Lipids (2008) 43:611–618
DOI 10.1007/s11745-008-3187-0

ORIGINAL ARTICLE

Peroxisome Proliferator-Activated Receptor α Agonists Regulate Cholesterol Ester Transfer Protein

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Received: 28 January 2008 / Accepted: 22 April 2008 / Published online: 15 May 2008

Abstract Peroxisome proliferator-activated receptor α (PPARα) belongs to the nuclear receptor superfamily that regulates multiple target genes involved in lipid metabolism. Cholesterol ester transfer protein (CETP) is a secreted glycoprotein that modifies high-density lipoprotein (HDL) particles. In humans, plasma CETP activity is inversely correlated with HDL cholesterol levels. We report here that PPARα agonists increase CETP mRNA, protein and accordingly its activity. In a human CETP transgenic animal model harboring the natural flanking regions (Jiang et al. in J Clin Investigat 90:1290–1295, 1992), both fenofibrate and a specific synthetic PPARα agonist LY970 elevated human CETP mRNA in liver, serum protein and CETP activity. In hamsters, the endogenous liver CETP mRNA level and the serum CETP activity were dose-dependently upregulated by fenofibrate. In addition Wy14643, a PPARα agonist, also significantly elevated CETP mRNA and activity. In a carcinoma cell line of hepatic origin, HepG2 cells, overexpression of PPARα resulted in increased CETP mRNA and agonist treatment further elevated CETP mRNA levels. We conclude that PPARα agonists upregulate CETP expression and activity and may play an important role in PPARα (agonist mediated HDL cholesterol homeostasis in humans.

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Introduction

Peroxisome proliferator-activated receptor α (PPARα) together with PPARγ and PPARδ form a subfamily of nuclear receptors that play a critical role in mediating lipid homeostasis, inflammation and metabolism. PPARγ plays a key role in adipocyte differentiation and is the molecular target of anti-diabetic thiazolidinediones (TZD) [1]. PPARδ modulates lipid metabolism and inflammation in multiple tissues [2]. PPARα primarily regulates fatty acid oxidation and also regulates many target genes such as apolipoprotein AI (ApoAI) [3], apolipoprotein CIII (ApoCIII) [4], apolipoprotein AV (ApoAV) [5], phospholipid transfer protein (PLTP) [6] and scavenger receptor class B, type I (SR-BI) in liver [7] that are intimately involved in lipoprotein metabolism.

Cholesterol ester transfer protein (CETP) is a 74 KDa glycoprotein that is primarily synthesized in liver [8]. The secreted protein is mainly associated with high-density lipoprotein (HDL) and catalyzes the neutral lipid exchange (cholesterol ester from HDL to triglyceride-rich lipoprotein particles and TG vice versa) among lipoprotein particles. Increased plasma CETP activity is inversely associated with HDL cholesterol levels [9]. While convincing epidemiological data strongly suggest the protective role of HDL cholesterol in cardiovascular diseases, the exact role of CETP in coronary artery diseases, however, remains controversial. In recent years, CETP has emerged as a major therapeutic target to modulate HDL cholesterol levels and cardiovascular diseases. CETP inhibitor CP-529,414 (torcetrapib) dose-dependently inhibited plasma CETP activity, elevated HDL cholesterol and reduced low-density lipoprotein (LDL) cholesterol levels in recent clinical studies [10–12]. However, the phase III study of torcetrapib was halted because of significantly increased cardiovascular and

total mortality [12]. While the exact reasons are being explored for the adverse mortality rate resulting from CETP inhibition using torcetrapib, torcetrapib was found to induce aldosterone levels and increase blood pressure in humans [12]. In addition, plasma electrolytes were also changed in patients treated with torcetrapib [12]. These observations on torcetrapib are believed to be off-target activity of torcetrapib, as other structurally divergent CETP inhibitors do not exhibit similar off-target activities [13, 14]. In addition, there have been no reports on blood pressure changes in human subjects with homozygous CETP deficiency.

To study the in vivo function of CETP, human CETP transgenic mice were generated using either metallothionein promoter [15] or its own genomic fragments (CETP minigene transgenic mice) [16]. The metallothionein promoter directed CETP transgenic mice did not have significant changes in HDL cholesterol although there was significant expression of human CETP under the basal condition. This was primarily due to the fact that CETP favors large HDL particles and in mice such HDL particles are relatively minor components of HDL [15]. The CETP minigene transgenic mice with its own natural flanking regions are responsive to diet-cholesterol, i.e. the increases in CETP transgene expression and its activity under hypercholesterolemic conditions [16]. A liver X receptor (LXR) basis was established recently for the observed cholesterol regulation of human CETP gene [17]. Synthetic LXR agonists thus regulate CETP expression in liver and possibly in adipose tissue as well [17].

Fatty acids are believed to be the natural ligands of PPARα. Fenofibrate as a hypolipidemic drug is also believed to act as a weak PPARα agonist [18]. In recent years, many potent synthetic pharmacological agonists have been made including LY970 that have demonstrated potent efficacy in reducing plasma triglyceride levels and elevating HDL cholesterol in an ApoAI transgenic animal model [19]. In humans, the fibrate class of drugs is known to effectively reduce plasma triglyceride levels but has only minimum to moderate effects in increasing HDL cholesterol levels [20, 21]. The modest change of HDL cholesterol mediated by PPARα agonists is thus intriguing in light of the combined regulations of ApoAI, PLTP and SR-BI that are expected to dramatically elevate HDL cholesterol levels. Human ApoAI transgenic mice have significantly elevated plasma HDL cholesterol [22]. PLTP is an HDL remodeling protein that contributes to PPARα agonist mediated HDL particle size increase [6] and SR-BI is an HDL receptor, reduced level of which leads to significantly elevated HDL cholesterol in mice [23].

We present evidence here in this report that PPARα agonists upregulate CETP. Because of the pleiotropic effects of PPARα agonists in regulating lipid homeostasis and also the limitation of the human CETP transgenic mice in studying the CETP activity link to its HDL cholesterol effect, it is difficult to assess the exact impact on HDL cholesterol homeostasis in our studies directly. However, based on the well established link of plasma CETP activity and HDL cholesterol levels in humans, our results suggest that this regulation may result in the attenuation of HDL cholesterol elevation mediated by PPARα agonists and thus may play a critical role in PPARα agonist mediated HDL cholesterol homeostasis in CETP species.

Methods

Materials

Fenofibrate, Wy14643, triolein and salts were obtained from Sigma-Aldrich (St Louis, MO). Cholesterol Oleate and POPC were obtained from Avanti Polar Lipids (Alabaster, AL). BODIPY-CE analogue was obtained from Molecular Probes (Eugene, OR). Purified human VLDL was obtained from Intracel (Frederick, MD). LY970, 2-(4-{3-[1-(3,4-Dimethyl-benzyl)-4-methyl-5-oxo-4,5-dihydro-1H-[1,2,4]triazol-3-yl]-propyl}-phenoxy)-2-methyl-propionic acid, was made at Lilly Research Laboratories as described [19].

Animals

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Eli Lilly and Company, Indianapolis, Indiana. The human CETP minigene transgenic mice were described previously [16]. Seven-week old male CETP human transgenic mice under the control of the natural human promoter were obtained from The Jackson Laboratory (Bar Harbor, ME, Stock No. 003904) and acclimated for 2 weeks prior to the start of the studies. Each study used 4–6 mice per group and the mice were individually caged. Light was controlled on a 12 h on 12 h off light/dark cycle. The mice were provided Purina 5001 chow ad libitum and had free access to water throughout the experiments. Compounds were dosed daily by oral gavage in CMC/Tween80 (1.0%/0.25%) vehicle for 7 days. The animals were sacrificed by CO_2 asphyxiation 3 h after the last dose. Blood samples for serum preparation were collected by cardiac puncture and livers were collected and frozen in liquid nitrogen. Eight-week old male Syrian Golden Hamsters (six per group) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and acclimated 2 weeks prior to the start of the study. The hamsters were housed 5 per cage. Light was controlled on a 12 h on 12 h off light/dark cycle. The hamsters were provided Purina 36315 chow ad libitum and had free access to water throughout the experiments. Compounds were dosed daily by oral gavage in CMC/Tween80 (1.0%/0.25%) vehicle for 14 days. The animals

were sacrificed by CO_2 asphyxiation 3 h after the last dose. Blood samples for serum preparation were collected by cardiac puncture and livers were collected and frozen in liquid nitrogen.

CETP Activity

A donor particle containing a fluorescent cholesterol ester analogue that self quenches its fluorescent signal was prepared similarly to what was described by Bisgaier et al. [24]. The particle contained 15 mol% BODIPY-CE analogue, 33 mol% cholesteryl oleate, 8 mol% triolein, and 44 mol% POPC. The donor particle was added to a Tris/NaCl buffer with an acceptor particle (purified human VLDL at final concentration of 25 μg/ml). Serum from the animals was added to the mixture and incubated 30 min at 37°C. Fluorescent signal at wavelengths 544 nm excitation, 595 nm emission with a 590 nm cutoff filter in a Gemini fluorescent plate reader (Molecular Devices, Sunnyvale, CA) represents CETP activity.

Plasma CETP Levels

An ELISA (Wako Diagnostics, Richmond, VA) was used to measure CETP protein concentration in mouse serum. The serum samples were serially diluted with PBS and the assay was performed as described according to the manufacturer's instructions.

Plasma Lipid Analysis

Serum triglyceride was measured in an automated clinical analyzer (Roche, Indianapolis, IN). Lipoproteins were analyzed by FPLC as described in Cao et al. [25].

mRNA Measurement

RNA was prepared with RNeasy Mini Kit (QIAGEN, Valencia, CA) or Total RNA Chemistry products for the ABI Prism 6100 Nucleic Acid PrepStation (PE Applied BioSystems, Foster City, CA, USA). Taqman real-time quantitative PCR (Q-PCR) was performed using an ABI Prism 7900 sequence detector (PE Applied BioSystems, Foster City, CA) with GAPDH as an internal control for HeG2 cells and 18S as an internal control for animal liver samples. Gene-specific primer probes were obtained from Applied BioSystems. Relative expression of the genes was determined by C_t method and calculated to fold induction against the controls.

Cell Culture Studies

Full length human PPARα cDNA was cloned from human liver and the sequence was confirmed by DNA sequencing. It was then subcloned into the mammalian expression vector pcDNA3.1(+) with *Kpn*I and *Not*I sites. The PPARα expression vector was used to establish a HepG2 Stable cell line in which PPARα was expressed at 80 fold (mRNA) over the basal expression level through standard Geneticin (G418, Invitrogen, Carlsbad, CA) selection procedure (800 μg/ml). HepG2 and HepG2 PPARα stable cells were grown in 3/1 DME/F12 + 10% FBS and seeded in 24-well plates at the density of 8×10^4 cells/well. Twenty-four hours later, the cells were treated with either DMSO or PPARα agonists for 48 h. RNAs were then prepared and analyzed as described above.

Statistics

Values are given as mean ± standard error of mean (SEM). Statistical differences, as defined as $P < 0.05$, from the vehicle control group were determined using Dunnett's test or *t* tests (JMP 5.1.1, SAS, Cary, NC).

Results

To explore whether PPARα agonists regulate CETP, we initially used a human CETP mini-gene transgenic mice. This mouse model utilized a DNA fragment containing the human CETP gene including coding regions, introns and flanking regulatory sequences to express the transgene product primarily in liver [16]. It has been shown previously that cholesterol rich diet induced hepatic CETP mRNA and activity in this model and the molecular basis for the observed regulation was traced to an LXR basis [16, 17]. We treated the animals ($n = 4$) using fenofibrate for 7 days and 3 h after the last dose, we sacrificed the animals and analyzed the data. Serum triglyceride level was reduced by 38% (Fig. 1a), suggesting the activation of PPARα in vivo. Serum CETP activity was assessed and demonstrated a significant increase (61%) (Fig. 1b). Measuring serum CETP protein levels using an ELISA indicated a 130% increase in serum CETP protein mass (Fig. 1c). A 2.7 fold increase in hepatic CETP mRNA was observed when liver samples were analyzed by the quantitative polymerase chain reaction (Fig. 1d). Next, we investigated specific PPARα mechanism by using a potent specific synthetic PPARα agonist LY970 that is structurally divergent from fenofibrate in the same model [19]. CETP mini-gene mice ($n = 4$–5) were treated with 1 mg/kg dose of LY970 for seven days and the serum lipid, protein and hepatic mRNA levels were analyzed similarly. As expected, LY970 reduced serum triglyceride level dramatically (75%, Fig. 2a), suggesting a very potent PPARα activation in this mouse model. Serum CETP activity was elevated 154% and the protein level increased 228% (Fig. 2b, c).

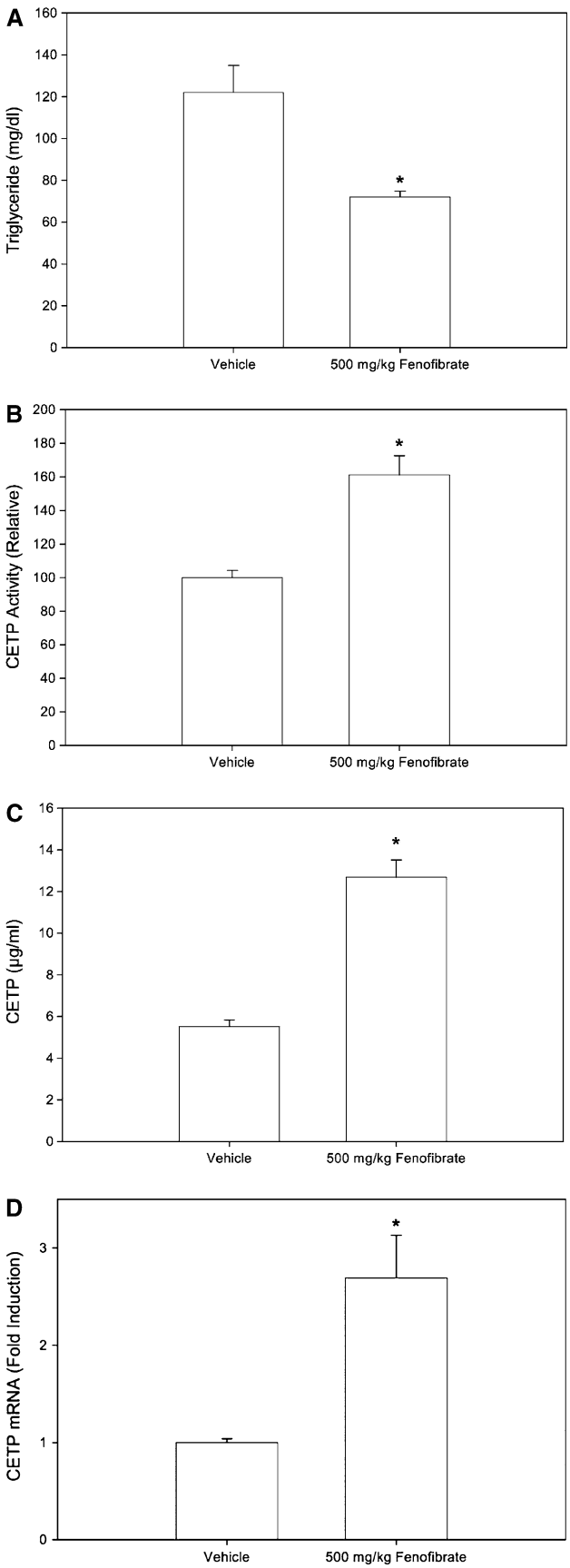
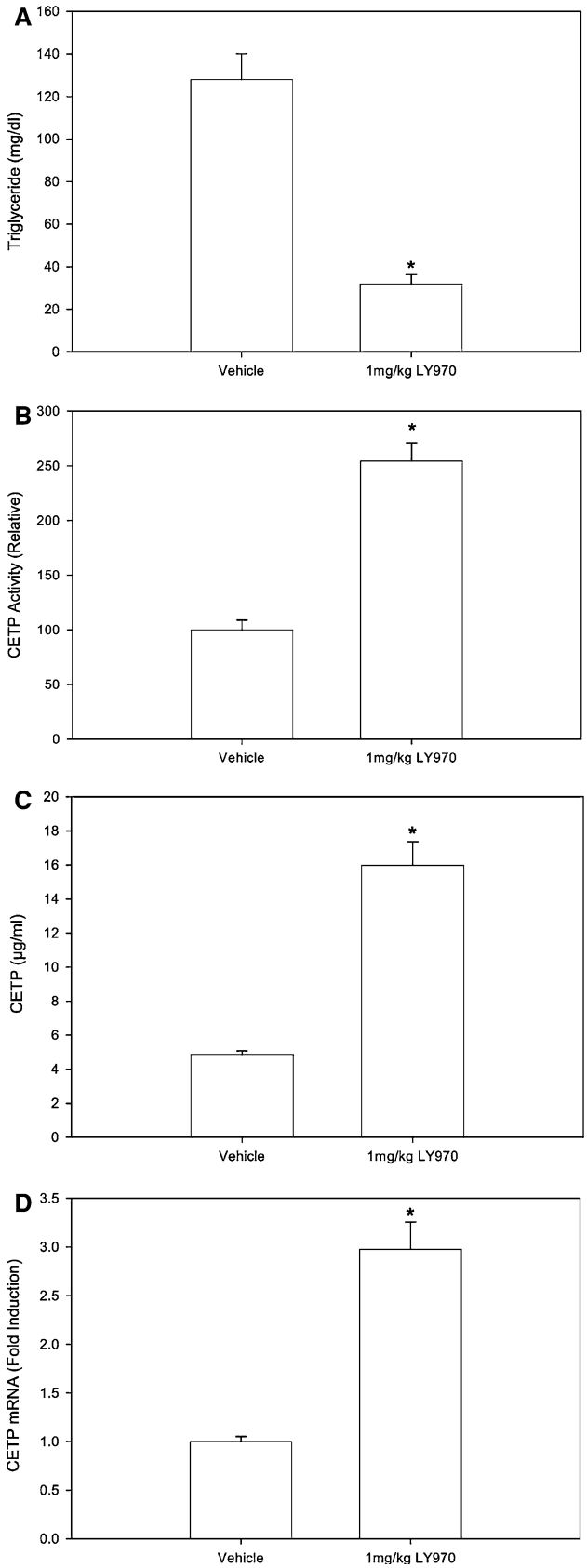


Fig. 1 Regulation of CETP by fenofibrate in CETP minigene transgenic mice. Four animals per group were dosed orally at 500 mg/kg for 7 days. Plasma and tissue samples were prepared 3 h after the last dose and analyzed as described in "Methods". **a** Fenofibrate reduced serum triglyceride levels. **b** Fenofibrate elevated CETP activity. Ex vivo CETP activity of serum samples was assessed with the assay described in "Methods". **c** Fenofibrate increased serum CETP protein mass. Serum CETP protein levels were measured by an ELISA kit for human CETP as described in "Methods". **d** Hepatic human CETP mRNA was increased upon PPARα activation by fenofibrate. Liver RNA was prepared and CETP mRNA level was assessed by the quantitative PCR as described in "Methods". $*P < 0.05$ versus vehicle (t test)

Hepatic mRNA was upregulated three fold as well (Fig. 2d). Significant reduction of total cholesterol (23%) that was attributable primarily to reductions of VLDL and LDL was observed. HDL cholesterol was not changed (data not shown).

To explore whether endogenous CETP could be regulated by PPARα agonists, we treated hamsters with increasing doses of fenofibrate or 10 mg/kg of Wy14634, a prototypic PPARα agonist for fourteen days and analyzed lipids, CETP mRNA and its activity. As were shown in Fig. 3, fenofibrate at the highest dose trended towards a reduction of serum triglyceride levels (nonsignificant, Fig. 3a). Fenofibrate elicited a dose-dependent increase in plasma CETP activity that was 221% at the highest dose compared to the vehicle group (Fig. 3b). Hepatic CETP mRNA was also dose-dependently elevated with the highest elevation around 60% with the highest fenofibrate dose and close to 90% increase with Wy14643 treatment (Fig. 3c). There was no significant change in HDL cholesterol levels as assessed by FPLC analysis (data not shown). Taken together, these data indicated that PPARα agonists upregulated CETP in vivo.

To explore the potential mechanisms of PPARα agonist regulation of CETP, we used a human liver carcinoma cell line HepG2 cells as a model. HepG2 cells have low PPARα expression [26] and not surprisingly, agonist treatment did not result in any significant change in CETP mRNA levels (Fig. 4b). We thus generated a stable cell line in which PPARα was overexpressed. PPARα overexpression led to an increase of more than 100% in CETP mRNA and a more than 5 fold elevation in carnitine palmitoyl transferase 1 (CPT1) mRNA as a control [27] (Fig. 4a). Treatment of the cells with PPARα agonists resulted in a further but moderate elevation in CETP mRNA compared to DMSO as the vehicle (20–60%, Fig. 4b). As controls, liver X receptor (LXR) agonist T0901317 robustly elevated CETP mRNA in these cells [17]. Thus, PPARα agonist regulation of CETP was direct on hepatocytes. In an attempt to map potential PPARα responsive elements within the regulatory regions of human CETP gene, we searched the promoter and intron one of the human CETP gene for potential direct



◀ **Fig. 2** Regulation of CETP by a specific synthetic PPARα agonist LY970 in CETP minigene transgenic mice. Five animals in vehicle group and four animals in compound group were treated with either vehicle or LY970 at 1 mg/kg for 7 days. Serum and tissue samples were prepared and analyzed similarly to "Methods" in Fig. 1. **a** Significant reduction of serum triglyceride levels. **b** Significant elevation of serum CETP activity. **c** Significant increase in serum CETP mass. **d** Significant increase in hepatic CETP mRNA levels by LY970. * $P < 0.05$ versus vehicle (*t*-test)

repeat of hormone responsive element AGGTCA separated by one nucleotide (DR1) and did not find any obvious DR1 elements (data now shown). Accordingly a five kilobase promoter fragment subcloned into a luciferase reporter did not show any PPARα agonist dependent increase in its reporter activity (data not shown). Thus, the molecular basis of PPARα agonist regulation of CETP remains to be defined.

Discussion

PPARα is a nuclear receptor that plays an important role in lipid homeostasis. Besides its major role in regulating ApoCIII [3], apoAV [5] and fatty acid oxidation [18], it has been well recognized that PPARα agonists regulate key molecular components involved in HDL cholesterol metabolism. Specifically, PPARα agonists upregulate human ApoAI [3] and PLTP [6] and reduce expression of SR-BI [7]. ApoAI is the major structural component of HDL particles and the amount of ApoAI is closely associated with HDL cholesterol levels. PPARα agonist treatment of human ApoAI transgenic mice resulted in dramatically elevated HDL cholesterol levels [3]. Although the role of PLTP in HDL metabolism is not entirely clear, it has been convincingly shown in rodent models that PLTP is essential for HDL biogenesis [28]. Further PPARα regulation of PLTP is necessary for PPARα agonist mediated HDL particle size increase in mice [6]. SR-BI is an HDL receptor, deficiency of which in mice leads to dramatically elevated plasma HDL cholesterol levels [23]. Despite the combined regulation of these important molecules involved in HDL cholesterol metabolism by PPARα agonists that are expected to raise HDL cholesterol level, the effect on HDL cholesterol by PPARα agonists in humans is minimum to modest [20, 21]. Although it has been suspected that potent synthetic PPARα agonists may elevate HDL cholesterol more dramatically, it has not been proven clinically and it appears that induction of HDL cholesterol by PPARα agonists may be bell-shaped [29]. A major missing link of PPARα regulation of ApoAI, PLTP and SR-BI and the associated impact on HDL cholesterol in humans versus

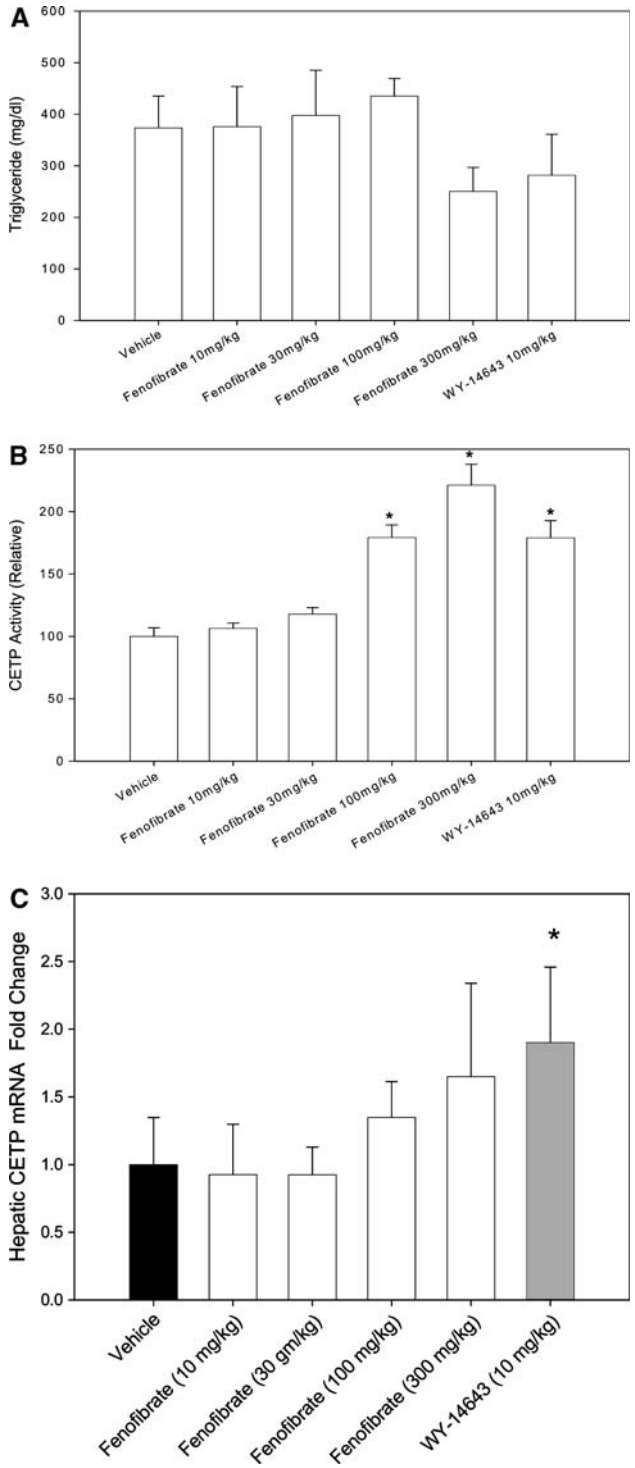


Fig. 3 Regulation of hamster CETP by PPARα agonists. Five animals per group were treated with either various doses of fenofibrate or Wy14643 at 10 mg/kg for 14 days. Serum lipid and CETP activity was analyzed similarly to the studies with CETP minigene transgenic mice. **a** Serum a triglyceride analysis. **b** Dose dependent increase in ex vivo CETP activity by fenofibrate and 10 mg/kg Wy14643. **c** Hepatic mRNA analysis by quantitative PCR. * $P < 0.05$ versus vehicle (Dunnett's)

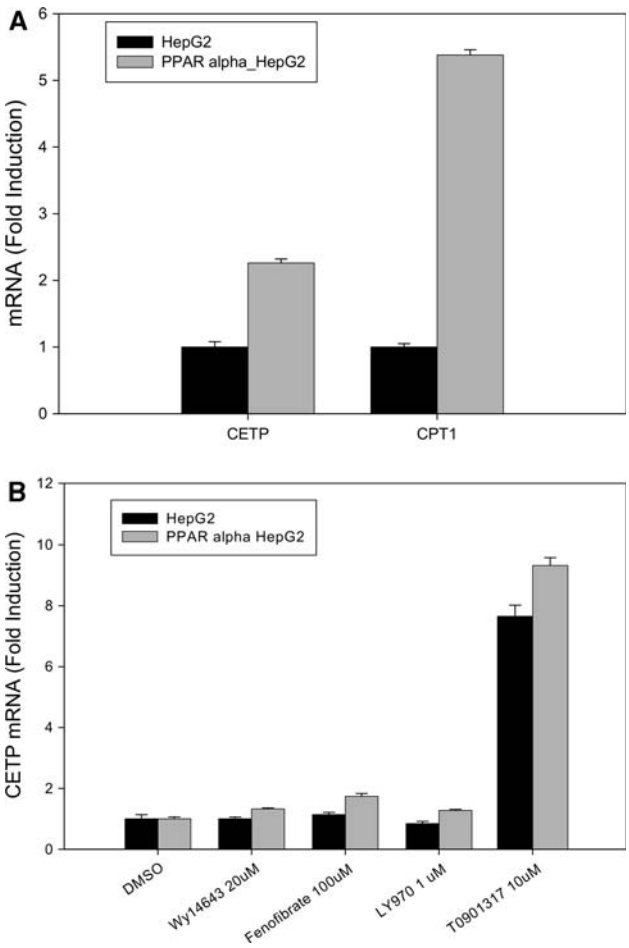


Fig. 4 Regulation of cellular CETP by PPARα. **a** Increased CETP expression by PPARα overexpression in HepG2 cells. A HepG2 stable cell line was established through stable overexpression of human PPARα driven by a CMV promoter. Relative CETP mRNA levels were assessed by quantitative PCR. **b** PPARα agonist regulation of CETP in hepatic cells. HepG2 stable cells overexpressing PPARα or the parental cell line were treated with different PPARα agonists. 48 h later, cells were lysed and mRNA levels were analyzed by the quantitative PCR. T0901317 was used as a control for LXR agonist mediated CETP regulation

the studies in mice is the presence of CETP in humans. CETP catalyzes the transfer of cholesterol ester from HDL particles to ApoB-containing lipoproteins in exchange for triglyceride. It has been well documented through extensive genetic studies with CETP deficiency and polymorphisms in humans that plasma CETP levels are inversely correlated with HDL cholesterol levels. Subjects with either heterozygous or homozygous CETP deficiency not only have significantly elevated HDL cholesterol levels but also have a significant increase in HDL particle size [30]. CETP has been shown to significantly attenuate or abolish completely lecithin cholesterol acyl-CoA acyltransferase (LCAT), or LXRα agonist mediated HDL

cholesterol and particle size increase [31, 32]. Significant attenuation of PPARα agonist mediated HDL cholesterol increase by CETP in humans is thus conceivable and may at least partly explain the relative anemic elevation of HDL cholesterol induced by PPARα agonists. Our data indicate that PPARα agonists can increase CETP mRNA, serum proteins and activity and thus may provide a plausible explanation to the HDL cholesterol response clinically to PPARα agonists. In our animal models, it has been impossible to study the direct effect of the changes of CETP level to the changes of HDL cholesterol because of pleiotropic effects of PPARα agonists on HDL cholesterol homeostasis and the limitation of the human CETP transgenic mice [15]. Our attempt to study the clinical effect of PPARα agonists on CETP levels, unfortunately, was inconclusive primarily because of the variations of the baseline plasma CETP levels and the extremely limited number of samples that were available for the study (data not shown). Thus, the exact pharmacological impact of PPARα agonists on plasma CETP protein levels and its activity in humans awaits further investigation.

Our data are in stark contrast with van der Hoogt et al. [33], which reported a decrease in plasma CETP activity in CETP mini-gene transgenic mice with PPARα activation by fenofibrate. At the moment, we do not know exactly the basis for the apparent opposite findings. In our studies we have used three different PPARα agonists, each structurally divergent, to demonstrate the upregulation of CETP in mRNA, plasma protein and its activity. The experiment reported by van der Hoogt et al. was done with high fat diet in a hypercholesterolemic apoE3 Leiden transgenic background. Whether the presence of apoE3 Leiden transgene or the diet influenced the results they reported is not clear at the moment. Future studies will clarify the nature of the PPARα regulation on CETP and its impact on HDL cholesterol.

Acknowledgments We would like to thank Dr. Shuyu Li for help with bioinformatics and Youyan Zhang for editorial support. We are indebted to Richard Tielking, Jack Cochran and Patrick Forler for excellent technical assistance. This work is supported by Lilly Research Laboratories.

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