Cross-Talk between Peroxisome Proliferator-Activated Receptor (PPAR) α and Liver X Receptor (LXR) in Nutritional Regulation of Fatty Acid Metabolism. II. LXRs Suppress Lipid Degradation Gene Promoters through Inhibition of PPAR Signaling

TOMOHIRO IDE, HITOSHI SHIMANO, TOMOHIRO YOSHIKAWA, NAOYA YAHAGI, MICHIO AMEMIYA-KUDO, TAKASHI MATSUZAKA, MASANORI NAKAKUKI, SHIGERU YATOH, YOKO IIZUKA, SACHIKO TOMITA, KEN OHASHI, AKIMITSU TAKAHASHI, HIROHITO SONE, TAKANARI GOTODA, JUN-ICHI OSUGA, SHUN ISHIBASHI, AND NOBUHIRO YAMADA

Department of Internal Medicine (T.I., H.S., T.M., M.N., S.Y., A.T., H.S., N.S.), Institute of Clinical Medicine, University of Tsukuba, Ibaraki 305-8575, Japan; and Department of Metabolic Diseases (T.Y., N.Y., M.A.-K., Y.I., S.T., K.O., T.G., J.O., S.I.), Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-8655, Japan

Fatty acid metabolism is transcriptionally regulated by two reciprocal systems: peroxisome proliferator-activated receptor (PPAR) controls fatty acid degradation, whereas sterol regulatory element-binding protein-1c activated by liver X receptor (LXR) regulates fatty acid synthesis. To explore potential interactions between LXR and PPAR, the effect of LXR activation on PPAR signaling was investigated. In luciferase reporter gene assays, overexpression of LXRα or β suppressed PPARα-induced peroxisome proliferator response element-luciferase activity in a dose-dependent manner. LXR agonists, T0901317 and 22(R)-hydroxycholesterol, dose dependently enhanced the suppressive effects of LXRs. Gel shift assays demonstrated that LXR reduced binding of PPARα/retinoid X receptor (RXR)α to peroxisome proliferator response element. Addition of increasing amounts of RXRα restored these inhibitory effects in both luciferase and gel shift assays, suggesting the presence of RXRα competition. In vitro protein binding assays demonstrated that activation of LXR by an LXR agonist promoted formation of LXR/RXRα and, more importantly, LXR/PPARα heterodimers, leading to a reduction of PPARα/RXRα formation. Supportively, in vivo administration of the LXR ligand to mice and rat primary hepatocytes substantially decreased hepatic mRNA levels of PPARα-targeted genes in both basal and PPARα agonist-induced conditions. The amount of nuclear PPARα/RXRα heterodimers in the mouse livers was induced by treatment with PPARα ligand, and was suppressed by superimposed LXR ligand. Taken together with data from the accompanying paper (Yoshikawa, T., T. Ide, H. Shimano, N. Yahagi, M. Amemiya-Kudo, T. Matsuza, S. Yatoh, T. Kitamine, H. Okazaki, Y. Tamura, M. Sekiya, A. Takahashi, A. H. Hasty, R. Sato, H. Sone, J. Osuga, S. Ishibashi, and N. Yamada, Endocrinology 144:1240–1254) describing PPARα suppression of the LXR-sterol regulatory element-binding protein-1c pathway, we propose the presence of an intricate network of nutritional transcription factors with mutual interactions, resulting in efficient reciprocal regulation of lipid degradation and lipogenesis. (Molecular Endocrinology 17: 1255–1267, 2003)

Recent progress in studying transcriptional regulation of lipid catabolism has unveiled the functions of orphan nuclear receptors such as peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs), whereas roles by sterol regulatory element-binding protein (SREBP) family have been established as lipid synthetic regulators. PPARs are members of a nuclear receptor superfamily and are described as key players in the regulation of lipid metabolism. PPARs include PPARα, PPARγ, PPARδ, and PPARβ/δ, each of which plays distinct roles in lipid and glucose metabolism. PPARα is mainly expressed in liver and white adipose tissue, where it promotes fatty acid oxidation and ketogenesis. PPARγ is highly expressed in adipose tissue, muscle, and liver, and plays a crucial role in lipid metabolism and insulin sensitivity. PPARδ is found in the liver, heart, and brain and is involved in the regulation of fatty acid metabolism. PPARβ/δ is expressed in a variety of tissues and is involved in the regulation of lipid and glucose metabolism. LXRα and LXRβ are two members of the nuclear receptor superfamily that play a key role in lipid metabolism. LXRα is primarily expressed in the liver and promotes the expression of genes involved in cholesterol and fatty acid degradation. LXRβ is expressed in a variety of tissues and promotes the expression of genes involved in cholesterol degradation. The interaction between PPARs and LXRs is essential for the efficient regulation of lipid metabolism. By understanding the mechanisms of PPAR-LXR interactions, we can develop new therapeutic strategies for the treatment of metabolic disorders such as obesity and type 2 diabetes.
structurally related to other members such as thyroid hormone receptor (TR) and vitamin D3 receptor (VDR) (1). PPARs are known to regulate expression of numerous genes involved in fatty acid metabolism and adipocyte differentiation (2, 3). PPARα is abundantly expressed in tissues, which have high lipid catabolic activity, such as liver, kidney, heart, skeletal muscle, and brown adipose tissue (4, 5). PPARα is activated by fatty acids, eicosanoids, and fibrates, a known class of hypolipidemic drugs. Like other nuclear receptors such as: TR, VDR, and retinoic acid receptor (RAR) (6–9), PPARα forms a heterodimer with RXRα, which enhances its binding to DNA sequence elements termed peroxisome proliferator response elements (PPRE) (10–12). PPREs have been recently identified in the 5′-flanking sequences of genes involved in lipid degradation such as the ACO [acyl-coenzyme A (CoA) oxidase] (13), mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA Syn) (14), l-carnitine palmitoyltransferase (CPTI) (15), and l-fatty acid binding protein genes (16). Studies using PPARα-deficient mice have established that PPARα plays a crucial role in fatty acid degradation (17–19).

LXRs (LXRα/NR1H3 and LXRβ/NR1H2) were identified as orphan nuclear receptors and are now thought to regulate the metabolism of several important lipids, including cholesterol and bile acid (20, 21). LXRs regulate intracellular cholesterol levels by induction of the gene expression of cholesterol 7α-hydroxylase (22, 23), which is the rate-limiting enzyme of the classic bile acid synthesis pathway, and ATP-binding cassette transporter A1 (ABCA1) (24), which modulates apolipoprotein mediated-efflux of cholesterol. Further evidence supporting an important role of LXRα in lipid homeostasis is provided by the loss of capacity to regulate catabolism of dietary cholesterol in LXRα deficient mice, an effect for which the isoform, LXRβ, could not compensate (25). Differences in the physiological functions between LXRα and LXRβ including target and tissue specificity have been suggested (26). LXRβ is ubiquitously expressed, whereas LXRα is restricted to metabolically active tissues, such as liver, kidney, intestines, and adrenal glands (27, 28). Recently, we (29) and others (30) reported that both LXRα and LXRβ are dominant activators for SREBP-1c/adipocyte determination and differentiation 1 (ADD1). Previous in vivo studies established that SREBP-1c plays a crucial role in the dietary regulation of most hepatic genes of fatty acid synthetic enzymes (31–34). Therefore, LXRs could be also important in fatty acid synthesis (35).

As PPARα and LXR-SREBP-1c are reciprocal regulators for fatty acid metabolism, nuclear receptors could interact with each other as was previously observed in a cross-talk between PPARα and TR (36, 37). The current study examined the effects of LXR activation on PPARα signaling. The results demonstrate that LXRα ligand activation represses PPARα signaling through reduction of stimulated-PPARα/RXR heterodimerization in the liver. Taken together with the accompanying paper (38) describing PPAR suppression of the LXR-SREBP-1c pathway, we propose a novel aspect of nutritional regulation with these mutual interactions forming a network of transcription factors regulating fatty acid metabolism.

Fig. 1. Inhibition of PPARα-Induced PPRE-Luc Activity by LXRα and β
HEK293 cells were transfected with a reporter plasmid (0.25 μg): pPPRE-Luc (A and B) or constitutive Luc plasmid, pTK-Luc (C), expression plasmids (0.05 μg): CMV-mPPARα with CMV-mLXRα (A) or CMV-mLXRβ (B), and a reference plasmid (0.2 μg): pSV-β-gal. Cells were treated with either ethanol as vehicle, 3 μM of 22(R)HC or T0901317. All Luc activities were corrected for transfection efficiency by measuring β-gal activities. Results are means ± se from three independent experiments.
RESULTS

LXR Activation Represses PPARα-Mediated Transactivation

To estimate the effect of LXR/RXRα activation on PPARα signaling, transfection studies with human embryonic kidney (HEK) 293 cells were performed using luciferase (Luc) reporter gene assays containing a PPRE from the ACO gene promoter [pPPRE-Luc (PPRE-Luc plasmid)], a representative PPARα target. As shown in Fig. 1, the Luc activity of PPRE-Luc was markedly (60-fold) induced by cotransfection of PPARα due to lack of endogenous PPAR expression in HEK293 cells (data not shown). The PPARα-inducible expression of Luc activity was slightly, but dose dependently suppressed by coexpression of LXRα (Fig. 1A) (50% inhibition at a DNA dose ratio of 1:10). Expression level of transfected PPARα or LXR gene in HEK293 cells were roughly comparable to that in mouse liver as estimated by Northern blotting, and thus was within a physiological range (data not shown). This observation is consistent with a previous report describing LXR interaction with PPARα signaling (39). More interestingly, addition of LXR ligands such as 22RHC and T0901317 markedly enhanced the inhibitory effects of LXRα alone, without an LXR ligand, substantially inhibit PPARα activation of PPRE-Luc in a dose-dependent manner (Fig. 1B). A 50% inhibition was observed at a DNA ratio of 1:5. Additional LXR ligands further augmented the inhibitory effect of LXRα. These effects by LXRs and LXR ligands are not due to direct inhibition of Luc activity because Luc activity from constitutive expression of Luc gene by TK promoter-Luc construct was not affected by LXR coexpression, by addition of LXR ligand, or a combination of both (Fig. 1C). These data demonstrate that LXRs and their ligands inhibit PPAR signaling with overexpressing nuclear receptors system.

LXR Cannot Bind to PPRE, but Inhibits PPARα/RXRα Binding to PPRE

The inhibitory effect of LXR was estimated in the light of the dose dependency of LXR ligands in Fig. 2. As a positive control, a dose-response curve of LXRE (LXR response element)-Luc activation by T0901317 and 22RHC with coexpression of LXRα is shown in the upper panel, which is consistent with previous data (29). With a similar efficiency, these LXR agonists inhibited PPARα activation of PPRE-Luc, making both curves a mirror image of each other. LXRβ coexpression gave similar results with a slightly higher efficiency.

To further investigate the mechanism for inhibition of PPARα by LXR, gel mobility shift assays were performed. The binding specificity of LXR/RXRα to LXRE, and PPARα/RXRα to PPRE was confirmed in the experiments shown in Fig. 3, A and B. RXRα is required for specific binding of both LXR and PPAR. Neither LXRα/RXRα nor LXRβ/RXRα bound to PPRE within the sensitivity of this assay. Coincubation of PPARα and LXRα or β essentially caused no shift using PPRE probe. These data exclude the possibility that LXR/RXRα competes with PPARα/RXRα binding to a response element. Next, PPARα/RXRα binding to PPRE was estimated in the presence of LXR and/or its ligand (Fig. 4). PPARα/RXRα binding to PPRE was partially inhibited by addition of LXR protein. LXR ligand en-

Fig. 2. Dose-Dependent Reversal Effects of LXR Ligands on LXR- and PPARα-Mediated Transactivation

HEK293 cells were transfected with 0.25 μg of pLXRE-Luc (A and B) or pPPRE-Luc (C and D) as a reporter plasmid, 0.05 μg of pCMVmLXRα (A and C), pCMVmLXRβ (B and D) as an expression plasmid and pSV-β-gal as a reference plasmid were cotransfected in the absence (A and B) or presence (C and D) of 0.05 μg of pCMV-mPPARα. Cells were treated with either ethanol as vehicle or the indicated concentrations of 22(R)HC or T0901317. All Luc activities were corrected for transfection efficiency by measuring β-gal activities.
enhanced this inhibition. These data are consistent with results from reporter assays, suggesting that suppression of PPARα/H9251 activity by LXR is mediated at least partly through inhibition of PPARα/H9251/RXRα/H9251 binding to PPRE.

**RXR Restores LXR Repression of PPARα/H9251-Mediated Transactivation**

Because PPARα and LXRs share RXRα as a heterodimer partner for their specific functions, it is conceivable that LXR inhibition of PPARα activation on PPRE-Luc is mediated through competition for RXRα when RXRα is in limiting amounts in cultured cells. Thus, the effect of supplementation with RXRα on the LXR inhibition of PPRE-Luc activity was evaluated (Fig. 5). By cotransfection of an increasing amount of RXRα, PPARα-activated PPRE-Luc activity was enhanced, indicating that the amount of RXRα is not saturated for PPARα/RXRα activation (Fig. 5A). Figure 5B shows effect of each amount of RXRα on the percent inhibition of PPARα-induced PPRE-Luc values by LXRα activation (LXRα coexpression with or without an LXR ligand, T0901317 or 22RHC). The inhibitory efficiency of LXR activation was attenuated by coexpressed RXRα in a dose-dependent manner (Fig. 5B). Similar results were obtained in the repression by LXRβ (Fig. 5C).

Next, we compared the inhibitory action of LXR on PPAR signaling with other RXR heterodimer partners, TR and farnesoid X receptor (FXR) (Fig. 6A). TRα/H9252 strongly suppressed PPRE-Luc activity, which is consistent with a previous report (37). Inhibition by FXR was modest. The ability of inhibitory action on PPARα-mediated transactivation appears to be different among LXR, TRβ, and FXR, suggesting their constitutive activity based on levels of intracellular natural ligands may influence cross-talk in heterodimeric receptors with RXRα.

cAMP response element binding protein-binding protein (CBP)/p300 is known to be involved in ligand-dependent activation of PPARs (40–42). However, unlike RXR, overexpression of CBP/p300 did not restore on LXR suppression of PPARα-induced PPRE-Luc activity (Fig. 6B).

The RXR restoration of LXR-dependent suppression was also evaluated in gel mobility shift assays. Without LXR, the signal of PPRE shifted by PPARα was enhanced with increasing amounts of RXRα, suggesting that the amount of RXRα is a limiting factor in the
range of PPARα/RXRα ratio used here (Fig. 7A). With the lowest amount of RXRα, addition of LXRs and T0901317 caused a significant decrease in PPARα/RXRα binding to PPRE (Fig. 7, A and B). The inhibitory effect of LXRα activation was abolished with increasing concentrations of RXRα. In the case of LXRβ, RXR restoration of the binding was partial even at the highest amount, reflecting stronger LXRβ inhibitory effect. These data are consistent with the results from cell reporter assays (Fig. 5), providing additional evidence for the hypothesis that LXR inhibits PPARα activation by competing with it for heterodimerization with RXRα.

**LXR Can Bind to PPARα and Inhibits Formation of PPARα/RXRα Heterodimer**

To explore the heterodimerization of the three nuclear receptors, mutual protein-protein interactions were in-
investigated (Fig. 8). First, LXRα protein was labeled with 35S-methionine, incubated with unlabeled RXRα protein, and precipitated with anti-RXRα antibody. Resolution of immunocomplex on SDS-PAGE detected labeled LXRα, indicating formation of an LXRα/RXRα heterodimer. The complex formation was markedly enhanced by addition of T0901317, suggesting that LXR ligand increases affinity for LXRα/RXRα het-

Fig. 8. LXR Inhibits PPARα/RXRα Heterodimerization by the Formation of LXR/RXRα and LXR/PPARα
In vitro translated receptor proteins as indicated were used for protein-protein interaction assay. Indicated proteins were used in the absence or presence of T0901317. Coimmunoprecipitation of the unlabeled RXRα and 35S-labeled LXRα (A) or RXRβ (B) with an anti-RXR antibody; unlabeled PPARα, and 35S-labeled LXRα (C) or RXRβ (D); 35S-labeled PPARα, unlabeled RXRα, and unlabeled LXRα (E) or RXRβ (F). After precipitation with protein G-sepharose, samples were run on SDS-PAGE.
erodimerization (Fig. 8A). We also tested the possibility of formation of PPARα/LXRα heterodimers, as was previously described (39). Labeled LXRα was incubated with PPARα and immunoprecipitated with anti-PPARα antibody. PPARα/LXRα complex was detected with an intensity comparable to LXRα/RXRα, suggesting that LXRα can heterodimerize with PPARα with similar efficiency to RXRα (Fig. 8C). T0901317 enhanced this interaction. LXRβ gave very similar results (Fig. 8, B and D). The gel shift assay (Fig. 3B) showed no retardation of the PPRE probe in the presence of both PPARα and LXRα, indicating that PPARα/LXRα heterodimers do not bind to PPRE. Therefore, LXRα could show inhibition of PPARα signaling by forming not only LXRα/RXRα, but also PPARα/LXRα in a competition with functional PPARα/RXR formation. To show this, effects of LXRs on PPARα/RXRα interaction were estimated in this protein interaction assay. PPARα protein was labeled, incubated with RXRα in the presence or absence of LXRα, precipitated with anti-RXRα antibody, and run on gels. PPARα/RXRα heterodimer formation was inhibited by addition of LXRα or LXRβ with T0901317 (Fig. 8, E and F), suggesting that PPARα/LXRα as well as LXR/RXRα could prevent formation of PPARα/RXRα and participate in repressing PPARα signaling.

**Fig. 9. PPARα Activator Inhibits LXR Ligand-Induced LXR/RXRα Binding to PPRE in Hepatic Nuclear Extracts**

A, Protein and mRNA levels of PPARα, LXRα, and RXRα in hepatic nuclear extracts from fasted or refed mice. For fasting and refeeding treatment, mice were fasted for 24 h and fed a high sucrose/fat-free diet for 12 h. Equal protein amounts (20 μg) in nuclear extracts were subjected to SDS-PAGE, and immunoblotted with an anti-PPARα, anti-LXRα, or anti-RXRα antibody. Total RNA was isolated from the livers of mice from each group, pooled, and subjected to Northern blot analysis with the indicated cDNA probes. B, Confirmation of presence of PPARα in Wy14,643 induced-complexes of nuclear proteins/ACO-PPRE in nuclear extracts from fasted mice. The 32P-labeled ACO-PPRE probe and hepatic nuclear extracts (1 mg) from fasted mice were incubated with Wy14,643 in the presence or absence of anti-PPARα (H-98, sc-9000, Santa Cruz Biotechnology Inc.) or anti-RXRα (D-20, sc-553, Santa Cruz Biotechnology Inc.) antibody. The DNA-protein complexes were resolved in a 4.5% PAGE. C, Wy14,643 induced-PPARα/RXRα binding to PPRE was inhibited by addition of T0901317. 32P-labeled ACO-PPRE probe and hepatic nuclear extracts from fasted mice were incubated with Wy14,643 and/or T0901317. The DNA-protein complexes were resolved in a 4.5% PAGE.

**LXR Activation Inhibits PPARα/RXRα Binding to PPRE in Hepatic Nuclear Extracts**

To examine suppressive action of LXR on PPAR/RXR binding to PPRE in a more physiological condition, gel mobility shift assays were performed using hepatic nuclear extracts from fasted mice. In nuclear extracts from fasted mice, LXRα and RXRα were abundant on immunoblot analysis, although PPARα was decreased after refeeding (Fig. 9A) as well as at mRNA level. Gel shift mobility assays demonstrated that the PPRE probe was shifted by coincubation with hepatic nuclear extracts. The signal of the complexes was stron-
ger from fasted mice than in a refed state, was enhanced by addition of Wy14,643 and declined after addition of anti-PPARα/RXRα or anti-RXRβ antibody, confirming the presence of PPARα/RXRα in the hepatic nuclear extracts from fasted mice and its specific binding to PPRE (Fig. 9B). The PPRE complex also contained a small amount of unidentified RXR heterodimer(s). T0901713 suppressed the signal of the PPARα/RXRα-PPRE complex induced by Wy14,643 (Fig. 9C). Thus, these data indicate that LXR ligand suppressed PPARα/RXRα-target genes, in a competitive manner with PPARα activation, supporting LXR interference with PPARα signaling in primary hepatocytes. To further evaluate the in vivo physiological significance of LXR interference to PPARα-mediated transactivation, similar conditions in mouse livers were recreated. Mice were fasted to induce hepatic endogenous PPARα and its downstream genes (17, 31) and were treated with T0901317 to activate LXR. Various PPARα-target genes in the livers such as mHMG CoA Syn, ACO, CPTI, and cytochrome P450A2 were estimated by Northern blot analysis. Wy14,643 was also administered to fasted mice as a positive control for activation of PPARα target genes. As shown in Fig. 11A, hepatic mRNA levels of these PPARα-regulated genes from T0901317 treated animals were considerably decreased, whereas Wy14643 treatment increased expression of these genes. The activation of LXR by T0901317 was confirmed by observational increases in SREBP-1 and ABCA1 mRNA levels, both of which are well-known LXR target genes (Fig. 11B). These data demonstrate that LXR activation...
by LXR ligand can affect PPARα signaling in mouse livers. In contrast, Wy14,643 alone slightly increased ABCA1 mRNA (Fig. 11B), which is consistent with recently reports of PPAR-LXR-ABCA1 pathway in macrophages (43–45). Moreover, we studied LXR interference in mice treated with both PPARα/H9251 and LXR ligands. As estimated by Northern blot analysis of mHMG-CoA syn and ACO mRNA levels, these PPARα-target genes were suppressed by T0901317 in both Wy14,643-induced and untreated conditions (Fig. 11D). Immunoprecipitation assays of liver nuclear extracts from these mice demonstrated that PPARα/RXRα heterodimers were increased by Wy14,643 treatment and this induction was suppressed by T0901317. These results suggested that LXRα ligand could repress hepatic expression of the PPARα target genes via reduction of PPARα/RXRα formation in liver nucleus (Fig. 11E), extending the hypothesis from transfection studies to in vivo conditions.

**DISCUSSION**

The current studies demonstrate that LXR activation by either overexpression of LXR or its ligand activation causes suppression of PPARα signaling. The Luc, gel shift, and protein-protein binding assays show that LXR activation inhibits formation of PPARα/RXRα, and thus, their binding to PPRE. RXRα supplementation restored this LXR inhibition of PPAR action, indicating that the mechanism could be at least partially explained by RXRα competition between PPAR and LXR. Protein-protein interaction experiments established the formation of PPARα/LXRα and PPARα/LXRβ as well as LXR/RXR heterodimers. Direct binding between PPARα and LXRα has been found in a two-hybrid system, and involvement of LXRα in PPAR signaling has been implicated (39). We further found that PPARα/LXR formation is enhanced by LXR ligand. PPARα/LXR complex cannot bind to either PPRE or LXRE. Therefore, this heterodimer can interfere with
both PPAR and LXR action and can contribute to PPAR inhibition of the LXR-SREBP-1c system as proposed in the accompanying paper (38) as well as LXR inhibition of PPAR signaling as described in the current study. Consistently, the combined treatment with both PPARα and LXR agonists resulted in simultaneous reduction of both PPARα/RXRα and LXR/RXRα [Fig. 11E in this paper and Fig. 9B in the accompanying paper (38)], implicating an important contribution of PPARα/LXR formation in reciprocal inhibition of LXR and PPARα signaling. This can also explain the restoration by supplementation with RXR, not only by providing sufficient RXR for functional RXR heterodimers, but also by preventing nonfunctional PPARα/LXR formation.

LXR inhibition of PPARα signaling observed from transfection studies with cultured cells is likely to be extendable to the in vivo regulation of the hepatic energy metabolism. It is noteworthy that administration of LXR agonist reduced hepatic nuclear PPARα/RXR and impaired expression of hepatic fatty acid degradation enzyme genes both in rat primary hepatocytes and livers of fasted mice (Figs. 10 and 11). In a fasted state, hepatic PPARα expression is highly induced and fatty acids are recruited as ligands for PPARα activation (17, 18). Nutritional condition does not markedly change the expression of hepatic LXRα (29). Therefore, LXR ligand can activate hepatic LXRα and cause cross-talk with PPARα/RXR leading to suppression of expression of PPARα target genes involved in lipid degradation. These data demonstrate that LXR interference with PPAR signaling can occur in vivo. In the accompanying paper (38), we observed a mirror image observation, that PPARα activation inhibits ligand-induced LXR signaling in hepatocytes.

The triangle relationship among LXR, PPAR, and RXR could be crucial for mutual regulation of both LXR and PPAR activities, and thus nutritional regulation of their downstream genes. PPARα is involved in fatty acid degradation as an adaptive control of energy depletion. Meanwhile, SREBP-1c, whose expression is dominated by LXR is involved in fatty acid synthesis for storage of excess energy. These opposite nutritional regulators are reciprocally up- and down-regulated depending upon energy states. As summarized in Fig. 12, our findings suggest that the mutual suppression efficiently facilitates these reciprocal actions of PPARα and LXR-SREBP-1c systems. These studies should open up a new paradigm of a novel cross-talk of nutritional transcription factors in energy metabolism where the nuclear concentrations of each receptor and ligand are crucial for nutritional regulation for fatty acid metabolism. It is also important to investigate whether the cross-talk of these receptors involves a potential competition for their coactivators such as CBP/p300 (42, 46, 47), steroid receptor coactivator-1 (48–50), and PPARγ coactivator-1 (51, 52). Further studies should focus on the precise mechanisms for this network.

MATERIALS AND METHODS

Materials

Anti-PPARα sc-9000, RXRα D20 sc-553, and LXRα C-19 sc-1201 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), Redivue [α-32P]deoxy-CTP (6,000 Ci/mmol) and Protein G Sepharose from Amersham Biosciences Inc. (Uppsala, Sweden), in vitro transcription and translation kit (TNT Coupled Reticulocyte Lysate System) from Promega (Madison, WI), and other chemicals from Sigma (St. Louis, MO). T0901317 (N-methyl-N-[4-(2, 2, 2-trifluoro-1-hydroxy-1-trifluoromethyl)phenyl]-benzenesulfonylamide) was provided by Kyorin Pharmaceutical Co. Ltd. (Tochigi, Japan).

Plasmids

Cytomegalovirus (CMV) promoter expression plasmid of human RXRα and the Luc reporter gene construct, PPRE from ACO gene promoter fused upstream from thymidine kinase (TK) promoter (pPPRE-Luc), and pCMV-RXR were kind gifts from Dr. D. J. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). pCMV-FXR, pCMX-TRβ, and pCMV-SP-β were from Dr. H. Fuji (Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo, Japan), Dr. R. M. Evans (The Salk Institute for Biological Studies, La Jolla, CA), and Dr. T. Nakajima (St. Marianna University School of Medicine, Kawasaki, Japan), respectively. The LXRE of SREBP-1c promoter-Luc construct (pLXRE-Luc), pCMV-mLXRα and pCMV-mLXRβ were prepared as previously described (29), CMV or T7 promoter expression plasmid of mouse (m) PPARα (1–468, amino acid) (16) was prepared by PCR, and the cDNA was introduced into pCMV7 or pBluescript II SK vector, respectively. T7 promoter expression plasmids of mLXRα and mLXRβ were prepared using pBluescript II SK vector.
Transfections and Luc Assays

HEK293 were grown at 37 C in an atmosphere of 5% CO2 in DMEM containing 25 mM glucose, 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate supplemented with 10% fetal bovine serum (FBS). Transfection studies were carried out with cells plated on 12-well plates as previously described (29). The indicated amount of each expression plasmid was transfected simultaneously with a Luc reporter plasmid (0.25 µg) and simian virus 40-β-galactosidase (β-gal) plasmid (0.2 µg) or (0.05 µg). The total amount of DNA in each transfection was adjusted to 1.5 µg/well with the vector DNA, pCMV7. 22RiHC and T0901713 were dissolved in ethanol. Each agent was treated to the cells immediately after transfection in DMEM with 10% FBS, and incubated for 24 h. After incubation, the amount of Luc activity in transfectants was measured and normalized to the amount of β-gal activity.

Gel Mobility Shift Assays

Gel mobility shift assays were performed as previously described (53). Briefly, mLXRalpha, mLXRbeta, mPPARalpha, and human (h) RXRalpha proteins were generated from the expression vectors using a coupled in vitro transcription/translation system (Promega). Double-stranded oligonucleotides used in gel mobility shift assays were the LXRalpha of the SREBP-1 promoter (29) and PPRE of the ACO promoter (16). These were used to generate proteins in vitro using a coupled transcription/translation system. Blot hybridization was performed with the cDNA probes labeled with [α-32P]CTP (6000 Ci/mmol) using the Megaprime DNA Labelling System (Amersham Biosciences Inc.). The cDNA probes for SREBP-1, fatty acid synthase, ACO, cytochrome P450A2, PPARalpha, LXRalpha and beta, ABCA1 and acidic ribosomal phosphoprotein PO (36B4) were prepared as previously described (29, 31). The cDNA probes for L-FABP, CPT1, and mHMG-CoA Syn were provided by Kyorin Pharmaceutical Co. LTD. Each signal was analyzed with BAS2000 and BASStation software (Fuji Photo Film). This study was supported by the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research.

Acknowledgments

We are grateful to A. H. Hasty for critical reading of the manuscript. We also thank T. Kitamine, H. Okazaki, Y. Tamura, and M. Sekiya for helpful discussion.

Received May 23, 2002. Accepted April 14, 2003.

Address all correspondence and requests for reprints to: Hitoshi Shimano, M.D., Ph.D., Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan. E-mail: shimano-ky@umin.ac.jp.

This study was supported by the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research.

T.I. and H.S. equally contributed to this work.

REFERENCES

2. Schoonjans K, Staels B, Auwerx J 1996 The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. Biochim Biophys Acta 1302:93–109

Hepatocyte Isolation and Culture

Primary hepatocytes were isolated from male Sprague-Dawley rats (160–180 g, Japan Clea, Tokyo, Japan) using the collagenase perfusion method as described previously (54). The viability of isolated cells was over 90% as determined by the trypan blue. Cells were resuspended in DMEM containing 100 U/ml penicillin and 100 µg/ml streptomycin sulfate supplemented with 5% FBS, seeded on collagen-coated dishes 100 mm at a final density of 4 x 10^6 cells/cm^2. After an attachment for 4 h, cells were cultured with medium containing the indicated agonists for 24 h.

Animals

Male mice (C57BL/6J were obtained from Charles River Japan (Yokohama, Japan). All mice were given a standard diet and tap water ad libitum. All institutional guidelines for animal care and use were applied in this study. Vehicle (0.5% carboxymethyl-cellulose) T0901317 (50 mg/kg), Wy14,643 (50 mg/kg), or both their agonists was orally administered to the mice before 18 h fasting. For fasting and refeeding treatment, mice were fasted for 24 h and fed a high sucrose/fat free diet for 12 h as described (55). Hepatic nuclear protein was prepared from the livers as previously described (56), were subjected to immunoblotting with the anti-PPARalpha, anti-LXRalpha, or anti RXRalpha antibodies.


