Suppression of Rat Thromboxane Synthase Gene Transcription by Peroxisome Proliferator-activated Receptor \(\gamma\) in Macrophages via an Interaction with NRF2*

We have studied the transcription regulation of the rat thromboxane synthase (TXS) gene by peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) in macrophages. The transcription activity of a cloned 5'-flanking region (1.6 kilobases) of the rat TXS gene (5'-FL-TXS) was examined by luciferase reporter gene assay. TXS mRNA expression and the transcription activity of 5'-FL-TXS were inhibited by PPAR\(\gamma\) ligands, 15-deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\) (PGJ\(2\)), and the thiazolidinedione troglitazone (TRO) in a dose-dependent manner. Overexpression of PPAR\(\gamma\) also significantly suppressed transcription, and further addition of PGJ\(2\) or TRO augmented the suppression. Deletion analysis showed that the element responsible for the PPAR\(\gamma\) effect is located in a region containing the nuclear factor E2 (NF-E2)/NF-E2-related factor 2 (NF-E2-related factor 2). Moreover, the complex was decreased with PGJ\(2\), TRO, or in vitro translated PPAR\(\gamma\).

The transcription suppression by PPAR\(\gamma\) was confirmed using this truncated NRF2-binding element (−98/−88) by the reporter gene assay. Finally, a direct interaction between PPAR\(\gamma\) and NRF2 was confirmed by glutathione S-transferase pull-down assay. In conclusion, the NRF2-binding site (−98/−88) is the major promoter of 5'-FL-TXS which can be suppressed by activated PPAR\(\gamma\) via a protein-protein interaction with NRF2 in macrophages. and a potent inducer of platelet aggregation and vasoconstriction (1) and cell proliferation (2). Thromboxane \(A_2\) has been known to play a pathophysiological role in atherosclerosis (3) and glomerulonephritis (4). Thromboxane synthase (TXS)\(\ast\) catalyzes the conversion of prostaglandin \(H_2\) to thromboxane \(A_2\) and is expressed in lung, liver, kidney, and blood cells including macrophages (5). The structures of human (6), mouse (7), and rat (8) TXS have been reported and are indicated to belong to a cytochrome P450 subfamily. We also have observed that TXS mRNA is induced in an inflammatory model of hydronephrosis characterized by infiltration of macrophages in the renal interstitium (8). Although the transcriptional regulatory regions of the human (9) and mouse TXS (10) genes have been cloned, analysis of their gene transcription has not yet been extensively performed, and any role of the peroxisome proliferator-activated receptor (PPAR) in TXS gene transcription has never been examined.

PPAR is one of the nuclear receptors that serves as a transcription factor (11). Three different types of PPAR isoforms are present: PPAR\(\alpha\), PPAR\(\beta\), and PPAR\(\gamma\). PPARs heterodimerize with the retinoid X receptor (RXR) (12) and regulate transcription through binding to the PPAR-response element, which is present in the transcriptional regulatory region in several genes. PPARs appear to exhibit distinct patterns of tissue distribution and have different ligands, suggesting that they have their own functions in different tissues. PPAR\(\gamma\) was initially identified in differentiated adipocytes, and its role has been determined in relation to the pathogenesis of insulin resistance because the thiazolidinedione class of antidiabetic drugs (insulin sensitizers), such as troglitazone (TRO), was identified with a ligand of PPAR\(\gamma\) (13) as well as 15-deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\) (PGJ\(2\)) (14). Recently, however, PPAR\(\gamma\) has been shown to be expressed not only in adipocytes, but also in vascular tissues, such as vascular smooth muscle cells (VSMCs) and endothelial cells, and in macrophages in atheromatous plaques (15). Moreover, PPAR\(\gamma\) activators inhibit matrix metalloproteinase-9 expression in VSMCs (16) and thrombin-induced endothelin-1 production in endothelial cells (17). In monocytes/macrophages, PPAR\(\gamma\) activators suppress production of inflammatory cytokines (18) and stimulate the ex-
pression of scavenger receptors and the uptake of oxidized low density lipoprotein, leading to differentiation of monocytes/macrophages to foam cells (19). Intimal hyperplasia of a balloon-injured rat aorta was also inhibited by TRO (20). These observations suggest that PPARγ plays a role in vascular metabolism.

In this study, we assessed the role of PPARγ in TXS gene regulation in macrophages since both TXS and PPARγ are expressed in macrophages and are possibly involved in atherosclerosis. We first cloned a 5′-flanking region of the rat TXS gene (5′FL-TXS), and identified its major promoter in macrophages. We then observed suppression of TXS gene transcription by PPARγ and revealed the mechanism. In conclusion, PPARγ can inhibit TXS gene transcription by a possible direct protein-protein interaction between NR2F2 and PPARγ via the NR2F2-binding site (−98/−88) in 5′FL-TXS.

### MATERIALS AND METHODS

**Cloning of 5′-FL-TXS—**5′-FL-TXS was cloned by the polymerase chain reaction (PCR) using Genome Walker kits (CLONTECH). Briefly, the first PCR was conducted with a combination of adaptor primer 1 (provided in the kit) and TXS cDNA primer 1 (5′-TCT TGA GAA CCG TGA TGT GGA GTA C-3′) using a rat genomic library provided in the kit as a template. Nested PCR was then carried out with a combination of adaptor primer 2 (provided in the kit) and TXS cDNA primer 2 (5′-ATT TCA GGG GCA AGA GAA GAA C-3′) using the first PCR product as a template. The resultant PCR product was subcloned into pBluescript SK (+) between the SalI and XhoI sites. Sequencing of the insert was performed in both directions by the previously reported PCR cycle sequence method (21) with an automatic sequence analyzer (ABI PRISM 310 Genetic Analyzer).

**Rapid Amplification of 5′-cDNA Ends—**This was carried out using Marathon-Ready cDNAs (CLONTECH). Briefly, PCR was conducted with adaptor primer 1 (provided in the kit) and TXS cDNA primer 2 using a rat kidney cDNA library as a template. Nested PCR was then performed with adaptor primer 2 and TXS cDNA primer 3 (5′-ACG GTA CCA ACT TCG AAC TTG A-3′) using the first PCR product as a template. The resultant PCR products were cloned into pCR2.1-TOPO with the TOPO TA cloning kit (Invitrogen). Ten clones were sequenced, and their 5′-ends were identified.

**Synthesis of Chimeric Luciferase Expression Vectors and Other Constructs—**To examine the transcription function of 5′-FL-TXS, we constructed chimeric expression vectors containing fragments of 5′FL-TXS fused upstream of firefly luciferase cDNA. Briefly, the fragment of 5′FL-TXS was inserted between MluI and XhoI of the luciferase reporter vector (pGL3-Basic). Deletion mutants of 5′FL-TXS were synthesized based on an exonuclease III deletion protocol (22). The 5′-end of each deletion fragment was determined by sequencing. The internal mutation was introduced in the full-length fragment (1598 bp) using the Transformer™ site-directed mutagenesis kit (CLONTECH). To analyze the transcription function of a putative NF-E2/AP-1 site, an oligonucleotide (−104/−86, 5′-TAA AGT TGC TGA TTC ATT C-3′) containing the NF-E2/AP-1 site (−98/−88) in 5′FL-TXS was inserted into the HindIII/XhoI fragment in the pT109 vector upstream of the thymidine kinase promoter to give NF-E2/AP-1-tkLuc.

Mouse PPARγ1 cDNA in pcDNA was kindly provided by Dr. K. Umezono (Kyoto University, Kyoto, Japan) (23). Mouse RXXRα cDNA (kindly provided by Dr. R. M. Evans, Salk Institute, San Diego, CA) (24) was subcloned into pcDNA1/Amp (Invitrogen). Human NR2F2 cDNA (1807 bp) (25) was subcloned into the pGEX-4T-2 vector (Amersham Pharmacia Biotech) and is designated as pGEX-NR2F2. The full-length human NR2F2 cDNA was inserted into pcDNA1/Amp to synthesize the human NR2F2 expression vector pcDNA1-hNR2F2.

**Cell Culture, Transfection, and Luciferase Assay—**Cultured rat VSMCs (22, 26) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin. Cells were cultured in a 12-well culture plate and transfected by the calcium phosphate precipitation method (3.0 μg of DNA/well) using N-[1-(2-hydroxyethyl)propyl- N,N,N-trimethylammonium salts liposomal transfection reagent (Roche Molecular Biochemicals). Cultured rat macrophages (NR8383) were maintained in Ham’s F-12K medium containing 2 mM l-glutamine, 1.5 g/liter sodium bicarbonate, 15% fetal bovine serum, and penicillin/streptomycin. Cells (5 × 105 cells/well) were cultured and transfected by the lipofection method (3.0 μg/well). Cultured rat lung endothelial cells (RLECs) (27) were maintained in RPMI 1640 medium containing 10% fetal calf serum and penicillin/streptomycin. Cells (106 cells/well) were cultured and transfected by a modification of the DEAE-dextran method. Briefly, 3.0 μg of DNA were mixed with 27 μl of 10 mg/ml DEAE-dextran and 0.85 ml of 100 ng of chloroquine, and the serum-free medium was added to a 10-ml volume/well. Cell culture was carried out over a mixture for 90 min and treated with 10% dimethyl sulfoxide/phosphate-buffer saline. Afterward, the medium was removed and replaced. As an internal control to normalize transfection efficiency, 1.0 μg of cytomegalovirus/β-galactosidase expression vector was cotransfected.

After transfection, the cells were cultured with a hypoxenur serum-free medium for 18 h and then treated with 0.5–5 μg PGE2 (Cayman Chemical Co., Inc.) or 1.0–20 μM TRO (kindly provided by Sankyo Co., Ltd.) for 12 h with 1.0 μg demamethasone (Wako), 700 IU/ml recombinant interferon-γ (Genzyme Corp.), 40 ng/ml recombinant interleukin-6 (BIO-SOURCE International), and 100 ng/ml tumor necrosis factor-α (Genzyme Corp.) for 24 h. In the overexpression study, 1.0 μg/well plasmid of PPARγ1 cDNA, human NR2F2 cDNA, or RXRα cDNA was cotransfected. Luciferase and β-galactosidase expression was analyzed by previously reported methods (22, 26).

**Determination of TXS mRNA Levels—**To analyze TXS mRNA expression levels, Northern blot analysis and reverse transcription (RT)-PCR were performed. Briefly, 7 × 105 cells were seeded in 100-mm dishes and maintained. Twelve hours after stimulation with PGE2 or TRO, the total RNAs were collected, and the total mRNAs were isolated by Trizol reagent (Life Technologies, Inc.). In the Northern blot analysis, the isolated RNAs (10 μg each) were electrophoresed on a 1.5% formaldehyde-agarose gel and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech) for hybridization with the radiolabeled rat TXS cDNA probe (706 bp). Integrity of RNA was verified by hybridization with the β-actin probe. Semiquantitative RT-PCR was performed with the One Step RNA PCR kit (TaKaRa) with a forward primer (5′-TTC ACA GGC TTG GCT GAT GAG AGG TGT CAT-3′) and a reverse primer (5′-GGC TTC TCA AGT TCG AAG TCA GTG GTA-3′) using a rat genomic library provided in the kit as a template. The resultant PCR products were cloned into pCR2.1-TOPO with the TOPO TA cloning kit (Invitrogen). Ten clones were sequenced, and their 5′-ends were identified.

**Analysis of TXS Promoter Activity—**Luminescent luciferase activity was quantified by the Luminous Imager 2.0, Aisin Cosmos R&D, Ltd.). The luciferase activity was normalized with the internal control (PRL promoter) and the expression of glyceraldehyde-3-phosphate dehydrogenase mRNA was also amplified with a reverse primer (5′-AGA TCA ACC AGG CAT ACA TT-3′) and a forward primer (5′-AGA TCA ACC AGG CAT ACA TT-3′). PCR was performed under the following conditions: 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min for 30 cycles. Under these PCR conditions, a linear correlation between the densitometric intensity units of the PCR product and amounts of template was confirmed. TXS mRNA levels were determined by a ratio between the densitometric intensity units of the PCR products and glyceraldehyde-3-phosphate dehydrogenase mRNA levels. TXS mRNAs in macrophages was also confirmed by RT-PCR with a pair of primers (forward primer, 5′-ATG AGG AAG TGT TCG AGG-3′; and reverse primer, 5′-ACT CTG GAT TCA GGT GCT CG-3′) for RXRα and (forward primer, 5′-TTT GCC AAG CTG CTG CTC-3′; reverse primer, 5′-TAA AGT TGC TGA TTC ATT C-3′) for PPARγ. PCR was performed under the following conditions: 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s for 40 cycles to detect RXRα mRNA and 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 30 cycles to detect PPARγ mRNA.

**Electrophoretic Mobility Shift Assay (EMSA)—**Macrophages were maintained in 100-mm dishes with 1% hypoxenur medium and treated with 1.0 μg PGE2 or 10 μM TRO for 12 h. Nuclear extracts were prepared from untreated or treated cells according to the previously described method (24), and stored at −70°C until used for the experiments. PPARγ and RXRα were synthesized in vitro using the TNT Quick Coupled Transcription/Translation system (Promega). The de novo translation product was confirmed on an SDS-polyacrylamide gel. EMSA was performed based on a previously reported method (24). Briefly, nuclear extracts from macrophages were mixed in a 20-μl reaction mixture containing 22.5 mM HEPS (pH 7.9), 2.6 mM MgCl2, 13.3% glycerol, 50 mM KCl, 0.125 mM EDTA, 0.5 mM dithiothreitol, 0.04 μg/ml sheared salmon sperm DNA, and 20,000 cpm end-labeled probe for 30 min at room temperature. After incubation, samples were subjected to electrophoresis through a 4.5% polyacrylamide gel in 45 mM Tris borate and 1 mM EDTA for 1.5 h at 4°C and analyzed by autorad-
diography. Double-stranded oligonucleotides (−104/−86, 5′-TAA AGT TGC TGA TTC ATT CTT GA/−104 to −86) containing the NF-E2/AP-1 site (−98/−88) in 5′-FL-TXS were labeled with 32P by a fill-in reaction with the Klenow fragment. Unlabeled oligonucleotides containing the NF-E2/AP-1 site as well as oligonucleotides harboring the mutated sequence of the NF-E2/AP-1 site (5′-TAA AGT TGC TGT TTC ATT CTT GA/−104 to −86) were used as competitors. To characterize the protein binding to the NF-E2/AP-1 site, antisera against transcription factors were incubated in the binding reactions at 10-fold dilution for 30 min on ice before the addition of the radiolabeled oligonucleotides. The antibodies used in this study were raised against NRF1 (c-19, Santa Cruz Biotechnology, Inc.), NRF2 (ECH) (28), BACH1/2 (29), NF-E2 (p45) (30), and MAP (31). Antibodies against c-Fos (sc-52) and c-Jun (sc-45) were obtained from Santa Cruz Biotechnology, Inc. The pre-absorption experiment in EMSA, anti-NRF2 serum was incubated with GST or GST-NRF2 fusion proteins loaded onto glutathione-Sepharose beads for 2 h at 4 °C. After incubation, the mixtures were briefly centrifuged, and supernatants were incubated with the nuclear extracts and probe.

In Vitro Binding Assay—GST fusion proteins were synthesized by the GST Gene Fusion system (Amersham Pharmacia Biotech). The GST fusion proteins were loaded onto glutathione-Sepharose beads, which were washed and resuspended in binding buffer (20 mM HEPES (pH 7.7), 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl2, 0.05% Nonidet P-40, 2 mM dithiothreitol, and 10% glycerol). The beads were incubated with 5 μl of in vitro translated 35S-labeled PPARγ or RXRα protein for 1 h at 4 °C, followed by washing seven times with binding buffer in the presence or absence of TRO (10 μM). They were then resuspended in 50 μl of 2× SDS sample buffer, and the supernatant was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Statistical Analysis—Data were analyzed using Stat View 4.0 (Aba

cus Concepts, Inc.). Analysis of variance was adopted to compare means among groups.

RESULTS

Structure of 5′-FL-TXS—We cloned a fragment of the 5′-flanking region of the rat TXS gene (1.6 kilobase) and revealed its sequence structure (Fig. 1). This sequence has 75% homology to murine 5′-FL-TXS (10). The transcription start site was indicated to be 136 bases upstream from the protein coding region (indicated with an asterisk). There are several putative transcription factor-binding sites: the TATA box, GATA-1 box, AP-1-binding site, AP-2-binding site, glucocorticoid-responsive element, γ-interferon-activated site, shear stress-responsive element, and NF-E2/AP-1-binding site. Three kinds of cells. For transcription study, −1598Luc was transfected into three kinds of cells, and luciferase expression was compared (Fig. 2B). The luciferase expression in macrophages was decreased with −1083Luc, but this was then reversed, suggesting that there would be a silencer element between positions −1083 and −773. Distinct from VSMCs and RLECs, significant luciferase expression was detected with −168Luc, whereas −96Luc and −34Luc transcription activities were not detected. It is therefore suggested that the major promoter region in 5′-FL-TXS in macrophages is different from that in RLECs and VSMCs and is located between positions −168 and −96 or its surrounding regions.

As 5′-FL-TXS has several putative cis-acting elements, the effect of some potential stimulators on transcription activity was examined in macrophages using −1598Luc (Fig. 4). PGJ2 (1.0 μM; PPARγ activator) significantly suppressed transcription activity. Interferon-γ (700 IU/ml) and dexamethasone (1.0 μM) also inhibited transcription, whereas tumor necrosis factor-α (100 ng/ml) and interleukin-6 (40 ng/ml) stimulated it. Effects of PGJ2 and TRO on TXS mRNA Expression—Northern blot analysis showed that TXS mRNA expression levels were significantly reduced by PGJ2 and TRO in a dose-depend
ent manner (Fig. 5A). The endogenous expression of both PPARγ and RXRa was also verified in macrophages by RT-PCR. The resultant PCR products were resolved on an ethidium bromide-stained 2% agarose gel. Isolated mRNA was treated (RT+) or not (RT−) with reverse transcriptase. RT-PCR products for TXS mRNA (466 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (308 bp) are indicated by arrows. B, the −1598Luc construct was transfected into macrophages (Mϕ), RLECs, and VSMCs. Bars represent means ± S.E. (n = 6) of luciferase expression after normalization of transfection efficiency by β-galactosidase expression expressed as relative luciferase activity. *, p < 0.001 compared with luciferase expression in VSMCs.

Transcription Inhibition of PPARγ in 5’FL-TXS—First, to examine the effect of PPARγ activators, macrophages transfected with −1598Luc were stimulated with PGJ2 or TRO. PGJ2 (Fig. 6A) and TRO (Fig. 6B) significantly suppressed luciferase expression in a dose-dependent manner. Second, to examine the role of PPARγ in transcription suppression, −1598Luc was cotransfected with PPARγ cDNA into macrophages. As shown in Fig. 6C, cotransfection with PPARγ significantly decreased the transcription activity of 5’FL-TXS, which was enhanced by PGJ2 (1.0 μM) or TRO (10 μM). These results suggest that PGJ2 and TRO can inhibit TXS gene expression mediated by PPARγ at the transcriptional level in macrophages.

Element Responsible for the Inhibitory Effect of PPARγ on 5’FL-TXS—We next tried to localize the potential element responsible for the PPARγ action. Macrophages transfected with deletion mutants were stimulated with PGJ2 (1.0 μM) or TRO (10 μM). Both agents significantly suppressed luciferase expression with −2506Luc, −2423Luc, −2304Luc, and −2168Luc, but no response was observed with −296Luc (Fig. 7). These results suggest that the element responsible for the transcription inhibitory effect of PPARγ activators is located between positions −168 and −96 or their surrounding regions, where the major promoter was shown to be present in macrophages (Fig. 3A). By sequence homology search, we found a putative NF-E2/AP-1 site at positions −298 to −288, whose sequence was incidentally disrupted in −296Luc (Fig. 8A), and no other potential cis-acting element including the PPAR-response element was identified between positions −168 and −96. We thus focused on the NF-E2/AP-1 site in the following experiments.

Characterization of the NF-E2/AP-1 Site in 5’FL-TXS—We conducted a mutation analysis to determine the role of the NF-E2/AP-1 site in transcription of the TXS gene. As a result, the transcription activity of −1598mLuc was markedly suppressed (Fig. 8B). Together with the previous observation that −96Luc (lacking the putative NF-E2/AP-1 site) (Fig. 8A) did not show significant transcription activity (Fig. 3A), it was thus
confirmed that the NF-E2/AP-1 site is a major promoter in macrophages.

We then examined a protein-DNA interaction in EMSA using radiolabeled oligonucleotides of the NF-E2/AP-1 site and nuclear proteins from macrophages (Fig. 9A). We observed a protein-DNA complex (arrow), which was decreased with oligonucleotides containing the intact NF-E2/AP-1 site, but not with an excess of oligonucleotides harboring the mutated NF-E2/ AP-1 site. Next, to identify the specific protein bound to this element, we treated the nuclear extracts with antisera against some candidate transcription factors (Fig. 9B). The addition of the anti-NRF2 serum, which had previously been shown to react with the NRF2-DNA complex (33), pronouncelly inhibited protein-DNA complex formation (lane 4). The inhibitory effect of anti-NRF2 serum was abrogated by preincubation of the serum with GST-NRF2 fusion protein (lane 10), but not with GST alone (lane 9), indicating the specific activity of anti-NRF2 serum. We also detected retarded bands in lane 4 (anti-NRF2), lane 5 (anti-p45), and lane 6 (anti-BACH) at the upper position of the NRF2-DNA complex. However, these bands comigrated with a band induced by preimmune serum treatment (lane 7). These results suggest that NRF2 is involved in the protein-DNA complex, but involvement of other NF-E2-related factors could not be excluded, even though the retarded bands appear to be nonspecific. To support the interaction between NRF2 and NF-E2/AP-1 in macrophages, endogenous expression of NRF2 was confirmed by detecting expression of NRF2 mRNA (1770 bp) by RT-PCR (Fig. 9C).

Effect of PPARγ on the Protein-DNA Interaction at the NF-E2/AP-1 Site—We further examined a possible interaction of PPARγ with the protein-DNA complex in EMSA. PGJ2 (1.0 μM) and TRO (10 μM) inhibited complex formation (Fig. 10A). Moreover, the addition of in vitro translated PPARγ reduced the intensity of the protein-DNA complex in a dose-dependent manner, but RXRa had no effect (Fig. 10B).

Next, to further verify the transcription function of the NF-E2/AP-1 site, the truncated element containing the NF-E2/ AP-1 site in 5′FL-TXS was fused upstream of the thymidine kinase promoter and luciferase cDNA (NF-E2/AP-1-tkLuc), and this construct was cotransfected with PPARγ, RXRa, or human NRF2 cDNA into macrophages. As shown in Fig. 11, the luciferase expression of NF-E2/AP-1-tkLuc significantly increased compared with that of the control vector (pT109) alone (bar 1

versus bar 12), and it was enhanced ~2-fold by cotransfection with human NRF2 cDNA (bar 1 versus bar 2). On the other hand, the luciferase expression of a control vector was not affected by cotransfection (bar 12 versus bar 13). The enhanced luciferase expression of NF-E2/AP-1-tkLuc with human NRF2 cDNA was attenuated by either PGJ2 (1.0 μM) (bar 2 versus bar 6) or TRO (10 μM) (bar 2 versus bar 7). The luciferase expression of NF-E2/AP-1-tkLuc was also suppressed by cotransfection with PPARγ cDNA (bar 2 versus bar 4) or PPARγ and RXRa cDNAs (bar 2 versus bar 5). Moreover, the suppression was further enhanced with the addition of PGJ2 (bars 8 and 10) or TRO (bars 9 and 11). However, cotransfection with RXRa cDNA did not affect the luciferase expression (bar 2 versus bar 3).

Physical Interaction between PPARγ and NRF2—Finally, we performed a GST pull-down assay to confirm the direct interaction between NRF2 and PPARγ. As shown in Fig. 12A, full-length NRF2 (amino acids 1–589) interacted with PPARγ, and the interaction was enhanced in the presence of TRO (10 μM). On the other hand, RXRa did not interact with NRF2 (Fig. 12B). These results suggest that PPARγ physically interacts with NRF2 and probably interferes with the transcription function of NRF2.
DISCUSSION

TXS gene transcription analysis with deletion mutants (Fig. 3A) and a construct harboring the mutated NF-E2/AP-1 site (−1598mLuc) (Fig. 3B) suggests that the major promoter in macrophages whose sequence is homologous to the NF-E2/AP-1 site is present in the −298/−288 region. Consistent with our observation, Lee et al. (9) have shown that the NF-E2/AP-1 site in the human TXS gene plays a crucial role in TXS gene transcription in the human promyelocytic leukemia cell line HL-60. They have also shown that transcription via the NF-E2/AP-1 site is activated by overexpression of NF-E2 (p45) and have concluded that the NF-E2 site is important for enhancing the TXS gene promoter activity in HL-60 cells, although they have not identified the transcription factor binding to the NF-E2/AP-1 site. In the present study, we observed inhibition of protein-DNA complex formation with anti-NRF2 serum in EMSA using nuclear extracts from macrophages, and the specificity of the inhibitory effect of anti-NRF2 serum was confirmed by a pre-absorption experiment. The results indicated that the NF-E2/AP-1 site (−98/−88) in 5′FL-TXS is possibly bound by NRF2 as an endogenous transcription factor in macrophages. This is the first evidence that NRF2 is involved.

![Fig. 6. PGJ2 and TRO treatment and PPARγ overexpression inhibit the transcription activity of 5′FL-TXS.](image)

**DISCUSSION**

TXS gene transcription analysis with deletion mutants (Fig. 3A) and a construct harboring the mutated NF-E2/AP-1 site (−1598mLuc) (Fig. 3B) suggests that the major promoter in macrophages whose sequence is homologous to the NF-E2/AP-1 site is present in the −98/−88 region. Consistent with our observation, Lee et al. (9) have shown that the NF-E2/AP-1 site in the human TXS gene plays a crucial role in TXS gene transcription in the human promyelocytic leukemia cell line HL-60. They have also shown that transcription via the NF-E2/AP-1 site is activated by overexpression of NF-E2 (p45) and have concluded that the NF-E2 site is important for enhancing the TXS gene promoter activity in HL-60 cells, although they have not identified the transcription factor binding to the NF-E2/AP-1 site. In the present study, we observed inhibition of protein-DNA complex formation with anti-NRF2 serum in EMSA using nuclear extracts from macrophages, and the specificity of the inhibitory effect of anti-NRF2 serum was confirmed by a pre-absorption experiment. The results indicated that the NF-E2/AP-1 site (−98/−88) in 5′FL-TXS is possibly bound by NRF2 as an endogenous transcription factor in macrophages. This is the first evidence that NRF2 is involved.
in the regulation of the TXS gene promoter in macrophages. However, we do not exclude a possibility that other factors, including BACH and p45, are also involved in protein-DNA complex formation. In megakaryocytes, the human TXS promoter was also shown to be bound by NF-E2 (p45) (34). Further analysis is needed to identify other factors than NRF2 that interact with the NF-E2/AP-1 site in macrophages.

NRF2 is a transcription factor with a leucine zipper structure and one of the Cap’n’Collar family transcription factors, including p45, NRF1, NRF3, BACH1, and BACH2 (36). Recently, it has been reported that NRF2 is involved in the induction of heme oxygenase-1 gene expression by oxidative stress (37, 38). Indeed, in NRF2-deficient macrophages, an important role of NRF2 has been demonstrated in the oxidative stress-induced response of heme oxygenase-1 gene expression (39). Moreover, NRF2 is also involved in gene transcription of phase II detoxifying enzyme genes, including the NAD(P)H:quinone oxidoreductase-1 gene (40) and the γ-glutamylcysteine synthetase subunit gene (41), via an interaction with the antioxidant response element or the electrophile response element, including BACH and p45, are also involved in protein-DNA complex formation.

**Fig. 9.** Possible binding of NRF2 to the fragment containing the −98/−88 site (NF-E2/AP-1). A, EMSA was performed using the radiolabeled oligonucleotides containing the NF-E2/AP-1 site and nuclear extracts from macrophages. Lane 1, incubation without nuclear extracts; lanes 2–8, incubation with nuclear extracts. A competition experiment was performed using unlabeled oligonucleotides containing the intact NF-E2/AP-1 site (−98/−88) at 10-, 50-, or 100-fold molar excess (lanes 3–5, respectively) or the mutated NF-E2/AP-1 site at a 10-, 50-, or 100-fold molar excess (lanes 6–8, respectively). B, EMSA was performed using various antibodies. Lanes 1 and 11, incubation without nuclear extracts; lanes 2, 8, and 12, nuclear extracts alone; lanes 7 and 13, preimmune serum; lane 3, anti-NRF1 serum; lane 4, anti-NRF2 serum; lane 5, anti-NP-E2 serum (anti-p45); lane 6, anti-BACH serum; lane 9, pre-absorption of anti-NRF2 serum with GST protein (GST); lane 10, pre-absorption of anti-NRF2 serum with GST-NRF2 protein (GST-NRF2); lane 14, anti-small MAF serum (anti-Maf); lane 15, anti-c-Fos serum; lane 16, anti-c-Jun serum. Arrows indicate specific bands. C, endogenous NRF2 mRNA expression was confirmed in macrophages. The RT-PCR product of NRF2 mRNA was detected at 1770 bp (arrow). RT(+) RT(−), RT-PCR with treatment of reverse transcriptase. RT(−), RT-PCR without treatment of reverse transcriptase.

**Fig. 10.** PGJ2, TRO, and in vitro synthesized PPARγ inhibit formation of the NRF2-DNA complex. A, EMSA was performed using the radiolabeled oligonucleotides containing the NF-E2/AP-1 site and nuclear extracts from macrophages stimulated with PGJ2 (1.0 µM) and TRO (10 µM). The arrow indicates specific bands. B, EMSA was performed in the presence of in vitro translated RXRα at 1.0 or 2.0 µl (lanes 3 and 4, respectively), PPARγ at 1.0 or 2.0 µl (lanes 5 and 6, respectively), or combination of both PPARγ and RXRα at 0.5 or 1.0 µl each (lanes 7 and 8, respectively). Lysate volumes were adjusted to be equal using lysate programmed with empty vector plasmid. Lane 1, incubation without nuclear extracts; lanes 2–9, incubation with nuclear extracts; lane 9, 2.0 µl of lysate programmed with empty vector plasmid (RL).

**Fig. 11.** PPARγ inhibits transcription activation by NRF2 at the NF-E2/AP-1 site. NF-E2/AP-1-tkLuc containing oligonucleotides of the NF-E2/AP-1 site was cotransfected with or without cDNA for human NRF2, RXRα, or PPARγ into macrophages. Transfected cells were also stimulated with PGJ2 (1.0 µM) and TRO (10 µM). Bars represent mean ± S.E. (n = 6) of luciferase expression expressed as relative luciferase activity (RLA). *, p < 0.01 compared with bar 1; ●, p < 0.01 compared with their controls (bars 4–7); ◊, not significant compared with bar 2; ▲, not significant compared with bar 12.
cytochrome P450 subfamily) is another new target gene trans-activated by NRF2.

The NF-E2/AP-1 site was shown to be the major promoter of the TXS gene in macrophages. On the other hand, in RLECs and VSMCs, the major promoter appears to be located in the region where a putative TATA box is present and downstream of the NF-E2/AP-1 site. Moreover, the transcription activity of 5′-FL-TXS is suggested to be most potent in macrophages, followed by RLECs and VSMCs, in proportion to the mRNA expression levels in these cells. Consistent with our present results, it has also been shown that the TXS protein is abundantly expressed in macrophages (5). 5′-FL-TXS is thus suggested to exert different transcription activities dependent on the tissues.

PPARγ has been shown to transactivate some genes, most typically the lipoprotein lipase gene (42), dependent on the PPAR-response element. Accumulated observations have shown, however, that activation of PPARγ can suppress trancription of some genes, including the angiotensin AT1 receptor (43) and endothelin-1 (17) genes. In the present study, we focused on the molecular mechanism of transcription suppression by PPARγ in the TXS gene. Treatment with PPARγ activators PGJ2 and TRO inhibited transcription of 5′-FL-TXS as well as mRNA expression levels. Moreover, overexpression of PPARγ also inhibited transcription of 5′-FL-TXS, and the addition of PGJ2 or TRO augmented the transcription inhibition. We thus confirmed the transcription suppression of 5′-FL-TXS by PPARγ activation. Next, we tried to identify the element responsible for the transcription inhibition. The negative regulation by PPARγ was not observed in 5′-flanking region constructs lacking the major promoter (−98/−88) in macrophages (Fig. 7). It was therefore hypothesized that PPARγ would be involved in the transcription activity of the NF-E2/AP-1 site. Using a reporter construct of the truncated NF-E2/AP-1 site, we examined the transcription suppression by PPARγ. As shown in Fig. 11, the transcription activity of the NF-E2/AP-1 site was stimulated by cotransfection with NRF2 cDNA, and the stimulation was inhibited by PGJ2 as well as by TRO. Overexpression of PPARγ also inhibited the transcription by NRF2, and further addition of PGJ2 or TRO augmented the inhibition. These inhibitory responses in gene transcription were identical when full-length 5′-FL-TXS was used. In EMSA with radiolabeled oligonucleotides containing the NF-E2/AP-1 site and nuclear extracts from macrophages, the formation of the protein-DNA complex was markedly inhibited by PGJ2 or TRO. Moreover, we observed pronounced inhibition of complex formation by in vitro synthesized PPARγ, but not by RXRα (Fig. 10). The results clearly indicate that activation of PPARγ inhibits the protein-DNA complex formation caused by the NF-E2/AP-1 site and nuclear proteins including NRF2. Finally, in the GST pull-down assay, PPARγ was shown to physically interact with NRF2. This is the first report of the direct interaction of PPARγ with NRF2 that possibly leads to gene transcription suppression. Taken together, the results suggest that PPARγ can interact with NRF2 and may suppress transcription of the TXS gene, probably interfering with the binding of NRF2 to the NF-E2/AP-1 site.

Thromboxane inhibitors have been reported to inhibit the progression of experimental diabetic nephropathy in rats (44) and to ameliorate microalbuminuria in diabetic patients (45). In inflammatory kidneys, infiltrating macrophages express abundant TXS protein (46), and enhanced thromboxane synthesis in monocytes/macrophages leads to glomerular injury (47). It is thus suggested that thromboxane synthesis from infiltrating macrophages plays an important role in inflammatory diseases. Thiazolidinediones are now widely used for the treatment of type 2 diabetes mellitus. It has also been reported that the agents would possess other clinical benefits such as inhibiting neointimal formation following balloon injury (20), decreasing blood pressure (48), and ameliorating microalbuminuria (49), although the causative mechanisms are still in need of examination. The present observations may provide an insight into the role of PPARγ in vascular and renal diseases in terms of regulation of gene expression.

In summary, we have revealed the structure of the functional transcriptional regulatory region of the rat TXS gene. In macrophages, 5′-FL-TXS is primarily dependent on the NRF2-binding element (−98/−88) in basal transcription. Activation of PPARγ may possibly suppress TXS gene expression at a transcriptional level. The NRF2-binding element is responsible for the transcription suppression by PPARγ. In EMSA, the formation of the protein-DNA complex caused by the NRF2-binding element and nuclear extracts from macrophages was clearly inhibited by PPARγ. The GST pull-down assay indicated the direct interaction between PPARγ and NRF2. RXRα was not implicated in the mechanism. In conclusion, activation of PPARγ can suppress transcription of the TXS gene via a possible interaction with NRF2 in macrophages.

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