Transcription Suppression of Thromboxane Receptor Gene by Peroxisome Proliferator-activated Receptor-\(\gamma\) via an Interaction with Sp1 in Vascular Smooth Muscle Cells*

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Thromboxane (TX) \(A_2\) exerts contraction and proliferation of vascular smooth muscle cells (VSMCs) via its specific membrane TX receptor (TXR), possibly leading to the progression of atherosclerosis. A nuclear hormone receptor, peroxisome proliferator-activated receptor (PPAR)-\(\gamma\), has recently been reported to be expressed in VSMCs. Here we examined a role of PPAR-\(\gamma\) in TXR gene expression in VSMCs. PPAR-\(\gamma\)-ligands 15-deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\) and troglitazone reduced TXR mRNA expression levels as well as cell growth as assessed by [\(^{3}H\)]thymidine incorporation. Transcriptional activity of the TXR gene promoter was suppressed with PPAR-\(\gamma\) ligands, and the suppression was augmented further by PPAR-\(\gamma\) overexpression. By deletion and mutation analyses, the transcription suppression was shown to be the result of a \(-22/-7\) GC box-related sequence (upstream of transcription start site). Electrophoretic mobility shift assays also showed that the sequence was bound by Sp1 but not by PPAR-\(\gamma\), and the formation of a Sp1-DNA complex was inhibited either by coinucitation with PPAR-\(\gamma\) or PPAR-\(\gamma\)-ligand treatment of VSMCs. Moreover, glutathione \(S\)-transferase pull-down assays demonstrated a direct interaction between PPAR-\(\gamma\) and Sp1. In conclusion, PPAR-\(\gamma\) suppresses TXR gene transcription via a protein-protein interaction with Sp1. PPAR-\(\gamma\) may possibly have an antiatheroscerotic action by inhibiting TXR gene expression in VSMCs.

Pathophysiological role of the inflammatory diseases such as atherosclerosis (5) and glomerulonephritis (6). The biological action of TX is mediated via its specific membrane TX receptor (TXR). We previously isolated a cDNA for rat TXR (7), localized it in either the kidney (8) or testis (9), and identified its chromosomal localization (10). Moreover, we have isolated \(-5'\)-flanking region (FL) of the rat TXR gene and studied its transcription regulation in VSMCs (11).

Peroxisome proliferator-activated receptor (PPAR)-\(\gamma\) is a nuclear hormone receptor that was shown to transactivate adipocyte-specific genes and induce adipocyte differentiation (12). Either insulin-sensitizing thiazolidinediones including troglitazone (TRO) or 15-deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\) (PGJ\(_2\)) (13, 14) has been identified as a ligand of PPAR-\(\gamma\). Recently, PPAR-\(\gamma\) has been shown to be present not only in adipocytes but also in vascular tissues including VSMCs (15), and an inhibitory effect of PPAR-\(\gamma\) on gene expression in atherosclerosis has been studied. Activation of PPAR-\(\gamma\) with its ligands suppressed expression of plasminogen activator inhibitor type 1 (16) in vascular endothelial cells and that of matrix metalloproteinase-9 in VSMCs (15). Moreover, we have observed that PPAR-\(\gamma\) can suppress TX synthase gene transcription in macrophages (17).

In the present study, we examined the role of PPAR-\(\gamma\) in TXR gene expression in VSMCs. We observed that PPAR-\(\gamma\) inhibited the TX-mediated cell growth of VSMCs and TXR mRNA expression. Suppression of TXR gene transcription was confirmed, and the suppression was shown to be dependent on a GC box-related sequence present at the \(-22/-7\) region of TXR gene promoter (upstream of transcription start site), which was bound by Sp1 but not by PPAR-\(\gamma\). PPAR-\(\gamma\) was shown to interact physically with Sp1 by glutathione \(S\)-transferase (GST) pull-down assays. Taken together, PPAR-\(\gamma\) was suggested to suppress TXR gene transcription via a protein-protein interaction with Sp1. An antiatherosclerotic action of PPAR-\(\gamma\) by inhibiting TXR gene expression in VSMCs may be suggested.

EXPERIMENTAL PROCEDURES

Plasmids—Previously reported (11) and newly subcloned chimeric constructs containing rat TXR gene promoter and luciferase cDNA were used for transient transfection studies: \(-989+/+184\)-lac (989-bp \(5'\)-flanking region (FL) and 184-bp \(5'\)-untranslated region (UTR) of rat TXR gene); \(-809/+184\)-lac (809-bp \(5'\)-FL and 184-bp \(5'\)-UTR); \(-489/+184\)-lac (489-bp \(5'\)-FL and 184-bp \(5'\)-UTR); \(-213/+184\)-lac (213-bp \(5'\)-FL and 184-bp \(5'\)-UTR); \(-78/+184\)-lac (78-bp \(5'\)-FL and 184-bp \(5'\)-UTR); \(-78/+120\)-lac (78-bp \(5'\)-FL and 120-bp \(5'\)-UTR); \(-47/+120\)-lac (47-bp \(5'\)-FL and 120-bp \(5'\)-UTR); \(-39/+120\)-lac (39-bp \(5'\)-FL and 120-bp \(5'\)-UTR); \(-22/+120\)-lac (22-bp \(5'\)-FL and 120-bp \(5'\)-UTR); \(-6/+120\)-lac (6-bp \(5'\)-FL and 120-bp \(5'\)-UTR); \(-78/+60\)-lac (78-bp \(5'\)-FL and 60-bp \(5'\)-UTR); \(-78/+1\)-lac (78-bp \(5'\)-FL); \(-78/-7\)-lac (78-bp \(5'\)-FL).
Suppression of TXR Gene Transcription by PPAR-γ

Lucid (-78/-48 and -6/-1 of rat TXR gene) and -47/-7 luc (-47/-7 of rat TXR gene). Moreover, Δ(-47/-29)/Δ(-47/-7) luc (-47/-7 of rat TXR gene whose GC box-related sequence at the -39/-29 region was mutated from CCG GCT GAG GGA CGA GGC TGG AGG GGG TGG TGG GGC TGG CG; mutated sites are underlined) and Δ(-22/-7)/Δ(-47/-7) luc (-47/-7 of rat TXR gene whose GC box-related sequence at the -22/-7 region was mutated from CCG GCT GAG GGA CGA GGC TGG AGG GGG TGG TGG GGC TGG CG; mutated sites are underlined) were also generated. Δ(-22/-7)/Δ(-47/-7) luc (-47/-7 of rat TXR gene whose GC box-related sequence at the -22/-7 region was mutated from CCG GCT GAG GGA CGA GGC TGG AGG GGG TGG TGG GGC TGG CG; mutated sites are underlined) was generated using a QuickChange™ site-directed mutagenesis kit (Stratagene). Mouse PPAR-γ expression plasmid in pCMX (18) was kindly provided by Dr. K. Umesono (Kyoto University, Kyoto, Japan). A previously described cDNA clone of mouse RXR-α in pBSK (19) (kindly provided by Dr. R. M. Evans, The Salk Institute, San Diego) was subcloned into pCDNA1/AMP (Invitrogen) in the right orientation. Full-length mouse PPAR-γ cDNA was subcloned into the pGEX-4T-2 vector (Amersham Biosciences, Inc.) in the right orientation (designated as pGEX-PPAR-γ). Sp1 in pCMV (20) was kindly provided by Dr. Y. Fujii (Tohoku University, Sendai, Japan), pGEX-Sp1, which contains full-length Sp1 cDNA subcloned into pGEX-2TK vector (Amersham Biosciences, Inc.) in the right orientation, was kindly provided by Dr. H. Rotheneder (University of Vienna, Vienna, Austria) (21). β-Galactosidase control plasmid in pCMV was purchased from Clontech.

Cell Culture—Rat VSMCs that were isolated from male Sprague-Dawley rat thoracic aorta were gifts from Dr. K. K. Griendling (Emory University, Atlanta, GA) and maintained as described previously (22). Passages between 7 and 15 were used for the following experiments.

3H]Thymidine Incorporation—When rat VSMCs grown in 3.5-cm plates became 70% confluent, media were changed to serum-free minimum Eagle’s medium and incubated for 2 days. Then they were incubated in the absence or presence of 1 μM U-46619 (2) (a stable TXA2 receptor agonist) (22/47) (CCG GCT GAT TTT TTA CAG TGC AGG GGG TGG CG; mutated sites are underlined) or the -47/-7 region mutated from CCA GTG CAG GGT GTG GGG CTG GCG AGA CGC AGC to CCA GTG CAG GGT GTG CTT CTT GTG AGA AGA CGC; mutated sites are underlined) was used for some experiments. For competition experiments, a 100-fold excess of unlabeled oligonucleotides for the [3H]thymidine incorporation (the value in line 1 as 100%) (n = 6). *p < 0.01 compared with the value in line 2.

Northern Blot Analysis—When rat VSMCs became 70% confluent, media were changed to Dulbecco’s modified Eagle’s medium with 1% resin and charcoal-treated calf serum (stripped medium) (23) and incubated for 5–6 h. The cells then were incubated either with or without 2.5 μM TRO for an additional 12 h. Their total RNAs were then extracted using an RNaseasy mini kit (Qiagen). 10 μg of isolated total RNA was subjected to electrophoresis in 1% agarose gel, stained with ethidium bromide, and analyzed by the manufacturer’s system (Amersham Biosciences, Inc.). The blot was hybridized with 32P-labeled TXR cDNA probe as described previously (7). The intensity of the blot was calculated using Luminous Image (ALC), and all values were normalized to the densities of ethidium bromide staining of 28S ribosomal RNA (rRNA).

Luciferase Reporter Gene Assay—When rat VSMCs became 70% confluent, media were changed to serum-free minimum Eagle’s medium and incubated for 5–6 h. Then the transfection using Lipofectin was performed according to the manufacturer’s instructions (Invitrogen). Briefly, 1.2 μg of reporter plasmid and 0.5 μg of β-galactosidase control plasmid in pCMV (Clontech) were mixed with 6 μl of Lipofectin/3.5-cm plate. In some experiments, 1 μg of PPAR-γ, RXR-α, and/or Sp1 expression plasmids were cotransfected. 12 h after transfection, media were changed to stripped medium, and the cells were incubated for an additional 12 h. The cells were then incubated either with or without several concentrations of PGJ2 or TRO for 12 h. In some experiments, the cells were incubated with 1 μM Iloquinol (LT) B4 (24) (Cayman Chemical) for 12 h. After harvesting, the cell extracts were analyzed for both luciferase and β-galactosidase activities (23). Transfection efficiency was normalized by the β-galactosidase expression.

Electrophoretic Mobility Shift Assay (EMSA)—In vitro transcription/translation of mouse PPAR-γ, mouse RXR-α, and Sp1 cDNA clones was performed using TNT™ kits (Promega). Unprogrammed reticulocyte lysate was also generated simultaneously. To confirm the generation of these proteins, in vitro transcription/translation in the presence of [35S]methionine was performed simultaneously. The proteins were then analyzed by SDS-PAGE as described previously (25). Rat VSMC nuclear extracts were prepared as described previously (25). EMSA was performed as described previously (25). Briefly, 32P-labeled double-stranded oligonucleotides containing either the intact −47/-7 region (CCG GCT GAG GGA CGA GGC TGG AGG GGG TGG TGG GGC TGG CG; mutated sites are underlined) or the −47/-7 region mutated from CCA GTG CAG GGT GTG GGG CTG GCG AGA CGC AGC to CCA GTG CAG GGT GTG CTT CTT GTG AGA AGA CGC; mutated sites are underlined) was used for either 2 μl of in vitro translated Sp1, PPAR-γ, RXR-α, reticulocyte lysate, or 2 μg of rat VSMC nuclear extracts for 30-min at room temperature, and was subjected to electrophoresis on 4% polyacrylamide gels. In some experiments, 2 μg of rat VSMC nuclear extracts was incubated with several amounts of in vitro translated PPAR-γ and/or reticulocyte lysate. Moreover, nuclear extracts prepared from rat VSMCs treated either with or without 2.5 μM PGJ2 or 50 μM TRO for 12 h were used for some experiments. For competition experiments, a 100-fold excess of unlabeled oligonucleotides for the −47/-7 region, SV40 early promoter Sp1 site (AGT TAG GGG CAT GGA AGT TAG) (26), or hydroxymethylguanine-CoA synthase gene PPRE (−111/−5) TTT TAT GAG ACC TTT GGC CAA CTT TTT (27) were coinubated.

Antibody Supershift Experiments—After a 30-min incubation of 2 μg of rat VSMC nuclear extracts or 2 μl of in vitro translated Sp1 with the intact −47/-7 region probe, further incubation with 1 μl of either polyclonal anti-Sp1 antibody (IC6, Santa Cruz), anti-Sp2 antibody (K-20, Santa Cruz), anti-Sp3 antibody (D-20, Santa Cruz), or anti-Sp4 antibody (V-20, Santa Cruz) at 4°C for 2 h was performed before electrophoresis as described previously (25).

GST Pull-down Assay—Full-length GST-PPAR-γ fusion protein was synthesized from pGEX-PPAR-γ, and full-length GST-Sp1 fusion protein was synthesized from pGEX-Sp1, using the GST Gene Fusion system (Amersham Biosciences, Inc.). The proteins were loaded onto glutathione-Sepharose beads, which were washed and resuspended in binding buffer (20 mM HEPES (pH 7.7), 75 mM KC1, 0.1 mM EDTA, 2.5 mM MgCl2, 0.05% Nonidet P-40, 2 mM dithiothreitol, and 10% glycerol) in the presence or absence of 10 or 50 μM TRO. The beads were incubated with 5 μl of in vitro translated 32P-labeled Sp1 or 32P-labeled PPAR-γ for 3 h at 4°C in the presence or absence of 10 or 50 μM TRO, followed by washing six times with binding buffer in the presence or absence of 10 or 50 μM TRO. They were then resuspended in 30 μl of SDS sample buffer and analyzed by SDS-PAGE.

Statistical Analysis—Statistical significance was calculated by one-factor analysis of variance using StatView 4.0 (ABACUS Concepts).

RESULTS

Effect of PPAR-γ Ligands on TXR Transcription by PPAR-γ Incorporation—We first examined the effect of PPAR-γ ligands PGJ2 and TRO on DNA synthesis in VSMCs. As shown in Fig. 1, an increase (−140% of basal) in [3H]thymidine incorporation
into VSMCs was induced by 1 μM TX mimetic U-46619 (lines 1 and 2) as reported previously (2). When VSMCs were coincubated with either 0.5 or 1 μM PGJ₂ or 1 or 10 μM TRO, the U-46619-stimulated increase in [³H]thymidine incorporation was inhibited in a dose-dependent manner (lines 3 and 4 for PGJ₂ and lines 5 and 6 for TRO). The results indicate that both PPAR-γ ligands can significantly inhibit the TX-stimulated DNA synthesis in VSMCs.

Effect of PPAR-γ Ligands on TXR mRNA Expression—We next performed Northern blot analysis to examine TXR mRNA expression regulation in VSMCs. TXR mRNA expression (3.7 kb, indicated by an arrow) was fully observed in VSMCs in the absence of PPAR-γ ligands (Fig. 2, lane 1). TXR mRNA expression levels were decreased significantly by PPAR-γ ligands such as PGJ₂ (2.5 μM) and TRO (50 μM) (Fig. 2, lanes 2 and 3, respectively). The results indicate that PPAR-γ ligands negatively regulate TXR mRNA expression, which may cause inhibition of TX-stimulated DNA synthesis, in VSMCs.

Effect of PPAR-γ Ligands on TXR Gene Promoter Activity—We then examined the effect of PPAR-γ ligands on transcription of the TXR gene. As shown in Fig. 3, PPAR-γ ligand PGJ₂ (lines 2–4) and TRO (lines 5–7) significantly decreased transcription of −989/+184-luc in a dose-dependent manner. In contrast, PPAR-γ ligand LTBP did not affect the transcription (line 8). These results suggest that PPAR-γ ligands specifically suppress TXR gene transcription.

Involvement of PPAR-γ in TXR Gene Suppression Independent of RXR-α—PPAR-γ overexpression in VSMCs was performed. As shown in Fig. 4, PPAR-γ overexpression decreased transcription of −989/+184-luc significantly in the absence (lines 1 and 2) or the presence of PPAR-γ ligands (lines 5 and 6 for PGJ₂ and lines 9 and 10 for TRO), whereas RXR-α overexpression did not affect it (lines 3 and 7). Moreover, overexpression of both factors did not affect the transcription suppression caused by that of PPAR-γ alone (lines 4 and 8). It is indicated that RXR-α is not involved in the TXR gene suppression.

A GC Box-related Sequence at the −22/−7 Region of the TXR Gene Promoter Responsible for the Suppression—The elements(s) responsible for the TXR gene transcription suppression by PPAR-γ was next localized. As shown in Fig. 5A, the suppression by 2.5 μM PGJ₂ or 50 μM TRO was observed in every construct (lines 1–3 for −989/+184-luc, lines 4–6 for −809/+184-luc, lines 7–9 for −489/+184-luc, lines 10–12 for −231/+184-luc, and lines 13–15 for −78/+184-luc). To localize the element further, we next created several deletion constructs (−78/+120-luc, −78/+60-luc, −78/−1-luc, −78/−7-luc, −78/−23-luc, −47/+120-luc, −39/+120-luc, −22/+120-luc, and −6/+120-luc) and performed transient transfection studies. As shown in Fig. 5B, both PPAR-γ ligands suppressed transcription of either −78/+184-luc (lines 1–3), −78/+120-luc (lines 4–6), −78/+60-luc (lines 7–9), −78/−1-luc (lines 10–12), −78/−7-luc (lines 13–15), −47/+120-luc (lines 19–21), −39/+120-luc (lines 22–24), or −22/+120-luc (lines 25–27). In contrast, transcription activities of −78/−23-luc (lines 16–18) and −6/+120-luc (lines 28–30) were not suppressed by both PPAR-γ ligands, suggesting that the region between −22 and −7 may be responsible for the transcription suppression. The possible involvement of the −22/−7 region for the TXR gene suppression was investigated further. We next created a construct Δ(−47/−7)(−78/−1-luc), which did not contain the −47/−7 region of the −78/−1-luc, and we examined its transcription activity compared with the control construct −47/−7-luc. As
shown in Fig. 5C, both PPAR-γ ligands suppressed transcription of −47/−7-luc (lines 7–9) as well as −78/−1-luc (lines 1–3), whereas transcription of ∆(−47/−7)/−78/−1-luc was not affected by PPAR-γ ligands (lines 4–6). These data confirm that some region between −47 and −7 is responsible for the suppression. The −47/−7 region contains two putative GC box-
related sequences. One is the above described –22/–7 region (GGG GGT GGG GCT GCC G), and the other is the region between –39 and –29 (GGG GGC AGC CC). We then created a construct \( \Delta(-22/-7)(-47/-7)-\text{luc} \) in which the \(-22/-7\) GC box-related sequence was mutated in the \(-47/-7\)-luc. As shown in Fig. 5C, transcription activity of \( \Delta(-22/-7)(-47/-7)-\text{luc} \) was unaffected by both PPAR-\( \gamma \) ligands (lines 13–15).

In contrast, when we transfected a construct \( \Delta(-39/-29)(-47/-7)-\text{luc} \) harboring the mutation at the –39/–29 GC box-related sequence in the –47/–7-luc, both PPAR-\( \gamma \) ligands could still suppress its transcription activity (Fig. 5C, lines 10–12) as well as –47/–7-luc (lines 7–9). Taken together, it is suggested that the –22/–7 GC box-related sequence is responsible for the transcription suppression. To confirm further that the –22/–7 GC box-related sequence is responsible for the transcription suppression, we mutated the –22/–7 region in full-length –989/184-luc. When we transfected \( \Delta(-22/-7)(-989/184)-\text{luc} \) in which the –22/–7 GC box-related sequence in full-length –989/184-luc was disrupted, transcription suppression by PPAR-\( \gamma \) ligands was completely abrogated (Fig. 5D, lines 4–6), further confirming that the –22/–7 GC box-related sequence is responsible for the suppression. Additionally, the basal transcription level of \( \Delta(-22/-7)(-989/184) \)-luc (Fig. 5D, line 4) was ~30% of that of wild type –989/184-luc (Fig. 5D, line 1), suggesting that basal transcription of the TXR gene is significantly dependent on the –22/–7 GC box-related sequence.

Supershifted Sp1—The functional interaction between Sp1 and PPAR-\( \gamma \) on the –22/–7 GC box-related sequence was next examined. As shown in Fig. 7, overexpression of Sp1 significantly increased transcription of \( \Delta(-39/-29)(-47/-7)-\text{luc} \) (in which the –22/–7 GC box-related sequence was intact) (lines 1 and 2). When PPAR-\( \gamma \) was also overexpressed, the transcription was decreased significantly (line 3).

In contrast, overexpression of Sp1 showed little effect on the transcription of \( \Delta(-22/-7)(-47/-7)-\text{luc} \) (in which the –22/–7 GC box-related sequence was disrupted) (lines 4 and 5), and additional PPAR-\( \gamma \) overexpression induced little effect (line 6). Taken together, it is suggested that PPAR-\( \gamma \) functionally antagonizes against Sp1 on the –22/–7 GC box-related sequence most likely by inhibiting Sp1 binding to the sequence.

Physical Interaction between PPAR-\( \gamma \) and Sp1—A GST pull-down assay was performed to study a possible protein-protein interaction between PPAR-\( \gamma \) and Sp1. As shown in Fig. 8A, no significant band was identified by incubation with GST alone with in vitro translated \( ^{35} \text{S}-\text{labeled Sp1} \) (lane 2), whereas by incubation of GST-PPAR-\( \gamma \) fusion protein with \( ^{35} \text{S}-\text{labeled Sp1} \) we observed a band indicating a physical interaction between PPAR-\( \gamma \) and Sp1 (lane 3), and the interaction was enhanced approximately 2.5-fold by incubation with 10 \( \mu \text{M} \) TRO (lane 4) and 3.1-fold by 50 \( \mu \text{M} \) TRO (lane 5). Similarly, as shown in Fig. 8B, incubation of GST-Sp1 fusion protein with \( ^{35} \text{S}-\text{labeled PPAR-\( \gamma \)} \) gave a band indicating an interaction between PPAR-\( \gamma \) and Sp1 (lane 3), which was enhanced 2.5-fold by incubation with 10 \( \mu \text{M} \) TRO by (lane 4) and 3.2-fold by 50 \( \mu \text{M} \) TRO (lane 5). These data suggest that PPAR-\( \gamma \) physically interacts with Sp1, and the interaction is enhanced by PPAR-\( \gamma \) ligand in a dose-dependent manner.
FIG. 6. **Protein-DNA interaction on the TXR gene promoter.** Panel A, generation of in vitro translated PPAR-γ and Sp1. Mouse PPAR-γ and Sp1 proteins were generated by in vitro translation in the presence of [35S]methionine and were analyzed by SDS-PAGE. Lane 1, unprogrammed reticulocyte lysate. 35S-Labeled PPAR-γ is observed in lane 2 (indicated by an arrow), and 35S-labeled Sp1 is observed in lane 3 (indicated by an arrow). Molecular size markers are also indicated. Panel B, interaction between Sp1 or PPAR-γ and TXR gene promoter. 32P-Labeled oligonucleotides containing the −47/−7 region of the TXR gene promoter (−47/−7) (lanes 1–3), Δ(−39/−29)/(−47/−7) oligonucleotides (−39/−29Mut), or Δ(−22/−7)/(−47/−7) oligonucleotides (−22/−7Mut) were incubated with 2 μg of in vitro translated Sp1 (lanes 2, 4, and 5), PPAR-γ (lane 3), or unprogrammed reticulocyte lysate (RL) (lane 1). The Sp1-DNA complex is indicated by an arrow. * represents nonspecific binding formed with unprogrammed reticulocyte lysate. Panel C, antibody supershift experiments using in vitro translated Sp1. 32P-Labeled oligonucleotides containing the −47/−7 region of the TXR gene promoter (−47/−7) were incubated with 2 μg of in vitro translated Sp1 and incubated sequentially with 1 μl of anti-Sp1 antibody (lane 2). The Sp1-DNA complex is indicated by an arrow (lane 1). A supershifted band by anti-Sp1 antibody (lane 2) is also indicated. Panel D, interaction between VSMC nuclear extracts and the TXR gene promoter. 32P-Labeled −47/−7 oligonucleotides (lanes 1–5 and 8–10), −39/−29Mut oligonucleotides (lane 6), or −22/−7Mut oligonucleotides (lane 7) were incubated with 2 μg of VSMC nuclear extracts (VSMC NE) (lanes 2–10). The indicated amounts of unprogrammed reticulocyte lysate (RL) or in vitro translated PPAR-γ were coincubated in lanes 8–10. Protein/DNA complexes are indicated by arrows. Unlabeled oligonucleotides (100-fold excess) containing the −47/−7 region (lane 2), SV40 early promoter Sp1 site (lane 4), or 3′ hydroxymethylglutaryl-CoA synthase gene PPRE (HMGS PPRE) (lane 5) were used for competition. Panel E, antibody supershift experiments using VSMC nuclear extracts. 32P-Labeled oligonucleotides containing the −47/−7 region of the TXR gene promoter (−47/−7) (lanes 1–3) were incubated with 2 μg of VSMC nuclear extracts and incubated sequentially with 1 μl of anti-Sp1 antibody (lane 2) or anti-Sp3 antibody (lane 3). Protein/DNA complexes are indicated by arrows. A supershifted band by anti-Sp1 antibody (lane 2) is also indicated by an arrow. Panel F, interaction between PPAR-γ ligands treated with VSMC nuclear extracts and the TXR gene promoter. 32P-Labeled −47/−7 oligonucleotides were incubated with 2 μg of VSMC nuclear extracts prepared from cells either not treated (lane 1) or treated with 2.5 μM PGJ2 (lane 2) or 50 μM TRO (lane 3) for 12 h. Note the significantly greater decrease of protein-DNA complexes in lanes 2 and 3 than in lane 1.

**DISCUSSION**

TX-stimulated DNA synthesis in VSMCs was inhibited by PGJ2 and an insulin-sensitizing drug TRO, both of which have been shown to be ligands for PPAR-γ (13, 14). Because TXR mRNA expression was also decreased by these PPAR-γ ligands, the inhibition of TX-stimulated DNA synthesis by PPAR-γ
fracor (GHF)-1 or coactivators such as cAMP response element-binding protein-binding protein (CBP) and SRC-1 (30). It also suppressed interleukin-6 gene transcription via an interaction with nuclear factor-κB or AP-1 (31). Regarding PPAR-γ, it suppressed interleukin-12 gene transcription via an interaction with nuclear factor-κB (32) and inducible nitric oxide synthase gene transcription via a direct interaction with CBP (33). Moreover, we have observed recently that PPAR-γ suppresses TX synthase gene transcription via a direct interaction with nuclear factor-κB (32). On the other hand, Sp1 was shown to interact physically with other nuclear hormone receptors such as retinoic acid receptor (34), estrogen receptor (35), and progesterone receptor (36), but all of these interactions enhanced their Sp1-induced gene transcription. In the present study, we have observed a direct physical interaction between PPAR-γ and Sp1 by GST-pull down assays. The interaction is specific because it has been augmented in the presence of PPAR-γ ligand. We thus propose a mechanism by which PPAR-γ inhibits the action of Sp1 on the GC box-related sequence. This is the first report describing an interaction between PPAR-γ and Sp1 causing the transcription inhibition by PPAR-γ.

The effects of PPAR-γ and its ligands on the cardiovascular system have been studied. PPAR-γ ligands suppress expression of plasminogen activator inhibitor type 1 (16) and inhibit neointimal formation after balloon injury (37) in vascular endothelial cells. In VSMCs, expression of matrix metalloproteinase-9 was suppressed by PPAR-γ ligands (15). We have reported recently the suppression of type-1 angiotensin II receptor gene transcription by PPAR-γ in VSMCs (38) and that of TX synthase gene transcription by PPAR-γ in macrophages (17). Moreover, in terms of atherosclerosis, PPAR-γ inhibited macrophage activation (39, 40) and stimulated cholesterol efflux from macrophages by inducing the ABCA1 gene (41, 42). Furthermore, in clinical studies, TRO was reported to lower blood pressure (43), ameliorate microalbuminuria (44), and prevent cardiac mass increase and cardiac function impairment (45) in diabetic patients. An inhibitory effect of TRO on carotid arterial wall thickening in diabetic patients was also reported (46). It thus appears that PPAR-γ has an antiatherosclerotic effect.

An increase in TX production is widely reported in patients with diabetes mellitus (DM) (47, 48) as well as in animal DM models (49, 50), which may induce the hypercoagulable state leading to cardiovascular complications including atherosclerosis. Moreover, TX-induced aortic contraction is augmented in diabetic rats (51). Therefore, both synthesis and action of TX are suggested to be up-regulated in DM. We have shown previously that PPAR-γ and its ligands also suppressed expression of the TX synthase gene (17), which is supported by the observation that TRO reduced TX production in human platelets and inhibited TX-induced cell proliferation in VSMCs. PPAR-γ can inhibit TX system possibly causing antiatherosclerotic and antiinflammatory effects on the vasculature.

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