Coadministration of a Liver X Receptor Agonist and a Peroxisome Proliferator Activator Receptor-\(\alpha\) Agonist in Mice: Effects of Nuclear Receptor Interplay on High-Density Lipoprotein and Triglyceride Metabolism in Vivo


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ABSTRACT

Liver X receptors (LXRs) are master transcription factors regulating cholesterol and fatty acid metabolism. Treatment of C57B6 mice with a specific synthetic LXR agonist, \(N\)-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1(trifluoromethyl)]ethyl][phenyl]-benzenesulfonamide (T0901317), resulted in elevated high-density lipoprotein (HDL) cholesterol as well as plasma and liver triglycerides. Peroxisome proliferator-activated receptor-\(\alpha\) (PPAR\(\alpha\)) agonists are known to induce peroxisomal fatty acid \(\beta\)-oxidation and also mediate HDL cholesterol metabolism. We have explored the hypothesis that simultaneous activation of PPAR\(\alpha\) and LXR may lead to additive effects on HDL cholesterol elevation as well as attenuation of triglyceride accumulation. Coadministration of T0901317 and the specific PPAR\(\alpha\) agonist [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (Wy14643)] in mice led to synergistic elevation of HDL cholesterol that was primarily associated with enlarged HDL particles enriched with apoE and apoAI. Liver phospholipid transfer protein (PLTP) mRNA and plasma PLTP activity were additively elevated, suggesting a role of PLTP in the observed HDL cholesterol elevation. Moderate increases in plasma triglyceride levels induced by LXR activation was reduced, whereas the accumulation of triglyceride in the liver was not altered upon coadministration of the PPAR\(\alpha\) agonist. Peroxosomal fatty acid \(\beta\)-oxidation in the liver was dramatically elevated upon PPAR\(\alpha\) activation as expected. Interestingly, activation of LXRs via T0901317 also led to a significant increase in peroxisomal fatty acid \(\beta\)-oxidation. Sterol regulatory element binding protein 1c expression was dramatically upregulated by the LXR agonist but was not changed with PPAR\(\alpha\) agonist treatment. Liver lipoprotein lipase expression was additively increased upon LXR agonist and PPAR\(\alpha\) agonist coadministration. Our studies mark the first exploration of nuclear receptor interplay on lipid homeostasis in vivo.

Plasma high-density lipoproteins (HDLs) are inversely correlated with coronary artery events and play a major role in mediating reverse cholesterol transport and anti-inflammatory actions. It is thus desirable to modulate HDL cholesterol level pharmacologically to achieve cardiovascular benefits. Recent studies have suggested that one potential way to elevate HDL cholesterol is through nuclear receptor modulation.

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Nuclear receptors are a distinct class of transcription factors that control a multitude of cellular processes. A pair of nuclear receptors named liver X receptors (LXR\(\alpha\) and LXR\(\beta\)) are instrumental in regulating reverse cholesterol transport (Repa and Mangelsdorf, 2002). LXR\(\alpha\)s and LXR\(\beta\)s were originally isolated as orphan nuclear receptors with distinct tissue distributions (Lu et al., 2001). Certain oxysterols were subsequently identified as LXR native ligands, suggesting their role in cholesterol homeostasis (Janowski et al., 1996, 1999; Fu et al., 2001). The spectrum of target genes identified to date indicates that these receptors are master transcription factors mediating cholesterol catabolism. First, LXRs

ABBREVIATIONS: HDL, high-density lipoprotein; LXR, liver X receptor; ABCA1, ATP-binding cassette transporter protein A1; PLTP, phospholipid transfer protein; SREBP, sterol regulatory element binding protein; PPAR, peroxisome proliferator activator receptor; ANOVA, analysis of variance; FPLC, fast protein liquid chromatography; LPL, lipoprotein lipase; T0901317, \(N\)-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1(trifluoromethyl)]ethyl][phenyl]-benzenesulfonamide; Wy14643, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid.
regulate ABCA1 (Costet et al., 2000; Repa et al., 2000b) that is both essential and rate limiting in mediating peripheral cholesterol efflux. LXRs also regulate several apolipoproteins that can mediate cholesterol efflux (Laffitte et al., 2001; Mak et al., 2002b). Second, LXRs regulate HDL-modifying enzymes, including cholesterol ester transfer protein and PLTP (Luo and Tall, 2000; Cao et al., 2002; Mak et al., 2002a; Laffitte et al., 2003b). Third, LXRs control cholesterol excretion into the bile in the liver and intestinal cholesterol absorption through regulating ABCG5 and ABCG8 (Repa et al., 2002; Yu et al., 2003). In addition, LXRs also play critical roles in mediating glucose homeostasis (Cao et al., 2003; Laffitte et al., 2003a). Thus, LXRs have emerged as prime targets through which to modulate cholesterol catabolism for metabolic diseases and atherosclerosis.

The pursuit of LXRs as therapeutic targets, however, has been hampered by the fact that LXRs regulate hepatic fatty acid biosynthesis pathway via the transcription factor SREBP1c (Repa et al., 2000a; Schultz et al., 2000). Studies performed in mice with specific synthetic LXR agonists produced marked liver triglyceride accumulation and hypertriglyceridemia (Greathorst et al., 2002). Accumulation of triglycerides in the liver significantly compromises liver function and thus is highly undesirable. Hypertriglyceridemia is associated with metabolic diseases and also regarded as an independent risk factor for cardiovascular disease (Ginsberg, 2002). These observations thus present major hurdles in developing therapies through LXR modulation.

Peroxisome proliferator-activated receptor-α (PPARα) belongs to a family of nuclear receptors that also plays critical roles in multiple physiopathological conditions. The fibrate class of drugs that are effectively clinically in reducing plasma triglyceride levels exert efficacy through PPARα activation (Staels et al., 1998). In rats, diet-induced liver triglyceride accumulation is effectively dissipated through the addition of a synthetic PPARα ligand (Foxworthy and Eacho, 1991). PPARα up-regulates apoAV (Vu-Dac et al., 2003) and down-regulates apoCIII (Staels et al., 1995), and the combined effects lead to plasma triglyceride reduction. PPARα also mediates liver fatty acid β-oxidation to dissipate fat accumulation (Staels et al., 1998). Fibrates also modestly elevate HDL cholesterol in humans (Staels et al., 1998). It has been reported that PPARα agonists can up-regulate human apoAI, which may be responsible for the observed HDL effect in humans (Berthou et al., 1996). Treatment of human apoAI transgenic mice with a PPARα agonist fenofibrate resulted in a dramatic HDL cholesterol increase (Berthou et al., 1996). PPARα also regulates PLTP, which is essential for HDL particle size increase (Bouly et al., 2001).

Our hypothesis was that liver triglyceride accumulation and hypertriglyceridemia mediated by LXR activation could be prevented through activation of PPARα via dissipation of triglycerides in the liver and plasma. We have also noted recent reports of cross talk between LXRs and PPARα that could impact HDL metabolism (Tobin et al., 2000; Chinetti et al., 2001). In this report, we have investigated the effects of simultaneous activation of both LXRs and PPARα through coadministration of a specific LXR agonist, T0901317, and the specific PPARα agonist Wy14643 or fenofibrate on both HDL cholesterol metabolism and triglyceride metabolism.

**Materials and Methods**

**Animals.** Eight-week-old C57BL6 mice (six per group) were purchased from Harlan (Indianapolis, IN) and acclimated for 1 week before the experiments. The mice were provided Purina 5001 food ad libitum throughout the experiments. Wy14643 (10 mg/kg) and fenofibrate (10, 75, and 300 mg/kg) were purchased from Calbiochem (San Diego, CA) and Sigma-Aldrich (St. Louis, MO) respectively. T0901317 (50 mg/kg) was available from Cayman Chemical (Ann Arbor, MI). Compounds were formulated in wet granulation vehicle [212.5 mg of povidone, 3.77 g of lactose anhydrous (granular), and 64.8 μl of polysorbate 80 (Tween 80) in 250 ml of water]. Animals
were treated through oral gavage with either vehicle or various doses of compounds daily for 7 days and sacrificed by CO₂ euthanasia 2 h after the last dose. Plasma and liver tissue were prepared for various analyses. Use of mice was approved by the Institutional Animal Care and Use Committees of the American Association for Accreditation of Laboratory Animal Care-accredited institutions and Lilly Research Laboratories in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. One-way ANOVA was used for statistical analysis.

**Plasma Lipid Analysis.** Plasma total cholesterol and triglyceride were measured using a Monarch Plus clinical chemistry analyzer. Lipoproteins were separated with fast protein liquid chromatography (FPLC), and cholesterol was quantified with an in-line detection system based on that described by Kieft et al. (1991). Briefly, 50 μl of pooled sample was applied to a Superox 6 HR 10/30 size exclusion column (Amersham Biosciences Inc., Piscataway, NJ) and eluted with phosphate-buffered saline, pH 7.4 (70011-044, dilution 1:10; Invitrogen, Carlsbad, CA), containing 5 mM EDTA, at 0.5 ml/min. Cholesterol reagent (Chol/HP 704036; Roche Diagnostics, Indianapolis, IN) at 0.16 ml/min was mixed with the column effluent. The colored product produced in the presence of cholesterol was monitored in the flow stream at 505 nm, and the analog voltage from the monitor was converted to a digital signal for collection and analysis.

**Western Blot Analysis.** Designated FPLC fraction samples (10 μl each) were separated on Tris-glycine gels (Novex, San Diego, CA) under denaturing conditions. Protein was transferred to nitrocellulose membranes and then blotted with antibodies specific for apolipoproteins A1 or E (Biodesign International, Kennebunk, ME) or apolipoprotein B48/100 (US Biological, Swampscott, MA). Blots were developed with enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences Inc.) and documented using X-OMAT film (Eastman Kodak, Rochester, NY).

**mRNA Measurement.** All mRNAs were measured by RNase protection assay (RPA). Total RNAs were prepared from frozen tissue samples using TRizol reagent (Invitrogen). Mouse PLTP and ABCA1 probes were described previously (Cao et al., 2002). The primer sets to amplify probes of mouse SREBP1c and lipoprotein lipase (LPL) were as follows: 5’-ATC GGC GCG GAA GCT GTC GGG GTA GCG TC-3’, 5’-ACT GTC TTG GTT GAT GAG CTT GAG CAT-3’ and 5’-GGA AGC CTT TGA GAA AGG-3’, 5’-GGT TGT GTT GCT TGC CAT TC-3’, respectively. The resulting polymerase chain reaction fragments were cloned into pGEM-T Easy (Promega, Madison, WI) and sequenced. The resulting construct was linearized, and RNA probe was synthesized using Promega’s T7/SP6 transcription kit. Specific activity was >10⁸ dpm/μg. After column purification, the probe was used for RPA analysis by using a kit from Ambion (Austin, TX). A probe for 28S RNA was used to normalize the RNA samples used in the experiments. The signal was quantified with an Amersham Biosciences PhosphorImager model 51.

**Determination of Liver Triglycerides.** Liver samples were frozen in liquid nitrogen and homogenized by Polytron on ice. Triglycerides in liver homogenates were determined enzymatically using a commercially available enzymatic kit (Wako Pure Chemicals, Tokyo, Japan) according to the manufacturer’s recommendations.

**Peroxisomal β-Oxidation Measurement.** Animals were sacrificed by carbon dioxide asphyxiation, and a portion of liver was homogenized in 9 volumes of cold 0.25 M sucrose. Samples were centrifuged at 600g for 10 min, and the supernatants were decanted. Triton X-100 was added to an aliquot of the supernatant (final concentration 1%). The samples were then assayed for peroxisomal β-oxidation in the presence of KCN, which inhibits mitochondrial β-oxidation. The oxidation of palmitoyl CoA was quantified by spectrophotometrically measuring the reduction of NAD⁺ at 340 nm. The rate of NAD⁺ reduction is directly related to the rate of fatty acid oxidation (Lazarow, 1981).

**PLTP Activity Measurement.** PLTP activity was measured with an assay kit according to the manufacturer’s instructions. (Cardiovascular Target Inc., New York, NY).

**Results**

Previously, it was shown that treatment of C57B6 mice with a specific synthetic LXR agonist, T0901317, at 50 mg/kg increased HDL cholesterol and particle size (Schultz et al., 2000; Cao et al., 2002). It was also known that treatment of mice with a specific PPARα agonist, Wy14643, would induce peroxisomal fatty acid β-oxidation and reduce plasma triglyceride levels. We treated C57B6 mice orally with the vehicle, 10 mg/kg Wy14643, and 50 mg/kg T0901317 alone or in combination daily for 7 days. Plasma samples from different groups were pooled and subjected to lipoprotein analysis by FPLC. Similar to what we reported previously, T0901317 treatment resulted in a significant increase in plasma HDL cholesterol and particle size. Wy14643 led to some moderate increase in HDL particle size. Combined treatment, however, resulted in a synergistic elevation of HDL cholesterol that largely resided in the enlarged HDL portion (Fig. 1A). These were confirmed through Western blot analysis to show that from fractions 28 to 36, where enlarged HDL was located,
there was a dramatic increase in apoE and apoAI content, whereas apoB level was not significantly changed (Fig. 1B). To further confirm our findings, we carried out dose-response studies with another PPARα ligand, fenofibrate, in combination with T0901317 in C57B6 mice (Fig. 2). We observed an increase in HDL size with increased doses of fenofibrate treatment. Compared with T0901317 alone, combined treatment of T0901317 with various doses of fenofibrate resulted in dose-dependent increases in HDL cholesterol levels without change in HDL particle size. Thus, simultaneous activation of LXRs and PPARα in mice led to synergistic elevation of HDL cholesterol that is largely residing in enlarged apoE- and apoAI-enriched HDL particles.

To explore the molecular mechanisms responsible for the effects observed on HDL, we examined the expression of two important target genes regulated by PPARα and LXRs—PLTP and ABCA1 in the liver that are intimately involved in HDL cholesterol metabolism. Wy14643 treatment resulted in a moderate increase in ABCA1 (43%), whereas T0901317 treatment led to a 270% increase in ABCA1 mRNA levels. The combined treatment, however, did not result in further elevation of ABCA1 mRNA (Fig. 3A). PLTP mRNA was significantly elevated upon activation of either PPARα or LXRs. The combination of two ligands in mice seemed to lead to a synergistic effect on PLTP mRNA levels (Fig. 3B). Consistent with liver mRNA data, plasma PLTP activity was increased 2.0- and 3.4-fold with Wy14643 and T0901317 treatment, respectively. The combined treatment elevated PLTP activity further to 4.4-fold ($p < 0.001$) (Fig. 3C). Thus, the synergistic elevation of HDL cholesterol was closely associated with the increases in hepatic PLTP mRNA and serum PLTP activity.

Previously, Schultz et al. (2000) reported the induction of plasma triglycerides upon LXR activation. Joseph et al. (2002) reported the transient elevation of plasma triglycerides after 3 days of dosing with T0901317 treatment at 50 mg/kg and minimal plasma triglyceride increases after 7 days of dosing. We have observed a moderate plasma triglyceride elevation after daily doses of T0901317 for 7 days (about 60% at 50 mg/kg versus vehicle, $p = 0.01$ by $t$ test and about 25% at 100 mg/kg, not significant) (Fig. 4A). Liver triglyceride accumulation, however, was more pronounced at the higher doses used (Fig. 4B). Treatment of mice with Wy14643 at 10 mg/kg for seven days trended toward a slight reduction of plasma triglycerides relative to vehicle treated animals ($p = 0.09$). Wy14643 coadministration with LXR agonist significantly reduced plasma triglycerides compared with T0901317 alone-treated animals (Fig. 5A, $p = 0.002$ of T0901317 + Wy14643 versus T0901317). Surprisingly, Wy14643 had no effect on liver triglyceride at basal level compared with vehicle control and seemed to only minimally reduce liver triglyceride accumulation induced by T0901317 treatment (Fig. 5B, no significance of T0901317 + Wy14643 versus T0901317). Similar results were obtained when fenofibrate was used in comparable studies (data not shown).

To investigate the molecular mechanisms of the above-mentioned observations, we first examined SREBP1c regulation. SREBP1c is the master transcription factor that controls the entire fatty acid biosynthetic pathway. T0901317 significantly up-regulated SREBP1c, which was consistent with the previous report (Schultz et al., 2000). Treatment of mice with Wy14643 did not significantly alter SREBP1c expression either at basal level or in the presence of LXR agonist (Fig. 6A). We then examined the peroxisome fatty acid β-oxidation in these mice. As expected, Wy14643 treatment resulted in a 6.8-fold induction of peroxisome fatty acid...
β-oxidation. Interestingly, T0901317 also resulted in a significant increase in β-oxidation (2.7-fold). The combined treatment of two agonists did not lead to any significant change in β-oxidation compared with Wy14643 treatment alone (Fig. 6B).

We then examined the expression of hepatic lipoprotein lipase, another critical gene involved in triglyceride metabolism. Adult liver expresses virtually no LPL. Wy14643 treatment induced liver LPL almost 4-fold. T0901317 treatment led to a nearly 5-fold elevation of LPL mRNA level, possibly through direct and indirect LXR regulation of LPL. Combined treatment resulted in an additive 10-fold increase in LPL mRNA levels (Fig. 6C). Because LPL plays a critical role in hydrolyzing triglyceride-rich lipoproteins, the dramatic regulation of LPL in the liver of mice treated with PPARα and/or LXR ligands may play a significant role in mediating plasma and liver triglyceride metabolism that is induced via PPARα and/or LXR activation.

Discussion

Nuclear receptors play prominent roles in regulating lipid homeostasis. Fibrates as a drug class effectively reduce plasma triglyceride levels and moderately elevate HDL cholesterol. These effects have been largely attributed to their activation of PPARα (Staels et al., 1998). LXRs on the other hand, are novel receptors that have been regarded as the master transcription factors mediating cholesterol catabolism (Repa and Mangelsdorf, 2002). In this report, we have investigated the effects of nuclear receptor interplay on lipid homeostasis in vivo. We have shown that simultaneous activation of PPARα and LXRs led to significant accumulation of enlarged HDL cholesterol that is enriched in apoE and apoAI. The enlarged HDL cholesterol is closely associated with liver PLTP mRNA induction and the elevation of plasma PLTP activity. Coadministration of PPARα and LXR agonists also reduced plasma triglycerides and produced vir-
Fig. 5. Effects on plasma and liver triglycerides with combined treatment of T0901317 and Wy14643 in C57B6 mice. C57B6 mice were treated as described in Fig. 1A, and plasma and liver triglycerides were measured as described under Materials and Methods. A, mild hypertriglyceridemia induced by LXR agonist was attenuated by a PPARα agonist. Plasma triglyceride measurement indicated attenuation of plasma triglyceride induced by T0901317 with Wy14643. D, minimal effect of a PPARα agonist on liver triglyceride accumulation induced by LXR activation. No significant liver triglyceride change was noted comparing the T0901317 group and the group with combined treatment. *, $p < 0.001$ against vehicle.

It has been shown that PPARα activation leads to increased apoAI transcription through a PPARα responsive element in human apoAI promoter (Berthou et al., 1996), thus increasing human plasma apoAI and HDL cholesterol level. In mice, however, ApoAI is reciprocally regulated (Berthou et al., 1996). Despite the down-regulation of apoAI in mice, PPARα agonists seem to slightly increase HDL cholesterol and particle size. These effects may be largely due to PPARα up-regulation of liver PLTP mRNA and plasma PLTP activity (Bouly et al., 2001), whereas down-regulation of SR-BI, the HDL receptor, slows the HDL particle catabolism (Mardones et al., 2003). The PLTP regulation by PPARα results in HDL particle size increases (Bouly et al., 2001).

LXR activation leads to increased HDL cholesterol as well as HDL particle size (Cao et al., 2002). The observed effects of LXR agonists on HDL metabolism have been attributed to primarily ABCA1 activation and liver PLTP regulation (Schultz et al., 2000; Cao et al., 2002). Previously, it was shown that in rats activation of PPARα led to increased LXRα expression in the liver (Tobin et al., 2000). It was also reported that in macrophage cells PPARα regulates LXRα and thus indirectly ABCA1 expression (Chinetti et al., 2001).

In our studies, we have observed synergistic elevation of HDL cholesterol upon simultaneous activation of both receptors. The cholesterol largely resides in enlarged HDL particles as analyzed by FPLC and Western blot analysis. We did not observe additive regulation of liver ABCA1 mRNA, rather PLTP mRNA was up-regulated in an additive manner. In addition, plasma PLTP activity was further augmented with both PPAR and LXR activation with their cognate agonists. Because PLTP plays critical roles in HDL biogenesis and remodeling, and its expression level is closely associated with the HDL cholesterol increase, we believe induction of PLTP mRNA in the liver and PLTP activity in the plasma were largely responsible for the observed HDL effect upon activation of both receptors.

LXR agonists regulate SREBP1c that controls the entire fatty acid biosynthetic pathway (Shimomura et al., 1998; Repa et al., 2000a; Schultz et al., 2000). Activation of LXRs thus leads to increased fatty acid synthesis and very low-density lipoprotein secretion (Greffhorst et al., 2002). Schultz et al. (2000) suggested a significant increase in plasma triglycerides in mice and hamsters with LXR activation. Joseph et al. (2002) reported that the induction of plasma triglycerides in mice was transient by LXR agonists. This transient induction of plasma triglycerides could be a result of increased lipid uptake because in apoE-deficient mice or LDL receptor-deficient mice with human apoB transgene expression, dramatically elevated plasma triglycerides persisted after 1 week oral treatment of T0901317 (our unpublished data). We observed moderate plasma triglyceride induction in mice upon LXR agonist T0901317 treatment for 7 days (Fig. 4A). We have also observed the reduction of plasma triglycerides with LXR activation upon PPARα agonist treatment in these mice (Fig. 4A). The exact molecular mechanism for these observations needs to be further defined. PPARα activation has been shown to reduce apoCIII levels in humans, thus increasing LPL activity and triglyceride hydrolysis (Staels et al., 1995). It was also reported recently that activation of PPARα increased the expression of apoAV, the newly identified secreted protein that plays a critical role in determining plasma triglyceride levels in humans (Vu-Dac et al., 2003). We observed no change in apoCIII mRNA in the liver when 10 mg/kg Wy14643 was administered to mice and paradoxical down-regulation of apoAV mRNA in mice by both LXR and PPAR activation (our unpublished data). The difference of apoAV regulation by PPARα in mice compared with that in humans is reminiscent of reciprocal apoAI reg-
Fig. 6. Additive induction of liver LPL mRNA with combined treatment of Wy14643 and T0901317. A, SREBP1c mRNA was measured by RPA as described under Materials and Methods. Significant induction was noted upon LXR activation, and no attenuation was observed with combined treatment. B, peroxisome fatty acid β-oxidation measurement. Animals were treated as described in Fig. 1. Individual liver samples were subjected to peroxisome fatty acid β-oxidation measurement as described under Materials and Methods. Values represent mean ± S.E.M. of six mice. a, statistically different from vehicle control, p < 0.001. b, statistically different from Tularik alone, p < 0.001. c, not statistically different from Wy14643 alone by one-way ANOVA. Note the significant induction of β-oxidation induced by T0901317 (p < 0.001 by one-way ANOVA). C, LPL mRNA was regulated by both Wy14643 and T0901317, and additive regulation in LPL mRNA was observed upon coadministration of Wy14643 and T0901317.