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Sterol-independent repression of low density lipoprotein receptor promoter by peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α)

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Abbreviations: E2, 17β-ethinyl estradiol; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; ERE, estrogen response element; LDLR, low density lipoprotein receptor; moi, multiplicity of infection; p38-MAPK, p38-mitogen activated protein kinase; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, PPARγ coactivator-1α; PPARγ, peroxisome proliferator activated receptor γ; SRE-1, sterol regulatory element-1; SREBP, SRE-1 binding protein; TZD, troglitazone

Abstract

Peroxisome proliferator activated receptor (PPAR) γ coactivator-1α (PGC-1α) may be implicated in cholesterol metabolism since PGC-1α co-activates estrogen receptor α (ERα) transactivity and estrogen/ERα induces the transcription of LDL receptor (LDLR). Here, we show that overexpression of PGC-1α in HepG2 cells represses the gene expression of LDLR and does not affect the ERα-induced LDLR expression. PGC-1α suppressed the LDLR promoter-luciferase (pLR1563-luc) activity regardless of cholesterol or functional sterol-regulatory element-1. Serial deletions of the LDLR promoter revealed that the inhibition by PGC-1α required the LDLR promoter regions between -650 bp and -974 bp. Phosphorylation of PGC-1α may not affect the suppression of LDLR expression because treatment of SB202190, a p38 MAP kinase inhibitor, did not reverse the LDLR down-regulation by PGC-1α. This may be the first report showing the repressive function of PGC-1α on gene expression. PGC-1α might be a novel modulator of LDLR gene expression in a sterol-independent manner, and implicated in atherogenesis.

Keywords: cholesterol; liver; peroxisome-proliferator-activated receptor-γ coactivator-1; PPARγ; promoter regions, genetic

Introduction

Peroxisome proliferator activated receptor gamma coactivator-1α (PGC-1α) is an emerging co-activator, which plays a role in regulation of adaptive thermogenesis, cellular respiration, and energy metabolism (Wu *et al*., 1999; Herzig *et al*., 2001; Rhee *et al*., 2003). Recent studies demonstrated that PGC-1α might be one of central regulators in transcription of various genes involved in glucose (Yoon *et al*., 2001; Rhee *et al*., 2003) or lipid (Barbera *et al*., 2001; Louet *et al*., 2002) metabolism. Overexpression of PGC-1α increases the biogenesis of mitochondria and energy expenditure program of cells. PGC-1α is induced in fasted or diabetic liver and activates the expression of key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (Yoon *et al*., 2001). Interestingly, PGC-1α is elevated in pancreatic islets of animal models of type 2 diabetes and negatively regulates insulin secretion. Based on this observation, Yoon *et al*. (2003) suggested that PGC-1α might play a key role in the pathogenesis of the diabetic phenotype. PGC-1α also induces the expression of liver carnitine palmitoyltransferase I (L-CPT I) (Louet *et al*., 2002), which catalyzes the transfer of long-chain fatty acids into mitochondria and is considered the rate-controlling enzyme in fatty acid oxidation.

It is possible for PGC-1α to be involved in cholesterol metabolism. Two reports demonstrated that the transcription of CYP7A1 encoding cholesterol 7α-hydroxylase, the rate limiting enzyme of bile acid biosynthesis from hepatic cholesterol, was activated by PGC-1α in cooperation with other transcription factors such as hepatocyte nuclear factor-4α (HNF-4α) and chicken ovary upstream promoter-transcription factor II (COUP-TFII) (De Fabiani *et al*., 2003; Shin *et al*., 2003). To achieve a fine control of cholesterol homeostasis, the sterol responsive genes involved in cholesterol biosynthesis (HMG-CoA reductase), uptake (low density

lipoprotein receptor, LDLR) and catabolism (CYP7A1) are coordinately regulated. In addition, PGC-1α is a co-activator of ERα on estrogen responsive element (ERE)-driven transactivity through direct protein-protein interaction (Tcherepanova *et al*., 2000). Since it is well known that treatment of estrogen *in vivo* or the activation of estrogen receptor-α (ERα) *in vitro* up-regulates LDLR gene expression at transcriptional level (Cooper *et al*., 1987; Distefano *et al*., 2002), we purport the hypothesis that PGC-1α may modulate the LDLR gene expression.

Sterol regulation of the LDLR promoter requires the concerted action of two proteins: the sterol-regulated SREBP and the generic co-regulator Sp1. The binding sites for SREBP and Sp1 are present on the proximal region (within +58 ~-234bp) of LDLR promoter. Cholesterol deficiency cleaves SREBP residing in endoplasmic reticulum, and the truncated SREBP increases the DNA binding of Sp1 to the LDLR promoter (Yieh *et al*., 1995; Xiong *et al*., 2000). Estrogen, a beneficial hormone for cardiovascular diseases, binds to ERα to up-regulate LDLR transcription. However, ERα does not seem to activate LDLR by direct binding to LDLR promoter because it does not contain any consensus or near-consensus estrogen response element (ERE). Instead, it was suggested that the LDLR promoter region near SRE-1 was responsible for the estrogen effects (Croston *et al*., 1997), indicating that ERα may activate the promoter indirectly, through transcriptional activation of SREBP or other factor(s) that regulate LDLR expression (Distefano *et al*., 2002; Bruning *et al*., 2003).

In this study, we investigated whether overexpression of PGC-1α regulated the LDLR promoter-driven transactivity in the relation with ER-α or SREBP using the LDLR promoter-luciferase (pLDLR1563-luc) reporter constructs. We demonstrated that PGC-1α did not co-activate the ER-α-mediated LDLR transcription, but repressed the transcriptional activity of LDLR promoter. The inhibitory mechanism of PGC-1α was independent of cholesterol and SRE-1. Furthermore, the serial deletion of LDLR promoter revealed that the distal LDLR promoter region between -650bp and -974bp was responsible for the PGC-1α-mediated inhibition. This promoter region contains a putative three E-box (CANNTG) and a TRE (AAAGGCGG) sequences. To our knowledge, this is the first report showing that PGC-1α is a negative regulator in LDLR gene expression and may play a role in atherogenesis.

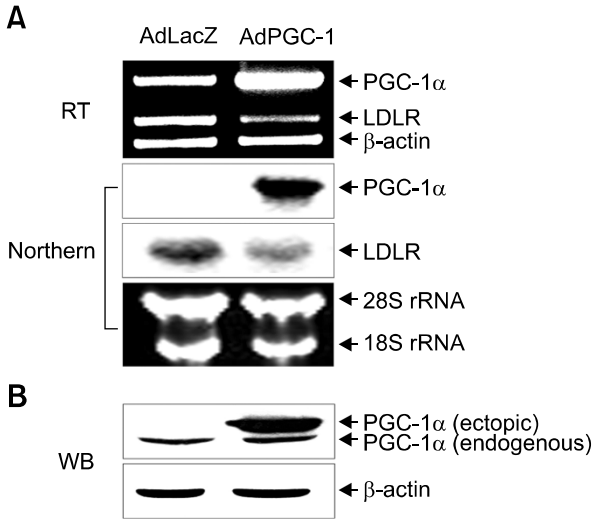

Figure 1. Repression of endogenous LDLR mRNA by overexpression of PGC-1α. HepG2 cells were infected with AdLacZ or AdPGC-1α for 48 h at a multiplicity of infection of 50. Total RNA or cell lysate was prepared from the cells. RT-PCR (RT), Northern blot (Northern) and Western blot (WB) analyses of PGC-1α or LDLR in HepG2 cells were executed as described in "Methods". A. The PCR fragments or hybridized signals of PGC-1α, LDLR and β-actin are shown. Equivalent loading of RNA was verified by the β-actin PCR product or 28S and 18S rRNAs on the agarose gel stained with ethidium bromide. B. The antibody-directed PGC-1α bands (upper band, the ectopically expressed PGC-1α with extra 35 amino acids; lower band, endogenous PGC-1α) are as shown (WB). Equivalent loading of protein was verified by β-actin protein.

Results

Ectopic expression of PGC-1α decreases the endogenous LDLR mRNA

To examine directly the ability of PGC-1α to regulate LDLR, we have utilized adenoviral vectors to express the PGC-1α protein in HepG2 cells. The cells grown in media containing 10% FBS were infected at a moi of 50 with AdLacZ or AdPGC-1. Forty-eight hours after infection, total RNA was extracted from the cells and RT-PCR and Northern blot were performed to detect mRNA for PGC-1α, LDLR or β-actin. As shown in Figure 1, PGC-1α mRNA was approximately 10-fold increased in the cells infected with AdPGC-1 compared to those with AdLacZ alone. However, the level of LDLR mRNA was 60% decreased in the PGC-1α- overexpressing cells, while the β-actin mRNA remained unchanged. The increase of PGC-1α mRNA was verified with the up-regulation of PGC-1α proteins.

PGC-1α represses LDLR promoter-dependent transcription in an estrogen-independent manner

PGC-1α works as a transcriptional co-activator of several nuclear receptors, such as ERα (Tche-

repanova *et al*., 2000), PPARs (Puigserver *et al*., 1998), and retinoid X receptor (Delerive *et al*., 2002). In order to check whether the cloned full-length PGC-1α, which contains 35 extra amino acids at N-terminus, enhances the estradiol- dependent transactivity of ERα as reported, we performed transient co-transfection assay with mammalian expression vectors of ERα and PGC-1α and pERE(3×)-luciferase in HepG2 cells. As expected, ERα or PGC-1α alone activates the reporter activity (Figure 2A). When PGC-1α and ERα were co-expressed, the ERE-dependent transcriptional activity was synergistically elevated. ERα activity was increased depending on the presence of estradiol.

However overexpression of PGC-1α inhibited the transcriptional activity of pLR1563-luc, a luciferase reporter bearing LDLR promoter region from +58 to -1563bp, up to 80% whereas ERα activated pLR1563-luc in the presence of estradiol (Figure 2B). Suppression of the transcriptional activity by PGC-1α remained unchanged by adding estradiol to the cells. Co-expression of ERα reversed the PGC-1α-mediated inhibition but there was no synergistic activation of LDLR promoter activity. The results suggested that despite the LDLR transcription was induced by estrogen or ERα overexpression, PGC-1α might not play any role as a co-regulator in the ERα-mediated LDLR activation.

Repression of LDLR promoter by PGC-1α is sterol-independent

Serum depletion or lipoprotein deficiency strongly induces the LDLR expression in cultured HepG2 cells (Pak *et al*., 1996). To examine if PGC-1α-mediated inhibition of LDLR promoter activity would be altered depending on the presence of serum factor(s) or cholesterol, the effects of PGC-1α overexpression were determined in HepG2 cells cultured in media containing 10% FBS, 0% FBS (serum-free), or 10% LPDS. As shown in Figure 3A, neither of culture conditions abolished the PGC-1α-induced LDLR inhibition. Serum depletion or LPDS, which was a signal for SREBP activation, recovered LDLR transcription as compared to that of 10% FBS. When 25-hydroxycholesterol, a turn-off signal for LDLR expression, was added in HepG2, the LDLR promoter activity was too low to measure the inhibitory effect of PGC-1α (data not shown). The data presented that the repression of LDLR promoter activity by PGC-1α might be independent of the cholesterol availability and provided another line of evidence that non-sterol factor(s) could regulate LDLR promoter activity.

SRE-1 is not necessary for the inhibitory effect of PGC-1α

The proximal region of LDLR promoter contains SRE-1 element that is essential for sterol-dependent regulation of LDLR. SREBP binds to SRE-1 to enhance the transcription of LDLR in response to deficiency of cholesterol. Although many factors other than cholesterol also regulate LDLR expression, the proximal region (below -234bp) including SRE-1 has been a major player to control the LDLR expression. We have previously shown that the distal region of LDLR promoter may be invol-

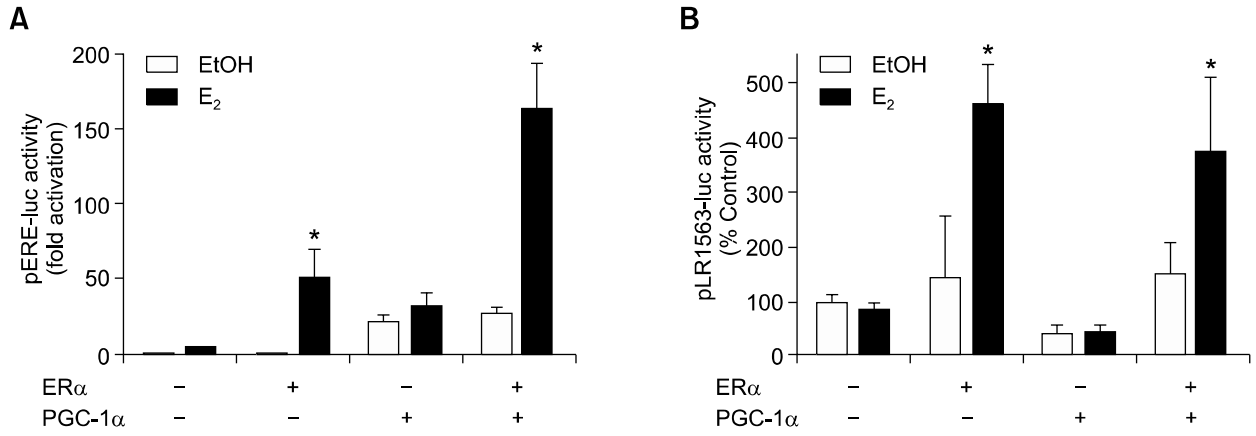

Figure 2. Repression of LDLR promoter activity by PGC-1α that co-activates the ERα/ERE-dependent transcription. HepG2 cells were transfected with *LacZ* expression vector (400 ng), expression vectors (400 ng) for PGC-1α and/or ERα, and a reporter gene (800 ng), pERE-luc (panel A) or pLR1563-luc (panel B), as indicated. The cells were treated for 24 h with either vehicle only (EtOH, ethanol) or 17β-ethinyl estradiol (E_2, 10^{-8} M) in phenol red-free MEM with 10% charcoal-treated FBS and harvested for luciferase assay. Normalized luciferase expressions from triplicate samples were calculated relative to the *LacZ* expressions, and the results were expressed as n-fold activation or % control over the value obtained with the reporter alone in EtOH. Values are mean ± SD of three independent duplicate experiments. *: $P < 0.05$ vs. the vehicle-treated.

ved in hepatocyte growth factor- or serum factor-induced upregulation of LDLR (Pak *et al*., 1996), but any *trans*-acting factor(s) or cis-acting element(s) for repression of LDLR expression have not been elucidated. Here, we studied whether SRE-1 was required for repression of LDLR transcription by PGC-1α. We constructed two mutants of LDLR promoter-luciferase reporter; dSRE-luc that lacks SRE-1 and mSRE-luc of which SRE-1 region was substituted for scrambled sequences to maintain the length of promoter. The basal promoter activities of both mutant constructs were only 20% of the wild type pLR1563-luc (WT). But the transcriptional activities of all three LDLR promoters were inhibited by overexpression of PGC-1α (Figure 4). These results clearly confirmed that SRE-1 or SREBP was not necessary for suppression of LDLR expression by PGC-1α, and suggested that PGC-1α might not affect the complex formation of SREBP and Sp1.

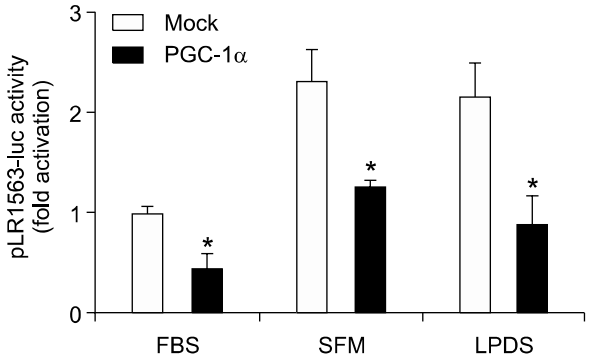

Figure 3. PGC-1α repressed the LDLR promoter activity regardless of cholesterol. HepG2 cells were transfected with *LacZ* expression vector (400 ng), expression vectors (400 ng) for PGC-1α or mock vector (pcDNA3.1/HisC), and pLR1563-luc, as indicated. The transfected cells were incubated for 24 h in media containing 10% FBS (FBS), 10% lipoprotein deficient serum (LPDS) or serum free media (SFM), and harvested for luciferase assay. Normalized luciferase expressions from triplicate samples were calculated relative to the *LacZ* expressions, and the results were expressed as n-fold activation over the value obtained with the reporter alone. Values are mean ± SD of three independent duplicate experiments. *: $P < 0.05$ vs. mock control.

PGC-1α required the LDLR promoter region between -650bp and -974bp for inhibition of LDLR transcription

We constructed the LDLR promoter-luciferase construct series (pLR1326, pLR974, pLR650, and pLR234) by sequential deletion to verify which LDLR promoter region was responsible for repression by PGC-1α co-expression. As shown in Figure 5, PGC-1α co-expression repressed luciferase activity of pLR1326 and pLR974 as same as pLR1563, while it did not repress the activity of pLR650 and pLR234. The result suggested that the LDLR promoter region between -650bp and -974bp was required for the PGC-1α-mediated repression of LDLR transcription. In addition, the known *cis*-acting elements of SRE-1 and Sp1 binding sites were not involved in the LDLR repression.

Dominant positive of SREBP-2 overrules the inhibitory effect of PGC-1α

Next, we explored the influence of dominant positive of SREBP-2 (N-SREBP-2, N-terminus of SREBP-2) on the inhibitory effect of PGC-1α. Addition of N-SREBP-2 on top of the PGC-1α overrode the

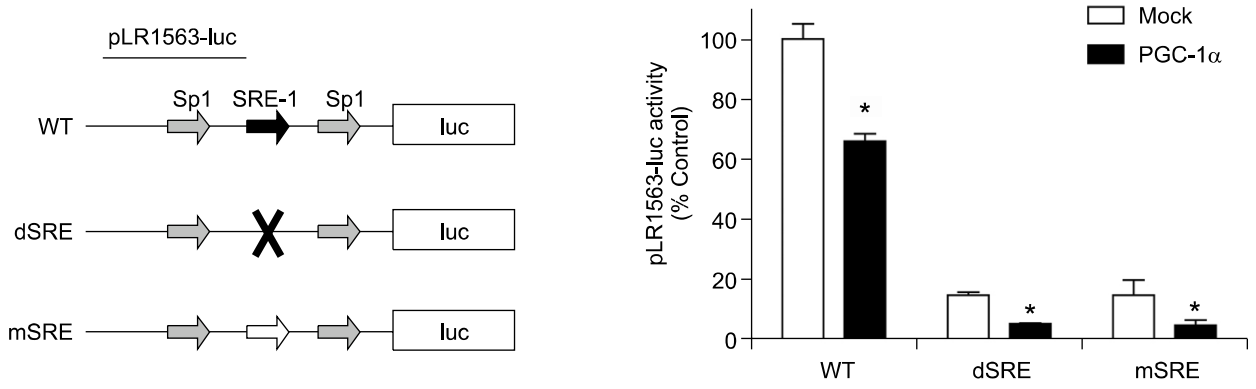

Figure 4. SRE-1 is not required for PGC-1α-mediated inhibition of LDLR promoter activity. HepG2 cells were transfected with *LacZ* expression vector, expression vectors (400 ng) for PGC-1α or mock vector (pcDNA3.1/HisC), and one of three LDLR promoter reporter genes (800 ng), wild type pLR1563-luc (WT), pLR1563 without SRE-1 (dSRE-luc), pLR1563 with scrambled SRE-1 (mSRE-luc), as indicated. Normalized luciferase expressions from triplicate samples were calculated relative to the *LacZ* expressions, and the results were expressed as % control over the value obtained with the reporter alone. Values are mean ± SD of three independent duplicate experiments. *: $P < 0.05$ vs. mock control.

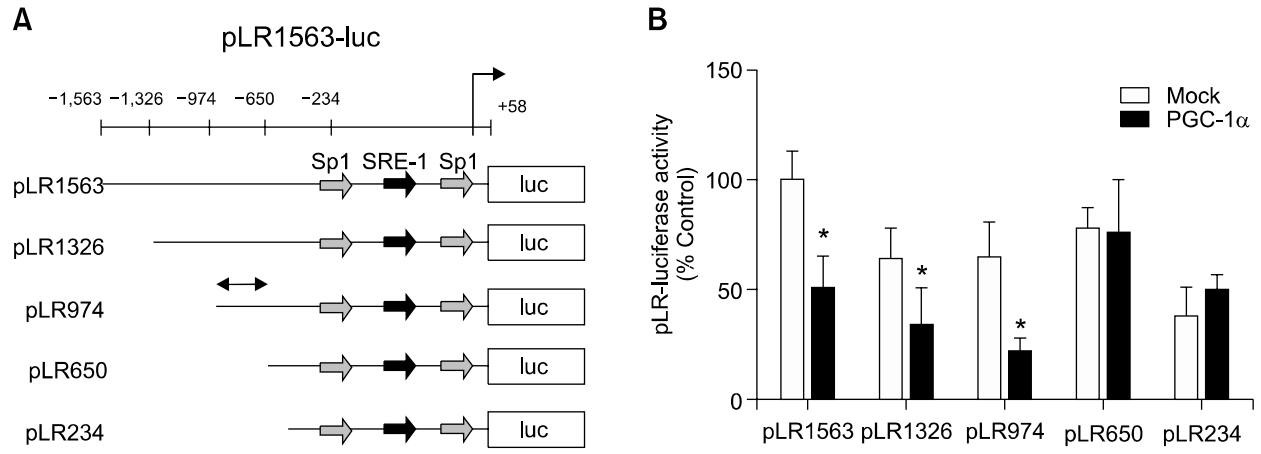

Figure 5. Identification of LDLR promoter region that is responsible for PGC-1α-mediated suppression. The original pLR1563-luc and the indicated series of 5'-deletion constructs linked to luciferase were cotransfected into HepG2 cells with *LacZ* expression vector, a PGC-1α expression vector or the pcDNA3.1/HisC mock vector alone. Normalized luciferase values (% Control) were averaged from three independent experiments. Arrows represent positions of two Sp1 and one SREBP binding sites of LDLR promoter. The results revealed that the PGC-1α-mediated suppression of LDLR promoter activity required the promoter region between -650 and -974. Values are mean ± SD of three independent duplicate experiments. *: $P < 0.05$ vs. mock control.

repressive effect by PGC-1α on LDLR transcription to the level of N-SREBP-2 alone (Figure 6A). Interestingly, a dose-dependent reactivation was not observed but small amount (200ng) of N-SREBP-2 could completely reverse the repression by 800ng PGC-1α and further maintained the LDLR promoter activity. Enhancement of LDLR transcription by N-SREBP-2 was 4 times stronger than the repression by PGC-1α in respect to molecular ratio. This result implies again that the LDLR repression by PGC-1α may exert separately from the SREBP-mediated LDLR induction. It was concluded that PGC-1α might be a novel sterol-independent modulator of LDLR regulation although its repressive function was mild compared to cholesterol itself.

PPARγ activation had no effect on PGC-1α-repressed transcription

PGC-1α co-activates the PPARγ activity. Activation of PPARγ by its ligand, such as troglitazone (TZD) or by overexpression may increase the activity of PGC-1α. However, the TZD treatment or PPARγ overexpression did not alter the repression of LDLR promoter activity by PGC-1α (Figure 6B). It

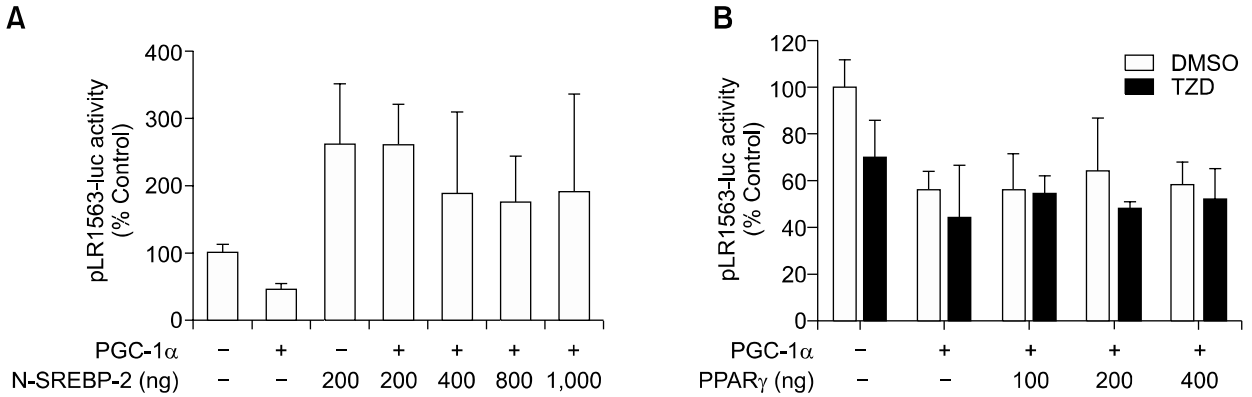

Figure 6. Effect of N-SREBP-2 dominant positive or PPARγ activation on the PGC-1α-repressed LDLR promoter activity. (A) Overexpression of N-SREBP2 dominates the repressive effect of PGC-1α. HepG2 cells were transfected with *LacZ* expression vector, expression vectors for PGC-1α (400 ng) and SREBP-2 dominant positive (N-SREBP-2), and pLR1563-luc (800 ng), as indicated. (B) Activation of PPARγ did not alter the inhibition by PGC-1α. HepG2 cells were transfected with *LacZ* expression vector, expression vectors for PGC-1α (400 ng) and PPARγ, and pLR1563-luc (800 ng), as indicated. The cells were treated for 24 h with either vehicle only (DMSO) or a PPARγ ligand troglitazone (TZD, 10 μM) in serum free media and harvested for luciferase assay. Normalized luciferase expressions from triplicate samples were calculated relative to the *LacZ* expressions, and the results were expressed as % control over the value obtained with the reporter alone. Values are mean ± SD of three independent duplicate experiments.

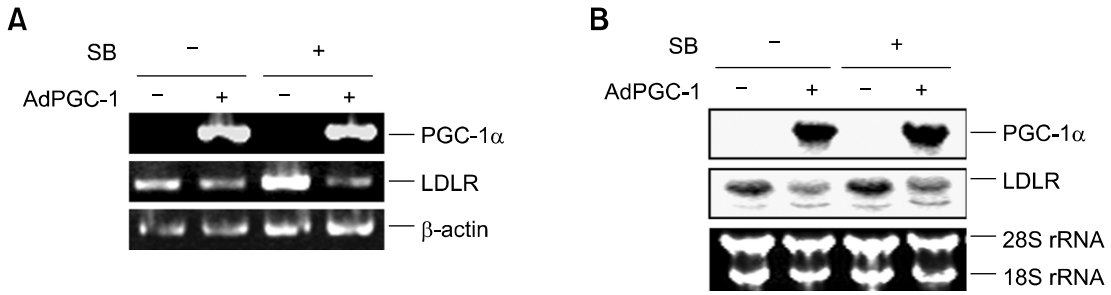

Figure 7. Inhibition of p38-MAPK had no effect on the PGC-1α-repressed LDLR mRNA expression. HepG2 cells were infected with AdLacZ alone (AdPGC -) or AdPGC-1 (AdPGC +) for 24 h at a multiplicity of infection of 50. The infected cells in serum free media were then incubated with p38-MAPK inhibitor SB202190 (20 μM) or vehicle DMSO for 24 h, and harvested for total RNA preparation. RT-PCR (A) and Northern blot (B) analyses of PGC-1α or LDLR in HepG2 cells. The PCR fragments or hybridized signals of PGC-1α, LDLR and β-actin are shown. Equivalent loading of RNA was verified by (A) the β-actin PCR product or (B) 28S and 18S rRNAs on the agarose gel stained with ethidium bromide.

was concluded that PGC-1α might function on LDLR promoter regardless of PPARγ activity.

PGC-1α still repressed the LDLR expression in the presence of p38-MAPK inhibitor

Knutti *et al*.(2001) reported that phosphorylation of PGC-1α by p38-MAPK might favor the release of the unidentified repressor, thereby enhancing the PGC-1α activity as a co- activator of glucocorticoid receptor. On the other hand, activation of p38-MAPK negatively regulated LDLR expression (Singh *et al*., 1999). In order to test the effect of p38-MAPK-mediated phosphorylation of PGC-1α on LDLR expression, HepG2 cells with or without PGC-1α overexpression were treated with a specific p38-MAPK inhibitor SB202190 (20 μM) in serum free media and effects on LDLR mRNA levels were assayed by RT-PCR and Northern blotting. Overexpression of PGC-1α represses the LDLR expression in the absence of SB202190, which is in agreement with the results shown in Figure 1. Inactivation of p38-MAPK by SB202190, which was reported to prevent phosphorylation of PGC-1α, induces the mRNA level of LDLR as reported (Figure 7). However, the treatment of SB202190 did not reverse the repression of LDLR expression by overexpression of PGC-1α. The result implies that the repressive activity of PGC-1α on LDLR promoter may be independent of

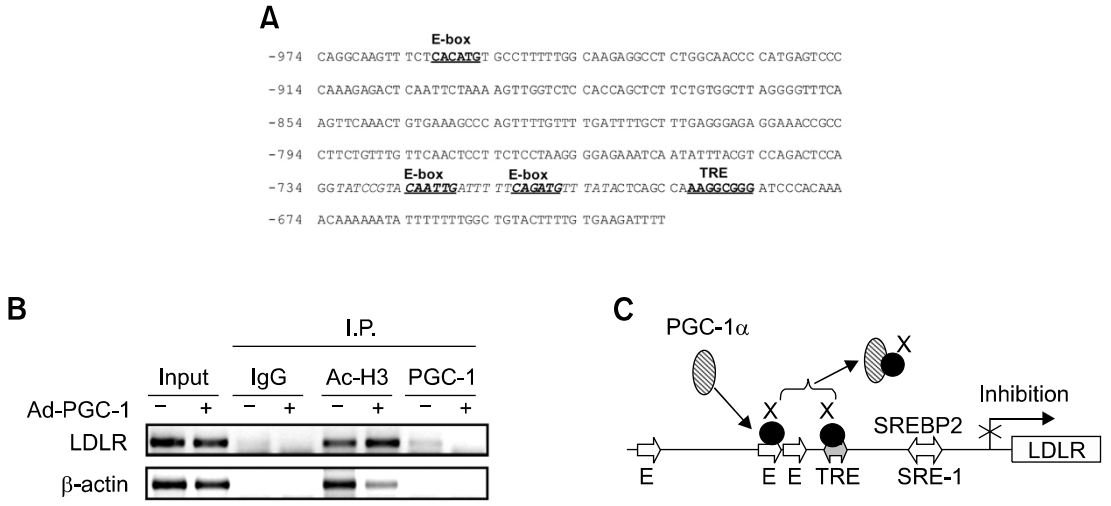

Figure 8. PGC-1α may interact with the LDLR distal promoter through unknown factor. (A) The distal LDLR promoter (-974 to -633) contains E-box and AP1 binding motifs. (B) ChIP assay. HepG2 cells were infected with Ad-PGC-1 or Ad-LacZ control. After 36 h, chromatin-bound DNA was immunoprecipitated with polyclonal antibodies against PGC-1α, normal rabbit IgG (IgG, negative control) or acetyl-histoneH3 (Ac-H3, positive control). Immunoprecipitated DNA was analyzed by PCR using primer sets for the LDLR promoter regions between -974 and -650bp and β-actin coding region as a negative control. Ten percent of the soluble chromatin used in the reaction was used as inputs. C. A putative model of LDLR gene suppression by PGC-1α via dissociation of unknown factor (X) is suggested.

p38-MAPK-mediated phosphorylation. Furthermore, it is possible that PGC-1α suppress the same signaling pathway of LDLR induction as those activated by p38-MAPK inactivation. It also suggests that the PGC-1α-repressed mechanisms and signals on LDLR expression may be different from those of other nuclear receptor-mediated transcription co-activation.

Three E-box and TRE consensus sequences are present between -974 and -681 bp region of LDLR promoter

Since the region between -974 and -681bp was responsible for the PGC-1α-mediated suppression of LDLR transcription, we searched for potential consensus sequences of transcription factors in the region to investigate the potential candidate repressor(s). Interestingly, one AP1 binding (TRE) and three E-box (CANNTG) sites were present between -974 and -681bp (Figure 8A). We assumed that the basic helix-loop-helix (bHLH) family of E-box binding proteins contain a group of transcription factors that includes fos-related antigen 2 (Fra-2) (Yang *et al*., 2002), Hairy Enhancer of Split (HES-1), DEC1 and CLOCK (Herzig *et al*., 2003). In gene profiling assay, none of these protein expressions were altered in AdPGC-1α-infected HepG2 cells relative to control (data not shown).

On the other hand, chromatin immunoprecipitation (ChIP) assay demonstrated that PGC-1α weakly interacted with LDLR distal promoter and this direct binding was abolished in AdPGC-1 infected cells (Figure 8B). The results imply that binding affinity of the unknown E-box or TRE binding protein to the promoter region may be decreased by PGC-1α overexpression (a putative scheme in Figure 8C). An intensive further study might be necessary to identify unknown factor(s) which might interact with E-box or form an AP1 transcription complex to induce the LDLR expression and dissociate from the promoter DNA after interaction with PGC-1α.

Discussion

In this work, we provide clear evidences for a repressive role of PGC-1α on LDLR gene expression using co-transfection and LDLR promoter reporter assay. Overexpression of PGC-1α was sufficient to suppress the levels of LDLR mRNA and LDLR promoter activity in HepG2 cells. The distal region of LDLR promoter between -974bp and -650bp was responsible for the gene suppression by PGC-1α. PGC-1α decreased the LDLR promoter binding affinity of Fra-2 which might bind to E-box of LDLR distal promoter. The mechanism of PGC-1α-mediated repression of LDLR expression seems to be separated from those of the known SREBP-dependent feedback regulation or the ERE/ERα-dependent stimulation.

The regulation of LDLR transcription by sterols has been extensively delineated (Hua *et al*., 1993; Wang *et al*., 1993, 1994). Lack of intracellular cholesterol signals to cleave high molecular weight SREBP on endoplasmic reticulum and let the cleaved N-SREBP move into nuclei to activate LDLR transcription. In contrast, abundance of intracellular cholesterol does not increase the levels of nuclear N-SREBP, and consequently turns off the LDLR transcription. Although SREBP is a dominant regulator for LDLR transcription, non-sterol factors such as hormones (Rudling *et al*., 1996), growth factors (Mazzone *et al*., 1990; Pak *et al*., 1996) and cytokines (Stopeck *et al*., 1993) are also suggested to control the LDLR expression. It is not yet clear whether the non-sterol regulators activate any other unidentified factor(s) or interact with SREBP to modulate the LDLR expression.

Signals to activate gene transcription can be in general transduced to three transcription regulators; transcription factors, basic transcription machinery such as RNA polymerase, and co-regulators. For LDLR regulation, not many *cis*-acting element(s) or trans-acting factor(s) except SRE-1 and SREBP have been identified. For example, Yin Yang 1 protein was reported to disrupt the complex of Sp1 and SREBP on LDLR promoter, resulting in reduction of LDLR transcription (Bennett *et al*., 1999). A promoter region near TATA box was identified as a *cis*-element for oncostatin M-induced, sterol-independent activation of LDLR (Liu *et al*., 2000). Another proximal promoter region between -234bp and -214bp was necessary for induction of LDLR transcription by a short term (2h) treatment of phorbol ester (Makar *et al*., 2000). It is interesting to note that most of the reported LDLR transcription regulators function at or near SRE-1, the proximal promoter, of LDLR. In this study, however, the *cis*-element (E-box) responsible for PGC-1α-inhibited LDLR expression is located in the distal region of LDLR promoter and does not require the presence of SREBP, which suggests that putative transcription regulatory factor(s) may bind to the *cis*-element in the distal region of LDLR promoter.

Various co-activators such as SHP-1, RIP140 and DAX-1 have shown to interact with the ligand-binding domain of ERα that is activated by estrogen. However, the role of co-activators in

ERα-mediated activation remains not understood. Basically, the co-activators interact with ligand-bound nuclear receptors and components of basal transcription machinery to activate target genes. PGC-1α also recruits several transcription factors to activate the gene transcription, which are involved in gluconeogenesis (Yoon *et al*., 2001; Rhee *et al*., 2003) and fatty acid metabolism (Louet *et al*., 2002) as well as ERα-mediated gene transcription (Tcherepanova *et al*., 2000). Therefore, we hypothesized at first that PGC-1α would up-regulate the LDLR gene expression through direct protein-protein interaction with ERα. Unexpectedly, however, PGC-1α did not co-activate ERα-induced LDLR transcription and repressed the LDLR transcription by itself. Our results suggested PGC-1α might play a novel repressive role in regulation of LDLR expression. As PGC-1α is a co-regulator which lacks the DNA-binding domain (Puigserver *et al*., 1999), there are two possible ways in which PGC-1α represses the transcription; i) PGC-1α enhances the activity of putative DNA-binding repressor(s) or ii) PGC-1α inactivates the putative transcription activator. If there is a putative silencing factor, PGC-1α would enhance the association of the silencing factor with the LDLR promoter to reduce the transcription. Or a transcription activator is involved; PGC-1α may deplete the putative transcription factor not to interact with LDLR promoter. To change the protein-protein interaction or the activity of the factors like the above, protein modification would be one of the plausible ways.

Knutti *et al*. (2001) provided some evidence for a putative repressor that interacts with and regulates PGC-1α. They showed that the phosphorylated PGC-1α by p38-MAPK lost the binding affinity with the repressor and enhanced the glucocorticoid hormone response. It was suggested that the repressor and nuclear receptors competed for recruiting PGC-1α to an inactive and active state, respectively, since the repressor and nuclear receptors might recognize overlapping sites of PGC-1α. But the mechanism of PGC-1α in LDLR repression is very different from the glucocorticoid hormone response. A specific inhibitor for p38-MAPK did not reverse the PGC-1α activity on LDLR expression, that is, the LDLR gene expression remained suppressed in the presence of p38-MAPK inhibitor. Therefore, the phosphorylation status of PGC-1α might not alter the association between the putative regulatory factor(s) and PGC-1α. The LDLR promoter region interacting with the putative factor(s) should be between -974 bp to -650bp. PPARα is one of the candidate transcription regulators working with PGC-1α. The results, however, demonstrated the PGC-1α activity on LDLR expression was independent of PPARα, which was expected as the PPARα response element is not present in the LDLR promoter region.

Screening of the consensus sequences for transcription factor binding in LDLR distal promoter between -974 bp to -650bp revealed that three E-box sequences might be involved in PGC-1α-induced suppression. None of putative E-box binding repressor proteins were involved in the suppression (data not shown). We suspect unknown factor(s) which exhibit affinity to TRE as transcription activators since we observed pLR1563-luc was induced greatly by AP1 complex (unpublished data). The subunit of the complex needs to be identified in detail.

In animal models, hepatic PGC-1α levels were increased in fasting status and diabetes, a pathological fasting status. Without increase of serum cholesterol it is possible that fasting or diabetic condition increases hepatic PGC-1α, resulting in decrease of LDLR expression and decrease of serum cholesterol clearance. Therefore, fasting condition may lead to increase of serum cholesterol. This postulate is well agreed with the observation showing that acute fasting increases serum cholesterol and LDL in healthy and non-obese human subjects (Savendahl and Underwood, 1999).

Methods

Materials

TRIzol and tissue culture supplies were purchased from GIBCO-BRL Co. Lipoprotein deficient serum (LPDS) fraction of FBS ($d > 1.21$ g/ml) was isolated by ultacentrifugation in KBr as previously described (Han and Pak, 1999). The enhanced chemiluminescence (ECL) detection kit was obtained from Amersham-Pharmacia Biotech. The mammalian expression plasmids of pcDNA3.1/HisC, pcDNA3.1/LacZ, and T-easy vector were purchased from Invitrogen and Promega, respectively. 17β-ethinyl estradiol (E_2) and 25-hydroxycholesterol were purchased from Sigma Co, St. Louis. The inhibitor of p38-MAPK, SB202190 was purchased from CalBiochem.

Cell culture and transient transfection

HepG2 cells (ATCC, HB-8085) were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS and 100 μg/ml penicillin and 100 μg/ml streptomycin at 37°C/5% CO_2. For transient transfections, the cells (4×10^5 cells/well) in 6 well plate were transfected with the indicated plasmid(s) and expression vector of β-galactosidase (pcDNA3.1/LacZ) by calcium phosphate co-precipitation method (Han *et al*., 2000; Kim *et al*., 2001). The total amounts of expression vectors were kept constant by

adding pcDNA3.1/HisC expression vector to transfection. After 24 h incubation in MEM with or without 10% FBS or LPDS, the cells were harvested and luciferase activity was measured using luciferase assay kit (Promega, Madison, WI) and luminometer (Berthold, German). The transfection efficiencies were normalized by the β-galactosidase activity. In some experiments, the transfected cells were incubated for 24 h in phenol red-free MEM supplemented with 10% charcoal dextran-treated FBS (Horwitz and McGuire, 1978) in the presence of vehicle (ethanol) or 17β-ethinyl estradiol (10^{-8} M) and harvested for luciferase assay.

Preparation of plasmids

A *Hin*dIII fragment (1563bp) of human LDLR promoter from pLDLR-CAT 1563 (Pak *et al*., 1996) was subcloned into *Hin*dIII site of pGL2-Basic vector (Promega), and named as pLR1563-luc. The serially truncated LDLR promoter-reporter vectors, pLR1326-luc, pLR974-luc, pLR650-luc, and pLR234-luc were also constructed by PCR as described previously (Oh *et al*., 2005). Vectors containing LDLR promoter mutants were generated using oligonucleotide-directed mutagenesis. A deletion mutant (dSRE-luc) that lacks the SRE-1 region, and a scrambled mutant (mSRE-luc) that SRE-1 sequences were randomly scrambled, were generated using a two-step PCR procedure. The full-length cDNA of human PGC-1α was cloned by RT-PCR as previously described (Choi *et al*., 2006). All products from PCR-based cloning were sequenced to ensure the fidelity of the resulting constructs. The expression vector of human N-terminus of SREBP-2 was a kind gift from Dr. Kyung-Sup Kim (Yonsei University, Korea). Dr. Mi-Ock Lee (Seoul National University, Korea) kindly provided us the pERE-luc, a luciferase reporter containing three copies of a canonical estrogen response element (ERE) under a TATA promoter, and pSV40-ERα expression vector.

Adenovirus infection

HepG2 cells were infected for 48 h with adenoviruses expressing either LacZ (AdLacZ) (Chung *et al*., 2002) or PGC-1α with $(His)_6$-tag at its N-terminus (AdPGC-1) at a multiplicity of infection (moi) of 50 (Rhee *et al*., 2003) and harvested for Western blotting or for RT-PCR analysis. In some cases, the infected cells in serum free media containing 0.5% FBS were treated with the inhibitor of p38-MAPK, SB202190 (20 μM), for 24 h and harvested for RT-PCR or Northern analysis.

Western blot analysis

Protein extracts (20 μg) from HepG2 cells infected with AdLacZ or AdPGC-1 adenovirus were prepared with lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, and 1% Triton X-100), separated by 10% SDS-PAGE, and analyzed by Western blot using rabbit anti-human PGC-1α polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech). After detection of PGC-1α, the membrane was stripped and reprobed with anti-actin antibody (Santa Cruz Biotechnology).

RNA isolation and RT-PCR

Total cellular RNA from rat liver or cultured cells was isolated using TRIzol reagent followed by manufacturer's instruction (Invitrogen) (Pak *et al*., 1996; Jang *et al*., 2006). Total cDNA synthesized from 2 μg of total RNA was amplified for 28-30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min. The primer sets for LDLR (5'-atgcatctcctacaagtgggt-3' and 5'-agtttccatcagagcactggaa-3'), PGC-1α (5'-atggcgtgggacatgtgcaa-3' and 5'-tccctcagttcaccggtc-3'), β-actin (5'-ttctacaatgagctgcgtgtggct-3' and 5'-gcttctccttaatgtcacgcacga-3') were used for amplification of 629 bp, 1,457bp, and 378bp fragments of human LDLR, PGC-1α, and β-actin, respectively. The reaction products were examined by 1.2% agarose gel electrophoresis and normalized by the RT-PCR products for β-actin (Kim *et al*., 2001).

Northern blot analysis

Twenty μg of total RNA were separated by gel electrophoresis and transferred to a Nytran membrane (Schleicher & Schuell Inc.) using TurboBlotter (Schleicher & Schuell Inc.). The membrane was hybridized with the ^{32}P-labeled specific cDNA probes for LDLR (629 bp PCR product) and PGC-1α (1,457 bp of PCR product) in Quickhyb hybridization solution (Stratagene) for 12 h at 65°C, washed twice for 15 min at room temperature with a 2 × SSC/0.1%SDS, then washed once for 30 min at 60°C with 0.1 × SSC/0.1% SDS. The membrane was exposed to K-type imaging screen and visualized using Molecular Imaging System FX (Bio-Rad).

Chromatin immunoprecipitation (ChIP)

HepG2 cells were infected with AdLacZ or AdPGC-1 and ChIP assay was performed (Puigserver *et al*., 2003). Briefly, cross-linked protein-DNA complex was immunoprecipitated with control IgG, anti-PGC-1α (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-acetylated histone 3 (AcH3) antibodies and the precipitated DNA fragments were analyzed by PCR using primers directed against human LDLR promoter (5'-caggcaagtttctcacatgtgccttttggcaaga-3' and 5'-agtacagccaaaaaaatatttttgttttg-3').

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was evaluated by paired or unpaired Student's *t*-test, and $P < 0.05$ was considered significant.

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