Identification and Functional Characterization of the Peroxisomal Proliferator Response Element in Rat GLUT2 Promoter

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We identified the peroxisomal proliferator response element (PPRE) in the +68/+89 region of the rat GLUT2 gene. To identify whether the putative PPRE in the GLUT2 gene (GLUT2-PPRE) is functional, GLUT2 promoter-luciferase reporter constructs were transfected into CV-1 cells. Promoter activities were increased by coexpression of peroxisomal proliferator-activated receptor (PPAR)-γ, retinoid X receptor (RXR)-α, and treatment of their ligands; troglitazone and 9-*cis* retinoic acid potentiated the transactivational effects. Introduction of mutations in GLUT2-PPRE resulted in loss of transactivational effects of the PPAR-γ/RXR-α heterodimer. Electrophoretic mobility shift assay using nuclear extracts of CV-1 cells, which were transfected with various combinations of PPARs or RXR-α expression plasmids, revealed that heterodimers of PPAR-γ and RXR-α preferentially bound to GLUT2-PPRE. In HIT-T15 cells, promoter activity of the rat GLUT2 gene was increased by troglitazone and 9-*cis* retinoic acid, and mutations of GLUT2-PPRE resulted in reduction of promoter activity. In addition, we observed increased GLUT2 transcription by troglitazone and 9-*cis* retinoic acid in isolated rat primary islets. These results suggested that the GLUT2-PPRE is functional and plays a significant role in gene expression of GLUT2 in pancreatic β-cells. This is the first report identifying PPRE in a gene involved in glucose homeostasis, linking the effect of troglitazone on the regulation of insulin secretion. *Diabetes* 49:1517–1524, 2000

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DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; PPAR, peroxisomal proliferator-activated receptor; PPRE, peroxisomal proliferator response element; RT, reverse transcriptase; RXR, retinoid X receptor.

Type 2 diabetes is a systemic disorder characterized by impairment of both insulin secretion and action. The cells of patients with diabetes become resistant to insulin, and their abilities to use carbohydrate, fat, and protein as energy sources are altered.

Recently, a new class of the antidiabetic agent troglitazone has been developed to improve glucose tolerance by enhancing insulin sensitivity and secretion in humans and rodents (1–4). The mechanism by which troglitazone exerts its effect involves the activation of peroxisomal proliferator-activated receptor (PPAR)-γ (5). PPAR-γ is a nuclear hormone receptor expressed predominantly in adipose tissue as well as other tissues (6,7). PPAR-γ is activated by binding of its ligand. The activated PPAR-γ forms a heterodimer with retinoid X receptor (RXR)-α and exerts biologic activity by binding to the peroxisomal proliferator response element (PPRE). Therefore, troglitazone enhances transcription of many genes involved in fatty acid or lipid metabolism and seems to be linked to adipocyte differentiation (8,9).

Because the antidiabetic effect of troglitazone is known, many researchers have been trying to identify the effects of troglitazone on glucose homeostasis and its mechanism of antidiabetic effects. Because the relative potency of troglitazone to bind to and activate PPAR-γ in vitro correlates perfectly with its antidiabetic potency in vivo, PPAR-γ is being considered to mediate the antidiabetic effects of troglitazone (10,11). However, there is no direct evidence to conclusively implicate PPAR-γ in the regulation of mammalian glucose homeostasis. Thus, it is not known whether the effect of troglitazone on glucose homeostasis is due to alterations in signaling molecules produced by adipose tissue secondary to the activation of PPAR-γ or to its direct action on PPAR-γ (8,9).

Because troglitazone is known to augment glucose disposal in peripheral tissue (12), many researchers have focused their interests on how GLUT1 and GLUT4 activities are regulated by PPAR-γ. In fact, troglitazone increases glucose transport and gene expression of both GLUT1 and GLUT4 in L6 myocytes (13) and isolated cardiac myocytes (14), which has proven to be differentiation independent (13). GLUT1 mRNA and protein were increased in the cultures of human skeletal muscle by troglitazone (15). Also, troglitazone analog YM268 could normalize the decreased GLUT4 protein in adipose tissue of Zucker diabetic fatty (ZDF) rats (16). These results strongly suggest that troglitazone can directly increase glucose transporter expression, which con-

tributes to glucose disposal in peripheral tissue. In addition, GLUT2, which acts as a glucose-sensing apparatus coupled with glucokinase in pancreatic β-cells, was reported to be increased in protein levels with troglitazone in ZDF rats (17). From these observations, it can be assumed that members of the glucose transporter family could be activated by troglitazone in the transcriptional level; thus, these genes may have a PPRE in their promoter region. However, so far, no PPRE has been identified in genes directly involved in glucose homeostasis including glucose transporters (8).

Here, we have identified a PPRE in the +68/+80 region of the rat GLUT2 gene, which was bound in a dose-dependent manner by heterodimers of PPAR-γ and RXR-α in response to troglitazone and 9-*cis* retinoic acid. Also, we have shown that combined treatment of these 2 ligands could activate the GLUT2 gene expression in pancreatic β-cells. Thus, this is the first report that may explain the role of troglitazone in GLUT2 gene expression, which could explain how troglitazone stimulates glucose-induced insulin secretion from β-cells.

RESEARCH DESIGN AND METHODS

Materials. Troglitazone was a gift from Sankyo (Tokyo). WY14643 was purchased from Cayman Chemical (Ann Arbor, MI), and 9-*cis* retinoic acid was purchased from Sigma-Aldrich (Milwaukee, WI). Troglitazone concentration was adjusted to 10 mmol/l in 19% bovine serum albumin and 5% dimethyl sulfoxide (DMSO) (vol/vol). WY14643 (20 mmol/l) and 9-*cis* retinoic acid (2 mmol/l) were prepared in 50% ethanol (vol/vol) and 50% dimethyl sulfoxide (DMSO) (vol/vol), respectively. Expression plasmids pCMX-mPPAR-α, pCMX-mPPAR-γ, pCMX-mRXR-α, and pCMX-mRXR-γ were gifts from Drs. R.M. Evans and D.J. Mangelsdorf (18,19). Control vector pCMX was prepared from pCMX-mRXR-γ by excising out the 1.5-kb fragment of mRXR-γ cDNA. PPRE3-tk-LUC reporter construct was a gift from Drs. R.M. Evans and H. Kang (20). The rat GLUT2 promoter region spanning from –732 to +189 was subcloned into the pGL3 basic vector and named pGL3-732. The region containing site A (+56/+189) was obtained by digesting the promoter with *Acc*I and *Eco*RI, was subcloned into the pGL3 basic luciferase reporter vector, and was named pGL3+56.

pGL3-381 and pGL3-126 were constructed by amplifying rat GLUT2 promoter regions of –381/+189 and –126/+189, respectively, and subcloned into the pGL3 basic vector. Mutant constructs pGL3-732m and pGL3+56m were produced by introduction of 3 bp substitutions using the QuickChange site-directed mutagenesis kit (Stratagene). DNA sequences of all constructs were confirmed by DNA sequencing. The primers used in site-directed mutagenesis were also used as a GLUT2-PPREmt probe in the electrophoretic mobility shift assay (EMSA).

Cell culture and transient transfection. CV-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. HIT-T15 cells (Hamster pancreatic β-cell line) were maintained in Ham's F12-K medium (Life Technologies) supplemented with 10% dialyzed horse serum, 2.5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Transient transfections were performed using LipofectAMINE PLUS reagent (Life Technologies) according to the manufacturer's protocol. Briefly, 800 ng luciferase reporter construct, 100 ng mPPAR, mRXR-α expression plasmids, 100 ng pCMV-β-galactosidase expression plasmids, and 4 μl PLUS reagent were gently mixed in 100 μl OPTI-MEM (Life Technologies) and incubated for 15 min at room temperature. To transfect constant amounts of DNA, sample DNAs were supplemented with control vector pCMX. LipofectAMINE (2.5 μl) in 100 μl OPTI-MEM was added and incubated for another 15 min. During this period, cells were washed with phosphate-buffered saline 2 times, and 800 μl OPTI-MEM was added. After 15 min of incubation, transfection mixtures were added to the cells and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 3 h. After incubation, the remaining DNA-liposome complexes were removed, and the medium was replaced with DMEM containing 10% charcoal-treated FBS. Twelve hours after transfection, the medium was replaced with DMEM containing appropriate ligands or their vehicles (1:1 mixture of DMSO and ethanol for 9-*cis* retinoic acid and WY14643, and 19% bovine serum albumin and 5% DMSO for troglitazone). After a 36-h culture, cells were harvested and lysed by 100 μl reporter lysis buffer (Promega), and cell debris was removed by centrifugation. Luciferase activities were measured using 10 μl cell extract and 50 μl luciferase assay reagent (Promega). β-Galactosidase activity was measured with 10 μl cell extract and 190 μl β-galactosidase assay reagent containing *O*-nitrophenol-β-D-galactopyranoside. Luciferase activities were normalized by β-galactosidase activities to adjust transfection efficiency. Luciferase activities exerted by the wild-type promoter constructs without expressing any receptors were taken as a control.

Nuclear extracts preparation. For nuclear extracts preparation, transfections were performed in a 10-cm plate. From each expression vector or control vector (pCMX), 3 μg was transfected into CV-1 cells using the LipofectAMINE PLUS reagent. At 3 h after transfection, cells were refed with DMEM containing 10% FBS and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 48 h. Nuclear extracts of transfected CV-1 cells were prepared by the method of Schreiber et al. (21). Protein concentration was determined by the method of Bradford (22).

EMSA and supershift assay. Single-stranded sense oligonucleotide (10 pmol) of PPAR response element in rat GLUT2 promoter (GLUT2-PPRE) was labeled with ^{32}P using T4 polynucleotide kinase (TaKaRa, Shiga, Japan) and annealed with 5 mol/l excess of antisense oligonucleotides. The resulting double-stranded oligonucleotides were purified by a Sephadex G50 spin column (Pharmacia). About 0.1 pmol (50,000 cpm) of probe and 4 or 8 μg of nuclear extracts were incubated for 20 min on ice in a buffer containing 10 mmol/l HEPES (pH 7.9), 60 mmol/l KCl, 10% glycerol (vol/vol), and 1 mmol/l dithiothreitol. Poly (dI-dC) (1 μg) was added to each reaction to suppress nonspecific bindings. The protein-DNA complexes were resolved from the free probe by electrophoresis at 4°C on a 5% polyacrylamide gel in 0.5 × TBE buffer (1 × TBE contained 9 mmol/l Tris, 90 mmol/l boric acid, and 20 mmol/l EDTA, pH 8.0). For competition assays, 50 mol/l excess (~5 pmol) of unlabeled cold oligonucleotides was added to the reaction mixture. For the supershift assay, 1 μg anti–PPAR-γ antibody (Santa Cruz Biotech) was added to the reaction. The dried gels were exposed to X-ray film at –70°C with an intensifying screen.

The oligonucleotides used in EMSA were as follows: GLUT-PPRE, 5′-CGTCATCCCAGGGCAAAGTACAAAGAGCCAGG-3′; GLUT-PPREmt, 5′-CGTCATCCCAGGGCtAtcTACAAAGAGCCAGG-3′; ARE (PPRE of aP2 gene) (23), 5′-CTTCTTACTGGATCAGAGTTCACAGATC-3′; CYP (PPRE of cytochrome p450 gene) (24), 5′-GCAAACACTGAACTAGGGCAAAGTTGAGGGCAGTG-3′. The PPRE sequence is underlined, and mutated bases are shown in lowercase letters. GLUT-PPRE contains the sequences of rat GLUT2 promoter region from +59 to +87 (25).

Islet isolation and culture. Pancreatic islets were isolated from male Sprague-Dawley rats (200–250 g) by the method of Lacy and Kostianovsky (26) with minor modifications. Briefly, under ether anesthesia, the common bile duct was clamped at the entrance to the duodenum and cannulated. The pancreas was distended by injecting M 199 medium containing 1 mg/ml collagenase P (Boehringer Mannheim, Mannheim, Germany) and excised out quickly. The isolated pancreas was incubated for 25 min at 37°C and shaken by hand intermittently to dissociate individual islets further. Then the collagen-digested pancreas was washed 3 times with M 199 medium supplemented with 10% FBS and passed through a mesh filter (500 μm) to eliminate exocrine and lymphatic tissue. Islets were separated by Histopaque density gradient centrifugation (Sigma Aldrich) and washed 2 times with M 199 medium. Islets were collected under a microscope. Isolated islets were cultured in 60-mm Petri dishes at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 5 days. The culture medium consisted of RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Two days after isolation, islets were treated with 20 μmol/l troglitazone and 2 μmol/l 9-*cis* retinoic acid or its vehicle for 3 days.

Extraction of total RNA and semiquantitation by reverse transcriptase–polymerase chain reaction. After 3 days of culture in the presence of troglitazone and 9-*cis* retinoic acid, total RNA was extracted from islets (~100) using the TRIzol reagent (Life Technologies) following the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg RNA in 20 μl volume using random hexamer and AccuPower RT PreMix (Bioneer, Seoul, Korea). Reverse transcription reaction mixture (1 μl) was amplified with primers specific for rat GLUT2 in a total volume of 50 μl. Linearity of the polymerase chain reaction (PCR) was tested by amplifying 50 ng total RNA in the amplification cycles between 20 and 50. According to this amplification profile, samples were amplified for 30 cycles using the following parameters: 92°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers were used as an internal control for quality and quantity of RNA. The PCR products were subjected to electrophoresis on 1.2% agarose gel, and the quantities of PCR products were analyzed by Molecular Analyst II (Bio-Rad). The PCR product was confirmed by DNA sequencing. Primers used in PCR were as follows: GLUT2-sense, 5′-CATTGCTGGAAGAAGCGTATCAG-3′; GLUT2-antisense, 5′-GGAGACCTTCTGCTCAGTCGACG-3′; G3PDH-sense, 5′-ACCACAGTCCATGCCATCAC-3′; and G3PDH-antisense, 5′-GGAGACCTTCTGCTCAGTCGACG-3′.

Statistical analysis. All transfection studies were performed in triplicate and repeated 3–5 times. The data were represented as means ± SE. Statistical analysis was carried out using SigmaStat software (Jandel Scientific).

RESULTS

Rat GLUT2 promoter has a functional PPRE. To identify the presence of PPRE in the promoter region of rat GLUT2, 5′ deleted fragments containing the promoter regions of –732/+189, 381/+189, and –126/+189 were prepared. The fragments were subcloned into pGL3-basic luciferase reporter vector and named pGL3-732, pGL3-381, and pGL3-126, respectively (Fig. 1). These clones were transiently transfected into CV-1 cells together with PPAR-α, PPAR-γ, or RXR-α expression plasmids as indicated (Fig. 2). The 5′ deleted promoter constructs did not show a significant difference in their basal activities compared with pGL3-732. Coexpression of PPAR-γ and RXR-α in the presence of appropriate ligands increased the promoter activities by 3.8-, 2.6-, and 2.3-fold in pGL3-732, pGL3-381, and pGL3-126, respectively. However, coexpression of PPAR-α and RXR-α did not activate the promoters, suggesting that functional PPRE is present in the region between –126 and +189 of the rat GLUT2 gene, and this PPRE preferentially responds to the heterodimer of PPAR-γ and RXR-α.

Site A of the rat GLUT2 promoter contains a PPRE. Using rat liver nuclear extract, we identified 8 DNase I footprinting regions in our earlier study (27) within the region of –732 to +190. Computer-based sequence analysis suggested that one of these protein binding sites, site A (+63/+110), has a putative PPRE (+68/+80) similar to the consensus sequence proposed by Palmer et al. (28). To confirm the identity of PPRE, we introduced 3 bp substitutions in the putative PPRE of pGL3-732 and compared the transactivational effects by PPAR-α, PPAR-γ, and RXR-α in CV-1 cells (Fig. 3*A*). Wild-type promoter was activated by coexpression of PPAR-γ and RXR-α (1.9-fold), and the transactivational effect was further potentiated by combined treatment of their ligands 9-*cis* retinoic acid and troglitazone (3.4- and 4.7-fold by 10 μmol/l troglitazone per 1 μmol/l 9-*cis* retinoic acid and 20 μmol/l troglitazone per 2 μmol/l 9-*cis* retinoic acid, respectively). Single expression of PPAR-α, PPAR-γ, or RXR-α did not activate the promoter, suggesting that PPAR-γ and RXR-α acted as heterodimers in activating the promoter. However, coexpression of PPAR-γ and RXR-α and/or treatment of their ligands did not activate the mutant type promoter construct pGL3-732m. These results further supported the conclusion that the +68/+80 region is a PPRE responding to the heterodimer of PPAR-γ and RXR-α in a ligand-dependent manner.

To minimize the effects of other factors possibly affecting the rat GLUT2 promoter, we constructed a luciferase reporter construct under the control of a minimal promoter containing the +56/+189 region of the rat GLUT2 gene (pGL3+56) and its mutant (pGL3+56m) and examined the transactivational effect of PPARs and RXR-α in CV-1 cells (Fig. 3*B*). Wild-type and mutant type promoter constructs did not show significant differences in their basal promoter activities. Combined expression of PPAR-γ and RXR-α in the presence of their ligands activated the wild-type minimal promoter (3.5-fold increase). In contrast, the mutant type promoter was not activated significantly. Coexpression of PPAR-α and RXR-α activated wild-type promoter slightly. These results agreed well with those observed in the transfection study using pGL3-732 and pGL3-732m and strongly supported the fact that heterodimer of PPAR-γ and RXR-α preferentially binds to and activates the PPRE in site A of the rat GLUT2 promoter.

PPAR-γ and RXR-α heterodimers bound to the GLUT2-PPRE. To determine whether PPAR-γ and RXR-α heterodimers activate rat GLUT2 promoter through direct DNA binding, we performed EMSA using GLUT2-PPRE (+59/+87 fragment) as a probe (Fig. 4). The nuclear extracts were prepared from groups of CV-1 cells transfected with PPAR-α, PPAR-γ, and RXR-α alone or in combination. EMSA using the

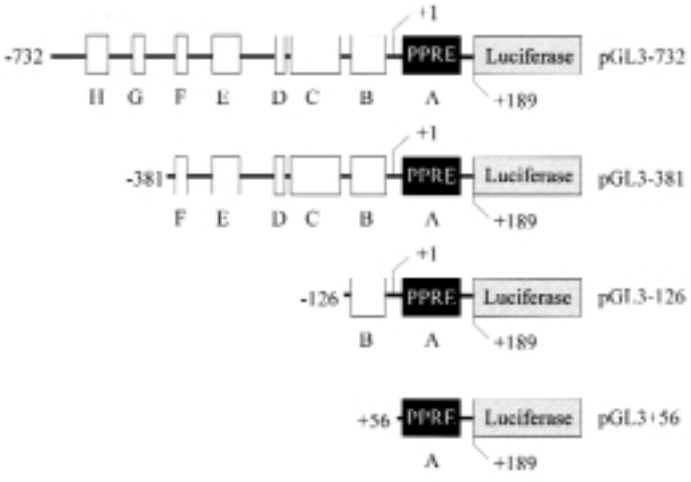


FIG. 1. Structure of the rat GLUT2 promoter. Boxes represent the protein binding sites reported by Kim and Ahn (27). Site A indicates the region containing a possible PPRE; +1 represents the transcription initiation site.

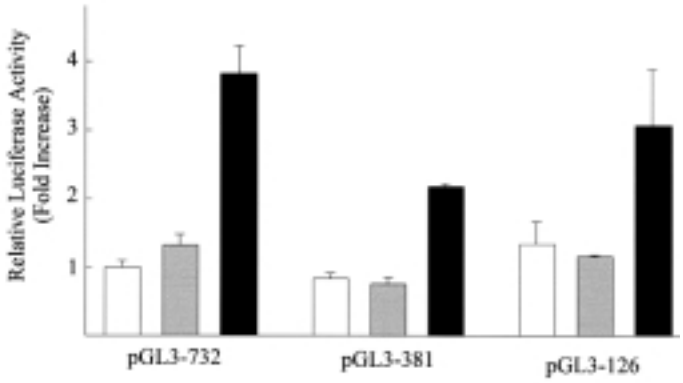
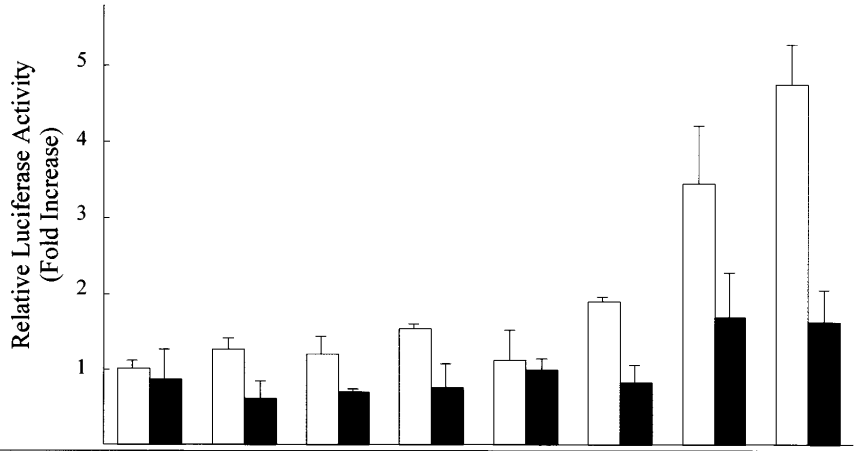


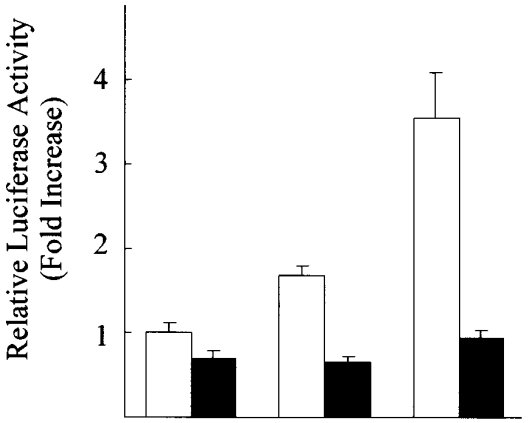
FIG. 2. Identification of the PPRE in the promoter region of the rat GLUT2 gene. Luciferase reporter constructs under the control of the rat GLUT2 promoter, each spanning from –732, –381, and –126 to +189, were cotransfected into CV-1 cells with or without PPARs and RXR-α expression vectors. The cells were incubated in the presence or absence of respective ligands as indicated. A total of 10 μmol/l WY14643 for PPAR-α, 10 μmol/l troglitazone for PPAR-γ, and 1 μmol/l 9-*cis* retinoic acid for RXR-α was used as ligand. Normalized luciferase activities are shown as means ± SE of 3 independent experiments in triplicate and are expressed as the fold-increase relative to the basal activity of pGL3-732 in the absence of expression vectors and ligands. □, Control; ▩, PPAR-α/RXR-α (vehicle, WY14643/9-*cis* retinoic acid); ■, PPAR-γ/RXR-α (vehicle, troglitazone/9-*cis* retinoic acid).

A



9-cis RA(μM)	-	-	-	-	1	-	1	2
WY-14643(μM)	-	-	-	-	10	-	-	-
Troglitazone(μM)	-	-	-	-	-	-	10	20
mRXRα	-	-	-	+	+	+	+	+
mPPARα	-	+	-	-	+	-	-	-
mPPARγ	-	-	+	-	-	+	+	+

B



9-cis RA(μM)	-	1	1
WY-14643(μM)	-	10	-
Troglitazone(μM)	-	-	10
mRXRα	-	+	+
mPPARα	-	+	-
mPPARγ	-	-	+

FIG. 3. *A*: Effect of mutations in the PPRE on GLUT2 promoter activities. Wild-type pGL3-732 (□) and its PPRE-mutated counterpart pGL3-732m (■) were cotransfected with or without expression vectors of PPARs and RXR-α. The cells were treated with their ligands or vehicles for 36 h as indicated. *B*: Effect of a mutation in the PPRE of pGL3+56. Rat GLUT2 minimal promoter pGL3+56 (□) and its PPRE-mutant counterpart (■) were cotransfected with or without expression vectors for PPARs and RXR-α. The cells were incubated for 36 h in the presence or absence of their ligands as indicated. Normalized luciferase activities are shown as means ± SE of 3 independent experiments in triplicate and are expressed as the fold-increase relative to the basal activity of pGL3-732 (*A*) or pGL3+56 (*B*) in the absence of expression vectors and ligands. RA, retinoic acid.

A

	5'flank	DR+1
Consensus	GG T	T
	CAAAACTAGGTCAAAGGTCA	
ARE	TCTTACTGGATCAGAGTTCA	
CYP	CTGAACTAGGGCAAAGTAGA	
GLUT2-PPRE	TCATCCCAGGGCAAAGTACA	
GLUT2-PPREmt	TCATCCCAGGGCtAtcTACA	

B

Amount of NE	4μg/lane						8μg/lane					
Number of Lane	1	2	3	4	5	6	7	8	9	10	11	12
Anti-PPARγ Ab	-	-	-	-	-	-	-	-	-	-	-	+
Competitor	-	-	-	-	-	-	-	Wild	Mt	ARE	CYP	-
PPAR overexpression	-	-	α	γ	α	γ	γ	γ	γ	γ	γ	γ
RXRα overexpression	-	+	-	-	+	+	+	+	+	+	+	+

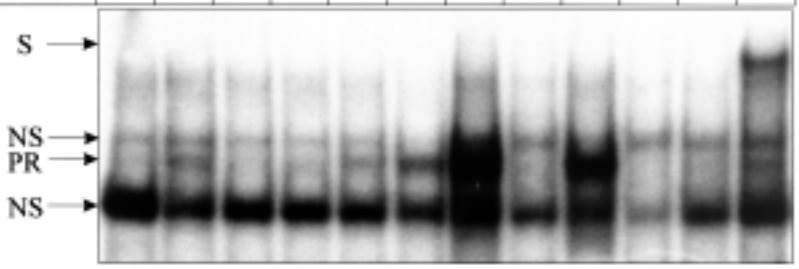


FIG. 4. *A*: Sequence comparison of PPREs from various genes. Sequences of GLUT2-PPRE and its mutant form GLUT2-PPREmt, ARE (23), and CYP (24) are compared with the consensus sequence of PPRE. PPRE sequence is underlined, and mutated bases are lowercase (28). *B*: EMSA of GLUT-PPRE. The ^{32}P-labeled double-strand oligonucleotide GLUT2-PPRE was incubated with nuclear extracts (4 or 8 μg) from CV-1 cells transfected with expression vectors of PPARs and/or RXR-α as indicated. For competition experiments, 50 mol/l excess of GLUT2-PPRE (Wild), GLUT2-PPREmt (Mt), ARE, and CYP were included in the binding reaction. For the supershift experiment, 1 μg anti–PPAR-γ antibody was used. PR indicates the shifted band by heterodimer of PPAR/RXR-α, and S indicates the supershifted band by anti–PPAR-γ antibody. Ab, antibody; DR+1, the hexamer sequence AGGTCA in a direct repeat formation spaced by 1 nucleotide; NE, nuclear extract; NS, nonspecific binding; PR, binding of PPAR/RXR-α heterodimer; S, supershifted band.

RXR-α–expressed nuclear extract showed a shifted band (Fig. 4B, lane 2, PR), which was thought to be the heterodimer of endogenous PPAR-γ and expressed RXR-α. In contrast, incubation with PPAR-α– or PPAR-γ–expressed nuclear extracts did not form specific DNA protein complexes (Fig. 4B, lanes 3 and 4). When nuclear extracts from CV-1 cells coexpressed with PPAR-α/RXR-α or PPAR-γ/RXR-α were used, a prominent DNA protein complex was observed (Fig. 4B, lanes 5 and 6, PR). Of these 2 groups, PPAR-γ/RXR-α seemed to form a more prominent DNA protein complex compared with PPAR-α/RXR-α coexpression. The binding of proteins to GLUT2-PPRE was increased in a dose-dependent manner (Fig. 4B, lanes 6 and 7). The specificity of the DNA protein complex was confirmed by the addition of cold oligonucleotides as wild competitors (Fig. 4B, lane 8) and mutant competitors (Fig. 4B, lane 9). Mutant competitors did not compete out, whereas wild competitors did well. The specificity of the GLUT2-PPRE was further confirmed by competition assay using oligonucleotides containing previously known PPREs ARE and CYP (Fig. 4B, lanes 10 and 11); ARE is the PPRE in the promoter of the mouse aP2 gene (23), and CYP is the PPRE in the promoter of the rabbit CYP4A6 gene (24). Because protein binding to GLUT2-PPRE was competed out by ARE and CYP, it could be generalized that GLUT2-PPRE belongs to PPREs. A supershift experiment using anti–PPAR-γ antibodies revealed that the protein binding to GLUT2-PPRE was PPAR-γ (Fig. 4B, lane 12). If the protein that bound to GLUT2-PPRE was the RXR-α homodimer, anti–PPAR-γ antibodies could not supershift the band. Thus, it could be concluded that PPAR-γ and RXR-α heterodimers bind to GLUT2-PPRE and transactivate the rat GLUT2 promoter.

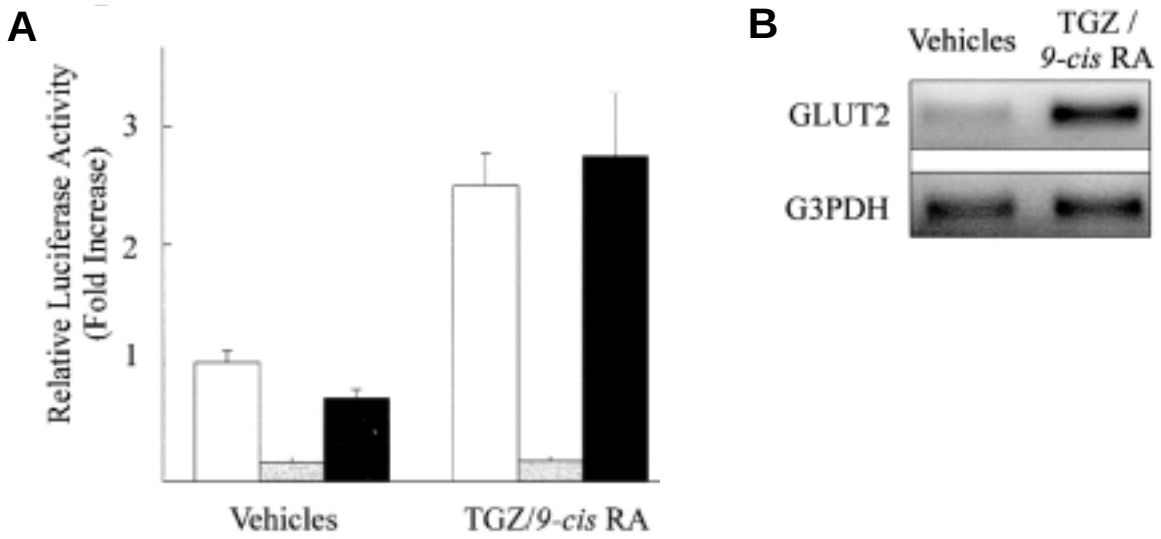


FIG. 5. ***A*****: Activation of the rat GLUT2 promoter by endogenous PPAR-γ and RXR-α in HIT-T15 cells. HIT-T15 cells were transfected with pGL3-732 (□), pGL3-732m (▩), or PPRE3-tk-LUC (■). The cells were then treated with 20 μmol/l troglitazone (TGZ) and 2 μmol/l 9-*cis* retinoic acid (RA) or their vehicles. The PPRE3-tk-LUC reporter was constructed by inserting 3 copies of an oligonucleotide encoding the PPRE (GTCGA CAGGGGACCAGGACAAAGGTCACGTTCGGGAGTCGAC) in direct orientation to the unique *SalI* site of the basal reporter construct tk-LUC (16). Normalized luciferase activities are shown as means ± SE of 3 independent experiments in triplicate and are expressed as the fold-increase relative to the basal activity of pGL3-732 in the absence of ligands. *B*: Effect of troglitazone and 9-*cis* retinoic acid on GLUT2 expression in the isolated primary islets of rats. Isolated primary islets were incubated for 3 days in the presence or absence of 20 μmol/l troglitazone and 2 μmol/l 9-*cis* retinoic acid, and total RNA was prepared. RT-PCR was adapted to measure the GLUT2 mRNA semiquantitatively. Quantitation of PCR products was performed by densitometric scanning using Molecular Analyst (Bio-Rad), and quantity of GLUT2 mRNA was normalized with respect to G3PDH mRNA.**

GLUT2-PPRE is responsive to the ligands of PPAR-γ and RXR-α in HIT-T15 cells. We observed the transactivational effect of PPAR-γ/RXR-α on the rat GLUT2 promoter in CV-1 cells. Although CV-1 cells are known to express PPAR-γ (29), endogenous PPAR-γ of CV-1 cells did not affect the transfected rat GLUT2 promoter (data not shown). Therefore, what will happen if the rat GLUT2 promoter is transfected into the cells in which GLUT2 is expressed and endogenous PPAR-γ is functionally active? To answer this question, we transfected pGL3-732 and pGL3-732m into HIT-T15 cells, which are known to express GLUT2. HIT-T15 cells can also secrete insulin by glucose stimulation and are known to increase glucose uptake and glucose-stimulated insulin secretion by troglitazone (30). To confirm that HIT-T15 cells have functional endogenous PPAR-γ, we transfected PPRE3-tk-LUC, which contains 3 copies of the PPRE of acyl-CoA oxidase promoter in the tk-LUC reporter vector and is known to be activated by PPARs (20). This promoter was activated 3.9-fold by combined treatment of troglitazone and 9-*cis* retinoic acid, which indicated the presence of functional PPAR-γ in HIT-T15 cells (Fig. 5*A*). We also transfected pGL3-732 and pGL3-732m to explore the role of PPAR-γ in the regulation of the rat GLUT2 promoter. The mutant type promoter (pGL3-732m) showed decreased basal promoter activity <20% of the wild-type promoter (pGL3-732). The activity of the wild-type promoter (pGL3-732) was increased 2.5-fold by combined treatment of troglitazone and 9-*cis* retinoic acid; however, no such increase was seen in its PPRE mutant (pGL3-732m). These results strongly suggest that endogenous PPAR-γ and RXR-α of HIT-T15 cells can activate the rat GLUT2 promoter in response to their ligands and that PPAR-γ might play a significant role in GLUT2 expression and glucose sensing in pancreatic β-cells.

Troglitazone induces transcription of the GLUT2 gene in primary rat islets. We demonstrated that PPAR-γ could activate the rat GLUT2 promoter in a ligand-dependent manner. We also identified the possibility that PPAR-γ has an important regulatory role for GLUT2 expression in pancreatic β-cells. If troglitazone can activate the rat GLUT2 promoter through GLUT2-PPRE, GLUT2 gene transcription should be increased by troglitazone in pancreatic β-cells. Therefore, we examined whether troglitazone could induce the transcription of GLUT2 in isolated rat primary islets. We prepared total RNA from isolated rat primary islets cultured in the presence or absence of troglitazone and 9-*cis* retinoic acid for 3 days and performed semiquantitative reverse transcription (RT)-PCR to measure mRNA levels of GLUT2, which resulted in increased GLUT2 transcription by 2.9-fold (Fig. 5*B*). This result clearly shows that this PPRE is truly functional, and the expression of the rat GLUT2 gene is induced by PPAR-γ.

DISCUSSION

Troglitazone has been known to reduce hyperglycemia, hyperinsulinemia, and hypertriglyceridemia in insulin-resistant humans and rodents (12). Although the mechanisms by which troglitazone exerts its antidiabetic action are not fully understood, it is generally accepted that it increases glucose transport activity and transporter expression in adipose tissue, muscle, and pancreatic islets (12,14,30,31). Because troglitazone is a synthetic ligand for PPAR-γ, it is possible that troglitazone mediates the gene expression of the glucose

transporter family through PPAR-γ. However, no PPRE has been identified in the promoters of glucose transporter families as well as other components directly involved in the glucose homeostasis so far (8). Thus, we could not exclude the possibility that the increased glucose transport mediated by troglitazone could be a secondary effect resulting from its metabolic effect on energy metabolism.

In this study, we were able to localize the PPRE in the rat GLUT2 promoter. The sequence AGGGCAAAGTACA contained in site A of the rat GLUT2 promoter worked as a PPRE that was responding to PPAR-γ and RXR-α in the presence of troglitazone and 9-*cis* retinoic acid. We also confirmed that the heterodimer of PPAR-γ and RXR-α preferentially bound to the GLUT2-PPRE. The 3 base pair mutations introduced in the GLUT2-PPRE abolished the transactivational effect of PPAR-γ and RXR-α in CV-1 cells and abolished the binding of PPAR-γ and RXR-α heterodimers. However, the basal promoter activities without coexpression of PPAR-γ and RXR-α were not affected by mutation of GLUT2-PPRE in CV-1 cells. Although CV-1 cells are known to express PPAR-γ, endogenous PPAR-γ of CV-1 cells did not affect the activity of the transfected promoters that were not active in CV-1 cells (32,33). Luciferase activity of pGL3-732 was not stimulated by treatment of troglitazone and 9-*cis* retinoic acid (data not shown), suggesting that endogenous PPAR-γ in CV-1 cells is not enough to drive the transfected rat GLUT2 promoter. Thus, CV-1 cells could be a suitable model to test the transactivational effect of PPAR-γ and RXR-α.

In the case of HIT-T15 cells, treatment with troglitazone and 9-*cis* retinoic acid without coexpression of their respective receptors increased the luciferase activities of pGL3-732 and PPRE3-tk-LUC. However, the PPRE mutated promoter (pGL3-732m) was not activated by troglitazone and 9-*cis* retinoic acid and showed decreased basal promoter activity compared with the wild-type promoter construct (<20% of the wild-type promoter). What is the difference between CV-1 and HIT-T15 cells? HIT-T15 cells are the hamster pancreatic β-cell line that expresses GLUT2 and secretes insulin in response to glucose. In the earlier report of Masuda et al. (30), troglitazone could increase glucose uptake and insulin secretion in HIT-T15 cells and primary rat islets, which means that HIT-T15 cells are functional pancreatic β-cells, and endogenous PPAR-γ in HIT-T15 cells is functionally active. We also confirmed the function of endogenous PPAR-γ of HIT-T15 cells by transfecting PPRE3-tk-LUC. Thus, it could be concluded that the rat GLUT2 promoter is active in HIT-T15 cells, and PPAR-γ plays a significant role in the activation of the rat GLUT2 promoter because only 3 bases pair point mutations of the promoter region resulted in an 80% decrease in promoter activity.

Higa et al. (17) have observed the reappearance of the GLUT2 protein in pancreatic β-cells of ZDF rats administered troglitazone. This observation suggests the potential role of PPAR-γ in the regulation of the GLUT2 gene in pancreatic β-cells. To explore this possibility, pancreatic islets were isolated from Sprague-Dawley rats and treated with troglitazone and 9-*cis* retinoic acid. Semiquantitative measurement of GLUT2 mRNA as determined by RT-PCR revealed that treatment of isolated rat primary islets with troglitazone and 9-*cis* retinoic acid increased GLUT2 transcription. These data support the fact that the increase of GLUT2 expression in protein level as observed by Higa et al. (17) was due to the increased GLUT2 transcription, and moreover, this process was achieved through binding of PPAR-γ/RXR-α heterodimers to GLUT2-PPRE. Taken together, these data support the conclusion that PPAR-γ heterodimerized with RXR-α controls the rat GLUT2 promoter in pancreatic β-cells, and the control of GLUT2 expression by PPAR-γ/RXR-α depends on their ligands.

Troglitazone can decrease the expression of genes that contribute to the accumulation of fat (34) and inhibit the production of inducible nitric oxide in β-cells (35). Thus, troglitazone plays the role of protecting β-cells from a lipotoxic environment (36). Troglitazone can also restore β-cell function, including glucose-stimulated insulin secretion in ZDF rats (17,36), suggesting that it can improve physiological regulation of insulin secretion. Currently, insulin secretion is known to be stimulated by glucose or its metabolites. In this pathway, GLUT2 may play a role as a glucose sensor (37). Based on these observations and our results, troglitazone is thought not only to protect β-cells from lipoapoptosis, but also to improve the glucose sensitivity of β-cells. Therefore, it would appear that increased GLUT2 gene expression by troglitazone contributes to the restoration of physiological regulation of insulin secretion in β-cells of ZDF rats.

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