur, S. N. (2001) Regulation of sterol regulatory element-binding proteins in hamster intestine by changes in cholesterol flux. *J. Biol. Chem.* **276**, 17576–17583
- Thewke, D. P., Panini, S. R. and Sinensky, M. (1998) Oleate potentiates oxysterol inhibition of transcription from sterol regulatory element-1-regulated promoters and maturation of sterol regulatory element-binding proteins. *J. Biol. Chem.* **273**, 21402–21407
- Worgall, T. S., Sturley, S. L., Seo, T., Osborne, T. F. and Deckelbaum, R. J. (1998) Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J. Biol. Chem.* **273**, 25537–25540
- Xu, J., Nakamura, M. T., Cho, H. P. and Clarke, S. D. (1999) Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. *J. Biol. Chem.* **274**, 23577–23583
- Kim, H. J., Takahashi, M. and Ezaki, O. (1999) Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. *J. Biol. Chem.* **274**, 25892–25898
- Hannah, V. C., Ou, J., Luong, A., Goldstein, J. L. and Brown, M. S. (2001) Unsaturated fatty acids down-regulate SREBP isoforms 1a and 1c by two mechanisms in HEK-293 cells. *J. Biol. Chem.* **276**, 4365–4372
- Ou, J., Tu, H., Shan, B., Luk, A., DeBose-Boyd, R. A., Bashmakov, Y., Goldstein, J. L. and Brown, M. S. (2001) Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 6027–6032
- Shiau, Y. F., Popper, D. A., Reed, M., Umstetter, C., Capuzzi, D. and Levine, G. M. (1985) Intestinal triglycerides are derived from both endogenous and exogenous sources. *Am. J. Physiol.* **248**, G164–G169
- Repa, J. J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J. M., Shimomura, I., Shan, B., Brown, M. S., Goldstein, J. L. and Mangelsdorf, D. J. (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRA and LXRbeta. *Genes Dev.* **14**, 2819–2830
- Willy, P. J., Umesono, K., Ong, E. S., Evans, R. M., Heyman, R. A. and Mangelsdorf, D. J. (1995) LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* **9**, 1033–1045
- Janowski, B. A., Willy, P. J., Devi, T. R., Falck, J. R. and Mangelsdorf, D. J. (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature (London)* **383**, 728–731
- Repa, J. J., Turley, S. D., Lobaccaro, J. A., Medina, J., Li, L., Lustig, K., Shan, B., Heyman, R. A., Dietschy, J. M. and Mangelsdorf, D. J. (2000) Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* **289**, 1524–1529
- Janowski, B. A., Grogan, M. J., Jones, S. A., Wisely, G. B., Kliewer, S. A., Corey, E. J. and Mangelsdorf, D. J. (1999) Structural requirements of ligands for the oxysterol liver X receptors LXRA and LXRbeta. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 266–271
- Boehm, M. F., Zhang, L., Zhi, L., McClurg, M. R., Berger, E., Wagoner, M., Mais, D. E., Suto, C. M., Davies, J. A., Heyman, R. A. and Nadzan, A. M. (1995) Design and synthesis of potent retinoid X receptor selective ligands that induce apoptosis in leukemia cells. *J. Med. Chem.* **38**, 3146–3155
- Willy, P. J. and Mangelsdorf, D. J. (1997) Unique requirements for retinoid-dependent transcriptional activation by the orphan receptor LXR. *Genes Dev.* **11**, 289–298
- Edwards, P. A., Kast, H. R. and Anisfeld, A. M. (2002) Baring it all. The adoption of LXR and FXR and their roles in lipid homeostasis. *J. Lipid Res.* **43**, 2–12
- Schultz, J. R., Tu, H., Luk, A., Repa, J. J., Medina, J. C., Li, L., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D. J. et al. (2000) Role of LXRs in control of lipogenesis. *Genes Dev.* **14**, 2831–2838
- Field, F. J., Albright, E. and Mathur, S. N. (1987) Regulation of cholesterol esterification by micellar cholesterol in CaCo-2 cells. *J. Lipid Res.* **28**, 1057–1066
- Murthy, S., Mathur, S. N., Varilek, G., Bishop, W. and Field, F. J. (1996) Cytokines regulate apolipoprotein B secretion by CaCo-2 cells: differential effects of IL-6 and TGF-beta1. *Am. J. Physiol. Gastrointest. Liver Physiol.* **270**, G94–G102
- Kumar, S. and Dodds, P. F. (1981) Fatty acid synthase from lactating bovine mammary gland. *Methods Enzymol.* **71**, 86–97
- Murthy, S., Albright, E., Mathur, S. N. and Field, F. J. (1990) Effect of eicosapentaenoic acid on triacylglycerol transport in CaCo-2 cells. *Biochim. Biophys. Acta* **1045**, 147–155
- Kim, J. B. and Spiegelman, B. M. (1996) ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev.* **10**, 1096–1107
- Bennett, M. K., Lopez, J. M., Sanchez, H. B. and Osborne, T. F. (1995) Sterol regulation of fatty acid synthase promoter. Coordinate feedback regulation of two major lipid pathways. *J. Biol. Chem.* **270**, 25578–25583
- Field, F. J., Albright, E. and Mathur, S. N. (1988) Regulation of triglyceride-rich lipoprotein secretion by fatty acids in CaCo-2 cells. *J. Lipid Res.* **29**, 1427–1437

- 42 Murthy, S., Albright, E., Mathur, S. N., Davidson, N. O. and Field, F. J. (1992) Apolipoprotein B mRNA abundance is decreased by eicosapentaenoic acid in CaCo-2 cells. Effect on the synthesis and secretion of apolipoprotein B. *Arterioscler. Thromb.* **12**, 691–700
- 43 Traber, M. G., Kayden, H. J. and Rindler, M. J. (1987) Polarized secretion of newly synthesized lipoproteins by the Caco-2 human intestinal cell line. *J. Lipid Res.* **28**, 1350–1363
- 44 Dashti, N., Smith, E. A. and Alaupovic, P. (1990) Increased production of apolipoprotein B and its lipoproteins by oleic acid in Caco-2 cells. *J. Lipid Res.* **31**, 113–123
- 45 Liang, G., Yang, J., Horton, J. D., Hammer, R. E., Goldstein, J. L. and Brown, M. S. (2002) Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. *J. Biol. Chem.* **277**, 9520–9528

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