Published in final edited form as:

Biochem J. 2015 December 15; 472(3): 275–286. doi:10.1042/BJ20150666.

A Novel Peroxisome Proliferator Response Element Modulates Hepatic Low Density Lipoprotein Receptor Gene Transcription in Response to PPAR δ Activation

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Abstract

The hepatic expression of LDLR gene is regulated primarily at the transcriptional level by a sterol-regulatory element (SRE) in its proximal promoter region which is the site of action of SRE-binding protein 2 (SREBP2). However whether additional cis-regulatory elements contribute to LDLR transcription has not been fully explored. We investigated the function of a putative PPAR-response element (PPRE) sequence motif located at –768 to –752 bases upstream of the transcription start site of human LDLR gene in response to PPAR δ activation. Promoter luciferase reporter analyses showed that treating HepG2 cells with PPAR δ agonist L165041 markedly increased the activity of a full-length LDLR promoter construct (pLDLR-1192) without any effects on the shorter promoter reporter pLDLR-234 that contains only the core regulatory elements SRE-1 and SP1 sites. Importantly, mutation of the PPRE sequence greatly attenuated the induction of the full-length LDLR promoter activity by L165041 without affecting rosvastatin mediated transactivation. Electrophoretic mobility shift and chromatin immunoprecipitation assays further confirmed the binding of PPAR δ to the LDLR-PPRE site. Treating HepG2 cells with L165041 elevated the mRNA and protein expressions of LDLR without affecting the LDLR mRNA decay rate. The induction of LDLR expression by PPAR δ agonist was further observed in liver tissue of mice and hamsters treated with L165041. Altogether, our studies identify a novel PPRE-mediated regulatory mechanism for LDLR transcription and suggest that combined treatment of statin with PPAR δ agonists may have advantageous effects on LDLR expression.

Keywords

LDL receptor; PPAR δ ; PPRE; Transcriptional regulation; SREBP2; Statin

INTRODUCTION

Homeostasis of circulating cholesterol is a complex mechanism involving several tissues and molecular intermediates. Hepatic low density lipoprotein receptor (LDLR) plays a major

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Author contribution: Vikram R. Shende initiated the study, performed the in vitro experiments and wrote the manuscript, Amar Bahadur Singh conducted the in vivo mice and hamster studies, and Jingwen Liu synthesized the concept, initiated the study, designed and supervised the experiments and wrote the manuscript.

role in regulation of cholesterol homeostasis by capturing and internalizing circulating LDL-cholesterol (LDL-C) from plasma (^{1,2}). Hepatic LDLR function is thus of clinical relevance because plasma LDL-C is a significant risk factor for coronary and metabolic disorders (³). The primary mode of LDLR regulation is at the transcriptional level by sterol response element bind protein 2 (SREBP2) which itself responds to intracellular cholesterol levels (^{4,5}). Decreases in cellular cholesterol levels result in nuclear translocation of the active mature form of SREBP2 where it transactivates target genes, primarily via sterol response elements (SREs) in their promoters (⁶).

The LDLR proximal promoter contains a SRE-1 motif, which has been shown to play a central role in regulating LDLR mRNA transcription (⁷). In addition, a sterol-independent regulatory element (SIRE) that mediates the cytokine oncostatin M-induced LDLR gene transcription is located downstream of the SRE-1 (⁸). Ancillary modes of post-translational regulation, mediated by PCSK9 and IDOL, as well as post-transcriptional modulation by mRNA-binding proteins have also been demonstrated in regulating LDLR protein turnover and mRNA half-life respectively (⁹⁻¹²). Recent studies also indicate that peroxisome proliferator activated receptors (PPARs) can modulate LDLR expression (^{13,14}).

Members of PPAR belong to the nuclear receptor superfamily and are a class of ligand-activated receptors. Three subtypes α , β/δ and γ have thus far been described sharing a characteristic type II zinc-finger DNA binding motif and a hydrophobic ligand binding domain (^{15,16}). Upon activation by endogenous ligands, including fatty-acids and eicosanoids, PPAR's heterodimerize with the retinoid X receptor (RXR) and can transactivate gene expression via peroxisome proliferator receptor element (PPRE) sequence motifs in the promoters of their target genes (^{17,18}). Although they share some overlapping characteristics, the three PPAR's have different tissue localization patterns, physiological roles and distinct activating ligands (^{17,19}). PPAR α is highly expressed in tissues with high metabolic rates, including the liver and muscle and is predominantly involved in regulation of fatty acid metabolism under fasting conditions (²⁰). PPAR γ is expressed primarily in adipose tissue and macrophages (¹⁹), functioning in adipocyte differentiation, lipid storage and glucose homeostasis (²¹). In comparison, PPAR δ is more ubiquitously expressed in several tissues including the liver, intestine, kidneys and skeletal muscle (¹⁹). Although the function of PPAR δ is less well understood, physiological roles include regulation of insulin sensitivity as well as glucose homeostasis (²²).

Earlier studies reported that PPAR α agonist fenofibrate and PPAR γ agonist pioglitazone increased LDLR mRNA and protein expression via a SREBP2 dependent mechanism (^{13,14}). Several synthetic agonists, L165041, GW0742 and GW501516 have been utilized to explore the potential roles of PPAR δ in regulating lipid and cholesterol metabolism (^{23,24}). Administration of L165041 to db/db mice resulted in elevated HDL-C and lowering of LDL-C compared to control animals (²⁵). Similarly, treatment of wild-type mice with GW0742 for 14 days caused modest reduction in serum TG with associated modest increases in serum HDL-C (²⁶). While the change in serum cholesterol in the latter report was attributed to increase in liver phospholipid transfer protein expression, whether LDLR played a role in any PPAR δ mediated effects on lipoprotein metabolism has not been reported.

In this current study, we have investigated the potential role of PPAR δ in regulating hepatic LDLR gene expression. We have identified a novel PPRE-like element located ~750 bp upstream of the transcription start site of the human LDLR gene. We further showed that PPAR δ can directly bind to this cis-regulatory motif of the LDLR promoter to drive expression of the mRNA and protein, both in cultured hepatic cells and in liver tissues of normolipidemic mice as well as hyperlipidemic hamsters.

MATERIALS AND METHODS

Cells and reagents

Human hepatoma HepG2 cells were obtained from ATCC. L165041 and GW0742 were purchased from TOCRIS Biosciences (Bristol, UK). Actinomycin D and anti- β -actin were purchased from Sigma. Anti-LDLR antibody was obtained from BioVision (Mountain View, CA). Mouse primary hepatocytes were isolated from male C57BL/6J mice at San Francisco General Hospital Liver Center.

Animals and drug treatment

All experimental analyses were conducted in accordance with animal use protocols approved by the Institutional Animal Care and Use Committee at the VAPAHCS. In the first in vivo experiment, male C57BL/6 mice between 8–10 weeks of age (Jackson Laboratories) were used. All animals were housed in the VA Palo Alto Health Care System (VAPAHCS) veterinary medical unit in a 12:12 Light:Dark cycle (lights on at 6:00 AM) with a regular rodent chow diet and water provided ad-libitum. Animals were housed 3–4 per cage and health parameters including body weight and food intake were monitored throughout the duration of the experiment. Starting the day of the experiment (day 0), mice (N=6 per treatment) were gavaged with either 20 mg/kg/day of L165041 suspended in 0.5% carboxymethyl cellulose (CMC) or an equivalent volume of 0.5% CMC alone as vehicle control. Blood was collected retro-orbitally from control and L165041 treated mice on days 0, 7 and 14. After euthanizing the animals on day 14, liver tissues were harvested and stored in liquid nitrogen until further analysis.

In the second in vivo experiments, to study the effect of PPAR δ activation on LDLR expression under hyperlipidemic conditions, ten male hamsters were fed a HFD (Harlan TD. 88137; ~42% of total calories from fat; 0.2% cholesterol) to induce hyperlipidemia. After 8-weeks on HFD, overnight fasting blood samples were taken to randomize hamsters into homogenous treatment groups according to total cholesterol (TC) levels. Hamsters were then maintained on the HFD and were treated orally with vehicle (0.5% hydroxypropyl methylcellulose in PBS) or L165041 (10 mg/kg, once a day) for 1 week. At the experimental termination, hamsters were fasted overnight before euthanization for serum and tissue collections. In this experiment, another group of hamsters (n=4) fed a normal chow diet (NCD) were included for serum lipid analysis and liver LDLR protein analysis.

Cell culture and transfections

HepG2 cells were cultured in minimal essential medium (MEM) supplemented with 10% FBS. Prior to experimentation, cells were seeded into 96-, 24- or 6- well plates at a density

of $0.5\text{--}0.7 \times 10^6$ cells/mL. After 24–48 h (at ~70% confluence), medium was changed to MEM supplemented with 0.5% FBS. Following overnight incubation in this low-serum containing medium, cells were treated with PPAR δ -agonists at desired concentrations. Cells were harvested at 24 h, or at other desired intervals post-treatment and processed for RNA and protein isolation. For experiments entailing estimation of mRNA half-life, after overnight incubation in low-serum medium, cells were pre-treated with 20 μM of L165041 for 16 h prior to administering 5 $\mu\text{g/mL}$ of Actinomycin D. Cells were harvested immediately after addition of Actinomycin D (0 h) and at indicated time points thereafter.

LDLR promoter luciferase reporter assay

The pLightSwitch_Prom vector containing a 1192 bp promoter region of human LDLR was purchased from Switchgear Genomics. The 1192 bp fragment was then sub-cloned into pGL3 basic promoter vector (Promega) and denoted pLDLR-1192. This plasmid contains 989 bases upstream and 203 bases downstream of the human LDLR gene transcription start site (–989 to +203). The pLDLR-PPRE mutant plasmid was generated by inducing mutations in four bases within the right half site of putative PPRE using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturers' guidelines. The LDLR-3' UTR reporter plasmid in pcDNA3.1 vector backbone has been previously described (27).

HepG2 cells were transfected with the designated reporter plasmids using FuGene 6 transfection reagent (Promega) according to manufacturer's guidelines. To control for differences in transfection efficiency, pRL-TK plasmid encoding *Renilla* luciferase gene was cotransfected with LDLR promoter vectors and was used to normalize firefly luciferase signal across all samples. Approximately 48–72 h after transfection or 24 h post-treatment with L165041 or rosuvastatin (RSV), cells were lysed with 50 μL of lysis buffer followed by measurements of firefly luciferase and renilla luciferase activities. The firefly luciferase activity was normalized to renilla activity. Four wells were assayed for each condition.

Electrophoretic mobility shift assay (EMSA)

Binding of PPAR δ to LDLR-PPRE promoter region was assessed using the LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturers' guidelines. Briefly, a 5' biotin end labeled probe identical to the 40 nucleotide region surrounding the putative PPRE element in the LDLR promoter was synthesized (Table 1). The single stranded biotin-labeled probe was hybridized to an unlabeled complement, and 20 fmole of the double-stranded oligonucleotide was incubated for 20 min with purified human recombinant PPAR δ (Cayman chemicals) plus RXR α (Active Motif) proteins (300 ng:100 ng; PPAR δ :RXR α ratio) mixture in vitro. The protein-DNA complex was resolved on 5% TBE gels and transferred to a nylon membrane. Following UV cross-linking and subsequent washing, the biotin signal was visualized using chemiluminescence. For competition experiments, a 100-fold higher concentration of unlabeled wild-type or PPRE-mutant oligos were incubated with the reaction mixture containing PPAR δ /RXR α and biotin-LDLR probe. For observation of the supershift complex, the reaction mixture was incubated for 10 min with anti-PPAR δ antibody (sc-7197X; Santa Cruz Biotechnology) prior to resolution on the TBE gels.

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed using ZymoSpin ChIP kit (Zymo Research, catalog # D5209) following the manufacturers' guidelines. Briefly, HepG2 cells were seeded in 10 cm dishes and treated with either L165041 (20 μ M) or DMSO (control) as described above. Following drug treatment for 24 h, cells were trypsinized, resuspended in PBS at a dilution of 8×10^6 cells/mL and fixed with 1% formaldehyde (Sigma, catalog # 8775) for 10 min. Fixed cells were collected by centrifugation. To obtain nuclear lysates, fixed cells were resuspended in 500 μ L chromatin shearing buffer and sonicated at 4°C in a Bioruptor 300 instrument (Diagenode, Inc.) for 12 cycles of 30sec ON: 30sec OFF at a "high" setting with intermittent vortexing. Chromatin containing nuclear lysates (100 μ L) were incubated overnight with 4 μ g of either rabbit anti-PPAR δ (sc-7197x) or isotype control IgG antibodies (Santa Cruz Biotechnologies) and complexes were immunoprecipitated using protein-A magnetic beads. ChIP DNA was eluted, cross-linking was reversed and protein-free DNA was purified before PCR amplification with site specific primers covering the LDLR-PPRE region (Table 1).

RNA isolation and quantitative real-time PCR

Total RNA was isolated from 20 mg of flash frozen liver tissue samples or from treated hepatic cells using the RNeasy PLUS mini kit (Qiagen) or Quick RNA kit (Zymo Research) respectively. Approximately 1.5 μ g of total RNA was reverse transcribed by random priming using the High-Capacity cDNA reverse transcription kit (Life technologies) according to the manufacturer's guidelines. Real-time PCR was then performed in an ABI 7900HT Sequence Detection system using SyBr Green PCR Master mix (Life Technologies) and PCR primers specific for each gene being amplified (Table 1). With duplicate or triplicate measurements from each cDNA sample, the data was analyzed using the $\Delta\Delta C_T$ method and relative expression of target mRNA's was normalized to that of GAPDH in each sample.

Western blotting from liver tissues of mice and hamsters

Total protein was extracted in RIPA buffer supplemented with 1 mM PMSF and protease inhibitor cocktail (Roche) from HepG2 cells or from flash-frozen liver samples by homogenizing ~50 mg of tissue. Protein content was quantified using BCA protein assay reagent (PIERCE) and 50 μ g of protein from individual samples was resolved by SDS-PAGE. Following transfer onto nitrocellulose membranes, LDLR and β -actin proteins were detected by immunoblotting using rabbit anti-LDLR (Biovision) and monoclonal anti- β -actin (Clone AC-15, Sigma) antibodies. Immunoreactive bands of predicted molecular mass were visualized using Super Signal West Femto Chemiluminescent Substrate (Pierce) and FluorChem E Western blot imaging system (Protein Simple). Background subtracted band densities were quantified using AlphaView SA Imaging Software (Protein Simple). Signal intensities of β -actin were used as normalization to control for protein loading differences between individual samples.

Analysis of Dil-LDL uptake in HepG2 cells

HepG2 cells were seeded into 48- or 6- well plates at a density of $0.5\text{--}0.7 \times 10^6$ cells/mL. After 24 h (at ~70% confluence), medium was changed to MEM supplemented with 0.5% FBS. Following overnight incubation in this low-serum containing medium, cells were

treated with PPAR δ -agonists at desired concentrations overnight. DiI-LDL (4 μ g/ml) was incubated with cells for 2 h and cells were washed three times by cold PBS. DiI-LDL uptake was examined with a fluorescent microscope and pictures were taken for 10–15 fields of view in each well. The fluorescence intensities were subsequently quantified using NIS-Elements imaging software.

Serum analysis

Serum total cholesterol, HDL-cholesterol and triglycerides were measured from serum samples collected at the time of termination of the experiments using StanBio kits according to the manufacturers' guidelines. Mouse serum PCSK9 was measured from the mouse serum samples using R&D Biosystems, mouse PCSK9 ELISA kit according to the manufacturers' guidelines.

Statistical analysis

All values are expressed as mean $\pm$ SEM. For multiple group comparisons, one way ANOVA with Students-Keuls pairwise post hoc test was performed using GraphPad Prism 5 software. For two group data analysis, unpaired two-tailed Student's *t* test was applied. A *p* value of < 0.05 was considered statistically significant for all analyses.

RESULTS

Human LDLR promoter contains a functional PPRE element

Utilizing the MatInspector software, we analyzed the upstream 5' flanking region of human LDLR gene to identify potential regulatory elements that may function either in association with or independent of the SRE-1 in the proximal promoter of LDLR. Transcription factor prediction indicated presence of a single PPRE-like sequence motif located at –768 to –752 bases upstream of the transcription start site. To assess the significance of this sequence motif, we procured a full length plasmid (pLDLR-1192) containing the promoter region from –989 to +203 relative to the transcription start site of human LDLR gene (Fig. 1A). First, we assessed the effects of the PPAR δ selective ligand L165041 on the full length LDLR promoter construct and the well-studied LDLR core promoter reporter construct pLDLR-234. We observed that L165041 treatment did not change the luciferase activity in HepG2 cells transfected with pLDLR-234, however, luciferase activity of pLDLR-1192 was significantly increased by ~2.5-fold in HepG2 cells treated with 20 μ M L165041 as compared to control (Fig. 1B). We utilized a PPRE reporter pPPRE-Luc)⁽²⁸⁾ and observed similar ~3-fold induction of luciferase signal intensity after treatment with L165041 (Fig. 1B). To address whether L165041 had any effect on PCSK9 gene transcription, we also included PCSK9 promoter construct (pGL3-PCSK9-D4) in our analyses. Contrary to effects on LDLR-1192 and PPRE-Luc reporter plasmids, treatment of the PCSK9-D4 luciferase plasmid with L165041 did not result in any changes in promoter activity (Fig. 1B).

Next, using site-directed mutagenesis we mutated the PPRE site (pLDLR-PPREmut) in the pLDLR-1192 (Fig. 1A) and assessed the effects of L165041 and RSV on the wild-type and PPRE-mutated full length LDLR promoter activity. As shown in Fig. 1C, mutation of PPRE site nearly abolished the inducing effect of L165041 but it did not diminish effect of RSV-

stimulated LDLR promoter activity. Taken together, these data suggest that the novel PPRE-like element positively mediates PPAR δ -induced LDLR transcription independent of the SREBP2 pathway that mediates statin induced changes in LDLR transcription via the SRE-1 site.

PPAR δ binds to LDLR-PPRE promoter region

It is well-demonstrated that PPAR δ forms heterodimers with RXR α and binds to the PPRE motif to activate its target gene transcription. To determine whether the observed increase in LDLR promoter activity by PPAR δ agonist treatment may be directly attributed to PPAR δ binding to the putative PPRE-like element, we first examined the interaction of PPAR δ with the LDLR-PPRE sequence by performing EMSA. Using biotin labeled probes we demonstrated the binding of purified PPAR δ -RXR heterodimers to the 40 base pair region encompassing the LDLR promoter PPRE sequence (Fig. 2, lane 2). Pre-incubation of the LDLR probe-PPAR δ protein complex with anti-PPAR δ antibody resulted in a supershift of the binding complex (Fig. 2, lane 3) thus verifying that the band detected in lane 2 was specifically a result of the binding of LDLR-PPRE probe with the PPAR δ -RXR heterodimer. Competition with wild-type unlabeled probe resulted in almost complete quenching of the binding with the biotin-labeled probe, whereas competition with an unlabeled probe harboring the mutant PPRE was less effective in repressing the binding of PPAR δ -RXR complex to the LDLR-promoter probe (Fig. 2A, lane 4 & 5). These results demonstrated the direct interaction of the LDLR-PPRE sequence with PPAR δ in vitro.

To assess the potential interaction of endogenous PPAR δ with the LDLR promoter with and without activation by an agonist (L165041), we performed ChIP assay in HepG2 cells using a PPAR δ -specific antibody. Compared to the control IgG antibody, we observed significantly higher immunoprecipitation of the LDLR promoter region surrounding the PPRE-motif with anti-PPAR δ antibody (Fig. 2B). Importantly we observed a ~2-fold increase in the relative amount of LDLR promoter immunoprecipitating with the PPAR δ antibody after treatment with the 20 μ M of L165041. We have previously demonstrated that PPAR δ binds to the ACSL3 promoter and activates its transcription in response to treatment with L165041 (28). Consistent with our previous report we observed a significant enrichment of the ACSL3 promoter region surrounding the ACSL3-PPRE motif in the same DNA sample of chromatin immunoprecipitated with the PPAR δ antibody (Fig. 2C).

Activation of PPAR δ by agonist L165041 elevates LDLR mRNA expression in hepatic cells

Our promoter results indicated a positive role of PPAR δ in LDLR gene transcription. Thus, we examined the effects of L165041 on endogenous levels of LDLR mRNA. We treated HepG2 cells with different concentrations of L165041 and observed a dose-dependent increase in steady state expression of LDLR mRNA at 24 h post-treatment with L165041 (Fig. 3A). In addition to HepG2 cells, we treated mouse primary hepatocytes with 10 μ M and 20 μ M L165041 for 24 h and observed a significant elevation of nearly 50% in LDLR mRNA expression (Fig. 3B).

Utilizing HepG2 cells, we next analyzed the temporal dynamics of L165041 action and observed that LDLR mRNA expression is elevated starting 2 h after addition of L165041.

The mRNA expression follows the upward trend until 12 h post-treatment, attaining peak levels of expression and staying close to the maxima thereafter (Fig. 3C). Importantly, we observed a similar trend in the mRNA expression time course of CPT1 α , a canonical PPAR δ -target gene (Fig. 3D) further supporting the premise that following activation by the agonist, PPAR δ can induce transactivation of LDLR gene expression. To examine the possibility of the post-transcriptional machinery also contributing to the observed increase in LDLR mRNA, we next exposed control (DMSO) or L165041 treated HepG2 cells to actinomycin D. Measurement of LDLR mRNA half-life indicated that L165041 treatment did not affect the turnover rate of endogenous LDLR transcripts (Fig. 3E). Further examination of luciferase-tagged full length LDLR 3' untranslated region (UTR) also indicated no change in 3' UTR reporter activity after treatment with the PPAR δ agonist L165041 (Fig. 3F).

To further investigate whether the increase in LDLR mRNA translates to increased LDLR protein abundance, we examined the time-dependent and dose-dependent effects of PPAR δ activation on LDLR protein levels in HepG2 cells treated with L165041 at different doses or different times. We observed a gradual increase in LDLR protein content with increasing dosage of L165041 with 10 μ M, 20 μ M and 25 μ M concentrations resulting in ~25%, ~100% and ~150% increase in LDLR protein abundance respectively (Fig. 4A). Similar to time-dependent increases in LDLR mRNA levels, the LDLR protein amount in HepG2 cells also increased in a time-dependent manner (Fig 4B). At 12 h and 24 h following treatment with 20 μ M concentration of L165041, LDLR protein levels were elevated by ~66% and ~80% respectively. We also confirmed the inducing effect of PPAR δ activation on LDLR protein amounts in HepG2 cells that were treated with GW0742, another PPAR δ agonist (Fig. 4C). Furthermore, LDLR-mediated DiI-LDL uptake assays showed that the DiI-LDL intracellular fluorescence intensity increased upon PPAR δ activation by L165041 (Supplemental Fig. 1A) and by GW0742 (Supplemental Fig. 1B) in HepG2 cells, suggesting that the increased LDLR protein abundance by PPAR δ activation translated into a higher functional activity of receptor-mediated uptake of LDL particles from the culture medium.

Activation of PPAR δ by L165041 led to elevated expression of hepatic LDLR mRNA and protein in normolipidemic mice

To assess whether the effects of PPAR δ agonists on LDLR mRNA and protein expression observed in HepG2 cells could also operate in vivo, first, C57BL/6 mice fed a standard chow diet were administered L165041 at a daily dose of 20 mg/kg body weight for 14 days. On the last day of treatment, mice were fasted for 4 h, euthanized, and liver tissue and blood were collected. Analysis of LDLR mRNA and protein levels from liver samples demonstrated that L165041 treatment significantly increased both, protein and mRNA expression of hepatic LDLR (Fig. 5A & 5B). Protein expression of LDLR in the liver was elevated by ~58% compared to that observed in vehicle-treated animals. We also profiled serum lipid and PCSK9 levels, but did not observe significant differences either of the parameters between L165041 treated and the control groups in these normolipidemic mice (data not shown).

Activation of PPAR δ by L165041 increased hepatic LDLR levels and reduced circulating cholesterol levels in hyperlipidemic hamsters

Next, we examined the impacts of ligand-induced activation of PPAR δ on LDLR expression and on circulating lipid levels in a hyperlipidemic hamster model. Hamsters were fed a HFD for 8-weeks prior to the treatment of L165041 or vehicle for 7 days. Figure 6A showed that compared to hamsters fed a normal diet, feeding hamsters with the HFD substantially reduced liver LDLR protein levels in control animals. In contrast, one week treatment of HFD-fed hamsters with L165041 at a daily dose of 10 mg/kg led to approximately 29% increase in hepatic LDLR protein amount ($p<0.01$) and almost reversed the suppression induced by the HFD feeding. Importantly, the increase in liver LDLR protein abundance by L165041 treatment was accompanied by significant reductions of serum total cholesterol (Fig. 6B), serum triglycerides (Fig. 6C), and non-HDL-C (Fig. 6E) in these HFD-fed animals. We did not detect differences in HDL-C levels between vehicle and L165041-treated group (Fig. 6D).

DISCUSSION

In this study, we have identified a novel PPRE motif on human LDLR promoter that mediates the transcriptional activation of LDLR gene by PPAR δ . We have provided five pieces of evidence demonstrating its functional role. First, we showed that mutation of this PPRE site did not affect statin-induced LDLR promoter activity but severely disrupted the stimulation of LDLR promoter activity by PPAR δ agonist. Second, we demonstrated the direct binding of PPAR δ to this LDLR-PPRE sequence motif by gel shift assay under in vitro conditions and by ChIP assay in intact hepatic cells. We further showed that activation of PPAR δ by two different selective agonists L165041 and GW0742 increased LDLR mRNA levels, and subsequently, LDLR protein abundance and the DiI-LDL uptake activity. Our in vivo study in mice showed that treatment of mice with PPAR δ agonist led to significant increases in LDLR mRNA and protein levels in liver tissue. Lastly, utilizing a hyperlipidemic hamster model, we demonstrated the elevated liver LDLR expression and the correlative reduction of circulating non-HDL-C by PPAR δ activation.

The proximal 177 base-pair region of the LDLR promoter (–234 to +58) is considered to constitute the primary functional region containing several cis-regulatory elements critical for LDLR gene transcription⁽²⁹⁾. This region contains an SRE, the site of action of SREBP2, which directly binds to SRE and activates LDLR gene transcription in response to depletion of intracellular cholesterol levels⁽⁷⁾. Also present are two Sp1 transcription factor binding sites that maintain the basal level of transcription and a sterol-independent regulatory element (SIRE), which mediates the cytokine oncostatin M-induced activation of the LDLR gene^(30,31). However, whether other upstream promoter regions play a role in regulation of LDLR transcription has not been fully explored. In this study, we have focused on a putative PPRE-like motif located ~750 bases upstream of the transcription start site. Comparison of pLDLR-234 and pLDLR-1192 promoter activities in cells untreated and treated with L165041 first indicated that sequences upstream of the proximal promoter may be involved in PPAR δ agonist, L165041, mediated induction of LDLR promoter activity. Further investigation by site-directed mutagenesis of the PPRE-like motif demonstrated that

the PPRE sequence has functional relevance; we observed significant reduction in activity of the LDLR-1192 PPRE mutant promoter construct in response to L165041 as compared to the wild-type promoter construct. Importantly, we observed that RSV continued to significantly and equally induce LDLR promoter activity in wild-type and the PPRE mutant constructs. To our knowledge this is the first report identifying a functional PPRE motif involved in transactivation of LDLR gene expression.

The LDLR family comprises a group of endocytic receptors on the cell surface, which bind and internalize lipoprotein ligands containing chylomicron, low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), or very low-density lipoprotein (VLDL). The family members include LDLR, VLDLR, ApoER2, LRP1, LRP2 and LRP6 (³²). A previous study reported that rosiglitazone a potent PPAR γ agonist enhanced hepatic LRP1 expression via regulation of the PPRE motif in the human LRP1 promoter (³³). Another study conducted in mice has demonstrated the role of PPAR α specific ligand fenofibrate in upregulation of hepatic VLDLR, which is essential for lowering plasma TG (³⁴).

With regard to the effects of PPAR activation on LDLR expression, a previous report investigating the effects of the PPAR γ agonist pioglitazone found that activation of PPAR γ results in induction of LDLR gene expression via enhancing SREBP2 processing (¹³). Another report suggested that PPAR α mediated phosphorylation of AKT increased SREBP2 maturation and subsequent modulating of LDLR transcription (¹⁴). To assess whether the transactivation of LDLR by L165041 is a result of direct binding of PPAR δ to the PPRE-LDLR promoter region or whether it may be indirectly mediated by another factor, we performed EMSA and ChIP assays for PPAR δ binding. Our results demonstrate that recombinant human PPAR δ protein binds to biotin-labeled LDLR-PPRE probe and that specific PPAR δ antibody can immunoprecipitate the LDLR-PPRE region in whole cell lysate. PPAR δ is known to associate with nuclear co-repressors and bind to PPRE sites on the promoters where it can either function as a repressor or remain functionally inactive until ligand induced conformational changes that initiate a switch out of quiescence (35,36). Thus our observed ~2-fold greater interaction between PPAR δ and the LDLR promoter PPRE-site after treatment of HepG2 cells with the PPAR δ agonist L165041 is important for further supporting the role of PPAR δ acting as an activator of LDLR gene transcription.

To assess whether the effects of PPAR δ agonists observed in HepG2 cells and mouse primary hepatocytes were preserved in vivo, we administered 20 mg/kg/day of L165041 to wild-type C57BL/6 mice fed a normal diet. Although several reports indicate beneficial effects of PPAR δ agonists on serum cholesterol, most studies have been performed in transgenic mice or in animal models under diet-induced obesity or diabetes (24,26,37). Under normolipidemic conditions, we did not observe significant changes in serum cholesterol or triglycerides with the two-week dosing paradigm used in our experiment. However, administration of L165041 significantly induced hepatic LDLR mRNA and protein expression without affecting serum PCSK9 levels. Interestingly, a PPRE-like sequence is present in the 5' flanking region, about 894 base pairs upstream from the SRE-1 site of the mouse LDLR gene. Thus, it is conceivable to assume the upregulations of LDLR expression in mouse liver as well as mouse primary hepatocytes (Fig. 3B) by L165041

treatment are mediated through this cis-regulatory element embedded in mouse LDLR promoter region.

Our previous studies in hamsters have demonstrated that hamsters are sensitive to high fat diet and their lipid levels can be elevated in a relative short time to reach a stable hyperlipidemic stage (38). Therefore, we further examined the impacts of ligand-induced PPAR δ on hepatic LDLR function and the consequential effects on circulating LDL-C levels in hamsters fed a HFD. Our results clearly supported a new functional role of PPAR δ in the attenuation of diet induced hyperlipidemia through its beneficial effect on enhancing hepatic LDLR expression.

In summary, while SRE-mediated regulation remains critical for maintaining basal expression and cholesterol response of LDLR gene expression, our results identify an ancillary, SRE-independent PPAR pathway that may be utilized to modulate LDLR expression in combination with statin or other LDL-C lowering therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This study was supported by the Department of Veterans Affairs (Office of Research and Development, Medical Research Service) and by grants (1R01 AT002543-01A1 and 1R01AT006336-01A1) from National Center of Complementary and Alternative Medicine.

Abbreviations

ChIP	chromatin immunoprecipitation
EMSA	electrophoresis mobility shift assay
LDLR	LDL receptor
PCSK9	proprotein convertase subtilisin/kexin type 9
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
RSV	rosuvastatin
RXR	retinoid X receptor
SRE	sterol regulatory element
SREBP	SRE binding protein

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SUMMARY STATEMENT

PPAR δ activation beneficially regulates lipid metabolism. We have now identified a novel function of PPAR δ that increases LDL receptor gene transcription in hepatic cells in vitro and in vivo through direct binding to a PPRE motif on LDLR promoter.

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Figure 1. Human LDLR promoter contains a functional PPRE motif

(A) Diagrammatic representation of LDLR promoter luciferase reporter constructs.

(B) Relative luciferase activities from HepG2 cells transfected with pLDLR-1192, pLDLR-234, PPRE-Luc or pPCSK9-D4 promoter luciferase plasmids. Data represent summarized results (mean $\pm$ SEM) of 4–6 replicates per treatment and are expressed as ratio of Firefly/Renilla activity from each sample where the relative luminescence from DMSO treated cells is set to 1. *** $P < 0.001$ compared with DMSO treated samples. The data shown are representative of 3–5 separate transfection experiments.

(C) Relative luciferase activities from HepG2 cells transfected with pLDLR-1192 or pLDLR-PPREmut luciferase reporter plasmids. Data represent summarized results (mean $\pm$ SEM) of 4–6 replicates per treatment and are expressed as ratio of Firefly/Renilla activity from each sample where the relative luminescence for DMSO treated cells from each group is set to 1. ** $P < 0.01$ and *** $P < 0.001$ compared with DMSO treated samples from the same group. # $P < 0.05$ compared to signal from pLDLR-1192 from same treatment group.

Figure 2. EMSA and ChIP analyses of PPAR δ association with PPRE site of LDLR promoter in vitro and in vivo

(A) Chemiluminescent signal from cross linked nylon membrane where biotin-5' end labeled LDLR-PPRE probe was incubated with 300 ng of PPAR δ and 100 ng of RXR α recombinant proteins in the absence (lane 2) or presence of 100-fold molar excess of unlabeled wild-type probe (lane 4) or mutated probe (lane 5). The binding reaction was also carried out in the presence of 1 μ g of anti-PPAR δ antibody (lane 3). The data shown are representative of 2 separate EMSA assays with similar results.

(B) ChIP analysis on HepG2 cells treated with DMSO or L165041 (20 μ M) where rabbit anti-PPAR δ or isotype IgG antibody immunoprecipitated DNA samples was PCR amplified

with primers specific for the LDLR-PPRE promoter region. The PCR product was separated on a 2% agarose gel and stained with ethidium bromide. Values represent ratio of measurements of relative densitometric intensity of PCR product amplified from PPAR δ /IgG antibodies normalized to the intensity observed in corresponding input samples, where the relative intensities from IgG immunoprecipitated samples was arbitrarily set to 1. The data shown are representative of 2 separate ChIP assays with similar results.

(C) ChIP analysis on HepG2 cells treated with DMSO or L165041 (20 μ M) where rabbit anti-PPAR δ or isotype IgG antibody immunoprecipitated DNA samples was PCR amplified with primers specific for the ACSL3-PPRE promoter region.

Figure 3. PPAR δ agonist L165041 induces LDLR mRNA expression

HepG2 cells were seeded in 24-well cell culture plates and treated with L165041 as described in the Methods section. Total RNA was collected from treated cells for real-time PCR analyses.

(A) Real-time PCR quantification of LDLR mRNA expression from HepG2 cells treated with L165041 at indicated concentrations and duplicate wells were used for each concentration. Bars represent (mean $\pm$ SEM) of two RNA samples with triplicate measurement per RNA sample. The data shown are representative of two independent assays.

(B) Mouse primary hepatocytes in triplicate wells were treated with indicated concentrations of L165041 or vehicle control DMSO, or untreated. LDLR expression levels were

normalized to GAPDH mRNA levels where the relative expression of LDLR mRNA in untreated cells was set at 1. * $P < 0.05$ compared with untreated samples.

(C & D) Bars represent summarized (mean $\pm$ SEM) real-time PCR quantification of LDLR (C) and CPT1 α (D) mRNA expression normalized to GAPDH levels from HepG2 cells treated with DMSO (control) or L165041 (20 μ M) where the relative expression of each gene at 0 h was set at 1. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to normalized expression levels of the corresponding gene at 0 h.

(E) HepG2 cells were treated with L165041 (20 μ M) or DMSO for 16 h. Actinomycin D was added to cells for different intervals. Total RNA was isolated and analyzed for the amount of LDLR mRNA and GAPDH mRNA by real-time PCR. The normalized LDLR mRNA levels were plotted as the percentage of the mRNA remaining. Decay curves were plotted versus time. The LDLR mRNA half-lives were calculated from three separate experiments (mean $\pm$ SEM, $n = 3$ per treatment).

(F) Relative luciferase activities from HepG2 cells transfected with pcDNA3.1 or LDLR-3' UTR reporter plasmids. Data represent summarized results (mean $\pm$ SEM) of 4–6 replicates per treatment and are expressed as ratio of Firefly/Renilla activity from each sample where the relative luciferase activity for DMSO treated cells is set to 1.

Figure 4. PPAR δ agonists induce LDLR protein expression

(A – C) Western blotting with antibodies to LDLR and β -actin was conducted by analyzing individual homogenates from HepG2 cells treated with DMSO (control), L165041 (at indicated doses and times) or GW0742 (10 μ M). Values represent mean of densitometric measurements of LDLR normalized to β -actin signal from duplicate samples per treatment.

Figure 5. Administering L165041 to mice increases hepatic LDLR mRNA and protein levels Wild-type, 8–10 week old, male C57BL/6 mice (n = 6 animals per treatment) were gavaged daily with 20 mg/kg of L165041. Four hour-fasted liver samples were collected 14 days after initiation of drug treatment.

(A) Western blotting with antibodies to LDLR and β-actin was conducted. Values are the mean ± SEM of five samples per group. ** $P < 0.01$, compared with the control group.

(B) Quantitative real-time PCR was used to determine the relative expression levels (mean ± SEM, n = 6) of LDLR mRNA after normalization with GAPDH mRNA levels. * $P < 0.05$, compared with the control group which was set at 1.

Figure 6. PPAR δ agonist induces hepatic LDLR protein expression and reduces serum non-HDL-C levels in hyperlipidemic hamsters

Hamsters fed a HFD were treated with 10 mg/kg of L165041 (n = 5) or with vehicle (n = 5) for 7 days. Another group of hamsters (n=4) were fed a normal chow diet for 8 weeks. At the end of drug treatment, all animals were sacrificed for serum and liver tissue collection. In A, total protein extracts were individually prepared from 4 randomly chosen liver samples of each group. Equal amounts of homogenate proteins (50 μ g) were resolved by SDS-PAGE and LDLR protein was detected by immunoblotting. The membrane was reprobed with anti-

β -actin antibody. The protein abundance of LDLR was quantified and normalized by signals of β -actin. Values are mean $\pm$ SEM of 4 samples per group.

(B–D) TC, TG and HDL-C levels in hamster sera were measured after the treatment of L165041 or the vehicle and in NCD group. The concentrations of non-HDL-C were derived after subtraction of HDL-C from total cholesterol. Data are mean $\pm$ SEM of 4–5 hamsters per group. ** $p < 0.01$ and *** $p < 0.001$ for comparing differences between HFD and NCD; # $p < 0.05$ and ## $p < 0.01$ for comparing the differences between L165041 and vehicle.

TABLE 1

List of primers used in this study

Human primers	5' – 3'
LDLR- Forward	GACGTGGCGTGAACATCTG
LDLR- Reverse	CTGGCAGGCAATGCTTTGG
GAPDH- Forward	ATGGGGAAGGTGAAGGTCG
GAPDH- Reverse	GGGGTCATTGATGGCAACAATA
Mouse primers	5' – 3'
LDLR- Forward	TCGTAGTGGACCCTGTGCAT
LDLR- Reverse	GGAAAGATCTAGTGTGATGCCATT
GAPDH- Forward	ATGGTGAAGGTCGGTGTGAA
GAPDH- Reverse	ACTGGAACATGTAGACCATGTAGT
EMSA probes	5' – 3'
LDLR-PPRE(WT)- Forward	TCTCTGTGGCTTAGGGGTTCAAGTTCAACTGTGAAAGCCC
LDLR-PPRE(WT)- Reverse	GGGCTTTCACAGTTGAACTTGAACCCCTAAGCCACAGAGA
LDLR-PPRE(mut)- Forward	TCTCTGTGGCTTAGGGGTTACCAACAACCTGTGAAAGCCC
LDLR-PPRE(mut)- Reverse	GGGCTTTCACAGTTGTTGGTGAACCCCTAAGCCACAGAGA
ChIP primers	5' – 3'
LDLR-PPRE-Forward	GCAACCCCATGAGTCCCC
LDLR-PPRE-Reverse	GAACAAACAGAAGGGCGGTTTC
ACSL3-PPRE-Forward	CAAGTTCTGGCGGCTTCCTG
ACSL3-PPRE-Reverse	CCCAGTACTAGTAGGATTGGTCTC