The Role of Liver X Receptor-α (LXRα) in the Fatty Acid Regulation of Hepatic Gene Expression.*

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The abbreviations used are: LXR, liver X receptor; LXRE, LXR regulatory element; PPAR, peroxisome proliferator activated receptor; RXR, retinoid X receptor; LBD, ligand binding domain; SREBP, sterol regulatory element binding protein; NEFA, non-esterified fatty acids; HEK, human embryonic kidney; PUFA, polyunsaturated fatty acids; ABC, ATP-binding cassette; GPAT, glycerophosphate acyl transferase; ACS-1, acyl CoA synthetase-1; CYP4A, cytochrome P450 4A; mtHMGCoAsyn, mitochondrial HMG-CoA synthase; FAS, fatty acid synthase, S14, S14 protein; L-PK, L-type pyruvate kinase; HNF-4, hepatic nuclear factor-4; CYP7A, 7α-hydroxylase; GiRR, glucose regulatory region; RLA, relative luciferase activity.

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**Abstract:** Liver X receptors (LXR, α and β) play an important role in regulating the expression of genes involved in hepatic bile and fatty acid synthesis, glucose metabolism as well as sterol efflux. Studies with human embryonic kidney 293 cells indicate that unsaturated fatty acids interfere with oxysterols binding to LXR and antagonize oxysterol-induced LXRα activity. In this report, we evaluated the effects of unsaturated fatty acids on LXR-regulated hepatic gene expression. The LXR agonist, T1317, induced mRNAs encoding sterol regulatory element binding protein-1c (SREBP-1c) and two SREBP-1c-regulated lipogenic genes, e.g., fatty acid synthase (FAS) and the S14 protein, in primary hepatocytes. Treatment of hepatocytes with eicosapentaenoic acid (20:5,n3) suppressed these mRNAs in the absence and presence of T1317. The cis-regulatory elements targeted by T1317 were not required for fatty acid suppression of FAS or S14 promoter activity. In contrast to SREBP-1-regulated lipogenic genes, 20:5,n3 had no effect on the T1317 induction of ABCG5 or ABCG8 in the rat hepatoma cell line, FTO-2B. These two genes require LXR, but not SREBP-1c, for their expression. Feeding rats a diet supplemented with fish oil suppressed hepatic SREBP-1c regulated genes and induced PPARα-regulated genes, but had no effect on the LXR-regulated transcripts, CYP7A1, ABCG5 or ABCG8. Transfection studies, using either full length hLXRα or a chimera containing only the LXRα ligand binding domain, indicate that a wide array of unsaturated fatty acids had little effect on LXRα activity in primary hepatocytes or FTO-2B. These studies suggest that LXRα is not a target for unsaturated fatty acid regulation in primary rat hepatocytes or in liver. Thus, oxysterol/LXR-mediated regulation of transcripts involved in bile acid synthesis or sterol efflux appear insensitive to dietary unsaturated fatty acids. The unsaturated fatty acid suppression of SREBP-1 and its targeted lipogenic genes is independent of LXRα.
Introduction.

Liver X receptors (LXR, α and β) are ligand regulated nuclear receptors that play an important role in hepatic bile acid and fatty acid synthesis, glucose metabolism and sterol efflux (1-3). Oxysterols, like 22[R]-hydroxycholesterol and 24,25-epoxycholesterol, bind to and activate liver X receptors. Together with RXR, LXR bind DNA regulatory elements, i.e., DR4, and induce the transcription of multiple genes involved in bile acid synthesis (CYP7A), sterol efflux (ABCA1, ABCG5 and ABCG8), glucose metabolism and de novo lipogenesis. Recent studies with human embryonic kidney 293 (HEK293) cells suggest that unsaturated fatty acids bind to LXRα (Kd~1-4 µM) and antagonize oxysterol-activation of the LXRα, but not LXRβ (4,5). Unsaturated fatty acids have also been reported to interfere with LXR/RXR binding to DNA regulatory elements (6). Fatty acid interference with oxysterol regulation of LXR has important physiological implications because of the potential to affect the expression of genes involved in bile acid, fatty acid, cholesterol and glucose metabolism.

The effect of LXR on lipogenesis involves both direct and indirect mechanisms. LXR/RXR heterodimers bind lipogenic gene promoters, e.g., fatty acid synthase (FAS), or regulate lipogenic gene expression by controlling levels of SREBP-1c (3,7). SREBP-1c is a basic helix-loop-helix-leucine zipper transcription factor that is translated as a ~125 kd precursor (pSREBP-1c) attached to the endoplasmic reticulum (8,9). After proteolytic processing in the Golgi, the active form, nSREBP-1c (~65 kd), accumulates in the nucleus where it binds sterol regulatory elements (SRE) in promoters of many genes involved in fatty acid and triglyceride synthesis. Transcription of the SREBP-1c gene is induced by insulin (10,11) and oxysterols through LXR (12). Insulin induction of LXRα gene transcription might also account for some of this control (13). Much of insulin action on lipogenic gene transcription has been ascribed to the insulin-mediated induction of SREBP-1c (9,14). Unsaturated fatty acid suppression of nuclear SREBP-1c levels is complex. In established cell lines, unsaturated fatty acids inhibit transcription
of the SREBP-1 gene (15), enhance mRNA_{SREBP-1} turnover (16) and interfere with proteolytic processing of SREBPs (17). In primary hepatocytes, unsaturated fatty acids have little impact on SREBP-1c gene transcription (18,19), but enhance mRNA_{SREBP-1c} turnover (16). Over expression of nSREBP-1c in primary hepatocytes eliminates the polyunsaturated fatty acid (PUFA) effects on several lipogenic genes indicating that SREBP-1c is a key target for PUFA action on de novo lipogenesis (19,20).

Because LXRα is a target for fatty acid inhibition in HEK293 cells, we were interested in evaluating the role LXRα played in the fatty acid regulation of hepatic gene expression. Our studies will show that under conditions sufficient to suppress SREBP-1c and lipogenic gene expression, certain LXR-regulated transcripts are resistant to PUFA regulation.

**Materials and Methods.**

**Animals:** Male Sprague Dawley rats (Charles River, Kalamazoo, MI) were maintained on a Tek-Lad chow diet, *ad lib*. For feeding studies, rats were meal-fed a high carbohydrate diet supplemented with olive oil at 10% w/w for 10 days. The high carbohydrate-fat free diet (glucose replaces sucrose) was obtained from ICN Biomedicals, Inc (Aurora, OH). Meal feeding involved allowing the rats to eat between 8 AM to noon daily. Animals were either maintained on this diet or switched to a high carbohydrate diet supplemented with fish oil (10%, w/w) (Dyets, Bethlehem, PA). After 5 days on the olive oil or fish oil diets, animals were euthanized ~2 hrs after completing the meal and livers were removed for RNA analysis (19).

**Primary Hepatocytes and Transfections.** Male Sprague-Dawley rats maintained on a Tek-Lad chow diet, *ad lib* were used for primary hepatocyte preparation (19). For RNA analysis, cells were plated onto 100 mm type I collagen-coated plates or Primaria plastic (BD Bioscience, Bedford, MA) dishes at $10^7$ cells/plate in Williams E with 10 mM lactate, 10 nM dexamethasone,
100 nM insulin and 10% fetal calf serum. For transfection studies, cells were plated in the same media onto 6 well type I-collagen coated plates or Primaria plastic dishes at 10^6 cells/well.

Transfection conditions have been described previously (19). The ratio of culture medium to cell number was maintained constant for the different plating conditions. After a 4-6 hr attachment period, media was changed to a serum-free medium, Williams E with 10 mM lactate, 10 nM dexamethasone, 100 nM insulin. Cells were transfected in this serum-free media using lipofectin or lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described (5). The media was changed the next morning to Williams E with 25 mM glucose, 10 nM dexamethasone, 100 nM insulin and 250 μM fatty acid (NuChek Prep, Elysian, MN), 50 μM bovine serum albumin—very low endotoxin and fatty acid free (Serological Proteins, Inc, Kankakee Ill) or drug treatment (TO-901317 (T1317), Cayman Chemicals, Ann Arbor, MI).

FTO-2B cells were obtained from L. Reid (University of North Carolina, Chapel Hill, NC) (21) and maintained in DMEM/F12 (Invitrogen) plus 7.5% fetal bovine serum on plastic culture dishes. HEK293 cells were described previously(5). Cells were grown in 6 well plastic culture plates for transfection and 100 mm plastic culture plates for RNA studies. At confluence, cells were transfected in serum free media as described above. For RNA studies, cells were incubated overnight in serum-free medium. At the time of treatment, FTO-2B cells received 100 nM insulin and 10 nM dexamethasone along with fatty acids and/or the LXR-agonist, T1317. Cells were transfected using lipofectamine 2000 or used for RNA extraction as described above.

RNA Isolation and Northern Analysis. RNA was extracted from rat liver, primary hepatocytes or FTO-2B cells using Triazol (Invitrogen, Carlsbad, CA). RNA was separated electrophoretically in denaturating agarose gels, transferred to nitrocellulose and probed with ^32P-cDNAs. Levels of hybridization were quantified using a Molecular Dynamics Phosphoimager 820 (Molecular Dynamics, Sunnyvale, CA).
Plasmids: cDNAs for SREBP-1c, fatty acid synthase and cytochrome P450 4A, (Cyp4A) were previously described (19,22,23). A cDNA for 7α-hydroxylase, CYP7A1 (24) was obtained from David Russell, University of Texas-Southwestern Medical Center, Dallas, TX. The plasmids containing ABC transporters G5 and G8 were obtained from Helen Hobbs, University of Texas-Southwestern Medical Center, Dallas TX (25). Plasmids containing GPAT and ACS1 were obtained from Roselind Coleman, University of North Carolina, Chapel Hill, NC (26,27). Inserts from plasmids were 32P-labeled and used to measure levels of specific mRNAs by northern analysis. CMX-Gal4-hLXRα, CMX-hLXRα, and TK-MH100X4-Luc were previously described (5). phRG-Luc was obtained from Promega (Madison, WI) and served as an internal control for transfection efficiency. The S14CAT and LPKCAT reporter genes were described previously (19,23,28,29). The FASCAT plasmids with its 5' end point at -2369 was obtained from Steven D. Clarke, Pennington, Baton Rouge, LA.

Cell Extracts and Western Blotting: Extracts of primary hepatocytes for analysis of protein levels (western blotting) were prepared by homogenizing cells in Buffer A (0.25 M sucrose, 10 mM Tris-Cl, pH 7.5, 3 mM MgCl₂ plus protease inhibitors (phenylmethylsulfonyl fluoride, 1 mM, pefabloc, 0.1 mM, pepstatin, 5 µg/ml, leupeptin, 5 µg/ml and aprotinin, 2 µg/ml)(30). The homogenate was centrifuged (1500 X g, 5 mins, 2°C.). The supernatant was centrifuged (100,000 X g for 1 hr, 4°C) to obtain microsomes. The pellet from the first centrifugation was resuspended in Buffer A, adjusted to 1% NP40 and homogenized. The homogenate was centrifuged (300 X g, 5 mins, 2°C.). The supernatant was retained for analysis. The nuclear pellet was resuspended in Buffer B (50 mM HEPES, pH 7.4, 0.1 M KCl, 3 mM MgCl₂, 1 mM EDTA, 10% glycerol, plus protease inhibitors (phenylmethylsulfonyl fluoride, 1 mM; pefabloc, 0.1 mM; pepstatin, 5 µg/ml; leupeptin; 5 µg/ml and aprotinin, 2 µg/ml) adjusted to 0.4 M ammonium sulfate and centrifuged at 25,000 x g for 15 mins. The supernatant was used for analysis of nuclear proteins.
Proteins (50-100 µg) were separated electrophoretically by SDS-polyacrylamide gel electrophoresis (NuPage 4-10% polyacrylamide-Bis-tris, Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. Membranes were incubated with antibodies for SREBP-1c (IgG-2A4) obtained from the supernatant of the hybridoma cell line CRL 2121 (American Type Culture Collection, Manassas, VA). The anti-mouse secondary antibody was obtained from Bio-Rad (Hercules, CA). The detection system employed the SuperSignal West Pico Chemiluminescence kit (Pierce, Rockford, Ill).

Results.

Effects of eicosapentaenoic acid (20:5,n3) and T1317 on SREBP-1c expression in primary hepatocytes. The LXR agonist, TO901317 (T1317) was used in all studies with primary hepatocytes and FTO-2B cells. The native oxysterols, 22[R]-hydroxycholesterol and 24,25-epoxycholesterol, have no effect on LXR activity or LXR-regulated transcripts in these cells, probably because of their rapid metabolism. T1317 at 5 µM was selected as the minimal dose to achieve reliable induction of LXR activity in primary hepatocytes and FTO-2B cells. In HEK293 cells, T1317-induced LXRα activity is attenuated by both 20:4,n6 and 20:5,n3, while PPARα activity was induced by these fatty acids. These effects on LXRα and PPARα activity correlated with significant changes in intracellular non-esterified unsaturated fatty acids (5). In the studies reported here, 20:5,n3 was used as the fatty acid for most studies with primary hepatocytes and FTO-2B cells. 20:5,n3 is a very low abundance unsaturated fatty acid in primary hepatocytes and FTO-2B cells. Its addition at 250 µM leads to a >10-fold increase in mass of 20:5,n3 in the intracellular non-esterified fatty acid pool of these cells. Changes in 20:5,n3 in the NEFA pool correlate with the suppression of SREBP-1c mRNA, as well as the activation of PPARα and PPARα-regulated genes (31).
Treating primary hepatocytes with the LXR agonist, T1317 induced mRNAsRBP-1c, as well as the precursor (pSREBP-1) and nuclear (nSREBP-1) forms by 3 to 4-fold (Fig. 1). Treating cells with 20:5,n3 in the presence or absence of T1317 suppressed mRNAsRBP-1c, pSREBP-1 and nSREBP-1 by 50-80%. This same pattern of control is seen for the 20:5,n3 and T1317 regulation of mRNAs encoding fatty acid synthase (FAS) and the S14 protein (S14) as well as reporter genes driven by the FAS and S14 promoters (Fig. 2A and B). While L-pyruvate kinase (L) mRNA and LPKCAT reporter gene were suppressed by 20:5,n3 treatment, T1317 did not induce this gene.

SREBP-1 binds the S14 promoter at the SRE located at -139/-129 bp; it is near a Y-box (-104/-99 bp) that binds NF-Y (19,28,32). Together, these elements play an obligatory role in S14 gene transcription. Mutation of either element essentially abrogates S14 gene transcription (28,32). The SRE/NF-Y elements are in the S14 PUFA-RR, a region previously identified as the principal target for PUFA suppression of S14 gene transcription (33). The FAS promoter has distinct regulatory elements for both SREBP and LXR (3). Our goal is to determine if the T1317 effect on S14 promoter activity is simply due to T1317/LXR-mediated induction of SREBP-1c. Accordingly, a detail promoter analysis was preformed to locate the cis-regulatory target for T1317.

Deletion and mutation analyses show that the T1317 regulatory region (T1317-RR) is located in a region previously identified as a glucose regulatory region (GlRR, -1.6/-1.4 kb) (Fig. 3A)(34). The E-box in the GlRR binds glucose-regulated binding proteins as well as SREBP-1c (35,36). Deletion of the GlRR (-1.6/-1.4 kb) or mutation of the E-box within (at -1440 bp) has no effect on PUFA suppression of S14CAT activity (Fig. 3B). In the context of the thymidine kinase promoter, neither the GlRR or the TRR are sensitive to 20:5,n3 suppression. Preliminary studies suggest that at least 2 T1317 elements are located within the GlRR. Studies are underway to define these elements. A similar analysis using FASCAT reporter genes indicted that the FASCAT activity is suppressed by 50% by 20:5,n3 treatment. Like the S14 promoter the presence
and absence of the LXRE at -669 bp (3) did not influence promoter sensitivity to 20:5, n3. These studies show that while S14 and FAS have distinct cis-regulatory elements for T1317/LXR and SREBP-1c, the T1317/LXR-cis regulatory elements are not required for PUFA control of these transcripts in rat primary hepatocytes.

**20:5, n3 effects on LXR-regulated transcripts in FTO-2B hepatoma cells.** While the expression of SREBP-1c and various lipogenic genes are easily examined in primary rat hepatocytes, certain LXR-regulated transcripts, e.g., CYP7A, ABCG5 and ABCG8, decrease to nearly undetectable levels when compared to their expression in liver. The reason for this decline is likely due to the loss of key transcription factors controlling expression of these transcripts. Fortunately, ABCG5 and ABCG8 are well-expressed and regulated by T1317 in FTO-2B cells (25). Because the T1317 regulation of these transcripts is independent of SREBP-1c, we examined the effect of 20:5, n3 on the T1317-mediated induction of SREBP-1c, ABCG5 and ABCG8 in FTO-2B cells (Fig. 4).

Previous studies indicated that the predominant SREBP-1 subtype in FTO-2B cells is SREBP-1c (4). RNA protection studies indicate the ratio of SREBP-1c to -1a is ≥4-fold. T1317 induced mRNA_{SREBP-1c} as well as the precursor and nuclear forms of SREBP-1c 2 to 3-fold in FTO-2B cells (Fig. 4A). 20:5, n3 suppressed mRNA_{SREBP-1c} and the nuclear form of SREBP-1c by 50-70% in the absence and presence of T1317. The 20:5, n3 and T1317 regulation of SREBP-1c in FTO-2B cells is similar to that seen in primary hepatocytes (Fig. 1). While T1317 induced mRNA_{ABCG5} and mRNA_{ABCG8} ~2-fold, 20:5, n3 had no effect on the level of these transcripts (Fig. 4B). This finding suggests that LXR, per se, is not a target for unsaturated fatty acid antagonism in FTO-2B cells.

**20:5, n3 regulation of LXRα activity in primary hepatocytes and FTO-2B hepatoma cells.** In an effort to examine the effect of 20:5, n3 on LXRα activity directly, we used a transfection
approach. Because our previous studies established that only LXR\(\alpha\), and not LXR\(\beta\), was affected by unsaturated fatty acid treatment (5), our studies focused on LXR\(\alpha\). Accordingly, primary hepatocytes were transfected with an expression vector containing the ligand-binding domain of LXR\(\alpha\) fused to the Gal-4 DNA binding domain (LXR\(\alpha\)-LBD) and the MH-TK-LUC reporter containing 4 Gal 4-regulatory elements (Fig. 5). The use of this chimeric receptor allows for an evaluation of fatty acid effects on LXR activity without the requirement for RXR heterodimerization. Following treatment of primary hepatocytes with T1317, LXR activity was induced 4-fold. No significant fatty acid-mediated antagonism of LXR\(\alpha\) activity was detected until 20:5,\(n_3\) levels reached 1 mM. At lower levels (0.25 mM), levels typically used to examine PUFA regulation of lipogenic gene transcription, 20:5,\(n_3\) augments LXR\(\alpha\) activity by 30%. A similar study with FTO-2B cells revealed no evidence of 20:5,\(n_3\) interference with T1317-mediated induction of LXR\(\alpha\) activity. In each case, fatty acid treatment had no effect on basal LUC activity in the absence of T1317. For comparison, a recent dose response analysis of 20:4,\(n_6\) effects on LXR activity in HEK293 cells showed that the IC\(_{50}\) for the antagonism of 20:4,\(n_6\) was 22 \(\mu\)M\(^2\). Clearly, LXR\(\alpha\) is considerably more sensitive to PUFA inhibition in HEK293 cells than in primary hepatocytes. More importantly, the inhibitory effect of 20:5,\(n_3\) on LXR\(\alpha\) activity is only seen when very high non-physiological levels of the fatty acid are added. Such levels are likely never reached in vivo.

To determine if other unsaturated fatty acids affect LXR\(\alpha\) activity in primary hepatocytes, transfected cells were treated with various mono- and poly-unsaturated fatty acids (at 250 \(\mu\)M) in the absence and presence T1317 (Fig. 6A). In no case did any fatty acid tested affect LXR\(\alpha\) activity. Of the fatty acids tested, all but 18:1,\(n_9\), suppress SREBP-1c mRNA levels in primary hepatocytes (19).

Others reported that, in addition to fatty acid effects on oxysterol binding, unsaturated fatty acids interfered with LXR-RXR heterodimerization and DNA binding (6). To determine if
unsaturated fatty acids interfered with the activity of the full length LXRα, primary hepatocytes were transfected with a full length LXRα expression vector and the LXRE-TK-Luc reporter vector (Fig. 6B). For the reporter plasmid to respond to T1317, endogenous RXR must be recruited to the promoter by LXRα. Accordingly, treatment of primary hepatocytes with T1317 induced LUC activity 15-fold. Treatment of cells with 18:1,n9 or 20:5,n3 in the absence of T1317 had no effect on basal LUC activity (not shown). Co-treatment with T1317 and either 18:1,n9 and 20:5,n3 did not inhibit LUC activity. Thus, the capacity of the full length LXRα to activated promoters containing LXREs is not impeded by the addition of unsaturated fatty acids to primary hepatocytes.

**Effects of fish oil on LXR-regulated gene expression in rat liver.** In an effort to assess the in vivo effects of fatty acids on LXR-regulated hepatic gene expression we fed rats fish oil. Fish oil is enriched in 20:5,n3 and 22:6,n3. Addition of fish oil to diets has well-established effects on PPARα and SREBP-1c-regulated gene expression in rat liver (19,23). In this study, rats were meal-fed a high carbohydrate diet containing either olive or fish oil for 5 days (Fig. 7). Fish oil feeding induced hepatic mRNA levels for several PPARα target genes, i.e., cytochrome P450-4A (CYP4A), mitochondrial HMG-CoA synthase (mtHMG-CoA syn), and acyl CoA synthetase-1 (ACS1) ≥3-fold (Fig. 7), as well as acyl CoA oxidase (AOX)². The mRNAs encoding SREBP-1c, FAS, S14, glycerophosphate acyl transferase (GPAT) and L-PK were suppressed by 50-80%. SREBP-1c regulates the transcription of FAS, S14 and GPAT, but not L-PK (9,19). Fish oil treatment had no effect on transcripts encoding PPARα and HNF4α. More importantly, fish oil feeding did not affect hepatic levels of the LXR-regulated transcripts, CYP7A1, ABC-G5 or ABC-G8. RT-PCR analysis for ABC-A1 also shows this transcript is insensitive to fish oil treatment². This in vivo analysis agrees favorably with our previous analysis of these transcripts in primary hepatocytes (Fig. 1 and 2) and FTO-2B hepatoma cells (Figure 4). In vivo, n3-PUFA induced PPARα-regulated transcripts and suppressed SREBP-1c-regulated transcripts, but had no
effect on those LXR regulated transcripts that do not require SREBP-1c. The in vivo studies corroborate the cell culture studies with primary hepatocytes and FTO-2B hepatoma cells.

**Discussion.**

Liver X receptors (LXR α and β) play a key role in regulating the transcription of multiple genes involved in bile acid and fatty acid synthesis, glucose metabolism and sterol efflux (1,3,7,12,25,37,38). As such these nuclear receptors influence a broad array of hepatic metabolic events that influence whole body glucose, lipid and cholesterol metabolism. Moreover, the liver is a major target for fatty acid regulated gene expression (39). Finding that unsaturated fatty acids antagonized LXRα, but not LXRβ, activity suggested that sphere of influence of unsaturated fatty acids may potentially be extended beyond their well known effects on the expression of genes involved in fatty acid synthesis and oxidation (39). In this report, we used several approaches to evaluate the fatty acid regulation of LXR in liver. The new information reported here includes: 1) the T1317-mediated induction of hepatic mRNA SREBP-1c, as well as the precursor and nuclear forms of SREBP-1, is suppressed by 20:5,n3 treatment (Fig. 1); 2) the T1317 regulation of SREBP-1c nuclear content parallels its effects on FAS and S14 gene expression and promoter activity (Fig. 2); 3) the T1317/LXR regulated cis-acting elements in the S14 and FAS genes are not required for fatty acid regulation of these promoters (Fig. 3); 4) the T1317 induction of LXR-regulated transcripts, i.e., CYP7A, ABCG5, ABCG8 and ABCA1, is not affected by unsaturated fatty acids in FTO-2B cells and in rat liver (Fig. 4 and 7); 5) hLXRα activity is antagonized by PUFA only at high, non-physiological levels, levels >20-fold of that found for the PUFA antagonism of hLXRα in HEK293 cells (Fig. 5). Based on these findings, we conclude that LXRα is not a target for fatty acid antagonism in rat liver, rat primary hepatocytes or rat FTO-2B hepatoma cells. Thus, unsaturated fatty acids activate PPARα regulatory networks and suppress
SREBP-1c regulatory networks, but do not impede hepatic LXR regulatory networks in primary hepatocytes or in vivo.

Because 20:5,n3 is a minor unsaturated fatty acid in the total lipid fraction and NEFA pool of hepatocytes, its addition to primary hepatocytes leads to >50- and >10-fold increase in mass of this fatty acid in the total lipid and intracellular NEFA fractions, respectively, within 90 minutes (31). The level of 20:5,n3 in the NEFA pool is sustained for ~6 hrs, after which it declines to levels ~3-fold above basal values by 24 hrs. The change in cellular 20:5,n3 in the NEFA fraction correlates well with the activation of PPARα and the induction of PPARα-regulated transcripts (31) and the suppression of SREBP-1c mRNA². The 250 µM dose of 20:5,n3 used here is sufficient to suppress mRNAs for SREBP-1c and lipogenic genes and to induce PPARα and PPARα-regulated transcripts, but is insufficient to affect LXRα-activity. Only very high, non-physiologic levels of 20:5,n3 inhibited LXRα activity (Fig. 5). Clearly, LXRα activity in primary hepatocytes is far less sensitive to PUFA than either PPARα or SREBP-1c or the PUFA regulation of LXRα in HEK293 cells (ED₅₀≤50 µM) (5). Finding that full length LXRα was insensitive to PUFA treatment (Fig. 6B) argues against effects of PUFA on LXR/RXR heterodimerization and DNA binding. Interestingly, in HEK293 cells, LXRα and PPARα are equally sensitive to PUFA action suggesting that both receptors might serve as sensors to intracellular NEFA levels (5). While PPARα can be considered as monitor of intracellular NEFA (31), the studies reported here indicate that LXRα is not responsive to major changes in intracellular NEFA in liver or primary hepatocytes.

The fact that we found no evidence for unsaturated fatty acid regulation of LXR activity in liver does not exclude LXRα from being sensitive to fatty acid control in another cell context. Clearly, fatty acids affect LXRα activity in HEK293 cells(4,5). While the mechanism for this control has been ascribed to competitive binding, in vivo mechanisms may be more complicated involving receptor-coactivator interaction or the regulation of other signaling mechanism that do
not exist in liver or primary hepatocytes, e.g., cyclooxygenase and lipoxygenase (29). Previous efforts to examine fatty acid effects on targets of LXR action, i.e., CYP7A, did not reveal specific effects of unsaturated fatty acids (40-42). In fact, effects of fatty acids on CYP7A have been attributed to PPAR\(\alpha\) (42), not LXR. A recent report on the LXR-regulated gene, ABCA1, in macrophage suggests that fatty acid treatment inhibited cholesterol efflux by increasing ABCA1 protein degradation, without effects on ABCA1 mRNA (43). These observations have clinical significance because if fish oil interfered with bile acid synthesis or sterol efflux, then serum cholesterol levels would rise significantly increasing the risk for atherosclerosis and coronary disease. If anything, fish oil or EPA/DHA treatment of rats and humans is cardio-protective having pronounced hypolipemic effects (44,45).

Finally, we have identified a prospective cis-regulatory region for LXR action in the S14 promoter. This region, tentatively identified as a T1317-regulatory region (T1317-RR) is located in the glucose-regulatory region of the S14 promoter located between -1.4 and -1.6 kb upstream from the transcription start site. This region contains an E-box that is known to bind glucose-regulated binding proteins as well as SREBP-1c (35,46). Additional studies will be required to identify the specific minimal cis-regulatory targets for T1317 action and whether LXR/RXR binds these elements. Despite this limitation, deletion of the entire GIRR eliminates T1317 regulation of S14 promoter activity, but fails to impact PUFA control of S14 promoter activity (Fig. 3). Previous studies established that the PUFA-regulatory region, binding both SREBP-1c and NF-Y, is indispensable for S14 gene transcription. Moreover, this region is the principal target for PUFA control of the S14 promoter activity (28,32,33). These findings indicate that the key elements involved in PUFA control of S14 are distinct from the GIRR containing T1317-regulated factors. The fatty acid synthase promoter contains an LXRE at -669 bp, that is distinct from two SREs (-150 and -65 bp) (3). Analysis of FASCAT reporter genes indicates that the LXRE is not required for PUFA suppression of FASCAT activity. Based on these studies,
T1317/LXR-regulatory elements in the FAS and S14 promoters are dispensable for PUFA control of these promoters.

In summary, we have examined the role LXRα plays in PUFA regulation of hepatic gene expression. While LXRα is clearly a target for PUFA antagonism in HEK293 cells (4,5), it is not a target for fatty acid regulation in rat liver, primary hepatocytes or FTO-2B hepatoma cells. This conclusion is based on the lack of 20:5,n3 antagonism on LXRα activity or the LXR-regulated transcripts, ABCG5 and G8 at doses sufficient to repress SREBP-1c mRNA or to induce PPARα activity. The fact remains that LXRα activity is well antagonized by PUFA in HEK293 cells. This antagonism might be important for LXR action in some tissues. However, the absence of any effect on LXR in liver suggests that fatty acid effects on bile acid and fatty acid synthesis, sterol efflux or glucose metabolism cannot be explain by abrogated LXR action.

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**Figure Legends.**

**Figure 1:** Eicosapentaenoic acid (20:5, n3) and T1317 regulation of SREBP-1 in rat primary hepatocytes. Primary hepatocytes were treated with 250 µM 20:5,n3 (black bars); 5 µM TO-901317 [T1317] (shaded bars); or both compounds (stripped bars) for 24 hrs. Vehicle treated cells (white bars) received 50 µM BSA. **[Upper panel]:** SREBP-1 northern analysis, RNA was extracted and separated by electrophoresis for northern analysis. Blots were probed with $^{32}$P-cDNA for SREBP-1c. Levels of hybridization were quantified by phosphoimager analysis. Results are expressed as Relative mRNA Abundance; the mRNA levels in the treated cells are normalized to the level of the corresponding RNA in a rat liver standard from a chow-fed male rat. These results are the mean ± SD, N=9 of three separate studies. **[Lower Panel]:** SREBP-1 Immunoblot. Cells were treated as above and extracted for microsomal and nuclear proteins (Materials and methods) for immunoblotting. The precursor form of SREBP-1, i.e., pSREBP-1 is found in the microsomal fraction, while the nuclear form, i.e., nSREBP-1, is recovered in the nuclear fraction. The figure is representative of at least 3 separate studies. Analysis of the nuclear extracts for HNF-4α revealed robust levels of HNF-4, with no consistent treatment effect (not shown).

**Figure 2:** Eicosapentaenoic acid (20:5, n3) and T1317 regulation of fatty acid synthase, S14 protein and L-pyruvate kinase expression in rat primary hepatocytes. Primary hepatocytes were treated with 250 µM 20:5,n3 (black bars); 5 µM T1317 (shaded bars); or both compounds (stripped bars) for 24 hrs. Vehicle treated cells (white bars) received 50 µM BSA. **[A]:** RNA was extracted and separated by electrophoresis for northern analysis. Blots were probed with $^{32}$P-cDNAs for FAS, S14 or LPK. Levels of hybridization were quantified by phosphoimager analysis. Results are expressed as Relative mRNA Abundance; the mRNA levels
in the treated cells are normalized to the level of the corresponding RNA in a rat liver standard from a chow-fed male rat. These results are the mean ± SD, N=9 of three separate studies. [B]: Primary hepatocytes were transfected with fatty acid synthase, S14 and L-pyruvate kinase reporter genes containing promoter elements fused to the chloramphenicol acetyltransferase (CAT) reporter gene. FASCAT contains -2363 to + 16 bp of the FAS promoter; S14CAT contains -2800 to + 19 bp of the S14 promoter and LPKCAT contains (-4300 to + 14 bp) of the L-pyruvate kinase promoter. After transfection, hepatocytes were treated with 20:5,n3 and T1317 as described above. After a 24 hrs treatment, cells were harvested for CAT and protein assays. Results are reported as Fold Change in CAT activity. The results are the mean of 3 independent studies involving triplicate samples.

Figure 3. Role of the T1317 regulatory region in the PUFA control of S14 promoter activity. [A] Mapping the T1317 regulatory element in the S14 promoter. Primary hepatocytes were transfected with CAT reporter genes containing various elements from the S14 promoter. A schematic of the S14 promoter is illustrated at the top of the figure and the composition of each promoter-reporter gene construct is illustrated in the figure. The thyroid hormone regulatory region (TRR) and the glucose regulatory region (GiRR) were excised from the S14 promoter and fused to the thymidine kinase (TK) promoter to yield TRR-TK-CAT and GiRR-TK-CAT, respectively. The reporter gene at the bottom of the figure has the GiRR (-1506 to -1408 bp) removed and replaced by a Nsi I restriction site. After transfection, hepatocytes were treated with 5 µM T1317 as described above. After a 24 hrs treatment, cells were harvested for CAT and protein assays. Results are reported as Fold Change in CAT activity. The results are the mean of 3 independent studies involving triplicate samples. T1317-RR, T1317 regulatory region; PUFA-RR, PUFA-regulatory region. [B] The S14 T1317-regulatory region is not involved in PUFA suppression of S14CAT activity. Primary hepatocytes were transfected with CAT reporter genes containing various elements from the S14 promoter or S14 promoter elements fused to the
thymidine kinase (TK)-promoter. The composition of the promoter elements is illustrated in the figure. The S14 reporter constructs lacking the entire GlRR or the E-box in the GlRR are replaced by the Nsi I restriction site. After transfection, hepatocytes were treated with 250 μM 20:5,n3 as described above. After a 24 hrs treatment, cells were harvested for CAT and protein assays. Results are reported as % Inhibition of CAT Activity by 20:5,n3. The results are the mean ± SD of 2 independent studies involving triplicate samples.

**Figure 4:** The effect of T1317 and 20:5,n3 on LXRα-regulated transcripts in rat FTO-2B hepatoma cells. Confluent FTO-2B cells were treated with insulin (100 nM) and dexamethasone (10 nM) for 48 hrs prior to the study. Cells then received T1317 (5 μM) and/or 20:5,n3 (300 μM) for 24 hrs. Cells were harvested for RNA extraction or microsomal and nuclear proteins as described in Figure 1. [A]: (Upper panel) Measurement of mRNASREBP-1 by northern analysis; (Lower panel) Measurement of pSREBP-1 and nSREBP-1 protein by western analysis. The lower panel displays duplicate samples for each treatment. As in Figure 1, Fold Change of SREBP-1 is included in the figure. Lane # is identified at the bottom of the figure. [B]: Measurement of mRNAABCG5 and mRNAABCG8 by northern analysis. Results are representative of 2 separate studies with duplicate samples; mean ± SD.

**Figure 5:** Fatty acid regulation of LXRα activity in primary hepatocytes and FTO-2B hepatoma cells. Primary hepatocytes [A] and FTO-2B hepatoma cells [B] were transfected with an expression vector containing LXRα-LBD fused to the Gal4 DNA binding domain (CMX-Gal4-hLXRα) and the reporter plasmid, TK-MH100X4-Luc. Cells also received phRG-Luc as an internal control for transfection efficiency. After an overnight transfection, cells were treated without or with an LXR agonist T1317 (5 μM), without and with 20:5,n3 ranging from 0.25 to 1 mM. The 20:5,n3:BSA ratio was kept constant at 5:1. Vehicle treated cells received the same
level of BSA. After 24 hr of treatment, cells were lysed and assayed for luciferase activity and protein. Relative luciferase activity is the ratio of firefly luciferase activity to renilla luciferase activity (internal control). Results are expressed as Relative Luciferase Activity and are representative of >2 studies. Mean ± SD; N = 6.

**Figure 6:** Effect of unsaturated fatty acids on LXRα activity in primary hepatocytes.

Primary hepatocytes were transfected with [A] CMX-Gal4-hLXRα and the TK-MH100X4-Luc reporter or [B] CMX-hLXRα and the LXREx3-Luc reporter plasmid. After an overnight transfection, cells were treated without or with the LXR agonist 5 µM T1317 and without and with various unsaturated fatty acids at 250 µM for 24 hrs. BSA was included at a 5:1 ratio of fatty acid to BSA. After the 24 hr treatment period, cells were lysed and assayed for luciferase activity. Results are expressed as Relative Luciferase Activity and are representative of >2 separate studies. Mean ± SD; N = 6.

**Figure 7:** Dietary fat regulation of hepatic gene expression. Male Sprague-Dawley rats were meal-fed high carbohydrate diets supplemented with 10% olive or 10% fish oil (w/w) for 5 days. RNA was extracted and assayed for various transcripts (Materials and Methods). Results are expressed as fold change in mRNA. The expression of the various transcripts was normalized to olive oil-fed rats. Mean ± SD, N=3. The results are representative of several studies.
References:

Figure 1

<table>
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<tr>
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<tr>
<td>pSREBP-1</td>
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<tr>
<td>nSREBP-1</td>
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Relative Abundance of SREBP-1 mRNA
Figure 3B

% Inhibition of CAT Activity by 20:5,n3
**Figure 4A**

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</table>

- **Relative Abundance of SREBP-1c mRNA**

- **Fold Change**

- **Lane #**
Figure 5

Gal4DBD-LXRα fusion protein

A. Primary Hepatocyte

Relative Luciferase Activity

[20:5,n3], mM

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7

Relative Luciferase Activity

~IC₅₀ ≥ 1 mM

T1317

Veh

B. FTO-2B Hepatoma

Relative Luciferase Activity

[20:5,n3], mM

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7

Relative Luciferase Activity

T1317

Veh
Figure 6A

Gal4DBD-LXRα fusion protein

Graph showing relative luciferase activity for different treatments.

- T1317
- +T1317
Figure 6B

**Full Length LXRα**

![Graph showing relative luciferase activity](http://www.jbc.org/)

- **Veh**
- **Veh**
- **18:1,n9**
- **20:5,n3**

+T1317
Figure 7

Fold Change in mRNA Abundance

PPARα

SREBP-1c

LXRα

HNF-4α

HNF-4α

CYP7A1

L-PK

HNF-4
The role of liver X receptor-alpha (LXR-alpha) in the fatty acid regulation of hepatic gene expression

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