Peroxisome Proliferator-Activated Receptor Gamma Activators Inhibit Gene Expression and Migration in Human Vascular Smooth Muscle Cells

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Abstract—Migration of vascular smooth muscle cells (VSMCs) plays an important role in atherogenesis and restenosis after arterial interventions. The expression of matrix metalloproteinases (MMPs), particularly MMP-9, contributes to VSMC migration. This process requires degradation of basal laminae and other components of the arterial extracellular matrix. Peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor family, regulate gene expression after activation by various ligands. Recent studies have suggested opposing effects of PPAR gamma (PPARγ) activation on atherogenesis. The present study tested the hypotheses that human VSMCs express PPAR alpha (PPARα) and PPARγ and that PPAR agonists in VSMCs modulate MMP-9 expression and activity, as well as VSMC migration. Human VSMCs expressed PPARα and PPARγ mRNA and protein. Treatment of VSMCs with the PPARγ ligands troglitazone and the naturally occurring 15-deoxy-d12,14-prostaglandin J2 (15d-PGJ2) decreased phorbol 12-myristate 13-acetate–induced MMP-9 mRNA and protein levels, as well as MMP-9 gelatinolytic activity in the supernatants in a concentration-dependent manner. Six different PPARα activators lacked such effects. Addition of prostaglandin F2α, known to limit PPARγ activity, diminished the MMP-9 inhibition seen with either troglitazone or 15d-PGJ2, further implicating PPARγ in these effects. Finally, troglitazone and 15d-PGJ2 inhibited the platelet-derived growth factor-BB–induced migration of VSMCs in vitro in a concentration-dependent manner. PPARγ activation may regulate VSMC migration and expression and activity of MMP-9. Thus, PPARγ activation in VSMCs, via the anti-diabetic agent troglitazone or naturally occurring ligands, may act to counterbalance other potentially proatherosclerotic PPARγ effects. (Circ Res. 1998;83:1097-1103.)

Key Words: gene expression ■ vascular smooth muscle cell ■ migration ■ peroxisome proliferator-activated receptor gamma ■ troglitazone

Vascular smooth muscle cell (VSMC) migration likely contributes to both atherogenesis and restenosis, complicating interventional treatments of atherosclerosis. Migration of VSMCs into and within the intima requires the degradation of basal laminae and interstitial extracellular matrix, processes that likely involve matrix metalloproteinases (MMPs). Among these, MMP-9, also known as gelatinase B, appears particularly important in migration of VSMCs after arterial injury. In situ hybridization demonstrated induction of MMP-9 mRNA in VSMCs within the first days after balloon injury in rat carotid arteries. Furthermore, systemic administration of an MMP inhibitor reduced early migration into the intima by 97%. Overexpression of MMP-9 occurs in vulnerable regions of human atheroma and in atherectomy specimens retrieved from humans with unstable coronary syndromes. Several mediators present in human atheroma, such as interleukin (IL)-1, tumor necrosis factor-α, and CD40 ligand can induce MMP-9 expression in VSMCs. The complexity of governing the balance of MMP activity is underscored by the presence of a family of inhibitors of MMP activity, proteins known as tissue inhibitors of MMPs (TIMPs). However, regulatory signals directly inhibiting MMP-9 gene expression in VSMCs remain poorly understood.

Troglitazone, a new anti-diabetic agent, reduces arterial injury-induced intimal hyperplasia, as well as migration and proliferation of rat VSMCs. Troglitazone acts as a ligand for peroxisome proliferator-activated receptor gamma (PPARγ). One of three members (alpha, delta, and gamma) of a family of ligand-activated nuclear receptor transcription factors. With ligand binding, PPARs form heterodimers with the retinoic X receptor and bind to PPAR response elements in the promoter region of specific target genes, thus regulating their expression. PPARα appears to interact with various fatty acids and eicosanoid derivatives.
whereas PPARγ ligands, in addition to troglitazone, include the naturally occurring prostaglandin D2 metabolite, 15-deoxy-12,14-prostaglandin J2 (15d-PGJ2).13,14

Macrophages in human atheroma express PPARγ.15,16 In vitro studies have revealed that PPARγ activation inhibits expression of proinflammatory genes as well as macrophage scavenger receptor A and inducible nitric oxide synthetase genes in cells of the monocyte lineage.16,17 PPARγ activation also decreases MMP-9 expression and gelatinolytic activity in these same cells.15 In contrast, other recent studies implicate PPARγ activation in promoting atherogenesis. Tontonoz et al18 and Nagy et al19 report that the scavenger receptor CD36 is a PPARγ-regulated gene, that oxidized LDL is a naturally occurring PPARγ ligand, and that PPARγ activation contributes to monocyte differentiation into foam cells. Given the role of VSMCs in atherosclerosis and restenosis, we hypothesized that PPARγ effects in VSMCs might oppose potentially proatherogenic actions induced by PPARγ activation in monocyte/macrophages. Specifically, we tested whether VSMCs express PPARα and if so, whether PPARα activation in these cells inhibits MMP-9 expression and activity. Furthermore, we investigated if PPAR ligands might inhibit human VSMC migration.

Materials and Methods

Cell Culture

Human VSMCs were isolated from the tunica media human saphenous veins and cultured in DMEM (BioWhittaker, Walkersville, Md) with 1% glucose (Sigma Chemical Co, St. Louis, Mo), 1% penicillin-streptomycin (Sigma), and 10% FCS. The cells were identified by their typical growth pattern and by immunofluorescence with anti-α-actin monoclonal antibodies (>99% positive cells). These experiments used cells at passages two to five. Cells were cultured in serum-free medium supplemented with insulin and transferrin (IT-medium) for 24 hours and then stimulated with phorbol 12-myristate 13-acetate (PMA) (50 μg/L) in the presence or absence of different activators of PPARα (lobocastanehaxanoic acid, eicosapentaenoic acid, fenofibrate, gemfibrozil, clofibrate [all from Sigma], and 10% FCS). The cells were lysed in 20 mmol/L HEPES (pH 7.9), 100 mmol/L KCl, and 0.5% NP-40. Nuclei were pelleted at 13 000g for 5 minutes, and the resulting supernatant was used as the cytosol fraction. Nuclei were lysed in 20 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl2, 420 mmol/L NaCl, and 0.2 mmol/L EDTA. After centrifugation at 13 000g for 5 minutes, the supernatant was diluted in equal volume of 20 mmol/L HEPES (pH 7.9), 100 mmol/L KCl, 0.2 mmol/L EDTA, and 20% glycerol and used as nuclear extract. Protein concentration of nuclear and cytosolic extracts was determined colorimetrically (Pierce, Rockford, Ill). Processed samples were applied to 10% SDS gels and transferred to nitrocellulose membranes (Millipore, Bedford, Mass) using semidry blotting, as described previously. Membranes were blocked overnight in TBS-Tween with 5% nonfat dry milk and incubated with goat anti-human PPARα or goat anti-human PPARγ antibodies (mAbs) (Santa Cruz, San Diego, Calif) for 1 hour. After washing, membranes were stained with horseradish peroxidase–conjugated rabbit anti-goat mAbs. Antigen detection was performed with a chemiluminescent detection system (NEN, Boston, Mass). Similar methods were used to perform Western blot analysis on MMP-9 or MMP-2 in VSMC supernatants using the respective anti-human mAbs (Oncogene Research, Cambridge, Mass). For the analysis of TIMP-1 and TIMP-2 in VSMC supernatants, we used anti-human TIMP-1 and anti-human TIMP-2 mAbs (Oncogene Research).
Migration Assay

Migration of VSMCs was investigated through the use of a standard in vitro wound assay. VSMCs were grown in 6-well plates to confluence, and after 24 hours of culture in IT-medium, a reusable template was used to create a standard wound (∼30 mm). Cells were then stimulated with platelet-derived growth factor (PDGF)-BB (50 µg/L) in the presence or absence of troglitazone or 15d-PGJ2, and wound closure rates followed. A reference point was created on the bottom of the plate in the field of the wound using direct microscopic visualization. This procedure permitted photographing the identical spot each time. The remaining cell-free area was determined via microphotography performed immediately after injury as well as 6 hours after stimulation. Differences were analyzed using NIH Image 1.6 software program, and the results were expressed as percent of migration compared with cultures stimulated with PDGF lacking any PPARγ activators.

Statistical Analysis

Results of the experimental studies are reported as mean±SEM. Differences were analyzed by paired Student t test. A P value <0.05 in the 2-tailed test was regarded as significant.

Results

Human VSMCs Express PPARα and PPARγ mRNA and Protein

Cultured human VSMCs express PPARα and PPARγ mRNA as detected by RT-PCR products of the predicted size (Figure 1A). Western blot analysis revealed PPARα and PPARγ protein expression in the nuclear fraction, whereas neither protein was detected in the cytosol fraction (Figure 1B). The identity of the bands was confirmed by its apparent molecular weight and its comigration with the signal from nuclei of PPARα- or PPARγ-transfected fibroblasts. Nuclei from untransfected fibroblasts exhibit no similar signal (data not shown).

PPARγ but not PPARα Activators Decrease Both Secreted MMP-9 Protein Levels and Gelatinolytic Activity in VSMCs

To investigate whether PPAR activation in VSMCs regulates MMP-9 protein expression and gelatinolytic activity, we stimulated human VSMCs with PMA in the presence or absence of PPARα or PPARγ activators and performed Western blot analysis as well as gelatin substrate zymography on supernatants. As previously reported, quiescent VSMCs secreted MMP-9 at very low levels, and stimulation with PMA markedly augmented MMP-9 protein levels in the supernatant. None of the PPARα activators used affected MMP-9 protein expression or gelatinolytic activity (Figure 2A). In contrast, treatment of PMA-stimulated VSMCs with the PPARγ activators troglitazone or 15d-PGJ2 decreased MMP-9 protein levels in a concentration-dependent manner (Figure 3A, upper panels). Moreover, gelatin zymography of the supernatants from VSMCs so treated revealed a decrease of MMP-9 gelatinolytic activity (Figure 3A, middle panels), with a maximal reduction to 44±3% at 10 µmol/L troglitazone (P<0.01; n=3) and 21±3% at 10 µmol/L 15d-PGJ2 (P<0.01; n=3) compared with PMA-stimulated VSMCs (Figure 3A, lower panels).

Neither PPARα activators nor PPARγ agonists affected the constitutively expressed 72-kDa gelatinase (MMP-2), as shown by zymography (Figures 2A and 3) and Western blot (Figure 2A, lower panel) analysis. To confirm the identity of the lytic band at 72 kDa in the zymography as being MMP-2, we performed immunoprecipitation and immunodepletion experiments on supernatants from PMA-treated VSMCs. As shown in the insert in Figure 2A, the band corresponding to MMP-2 could be depleted from the supernatant with anti–MMP-2 antibodies, whereas the pellet continues to show a 72-kDa signal. Treatment of VSMCs with either PPARα or PPARγ activators in the absence of PMA did not change MMP-9 protein levels or gelatinolytic activity in supernatants (data not shown). Determination of lactate dehydrogenase in the supernatants revealed no significant differences between the samples, indicating that the effects observed did not result from cell death (data not shown).

Western blot analysis for TIMP-1 and TIMP-2 on supernatants from PMA-stimulated (Figure 3B, upper panels) and PMA-treated (Figure 3B, middle panels) VSMCs revealed increased TIMP-1 expression and unchanged TIMP-2 expression in response to PMA. Thus, the observed decrease in MMP-9 protein levels was not due to upregulated expression of TIMP-1 or TIMP-2, which could inhibit gelatin degradation.
stimulated VSMCs (Figures 2B and 3B) revealed no changes after treatment with PPAR\textsubscript{a} or PPAR\textsubscript{g} activators.

**PPAR\textsubscript{g} Activation Decreases MMP-9 mRNA in VSMCs**
Northern blot analysis of PMA-stimulated VSMCs treated with or without PPAR\textsubscript{g} activators for 18 hours demonstrated a marked reduction of MMP-9 mRNA (size, 2.3 kb) levels by either troglitazone or 15d-PGJ\textsubscript{2} (Figure 3C, upper panel). Ethidium bromide staining of the gels showed equivalent loading in each lane (Figure 3C, lower panel).

**Inactivation of PPAR\textsubscript{g} Through PGF\textsubscript{2\alpha} Diminishes the Inhibition of MMP-9 Expression by Troglitazone and 15d-PGJ\textsubscript{2}**
To determine whether PPAR\textsubscript{g} mediates the effects of troglitazone and 15d-PGJ\textsubscript{2}, we performed similar experiments in the presence of PGF\textsubscript{2\alpha}, an agent known to inactivate PPAR\textsubscript{g} by causing its phosphorylation.\textsuperscript{21} Addition of PGF\textsubscript{2\alpha} (200 nmol/L) diminished the inhibitory effect of troglitazone or 15d-PGJ\textsubscript{2} on PMA-induced MMP-9 expression (Figure 4A) and gelatinolytic activity (Figure 4B). PGF\textsubscript{2\alpha} alone had no effect on MMP-9 protein expression or gelatinolytic activity.

**Troglitazone and 15d-PGJ\textsubscript{2} Inhibit PDGF-BB–Induced Migration of VSMCs**
To explore whether PPAR\textsubscript{g} activation directly affects PDGF-BB–induced VSMC migration, we performed an in vitro wound closure assay. Treatment of human VSMCs with troglitazone significantly decreased the PDGF-BB–induced migration to 39±3% at 5 μmol/L and to 34±12% at 10 μmol/L (P<0.05), respectively (Figure 5). 15d-PGJ\textsubscript{2} reduced the migration significantly to 42±7% at 5 μmol/L and to 25±6% at 10 μmol/L 15d-PGJ\textsubscript{2} (P<0.05), respectively (Figure 5). Medium with solvent alone had no influence on VSMC migration (data not shown).

**Discussion**
The present study reports expression of both PPAR\textsubscript{a} and PPAR\textsubscript{g} by human VSMCs. Although PPAR\textsubscript{a} activation had no effect on MMP-9 expression, stimulation of PPAR\textsubscript{g} inhibited MMP-9 mRNA and protein expression, gelatinolytic activity, and PDGF-BB–induced migration of human VSMCs. Neither PPAR\textsubscript{a} nor PPAR\textsubscript{g} activators appear to influence TIMP expression.

The absence of an effect of PPAR\textsubscript{a} agonists on MMP-9 expression or enzymatic activity could be explained by either low PPAR\textsubscript{a} levels in these cells or a lack of transcriptional regulation by PPAR\textsubscript{a} on this gene. Our data showing approximately similar levels of PPAR\textsubscript{a} and PPAR\textsubscript{g} in VSMCs, within the limitations of RT-PCR and Western blotting techniques, argue against low PPAR\textsubscript{a} levels as an explanation for these findings. Furthermore, while this article was in review, Staels et al\textsuperscript{20} also found that PPAR\textsubscript{a} and PPAR\textsubscript{g} were present in VSMCs, at a modestly higher level of PPAR\textsubscript{a} than...
PPARγ under the conditions of their experiments. These investigators also reported that PPARα, but not PPARγ, agonists inhibited the IL-1–induced production of IL-6 and prostaglandin and the expression of the cyclooxygenase-2 gene. As such, although PPARα appears to be functionally active in VSMCs, our work suggests that it is not involved in MMP-9 regulation.

In contrast, PPARγ activators do inhibit MMP-9 expression and gelatinolytic activity in stimulated VSMCs. Our data suggest PPARγ activation as the mechanism for the effects of these activators. Troglitazone is a synthetic compound known to have a high affinity for PPARγ as evidenced by ligand binding assays. 15d-PGJ2 also has significant binding capacity to PPARγ, although it may also have some activity on other PPAR isoforms. However, because none of 6 established PPARα activators demonstrated any effect on MMP-9, PPARα activation by 15d-PGJ2 would seem to be an unlikely explanation for our findings. Furthermore, the ability to reverse the effects of troglitazone and 15d-PGJ2 on MMP-9 by PGF2α costimulation also supports that these agonists are acting through PPARγ; PGF2α has been shown to induce inhibitory phosphorylation of PPARγ by activation of mitogen-activated protein kinase.

The mechanism through which PPARγ activators inhibit MMP-9 expression is likely to be transcriptional, although posttranscriptional modulation cannot be ruled out. Certainly, nuclear hormone receptors, including PPARs, negatively regulate expression of other genes. The transcriptional suppression of MMP-9 could be due to a negative PPAR-response element, as previously described for the apolipoprotein A-1 gene and for thyroid hormone regulation of thyroid-stimulating hormone. Alternatively, inhibitory ef-
Effects might occur independent of a PPAR binding site, through competitive binding and “squelching” of transcriptional coactivators by liganded PPAR. This has been suggested as the mechanism of negative cross talk between other nuclear receptors and activator protein-1 as well as other transcription factors. The expression of PPARs in VSMCs, and its regulation of MMP-9 expression and activity, and VSMC migration have potentially important implications for atherosclerosis. Matrix degradation by MMPs and medial VSMC movement into the intima occur early during intimal hyperplasia in injured rat arteries. Similar processes are very likely to contribute to human atherogenesis. Interestingly, troglitazone inhibits intimal thickening after arterial injury in rats, a process that might be influenced by the MMP-9 effects described in the present study.

Beyond the part of PPARγ in VSMC biology is the question of its role in the pathology of the arterial wall. Several lines of evidence would suggest an antiatherosclerotic effect of PPARγ activation: inhibition of MMP-9 in VSMCs and monocyte/macrophages, decreased expression of cytokines and inducible nitric oxide synthetase, and decreased LDL oxidation in response to troglitazone. Furthermore, preliminary findings of decreased intimal and medial carotid thickening in diabetic patients treated with troglitazone also suggest that PPARγ activation might limit atherosclerosis.

In contrast, Tontonoz et al and Nagy et al recently reported that oxidized LDL increases scavenger receptor (CD36) expression via direct PPARγ binding and transcriptional activation. They found, as did we, high levels of PPARγ in lesional macrophages, although our data also suggested inhibition of macrophage MMP-9 expression via PPARγ, which might limit atherosclerosis. The evidence to date in monocyte/macrophages might suggest complex regulatory effects of PPARγ, with mediation of macrophage development and oxidized LDL signaling on one hand and decreased MMP-9 and inflammatory cytokine production on the other. Reconciling these findings with the overall benefits seen with troglitazone in the clinical setting might suggest PPARγ effects in other arterial wall cells that limit the atherogenic response. The data in the present study suggest that PPARγ activation in human VSMCs might provide one such counterbalancing antiatherogenic mechanism.

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