Induction of Adiponectin, a Fat-Derived Antidiabetic and Antiatherogenic Factor, by Nuclear Receptors

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Adiponectin is a fat-derived hormone with antidiabetic and antiatherogenic properties. Hypoadiponectinemia seen in obesity is associated with insulin-resistant diabetes and atherosclerosis. Thiazolidinediones, peroxisome proliferator–activated receptor-γ (PPAR-γ) agonists, have been shown to increase plasma adiponectin levels by the transcriptional induction in adipose tissues. However, the precise mechanism of such action is unknown. In this study, we have identified a functional PPAR-responsive element (PPRE) in human adiponectin promoter. PPAR-γ/retinoid X receptor (RXR) heterodimer directly bound to the PPRE and increased the promoter activity in cells. In adipocytes, point mutation of the PPRE markedly reduced the basal transcriptional activity and completely blocked thiazolidinedione-induced transactivation of adiponectin promoter. We have also identified a responsive element of another orphan nuclear receptor, liver receptor homolog-1 (LRH-1), in adiponectin promoter. LRH-1 was expressed in 3T3-L1 cells and rat adipocytes. LRH-1 bound specifically to the identified responsive element (LRH-RE). LRH-1 augmented PPAR-γ–induced transactivation of adiponectin promoter, and point mutation of the LRH-RE significantly decreased the basal and thiazolidinedione-induced activities of adiponectin promoter. Our results indicate that PPAR-γ and LRH-1 play significant roles in the transcriptional activation of adiponectin gene via the PPRE and the LRH-RE in its promoter. *Diabetes* 52: 1655–1663, 2003

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DMEM, Dulbecco's modified Eagle medium; DR1, direct repeat 1; EMSA, electrophoretic mobility shift assay; FXR, farnesoid X receptor; HB-EGF, heparin-binding epidermal growth factor; IRS-1, insulin receptor substrate-1; LRH-1, liver receptor homolog-1; LRH-RE, LRH responsive element; LXR, liver X receptor; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor; PI-3K, phosphatidylinositol-3 kinase; PPAR, peroxisome proliferator–activated receptor; PPRE, PPAR responsive element; RXR, retinoid X receptor; SF-1, steroidogenic factor 1; SHP, small heterodimer partner; TNF-α, tumor necrosis factor-α.

The adipose tissue has been shown to be an endocrine organ that produces and secretes many bioactive substances (1–4), conceptualized as adipocytokines (3). Controlled production of adipocytokines plays a crucial role in maintaining homeostasis of glucose and lipid metabolism. Dysregulation of adipocytokine production is directly involved in the pathophysiology of metabolic syndrome, while normalization of plasma concentrations of dysregulated adipocytokines reverses the phenotype of metabolic syndrome (1,5–8). Adiponectin is the adipocytokine we identified from human fat cDNA (9). Adiponectin exhibits an adipose-specific expression and it is the most abundant transcript in human fat. Mouse homolog for adiponectin, Acrp30 and AdipoQ, was cloned by Scherer et al. (10) and Hu et al. (11), independently. Adiponectin exists abundantly in human blood (5–20 μg/ml), and its plasma concentration decreases with fat accumulation in the body (12). Plasma adiponectin concentrations are lower in patients with diabetes (13) and ischemic heart disease (14). Significantly, the genetic mutation of the adiponectin gene, which causes low plasma adiponectin levels, is associated with metabolic syndrome, including insulin-resistant diabetes and atherosclerotic disease (15). In Pima Indians, hypoadiponectinemia is a strong predictor for the development of type 2 diabetes (16). In the monkey model of overcalorie-induced diabetes, reduction of plasma adiponectin precedes the development of hyperinsulinemia and hyperglycemia (17).

Adiponectin has antidiabetic properties (18–21). It activates insulin receptor substrate-1 (IRS-1)-mediated phosphatidylinositol-3 kinase (PI-3K) and glucose uptake in skeletal muscle cells (18,20,21), enhances muscle β-oxidation via the activation of AMP-kinase (21), and suppresses hepatic glucose production (19,21). Adiponectin exhibits antiatherogenic effects in tissue cultures (14,22–24). Adiponectin suppresses monocyte adhesion to endothelial cells by reducing the nuclear factor-κB (NF-κB) signaling and the mRNA expression of adhesion molecules in endothelial cells (14,22). Adiponectin inhibits foam cell formation from macrophages by reducing the expression of class A macrophage scavenger receptor and lipid accumulation (23). It also suppresses the proliferation and migration of vascular smooth muscle cells by reducing the effects of various growth factors, including platelet-derived growth factor (PDGF)-AA, PDGF-BB, and heparin-binding epidermal growth factor (HB-EGF), on the cells (24). In apolipoprotein

E–deficient mice, infection of adenovirus-expressing human adiponectin ameliorates atherosclerosis (25). Finally, adiponectin deficiency in knockout mice causes diet-induced insulin resistance and diabetes and increases neointimal thicking in the arterial wall by mechanic injury (20,26,27). Adiponectin supplement completely reverses the diabetic and atherosclerotic phenotypes of the knockout mice (20,26).

These observations implicate that hypoadiponectinemia is a precedent pathological condition for the development of obesity-related metabolic syndrome including insulin-resistant diabetes and atherosclerosis. Therefore, the therapy that can increase the production and secretion of adiponectin should be useful to treat metabolic syndrome.

We and others have reported that treatments with thiazolidinediones, which are known as peroxisome proliferator–activated receptor-γ (PPAR-γ) agonists, can increase plasma adiponectin levels in humans and mice (28–30). Studies using mice and cultured adipocytes have revealed that thiazolidinediones increase adiponectin mRNA levels in adipose cells, resulting in increased production and secretion of adiponectin protein (28,29). However, direct repeat 1 (DR1) sites, to which PPAR-γ/retinoid X receptor (RXR) heterodimer is thought to bind, have not been identified in the promoter region of adiponectin gene (31,32). Therefore, the question of whether the increase in adiponectin mRNA levels by PPAR-γ ligands is a direct effect via a PPAR-responsive element (PPRE) in its flanking region, or is an indirect effect mediated by accelerated differentiation of adipocytes or other target genes of PPAR-γ, remains to be resolved. The current study reveals the existence of PPRE in human adiponectin promoter, PPAR-γ/RXR heterodimer-mediated transactivation of adiponectin gene through the PPRE, and involvement of liver receptor homolog-1 (LRH-1) in enhancing PPAR-γ–mediated transactivation of adiponectin gene in adipocytes. Revealing the transactivation machinery of adiponectin gene regulated by PPAR-γ, RXR and LRH-1 should be useful to identify new compounds to enhance the adiponectin production.

RESEARCH DESIGN AND METHODS

Plasmids. The luciferase reporter plasmids of human adiponectin promoter were generated by excising the promoter fragment from the genomic clone of adiponectin (31) and inserting it into the *Kpn*I and *Sac*I sites of the pGL3 basic luciferase expression vector (Promega). Serial deletion of the pGL3-adiponectin luciferase plasmid was performed by PCR. Point mutation of the pGL3-adiponectin luciferase plasmid was introduced using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Expression plasmids encoding mouse PPAR-γ (pCMX-mPPAR-γ), mouse RXRα (pCMX-mRXRα), mouse LRH-1 (pCMX-mLRH-1), VP16 (pCMX-VP16), VP16-mouse PPAR-γ chimera protein (pCMX-VP16-mPPAR-γ), VP16-human RXRα chimera protein (pCMX-VP16-hRXRα), and β-galactosidase (pCMX-β-gal) were generous gifts from Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). Expression plasmids encoding human PPAR-γ (pcDNA3.1-hPPAR-γ) and human RXRα (pcDNA3.1-hRXRα) were previously described (33). Human LRH-1 (AF146343) was cloned and inserted into pcDNA3.1 to generate the expression plasmid for human LRH-1 (pcDNA3.1-hLRH-1).

Animal studies. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University. Male 13-week-old C57BL/KsJ (*db+/db+*) mice (Clea, Tokyo, Japan) were fed powder diet (CRF-1; Oriental Kobo) containing 0.2% troglitazone or 0.01% pioglitazone, respectively. The mice were killed 2 weeks later. Total RNA extracted from subcutaneous adipose tissues was electrophoresed and transferred to nylon membrane (6). The membranes were hybridized with mouse adiponectin and 36B4 cDNA probe labeled with [α-^{32}P]dCTP. Plasma adiponectin levels were measured by Western blot analysis. Plasma samples were resolved on 12.5% SDS-PAGE gel, followed by electrophoretic transfer to a nitrocellulose membrane. The signal was detected with a rabbit polyclonal antibody against mouse adiponectin using the enhanced chemiluminescence system (Amersham Biosciences).

Cotransfection assays in HEK293 cells. Human embryonic kidney (HEK) 293 cells were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS. Transfections were performed by calcium phosphate coprecipitation as previously described (34). A ligand compound was added at 8 h after transfection. Cells were harvested after 18 h for luciferase and β-galactosidase assays. Luciferase data were normalized relative to an internal β-galactosidase control and expressed as the relative luciferase activity.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assay (EMSA) was performed as described previously (35). PPAR-γ, RXRα, and LRH-1 proteins were synthesized from pcDNA3.1-hPPAR-γ, pcDNA3.1-hRXRα, and pcDNA3.1-hLRH-1 expression plasmids, respectively, using the T_NT T7 Quick Coupled Transcription/Translation Systems (Promega). To analyze the specific binding of PPAR-γ/RXR heterodimer to the PPRE, the double-strand oligonucleotide spanning from −291 to −267 of human adiponectin promoter was ^{32}P-radiolabeled using T4 polynucleotide kinase (TaKaRa) with [γ-^{32}P]ATP. To analyze the specific binding of LRH-1 to the LRH-RE, the double-strand oligonucleotide spanning from −245 to −221 of human adiponectin promoter was labeled. Double-strand oligonucleotides composed of the following sequences were used for competition assays: human adiponectin PPRE wild-type, 5′-TGG TTT *TGA CTT TTG CCC C*AT CTT C-3′ and human adiponectin PPRE mutant, 5′-TGG TTT *TGA CTT TTG ttC C*AT CTT C-3′; mouse aP2 PPRE (36), 5′-GAT CTG *TGA ACT CTG ATC C*AG TAA G-3′; human adiponectin LRH-RE wild-type, 5′-AAT AAG GG*T CAA GGC CT*G GAA ACA C-3′; human adiponectin LRH-RE mutant, 5′-AAT AAG GG*T CAA ccC CT*G GAA ACA C-3′ and rat cytochrome P450 (Cyp) 7a1 LRH-RE (34), 5′-GAC TTA GT*T CAA GGC CG*G GTA ATG C-3′. PPRE and LRH-RE sequences are italicized. The mutated bases are shown in lower case.

RNA preparations from adipocytes. Rat stromal vascular cells and mature adipocytes were isolated by collagenase digestion as described previously (37). Epididymal fat pads were excised from male 8-week-old Sprague-Dawley rats (Japan SLC) and immediately minced in ice-cold Krebs-Ringer HEPES buffer (pH 7.4) containing 1% BSA. The minced tissue (2 g) was digested at 37°C for 30 min in 6 ml of Krebs-Ringer HEPES buffer (pH 7.4) containing 1% BSA and 20 mg of type I collagenase (Worthington Biochemical) with continuous agitation. Digested tissue was filtered through a nylon mesh and centrifuged at 500*g* for 3 min. Mature adipocytes, found at the top of the supernatant, and stromal vascular cells, found in the pellet, were transferred to new tubes, respectively. The collected cells were mixed thoroughly by vortexing with RNA STAT-60 solution (TEL-TEST). Total RNA was extracted using the protocol provided by the manufacturer.

Mouse preadipocyte 3T3-L1 cells were maintained in DMEM containing 10% FBS. For differentiation, cells (3 days after reaching confluence) were cultured for 2 days with 10% FBS-supplemented DMEM containing 5 μg/ml of insulin, 0.5 mmol/l 1-methyl-3-isobutyl-xanthine, and 1 μmol/l dexamethasone. The cells were further incubated with DMEM containing 10% FBS to differentiate into adipocytes. Total RNA was extracted from undifferentiated 3T3-L1 preadipocytes and differentiated 3T3-L1 adipocytes (on day 6 after induction of differentiation) using RNA STAT-60 according to the method recommended by the manufacturer.

RT-PCR. First-strand cDNA was synthesized from total RNA using Thermoscript RT (Invitrogen) primed by an oligo dT primer. Real-time PCR amplification of PPAR-γ, LRH-1, and β-actin was performed on the LightCycler (Roche Diagnostics) using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics). Primer sets were the following: PPAR-γ, 5′-CCA GAG TCT GCT GAT CTG CG-3′ and 5′-GCC ACC TCT TTG CTC TGA TC-3′; LRH-1, 5′-GCC CTC ATT CGA GCC AAT GG-3′ and 5′-CTG GGT ACT CAG ACT TGA TGG C-3′; and β-actin, 5′-TGA CAG GAT GCA GAA GGA GAT-3′ and 5′-CTC CTG CTT GCT GAT CCA CAT-3′. The levels of PPAR-γ and LRH-1 mRNA were normalized relative to the amount of β-actin mRNA and were expressed in arbitrary units.

Transfection studies of 3T3-L1 cells. On day 6 after induction of differentiation, the media of 3T3-L1 cells in 6-well plates were changed to OPTI-MEM (Invitrogen), and the cells were transfected with luciferase reporter plasmids using LipofectAMINE 2000 reagent (Invitrogen) according to the instructions provided by the manufacturer. Transfection was performed using 1 μg of pCMX-β-gal (internal standard) along with 2 μg of pGL3-basic plasmid containing human adiponectin promoter or with control pGL3-basic plasmid alone. An equal amount of 20% FBS-supplemented DMEM with or without pioglitazone was added into the media 3.5 h later. At 44 h after ligand treatment, luciferase reporter assays were performed using Luciferase Assay

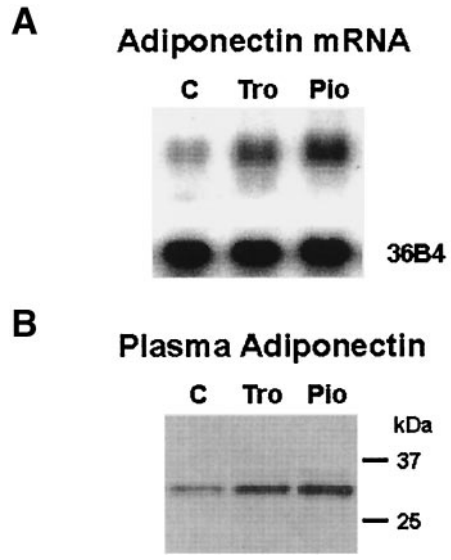


FIG. 1. Effects of thiazolidinediones on adipose mRNA expression and plasma protein levels of adiponectin in mice. Male 13-week-old obese (*db*+/*db*+) mice were fed powder diet containing various thiazolidinediones for 2 weeks. *A*: Northern blot analysis for adiponectin was performed using total RNA extracted from subcutaneous adipose tissues. *B*: Plasma adiponectin was detected by Western blot analysis with anti-mouse adiponectin antibody. Representative data from individual animals are demonstrated among three to four animals for each group. Tro, troglitazone; Pio, pioglitazone; C, control group fed powder diet containing no compounds.

System (Promega). Luciferase values were normalized by an internal β-galactosidase control and expressed as the relative luciferase activity.

RESULTS

Thiazolidinediones increase adipose mRNA expression and plasma protein levels of adiponectin in mice. Consistent with the previous reports (28,29), PPAR-γ ligands augmented adiponectin mRNA levels in white adipose tissues (Fig. 1*A*). The mRNA induction of adiponectin was associated with an increase in its plasma concentrations (Fig. 1*B*).

PPAR-γ and RXR transactivate human adiponectin promoter. PPAR-γ acts as a nuclear receptor-transcription factor by forming a heterodimer with RXR. We examined the direct effect of PPAR-γ/RXR heterodimer on the transcription of adiponectin gene by luciferase reporter assays. The 5′-flanking region of human adiponectin gene (−908 to +14) was cloned by PCR and inserted into the luciferase cassette construct. Assays were performed in human kidney HEK293 cells, which seemingly lack most of the adipogenic transcription factors. As shown in Fig. 2*A*, coexpression of PPAR-γ and RXRα increased human adiponectin promoter activity, although expression of PPAR-γ or RXRα alone failed to enhance the promoter activity. Pioglitazone augmented the luciferase activity induced by coexpression of PPAR-γ and RXRα. Next, to strengthen the response, we utilized VP16-chimera nuclear receptors, in which the activation domain of herpes virus VP16 protein was fused with receptors. VP16-PPAR-γ and VP16-RXRα are ligand-independent and constitutively active for the responsive genes (38). As shown in Fig. 2*B*, expression of VP16-PPAR-γ alone slightly increased the adiponectin promoter activity. Expression of VP16-RXRα alone did not affect the luciferase activity compared with the VP16 control. However, coexpression of VP16-PPAR-γ and VP16-RXRα markedly increased the promoter activity. These results demonstrated that PPAR-γ/RXR heterodimer acted on the promoter of human adiponectin gene.

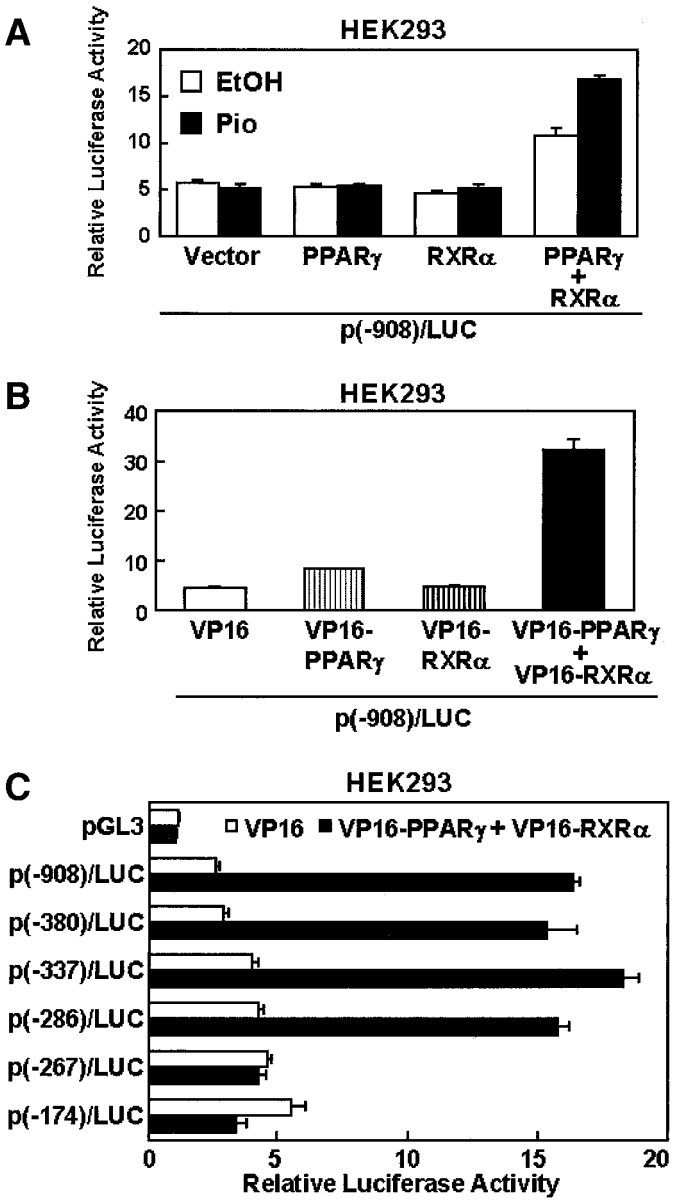


FIG. 2. Promoter analysis of human adiponectin gene. *A*: HEK293 cells were transfected with nuclear receptor expression plasmids and luciferase reporter constructs containing the 5′-flanking region of human adiponectin gene. The reporter construct, p(−908)/LUC, consists of the nucleotide sequence between −908 and +14 upstream of the transcription start site. Full-length PPAR-γ and/or RXRα were expressed. Following transfection, cells were treated for 18 h with 1 μmol/l pioglitazone (Pio) or vehicle (EtOH). Normalized luciferase activities are shown as means ± SE (n = 3). *B*: HEK293 cells were transfected with VP16-PPAR-γ and/or VP16-RXRα expression plasmids and the luciferase reporter vector containing human adiponectin promoter. No ligand was added. Normalized luciferase activities are shown as means ± SE (n = 3). *C*: HEK293 cells were transfected with VP16-PPAR-γ– and VP16-RXRα–expression plasmids and luciferase reporter constructs containing serial deletions of the 5′-flanking region of human adiponectin gene. No ligand was added. Normalized luciferase activities are shown as means ± SE (n = 3).

Identification of PPRE in human adiponectin promoter. To identify the element responsible for PPAR-γ/RXR heterodimer–induced transactivation of adiponectin promoter, we analyzed the effects of serial deletions of

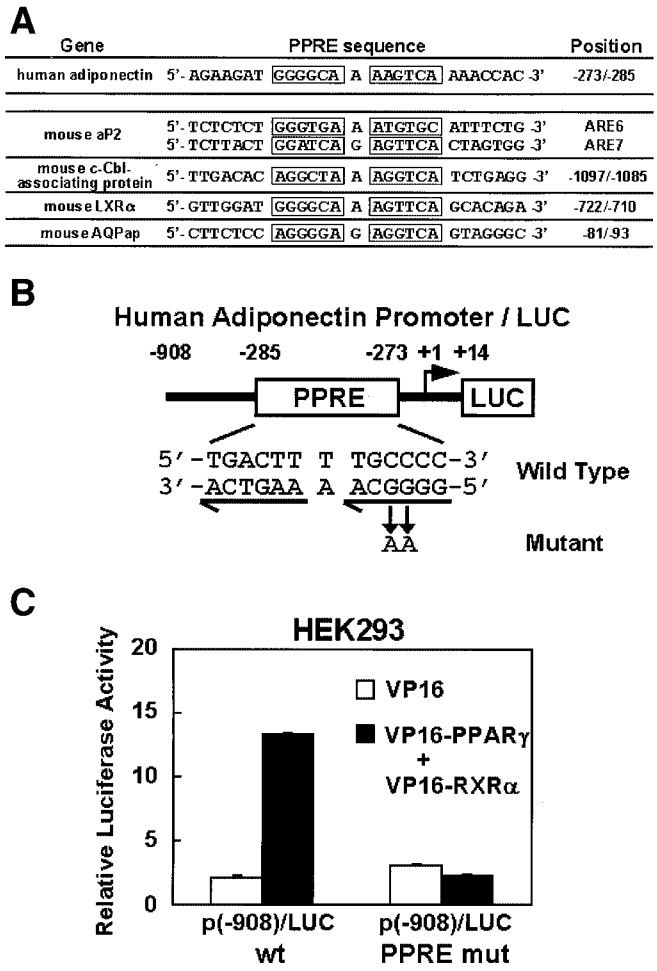


FIG. 3. Identification of the functional PPRE in human adiponectin promoter. *A*: The PPRE sequence in human adiponectin promoter is compared with the PPRE sequences in the promoters of various PPAR-γ–responsive genes: mouse aP2 PPRE, *ARE6*, and *ARE7* (36); mouse c-Cbl–associating protein PPRE (39); mouse LXRα PPRE (40); and mouse AQPap (aquaporin adipose) PPRE (35). DR1 motifs are boxed. *B*: Schematic illustration of the human adiponectin promoter and luciferase reporter construct. The PPRE sequence at position from −285 to −273 is indicated. Point mutation from GG to AA was introduced at −276/−275. *C*: Transcriptional activities were compared between wild-type [p(−908)/LUC wt] and mutant reporters [p(−908)/LUC PPRE mut], as described in *B*. HEK293 cells were transfected with VP16-PPAR-γ and VP16-RXRα expression plasmids and luciferase reporter constructs. No ligand was added. Normalized luciferase activities are shown as means ± SE (n = 3).

adiponectin promoter on luciferase activities (Fig. 2*C*). VP16-PPAR-γ– and VP16-RXRα–induced transactivation was completely abolished when the region between −286 and −267 was deleted. Inspection of this region revealed a putative PPRE of the DR1 type at −273 to −285. Figure 3*A* shows the similarity of the putative PPRE sequence in human adiponectin promoter to the previously described PPRE sites in various gene promoters (35,36,39,40).

To validate this putative PPRE, we introduced point mutation from GG to AA at −276/−275 position (Fig. 3*B*). This replacement of two nucleotides completely abolished transactivation of human adiponectin promoter by VP16-PPAR-γ and VP16-RXRα (Fig. 3*C*). These results showed that PPAR-γ/RXR heterodimer activated the transcription of adiponectin gene via the identified PPRE in its promoter.

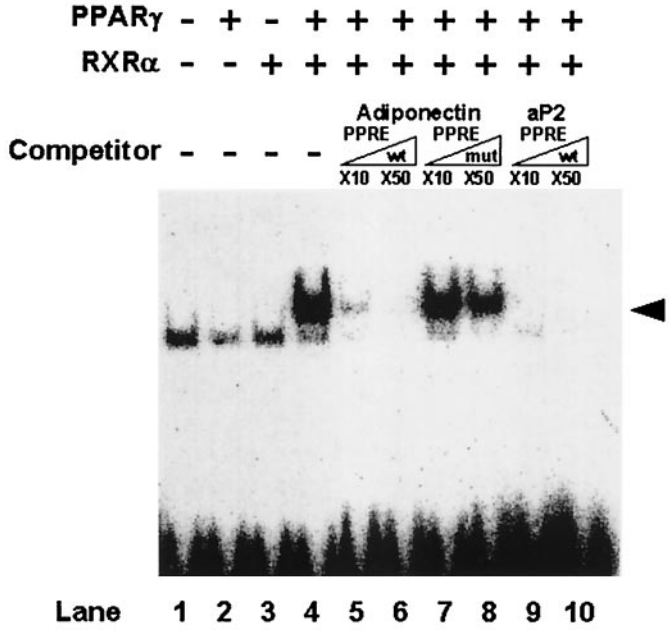


FIG. 4. Specific binding of PPAR-γ/RXR heterodimer to the PPRE in human adiponectin promoter. EMSAs were performed with the [^{32}P]-labeled oligonucleotide containing the PPRE in human adiponectin promoter. The labeled probe was incubated with in vitro synthesized PPAR-γ and/or RXRα. The position of the shifted PPAR-γ/RXRα complex with the labeled oligonucleotide is indicated by the arrow. Competitive assays were performed using unlabeled oligonucleotides containing human adiponectin PPRE wild-type (*lanes 5* and *6*), PPRE mutant (−276/−275, GG to AA) (*lanes 7* and *8*), or mouse aP2 PPRE (*lanes 9* and *10*), as competitors by 10- and 50-fold molar excess.

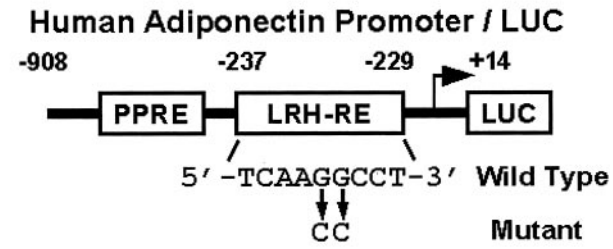
PPAR-γ/RXR heterodimer binds to human adiponectin PPRE. To confirm that PPAR-γ/RXR heterodimer binds to the identified PPRE in human adiponectin promoter, EMSAs were performed with the double-stranded oligonucleotide containing human adiponectin PPRE. ^{32}P-radiolabeled PPRE oligonucleotide was incubated with in vitro translated PPAR-γ and/or RXRα (Fig. 4). Neither PPAR-γ nor RXRα alone bound to human adiponectin PPRE (Fig. 4, *lanes 2* and *3*). When PPAR-γ and RXRα were incubated together, the mobility of the labeled probe was shifted (Fig. 4, *lane 4*). The signal of the shifted band disappeared by competition with an excess of the homologous oligonucleotide (Fig. 4, *lanes 5* and *6*), but not with the PPRE mutant (Fig. 4, *lanes 7* and *8*), which contains point mutation (GG to AA, −276/−275), as described in Fig. 3*B*. Another unlabeled oligonucleotide based on the PPRE present in mouse aP2 gene enhancer (36) was also an effective competitor (Fig. 4, *lanes 9* and *10*). These results indicated that PPAR-γ/RXR heterodimer bound specifically to the identified PPRE in human adiponectin promoter.

LRH-1 enhances PPAR-γ–induced transactivation of human adiponectin promoter via LRH-RE. To identify other transcription factors that could enhance the transcription of human adiponectin gene, we examined the binding elements of transcription factors in the adiponectin promoter. We identified a putative LRH-RE located at −237 to −229 (Fig. 5*A*), downstream of the PPRE in human adiponectin promoter (Fig. 5*B*). LRH-RE is a binding element of another orphan nuclear receptor, LRH-1. Figure 5*A* shows the similarity of the putative LRH-RE sequence in human adiponectin promoter to the

A

Gene	LRH-RE sequence	Position
human adiponectin	5'- TCAAGGCCT -3'	-237/-229
rat Cyp7a1	5'- TCAAGGCCG -3'	-136/-128
human Cyp7a1	5'- TCAAGGCCA -3'	-134/-126
human CETP	5'- GCAAGGTCC -3'	-75/-83
rat Cyp8b1	5'- GCAAGGTCC -3'	-63/-55
	5'- CCAAGGGCA -3'	-56/-48

B



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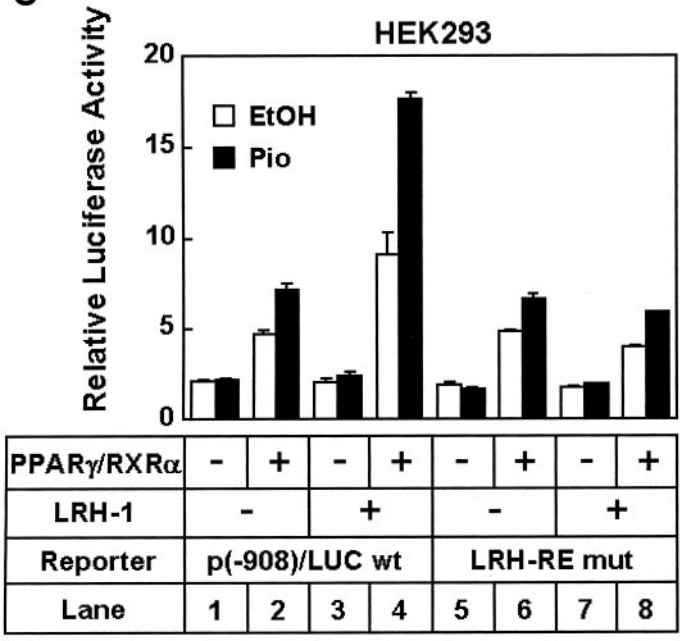

FIG. 5. Effect of LRH-1 on PPAR-γ–induced transactivation of human adiponectin promoter. ***A*****: The LRH-RE sequence exists at position from −237 to −229 in human adiponectin promoter. The LRH-RE sequence in human adiponectin promoter is compared with the LRH-RE sequences in the promoters of various LRH-1 responsive genes: rat Cyp7a1 LRH-RE (34); human Cyp7a1 LRH-RE (41); human CETP LRH-RE (42); and rat Cyp8b1 LRH-RE (43).** ***B*****: Schematic illustration of the human adiponectin promoter and luciferase reporter construct. The LRH-RE sequence between −237 and −229, downstream of the PPRE, is indicated. Point mutation from GG to CC was introduced at −233/−232.** ***C*****: HEK293 cells were cotransfected with nuclear receptor expression plasmids and luciferase reporter constructs containing human adiponectin promoter. Full-length PPAR-γ, RXRα, and/or LRH-1, none of which contained the VP16 component, were expressed. Transcriptional activities were compared between wild-type [p(−908)/LUC wt] and LRH-RE mutant reporter (LRH-RE mut), as described in** ***B*****. Following transfection, cells were treated for 18 h with 1 μmol/l pioglitazone (Pio) or vehicle (EtOH). Normalized luciferase activities are shown as means ± SE (*n* = 3).**

previously described LRH-RE sites in various LRH-1–responsive gene promoters (34,41–43).

To examine whether LRH-1 affects PPAR-γ–induced transactivation of human adiponectin promoter, cotransfection assays with the luciferase reporter construct, expression plasmids of PPAR-γ, RXRα (without the VP16 component) and LRH-1 were performed (Fig. 5*C*). Coexpression of PPAR-γ and RXRα activated human adiponectin promoter activity (Fig. 5*C*, *lane 2* vs. *lane 1*). Pioglitazone augmented the luciferase activity induced by PPAR-γ/RXR (Fig. 5*C*, *lane 2*). LRH-1 alone did not affect the promoter activity (Fig. 5*C*, *lane 3*). However, coexpression of PPAR-γ/RXRα and LRH-1 significantly augmented the luciferase activity in either the presence or absence of pioglitazone (Fig. 5*C*, *lane 4*). These results demonstrated that LRH-1 enhanced PPAR-γ–stimulated transactivation of human adiponectin promoter.

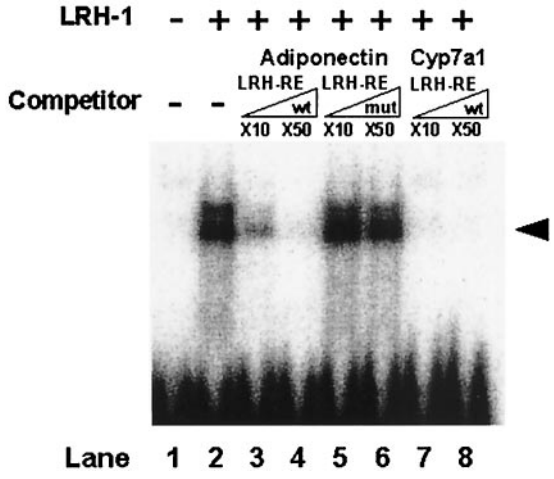

FIG. 6. Specific binding of LRH-1 to LRH-RE in human adiponectin promoter. EMSAs were performed with the [^{32}P]-labeled oligonucleotide containing the LRH-RE in human adiponectin promoter and in vitro synthesized LRH-1. The position of the shifted LRH-1 complex with the labeled oligonucleotide is indicated by the arrow. Competitive assays were performed using unlabeled oligonucleotides containing human adiponectin LRH-RE wild-type (*lanes 3* and *4*), LRH-RE mutant (−233/−232, GG to CC) (*lanes 5* and *6*), or rat Cyp7a1 LRH-RE (*lanes 7* and *8*), as competitors by 10- and 50-fold molar excess.

To determine the significance of the putative LRH-RE in human adiponectin promoter, we introduced point mutation from GG to CC at −233/−232 position (Fig. 5*B*). This point mutation did not affect PPAR-γ/RXR–induced transactivation (Fig. 5*C*, *lane 6* vs. *lane 2*). However, the effect of LRH-1 on PPAR-γ–induced transactivation was completely abolished (Fig. 5*C*, *lane 8* vs. *lane 6* and *lane 8* vs. *lane 4*). These results indicated that the effect of LRH-1 on PPAR-γ–induced transactivation of adiponectin promoter was mediated through the identified LRH-RE.

LRH-1 binds to human adiponectin LRH-RE. To determine whether LRH-1 binds to the LRH-RE in human adiponectin promoter, EMSAs were performed with the double-stranded oligonucleotide containing human adiponectin LRH-RE. Incubation of ^{32}P-radiolabeled LRH-RE oligonucleotide with in vitro translated LRH-1 resulted in a shift in the mobility of the labeled probe (Fig. 6, *lane 2*). The signal of the shifted band disappeared by competition with an excess of the homologous oligonucleotide in a dose-dependent manner (Fig. 6, *lanes 3* and *4*) but not with the LRH-RE mutant (Fig. 6, *lanes 5* and *6*), which contains point mutation (GG to CC, −233/−232) described in Fig. 5*B*. Another unlabeled oligonucleotide based on the LRH-RE present in rat Cyp7a1 gene promoter (34) was also an effective competitor (Fig. 6, *lanes 7* and *8*). These

results indicated that LRH-1 bound specifically to the identified LRH-RE in human adiponectin promoter.

Expression of LRH-1 in adipocytes. To address the physiological role of LRH-1 in adiponectin gene expression, we measured the mRNA levels of LRH-1 in stromal vascular cells, containing preadipocytes, and in mature adipocytes, isolated from rat epididymal fat pads (Fig. 7*A*). Reverse transcription and real-time PCR were performed with total RNA prepared from these cells. PPAR-γ mRNA was expressed predominantly in mature adipocytes as described previously (44), and LRH-1 mRNA was expressed more abundantly in mature adipocytes than in stromal vascular cells.

Next, we performed RT-PCR using total RNA prepared from mouse 3T3-L1 cells (Fig. 7*B*). The expression of LRH-1 mRNA was observed in both undifferentiated preadipocytes and differentiated adipocytes. There was no significant difference in the level of LRH-1 mRNA in 3T3-L1 cells before and after differentiation.

Significance of PPRE and LRH-RE on transcription of adiponectin gene in 3T3-L1 adipocytes. To assess the significance of PPRE and LRH-RE in adiponectin promoter in adipocytes, we transfected luciferase reporter constructs containing wild-type or mutated human adiponectin promoter (−908 to +14) into 3T3-L1 adipocytes (day 6 after induction of differentiation) (Fig. 7*C*). The basal luciferase activity of the wild-type construct of adiponectin promoter was 19-fold higher than that of the pGL3-basic luciferase vector in 3T3-L1 adipocytes. Incubation with pioglitazone markedly enhanced the transcriptional activity of adiponectin promoter by 8.8-fold compared with the basal condition. However, transfection of the PPRE-mutated reporter construct (GG to AA, −276/−275; Fig. 3*B*) caused a marked reduction of transcriptional activity in the basal state, and no further stimulation was seen by treatment with pioglitazone.

On the other hand, transfection of the LRH-RE–mutated reporter construct (GG to CC, −233/−232; Fig. 5*B*) significantly decreased the transcriptional activity in the basal state, while the response to pioglitazone was maintained (7.5-fold in LRH-RE mutation vs. 8.8-fold in wild-type). These results indicated that both PPRE and LRH-RE in adiponectin promoter played significant roles in the transcriptional activation of adiponectin gene in adipocytes.

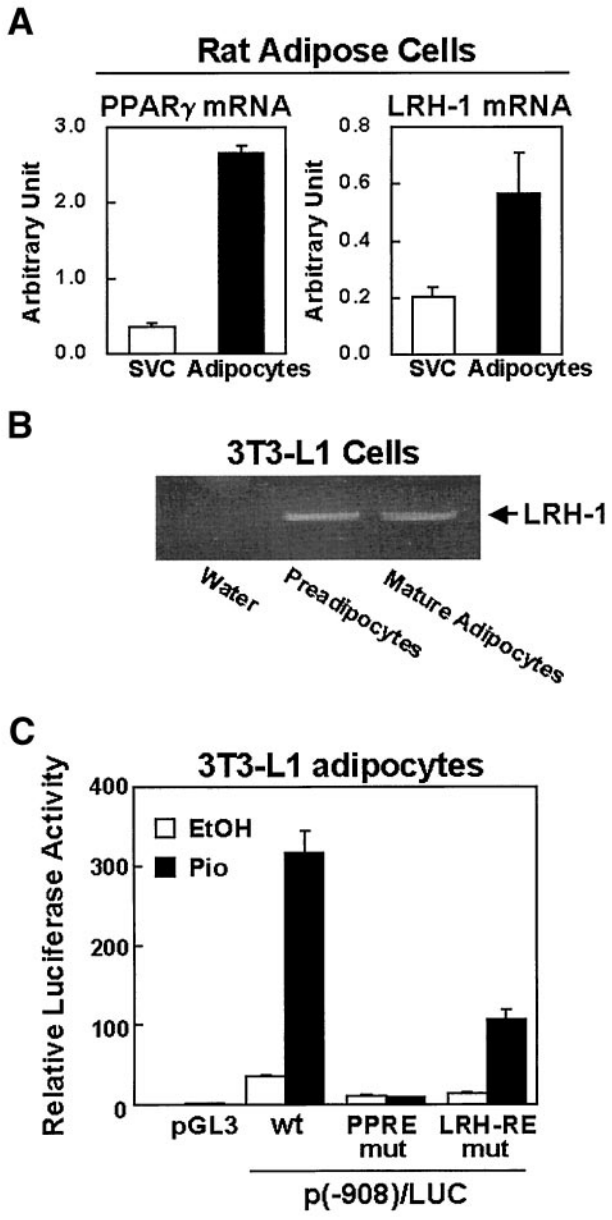

FIG. 7. Adipose expression of LRH-1 and significance of PPRE and LRH-RE on transcription of adiponectin gene in adipocytes. *A*: mRNA levels of PPAR-γ and LRH-1 were quantified by reverse transcription and real-time PCR. Total RNA was extracted separately from stromal vascular cells and mature adipocytes, isolated from rat epididymal fat pads by collagenase digestion and fractionation as described in RESEARCH DESIGN AND METHODS. First-strand cDNA was synthesized from 180 ng of total RNA, and real-time PCR amplification of PPAR-γ, LRH-1, and β-actin was performed as described in RESEARCH DESIGN AND METHODS. PPAR-γ and LRH-1 mRNA levels were normalized to the amount of β-actin mRNA. Data are shown as means ± SE (n = 3) in arbitrary units. SVC, rat stromal vascular cells; Adipocytes, rat mature adipocytes. *B*: Expression of LRH-1 was detected at similar levels in undifferentiated 3T3-L1 preadipocytes and differentiated 3T3-L1 adipocytes. RT-PCR was performed using total RNA extracted from 3T3-L1 preadipocytes and adipocytes (on day 6 after differentiation). cDNA samples were subjected to PCR using primers specific for LRH-1. *C*: Differentiated 3T3-L1 cells were transfected with the luciferase reporter constructs of human adiponectin promoter. Transcriptional activities were compared among wild-type [p(−908)/LUC wt], PPRE mutant [p(−908)/LUC PPRE mut, described in Fig. 3*B*], and LRH-RE mutant [p(−908)/LUC LRH-RE mut, described in Fig. 5*B*]. Following transfection, cells were treated for 44 h with 1 μmol/l pioglitazone (Pio) or vehicle (EtOH). Normalized luciferase activities are shown as means ± SE (n = 3).

DISCUSSION

The present study has identified a functional PPRE in the adiponectin promoter and has demonstrated that it plays a significant role in the transcriptional activation of adiponectin gene in adipocytes. PPAR-γ is one of key transcription factors that regulate adipogenesis and glucose and lipid metabolism (45). PPAR-γ regulates the expression of adipocyte-secreted proteins, adipocytokines, which include leptin, tumor necrosis factor-α (TNF-α), and adiponectin (28,29,46,47). Previous studies have shown that PPAR-γ agonists downregulate adipose mRNA expression of leptin and TNF-α (46,47). However, the precise mechanisms of how PPAR-γ decreases the mRNA levels of these adipocytokines have not been delineated. We and others (28,29) have reported that treatment with thiazolidinediones, PPAR-γ agonists, increase adiponectin mRNA levels in adipocytes and adipose tissues. In the

current study, the use of adiponectin promoter-reporter constructs and expression plasmids of VP16-chimera nuclear receptors in nonadipose HEK293 cells has led us to identify the functional PPRE in the promoter. This is the first evidence demonstrating the direct transcriptional regulation of adipocytokines by PPAR-γ.

In 3T3-L1 adipocytes, pioglitazone markedly induced the luciferase activity of adiponectin promoter. However, pioglitazone treatment of nonadipose HEK293 cells transfected with PPAR-γ and RXR increased the transcriptional activity to a much lesser extent than in 3T3-L1 adipocytes. These findings suggest that the expression of adiponectin gene in adipocytes would require tissue-specific competence factors for full-activation. In this study, we have found a binding element of LRH-1 (LRH-RE) in adiponectin promoter. LRH-1 is a monomeric orphan nuclear receptor expressed in the liver, pancreas, intestine, and ovary (48). LRH-1 binds to the LRH-RE in the promoter of Cyp7a1 gene, the rate-limiting enzyme for the conversion of cholesterol into bile acids in the liver. LRH-1 is necessary for transactivation of Cyp7a1 gene by liver X receptor (LXR)/RXR heterodimer (34). In another case, LRH-1 enhances the induction of small heterodimer partner (SHP) gene mediated by farnesoid X receptor (FXR)/RXR heterodimer (34). Hence LRH-1 is considered to serve as a competence factor for the nuclear receptors that form a heterodimer with RXR in the liver (48). Based on this background, we hypothesized that LRH-1 might also exert a function as a competence factor for PPAR-γ/RXR heterodimer in adipocytes.

In nonadipose HEK293 cells, the endogenous LRH-1 expression is negligible (41). In our study, transfection of LRH-1 alone did not affect the transcriptional activity of adiponectin promoter in HEK293 cells. However, coexpression of LRH-1 augmented PPAR-γ/RXR–induced transactivation of adiponectin promoter. In 3T3-L1 adipocytes, which should contain endogenous PPAR-γ ligands, the luciferase activity of adiponectin promoter significantly decreased when the LRH-RE was mutated. We also observed that LRH-1 was expressed in 3T3-L1 adipocytes and mature adipocyte fraction obtained from rat fat pads. These results suggest that LRH-1 plays an important role in PPAR-γ–stimulated transcription of adiponectin gene as a competence factor in adipocytes, although the precise mechanism of LRH-1 action remains unknown. The direct interaction of PPAR-γ and LRH-1 was not seen by protein pull-down assay (data not shown). LRH-1 might be associated with a cofactor complex interacting with PPAR-γ/RXR as well as LXRα/RXR (34).

Steroidogenic factor 1 (SF-1) is expressed from the inception of adrenal and gonadal development and plays an important role in regulating the expression of multiple components of steroidogenesis (49). As SF-1 has been shown to share the binding site with LRH-1 (50), it is possible that SF-1 might also be involved in transactivation of adiponectin gene via the LRH-RE identified here. By RT-PCR, SF-1 was highly expressed in adrenal gland and testis in adult male mice, but SF-1 expression was not detected in mouse adipose tissues or 3T3-L1 cells (data not shown). Therefore, in adipocytes, we assume that LRH-1 should be more physiologically relevant for the LRH-RE in adiponectin promoter.

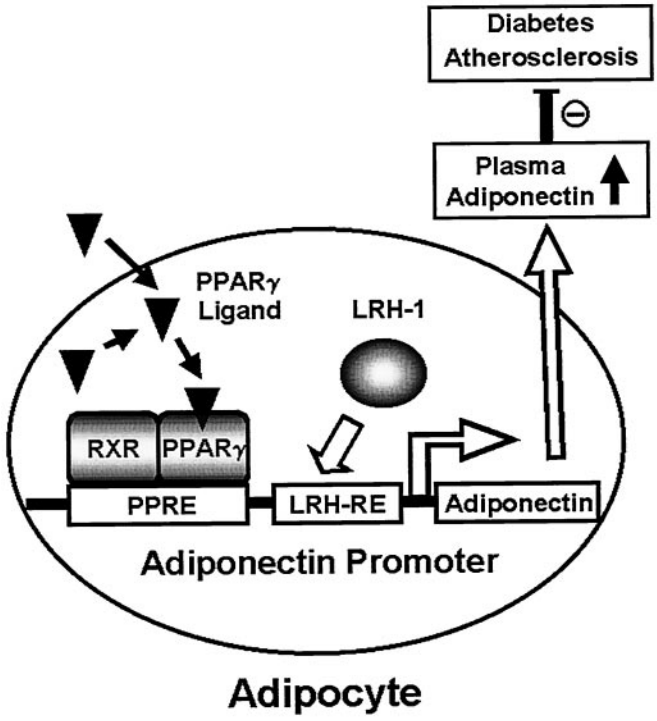

FIG. 8. Model illustrating transactivation of adiponectin promoter. Adipose expression of adiponectin gene is maintained and induced by endogenous or exogenous PPAR-γ ligands via the direct binding of PPAR-γ/RXR heterodimer to the PPRE (−273/−285) in adiponectin promoter. Another orphan nuclear receptor, LRH-1, also binds to the LRH-RE (−237/−229), enhancing transactivation of adiponectin promoter, in concert with PPAR-γ/RXR heterodimer. Increased transcription of adiponectin gene via the PPRE and the LRH-RE in the promoter results in enhanced production and secretion of adiponectin from adipocytes.

In the previous study by Das et al. (32), alignment of the 3-kb mouse adiponectin/Acrp30 promoter sequence with its human counterpart shows ~70% identity. However, in their article, the PPRE and the LRH-RE identified here are not described. We compared human and mouse adiponectin promoter sequences closely, and identified the PPRE-like sequence (−255/−267) and the LRH-RE–like sequence (−230/−222) in mouse adiponectin promoter. It will be of interest to determine whether these elements in mouse adiponectin promoter are functional as well.

In the present study, we have shown the molecular mechanisms of stimulatory effects of PPAR-γ agonists on the transcription of adiponectin gene (Fig. 8). Adipose expression of adiponectin gene is maintained and induced by endogenous or exogenous PPAR-γ ligands via the direct binding of PPAR-γ/RXR heterodimer to the PPRE (−273/−285) in adiponectin promoter. Another nuclear receptor, LRH-1, also binds to the LRH-RE (−237/−229) and functions as a competence factor, enhancing transactivation by PPAR-γ/RXR. Increased transcription of adiponectin gene via the PPRE and the LRH-RE in its promoter results in enhanced production and secretion of adiponectin from adipocytes. Therapy manipulating the transcriptional machinery regulated by PPAR-γ, RXR, and LRH-1 should enhance the production of adiponectin, a fat-derived antidiabetic and antiatherogenic factor.

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