The PPARγ ligand rosiglitazone attenuates hypoxia-induced endothelin signaling in vitro and in vivo

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Kang BY, Kleinhenz JM, Murphy TC, Hart CM. The PPARγ ligand rosiglitazone attenuates hypoxia-induced endothelin signaling in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol 301: L881–L891, 2011. First published September 16, 2011; doi:10.1152/ajplung.00195.2011.— Peroxisome proliferator-activated receptor (PPARγ) activation attenuates hypoxia-induced pulmonary hypertension (PH) in mice. The current study examined the hypothesis that PPARγ attenuates hypoxia-induced endothelin-1 (ET-1) signaling to mediate these therapeutic effects. To test this hypothesis, human pulmonary artery endothelial cells (HPAECs) were exposed to normoxia or hypoxia (1% O2) for 72 h and treated with or without the PPARγ ligand rosiglitazone (RSG, 10 μM) during the final 24 h of exposure. HPAEC proliferation was measured with MTT assays or cell counting, and mRNA and protein levels of ET-1 signaling components were determined. To explore the role of hypoxia-activated transcription factors, selected HPAECs were treated with inhibitors of hypoxia-inducible factor (HIF)-1α (chemotxin) or nuclear factor (NF)-κB (caffeic acid phenethyl ester, CAPE). In parallel studies, male C57BL/6 mice were exposed to normoxia (21% O2) or hypoxia (10% O2) for 3 wk with or without gavage with RSG (10 mg·kg⁻¹·day⁻¹) for the final 10 days of exposure. Hypoxia increased ET-1, endothelin-converting enzyme-1, and endothelin receptor A and B levels in mouse lung and in HPAECs and increased HPAEC proliferation. Treatment with RSG attenuated hypoxia-induced activation of HIF-1α, NF-κB activation, and ET-1 signaling pathway components. Similarly, treatment with chemotxin or CAPE prevented hypoxia-induced increases in HPAEC ET-1 mRNA and protein levels. These findings indicate that PPARγ activation attenuates a program of hypoxia-induced ET-1 signaling by inhibiting activation of hypoxia-responsive transcription factors. Targeting PPARγ represents a novel therapeutic strategy to inhibit enhanced ET-1 signaling in PH pathogenesis.”

Address for reprint requests and other correspondence: C. M. Hart, Division of Pulmonary and Critical Care Medicine, Atlanta VAMC (151), 1670 Clairmont Rd., Decatur, GA 30033 (e-mail: Michael.hart3@va.gov).
defined. Once activated, PPARγ forms a heterodimer with the retinoid X receptor, RXR, and binds to PPAR response elements in the promoter region of responsive genes to increase their expression. PPARγ activation can also reduce expression of selected genes through transrepression mechanisms (43). For example, activation of PPARγ inhibited hypoxia-inducible factor-1α (HIF-1α) (29) and nuclear factor-κ light-chain-enhancer of activated B cells (NF-κB) (29, 34) through PPARγ-induced transrepression. In this study, we postulate that these transrepression effects of PPARγ are critical for modulation of ET-1 signaling in PH.

The current study examines the hypothesis that PPARγ regulates hypoxia-induced PH by modulating ET-1 signaling. To explore this hypothesis, mice or human pulmonary artery endothelial cells (HPAECs) were exposed to hypoxia and then treated with or without the PPARγ ligand rosiglitazone. The results demonstrate that hypoxia activates transcriptional mechanisms that upregulate multiple components in the ET-1 signaling pathway. Rosiglitazone attenuates hypoxia-induced PH (39) and concomitantly prevents enhanced ET-1 signaling. Collectively, these findings suggest that targeting PPARγ represents a novel strategy to successfully inhibit multiple steps of ET-1 signaling that contribute to PH pathogenesis.

MATERIALS AND METHODS

Reagents. Human ET-1 and mouse ET-1 enzyme-linked immunoassorberent assay (ELISA) kits were purchased from R & D Systems (Minneapolis, MN). HPAECs were obtained from Invitrogen (Carlsbad, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from ATCC (Manassas, VA). ET-1, ECE-1, ETAR, ETBR, HIF-1α, NF-κB, and CDK4 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The following reagents were supplied by Sigma-Aldrich (St. Louis, MO): Trypan blue, FBS, dimethyl sulfoxide (DMSO), methyl cellulose, HIF-1α inhibitor chetomin, and NF-κB inhibitor caffeic acid phenethyl ester (CAPE). The PPARγ ligand rosiglitazone was obtained from Cayman Chemical (Ann Arbor, MI).

HPAECs. Pulmonary artery ECs were isolated from the lungs of control or idiopathic pulmonary arterial hypertension (IPAH) patients as described (35), and lysates of these cells were generously provided by Dr. Suzy Comhair (Cleveland Clinic, Cleveland, OH).

Exposure of C57BL/6 mice to hypoxia. Male C57BL/6 mice aged 8–12 wk old were purchased from the Jackson Laboratory (Bar Harbor, ME) and exposed to 10% O2 (hypoxia) or room air (control) for 3 wk as reported (39). All animals were given unrestricted access to water and standard mouse chow. Mice were housed socially in groups of five. During the last 10 days of the experiment, each animal was gavaged daily with rosiglitazone (10 mg·kg⁻¹·day⁻¹) in 100 μl 0.5% methyl cellulose) or with an equivalent volume of vehicle alone as reported (39). All animal studies were approved by the Institutional Animal Care and Use Committee of the Atlanta Veterans Affairs Medical Center.

Hypoxia-exposed HPAEC and cell proliferation assay. HPAECs (passages 2–7) were exposed to hypoxia in a Biospherix (Lacona, NY) exposure chamber. HPAECs were placed in a standard incubator or hypoxia chamber that maintains temperature (37°C), O2 concentration (1%), and CO2 levels (5%) for 72 h. To examine the effect of rosiglitazone on hypoxia-induced cell proliferation, HPAECs were treated during the last 24 h of normoxia or hypoxia exposure by adding rosiglitazone (0–20 μM) or an equivalent volume of vehicle directly to the culture media, and cell proliferation was measured using MTT assays. In selected studies, HPAECs were pretreated with DMSO or the HIF-1α inhibitor chetomin (25 nM) or the NF-κB inhibitor CAPE (20 μM) for 3 h and then for the entire 72-h exposure to normoxia or hypoxia. All manipulations of cells exposed to hypoxia were performed in a glove box that maintains the hypoxic environment to avoid effects of reoxygenation during sample processing. The impact of hypoxia with or without rosiglitazone on HPAEC function was determined by assessing HPAEC proliferation using cell counting and MTT assays. HPAECs were counted on a hemocytometer. Cell viability was determined by Trypan blue exclusion assay. HPAECs were stained with 0.08% Trypan blue at room temperature for 5 min. The number of live cells was calculated as: total number of cells − number of stained cells. The data were then normalized to the cells in the control preparation. In selected experiments, proliferation was determined using a colorimetric method based on metabolic reduction of the soluble yellow tetrazolium dye MTT to its insoluble purple formazan as recently reported (17, 33).

ET-1 ELISA. C57BL/6 mouse lung tissue was homogenized in tissue protein extraction reagent buffer (Thermo Scientific) and analyzed with a quantitative sandwich enzyme immunoassay for ET-1 (R&D Systems). Media from HPAECs exposed to normoxia or hypoxia with or without rosiglitazone were collected, and human ET-1 levels in HPAEC supernatants were measured using a high-sensitivity chemiluminescence ELISA kit (R&D Systems). ET-1 levels were normalized to total protein content of samples measured with a bicinchoninic acid assay (Pierce, Rockford, IL).

Immunohistochemistry. Lungs from normoxia- or hypoxia-exposed C57BL/6 mice following treatment with vehicle or rosiglitazone were subjected to histological examination as described previously (39). Briefly, the lungs were isolated, pressure perfused with calcium-free EDTA-containing buffer followed by optimum-cutting temperature compound (Fisher), frozen, sectioned, fixed, and stained with primary antibodies to ET-1. Lung sections (8 μm) were incubated with biotinylated donkey antirabbit secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) followed by horseradish peroxidase-streptavidin (Vectastain kit; Vector Laboratories, Burlingame, CA). Color was developed with 3,3′-diaminobenzidine tetrahydrochloride substrate (Vector Laboratories). Nonimmune rabbit IgG isotype control primary antibody was used to control for nonspecific antibody binding. Multiple high-power photomicrographs were obtained using a Leica DM4000B microscope (Wetzlar, Germany).

Quantitative real-time PCR analysis. RNA was extracted from lung tissue or HPAEC using an RNeasy kit (Quiaigen, Valencia, CA) according to the manufacturer’s protocol. The concentration of mRNA was quantified using an ND-1000 Spectrophotometer (NanoDrop Technology, Wilmington, DE). One microgram of RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR (qRT-PCR) was performed on samples using a Bio-Rad iCycler and analyzed by its software. The qRT-PCR specific primers were designed using ProbeFinder web-based software (Roche, Indianapolis, IN). ET-1, ECE-1, ETAR, ETBR and CDK4 mRNA levels were normalized to 18S rRNA reference gene (Table 1). The comparative threshold cycles (Ct) values were normalized to the 18S rRNA reference gene using the 2ΔΔCt method (32).

Western blot analysis. After treatment with normoxia or hypoxia with or without rosiglitazone, mouse lung or HPAEC protein lysates were subjected to Western blot analysis as reported (39). Primary antibodies included ET-1, ECE-1, ETAR, ETBR, and CDK4 antibodies. Proteins were visualized using a peroxidase-coupled anti-goat, anti-rabbit, or anti-mouse IgG in the presence of LumiGlo reagent (Thermo Scientific). Bands were identified by chemiluminescence, quantified by laser densitometry (Bio-Rad), and normalized to CDK4 levels within the same lane.

Analysis of transcription factor activation. Electrophoretic mobility shift assay (EMSA) or p65 nuclear translocation were employed to examine hypoxia and rosiglitazone-induced alterations in the nuclear binding of NF-κB or HIF-1α. Nuclear extracts from HPAECs were prepared using a commercial kit (ActiveMotif, Carlsbad, CA). Double-stranded consensus oligonucleotides or mutated probes for


**Table 1. Primer information for real-time quantitative PCR**

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<th>mRNA Product Length, bp</th>
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HIF-1α (50, 51) and NF-κB (30) were radiolabeled with [32P-γ]ATP using T4 polynucleotide kinase enzyme. Nuclear protein (5 μg) was incubated with radiolabeled probes, and then DNA-protein complexes were separated on 6% native polyacrylamide. Gels were fixed and exposed to X-ray film. DNA-protein bands were quantified by densitometric scanning using a GS-800 laser densitometer (Bio-Rad). To analyze NF-κB p65 activation and nuclear translocation, HPAEC were seeded in eight-well chamber slides (Lab-Tek, Vernon Hills, IL) and grown to confluency. After treatment with control or hypoxic conditions with or without rosiglitazone, HPAEC were washed, fixed with 4% (vol/vol) paraformaldehyde, and then permeabilized with 0.1% (vol/vol) Triton X-100. After being blocked with 0.5% BSA/PBS, primary anti-p65 rabbit polyclonal antibody (1:100 dilution; Santa Cruz Biotechnology) was added. HPAEC were then incubated overnight at 4°C followed by incubation with DyLight 488 conjugated secondary goat anti-rabbit antibody (1:500 dilution; Thermo Scientific, Waltham, MA). The chamber slides were then washed and mounted with Vectashield mounting medium with DAPI reagent (Vector Laboratories). Confocal images were captured on an Olympus laser scanning microscope equipped with the appropriate filter sets.

**Statistical analysis.** In all experiments, data were analyzed by one-way ANOVA followed by post hoc analysis with Student-Newman-Keuls test to examine differences between specific groups using the software GraphPad Prism, version 4.0 (La Jolla, CA). The level of statistical significance was taken as \( P < 0.05 \).

**RESULTS**

Rosiglitazone attenuates hypoxia-induced ET-1 levels in *C57BL/6* mouse lung in vivo and in HPAEC in vitro. Recent studies showed that ET-1 mRNA levels were increased in pulmonary microvascular ECs isolated from patients with IPAH compared with control groups (8). To our knowledge, the present study is the first to present that pulmonary artery ECs isolated from patients with IPAH demonstrated increased ET-1 mRNA levels compared with control groups (Fig. 1A). Because rosiglitazone administered by gavage attenuated hypoxia-induced PH, right ventricular hypertrophy, and pulmonary vascular remodeling in mice (39), we used this model in the current studies, as shown in Fig. 1, to examine ET-1 signaling in the hypoxic mouse model. Hypoxia increased ET-1 levels in *C57BL/6* mouse lung nearly sevenfold, and treatment with the PPARγ ligand rosiglitazone reduced hypoxia-mediated increases in lung ET-1 (Fig. 1B). Similarly hypoxia (1% \( O_2 \times 72 \) h)-induced ET-1 release from HPAECs was attenuated by rosiglitazone treatment (Fig. 1C). As shown in Fig. 1D, lungs from hypoxia-exposed *C57BL/6* mice demonstrated increased intensity of staining with the ET-1 antibody, and staining intensity was attenuated in lungs from hypoxia-exposed mice treated with rosiglitazone. Immunostaining (brown coloration) was observed in small arterioles and alveolar structures in each treatment group. These findings provide additional evidence that hypoxia significantly increases ET-1 expression in the pulmonary vasculature. There was no staining with nonimmune IgG isotype control primary antibody (data not shown). To examine the effects of rosiglitazone on hypoxia-induced cell proliferation, HPAECs were treated with rosiglitazone (0–20 μM) and examined using MTT assays. Figure 1E shows that a low concentration of rosiglitazone (1 and 5 μM) had no effect on hypoxia-induced HPAEC proliferation, whereas 10 μM rosiglitazone treatment significantly decreased hypoxia-induced HPAEC proliferation compared with control conditions. Therefore, we used 10 μM concentrations of rosiglitazone in all subsequent experiments.
were attenuated by rosiglitazone (Fig. 3). Although rosiglitazone attenuated hypoxia-induced ETAR mRNA and protein levels, at baseline, ETAR mRNA levels were only 1/30th the level of ETBR, indicating that ETBR is the major ET receptor expressed in HPAECs (data not shown).

Rosiglitazone inhibits hypoxia-induced HPAEC cell proliferation. To investigate whether PPARγ activation could inhibit hypoxia-induced proliferation of HPAECs, HPAECs were placed in normoxic or hypoxic conditions for 72 h. During the last 24 h, dishes were exposed to vehicle (DMSO) or rosiglitazone (10 μM). As shown in Fig. 3E, hypoxia-induced increases in endothelin signaling pathway components were associated with increased HPAEC cell proliferation. In contrast, these alterations were attenuated by the PPARγ ligand rosiglitazone.

Rosiglitazone decreases nuclear binding of transcription factors involved in ET-1 expression. The comparable regulation of multiple components within the ET-1 signaling pathway by PPARγ following hypoxic activation suggested that PPARγ targets common proximal upstream regulators of ET-1 pathway components and led us to consider PPARγ-mediated transcriptional regulation. The ET-1, ECE, ETAR, and ETBR promoters have binding sites for transcription factors known to be activated by hypoxia such as HIF-1α and NF-κB (26, 38, 42, 48, 52). Because previous reports have demonstrated that chronic hypoxia upregulates HIF-1α through transcriptional mechanisms (48) (in addition to the originally described hypoxia-mediated prolyl hydroxylase inhibition and HIF-1α protein stabilization) (3), EMSAs were performed to evaluate the effect of hypoxia and rosiglitazone treatment on HIF-1α expression.
NF-κB nuclear binding in HPAECs. As shown in Fig. 4, hypoxia increased nuclear binding of HIF-1α (Fig. 4, A and B) and NF-κB (Fig. 4, C and D) compared with control conditions, and rosiglitazone decreased HIF-1α and NF-κB nuclear binding. Consistent with these findings, hypoxia-induced nuclear translocation of NF-κB p65 was attenuated by rosiglitazone (Fig. 4E).

Roglitazone attenuates HIF-1α and NF-κB mRNA expression. We also investigated whether exposure to hypoxia would increase HIF-1α and NF-κB mRNA levels. As shown in Fig. 5, rosiglitazone attenuated hypoxia-induced increases in HIF-1α (Fig. 5A) and NF-κB (Fig. 5B) mRNA levels in mouse lung. Similarly, hypoxia-induced increases in HPAEC HIF-1α (Fig. 5C) and NF-κB (Fig. 5D) mRNA levels were also attenuated by rosiglitazone.

To further establish the importance of hypoxic activation of HIF-1α and NF-κB in enhanced ET-1 signaling, the ability of rosiglitazone to inhibit hypoxic induction of ET-1 was compared with the HIF-1α inhibitor chetomin and with the NF-κB inhibitor CAPE (Fig. 6). Consistent with the findings in Fig. 1C, rosiglitazone attenuated hypoxic increases in HPAEC ET-1 mRNA and protein levels. Treatment with chetomin (25 nM) or CAPE (20 μM) similarly attenuated hypoxia-induced ET-1 mRNA (Fig. 6A) and protein (Fig. 6B) levels. These results confirm that modulation of hypoxia-activated transcription factors regulates ET-1 signaling in human ECs and that targeting these transcription factors with PPARγ ligands may represent a novel and selective approach to attenuate ET-1-induced alterations in the pulmonary circulation.
DISCUSSION

ET-1 plays a critical role in endothelial dysfunction, an early event in the pathogenesis of PH. Pharmacological blockade of ET receptors constitutes one of the currently available therapies for PH; however, therapeutic trials with ET receptor antagonists in patients with PH have met with mixed results (11). Furthermore, treatment with ET receptor antagonists is not recommended in subsets of PH patients such as those with PH related to chronic obstructive pulmonary disease (11, 41). While a recent meta-analysis demonstrates that ET receptor blockers provide benefits in some PH patients (11, 13, 44), PH morbidity and mortality remain high. Collectively, these findings support an important pathophysiological role for ET-1 signaling in PH pathogenesis and suggest that new strategies beyond ET receptor antagonism that regulate ET-1 signaling may contribute to novel approaches to PH therapy.

Recent studies indicate that stimulating PPARγ with TZD ligands, such as rosiglitazone or pioglitazone, attenuates PH in several experimental models (6, 19, 27, 36, 39). Because previous evidence suggests that PPARγ can regulate ET-1 signaling in the systemic circulation (21, 22, 40), the current study examines PPARγ and its ability to regulate ET-1 signaling in mouse lung and HPAECs. Our studies provide novel evidence that hypoxia activates transcriptional mechanisms involved in upregulating multiple components of the ET-1 signaling pathway and that treatment with rosiglitazone mediates coordinated reductions in hypoxia-induced ET-1 signaling pathway components. These findings suggest that targeting PPARγ may represent a novel strategy to successfully inhibit
Fig. 4. RSG attenuates HYP-induced HYP-inducible factor (HIF)-1α and nuclear factor (NF)-κB nuclear binding in HPAEC. HPAEC were exposed to NOR (21% O₂) or HYP (1% O₂) for 72 h. During the final 24 h of exposure, selected cells were treated with RSG (RSG, 10 μM). Nuclear proteins were extracted from HPAECs and then incubated with radiolabeled HIF-1α (A) and NF-κB (C) oligonucleotides and subjected to electrophoretic mobility shift assay analysis. DNA-protein complexes were separated on a native polyacrylamide gel, and densitometric analysis of bands was performed (B and D). Arrows, probe shift due to HIF-1α and NF-κB protein binding and unbound free probe; P, probe alone; C, control 50× unlabeled probe; Cm, control mutated 50× probe. Each bar represents the mean ± SE. *P < 0.05 vs. NOR (+) and vs. HYP (+); n = 3. In E, the intracellular localization of the NF-κB subunit p65 was investigated by immunofluorescence by using specific p65 antibody. Images are representative of 3 experiments. Scale bar = 10 μm. There was no staining with nonimmune IgG isotype control primary antibody (data not shown).
multiple steps in ET-1 signaling that contribute to PH pathogenesis.

Hypoxia stimulates PH pathogenesis in human (15) and animal subjects (6, 27, 39). Chronic hypoxia-induced PH is a frequent clinical problem that contributes to pulmonary vasoconstriction, pulmonary vascular remodeling, endothelial dysfunction, right ventricular failure, and death. We recently reported that C57BL/6 mice exposed to chronic hypoxia developed increases in right ventricular systolic pressure, right ventricular hypertrophy, and pulmonary vascular remodeling that were attenuated by treatment with rosiglitazone (39). In the current study using this same model, hypoxia (10% O2 for 3 wk) increased ET-1 levels in C57BL/6 mouse lung, and treatment with rosiglitazone attenuated hypoxia-induced lung

Fig. 5. RSG attenuates HYP-induced increases in HIF-1α and NF-κB mRNA levels in mouse lung and in HPAEC. C57BL/6 mice (A and B) or HPAEC (C and D) were exposed to NOR or HYP ± RSG as described in Fig. 1. Each bar represents the mean ± SE HIF-1α or NF-κB mRNA level relative to ribosomal 9S and expressed relative to control. *P < 0.05 vs. NOR (*) and 9s. HYP (+); n = 5–9.

Fig. 6. Modulation of HYP-induced ET-1 mRNA and protein levels by inhibitors of HIF-1α or NF-κB in HPAECs. HPAECs were pretreated with DMSO, with the HIF-1α inhibitor chetomin (CTM, 25 nM), or with the NF-κB inhibitor caffeic acid phenethyl ester (CAPE, 20 μM) for 3 h. HPAECs were then exposed to NOR (21% O2) or HYP (1% O2) for 72 h in the presence of absence of CTM or CAPE. During the final 24 h of exposure, selected HPAEC were treated with RSG (10 μM). In A, each bar represents the mean ± SE ET-1 mRNA level relative to 9S and expressed relative to control. *P < 0.05 vs. NOR (*) and vs. HYP (†); n = 3. In B, representative Western blots for ET-1 and CDK4 are presented. In C, average ET-1 densitometric values ± SE are presented. *P < 0.05 vs. NOR (*) and vs. HYP (+); n = 3.
ET-1 levels. These observations are consistent with recent reports demonstrating that treatment with rosiglitazone reduced ET-1 levels in Sprague-Dawley rats exposed to 12% O2 for 4 wk (27). To our knowledge, the current studies provide the first demonstration that PPARγ ligands also attenuate hypoxic increases in other components of the ET-1 signaling pathway, including ECE, ETAR, and ETBR. Our in vitro studies further indicate that hypoxia-induced ET-1 release from HPAECs was attenuated by rosiglitazone treatment. These findings provide novel evidence that PPARγ can coordinately regulate the expression of a program of ET-1 signaling components. Because pharmacological ligands for the PPARγ receptor are currently employed in the treatment of type 2 diabetes in the United States, these findings suggest that PPARγ ligands could be examined in clinical trials of PH therapy. The relevance of these observations is supported by the current finding that ET-1 mRNA levels are increased in HPAECs derived from patients with PH and by previous reports of HIF activation (12) and impaired NO production (53, 54) in IPAH ECs.

PPARγ not only regulated hypoxia-induced ET-1 levels, but also other ET-1 signaling pathway components, such as ECE-1, ETAR, and ETBR mRNA and protein in vitro and in vivo. Furthermore, hypoxia-induced increases in ET-1 signaling components were associated with increased HPAEC proliferation and were attenuated by treatment with rosiglitazone. Significant effects of rosiglitazone treatment on components of the ET-1 signaling pathway were observed only in hypoxia-exposed mice or cells and were not observed following exposure to control conditions. Furthermore, these in vitro and in vivo effects on the ET-1 signaling pathway were accomplished by administering rosiglitazone only during the latter third of the hypoxia exposure period. These findings indicate that PPARγ activation with rosiglitazone can attenuate and reverse a diverse spectrum of hypoxia-induced increases in ET-1 signaling pathway components even when introduced late in the course of hypoxia exposure.

The molecular mechanisms whereby PPARγ inhibits gene expression in response to hypoxia are not fully defined. PPARγ activation can inhibit gene expression through transrepression mechanisms (43) that involve inhibitory cross talk between PPARγ and other proinflammatory transcription factors, such as HIF-1α (29) or NF-κB (29, 34). Our studies provide several lines of evidence supporting involvement of PPARγ-mediated transrepression in the effects of rosiglitazone on hypoxic stimulation of ET-1 signaling. First, previous reports demonstrated that hypoxia stimulates HIF-1α binding to the ET-1 promoter (29, 55). Our findings not only confirm that hypoxia stimulates HIF-1α and NF-κB activation but demonstrate that rosiglitazone inhibits hypoxia-induced activation of these transcription factors. These results suggest that PPARγ represses HIF-1α or NF-κB transcriptional activity. Second, in silico analysis of the promoter elements of the ET-1 signaling components examined demonstrated that, while the ETBR promoter contains putative PPAR response elements, the ET-1 and ETAR promoters did not. Absence of direct regulation of ET-1, ECE, ETAR, or ETBR expression by rosiglitazone alone further suggests that the observed effects of rosiglitazone are unlikely mediated through activation and binding of PPARγ to ET-1 signaling component promoters. In addition, the ET-1, ECE, ETAR, and ETBR promoters contain binding sites for transcription factors known to be activated by hypoxia (26, 38, 42, 48, 52) such as HIF-1α (45, 55) or NF-κB (reviewed in Ref. 46) (49), indicating that indirect, PPARγ-mediated regulation of HIF-1α and NF-κB transcriptional activity provides plausible mechanisms for the observed regulatory effects of rosiglitazone. Third, applying pharmacological interventions to inhibit hypoxic-mediated activation of HIF-1α or NF-κB produced comparable reductions in hypoxia-induced increases in ET-1 mRNA and protein levels. Collectively, these findings indicate that rosiglitazone attenuated hypoxic upregulation of ET-1 signaling pathway components through PPARγ-mediated transrepression of hypoxic transcriptional activation involving HIF-1α and NF-κB. These hypothetical relationships are depicted in schematic form in Fig. 7.

The current study has several important limitations that should be recognized. First, hypoxia-induced PH in rodents does not reproduce the characteristic obliterative plexogenic arteriopathy seen in patients with severe forms of PAH. Thus extrapolation of the current findings that targeting PPARγ effectively reduces upregulation of ET-1 signaling pathway components will require confirmation in models that more accurately recapitulate the pathology of PAH. Second, our results provide indirect evidence that hypoxia-induced increases in ET-1 signaling pathway components are mediated

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![Diagram](http://ajplung.physiology.org/)
by HIF-1α or NF-κB. Ongoing studies in our laboratories will utilize chromatin immunoprecipitation assays to confirm that: 1) hypoxia stimulates binding of either HIF-1α or NF-κB to the promoter elements of ET-1, ECE, ETAR, and ETBR and 2) that PPARγ ligands inhibit HIF-1α or NF-κB binding and promoter activation. Finally, the ultimate application of strategies to target PPARγ to inhibit the ET-1 signaling program in PH would ideally be based on an analysis comparing the effectiveness of that strategy with current endothelin receptor antagonists. Nonetheless, it seems reasonable to postulate that PPARγ ligands, by inhibiting multiple steps in the ET-1 signaling pathway, might provide more effective endothelin signaling inhibition and simultaneously reduce other pathogenic pathways (39) to more effectively attenuate PH pathogenesis.

In summary, the current study extends previous reports to demonstrate that hypoxia increases the expression of ET-1, ECE, ETAR, and ETBR in mouse lung and in HPAECs and provides novel evidence that pharmacological PPARγ ligands can effectively attenuate the upregulation of this program of ET-1 signaling. Furthermore, the current findings provide additional evidence that rosiglitazone mediates its effects through transrepression of the activity of other hypoxia-sensitive transcription factors. These results and related studies provide additional rationale for the continued exploration of nuclear hormone receptors as therapeutic targets in lung disease and vascular biology. The current availability of pharmacological ligands for the PPARγ receptor and their widespread application in the treatment of type 2 diabetes suggest that confirmation of therapeutic benefits in preclinical models could be readily translated to clinical trials. Thus the current report provides additional insight into mechanisms by which targeting the PPARγ receptor modulates PH pathogenesis.

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DISCLOSURES

None

REFERENCES


