Phospholipid Transfer Protein Is Regulated by Liver X Receptors in Vivo*

Received for publication, July 18, 2002, and in revised form, August 8, 2002
Published, JBC Papers in Press, August 9, 2002, DOI 10.1074/jbc.M207187200

Guoqing Caossil1, Thomas P. Beyer*, Xiao Ping Yang*, Robert J. Schmidt†, Yoyuan Zhang†, William R. Bensch‡, Raymond F. Kauffman‡, Hong Gao‡, Timothy P. Ryan‡, Yu Liang‡, Patrick I. Eacho‡, and Xian-Cheng Jiang§

From the §Lilly Research Laboratories, Eli Lilly & Company, Indianapolis, Indiana 46285 and the ¶Department of Anatomy and Cell Biology, State University of New York (SUNY) Downstate Medical Center, Brooklyn, New York 11203

Liver X receptors (LXR) belong to the nuclear receptor superfamily that can regulate important lipid metabolic pathways. The plasma phospholipid transfer protein (PLTP) is known to mediate transfer of phospholipids from triglyceride-rich lipoproteins to high density lipoprotein (HDL) and plays a critical role in HDL metabolism. We report here that a specific LXR agonist, T0901317, elevated HDL cholesterol and phospholipid in C57/Bl6 mice and generated enlarged HDL particles that were enriched in cholesterol, ApoAI, ApoE, and phospholipid. The appearance of these HDL particles upon oral dosing of T0901317 in C57/Bl6 mice was closely correlated with the increased plasma PLTP activity and liver PLTP mRNA levels. Nuclear run-on assay indicated that the effect of LXR agonist on PLTP expression was at the transcriptional level. In mouse peritoneal macrophage cells, PLTP expression was also up-regulated by the LXR/RXR (retinoid X receptor) heterodimer. However, cholesterol efflux in mouse peritoneal macrophage cells from PLTP-deficient mice (PTLP0) was not significantly different from wild type animals. Although in PLTP-deficient mice, the induction of HDL cholesterol as well as HDL particle size increase persisted, the extent of the induction was greatly attenuated. We conclude that PLTP is a direct target gene of LXRs in vivo and plays an important role in LXR agonist-mediated HDL cholesterol and size increase in mice.

Epidemiological studies have revealed that plasma HDL cholesterol is inversely correlated to coronary artery disease in humans. Several hypotheses have been proposed to explain the benefits of HDL. Among these, reverse cholesterol transport concept has been widely accepted. This notion, proposed more than 30 years ago by Glomset (1), is defined as the process through which nascent HDL particles remove excessive free cholesterol from peripheral tissues and carry it back to the liver for catabolism. The studies on cellular cholesterol efflux pathway were highlighted by the recent breakthrough defining the genetic defects associated with Tangier disease and hypolipidoproteinemia (2–5). The mutations of ATP-binding cassette transport protein 1 (ABCA1) were identified as the underlining cause of the rare genetic disorder that leads to almost total absence of plasma ApoAI and HDL cholesterol and to massive accumulation of cholesterol esters in macrophage cells.

Plasma phospholipid transfer protein (PLTP) activity is also closely related to HDL levels. PLTP transfers phospholipids from triglyceride-rich lipoproteins to HDL during lipolysis. Moreover, it also participates in the phospholipid exchanges between HDL particles (21). Disruption of PLTP in mice dramatically reduces plasma HDL cholesterol and phospholipid levels (6). Although its role in the circulation has been studied extensively, its potential function in the reverse cholesterol transport pathway and HDL biogenesis awaits further elucidation.

Liver X receptors (LXRs) belong to the orphan nuclear receptor superfamily and exist in two isoforms, LXRα and LXRβ. LXRs are major transcription factors controlling cholesterol catabolism (13, 15, 16). Administration of LXR agonist increased HDL cholesterol in mice (13). ABCA1, as a target gene of LXR (16, 17), is considered to play an important role in this process. However, ABCA1 expression is thought to be closely related to the generation of nascent HDL through both phospholipid and cholesterol efflux to free apoAI (18, 19). In mice HDL maturation or remodeling is dependent on at least two other proteins, PLTP and LCAT (20). Here we report that a specific LXR agonist, T0901317, regulates transcriptionally PLTP in vivo. Our studies indicate, for the first time, that PLTP is a direct target gene of LXRs and plays an important role in LXR agonist-mediated HDL cholesterol and size increase in mice.

* This study was supported in part by National Institutes of Health Grant HL 69817 (to X.-C. J.). 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.
‡ To whom correspondence should be addressed: Cardiovascular Research, Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, IN 46285. Tel.: 317-433-3535; Fax: 317-276-1417; E-mail: Guoqing_Cao@lilly.com.
§ The abbreviations used are: HDL, high density lipoprotein; LDL, low density lipoprotein; ABC, ATP-binding cassette transporter; CETP, cholesteryl ester transport protein; ApoE, apolipoprotein E; LXR, liver X receptor; RXR, retinoid X receptor; PLTP, phospholipid transfer protein; PPARα, peroxisome proliferator-activated receptor α; DMEM, Dulbecco’s modified Eagle’s medium; FPLC, fast protein liquid chromatography.

MATERIALS AND METHODS

Animals—Eight-week-old C57/Bl6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and acclimated for 1 week before the experiments. The mice were provided Purina 5001 food ad libitum throughout the experiments. PLTP0 mice (6) were maintained in a similar fashion as wild type C57/Bl6 mice. Genotyping and PLTP activity assay were both used to ensure the genotype of the knock-out mice. The compound was dosed orally. Mice were sacrificed at the end of the studies with CO2 euthanasia. Plasma samples were prepared for lipid analysis, and tissues were collected for RNA study.

Plasma Lipid Analysis—Total cholesterol and triglyceride levels

This paper is available on line at http://www.jbc.org

39561
C57/BL6 mice were treated orally with the prepared from each animal (6/group) and pooled for FPLC analysis as LXR agonist T0901317 once daily for 7 days. Plasma samples were concentration was plotted versus voltage from the monitor was converted to a digital signal for collection cholesterol was monitored in the flow stream at 505 nm, and the analog

were measured utilizing a Monarch Plus clinical chemistry analyzer. Plasma phospholipid was measured as described (6). Lipoproteins were separated with FPLC and cholesterol quantitated with an in-line de-

-glycollate medium (BD Pharmingen) into C57BL/6 or PLTP0 mice. At
cells were finally lysed in 0.5 ml of 0.1M sodium hydroxide, 0.1% SDS,
of supernatant was determined by liquid scintillation counting. The
cells were washed with phosphate-buffered saline, equilibrated with

designated FPLC fraction samples were separated on

were centrifuged at 4°C for 10 min at 2000 rpm. Cells were resus-

PLTP Activity Measurement—PLTP activity was measured with an

were counted and plated into 96-well plates. After 2 h the medium was

were measured utilizing a Monarch Plus clinical chemistry analyzer.

were washed with phosphate-buffered saline, equilibrated with

peritoneal lavage with 10 ml of ice-cold phosphate-buffered saline. Macrophages

trinucleotide membrane for Western blot analysis of ApoAI, ApoE, and ApoB.

were run on Tris-glycine gels and transferred to nitrocellulose membrane for Western blot analysis of

were separated according to a previously published procedure (22). The in vivo elongation

were denatured in 0.1 M NaOH for 30 min at room temperature, neutralized in 6x SSC, and applied to Hybond-N membrane (10 μg/slot) using a slot-blot apparatus. 32P-labeled RNA (1–4 × 10⁶ cpm/ml) was hybridized to the membranes in a buffer containing 10 ml Hepes, pH 7.5, 10 ml EDTA, 0.3 M NaCl, 1% SDS, 1X Denhardt’s solution (0.02% polyvinylpyrrolidea-
done, 0.02% Ficoll, 0.02% bovine serum albumin), and 250 μg/ml tRNA at 45°C for 24 h. The membranes were washed four times for 5 min each in 2X SSC at room temperature, incubated in 2X SSC containing 10 μg/ml RNaseA for 30 min at 37°C, and then washed twice for 30 min each in 0.5X SSC, 0.1% SDS at 65°C. The signal was detected by autoradiography and quantitated by a PhosphorImager (Fuji, Stamford, CT).

Western Blot—Designated FPLC fraction samples were separated on

were transferred to nitrocellulose membrane and then blotted with antibodies
to apolipoproteins AI and E (Biosdesign) and apolipoprotein B48/100 (U. S. Biological). Blots were developed with ECL Western blotting detection reagents (Amersham Biosciences) and documented using X-Omat film (Kodak).

Cholesterol Efflux—Mouse peritoneal macrophages were labeled with 1H]cholesterol carried by acetylated LDL. After the labeling, the cells were washed with phosphate-buffered saline, equilibrated with DMEM, 0.2% bovine serum albumin for 1 h, and then incubated with 10 μg/ml purified human apoAI in 0.5 ml of DMEM, 0.2% bovine serum albumin with or without the LXR agonist. The medium was collected at 2, 4, 6, and 8 h time points and centrifuged at 6000 × g for 10 min to remove cell debris and cholesterol crystals. Radioactivity in an aliquot of supernatant was determined by liquid scintillation counting. The cells were finally lysed in 0.5 ml of 0.1% sodium hydroxide, 0.1% SDS, and the radioactivity in an aliquot was determined. Cholesterol efflux was expressed as the percentage of the radioactivity released from the cells into the medium relative to the total radioactivity in cells and medium.

Fig. 2. Apolipoprotein analysis by Western blot. Fractions equivalent to those indicated in Fig. 1 were run on Tris-glycine gels and transferred to nitrocellulose membrane for Western blot analysis of ApoAI, ApoE, and ApoB.
RESULTS

C57BL6 mice fed on a chow diet were dosed orally with various amounts of T0901317 (13) once daily for 1 week. Plasma samples were prepared and subjected to lipid analysis. Total cholesterol and phospholipid were increased in a dose-dependent fashion (Table I). At the highest dose used (100 mg/kg), total cholesterol increased 257% and phospholipid 220% compared with vehicle control. Plasma samples were then subjected to FPLC lipoprotein analysis (Fig. 1). The increase in total cholesterol caused by T0901317 was due to the increase of cholesterol in HDL and LDL-like particles. The plasma fractions from mice treated with 100 mg/kg T0901317 were analyzed further for their apolipoprotein contents. Plasma fractions separated by FPLC (Fig. 1, A–D) were subjected to SDS-PAGE Western blot analysis for ApoE, ApoAI, and ApoB. The results indicated that the fractions from T0901317-treated mice contained much more ApoE and ApoAI, but not ApoB, than those from controls (Fig. 2). Measurement of phospholipid levels indicated that these fractions also contained much more phospholipid than did the controls (data not shown). We concluded that the LDL-like fraction actually contained enlarged HDL particles. Moreover, the overall HDL cholesterol increase upon LXR agonist T0901317 treatment appeared to be biphasic. At 1 and 10 mg/kg, the cholesterol increase was observed largely in the fractions that were similar in size to those from the wild type. Higher doses resulted in a significant induction of enlarged HDL particles.

Recently, it was shown that PPARα agonist-mediated HDL particle size increase in mice required PLTP (24). Thus we examined plasma PLTP activity in our studies. Plasma PLTP activity was increased in a dose-dependent fashion by T0901317 (Fig. 3A). At the highest dose (100 mg/kg), plasma PLTP activity was increased 201% compared with vehicle control. To determine whether the increased PLTP activity was a result of increased gene transcription, we measured liver PLTP mRNA by RNase protection. PLTP mRNA was increased by LXR agonist in a dose-dependent manner up to 6-fold at the 100 mg/kg dose (Fig. 3B). We noticed that the distinct induction of PLTP mRNA, its plasma activity, and the appearance of large HDL particles were all closely correlated well with the dose of 50 mg/kg, suggesting that PLTP regulation was critical for the enlarged HDL production. The in vivo regulation was also examined in other tissues (heart, lung, adipose tissue, and small intestine), and only modest increases were observed (data not shown), suggesting that the liver is the major contributor to the phenotype. We further utilized a nuclear run-on
In Vivo Regulation of PLTP by LXRs

Table II

Plasma total cholesterol and phospholipid level in wild type and PLTP knock-out mice treated with or without T0901317

|                      | Vehicle       | PLTP knock-out |
|----------------------|---------------|----------------|
|                      | C57 BL/6      | 100 mg/kg      | Vehicle       | PLTP knock-out |
|                      |               | 100 mg/kg      |               |               |
| Total cholesterol (mg/dl) | 109 ± 14     | 264 ± 18*      | 61 ± 7        | 115 ± 13*     |
| Phospholipid (mg/dl)    | 156 ± 20      | 382 ± 35*      | 111 ± 9       | 202 ± 9*      |

* p < 0.0001 versus vehicle. N = 6/group.

Fig. 5. PLTP regulation in mouse peritoneal macrophage cells by LXR agonists. Mouse peritoneal macrophage cells were collected and cultured as described under “Materials and Methods.” A, cells were treated with various concentrations of T0901317 for 24 h and RNAs were isolated and subjected to RNase protection assay. B, cells were treated with either 22-(R)-hydroxycholesterol or 9-cis-retinoic acid or with both, and PLTP mRNAs were detected by RNase protection assay. C, cells were labeled with [3H]cholesterol and loaded with 50 μg/ml acetylated LDL. Cholesterol efflux was measured as described under “Materials and Methods.”

Because LXRs regulate a spectrum of important genes involved in cholesterol efflux in macrophage cells, we speculated that PLTP might also be regulated in macrophage cells and potentially contribute to macrophage cholesterol efflux. Thus, we further investigated PLTP expression and its regulation in mouse peritoneal macrophage cells. C57BL/6 mice were injected with thioglycollate, and peritoneal macrophage cells were collected and analyzed. There was basal level expression in these cells. In vitro treatment of these cells with T0901317 caused a concentration-dependent increase of PLTP mRNA of up to 5-fold at 1 μM (Fig. 5A). Treatment of macrophage cells with 22-(R)-hydroxycholesterol or 9-cis-retinoic acid also induced PLTP expression (Fig. 5B). The combination of these two reagents resulted in additive activation of PLTP mRNA, suggesting that PLTP was regulated by the LXR/RXR heterodimer. To further elucidate the function of PLTP in macrophages, we measured cholesterol efflux from peritoneal macrophage cells of wild type and PLTP-deficient mice. The deficiency of PLTP caused only minimal reduction in cholesterol efflux capacity under both basal and LXR agonist stimulated conditions (Fig. 5C). Examination of phospholipid efflux in these cells yielded similar results (data not shown). Thus PLTP is not essential for either cholesterol or phospholipid efflux in mouse peritoneal macrophage cells, or there is a compensatory mechanism to complement PLTP deficiency.

**DISCUSSION**

In this study, we have demonstrated that the LXR agonist T0901317 increases HDL cholesterol and causes enlargement of HDL particles. The LXR agonist transcriptionally up-regulates PLTP expression that contributes to the increase of HDL cholesterol, because the increase was greatly attenuated in PLTP-deficient mice. PLTP had no direct effect on cholesterol efflux from mouse peritoneal macrophages, although the LXR agonist-mediated up-regulation of PLTP was also observed in these cells.

Oxysterols were initially identified and proposed as endogenous LXR ligands (25). Gene disruption of LXRα in mice resulted in massive lipid accumulation in the liver in response to a lipid-rich diet (15). With several critical gene products involved in lipid metabolism identified as direct target genes for LXRs, the concept was proposed that LXRs acted as master transcription factors mediating cholesterol catabolism (7, 17). Indeed, the ensuing reports convincingly showed that a specific nonsteroidal synthetic LXR agonist, T0901317, increased HDL cholesterol and regulated ABCA1 in vivo (13, 16). LXRs also regulate sterol-responsive element-binding protein 1c (SREBP1c), which controls lipogenesis (13, 26). In this study we showed that the increase in HDL cholesterol is associated with two distinct HDL particles: a normal size HDL and an enlarged HDL. The increase of the HDL cholesterol appears to be biphasic. A low dose of LXR agonist caused normal size HDL to increase, and higher doses resulted in a robust increase of a distinct class of HDL particles in the LDL size range.

We report here for the first time that PLTP is an LXR target gene. Although the mouse promoter region does not contain a

To explore whether PLTP plays a role in the above LXR-mediated effect, LXR agonist T0901317 (100 mg/kg) was administered to PLTP-deficient mice. Plasma samples from wild type and PLTP-deficient mice were subjected to FPLC analysis (Fig. 4). Although increases of HDL cholesterol and particle size persisted in PLTP-deficient mice, the extent of induction of HDL cholesterol levels was greatly attenuated (see also Table II). We concluded that the presence of PLTP contributed significantly to LXR agonist-mediated HDL cholesterol and size increase in mice.

PLTP is not essential for either cholesterol or phospholipid efflux in mouse peritoneal macrophage cells, or there is a compensatory mechanism to complement PLTP deficiency.
In Vivo Regulation of PLTP by LXRσ

...critical role that PLTP plays in HDL biogenesis. Our studies further suggest the reduced expression of PLTP and the enhanced PLTP activity by the LXR agonist is not a primary effect of tissue-specific repressors or activators are associated specifically with LXR/RXR heterodimer action.

The LXR agonist caused a dose-related enlargement of HDL. This effect was paralleled by the PLTP induction. However, up-regulation of PLTP by the LXR agonist is not a primary factor for HDL enlargement, because this effect was also observed in PLTP-deficient mice. This scenario is different from PLTP regulation by PPARα, where fenofibrate treatment increased plasma PLTP activity through a PPARα-dependent mechanism and increased plasma PLTP levels accounting for the marked enlargement of HDL in fenofibrate-treated mice (24).

The enlargement of HDL after LXR agonist administration to the mouse may largely involve the induction of ABCA1 and apoE, because both are regulated by LXRα (8, 17). ABCA1 deficiency causes hypercholesterolemia in humans (2–5) and mouse (29). ABCA1 overexpression led to increased HDL cholesterol (30, 31). It has also been reported that apoE is responsible for larger HDL formation (32). The other factor involved in HDL enlargement in humans is CETP. In patients with complete CETP deficiency, HDL is increased in size and enriched in apoE and cholesteryl ester (33). Because mouse has no CETP activity in plasma (34), the accumulation of enlarged HDL particles is preferential substrates for CETP.

Although PLTP was expressed and regulated in macrophage cells, which suggests its potential involvement in phospholipid and cholesteryl efflux, we were surprised to find that the absence of PLTP did not produce significant changes in cell cholesteryl efflux capacity. It is very likely that other alternative molecules are also operating in macrophage cells to compensate for PLTP action. Indeed, a redundancy of LXR-regulated apolipoprotein secretion from macrophages has been suggested (35). It has also been reported that phosphatidylcholine transfer protein is involved in phospholipid and cholesteryl efflux from Chinese hamster ovary cells (36). As the cholesteryl-carrying capacity of lipoproteins is a function of their phospholipid content, it can be expected that variations in PLTP levels in plasma would be associated with variations in their capacity to cause cholesteryl efflux from tissues. Thus, phospholipid homeostasis is closely linked to cholesteryl homeostasis.

In summary, we report here the in vivo regulation of PLTP mediated by LXR agonists, which contributes significantly to HDL cholesterol metabolism. Our studies further suggest the critical role that PLTP plays in HDL biogenesis.

Acknowledgments—We thank Richard Teikong, Pat Foler, Jack Cochran, and Philis Cross for technical support and Tim Grese, George Cullinan, and Steve Yu for making the compound available for our studies.

REFERENCES

...other tissues. This effect is similar to lipoprotein lipase regulation by LXR agonists. These results suggest that tissue-specific repressors or activators are associated specifically with LXR/RXR heterodimer action.

The LXR agonist caused a dose-related enlargement of HDL. This effect was paralleled by the PLTP induction. However, up-regulation of PLTP by the LXR agonist is not a primary factor for HDL enlargement, because this effect was also observed in PLTP-deficient mice. This scenario is different from PLTP regulation by PPARα, where fenofibrate treatment increased plasma PLTP activity through a PPARα-dependent mechanism and increased plasma PLTP levels accounting for the marked enlargement of HDL in fenofibrate-treated mice (24).

The enlargement of HDL after LXR agonist administration to the mouse may largely involve the induction of ABCA1 and apoE, because both are regulated by LXRα (8, 17). ABCA1 deficiency causes hypercholesterolemia in humans (2–5) and mouse (29). ABCA1 overexpression led to increased HDL cholesterol (30, 31). It has also been reported that apoE is responsible for larger HDL formation (32). The other factor involved in HDL enlargement in humans is CETP. In patients with complete CETP deficiency, HDL is increased in size and enriched in apoE and cholesteryl ester (33). Because mouse has no CETP activity in plasma (34), the accumulation of enlarged HDL particles are preferential substrates for CETP.

Although PLTP was expressed and regulated in macrophage cells, which suggests its potential involvement in phospholipid and cholesteryl efflux, we were surprised to find that the absence of PLTP did not produce significant changes in cell cholesteryl efflux capacity. It is very likely that other alternative molecules are also operating in macrophage cells to compensate for PLTP action. Indeed, a redundancy of LXR-regulated apolipoprotein secretion from macrophages has been suggested (35). It has also been reported that phosphatidylcholine transfer protein is involved in phospholipid and cholesteryl efflux from Chinese hamster ovary cells (36). As the cholesteryl-carrying capacity of lipoproteins is a function of their phospholipid content, it can be expected that variations in PLTP levels in plasma would be associated with variations in their capacity to cause cholesteryl efflux from tissues. Thus, phospholipid homeostasis is closely linked to cholesteryl homeostasis.

In summary, we report here the in vivo regulation of PLTP mediated by LXR agonists, which contributes significantly to HDL cholesterol metabolism. Our studies further suggest the critical role that PLTP plays in HDL biogenesis.

Acknowledgments—We thank Richard Teikong, Pat Foler, Jack Cochran, and Philis Cross for technical support and Tim Grese, George Cullinan, and Steve Yu for making the compound available for our studies.

REFERENCES

...other tissues. This effect is similar to lipoprotein lipase regulation by LXR agonists. These results suggest that tissue-specific repressors or activators are associated specifically with LXR/RXR heterodimer action.

The LXR agonist caused a dose-related enlargement of HDL. This effect was paralleled by the PLTP induction. However, up-regulation of PLTP by the LXR agonist is not a primary factor for HDL enlargement, because this effect was also observed in PLTP-deficient mice. This scenario is different from PLTP regulation by PPARα, where fenofibrate treatment increased plasma PLTP activity through a PPARα-dependent mechanism and increased plasma PLTP levels accounting for the marked enlargement of HDL in fenofibrate-treated mice (24).

The enlargement of HDL after LXR agonist administration to the mouse may largely involve the induction of ABCA1 and apoE, because both are regulated by LXRα (8, 17). ABCA1 deficiency causes hypercholesterolemia in humans (2–5) and mouse (29). ABCA1 overexpression led to increased HDL cholesterol (30, 31). It has also been reported that apoE is responsible for larger HDL formation (32). The other factor involved in HDL enlargement in humans is CETP. In patients with complete CETP deficiency, HDL is increased in size and enriched in apoE and cholesteryl ester (33). Because mouse has no CETP activity in plasma (34), the accumulation of enlarged HDL became evident in the animal after administration of the LXR agonist (Figs. 1 and 2). It is very interesting to note from our preliminary results that LXR agonist-induced enlarged HDL particles were preferential substrates for CETP.

Although PLTP was expressed and regulated in macrophage cells, which suggests its potential involvement in phospholipid and cholesteryl efflux, we were surprised to find that the absence of PLTP did not produce significant changes in cell cholesteryl efflux capacity. It is very likely that other alternative molecules are also operating in macrophage cells to compensate for PLTP action. Indeed, a redundancy of LXR-regulated apolipoprotein secretion from macrophages has been suggested (35). It has also been reported that phosphatidylcholine transfer protein is involved in phospholipid and cholesteryl efflux from Chinese hamster ovary cells (36). As the cholesteryl-carrying capacity of lipoproteins is a function of their phospholipid content, it can be expected that variations in PLTP levels in plasma would be associated with variations in their capacity to cause cholesteryl efflux from tissues. Thus, phospholipid homeostasis is closely linked to cholesteryl homeostasis.

In summary, we report here the in vivo regulation of PLTP mediated by LXR agonists, which contributes significantly to HDL cholesterol metabolism. Our studies further suggest the critical role that PLTP plays in HDL biogenesis.