THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 277, No. 3, Issue of January 18, pp. 1705–1711, 2002
Printed in U.S.A.

Polyunsaturated Fatty Acids Suppress Sterol Regulatory Element-binding Protein 1c Promoter Activity by Inhibition of Liver X Receptor (LXR) Binding to LXR Response Elements*

Received for publication, June 20, 2001, and in revised form, November 1, 2001
Published, JBC Papers in Press, November 2, 2001, DOI 10.1074/jbc.M105711200

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Previous studies have demonstrated that polyunsaturated fatty acids (PUFAs) suppress sterol regulatory element-binding protein 1c (SREBP-1c) expression and, thus, lipogenesis. In the current study, the molecular mechanism for this suppressive effect was investigated with luciferase reporter gene assays using the SREBP-1c promoter in HEK293 cells. Consistent with previous data, the addition of PUFAs to the medium in the assays robustly inhibited the SREBP-1c promoter activity. Deletion and mutation of the two liver X receptor (LXR)-responsive elements (LXREs) in the SREBP-1c promoter region eliminated this suppressive effect, indicating that both LXREs are important PUFA-suppressive elements. The luciferase activities of both SREBP-1c promoter and LXRE enhancer constructs induced by co-expression of LXRα or -β were strongly suppressed by the addition of various PUFAs (arachidonic acid > eicosapentaenoic acid > docosahexaenoic acid > linoleic acid), whereas saturated or monounsaturated fatty acids had minimal effects. Gel shift mobility and ligand binding domain activation assays demonstrated that PUFA suppression of SREBP-1c expression is mediated through its competition with LXR ligand in the activation of the ligand binding domain of LXR, thereby inhibiting binding of LXR/retinoid X receptor heterodimer to the LXREs in the SREBP-1c promoter. These data suggest that PUFAs could be deeply involved in nutritional regulation of cellular fatty acid levels by inhibiting an LXR-SREBP-1c system crucial for lipogenesis.

Sterol regulatory element (SRE)[1]-binding proteins (SREBPs) are membrane-bound transcription factors that belong to the basic helix-loop-helix leucine zipper family (1–3). In the absence of sterols, by means of sterol-regulated cleavage, SREBP enters the nucleus and activates the transcription of genes involved in cholesterol and fatty acid synthesis by binding to an SRE or its related sequences including SRE-like sequences and E-boxes, within their promoter regions (4, 5). There are three forms of SREBP, SREBP-1a and -1c (also known as ADD1) and -2 (6–8). Most organs, including the liver and adipose tissue, predominantly express SREBP-2 and the -1c isoform of SREBP-1 (9). Recent *in vivo* studies demonstrate that SREBP-1c plays a crucial role in the dietary regulation of most hepatic lipogenic genes, whereas SREBP-2 is actively involved in the transcription of cholesterogenic enzymes (10). These include studies of the effects of the absence or overexpression of SREBP-1 on hepatic lipogenic gene expression (10–12) as well as physiological changes of SREBP-1c protein in normal mice refed after fasting (13–17).

Polyunsaturated fatty acid (PUFA) administration has been well established as a negative regulator of hepatic lipogenesis as well as an activator of peroxisome proliferator-activated receptor (PPAR) α, which is crucial for lipid degradation. Consistent with the notion that SREBP-1c is a dominant regulator for lipogenesis, there are several reports demonstrating that administration of PUFA suppresses SREBP-1c protein and mRNA both in cultured cells and in animal livers (14–16, 18). PUFA inhibition of SREBP-1c gene expression has been reported to be at cleavage, transcriptional, and post-transcriptional levels (14–16, 18–21); however, the precise mechanism for this effect remains unknown.

Recent promoter analysis reveals that the expression of the SREBP-1c gene is regulated by two factors; they are SREBP itself, forming an autoloop, and the liver X receptor (LXR)/retinoid X receptor (RXR) (22, 23). LXRs belong to a subclass of nuclear hormone receptors that form obligate heterodimers with RXRs and are activated by oxysterols (24–27). It has been established that LXRs regulate intracellular cholesterol levels by transactivating the expression of cholesterol 7α-hydroxylase (26–28), cholesterol ester transfer protein (29), and ATP binding cassette transporter 1, which modulates cholesterol efflux from cells with excess cellular cholesterol and mediates reverse cholesterol transport from peripheral tissues. LXR/RXR may

* This study was supported by the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: SRE, sterol regulatory element; SREBP, SRE-binding protein; PUFA, polyunsaturated fatty acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; LBD, ligand binding domain; LXR, liver X receptor; LXRE, LXR responsive element; RXR, retinoid X receptor; 22RHC, 22(R)-hydroxycholesterol; 9CRA, 9-*cis*-retinoic acid; kb, kilobases; CMV, cytomegalovirus; HEK, human embryonic kidney.

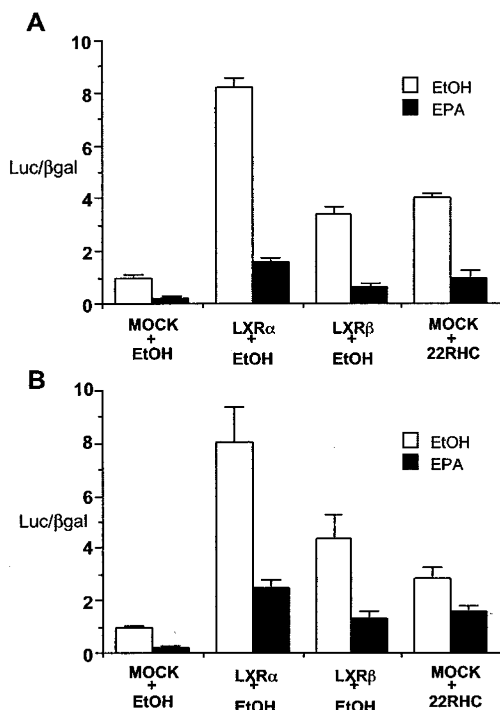

FIG. 1. **Polyunsaturated fatty acids suppress SREBP-1c promoter activity in HepG2 and HEK293 cells.** A luciferase reporter gene containing the mouse SREBP-1c promoter (2.6 kb); pBP1c2600-Luc was co-transfected into HepG2 (*A*) and HEK293 (*B*) cells with LXR (0.1 μg) or an empty vector CMV-7, as a control, and pSV-βgal, as a reference plasmid. Either EPA (100 μM) or ethanol (*EtOH*) as a control was added to the cells after transfection in medium with 10% fetal bovine serum 24 h before the assay. After incubation, luciferase activity was measured and normalized to β-galactosidase activity. The relative fold change in luciferase activity as compared with a mock-transfected control is shown (means ± S.D., three independent experiments in a duplicate assay).

also be involved in cholesterol absorption in intestine (30). Furthermore, LXR/RXR was identified as an activator of the SREBP-1c promoter (23, 31), implicating a new link of cholesterol and fatty acid metabolism.

Extending our previous promoter analysis studies, we attempted to explore the mechanism for the transcriptional inhibition of SREBP-1c by PUFA. In the current study, we analyzed the suppressive effect of PUFAs on SREBP-1c promoter activity. The data indicate that PUFA inhibit binding of the LXR/RXR heterodimer to the LXR response elements (LXREs) in the SREBP-1c promoter, a process crucial for SREBP-1c expression.

EXPERIMENTAL PROCEDURES

Materials—We obtained 22(*R*)-hydroxycholesterol (22RHC), 9-*cis*-retinoic acid (9CRA), Wy-14,643, stearic acid sodium salt (SA), oleic acid sodium salt (OA), linoleic acid sodium salt (LA), eicosapentaenoic acid sodium salt (EPA), docosahexaenoic acid sodium salt (DHA), and arachidonic acid sodium salt (AA) from Sigma, Redivue [α-^{32}P]dCTP (6,000 Ci/mmol) from Amersham Biosciences, Inc., and restriction enzymes from New England Biolabs. T0901317 (*N*-methyl-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethylethyl)-phenyl]-benzenesulfonamide), fenofibric acid and pioglitazone were provided by Kyorin Pharmaceutical Co. LTD., Laboratories Fournier (Paris, France), and Takeda pharmaceutical (Osaka, Japan), respectively.

Plasmids—Luciferase gene constructs containing a 2.6-kb fragment of the mouse SREBP-1c promoter (pBP1c2600-Luc), and other SREBP-1 promoter luciferase constructs were prepared as previously described (23). CMV and T7 promoter expression plasmids of human RXRα (pRXR) and PPAR response element (PPRE) luciferase reporter plasmid (pPPRE-Luc) were kind gifts from Dr. D. J. Mangelsdorf. The expression plasmid of the Gal4 DNA binding domain fused to the human LXRα-ligand binding domain (LBD) (pM-LXRα) was provided

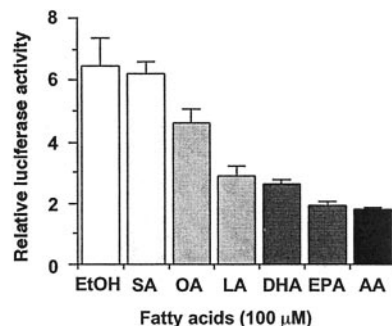

FIG. 2. **Inhibitory effects of various polyunsaturated fatty acids on SREBP-1c promoter activity.** pBP1c2600-Luc was co-transfected into HEK293 cells with LXRα (0.1 μg), and pSV-βgal (0.2 μg) as a reference plasmid. Various PUFAs (100 μM) dissolved in ethanol or ethanol only (*EtOH*) as a control were added to the cells after transfection in medium with 10% fetal bovine serum 24 h prior to the assay. After incubation, luciferase activity was measured and normalized to β-galactosidase activity. The relative fold change in luciferase activity as compared with a mock-transfected control is shown (means ± SD, three independent experiments in a duplicate assay).

from Mochida Pharmaceutical co. ltd. (Tokyo, Japan). A luciferase reporter plasmid containing Gal4 binding sites (p17 m8) was a gift from Dr. S. Kato.

Transfections and Luciferase Assays—Human embryonic kidney (HEK) 293 and HepG2 cells were grown at 37 °C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium containing 25 mM glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate supplemented with 10% fetal bovine serum. Transfection studies were carried out with cells plated on 12-well plates as previously described (22). The indicated amount of each expression plasmid was transfected simultaneously with a luciferase reporter plasmid (0.25 μg) and pSV-βgal (0.2 to 0.4 μg). The total amount of DNA in each transfection was adjusted to 1.5 μg/well with the vector DNA, pCMV7-NotI. Each fatty acid was dissolved in water or ethanol, 22RHC and T0901317 were dissolved in ethanol, and PPAR ligands were dissolved in dimethysulfoxide. Each agent was added to the cells immediately after transfection in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and incubated for 24 h. After incubation, the amount of luciferase activity in transfectants was measured and normalized to the amount of β-galactosidase activity as measured by standard kits (Promega).

Gel Mobility Shift Assays—Gel shift assays were performed as previously described (22). Briefly, the entire open reading frames of mLXRα and mPPARα were amplified from the pCMV-LXRα and pCMV-mPPARα by PCR (forward primers, 5′-TTGGTAATGTCCAGGG and 5′-GCCATACACTTGAGTGACAAT; reverse primers, 5′-CTTCCA-AGGCCAGGAGA and 5′-AGATCAGTACATGTCTCTGTAGA) and cloned into the *Eco*RI and *Not*I sites, and *Sal*I and *Not*I sites of the pBluescript II SK plasmid, respectively. mLXRα, mPPARα, and hRXRα proteins were generated from the expression vectors using a coupled *in vitro* transcription/translation system (Promega). Double-stranded oligonucleotides used in gel shift assays were prepared by annealing both strands of the LXREb in the LXRE complex of the SREBP-1c promoter (23) or rat fatty acyl-CoA oxidase PPRE (32). These were then labeled with [α-^{32}P]dCTP by Klenow enzyme followed by purification on G50-Sephadex columns. The labeled probes (3,000–10,000 cpm) were incubated with nuclear receptor lysates (1–1.5 μl) in a mixture (20 μl) containing 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.5 μg/ml poly(dI-dC), 0.1% Triton X-100, and 1 mg/ml nonfat milk for 30 min on ice. The DNA-protein complexes were resolved on a 4.6% polyacrylamide gel at 140 V for 1 h at 4 °C. Gels were dried and exposed to BAS2000 with BAStation software (Fuji Photo Film).

RESULTS

PUFA Suppression of Mouse SREBP-1c Promoter Activity in HepG2 and HEK293 Cells—To investigate the molecular mechanism by which dietary PUFAs decrease hepatic SREBP-1c expression, we established mouse SREBP-1c promoter luciferase reporter gene assays in HepG2 and HEK293 cells. As an initial study, we estimated the effect of supplementation of EPA to the medium (100 μM) on mouse SREBP-1c promoter (2.6-kb 5′-flanking region) activity in HepG2 cells. Cells were

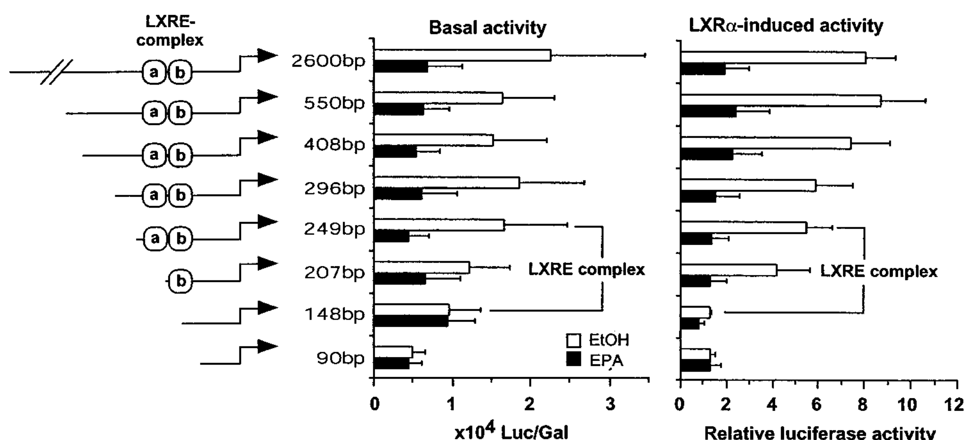

FIG. 3. **Identification of the polyunsaturated fatty acid-suppressive region in the SREBP1c-promoter by deletional analysis.** SREBP-1c promoter luciferase reporters of various lengths (as indicated) were constructed (*left panel*). The HEK293 cells were transfected with each reporter plasmid, pCMV-LXRα, and reference plasmid, pSV-βgal. Either EPA (300 μM) or ethanol (*EtOH*) as a control was added to the cells after transfection in medium with 10% fetal bovine serum 24 h before the assay. After incubation, luciferase activity was measured and normalized to β-galactosidase activity. The effect of EPA in each construct without LXRα co-expression (*Basal activity*) is expressed as normalized luciferase activity (means ± S.D., three independent experiments in a duplicate assay) (*middle panel*). The data from LXRα co-expression (0.1 μg of pCMV-LXRα, *LXRα-induced activity*) are shown as fold change relative to mock-transfected control (means ± S.D., three independent experiments in a duplicate assay) (*right panel*). *bp*, base pairs.

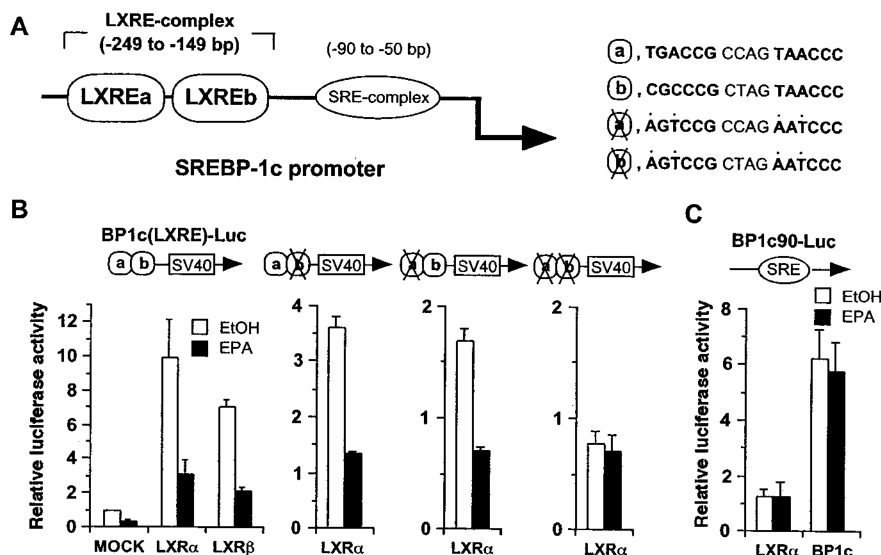

FIG. 4. **Inhibitory effect of polyunsaturated fatty acid on SREBP-1c promoter activity is mediated by the LXRE complex in the SREBP-1c promoter.** *A*, the LXRE complex containing two LXREs (LXREa and -b) was located at −249 to −148 bp in the SREBP-1c promoter as described previously (23). *B*, the LXRE complex in the SREBP-1c promoter was fused to a luciferase reporter plasmid, which contained an SV40 promoter (pGL2 promoter vector). This enhancer construct (pLXRE-Luc) or the indicated mutant construct was co-transfected into HEK293 cells with pCMV-LXRα, -β, or an empty vector, CMV-7 as a control, and pSV-βgal as a reference plasmid. *C*, pBP1c90b-Luc, which contained an SRE complex but no LXRE complex, was co-transfected into HEK293 cells with pCMV-SREBP-1c or an empty vector (CMV-7) as a control and pSV-βgal as a reference plasmid. Either EPA (100 μM) or ethanol (*EtOH*) as a control was added to the cells after transfection in medium with 10% fetal bovine serum 24 h before the assay. After incubation, luciferase activity was measured and normalized to β-galactosidase activity. The fold change by LXRs or their ligands in the luciferase activity (means ± S.D., three independent experiments in a duplicate assay) as compared with the respective control is shown. *bp*, base pairs.

co-transfected with LXRα or -β. These conditions have been shown to activate SREBP-1c promoter through LXREs (23). Consistent with previous *in vivo* observations that PUFA suppresses SREBP-1c expression (14), current data indicate that EPA considerably decreases the SREBP-1c promoter activity when LXR/RXR is activated (Fig. 1*A*). This suppressive effect of EPA was similarly observed in HEK293 cells (Fig. 1*B*). After these studies, effects of various PUFAs were tested in HEK293 cells co-transfected with LXRα. As shown in Fig. 2, SREBP-1c promoter activity was reduced by the addition of each PUFA (AA > EPA > DHA > LA). In contrast, saturated fatty acid (SA) had no effect, and the result of the addition of OA was minimal. These data indicate that SREBP-1c promoter assays can reflect PUFA suppression of SREBP-1c expression reported by us and others (14–16, 18) and that the cis-element(s) responsible for this PUFA effect should be located within this 2.6-kb 5′-flanking sequence of the mouse SREBP-1c gene.

LXREs as PUFA-suppressive Elements in the SREBP-1c Promoter—To locate the PUFA-suppressive element in the SREBP-1c promoter, we estimated the inhibitory effects of EPA on reporter genes containing the SREBP-1c promoter of various sizes. As shown in Fig. 3, the lack of LXREa, the upstream LXRE site of the two LXREs in the SREBP-1c promoter, caused a partial decrease in the inhibitory effect of EPA, and deletion of both LXREa and -b abolished the effect completely. These data suggest that the region containing the two LXREs are the PUFA-responsive elements of the SREBP-1c promoter. To explore this more precisely, we constructed an enhancer lucifer-

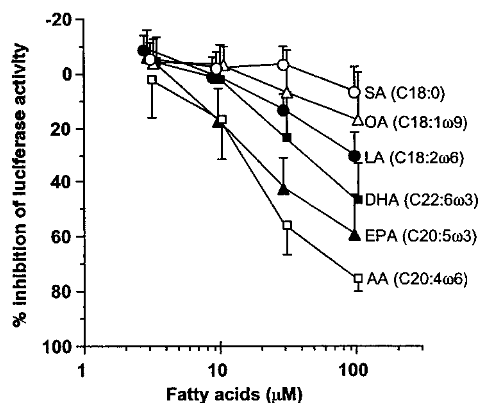

FIG. 5. **Dose-dependent suppression of the LXRE enhancer complex in the SREBP-1c promoter by various polyunsaturated fatty acids.** Indicated PUFA or ethanol (*control*) was added to the HEK293 cells after transfection of pLXRE-Luc, pCMV-LXRα, and pSV-βgal. After a 24-h incubation, luciferase activity was measured and normalized to β-galactosidase activity. The percent inhibition by PUFAs in the luciferase activity (means ± S.D., three independent experiments in a duplicate assay) as compared with the LXRα (0.1 μg)-induced control is shown.

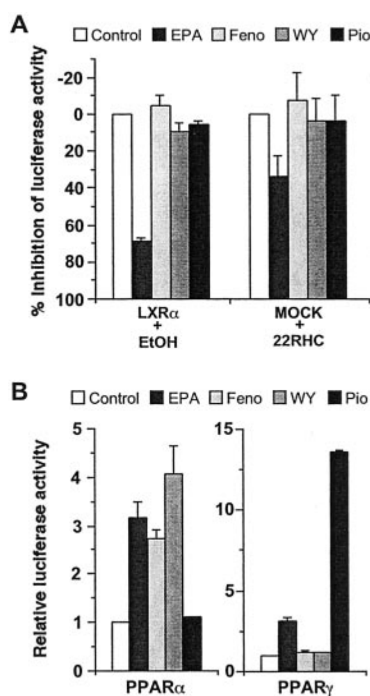

FIG. 6. **No suppression of the SREBP-1c promoter by pharmacological PPAR activators.** pLXRE-Luc was co-transfected into HEK293 cells with pCMV-LXRα or an empty vector, CMV-7 as a control and pSV-βgal as a reference plasmid (*A*). pPPRE-Luc was co-transfected into HEK293 cells with pCMV-PPARα, pCMV-PPARγ, or an empty vector, CMV-7 as a control, and pSV-βgal as a reference plasmid (*B*). LXR ligand 22RHC (10 μM), EPA (100 μM), PPARα pharmacological ligands fenofibric acid (*Feno*, 10 μM) and Wy-14,643 (*WY*, 10 μM), PPARγ pharmacological ligand pioglitazone (*Pio*, 1 μM), and ethanol (or dimethysulfoxide) as a control were added to the cells after transfection of pLXRE-Luc and pSV-βgal 24 h before the assay. After incubation, luciferase activity was measured and normalized to β-galactosidase activity. The percent inhibition of luciferase activity by EPA or PPAR ligands in the luciferase activity (means ± S.D., $n = 3$) as compared with the LXRα (0.1 μg)- or 22RHC (10 μM)-induced controls (*A*) and fold change by EPA or PPAR ligands in the luciferase activity (means ± S.D., three independent experiments in a duplicate assay) as compared with the PPARα (0.01 μg)- or PPARγ (0.01 μg)-induced controls, are shown.

ase construct containing the two LXREs (pLXRE-Luc). As shown in Fig. 4, pLXRE-Luc was activated by overexpression of either LXRα or -β. EPA suppression was observed in both LXRα- and LXRβ-activated LXRE-Luc activities. Introduction of a mutation in either LXREa or LXREb caused a partial impairment in this EPA inhibitory effect. Disruption of both elements abolished the EPA suppression completely. Another SREBP-1c promoter luciferase reporter (pBP1c90-Luc) containing an SRE, but no LXREs, was activated by co-expression of nuclear SREBP-1c. No EPA inhibition was observed in this construct, even when SREBP-1c was overexpressed. These data confirm that both LXREs are responsible for PUFA suppression of the SREBP-1c promoter.

Using the LXRE complex construct, we demonstrated a dose-dependent inhibition of SREBP-1c promoter activity by a variety of fatty acids. As shown in Fig. 5, PUFA, AA, EPA, DHA, and LA all suppressed pLXRE-Luc in a dose-dependent manner. OA showed a weak suppression, whereas SA did not have any effect. These data are consistent with results from the original 2.6-kb SREBP-1c construct and indicate that two LXREs are responsible for PUFA suppression.

PUFA are known to be PPAR ligands (33, 34). However, previous *in vivo* observations on PUFA inhibition of SREBP-1c suggest that the effect was PPAR-independent (35). The present study indicates that the element responsible for PUFA suppression is the LXREs. To rule out the possibility that PUFA inhibition of pLXRE-Luc is mediated through PPARα, we used synthetic PPAR ligands such fenofibric acid and Wy-14,643 for PPARα and pioglitazone for PPARγ (Fig. 6). In contrast to EPA, these pharmacological PPAR agonists did not suppress pLXRE-luc, whereas these ligands did activate their respective target pPPRE-luc constructs. These data indicate that PPAR activation is not involved in PUFA inhibition of LXRE and SREBP-1c.

PUFA Inhibition of Ligand-activated LXR-RXR Binding to the LXRE—To further investigate the molecular mechanism by which PUFAs suppress LXREs in the SREBP-1c promoter, gel shift mobility assays were performed. *In vitro* translated LXRα and RXR recombinant proteins were used to confirm binding of LXRα/RXR heterodimer to the LXREb probe as estimated by the shifted band. Fig. 7*A* shows that the shifted signal was enhanced by the direct addition of T0901713, an artificial LXR ligand, in a dose-dependent manner, demonstrating ligand activation of LXR binding to LXRE. The addition of PUFA inhibited the shifted bind, whereas SA and OA had a minimal effect. The rank order of the potency of the inhibitory effect of each fatty acid was similar to that observed in the luciferase assays. The inhibitory effect of AA was the strongest (Fig. 7*B*). Fig. 7*C* shows the competition between AA and LXR ligand. The signal from the LXR/RXR·LXRE complex was inhibited by the addition of AA in a dose-dependent manner, and further addition of T0901713 dose-dependently blocked the inhibitory effect of AA. These results strongly suggest that inhibition of LXR/RXR binding to LXRE by PUFA was mediated through antagonizing the ligand effect on the LBD of LXR. For comparison, effects of PUFA on shifts of a PPRE probe by PPARα and RXR proteins were also tested (Fig. 7*D*). PPARα/RXR binding to PPRE was not affected by SA but was enhanced by OA, PUFA, and PPAR ligands, demonstrating that blocking the effects of PUFA on LXR/RXR-LXRE binding are not due to nonspecific inhibition of the assays by the fatty acid moiety. The data suggest that PUFA directly inhibits LXR/RXR binding to LXRE or LXR/RXR heterodimers.

PUFA Competition with an LXR Ligand in the Activation of the LBD of LXR—To clarify the molecular mechanism by which PUFA inhibits LXR/RXR binding to LXRE, LBD activation assays of LXRα were performed. In these assays, an expression plasmid of the LBD of LXRα fused to the Gal4 DNA binding domain was co-transfected with a luciferase reporter containing Gal4 binding sites to estimate the specific ligand binding of the samples to LBD of LXRα. The addition of 22RHC, a well

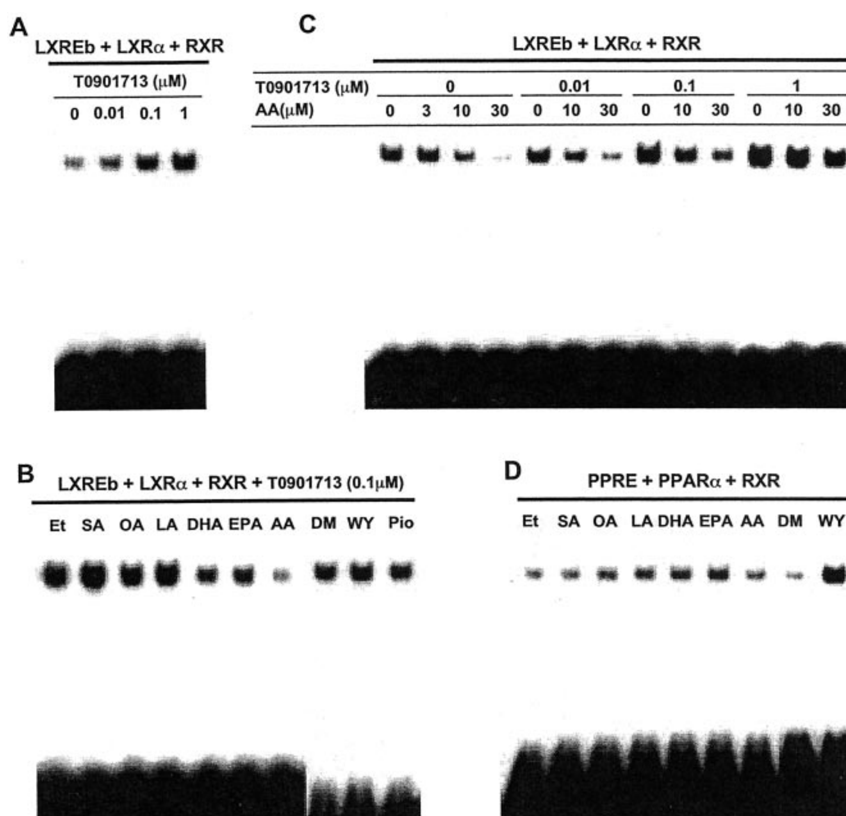

FIG. 7. **Polyunsaturated fatty acids inhibit LXR-RXR binding to LXREs in the SREBP1c-promoter as measured by gel-mobility shift assays.** The indicated fatty acid, PPAR ligands, or ethanol (or dimethysulfoxide) as controls were incubated with *in vitro* synthesized LXRα, PPARα, and RXR (1–1.5 μl of programmed reticulocyte lysate, TNT Quick Coupled Transcription/Translation Systems, Promega) for 30 min on ice. After incubation, labeled LXR-response element (LXREb) in the SREBP-1c promoter (*A–C*) or labeled PPRE in the acyl-CoA oxidase promoter (*D*) was added and incubated for 30 min on ice. 30 μM PUFA, 10 μM Wy-14,643 (*WY*), and 1 μM pioglitazon (*Pio*) were used in *panels B* and *D*. The DNA-protein complexes were resolved in a 4.8% polyacrylamide gel. *DM*, dimethyl sulfoxide.

known LXR ligand, increased the Gal4 activity. As shown in Fig. 8, the addition of each PUFA showed a dose-dependent inhibition of the LBD activation in a very similar pattern to the effects observed in the LXRE luciferase assay under 22RHC-activated conditions, suggesting that PUFA inhibits LXR ligand binding to LXR/RXR.

Subsequently, the PUFA inhibitory effect on SREBP-1c promoter activity was re-estimated by competition between EPA and 22RHC in the presence of an abundant amount of LXRα by co-transfection (0.1 μg DNA). The addition of 22RHC without EPA resulted in a dose-dependent increase in the luciferase activity (Fig. 9, *left*). As shown in Fig. 9, *right*, in the presence of 10 and 30 μM of 22RHC, the percent inhibition curve of LXRE-LBD binding activity by EPA was shifted to the right, suggesting a competition between 22RHC and EPA in the activation of LXR.

No Involvement of RXR on PUFA Suppression of the SREBP-1c Promoter—We also investigated the possibility that PUFA inhibition of LXR/RXR binding to LXRE might be mediated through an interaction of PUFA to RXR. Fig. 10 shows that overexpression of RXR by co-transfection minimally changed LXRE-enhancer luciferase activity, suggesting that RXR is not a limiting factor for LXR/RXR binding to LXRE in this system. If PUFA could interact with RXR to modify LXR/RXR binding to LXRE, overexpression of RXR should absorb and repress this PUFA effect on LXR/RXR. However, inhibitory effects of PUFA on LXRE-enhancer activity (Fig. 10) and the 2.6-kb SREBP-1c promoter activity (data not shown) were not affected by RXR overexpression in the RXR-co-transfected cells. The addition of 9CRA, an RXR ligand, increased the effect of LXR/RXR but did not markedly affect the inhibitory efficiency of PUFA. These results indicate that the inhibitory effect of PUFA may be independent of the RXR portion of the LXR/RXR heterodimer.

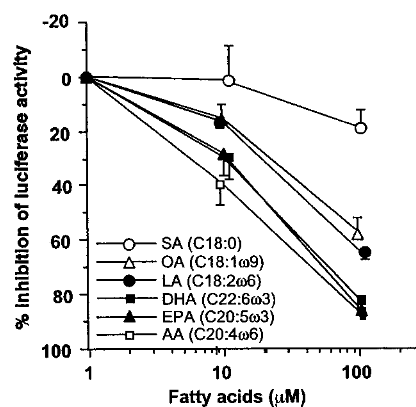

FIG. 8. **Polyunsaturated fatty acids inhibit the LBD activation assay of LXR.** The Gal4-driven luciferase reporter construct, p17m8 was co-transfected into HEK293 cells with an expression vector containing the Gal4 DNA binding domain fused to the ligand binding domain of LXRα, pM-LXRα, and a control plasmid, pSV-βgal. After the transfection, the cells were incubated with various PUFAs in the presence of their ligand, 22RHC for 24 h. After incubation, luciferase activity was measured and normalized to β-galactosidase activity. The percent inhibition in luciferase activity by polyunsaturated fatty acids (means ± S.D., three independent experiments in a duplicate assay) in the presence of 22RHC (10 μM) is shown.

DISCUSSION

In the current study, we located PUFA suppressive elements in the mouse SREBP-1c promoter. The responsible elements correspond to two LXREs that were previously identified as LXR/RXR activation sites (23). Further luciferase studies, gel shift assays, and LBD activation assays demonstrated that PUFAs suppress SREBP-1c expression through interacting with the LBD of LXR and inhibiting LXR/RXR binding to the LXREs crucial for SREBP-1c expression as schematized in Fig. 11.

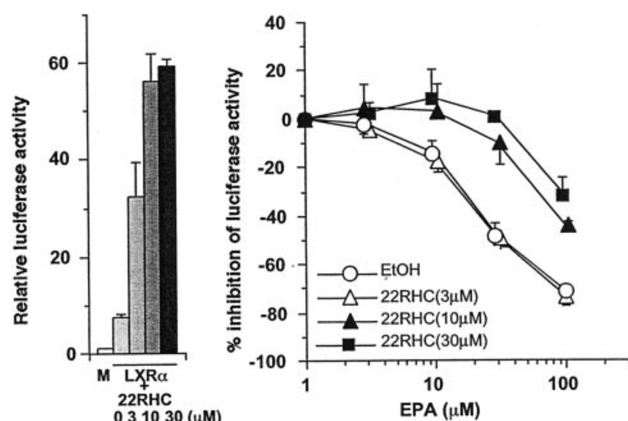

FIG. 9. **Competition between 22(R)-hydroxycholesterol and EPA in LXR-induced SREBP-1c promoter activity.** pLXRE-Luc (0.25 μg) was co-transfected into HEK293 cells with pCMV-LXRα (0.1 μg) or an empty vector, CMV-7, as a control, and pSV-βgal, as a reference plasmid. An indicated concentration of 22RHC or ethanol (*EtOH*) as a control was added to the cells after transfection in medium with 10% fetal bovine serum 24 h before the assay (*left panel*). In the *right panel*, an indicated amount of EPA was also added. After incubation, luciferase activity was measured and normalized to β-galactosidase activity. The fold induction by LXRα and 22RHC in luciferase activity as compared with control (mock-transfected cells without 22RHC addition) is shown in the *left panel*. The percent inhibition by EPA in the luciferase activity is shown (*right panel*). *M*, mock.

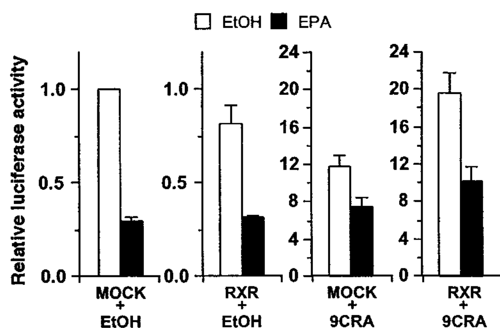

FIG. 10. **Inhibitory effect of eicosapentaenoic acid on SREBP-1c promoter activity is not affected by the overexpression of RXR or 9CRA addition.** pLXRE-Luc was co-transfected into HEK293 cells with pCMV-LXRα (0.1 μg), pCMV-RXR (0.5 μg), or an empty vector, pCMV-7 as a control, and pSV-βgal as a reference plasmid. EPA (100 μM), 9-*cis* retinoic acid (*9CRA*, 10 μM), or ethanol (*EtOH*) as a control were added to the cells after transfection in medium with 10% fetal bovine serum 24 h before the assay. After incubation, luciferase activity was measured and normalized to β-galactosidase activity. The fold induction by RXR and 9CRA in luciferase activity (means ± S.D., three independent experiments in a duplicate assay) as compared with control (*MOCK* and ethanol) is shown.

The order of inhibitory magnitude of each long chain fatty acid on SREBP-1c expression is as follows: AA > EPA > DHA > LA ≫ OA > SA = 0. This order was essentially consistent among the luciferase assays with the 2.6-kb SREBP-1c promoter and LXRE-enhancer as well as in gel shift and LBD activation assays. Furthermore, the same order of long chain fatty acid effects on SREBP-1c suppression has been shown in diet studies with mice.[2] Furthermore, these data suggest that the inhibitory effect of PUFA is primarily attributed to their blocking effect on the LBD of LXR. The degree of unsaturation of the fatty acids might be a factor for this inhibitory effect, but whether they are n-3 or n-6 appears to be irrelevant.

The LXR/RXR complex has been established as a nuclear receptor for oxysterols, controlling regulation of excess cellular

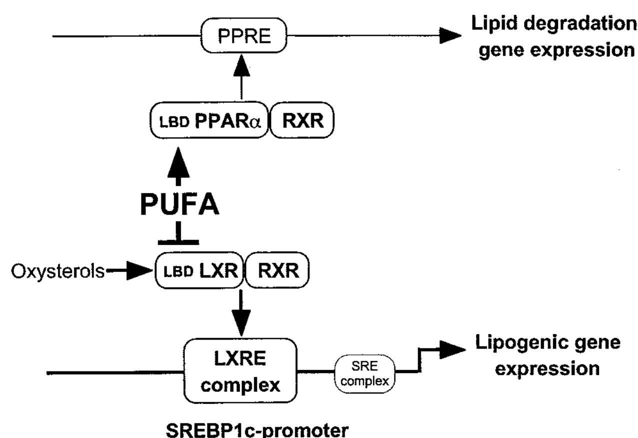

FIG. 11. **Mechanism by which polyunsaturated fatty acids suppresses the SREBP-1c promoter activity through affecting LXR-RXR activation pathway.** PUFAs suppress SREBP-1c gene expression crucial for lipogenesis by inhibiting LXR-RXR binding to the LXREs. Reciprocally, PUFAs promote PPAR-activated genes expression crucial for lipid degradation through activation of PPAR-RXR binding to the PPREs.

cholesterol (24–27, 30). It is interesting to speculate that oxidative modification of PUFA during incubation might make them eligible to interact with LXR. However, the addition of several kinds of antioxidants such as probucol and vitamin E did not change the ability of PUFA to inhibit SREBP-1c activation (data not shown). Finally, even after direct addition of PUFA to the incubation mixture for gel shift assays, PUFA still showed inhibitory effects on LXR/RXR binding to LXRE, strongly suggesting a direct action of PUFA. The current study demonstrates that PUFA can be an antagonist for LXR/RXR. It seems that PUFA binds to the LBD of LXRs in a fashion that is competitive with an endogenous LXR ligand, thereby repressing LXR/RXR transactivity. During the process of preparing this manuscript, an antagonizing effect of PUFA in competition with an LXR pharmacological ligand, as measured by SREBP-1c expression in RNA protection and LXR coactivator recruitment assays in rat hepatoma cells, was reported (21). The conclusion was compatible with our present data. Further studies on LXR ligands in relation to oxysterols and PUFA are needed. Structure analysis on ligand binding and DNA binding domains of LXR in the presence of LXR ligands and LXRE should assist in understanding the complex nature of this system.

Our new finding on PUFA inhibition of LXR-SREBP-1c brings up an intriguing speculation for a mechanism of energy regulation as depicted in Fig. 11. Previous work by our laboratory (23) and others (31, 36) suggests that LXR/RXR is a dominant activator for expression of SREBP-1c, a transcription factor that is a crucial factor for hepatic lipogenesis that is necessary for storage of excess energy as observed in a refed state. Meanwhile, PUFAs can function as ligands for PPARα (33, 34), another transcription factor that plays a crucial role for fatty acid oxidation in an energy-depleted state such as fasting (37, 38). In a fasted state, PUFAs can be released from adipose tissue by lipolysis. Taken up by the liver, PUFAs can bind to and activate PPARα to induce β-oxidation of other saturated or monounsaturated fatty acids. At the same time, PUFA antagonize LXR/RXR, leading to suppression of SREBP-1c and minimizing lipogenesis. Therefore, PUFA might have efficient regulatory roles for adaptic control of two extreme nutritional states by having reciprocal effects on LXR-SREBP-1c and PPARα (Fig. 11). In addition, post-transcriptional regulation of the SREBP-1c gene by PUFA has been also proposed (15, 20, 35). Further studies are needed to clarify the

[2] N. Yahagi, unpublished data.

relative roles of transcriptional, post-transcriptional, and cleavage regulation of SREBP-1c by PUFA *in vivo*.

The current data suggest that PUFAs could be intricately involved in nutritional regulation by affecting the LXR-SREBP-1c system that is crucial for lipogenesis as well as having a well established role as ligands for PPARα. This might open up a new aspect of nutritional regulation involving essential fatty acids as well as energy fuels.

Acknowledgment—We thank A. H. Hasty for critical reading of the manuscript.

REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1997) *Cell* **89,** 331–340
2. Brown, M. S., and Goldstein, J. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11041–11048
3. Brown, M. S., Ye, J., Rawson, R. B., and Goldstein, J. L. (2000) *Cell* **100,** 391–398
4. Briggs, M. R., Yokoyama, C., Wang, X., Brown, M. S., and Goldstein, J. L. (1993) *J. Biol. Chem.* **268,** 14490–14496
5. Kim, J. B., Spotts, G. D., Halvorsen, Y. D., Shih, H. M., Ellenberger, T., Towle, H. C., and Spiegelman, B. M. (1995) *Mol. Cell. Biol.* **15,** 2582–2588
6. Yokoyama, C., Wang, X., Briggs, M. R., Admon, A., Wu, J., Hua, X., Goldstein, J. L., and Brown, M. S. (1993) *Cell* **75,** 187–197
7. Tontonoz, P., Kim, J. B., Graves, R. A., and Spiegelman, B. M. (1993) *Mol. Cell. Biol.* **13,** 4753–4759
8. Hua, X., Yokoyama, C., Wu, J., Briggs, M. R., Brown, M. S., Goldstein, J. L., and Wang, X. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11603–11607
9. Shimomura, I., Shimano, H., Horton, J. D., Goldstein, J. L., and Brown, M. S. (1997) *J. Clin. Invest.* **99,** 838–845
10. Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L., and Shimano, H. (1998) *J. Clin. Invest.* **101,** 2331–2339
11. Shimano, H., Horton, J. D., Hammer, R. E., Shimomura, I., Brown, M., and Goldstein, J. L. (1996) *J. Clin. Invest.* **98,** 1575–1584
12. Shimano, H., Yahagi, N., Amemiya-Kudo, M., Hasty, A., Osuga, J., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., Harada, K., Gotoda, T., Ishibashi, S., and Yamada, N. (1999) *J. Biol. Chem.* **274,** 35832–35839
13. Horton, J. D., Bashmakov, Y., Shimomura, I., and Shimano, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5987–5992
14. Yahagi, N., Shimano, H., Hasty, A., Amemiya-Kudo, M., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., and Yamada, N. (1999) *J. Biol. Chem.* **274,** 35840–35844
15. Xu, J., Nakamura, M. T., Cho, H. P., and Clarke, S. D. (1999) *J. Biol. Chem.* **274,** 23577–23583
16. Kim, H. J., Takahashi, M., and Ezaki, O. (1999) *J. Biol. Chem.* **274,** 25892–25898
17. Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., Lowell, B. B., and Spiegelman, B. M. (1998) *J. Clin. Invest.* **101,** 1–9
18. Worgall, T. S., Sturley, S. L., Seo, T., Osborne, T. F., and Deckelbaum, R. J. (1998) *J. Biol. Chem.* **273,** 25537–25540
19. Hannah, V. C., Ou, J., Luong, A., Goldstein, J. L., and Brown, M. S. (2001) *J. Biol. Chem.* **276,** 4365–4372
20. Xu, J., Teran-Garcia, M., Park, J. H., Nakamura, M. T., and Clarke, S. D. (2001) *J. Biol. Chem.* **276,** 9800–9807
21. Ou, J., Tu, H., Shan, B., Luk, A., DeBose-Boyd, R. A., Bashmakov, Y., Goldstein, J. L., and Brown, M. S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 6027–6032
22. Amemiya-Kudo, M., Shimano, H., Yoshikawa, T., Yahagi, N., Hasty, A. H., Okazaki, H., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., Osuga, J.-i., Harada, K., Gotoda, T., Sato, R., Kimura, S., Ishibashi, S., and Yamada, N. (2000) *J. Biol. Chem.* **275,** 31078–31085
23. Yoshikawa, T., Shimano, H., Amemiya-Kudo, M., Yahagi, N., Hasty, A. H., Matsuzaka, T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Kimura, S., Ishibashi, S., and Yamada, N. (2001) *Mol. Cell. Biol.* **21,** 2991–3000
24. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell* **83,** 835–839
25. Janowski, B. A., Willy, P. J., Devi, T. R., Falck, J. R., and Mangelsdorf, D. J. (1996) *Nature* **383,** 728–731
26. Lehmann, J. M., Kliewer, S. A., Moore, L. B., Smith-Oliver, T. A., Oliver, B. B., Su, J. L., Sundseth, S. S., Winegar, D. A., Blanchard, D. E., Spencer, T. A., and Willson, T. M. (1997) *J. Biol. Chem.* **272,** 3137–3140
27. Russell, D. W. (1999) *Cell* **97,** 539–542
28. Peet, D. J., Turley, S. D., Ma, W., Janowski, B. A., Lobaccaro, J. M., Hammer, R. E., and Mangelsdorf, D. J. (1998) *Cell* **93,** 693–704
29. Luo, Y., and Tall, A. R. (2000) *J. Clin. Invest.* **105,** 513–520
30. 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) *Science* **289,** 1524–1529
31. 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) *Genes Dev.* **14,** 2819–2830
32. Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) *Nature* **358,** 771–774
33. Forman, B. M., Chen, J., and Evans, R. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4312–4317
34. Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4318–4323
35. Mater, M. K., Thelen A. P., Pan, D. A., and Jump, D. B. (1999) *J. Biol. Chem.* **274,** 32725–32732
36. DeBose-Boyd, R. A., Ou, J., Goldstein, J. L., and Brown, M. S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1477–1482
37. Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B., and Wahli, W. (1999) *J. Clin. Invest.* **103,** 1489–1498
38. Leone, T. C., Weinheimer, C. J., and Kelly, D. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7473–7478