Peroxisome Proliferator-Activated Receptor Activators Inhibit Thrombin-Induced Endothelin-1 Production in Human Vascular Endothelial Cells by Inhibiting the Activator Protein-1 Signaling Pathway

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Abstract—Endothelin-1 (ET-1), a 21-amino acid vasoactive peptide mainly produced by vascular endothelial cells, is involved in the regulation of vascular tone and smooth muscle cell proliferation. Peroxisome proliferator-activated receptors (PPARs), key players in lipid and glucose metabolism, have been implicated in metabolic disorders that are predisposing to atherosclerosis. Because of the potential role of ET-1 in vascular disorders such as hypertension and atherosclerosis, we investigated the regulation of ET-1 expression by PPAR activators. Western blot and reverse transcription–polymerase chain reaction analyses demonstrated that both PPARα and PPARγ are expressed in human coronary artery endothelial cells as well as in endothelial cell lines such as HMEC-1 and ECV304. In bovine aortic endothelial cells and HMEC-1 cells, both PPARα and PPARγ ligands inhibited thrombin-induced ET-1 secretion, whereas basal ET-1 secretion was only slightly suppressed. Reverse transcription–polymerase chain reaction experiments showed that this inhibition of ET-1 production occurs at the gene expression level. Using transient transfection assays, we demonstrated that PPARs downregulate thrombin-activated transcription of the human ET-1 promoter. Transactivation studies with c-Jun and c-Fos expression plasmids indicated that PPARs negatively interfere with the activator protein-1 signaling pathway, which mediates thrombin activation of ET-1 gene transcription. Furthermore, electrophoretic mobility shift assays demonstrated that PPAR activators reduce the thrombin-stimulated binding activity of bovine aortic endothelial cell nuclear extracts as well as c-Jun binding to an activator protein-1 consensus site. Taken together, these data indicate that (1) both PPARα and PPARγ are expressed in human vascular endothelial cells and (2) PPAR activators inhibit thrombin-induced ET-1 biosynthesis, indicating a novel role for PPARs in vascular endothelial function. (Circ Res. 1999;85:394-402.)

Key Words: peroxisome proliferator-activated receptor ■ endothelin ■ thrombin ■ atherosclerosis ■ endothelium

Endothelium injury is a primary event in atherogenesis, which is followed by monocyte recruitment, macrophage differentiation, and smooth muscle cell (SMC) activation and proliferation. This proliferation of vascular SMCs is, at least in part, the consequence of the release of growth factors and different mitogenic agents, such as endothelin-1 (ET-1), by activated endothelial cells. ET-1 is a 21-amino acid vasoconstrictor peptide originally isolated from the supernatant of cultured porcine aortic endothelial cells. Subsequently, ET-1 has been shown to possess a number of different biological activities. ET-1 displays chemotactic properties on monocytes and is a potent inducer of cell adhesion molecules in endothelial cells. Furthermore, ET-1 is thought to act locally in a paracrine and autocrine fashion to regulate vascular tone and endothelial functions. Several groups reported that ET-1 is highly expressed in atherosclerotic lesions, suggesting that ET-1 may participate in the atherogenic process. The expression of mRNA and the release of the peptide are stimulated by activators of endothelial cells such as thrombin, tumor necrosis factor-α and interferon-γ, angiotensin II, as well as activators of second messenger pathways such as phorbol ester and calcium ionophore. By using reporter gene experiments, two major regulatory regions located at base pairs −148 to −117 (region A) and −117 to −98 (region B) of the ET-1 gene promoter have been identified that drive ET-1 transcription in endothelial cells. Region A interacts with GATA factors, whereas region B is capable of binding proteins of the activator protein-1 (AP-1) family.

Recent studies have emphasized the importance of protein–protein interactions of transcription factors in the regulation...
of gene expression. For instance, transcription of the ET-1 gene has been shown to be regulated through a cooperative interaction of GATA-2 and AP-1 transcription factors.16 Interestingly, several members of the nuclear receptor superfamily of ligand-activated transcription factors have also been reported to interact with members of the Jun and Fos families.17,18 Peroxisome proliferator-activated receptors (PPARs) constitute a subfamily of the nuclear receptor superfamily.19 PPARs stimulate the β-oxidative degradation of fatty acids, whereas PPARγ promotes lipid storage via its effects on adipocyte differentiation and function.20 PPARs are activated by natural ligands such as fatty acids and eicosanoids.21–23 Furthermore, the lipid-lowering fibrates and the antidiabetic thiazolidinediones are synthetic ligands for PPARα and PPARγ, respectively. PPARs regulate gene expression by binding with RXR as a heterodimeric partner to specific DNA sequence elements termed PPAR response elements (PPREs).25 In addition to regulating gene transcription via PPREs, PPARs have recently been shown to modulate gene transcription activity by interfering with other transcription factor pathways in a DNA binding–independent manner. We reported previously26 that PPARα activators have anti-inflammatory activities in SMCs by interfering negatively with the nuclear factor-κB (NF-κB) signaling pathway, whereas Ricote et al27 and Jiang et al28 reported that PPARγ could downregulate inflammatory responses in monocytes/macrophages, likely by interfering negatively with the NF-κB, Stat, and AP-1 signaling pathways.

In the present study, we first investigated PPAR expression in endothelial cells. We report that both PPARα and PPARγ are expressed in human endothelial cells from macrovascular (coronary artery endothelial cells [CAECs] and ECV304 cells) and microvascular (HMEC-1 cells) origin. Subsequently, we examined whether PPAR activators regulate the expression of ET-1. Our results demonstrate that PPAR activators inhibit thrombin-induced ET-1 gene expression as well as its secretion. This inhibition occurs at the transcriptional level and occurs via an interference with the AP-1 signaling pathway by preventing the binding of AP-1 proteins to its target sequence.1

Materials and Methods

Cell Culture

Bovine aortic endothelial cells (BAECs) were isolated as previously described28 and cultured in DMEM supplemented with 10% FCS, 600 mg/mL glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. BAECs were used at passages 4 to 8 for transfection and ET-1 secretion studies. Human CAECs (Clonetics) were grown in endothelial cell basal medium supplemented with 5% FCS and various growth factors as described by the manufacturer. ECV304 cells (a human umbilical vein endothelial cell line; American Type Culture Collection, Manassas, Va) were grown in medium (M199; Gibco BRL) supplemented with 5% FCS, 1 μg/mL hydrocortisone, and 10 ng/mL of epidermal growth factor (Boehringer Ingelheim Bioproducts).

Protein Extraction and Western Blot Analysis

Cells were washed twice in ice-cold PBS and harvested in ice-cold lysis buffer containing PBS, 1% Triton X-100, and a freshly prepared protease inhibitor cocktail (ICN) (10 mg/mL AEBSF, 1 mg/mL leupeptin, 1 mg/mL pepstatin, and 5 mg/mL EDTA-Na2) to which 1 mmol/L PMSF was added. Cell homogenates were collected by centrifugation at 13 000g for 4°C, and protein concentrations were determined using the bicinchoninic acid assay (Pierce Interchim). Electrophoresis of the indicated amount of protein lysate was performed through a 10% polyacrylamide gel under reducing conditions (sample buffer containing 10 mmol/L DTT). Proteins were transferred onto nitrocellulose membranes, and membranes were checked for equal loading by Ponceau red staining. Nonspecific binding sites were blocked overnight at 4°C with 10% skim milk powder in TBST (20 mmol/L Tris-HCl, 55 mmol/L NaCl, and 0.1% Tween 20). Membranes were subsequently incubated for 4 hours at room temperature in 5% skim milk-TBST containing rabbit polyclonal antibodies raised against N-terminal PPARα or PPARγ peptides.31 After incubation with a secondary peroxidase-conjugated antibody, signals were visualized by chemiluminescence (Amersham).

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis

Total cellular RNA was extracted using the guanidium thiocyanate/phenol chloroform method.32 For RT-PCR analysis of PPARα and PPARγ expression, total RNA was reverse-transcribed using random hexamer primers and Superscript reverse transcriptase (Life Technologies) and subsequently amplified by PCR using the following primers: for PPARα, 5′-GAC GAA TGC CAA GAT CTG AGA AGG C-3′ and 5′-CTG CTG AGC CAG GTT GCC GGC-3′ (fragment size 948 bp); for PPARγ, 5′-GAC TGC CAC CAT ATG AGC TCT GTG AGG TAT GGA-3′ and 5′-AGC ATG ACC CTT GAT ACC ATG TGC TCC TCG CTC ATG GAT AAA GAG-3′ (fragment size 900 bp). ET-1 mRNA was measured using the following primers: 5′-TGC TCC TCG TGC TCC TCG ATG GAT AAA GAG-3′ and 5′-GCT CAC CAT ATG AGC TCT GTG AGG TTT CTT GCT-3′ (fragment size 461 bp).33 GAPDH-specific primers (sense primer: 5′-ATG CAG CCC CGA ATG CTC ATC ATG GCC-3′; antisense primer: 5′-TTC TTT GAC GAG ATG TGG GCC ATC AT-3′) were used as control (fragment size 239 bp). The resulting products were separated on a 1% agarose gel stained with ethidium bromide.

ET-1 Secretion

BAECs and HMEC-1 cells were subcultured in 24-well plates containing ∼5×10⁶ cells/well. Confluent cells were treated for 24 hours with various PPAR activators or vehicle (0.1% DMSO) and were stimulated with thrombin (10 U/mL) (Roche Laboratories) for 24 hours in lipoprotein-deficient serum containing the various PPAR activators or vehicle (0.1% DMSO). At the end of the treatment period, medium was collected, and the ET-1 concentration was quantified using a radioimmunoassay kit (Amersham). Results were normalized to cellular protein content in all the experiments.

Plasmids

To construct the human ET-1 promoter/luciferase reporter construct, a –250/+55 promoter fragment was amplified by PCR from human genomic DNA with the oligonucleotides 5′-GGA AAA CTC GAG GCC GC AGT TTT AGC-3′ and 5′-TTC GTT AAG CCT GCT GGT TCA GGT CCC-3′. The resulting PCR product was digested and inserted in the XhoI/HindIII sites of the pGL2 basic luciferase vector (Promega) yielding pGL2-hET-1. The internal mutation of the ET-1 promoter AP-1 site was introduced using the site-directed mutagenesis kit (Stratagene) and the following oligonucleotides: 5′-CTG CAC GTT GCC TGT TGG GTA CTA ATA ACA C-3′ and 5′-CTG CTG AGC CTT GAG CCT GGT TCA GGT C-3′. The resulting PCR product was digested and inserted in the XhoI/HindIII sites of the pGL2 basic luciferase vector (Promega) yielding pGL2-hET-1. The internal mutation of the ET-1 promoter AP-1 site was introduced using the site-directed mutagenesis kit (Stratagene) and the following oligonucleotides: 5′-CTG CAC GTT GCC TGT TGG GTA CTA ATA ACA C-3′ and 5′-ATG CAG CCC CGA ATG CTC ATC ATG GCC-3′. All constructs were verified by DNA sequencing analysis. Expression plasmids for human PPARα and PPARγ were described previously.29,31 The pAP-1-3-Luc (Stratagene) contains 3 copies of an AP-1 enhancer. The Rous sarcoma virus (RSV)-driven c-Fos and c-Jun expression plasmids as well as the pBS-Jun plasmids were a
kind gift from Drs Bakiri and Yaniv (Institut Pasteur de Paris, France).

Transient Transfection Assays

BAECs were grown to between 50% and 60% confluence on 6-well plates. Cells were transiently transfected using the calcium phosphate method with 1 μg of wild-type pGL2-hET-1 or mutated pGL2-hET-1 mut or (AP-1)-3-Luc in the presence or absence of 1 μg of pSG5-hPPARα or pSG5-hPPARγ2 and 2 μg of c-Fos (RSV-Fos) and c-Jun (RSV-Jun) expression vectors or corresponding empty vectors. Mock DNA was added to obtain identical amounts of DNA in each well. To correct for variability in transfection efficiency, 200 ng of pCMV-βGal plasmid DNA was cotransfected in all experiments. Cell extracts were prepared 48 hours after transfection, and the luciferase and β-galactosidase assays were performed as previously described. Each experiment was performed at least 3 times, and each transfection was performed in triplicate.

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

BAECs (3×10^6) were cultured in DMEM medium supplemented with 0.2% FCS. Cells were pretreated with different PPAR activators or vehicle (0.1% DMSO) for 2 hours before stimulation with thrombin (10 U/mL) for 1 hour. Cells were harvested, and nuclear extracts were obtained as previously described. Nuclear proteins were quantified using the bicinchoninic acid assay and stored at -80°C. pSG5-hPPARα, pSG5-hPPARγ2, and pBS-Jun were in vitro–translated with T7 polymerase and translated using the rabbit reticulocyte lysate system (Promega). For EMSA, an AP-1 double-stranded oligonucleotide (Promega) was end-labeled with [γ-32P]-ATP using T4 polynucleotide kinase according to standard protocols. Nuclear extracts (5 μg) from BAECs or in vitro–translated proteins were incubated with 50 000 cpm of labeled probes for 20 minutes at room temperature in 20 μL of buffer containing 10 mmol/L Tris (pH 7.5), 50 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, 5% glycerol, 0.3 μg BSA, and 2 μg of poly(dI-dC). The reactions were analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel in 0.5×Tris-Borate-EDTA. The gels were dried and exposed at -80°C for autoradiography.

Results

PPARα and PPARγ Are Expressed in Human Macrovacular (CAECs and ECV304) and Microvascular (HMEC-1) Endothelial Cells

First, it was determined whether PPARs, and which ones, are expressed in human endothelial cells from different origins. By using specific primers for PPARα and PPARγ, RT-PCR analysis demonstrated the presence of both PPARα and PPARγ mRNAs in human CAECs, ECV304 cells, and HMEC-1 cells (Figure 1A). Although PPARγ mRNA could be detected in every different endothelial cell type, its expression levels appeared lower than PPARα. Western blot analysis using specific antibodies for PPARα and PPARγ demonstrated the presence of appreciable amounts of PPARα, whereas only low amounts PPARγ protein were detected even after a prolonged exposure (15 minutes compared with 2 minutes for PPARα) (Figure 1B). CAECs expressed the highest levels of PPARα, whereas PPARγ protein level was lowest compared with the other endothelial cell types. Taken together, these data indicate that both PPARα and PPARγ are expressed in human endothelial cells from macrovascular (CAECs and ECV304 cells) and microvascular origin (HMEC-1 cells).

PPAR Activators Inhibit Thrombin-Induced ET-1 Secretion in HMEC-1 Cells and BAECs

In endothelial cells, ET-1 secretion is detectable under basal conditions, whereas thrombin induces its secretion. Therefore, the effect of PPAR activators on basal and thrombin-induced ET-1 release by human endothelial cells was examined (Figure 2). As previously described for HMEC-1 cells, thrombin induced an approximately 1.5-fold increase in ET-1 secretion by HMEC-1 cells (basal and thrombin-stimulated ET-1 levels were 12.7±0.22 and 39.6±1.7 ng/mL, respectively). PPAR activators inhibited ET-1 production in a dose-dependent manner, with the highest efficiency when cells were pretreated with 100 μmol/L PPAR activators for 2 hours before thrombin stimulation.
PPAR Activators Inhibit Thrombin-Induced ET-1 Production at the Gene Expression Level

Given that it has been reported that thrombin induces ET-1 expression by increasing its mRNA levels, it was determined by use of semiquantitative RT-PCR analysis whether PPAR activators regulate ET-1 gene expression in BAECs. As previously described, thrombin significantly induced ET-1 mRNA levels (Figure 4). This induction was totally abolished in the presence of both PPARα (Wy-14643) and PPARγ (BRL49653) activators. These results indicate that PPAR ligands inhibit thrombin-induced ET-1 production in BAECs at the gene expression level.

PPARs Repress Transcriptional Activation of the Human ET-1 Gene Promoter

Because PPARs act by modulating gene transcription, a potential inhibitory effect of PPAR activators on ET-1 promoter activation by thrombin was investigated. Therefore, the human ET-1 gene promoter was PCR-amplified from human genomic DNA and subcloned, and transient transfection experiments were performed in BAECs (Figure 5). On the basis of previous studies demonstrating that the transcriptional regulation of genes by thrombin occurs mainly via the AP-1 signaling pathway, a mutation in the AP-1 site of the human ET-1 gene promoter was generated. As expected, thrombin stimulation increased ET-1 promoter activity (Figure 5) (P=0.0001). Coincubation with either Wy-14643 or BRL49653 significantly inhibited this transcriptional induction (P=0.0001 and P=0.0004, respectively). Furthermore, cotransfection with either a PPARα or a PPARγ expression vector significantly repressed both basal (P=0.002 and P=0.007, respectively) and thrombin-induced ET-1 promoter activity, an effect which was further enhanced in the presence of their respective ligands (P=0.001 and P=0.002, respectively) (Figure 5A and 5B). Whereas treatment with thrombin alone resulted in the induction of ET-1 promoter activity (Figure 5A and 5B), the AP-1–mutated promoter was no longer activated, thereby suggesting that the AP-1 site is essential for the activation of ET-1 promoter by thrombin (Figure 5A and 5B). Furthermore, PPAR cotransfection and
ligand treatment did not have major effects on the activity of the mutated ET-1 promoter. These data indicate that thrombin induces ET-1 promoter activation via a mechanism involving the AP-1 site and that PPARs repress both basal and thrombin-induced ET-1 expression by downregulating ET-1 promoter activity.

**PPARs Downregulate Human ET-1 Gene Promoter Activity by Interfering Negatively With AP-1 Transcription Activity**

Having shown that thrombin induces ET-1 promoter activity via the AP-1 site, a direct interference between PPAR and AP-1 signaling pathways was investigated by cotransfection experiments with c-Fos and c-Jun expression plasmids. Whereas cotransfection of c-Jun/c-Fos resulted in a 3.5-fold increase of wild-type ET-1 promoter activity ($P < 0.024$), the mutated promoter was no longer activated (Figure 6A and 6B). Cotransfection of both PPARα and PPARγ expression plasmids inhibited basal activity of the wild-type ET-1 promoter ($P < 0.007$ and $P < 0.008$, respectively) and prevented its activation by c-Jun/c-Fos ($P < 0.013$ and $P < 0.02$, respectively). By contrast, PPAR cotransfection did not have any major effect on the activity of the mutated ET-1 promoter. These data indicate that PPAR activators repress ET-1 gene expression by interfering negatively with AP-1 transcriptional activity.

**PPARs Repress Activity of a Heterologous Promoter Driven by AP-1 Response Elements**

Second, it was determined whether PPARs could interfere with the activity of a heterologous promoter driven by AP-1 response elements (Figure 7). As expected, cotransfection of both c-Jun and c-Fos led to a strong activation (>15-fold; $P < 0.018$) of the reporter construct (Figure 7A and 7B). This induction tended to be reduced in the presence of both PPARα ($P < 0.044$) and PPARγ activators ($P < 0.16$). Cotransfection of both PPARα and PPARγ strongly repressed AP-1 transcriptional activity, and this repression was more pronounced in the presence of their respective ligands ($P = 0.04$ and $P = 0.038$, respectively) (Figure 7A and 7B). These results indicate that both PPARα and PPARγ interfere...
with AP-1 transcriptional activity in a manner independent of the promoter context.

PPAR Activators Reduce Fos/Jun DNA Binding Activity

Finally, it was analyzed by EMSAs whether PPAR activators interfere with AP-1 activity by inhibiting the binding of transcription factors present in nuclear extracts of thrombin-activated BAECs to the AP-1 consensus site. Unstimulated BAECs exhibited a basal AP-1 binding activity, which was approximately 2-fold stimulated by thrombin (Figure 8A). Treatment of BAECs with PPAR activators significantly reduced the basal AP-1 binding activity. Furthermore, in thrombin-stimulated BAECs, both PPARα and PPARγ activators strongly reduced the binding of nuclear proteins to the AP-1 oligonucleotide. EMSA with in vitro–translated PPAR and c-Jun proteins indicated that both PPARα and PPARγ decrease in a dose-dependent manner c-Jun binding activity to an AP-1 consensus sequence (Figure 8B). These observations suggest that PPARs negatively interfere with the AP-1 signaling pathway, at least in part, by preventing the binding of transcription factors of the Fos/Jun family to the AP-1 site.

Discussion

In the present study, we report that both PPARα and PPARγ are expressed in human endothelial cells of macrovascular (human CAECs and ECV304 cells) as well as microvascular (HMEC-1) origin. Furthermore, we demonstrate that PPAR activators inhibit the thrombin-induced release of ET-1 by endothelial cells by interfering negatively with ET-1 gene expression. Finally, we show that PPAR activators act at the transcriptional level and downregulate human ET-1 promoter activity by negatively interfering with the AP-1 signaling pathway. PPAR activators act, at least in part, by reducing AP-1 DNA binding activity after thrombin stimulation.

RT-PCR and Western blot analysis demonstrated that PPARα and PPARγ are expressed in human endothelial cells. PPARα protein was detected in appreciable amounts, whereas PPARγ appeared to be expressed at lower basal levels. These observations confirm and extend those reported by Inoue et al.36 who showed the presence of PPARα mRNA in human aortic and venous endothelial cells. Furthermore, while this work was in progress, human saphenous vein endothelial cells were shown to express PPARγ mRNA and protein.37 Recently, it was reported that PPARα but not PPARγ is expressed in human aortic SMCs,38 whereas PPARα and PPARγ protein are expressed in vascular SMCs isolated from human saphenous veins.38 Furthermore, both PPARα and PPARγ are expressed in differentiated human monocyte-derived macrophages, which participate in inflammation control and atherosclerotic plaque formation.27,31,39 Finally, PPARγ has been detected in human and mouse atherosclerotic lesions.39–41 The expression of PPARs in all 3 major cell types of the atherosclerotic lesion suggests a potential regulatory role for PPARs in the pathogenesis of atherosclerosis. In line with this hypothesis is the demonstration that PPAR activation modulates macrophage foam cell formation and apoptosis as well as the inflammatory response of SMCs and macrophages.26,27,31,38–40
To determine whether PPARs could also be involved in the regulation of endothelial function, the influence of PPARα activators (fenofibric acid and Wy-14643) and PPARγ (troglitazone and BRL49653) activators on ET-1 production was evaluated in the present study. Furthermore, because ET-1 expression is upregulated at the transcriptional level by thrombin, the influence of PPAR agonists on thrombin-induced ET-1 production was also analyzed. Our results confirm previous reports showing that thrombin induces ET-1 release in BAECs and HMEC-1 cells. PPARα activators did not induce unstimulated ET-1 secretion, suggesting the absence of a PPRE in the ET-1 gene promoter. PPARα activators, however, repressed the thrombin-induced ET-1 secretion in a dose-dependent manner at concentrations within the range of the Ki for PPARα and the steady-state plasma concentrations observed in humans. By contrast, in HMEC-1 cells, the PPARγ ligand BRL49653 only slightly repressed the thrombin-induced ET-1 release. This is concordant with the low amount of PPARγ protein detected in these endothelial cells. Interestingly, in BAECs, PPARγ activators more pronouncedly reduced ET-1 production. This difference in response between HMEC-1 cells and BAECs might be due to differences in PPARγ protein levels. Moreover, in BAECs, BRL49653 was more active than troglitazone in downregulating ET-1 production, which is coherent with their relative affinities for PPARγ, BRL49653 being a higher-affinity ligand than troglitazone. These data indicate that PPAR activators regulate ET-1 secretion in both microvascular and macrovascular endothelial cells. Because ET-1 is involved in SMC proliferation and because ET-1 displays chemotactic activities on blood monocytes that are involved in the earliest events of atherogenesis, PPAR activators might influence the pathogenesis of atherosclerosis by reducing endothelial ET-1 secretion. Additional studies are required to test this hypothesis.

Our results demonstrate that PPAR activators inhibit thrombin-induced ET-1 production by acting at the level of ET-1 gene expression. To gain further insight into the mechanism of the PPAR-mediated inhibition of ET-1 gene expression, the effect of PPARs on human ET-1 promoter activity was studied. In parallel to ET-1 secretion, thrombin induced ET-1 promoter activity in BAECs, and treatment with both the PPARα and PPARγ activators Wy-14643 and BRL49653 prevented this activation. Furthermore, cotransfection of PPAR expression plasmids totally abolished this induction, indicating that the effects of fibrates and BRL49653 occur via the nuclear receptors PPARα and PPARγ, respectively. Morey et al reported that estrogens and progesterone also inhibit the stimulated production of ET-1 and that this inhibition occurs at the transcriptional level, probably via activation of their respective receptors. Therefore, inhibition of ET-1 production could be a mechanism shared by a number of nuclear receptors.

The analysis of the ET-1 promoter did not reveal the presence of any putative PPAR response elements, which is consistent with the absence of its activation by PPAR transfection. ET-1 promoter activity has been shown to be controlled by a complex interaction of GATA and AP-1 transcription factors binding to closely apposed sites on the proximal ET-1 promoter. Because thrombin has been shown to increase both c-Jun mRNA levels and AP-1 binding activity, we hypothesized that PPARs could exert their effect on ET-1 gene transcription by interfering with the AP-1 signaling pathway. Results from transactivation studies with c-Jun and c-Fos expression plasmids confirmed a strong activation of the ET-1 promoter by c-Jun and c-Fos.
Cotransfection of PPARs in the presence of c-Jun and c-Fos repressed the strong transactivation of the ET-1 promoter as well as the transactivation of a heterologous AP-1–driven promoter. A similar negative interference with AP-1 activity has been described for other nuclear receptors, such as the retinoic acid receptor (RAR) and the glucocorticoid receptor (GR). In these reports, both GR and RAR were shown to form a nonproductive complex with c-Jun, leading to a decrease of AP-1 binding activity. Our results from EMSAs showing that PPAR activators decrease AP-1 binding activity in BAECs in vivo and that PPARα and PPARγ can inhibit c-Jun binding to an AP-1 site in vitro suggest that a similar mechanism is operative for PPARs. These data are in line with a report from Sakai et al suggesting that PPARs may physically interact with c-Jun in a manner similar to GR. Taken together, these data show that PPARs negatively regulate ET-1 expression, at least in part, by interfering with its activation by AP-1. Kawana et al demonstrated that ET-1 promoter is cooperatively regulated by GATA-2 and AP-1, which have been shown to interact physically, resulting in a synergistic regulation of the ET-1 promoter by these factors, even in the absence of their respective binding sites. Although the results from the transfections on the wild-type and AP-1–mutated promoter constructs suggest that PPARs interfere primarily via the AP-1 site, our results do not allow us to rule out a potential interference between PPARs and GATA factors as well. Indeed, in a recent study, it was reported that both AP-1 and GATA-2 sites contained in the ET-1 promoter are essential for retinoid repression. Caelles et al proposed a mechanism by which hormone-activated receptors may block AP-1 activity by interfering with the transactivation of a heterologous AP-1–driven promoter. A similar negative interference with AP-1 activity as well as the transactivation of a heterologous AP-1–driven promoter is cooperatively regulated by GATA-2 and AP-1, showing that PPAR activators decrease AP-1 binding activity in advanced atherosclerosis. In these reports, both GR and RAR were shown to physically interact with c-Jun in a manner similar to GR. Our results do not allow us to rule out the participation of such mechanisms in the downregulation of ET-1 gene transcription by PPARs, and additional studies are necessary to delineate in more detail the molecular mechanism involved in the negative regulation of Fos/Jun transactivation by PPARs.

In conclusion, in addition to their expression and activity in SMCs and macrophages, the results from the present study demonstrate that PPARs are also expressed in endothelial cells where they modulate endothelial function. In these different cell types, PPARs interfere negatively with the AP-1, Stat, and NF-κB signaling pathways. Because these pathways are implicated in the activation of inflammatory response genes in the atherosclerotic lesion, such activity of PPARs may be considered beneficial in the pathogenesis of atherosclerosis. More studies are needed to test the action of PPAR agonists on atherosclerosis development in vivo.

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References


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