Peroxisome Proliferator-Activated Receptor γ Ligands Stimulate Endothelial Nitric Oxide Production Through Distinct Peroxisome Proliferator-Activated Receptor γ–Dependent Mechanisms

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Objective.—We recently reported that the peroxisome proliferator-activated receptor γ (PPARγ) ligands 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) and ciglitazone increased cultured endothelial cell nitric oxide (NO) release without increasing the expression of endothelial nitric oxide synthase (eNOS). The current study was designed to characterize further the molecular mechanisms underlying PPARγ-ligand–stimulated increases in endothelial cell NO production.

Methods and Results.—Treating human umbilical vein endothelial cells (HUVEC) with PPARγ ligands (10 μmol/L 15d-PGJ2, ciglitazone, or rosiglitazone) for 24 hours increased NOS activity and NO release. In selected studies, HUVEC were treated with PPARγ ligands and with the PPARγ antagonist GW9662 (2 μmol/L), which fully inhibited stimulation of a luciferase reporter gene, or with small interfering RNA to PPARγ, which reduced HUVEC PPARγ expression. Treatment with either small interfering RNA to PPARγ or GW9662 inhibited 15d-PGJ2-, ciglitazone- and rosiglitazone-induced increases in endothelial cell NO release. Rosiglitazone and 15d-PGJ2, but not ciglitazone, increased heat shock protein 90-eNOS interaction and eNOS ser1177 phosphorylation. The heat shock protein 90 inhibitor geldanamycin attenuated 15d-PGJ2- and rosiglitazone-stimulated NOS activity and NO production.

Conclusions.—These findings further clarify mechanisms involved in PPARγ-stimulated endothelial cell NO release and emphasize that individual ligands exert their effects through distinct PPARγ-dependent mechanisms. (Arterioscler Thromb Vasc Biol. 2005;25:1810-1816.)

Key Words: peroxisome proliferator-activated receptor γ ■ nitric oxide ■ endothelium ■ endothelial nitric oxide synthase ■ thiazolidinedione

Endothelium-derived nitric oxide (NO) is a key molecule in vascular biology that decreases vascular tone, smooth muscle cell proliferation, leukocyte adhesion, and platelet aggregation.1–6 Endothelial dysfunction, characterized by impaired endothelial NO production, participates in the pathogenesis of atherosclerotic disease and is associated with risk factors for vascular disease, including hypercholesterolemia, diabetes mellitus, insulin resistance, and obesity.7 Our recent studies demonstrate that the peroxisome proliferator-activated receptor γ (PPARγ) ligands 15-deoxy-Δ12,14-πG J2 (15d-PGJ2) and ciglitazone stimulate NO release from endothelial cells (ECs).8 Understanding the mechanisms of PPARγ ligand-induced stimulation of EC NO release may provide novel insights into the vascular protective effects of PPARγ ligands.

In ECs, type III endothelial nitric oxide synthase (eNOS) produces NO from the amino acid l-arginine. eNOS is regulated not only at the level of expression,9–11 but also post-translationally by mechanisms including interactions of eNOS with other proteins12–15 and eNOS phosphorylation.16–19 For example, specific stimuli including vascular endothelial growth factor (VEGF), histamine, and shear stress have been shown to activate eNOS by promoting the interaction of eNOS with heat shock protein 90 (hsp90), a molecular chaperone protein.14 Hsp90 has been shown to increase eNOS activity by (1) recruiting Akt, the serine protein kinase B, to phosphorylate eNOS at ser1177,20 (2) facilitating the displacement of eNOS from inhibitory interactions with caveolin,21 and (3) increasing the affinity of eNOS for calmodulin.22 In addition to protein-protein interactions, several specific sites of phosphorylation also regulate eNOS activity. For example, phosphorylation of eNOS at ser1177 increases electron flux from the reductase to the oxygenase domain of eNOS and also increases enzyme activity.16,23

Our laboratory recently demonstrated that (1) overexpression of PPARγ or treatment with 9-cis retinoic acid, the ligand for the PPAR heterodimer RXR, enhanced EC NO release, (2) the PPARγ ligands 15d-PGJ2 and ciglitazone, without altering PPARγ expression, stimulated a PPAR
response element-luciferase reporter construct in transfected ECs and significantly increased basal as well as calcium ionophore-induced endothelial NO release, and (3) neither 15d-PGJ2 nor ciglitazone altered eNOS mRNA levels, whereas 15d-PGJ2, but not ciglitazone, decreased eNOS protein expression. These findings led us to further characterize the molecular mechanisms underlying the ability of PPARγ ligands to stimulate endothelial NO production. The current study demonstrates that 15d-PGJ2, ciglitazone, and rosiglitazone increase NO release in ECs by distinct PPARγ-dependent signaling pathways.

Methods

HUVEC Treatment Protocols

HUVECs were grown on 100-mm dishes or 6-well plates and maintained in endothelial cell growth media according to the protocols provided by the manufacturer (Clonetics). When 90% confluent, HUVECs were treated with an equal volume of vehicle, 5 μmol/L A23187 (Alexis Biochemicals), or with the PPARγ ligands, 10 μmol/L 15d-PGJ2 (Calbiochem), 10 μmol/L ciglitazone (Biomol Research Laboratories), or 10 μmol/L rosiglitazone (Cayman Chemicals) for 24 hours at 37°C in a 5% CO2 incubator, a concentration previously shown to increase HUVEC NO release. In selected experiments, HUVECs were also cotreated for 24 hours with the PPARγ antagonist GW9662 (2 μmol/L; Cayman Chemicals), which covalently modifies Cys285 in helix 3 of the PPARγ ligand-binding domain, or the hsp90 inhibitor geldanamycin (GA) (1 μmol/L; Calbiochem). All treatments were prepared as a stock solution in water, ethanol, or dimethyl sulfoxide, and diluted in endothelial cell growth media to their final concentrations. Control conditions included HUVECs treated with vehicle alone.

Analysis of HUVEC NO Release and NOS Activity

NO release from HUVEC was determined by subjecting culture media from treated monolayers to chemiluminescence analysis with a Sievers Nitric Oxide Analyzer as previously reported. Total NOS activity was quantified by measuring conversion of [14C]-l-arginine to [14C]-l-citrulline by the Nitril Oxide Synthase Assay Kit (Calbiochem) as previously reported.

Transfection Protocols

Following protocols provided by the manufacturer, HUVECs (50% confluence) were transfected with optimized concentrations of either human PPARγ small interfering RNA (siRNA) Cat. # sc-29455), control nonsense fluorescein conjugate siRNA (Cat. # sc-36869), or with mock conditions using siRNA transfection reagent alone (Santa Cruz Biotechnology, Inc.). Forty-eight hours after transfection, cells were treated with PPARγ ligands. Whole cell lysates were subjected to immunoblotting with anti-PPARγ antibodies (Santa Cruz Biotechnology, Inc.) to confirm small interfering RNA to PPARγ-induced alterations in PPARγ expression. In separate studies, HUVECs were transfected with a PPARγ reporter vector containing 3 PPAR response elements (PPREs) which luciferase expression is induced by PPARγ agonists, or with the control vector (Panomics, Inc), using the FuGENE 6 transfection reagent (Roche). Twelve hours after transfection, HUVECs were treated with PPARγ ligands for 24 hours. Cells were then harvested and luminescence was measured in a microplate luminometer.

Western Blotting and Immunoprecipitation Techniques

After treatment and washing, HUVEC monolayers were collected into lysis buffer (20 mmol/L Tris pH 7.4, 2.5 mmol/L EDTA, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 100 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L NaVO3, and anti-protease cocktail pill), sonicated, and centrifuged. Each sample (20 μg protein) was subjected to SDS-PAGE (4% to 12% gradient gels) (Invitrogen), and proteins were transferred to polyvinylidene fluoride membranes and immunoblotted with primary antibodies (1:1000) to phospho-eNOS at ser1177 (Cell Signaling), hsp90 (Stressgene), or eNOS (BD Transduction Laboratories) in TBS-T (10 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 0.1% Tween) containing 5% powdered non-fat dry milk or 3% bovine serum albumin for the phospho-antibody. Hsp90-eNOS interactions were examined by incubating whole cell lysates (200 μg protein) with 5 μg monoclonal eNOS antibody overnight at 4°C on a rocker. Antibody-eNOS complexes were collected by incubation with GammaBind sepharose beads (Amersham Pharmacia), and the immunocomplexes were precipitated by centrifugation. Immunoprecipitated proteins were then separated with SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunoblotted for eNOS and hsp90. Protein bands were identified with chemiluminescence and quantified with laser densitometry.

Statistical Analysis

Overall treatment effects were examined by ANOVA. Post hoc analysis to detect differences between specific groups was accomplished with the Student Neuman Keuls test. The level of statistical significance was taken as P<0.05.

Results

We have previously reported that treatment with PPARγ agonists increased NO release from human umbilical vein or aortic endothelial cells without increasing PPARγ or eNOS expression. To verify that 15d-PGJ2, ciglitazone, and rosiglitazone increased endothelial NO release through PPARγ-dependent signaling, HUVECs were transfected with PPARγ-specific siRNA and then assayed for PPARγ expression and NO release in the presence of these PPARγ ligands. Western blotting verified that the expression of PPARγ was specifically and significantly reduced by the cognate PPARγ siRNA duplex compared with mock transfections or transfections with a control fluorescein conjugate-scrambled siRNA whose sequence is unrelated to PPARγ (Figure 1A and 1B). Importantly, compared with mock transfections, PPARγ siRNA significantly reduced 15d-PGJ2-, ciglitazone-, and rosiglitazone-dependent NO release (Figure 1C).

By transfecting HUVECs with a luciferase reporter gene containing 3 PPREs as an index of PPARγ transactivation, Figure 2A demonstrates that the PPARγ antagonist GW9662 (2 μmol/L) fully prevented PPARγ transactivation caused by 15d-PGJ2, ciglitazone, or rosiglitazone. Furthermore, treating HUVECs with 2 μmol/L GW9662 significantly reduced 15d-PGJ2-, ciglitazone-, and rosiglitazone-dependent NO release (Figure 2B). In contrast, GW9662 failed to inhibit NO release stimulated by the calcium ionophore A23187, indicating that GW9662 specifically inhibits PPARγ ligand-stimulated NO release. The Toxilight bioassay kit (Cambrex), which measures the release of adenylate kinase from damaged cells, was used to demonstrate that the inhibitory effects of neither PPARγ siRNA nor GW9662 were attributable to cell injury (data not shown). Because PPARγ ligands increased EC NO release without increasing eNOS expression, post-translational mechanisms of eNOS regulation were examined. As illustrated in Figure 3A and 3B, treatment with 15d-PGJ2, but not ciglitazone or rosiglitazone, significantly increased the overall cellular content of hsp90 when normalized to actin expression and decreased eNOS expression (Figure 3C), as previously re-
15d-PGJ2- and rosiglitazone-stimulated eNOS activity was available online at http://atvb.ahajournals.org. Similarly, effect on ciglitazone-stimulated NO production (Figure 1), rosiglitazone treatments (Figure 5A), whereas GA had no effect with vehicle alone, 15d-PGJ2 and rosiglitazone, but not ciglitazone, significantly increased the phosphorylation of eNOS, eNOS phosphorylation after treatment with PPARγ antagonist GW9662 attenuated these increases in hsp90-eNOS interactions (Figure 4). The importance of hsp90-eNOS interactions in 15d-PGJ2- and rosiglitazone-stimulated HUVEC NO release is further supported by the demonstration that the hsp90 inhibitor GA completely abolished the increase in NO production seen after 15d-PGJ2 and rosiglitazone treatments (Figure 5A), whereas GA had no effect on ciglitazone-stimulated NO production (Figure I, available online at http://atvb.ahajournals.org). Similarly, 15d-PGJ2- and rosiglitazone-stimulated eNOS activity was also inhibited by GA treatment (Figure 5B).

Because hsp90-eNOS interaction can recruit kinases that phosphorylate eNOS, eNOS phosphorylation after treatment with PPARγ ligands was examined. Compared with treatment with vehicle alone, 15d-PGJ2 and rosiglitazone, but not ciglitazone, significantly increased the phosphorylation of eNOS at ser1177, an effect attenuated by the PPARγ antagonist GW9662. None of the PPARγ ligands examined caused significant alterations in the phosphorylation of eNOS at thr495 (data not shown).

**Discussion**

The molecular action of the thiazolidinedione (TZD) class of insulin-sensitizing medications, currently used with patients with type 2 diabetes, involves direct activation of the PPARγ receptor. PPARs are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. PPARγ is expressed at low levels in many tissues, where its activation produces diverse tissue-specific effects. In the vessel wall, PPARγ is expressed in smooth muscle and endothelial cells. Current evidence suggests that activation of PPARγ exerts beneficial effects on the vasculature. For example, studies in non-diabetic mouse models of atherosclerosis demonstrated that TZDs reduced lesion formation. TZD therapy has also been associated with improved endothelial function, reduced carotid intimal thickening, and neointimal formation after coronary stent placement. The vascular protective effect of PPARγ ligands in humans was recently extended to non-diabetic subjects with documented coronary disease wherein rosiglitazone reduced common carotid arterial intima-media thickness progression. Taken together, these reports indicate that PPARγ activation modulates the biology of the vascular wall through mechanisms that are incompletely defined.
Several studies have demonstrated that PPARγ ligands exert direct effects on vascular wall cells in vitro. Troglitazone increased endothelial NO release through both PPARγ-dependent and -independent signaling pathways involving differential eNOS phosphorylation and alterations in VEGF and VEGF receptor expression, confirming previous evidence that 15d-PGJ2 and ciglitazone increased EC NO release. The current study extends these reports by demonstrating that rosiglitazone also increased EC NO production. Neither 15d-PGJ2, ciglitazone, nor rosiglitazone, however, altered the expression of VEGF or its receptor in the current study (Figure II, available online at http://atvb.ahajournals.org). In addition, 10 μmol/L 15d-PGJ2 was previously reported to lower glutathione levels and cell viability in cultured HUVECs, effects not observed in the current model (data not shown). These apparent discrepancies in endothelial response to various PPARγ ligands may relate to differences between studies in culture conditions that modulate PPARγ effects, such as culture media serum concentrations. The current study was therefore designed to examine several PPARγ ligands under identical culture conditions to determine whether individual PPARγ ligands exert their effects on endothelial NO production through common pathways.

The most important findings in the current study are that under identical culture conditions, the same concentration of 3 different PPARγ ligands activated EC PPARγ and increased EC NO release to a comparable degree (Figures 1 and 2). Furthermore, the ability of each PPARγ ligand to stimulate EC NO release was PPARγ-dependent because it was inhibited by treatment with either siRNA or the PPARγ antagonist GW9662. These findings demonstrate that the current model represents an appropriate system to examine potential ligand-specific mechanisms of PPARγ-induced alterations in endothelial NO production.

The current study also provides novel evidence that selected PPARγ ligands modulate EC NO release through hsp90-related mechanisms. Hsp90 increases eNOS activity and NO release in a dose-dependent manner. Of the PPARγ ligands studied, only 15d-PGJ2 increased overall HUVEC hsp90 expression (Figure 3), an effect that was not blocked by GW9662, suggesting a PPARγ-independent mechanism. Although the hsp90 gene has not been reported to contain a PPRE, cyclopentenone prostaglandins similar to 15d-PGJ2 stimulated heat shock protein expression through activation of heat shock transcription factor, providing a plausible explanation for the ability 15d-PGJ2 but not
ciglitazone or rosiglitazone to increase hsp90 expression. Treatment with 15d-PGJ$_2$ or rosiglitazone, but not ciglitazone, however, increased hsp90 binding to eNOS (Figure 4) suggesting a potential role for this protein-protein interaction in enhanced NO production. The role of hsp90 was further supported by studies using the hsp90 inhibitor GA. As previously reported for VEGF- and bradykinin-induced NO release,52,53 GA attenuated 15d-PGJ$_2$- or rosiglitazone-mediated NO production (data not shown). Additional mechanistic studies are currently ongoing in our laboratory to identify upstream signaling pathways activated by PPAR$\gamma$ ligands that promote eNOS-hsp90 interaction and eNOS phosphorylation.

Although 15d-PGJ$_2$, ciglitazone, and rosiglitazone each stimulated EC NO release and caused comparable degrees of PPAR$\gamma$ activation, ciglitazone, unlike the other PPAR$\gamma$ ligands, failed to stimulate hsp90 expression, eNOS-hsp90 association, or eNOS ser$^{1177}$ phosphorylation. The mechanisms of ciglitazone-stimulated NO release continue to be defined, but are likely attributable in part to ciglitazone-induced reductions in the expression of endothelial NADPH oxidase components and superoxide generation, as well as increased superoxide dismutase expression.56 By decreasing superoxide levels, PPAR$\gamma$ ligands can reduce superoxide-mediated interactions with NO to increase NO release and enhance NO bioavailability. These studies demonstrate that

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activity.23,47 Although the dephosphorylation of eNOS at thr$^{495}$ can also increase enzyme activity, neither 15d-PGJ$_2$, ciglitazone, nor rosiglitazone altered eNOS thr$^{495}$ phosphorylation (data not shown). The precise mechanism of 15d-PGJ$_2$- and rosiglitazone-induced eNOS phosphorylation remains to be defined but could involve enhanced kinase activity, reduced phosphatase activity, or both. Several kinase pathways are known to phosphorylate eNOS at ser$^{1177}$ including protein kinase B, extracellular signal regulated kinase, AMP-activated protein kinases, calmodulin-dependent kinase II, protein kinase G, and protein kinase A.44,55 To date, we have determined that wortmannin, a specific inhibitor of PI-3 kinase/protein kinase B signaling, does not attenuate PPAR$\gamma$ ligand-mediated NO production (data not shown). Additional mechanistic studies are currently ongoing in our laboratory to identify upstream signaling pathways activated by PPAR$\gamma$ ligands that promote eNOS-hsp90 interaction and eNOS phosphorylation.

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Figure 5. GA inhibits 15d-PGJ$_2$- and rosiglitazone-stimulated NO release and NOS activity. HUVECs were treated with vehicle (Control), 10 $\mu$mol/L 15d-PGJ$_2$, or 10 $\mu$mol/L rosiglitazone (Rosi) for 24 hours. Where indicated, HUVECs were also treated with 1 $\mu$mol/L GA for 24 hours. In A, HUVEC media were collected, and NO release was determined as described in Methods. Each bar represents the mean NO release $\pm$ SEM as % Control (n=4). $^*P<0.05$ vs Control, $^{**}P<0.05$ vs similarly treated group ($-$) GA. In B, NOS activity was measured in HUVEC lysates as described in Methods. Each bar represents the mean amount of L-citrulline formed in counts per minute (cpm) per mg protein. $^*P<0.05$ vs Control, $^{**}P<0.05$ vs similarly treated group ($-$) GA.

Figure 6. Specific PPAR$\gamma$ ligands enhance eNOS ser$^{1177}$ phosphorylation. HUVECs were treated with vehicle (Control), 10 $\mu$mol/L 15d-PGJ$_2$, 10 $\mu$mol/L ciglitazone (Cig), or 10 $\mu$mol/L rosiglitazone (Rosi) for 24 hours. Where indicated, HUVECs were also treated with 1 $\mu$mol/L GA for 24 hours. In A, HUVEC media were collected, and NO release was determined as described in Methods. Each bar represents the mean NO release $\pm$ SEM as % Control (n=5). $^*P<0.05$ vs Control, $^{**}P<0.05$ vs similarly treated group ($-$) GA.

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PPARγ ligands regulate several pathways involved in endothelial NO production and bioavailability.

Although PPARγ-independent effects of specific ligands could potentially account for differing biological effects of PPARγ ligands, the current study demonstrates that PPARγ-ligand–stimulated NO release from HUVECs was fully inhibited by either PPARγ siRNA (Figure 1) or GW9662 (Figure 2). Therefore, we speculate that these ligand-specific effects are attributable to characteristics of the PPARγ receptor itself. PPARγ receptor activation involves not only ligand binding but also the association or dissociation of coactivator and corepressor complexes. Activation of PPARγ involves ligand-specific conformational changes in the receptor that recruit distinct populations of coactivator and corepressor proteins to induce ligand-specific patterns of gene expression. These considerations suggest that whereas PPARγ activation in vascular endothelial cells may control an overall pattern of gene expression that promotes NO production, the biological effect of individual ligands may be mediated through discreet pathways.

In summary, our findings demonstrate that activation of PPARγ in vascular endothelial cells provides a novel mechanism for stimulating endothelial NO release. 15d-PGJ2, ciglitazone, and rosiglitazone increased NO production by distinct signaling pathways that are PPARγ-dependent. These results provide further evidence that PPARγ ligands have the potential to directly modify vascular endothelial function and to modulate the production of NO, a critical mediator in maintenance of normal vascular physiology. These findings further refine our understanding of novel targets for pharmacological intervention in vascular disease and contribute to the definition of the molecular targets for TZDs and related PPARγ ligands in the vasculature.

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Peroxisome Proliferator-Activated Receptor γ Ligands Stimulate Endothelial Nitric Oxide Production Through Distinct Peroxisome Proliferator-Activated Receptor γ–Dependent Mechanisms


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Figure I. *Geldanamycin (GA) does not inhibit ciglitazone-stimulated NO release.*

HUVEC were treated with vehicle (Control) or 10 μM ciglitazone (Cig) for 24 hours. Where indicated, HUVEC were also treated with 1 μM GA for 24 h. HUVEC media were collected, and NO release was determined as described in Methods. Each bar represents the mean NO release ± SEM as % Control (n=4). *p<0.05 vs. Control.

Figure II. *PPARγ ligands have no effect on VEGF or VEGF receptor expression.* HUVEC were treated with vehicle (Control), 10 μM 15d-PGJ$_2$, 10 μM ciglitazone (Cig), or 10 μM rosiglitazone (Rosi) for 24 hours. Cell lysates were then prepared and subjected to SDS-PAGE, followed by immunoblotting for VEGF, the VEGF receptor, flt-1, (VEGF-R), or actin. Representative immunoblots are depicted (n=3).

Figure III. *PPARγ ligands reduce caveolin-eNOS association.* HUVEC were treated with vehicle (Control), 15d-PGJ$_2$, ciglitizone (Cig), or rosiglitazone (Rosi) for 24 hours. Cell lysates were prepared and immunoprecipitated with monoclonal antibodies to caveolin I. Immunoprecipitates were then subjected to SDS-PAGE and immunoblotting for eNOS. In A, a representative immunoblot is shown. In B, cell lysates were subjected to SDS-PAGE, Western blotting, and probed for eNOS and caveolin 1. In C, each bar represents the mean ± SEM abundance of eNOS immunoprecipitated with caveolin 1 antibodies as % Control (n=4). *p<0.05 vs Control.
Figure I
Figure II
A

IP: Caveolin 1
IB: eNOS

Control  15d-PGJ2  Cig  Rosi

B

IB: eNOS

IB: Caveolin 1

Control  15d-PGJ2  Cig  Rosi

C

![Bar chart](image)

Figure III