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Hypoxia downregulates PPAR γ via an ERK 1/2-NF- κ B-Nox4-dependent mechanism in human pulmonary artery smooth muscle cells

Xianghuai Lu*, Kaiser M. Bijli*, Allan Ramirez, Tamara C. Murphy, Jennifer Kleinhenz, and C. Michael Hart

Department of Medicine, Atlanta Veterans Affairs and Emory University Medical Centers, Atlanta, GA 30033

Abstract

The ligand-activated transcription factor, peroxisome proliferator-activated receptor gamma (PPAR γ), regulates metabolism, cell proliferation, and inflammation. Pulmonary hypertension (PH) is associated with reduced PPAR γ expression, and hypoxia exposure regimens that cause PH reduce PPAR γ expression. The current study examines mechanisms of hypoxia-induced PPAR γ downregulation *in vitro* and *in vivo*. Hypoxia reduced PPAR γ mRNA and protein levels, PPAR γ activity, and the expression of PPAR γ regulated genes in human pulmonary artery smooth muscle cells (HPASMC) exposed to 1% oxygen for 72 hours. Similarly, exposure of mice to hypoxia (10% O₂) for 3 weeks reduced PPAR γ mRNA and protein in mouse lung. Inhibiting ERK1/2 with PD98059 or treatment with siRNA directed against either NF- κ B p65 or Nox4 attenuated hypoxic reductions in PPAR γ expression and activity. Furthermore, degradation of H₂O₂ using PEG-catalase prevented hypoxia-induced ERK 1/2 phosphorylation and Nox4 expression suggesting sustained ERK 1/2-mediated signaling and Nox4 expression in this response. Mammalian two hybrid assays demonstrated that PPAR γ and p65 bind directly to each other in a mutually repressive fashion. Taken together, we conclude that hypoxic regimens that promote PH pathogenesis and HPASMC proliferation reduce PPAR γ expression and activity through ERK1/2-, p65-, and Nox4-dependent pathways. These findings provide novel insights into mechanisms by which pathophysiological stimuli such as hypoxia cause loss of PPAR γ activity and pulmonary vascular cell proliferation, pulmonary vascular remodeling, and PH. These results also indicate that restoration of PPAR γ activity with pharmacological ligands may provide a novel therapeutic approach in selected forms of PH.

Keywords

Hypoxia; transcription factors; signaling mechanism; pulmonary hypertension; vascular wall cells

Address correspondence to: C. Michael Hart, M.D., Professor of Medicine, Division of Pulmonary and Critical Care Medicine, Atlanta VAMC (151-P), 1670 Clairmont Rd., Decatur, GA 30033, Phone: 404 – 321 – 6111 ext 7278, Fax: 404 – 728 - 7750, michael.hart3@va.gov.

*Drs. Lu and Bijli contributed equally to this paper.

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors that regulate a large number of gene networks involved in cell metabolism, growth, differentiation, and inflammation [1, 2]. There are three PPAR isoforms, α , β , and γ whose expression patterns vary among tissues. Ligand-activated PPARs form heterodimers with the 9-cis retinoic acid receptor (RXR- α) and bind to peroxisome proliferator response elements (PPRE) in the promoter regions of target genes to stimulate their expression [3, 4]. In addition, PPAR γ activation can inhibit the activity of other pro-inflammatory transcription factors through transrepression mechanisms [5]. PPAR γ is expressed in numerous cells including pulmonary vascular endothelial and smooth muscle cells where it plays a critical role in normal pulmonary vascular function [6, 7].

Growing evidence supports the role of PPAR γ in pulmonary vascular regulation. PPAR γ activation with exogenous synthetic thiazolidinedione ligands attenuates PH and pulmonary vascular remodeling in several experimental models of PH [8–12]. On the other hand, loss of PPAR γ expression is associated with pulmonary hypertension (PH). PPAR γ expression is reduced in the lungs of rodents with PH caused by chronic hypoxia although the mechanisms for these reductions have not been defined [10, 12]. Reduced PPAR γ expression has also been observed in the vascular lesions of patients with idiopathic pulmonary arterial hypertension, in a rat model of severe PH caused by treatment with hypoxia and a vascular endothelial growth factor receptor antagonist [13], and in pulmonary artery endothelial cells isolated from patients with idiopathic pulmonary arterial hypertension [6]. Furthermore, targeted genetic deletion of PPAR γ from endothelial [14] or vascular smooth muscle cells [15] is associated with the development of spontaneous PH in mice. Taken together, these reports suggest that activation of PPAR γ attenuates pulmonary vascular dysfunction and PH whereas reductions in PPAR γ may contribute to PH pathogenesis. The mechanisms by which PPAR γ expression is reduced in PH remain to be defined and constitute the focus of the current study.

Hypoxia, which constitutes a commonly employed stimulus to induce experimental PH in rodent models [16] activates a variety of signaling mechanisms that may contribute to alterations in PPAR γ expression and activity. For example, hypoxia activates mitogen-activated protein kinases (MAPKs) [17, 18] that regulate the activity of transcription factors such as PPAR γ and NF- κ B. Hypoxia also activates pro-inflammatory and oxidative stress signals that reduce PPAR γ expression and activity [19, 20]. Hypoxia selectively increased Nox4 expression in the pulmonary vasculature [21]. Nox4 generates H₂O₂ [22, 23] which reduces PPAR γ expression and activity in endothelial cells *in vitro* [24]. Consistent with the putative role of Nox4 and H₂O₂ in hypoxic reductions in PPAR γ , a novel Nox4 inhibitor, GKT137831, attenuated hypoxia-induced H₂O₂ release, proliferation and reductions in PPAR γ expression in human pulmonary artery endothelial cells (HPAEC) and smooth muscle cells (HPASMC) *in vitro* and in mouse lung *in vivo* [6]. Hypoxia-induced increases in Nox4 expression in HPASMC were caused by NF- κ B p65 binding to and activating the Nox4 promoter [25]. The NF- κ B family of transcription factors thus represents another signaling pathway activated by hypoxia [26, 27]. Collectively, these reports led us to further explore the role of MAPK, NF- κ B, and Nox4 in the molecular regulation of PPAR γ expression and activity under hypoxic conditions.

Our findings demonstrate that ERK1/2, p65, and Nox4 participate in hypoxic downregulation of PPAR γ expression and that PPAR γ physically interacts with NF- κ B p65 in a mutually repressive fashion to reduce PPAR γ transcriptional activity. These findings provide novel evidence for specific signaling mechanisms involved in hypoxia-induced

reductions in PPAR γ expression and activity. Suppression or reversal of these pathways may preserve PPAR γ function in the pulmonary vascular wall and provide a novel therapeutic strategy in PH.

Materials and Methods

Reagents

Rosiglitazone, ERK 1/2 inhibitor (PD98059), p38 inhibitor (SB203580) and JNK inhibitor (SP600125) were purchased from Calbiochem (La Jolla, CA). Antibodies against PPAR γ were purchased from Bethyl Laboratories (Montgomery, TX) or from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-(Thr202/Tyr204)-ERK 1/2, total ERK 1/2, phospho-(Ser536)-NF- κ B, total NF- κ B p65, CDK4, Nox4, GAPDH and actin were purchased from Santa Cruz Biotechnology or Cell Signaling Technology (Beverly, MA). All other materials were purchased from VWR Scientific Corp. (Gaithersburg, MD).

Cell Culture

Human pulmonary artery smooth muscle cells (HPASMC) were purchased from Lonza (Basel, Switzerland). HPASMC monolayers were grown at 37°C in a 5% CO₂ atmosphere in culture media (SmGM-2, Lonza) containing 2% fetal calf serum, growth factors, and antibiotics as previously reported [25]. In selected studies, human embryonic kidney (HEK 293) cells were grown in basal modified Eagle's medium with 10% fetal bovine serum. HPASMC or HEK 293 cells were incubated in a hypoxia chamber (Biospherix, Lacona, NY) (1% O₂, 5% CO₂) or cell culture incubator (21% O₂, 5% CO₂) at 37°C for 72 hours.

Measurement of HPASMC proliferation

HPASMC proliferation was determined with a quantitative colorimetric assay employing dimethylthiazol (MTT assay; ATCC) as described earlier [25]. Briefly, after exposure to control or hypoxia with or without treatment with PEG-catalase (1000 u/ml), cells were incubated with the MTT reagent for 4 hours. The mitochondrial reductase present in living cells reduces MTT to purple formazan, which is detected by spectrophotometry. Samples were then analyzed using an ELISA plate reader ($\lambda = 570$ nm), and values from treated cells were normalized to values from corresponding control cells. We previously reported that MTT assays and cell counting methods produced similar results in hypoxia-exposed HPASMC [28].

Animals

Male C57Bl/6 mice (age 8–10 weeks, Jackson Laboratory, Bar Harbor, ME) were exposed to hypoxia (10% O₂) or room air for 3 weeks as reported [12]. All animals had access to standard mouse chow and water *ad libitum* and were housed socially in groups of 5 mice per cage. All animal studies were approved by the Institutional Animal Care and Use Committee of the Atlanta VA.

RNA isolation, reverse transcription, and quantitative PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA), and RNA was quantified by Nanodrop spectrophotometry (Thermo Scientific, Wilmington, DE). cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed to assess the expression levels of PPAR γ 1, aP2, C/EBP α , and GAPDH using primers based on human mRNA sequences: F, 5'-gtggc cgcag atttg aaaga ag-3' and R, 5'-tgtca accat ggtca ttctg-3' (hPPAR γ 1); F, 5'-actgg gccag gaatt tgacg-3' and 5'-cccca tctaa ggtta tgggtg ctc-3' (aP2); F, 5'-gaggg accgg agtta tgaca-3' and 5'-ttcac attgc acaag

gcact-3' (C/EBP α); F, 5'-gccca atacg accaa atcc-3' and R, 5'-agcca catcg ctcag acac-3' (GAPDH), or mouse mRNA sequences: F, 5'-gaaag acaac ggaca aatca cc-3' and 5'-ggggg tgata tgttt gaact tg-3' (mPPAR γ 1). Real-time PCR was performed using iQ SYBR Green Supermix and the iCycler real-time PCR detection system (Bio-Rad). Amplicon expression in each sample was normalized to GAPDH mRNA levels. The relative abundance of target mRNA in each sample was calculated using $\Delta\Delta$ CT methods (Applied Biosystems, Carlsbad, CA).

Western blot analysis

HPASMC lysates or mouse lung homogenates were prepared and equivalent amounts of protein were resolved by SDS-PAGE and immunoblotted with appropriate antibodies. Relative levels of immunoreactive proteins were quantified using the ChemiDoc XRS imaging system and Quantity One software (Bio-Rad Laboratories).

Transient transfection and luciferase assays

A mammalian expression vector for NF- κ B p65 was employed in transient transfection studies as described previously [29]. HEK 293 cells were transiently transfected with the PPREx3-TK-luc (Addgene, Cambridge, MA), with full-length p65, with κ Bx6-TK-luc, or with PPAR γ expressing plasmid using Superfect transfection reagent (Qiagen). To normalize the transfection efficiency, cells were cotransfected with 0.1 μ g/well pRL-TK renilla luciferase. After transfection, cells were incubated for 72 hours in either normal or hypoxic conditions, washed twice with PBS, and then lysed with 300 μ l of passive lysis buffer (Promega, Madison, WI). Luciferase activities were measured with the Luciferase Reporter Assay System (Promega) using a luminometer (Perkin Elmer, Covina, CA). Relative light units were examined in triplicate and normalized to renilla.

Gene silencing with siRNA

NF- κ B p65 or Nox4 were reduced using siRNA to p65 (Santa Cruz Biotechnology) or to Nox4 (Qiagen) respectively. Cells were incubated for 24 hr in antibiotic-free medium containing 10% serum before incubating with the transfection reagent, Oligofectamine (Invitrogen) for 48 hr. For co-transfection studies, Attractene (Qiagen) was employed as the transfection agent, and empty vector pcDNA3 and control siRNA were used as experimental controls. Previous studies using this siRNA approach to deplete NF- κ B p65 and Nox4 confirmed that compared to control siRNA, target mRNA levels were reduced by at least 50% as determined with real-time PCR [25]. These conditions were therefore employed in the current study to deplete NF- κ B p65 or Nox4 in HPASMC.

Mammalian two-hybrid assays

The mammalian two-hybrid assay (Agilent Technologies, Santa Clara, CA) was employed using PPAR γ fused to the DNA-binding domain of the yeast protein, GAL4, in the pCMV-BD vector (bait). The full length NF- κ B p65 protein in the pCMV vector was used as prey. pFR-Luc was used as a reporter vector, which contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites that control the expression of a luciferase gene. In this system, physical interaction between PPAR γ and p65 stimulates luciferase activity. The assay was performed strictly following the manufacturer's instructions.

Statistical analysis

When comparing more than 2 groups, data were analyzed using analysis of variance (ANOVA). Post hoc analysis using the Student Neuman Keuls test was employed to detect differences between individual groups. In studies comparing only two experimental groups,

data were analyzed with Student's t-test to determine significance of treatment effects. The level of statistical significance was taken as $p < 0.05$.

Results

Hypoxia decreases PPAR γ expression in mouse lung *in vivo* and in HPASMC *in vitro*

Hypoxia reduced PPAR γ protein expression in pulmonary artery endothelial cells [12] *in vitro* and rodent lungs *in vivo* [10]. We analyzed PPAR γ mRNA and protein levels in mouse lungs from male C57Bl/6 mice exposed to room air or hypoxia (10% O₂) for 3-weeks. We previously reported that this hypoxia exposure regimen causes significant pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular remodeling in mice [12]. Compared to control conditions, hypoxia exposure reduced PPAR γ mRNA (Fig. 1A) and protein (Fig. 1B) levels in lung homogenates. To further examine hypoxia-induced alterations in PPAR γ expression and activity, HPASMC were exposed to normoxia or hypoxia (1% O₂) for 72 hours. This hypoxia regimen stimulated significant proliferation of HPASMC and HPAEC *in vitro* [6]. Compared to control conditions, hypoxia reduced HPASMC PPAR γ mRNA (Fig. 1C) and protein (Fig. 1D) levels by roughly 50%, comparable in magnitude to the hypoxic reductions in PPAR γ observed in the mouse lung. Hypoxia not only reduced HPASMC PPAR γ mRNA and protein levels, but also reduced the expression of two PPAR γ target genes, *aP2* and *C/EBP α* (Fig. 1E). To examine the impact of hypoxia on PPAR γ activity, HPASMC were transiently transfected with a PPRE-Luc vector, which contains three copies of consensus PPAR γ response elements that drive luciferase expression [30]. After transfection, the cells were exposed to either normoxia (21% O₂) or hypoxia (1% O₂) for 72 hours. Compared to normoxic conditions, hypoxia reduced constitutive PPAR γ activity in HPASMC (Fig. 1F) as measured by luciferase activity.

Hypoxia-induced reductions in PPAR γ are attenuated by ERK1/2, NF- κ B or Nox4 inhibition

To further explore the mechanisms by which hypoxia reduced PPAR γ expression and function, the role of MAPKs, NF- κ B, and Nox4 in hypoxic effects on PPAR γ expression and activity were examined. HEK 293 cells were employed because of their higher transfection efficiency compared to HPASMC. HEK293 cells were transiently transfected with PPRE-Luc and renilla vectors and then exposed to normoxia or hypoxia for 72 hours. During the last 24 hours of exposure to normoxic or hypoxic conditions, selected cells were treated with DMSO vehicle, the ERK1/2 inhibitor (PD98059, 40 μ M), the p38 inhibitor (SB203580, 20 μ M) or the JNK inhibitor (SP600125, 50 nM). The concentrations of MAP kinase inhibitors were selected based on published reports [31–33]. As shown in Figure 2A, hypoxia reduced PPAR γ activity in HEK 293 cells similar to hypoxic reductions in HPASMC PPAR γ activity reported in Figure 1. Treatment with the ERK1/2 inhibitor, PD98059, attenuated hypoxia-induced reductions in PPAR γ transcriptional activity while treatment with inhibitors of p38 or JNK had no significant effect. These findings suggested that ERK1/2 was involved in hypoxic inhibition of PPAR γ activity. The hypoxic conditions employed in these *in vitro* studies stimulated ERK activation as demonstrated by increased levels of phosphorylated ERK1/2, but had no effect on total ERK1/2 levels (Fig. 2B). As expected, the increase in phosphorylated ERK1/2 was attenuated by treatment with PD98059 in HPASMC (Fig. 2B). Furthermore, inhibiting ERK1/2 with PD98059 attenuated hypoxic reductions in PPAR γ protein as determined by western blot (Fig. 2C). As shown in Figure 2D, compared to control conditions, hypoxia increased p65 protein levels in HPASMC, and ERK1/2 inhibition with PD98059 attenuated hypoxia-induced increases in p65. Collectively these findings indicate that hypoxia-induced ERK activation contributes to increased expression of p65 and reduced PPAR γ expression and activity.

Hypoxia increased Nox4 expression and activity in HPASMC *in vitro* [25] and mouse lung *in vivo* [12], and Nox4 produces H₂O₂ [22] which reduces PPAR γ expression [24]. Therefore, the role of Nox4 in hypoxia-induced inhibition of PPAR γ expression and activity was further examined. HEK 293 cells or HPASMC were transfected with control siRNA or Nox4 siRNA as described in “Materials and Methods” and exposed to normoxia for 72 hours. Figure 3A demonstrates the depletion of Nox4 protein in these cells. We previously reported that silencing Nox4 by this approach reduced H₂O₂ production in HPASMC exposed to hypoxia [25]. These conditions for Nox4 depletion were therefore adopted in subsequent experiments. HEK 293 cells were transfected with PPRE-Luc and with control or Nox4 siRNA prior to exposure to control or hypoxic conditions. Transfection with neither control nor Nox4 siRNA altered PPAR γ activity in HEK 293 cells exposed to normoxic conditions (Fig. 3B). Hypoxia reduced PPAR γ activity in cells treated with control siRNA, but treatment with Nox4 siRNA attenuated hypoxia-induced reductions in PPAR γ activity (Fig. 3B). As illustrated by Figure 3C, transfection with Nox4 siRNA also attenuated hypoxia-induced reductions in PPAR γ protein expression in HPASMC. Hypoxia not only stimulated increased levels of p65 protein in HPASMC, but it also stimulated p65 phosphorylation consistent with hypoxia-induced NF- κ B p65 activation that was attenuated by transfection with Nox4 siRNA (Fig. 3D). To further confirm the role of Nox4-derived H₂O₂ in hypoxia-induced HPASMC proliferation [25], selected HPASMC were treated with PEG-catalase to enhance H₂O₂ degradation. As expected, hypoxia-induced HPASMC proliferation was significantly attenuated by treatment with PEG-catalase (Fig. 3E) consistent with our previous observations with Nox4 siRNA [25].

To further examine the role of hypoxia-induced H₂O₂ generation in feed forward signaling mechanisms associated with hypoxia-induced HPASMC proliferation, we analyzed the impact of PEG-catalase treatment on ERK 1/2 activation and Nox4 expression. Degradation of H₂O₂ by PEG-catalase during the final 24 hours of a 72 hour hypoxia exposure regimen attenuated hypoxia-induced ERK 1/2 phosphorylation and Nox4 expression (Fig. 4A and 4B).

Because hypoxia activates NF- κ B to stimulate Nox4 expression, the role of the NF- κ B pathway in hypoxia-induced alterations in PPAR γ activity and expression was further examined. As shown in Figure 5A, depletion of NF- κ B p65 attenuated hypoxia-mediated reductions in PPAR γ activity in HEK293 cells. Similarly, NF- κ B p65 depletion also attenuated hypoxia-induced reductions in PPAR γ protein in HPASMC (Fig. 5B). To better understand how NF- κ B p65 mediates hypoxia-induced inhibition of PPAR γ activity, mammalian two hybrid assays were employed to examine direct p65-PPAR γ interactions. HEK 293 cells were transiently transfected with pCMV-Gal4-PPAR γ -BD (ligand-dependent activation functionality to coordinate transcription with Gal4), pCMV-p65, pFR-luciferase (readout of the protein interaction) and control renilla luciferase vectors. Selected cells were treated with the synthetic PPAR γ ligand, rosiglitazone (10 μ M) or an equivalent volume of vehicle, to determine if ligand-dependent PPAR γ activation promotes interactions with NF- κ B p65. As illustrated in Figure 6A, baseline luciferase activity which indicates p65-PPAR γ interaction was stimulated by treatment with rosiglitazone and further increased by co-transfection with exogenous p65. Graded increases in the amount of transfected p65 caused a roughly dose-dependent increase in luciferase activity (Fig. 6B). To further characterize the impact of p65 on PPAR γ , PPAR γ activity assays were performed in HEK 293 cells co-transfected with a PPRE luciferase construct and pCMV p65. As shown in Figure 6C, compared to a control vector, expression of exogenous p65 reduced luciferase activity indicating that NF- κ B suppresses PPAR γ activity. To further explore if PPAR γ transactivation caused reciprocal repression of p65, HEK293 cells were transiently transfected with an NF- κ B luciferase reporter along with either control or PPAR γ expression constructs. Transfection with the PPAR γ construct reduced NF- κ B activity (Fig.

6D) as did stimulation of endogenous PPAR γ with rosiglitazone (Fig. 6E). Collectively, these findings demonstrate that NF- κ B and PPAR γ physically interact to mutually repress each other's transcriptional activity.

Discussion

The pathogenesis of pulmonary hypertension involves excessive proliferation and decreased apoptosis of pulmonary vascular wall cells, and imbalances in the production of vasoconstrictor and vasodilator molecules [34–36]. These abnormalities promote remodeling of the pulmonary vasculature resulting in increased pulmonary vascular resistance and pulmonary hypertension. Clinical conditions that trigger pulmonary hypertension include those associated with chronic alveolar hypoxia such as living at altitude, chronic obstructive pulmonary disease, and obstructive sleep apnea [37, 38]. The precise molecular pathogenesis by which hypoxia causes pulmonary hypertension remains to be defined, and the identification of new pathogenic pathways can potentially enable new therapeutic targets for this disorder.

The nuclear hormone receptor, PPAR γ , not only regulates glucose and lipid metabolism, but also participates in diverse physiological and pathological processes, including cell signaling, inflammation, and wound healing [39, 40]. For example, PPAR γ activation attenuated signaling pathways associated with vascular smooth muscle cell proliferation and remodeling [12]. Although previous studies have examined hypoxia-induced reductions in PPAR γ expression in a variety of models [8, 41, 42], the current study provides new mechanistic insights into cellular pathways involved in hypoxic reductions in PPAR γ focusing particularly on PPAR γ reductions in HPASMC. The current study demonstrates that hypoxia decreased PPAR γ mRNA and protein levels in HPASMC *in vitro* and in the mouse lung *in vivo*. These hypoxia-induced reductions in PPAR γ mRNA and protein were mediated through activation of ERK1/2, NF- κ B and Nox4, and pharmacological inhibition or knockdown of each of these targets was sufficient to attenuate reductions in PPAR γ . We previously reported that hypoxia increased HPASMC Nox4 expression by stimulating NF- κ B activation and p65 binding to the Nox4 promoter thereby increasing Nox4 expression and activity [25]. Furthermore H₂O₂, the product of Nox4 activity, reduces PPAR γ expression [24]. In the current study we further demonstrate that Nox4 derived H₂O₂ plays an important role in hypoxic HPASMC proliferation (Figure 3E). Coupled with the current results that inhibition of either ERK1/2 or Nox4 attenuated hypoxic induction of NF- κ B p65, these results support a hypoxia-induced signaling cascade involving ERK1/2, NF- κ B p65, and Nox4 that reduces PPAR γ expression and its regulation of PPAR γ -target genes (Fig. 7). The current results also provide novel evidence that hypoxia-induced Nox4 induction contributes to sustained p65 activation during chronic hypoxia exposure. Furthermore, the requirement of hypoxia-induced H₂O₂ generation for activation of ERK 1/2 and Nox4 expression (Fig. 4A and B) strongly supports the existence of a feed forward cycle of NF- κ B p65 activation and Nox4 induction in HPASMC in response to hypoxia that lead to increases in H₂O₂ generation and reductions in PPAR γ . Coupled with previous reports that PPAR γ depletion increases basal and stimulated SMC proliferation [43], these findings provide mechanistic insights into the role of PPAR γ in hypoxic pulmonary vascular cell proliferation and pulmonary hypertension (Fig. 7).

ERK1/2 activity participates in the regulation of cell proliferation and growth [44], and increased ERK1/2 signaling stimulated PPAR γ phosphorylation and proteosomal degradation leading to decreased PPAR γ expression and transcriptional activity [45, 46]. While it is likely that this pathway participates in reduced levels of PPAR γ in hypoxic HPASMC, our data provide clear evidence for the involvement of additional pathways, including transcriptional or post-transcriptional suppression of PPAR γ resulting in reduced

levels of PPAR γ mRNA. In addition, our findings provide evidence that hypoxic NF- κ B activation can stimulate post-translational inhibition of PPAR γ through direct and mutually repressive interactions between p65 and PPAR γ . These findings are consistent with those recently reported in human colonic HT29 cells and mouse embryonic fibroblasts that PPAR γ acts as an E3 ligase that physically associates with NF- κ B p65 to promote its proteosomal degradation [47]. Taken together these findings suggest that mutually repressive effects of PPAR γ and NF- κ B can be observed in diverse cell types. Based on the inhibitory p65-PPAR γ interactions observed in HEK293 cells using mammalian two-hybrid assays in the current study, we speculate that similar interactions occur in hypoxia-exposed HPASMCs. Confirmation of these pathways in HPASMCs and other cells will require additional studies in the future using approaches such as co-immunoprecipitation and/or FRET.

The findings in the current study indicate that hypoxic conditions that are sufficient to promote HPASMC proliferation both activate ERK1/2 and reduce PPAR γ expression. Inhibiting ERK1/2 with the specific pharmacological inhibitor, PD98059, attenuated hypoxic reductions in PPAR γ expression, whereas treatment with inhibitors of JNK or p38 failed to attenuate hypoxic reductions in PPAR γ . Hypoxia also increased levels of HPASMC p65 that were attenuated by ERK1/2 inhibition. These findings indicate that activation of ERK1/2 signaling plays a critical upstream role in hypoxic downregulation of PPAR γ . However, it must be emphasized that our findings do not determine if ERK 1/2 is both necessary and sufficient to induce downregulation of PPAR γ . Future studies to effect genetic gain and loss of ERK 1/2 function are planned to further explore the specific role of ERK 1/2 in modulating hypoxia-inducible transcription factor activation.

NF- κ B, a member of the Rel family of dimeric transcription factors, is activated by hypoxia [27, 48, 49] and mediates hypoxia-induced expression of inflammatory target genes [48]. Our findings demonstrate that both ERK1/2 and Nox4 contribute to activation of NF- κ B following sustained hypoxia and that p65 knockdown attenuates hypoxic reductions in PPAR γ . We previously reported enhanced NF- κ B activity in the aortas of endothelial-targeted PPAR γ -/- mice [50]. These findings suggest that reductions in PPAR γ expression and activity may contribute to NF- κ B activation contributing to the feed forward signaling cascade illustrated in Figure 6. Our findings and reports in other systems [51, 52] indicate that NF- κ B and PPAR γ are mutually repressive.

In conclusion, the current study emphasizes that hypoxic activation of ERK1/2 and NF- κ B can stimulate Nox4-mediated H₂O₂ generation that reduces PPAR γ expression and activity. We postulate that reduced PPAR γ expression and activity fosters sustained NF- κ B activation and persistent derangements in redox signaling that contribute to HPASMC proliferation and pulmonary hypertension pathogenesis. By identifying these pathogenic mechanisms, targeted interventions may permit interruption of these feed-forward signaling cascades thereby permitting a restoration of balance between NF- κ B and PPAR γ signaling. These findings can thereby facilitate the development of novel strategies for the prevention or treatment of PH.

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Abbreviations

PH	Pulmonary hypertension
ANOVA	Analysis of variance
aP2	Activating protein-2
CDK4	Cyclin-dependent kinase 4
C/EBPα	CCAAT/enhancer binding protein-alpha
ELISA	Enzyme-linked immunosorbent assay
ERK 1/2	Extracellular signal-regulated kinase 1/2
GAL4	Transcription factor for galactose-inducible genes in yeast
GAPDH	Glyceraldehyde phosphate dehydrogenase
HEK 293	Human Embryonic Kidney 293 cells
HPAEC	Human pulmonary artery endothelial cells
HPASMC	Human pulmonary artery smooth muscle cells
MAPK	Mitogen activated protein kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nox4	NADPH oxidase 4
NF-κB	Nuclear factor-kappaB
pCMV	Mammalian expression vector containing CMV promoter
PPRE	Peroxisome proliferator response element
PPARγ	Peroxisome proliferator-activated receptor-gamma
PPREx3-Tk-luc	Thymidine kinase luciferase containing reporter plasmid with a PPAR γ response element
pRL-TK	Renilla luciferase reporter containing plasmid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SmGM-2	Smooth muscle growth medium-2

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FIG. 1. Hypoxia decreases PPAR γ expression *in vivo* and *in vitro*

Male C57Bl/6 mice were exposed to room air (C) or hypoxia (H, 10% O₂) for 3-weeks, and mRNA and protein were isolated from the lungs. **(A)** PPAR γ and GAPDH mRNA levels were determined with real-time PCR. Each bar represents the mean $\pm$ SEM copies of PPAR γ mRNA normalized to GAPDH in the same sample and expressed as fold-change relative to control. n=3. **(B)** The levels of PPAR γ and cyclin-dependent kinase 4 (CDK4) in the lungs were analyzed with western blotting and laser densitometry. PPAR γ levels were calculated relative to the densitometric intensity of CDK4 in the same sample. A representative blot for PPAR γ and CDK4 is presented above the graph. Each bar represents the mean $\pm$ SEM PPAR γ densitometric intensity relative to CDK4 expressed as fold-change relative to control. n=5. HPASMC were exposed to control (C) normoxic (21% O₂) or

hypoxic (H, 1% O₂) conditions for 72 hours followed by isolation of mRNA and protein. **(C)** HPASMC PPAR γ and GAPDH mRNA levels were determined with real-time PCR. Each bar represents the mean $\pm$ SEM copies of PPAR γ mRNA normalized to GAPDH in the same sample and expressed as fold-change relative to control from two experiments performed in triplicate. **(D)** The levels of HPASMC PPAR γ and CDK4 protein were analyzed with western blotting and laser densitometry. PPAR γ levels were calculated relative to the densitometric intensity of CDK4 in the same sample. A representative blot for PPAR γ and CDK4 is presented above the graph. Each bar represents the mean $\pm$ SEM PPAR γ densitometric intensity relative to CDK4 expressed as fold-change relative to control. n=3. **(E)** HPASMC aP2, C/EBP α , and GAPDH mRNA levels were determined with real-time PCR. Each bar represents the mean $\pm$ SEM copies of aP2 or C/EBP α mRNA normalized to GAPDH in the same sample and expressed as fold-change relative to control from four experiments performed in triplicate. **(F)** HPASMC were transiently transfected with PPRE-Luc and control renilla luciferase vectors, then exposed for 72 hours to control or hypoxic conditions. Cell lysates were subjected to luciferase activity assays. Each bar represents the mean $\pm$ SEM luciferase activity relative to renilla in each sample, expressed as fold-change relative to control from three experiments performed in triplicate. *p<0.05 vs C.

FIG. 2. ERK1/2 activation contributes to hypoxic reductions in PPAR γ activity and expression and increases in p65

HEK 293 cells were transiently transfected with PPRE-Luc and control renilla luciferase vectors, then exposed to control (C) normoxic (21% O₂) or hypoxic (H, 1% O₂) conditions for 72 hours. (A) The ERK1/2 inhibitor (PD98059, 40 μ M), the p38 inhibitor (SB203580, 20 μ M), the JNK inhibitor (SP600125, 50 nM) or an equivalent volume of DMSO vehicle were added to selected cells 24 hours before harvesting and measurement of luciferase activity. Each bar represents the mean $\pm$ SEM luciferase activity relative to renilla in each sample, expressed as fold-change relative to control from three experiments performed in triplicate. * p <0.001 vs C-DMSO, ** p <0.001 vs all other H groups. (B) HPASMC were exposed to C or H conditions $\pm$ treatment with PD98059 as described in A. Cell lysates were analyzed by western blotting using antibodies to phospho-(Thr202/Tyr204)-ERK1/2 (p-ERK1/2) and total ERK1/2. A representative blot of p-ERK1/2 and total ERK1/2 is presented. Each bar represents the mean $\pm$ SEM p-ERK1/2 protein levels measured with densitometry and normalized to total ERK1/2, expressed as fold-change relative to control from three experiments. * p <0.05 vs C, ** p <0.05 vs H. (C) HPASMC were exposed to C or H conditions for 72 hours $\pm$ treatment with PD98059 during the last 24 hours and the cell lysates were analysed by western blotting using antibodies to PPAR γ and β -actin. A representative blot of PPAR γ and β -actin is presented. Each bar represents the mean $\pm$ SEM PPAR γ protein levels measured with densitometry and normalized to β -actin, expressed as fold-change relative to control. $n=3$, * p <0.05 vs C, ** p <0.05 vs H. (D) HPASMC were exposed to C or H conditions for 72 hours $\pm$ treatment with PD98059 during the last 24

hours and the cell lysates were analyzed by western blotting using antibodies to p65 and β -actin. A representative blot is presented. Each bar represents the mean $\pm$ SEM NF- κ B p65 protein levels measured with densitometry, normalized to β -actin, and expressed relative to C, performed in triplicate. * $p < 0.05$ vs C.

FIG. 3. Nox4 knockdown attenuates hypoxia-induced reductions in PPAR γ activity, expression, and p65 activation

(A) HEK 293 cells or HPASMC were transfected with either control siRNA (siC) or Nox4 siRNA (siNox4) as described in "Materials and Methods" and then exposed for 72 hours to normoxic (21% O₂) conditions. Cell lysates were prepared and analyzed for Nox4 protein depletion. These conditions for Nox4 depletion were followed for subsequent experiments. (B) HEK 293 cells were transiently transfected with PPRE-Luc and control renilla luciferase vectors along with either control siRNA (siC) or Nox4 siRNA (siNox4), then exposed for 72 hours to control (C) normoxic (21% O₂) or hypoxic (H, 1% O₂) conditions. Cells lysates were subjected to luciferase activity assays. Each bar represents the mean $\pm$ SEM luciferase activity relative to renilla in each sample, expressed as fold-change relative to control from three experiments performed in triplicate. (C) HPASMC were transfected with control or Nox4 siRNA and then exposed to C or H conditions for 72 hours. Cell lysates were analyzed

by western blotting using antibodies to PPAR γ and β -actin. A representative blot of PPAR γ and β -actin is presented. Each bar represents the mean $\pm$ SEM PPAR γ protein levels measured with densitometry and normalized to β -actin, expressed as fold-change relative to control from three experiments. **(D)** HPASMC were transiently transfected with control or Nox4 siRNA and then exposed to C or H conditions for 72 hours. Cell lysates were analyzed by western blotting using antibodies to phospho-(Ser536)-p65 (p-p65) and total p65. A representative blot is presented. Each bar represents the mean densitometric intensity $\pm$ SEM of p-p65 protein levels normalized to total p65 and expressed relative to control, performed in triplicate. * $p < 0.05$ vs Control-siC, ** $p < 0.05$ vs Hypoxia-siC. **(E)** HPASMC were exposed to C or H conditions for 72 hours and treated with PEG-catalase (Pcat, 1000 u/ml) or equivalent volume of vehicle (Veh) during the last 24 hours. Cells were then analyzed for proliferation using MTT assay, and the data were recorded in absorbance units. Each bar represents HPASMC proliferation as the mean $\pm$ SE fold change relative to control. $n = 3$. * $p < 0.05$ for H+Veh vs C+Veh; ** $p < 0.05$ for H+Pcat vs. H+Veh.

FIG. 4. Degradation of H₂O₂ attenuates hypoxia-induced phosphorylation of ERK 1/2 and Nox4 expression

HPASMC were exposed to C or H conditions for 72 hours and treated with PEG-catalase (Pcat, 1000 u/ml) or equivalent volume of vehicle (Veh) during the last 24 hours. Cell lysates were analyzed by western blotting using antibodies to phospho-(Thr202/Tyr204)-ERK1/2 (p-ERK1/2) and total ERK1/2 (**A**) or Nox4 and β-actin (**B**). In **A**, each bar represents ERK 1/2 phosphorylation as the mean + SE fold change relative to control. n=6. ** p<0.05 for H+Veh vs C+Veh; *** p<0.05 for H+Pcat vs. H+Veh. In **B**, each bar represents Nox4 expression as the mean + SE fold change relative to control. n=3–4. ** p<0.05 for H+Veh vs C+Veh; * p<0.05 for H+Pcat vs. H+Veh.

FIG. 5. Depletion of NF- κ B attenuates hypoxia-induced reductions in PPAR γ transcriptional activity and protein levels

(A) HEK 293 cells were transiently transfected with PPRE-Luc and control renilla luciferase vectors along with either control siRNA (siC) or NF- κ B p65 siRNA (sip65), then exposed for 72 hours to Control (21% O₂) or Hypoxia (1% O₂) conditions. Cells lysates were subjected to luciferase activity assays. Each bar represents the mean $\pm$ SEM luciferase activity relative to renilla in each sample, expressed in arbitrary units from three experiments performed in triplicate. (B) HPASMC were transiently transfected with either siC or sip65 and then exposed to control or hypoxia conditions for 72 hours. Cell lysates were analyzed by western blotting with antibodies to PPAR γ , NF- κ Bp65 and GAPDH (loading control). A representative blot is presented. Each bar represents the mean $\pm$ SEM PPAR γ protein levels measured with densitometry, normalized to GAPDH, and expressed relative to siC-Control, performed in triplicate. *p<0.05 vs siC-Control; **p<0.05 vs siC-Hypoxia.

FIG. 6. PPAR γ binds to NF- κ B p65 in a mutually repressive fashion

(A) HEK 293 cells were transiently transfected with pCMV-Gal4-PPAR γ -BD, pCMV-p65, pFR-luciferase and control renilla luciferase vectors. Selected cells were treated with the synthetic PPAR γ ligand, rosiglitazone (Rosi, 1 μ M) or an equivalent volume of vehicle for 48h. Cell lysates were subjected to luciferase activity assays. Each bar represents the mean $\pm$ SEM luciferase activity relative to renilla in each sample, expressed as fold-change versus control from three experiments performed in triplicate. * $p < 0.05$ vs PPAR γ -BD ** $p < 0.05$ vs PPAR γ -BD + Rosi. (B) HEK 293 cells were transiently transfected with the vectors as described in A, which involved three different doses of pCMV-p65 (0.1, 0.2, or 0.5 μ g). Cell lysates were subjected to luciferase activity assays. Each bar represents the mean $\pm$ SEM luciferase activity relative to renilla in each sample, expressed as fold-change versus control

from three experiments performed in triplicate. * $p < 0.05$ vs all other groups. **(C)** HEK 293 cells were transiently transfected with PPRE-luciferase and control renilla luciferase vectors with or without a p65 expression vector (p65 or C, respectively). Cell lysates were subjected to luciferase activity assays. Each bar represents the mean $\pm$ SEM luciferase activity relative to renilla in each sample, expressed as fold-change versus control from three experiments performed in triplicate. * $p < 0.05$ vs C. **(D)** HEK 293 cells were transiently transfected with pCMV PPAR γ (PPAR γ), pcDNA3 (control, C) and (κ B)x6-luciferase and control renilla luciferase vectors. Cell lysates were subjected to luciferase activity assays. Each bar represents the mean $\pm$ SEM luciferase activity relative to renilla in each sample, expressed as fold-change versus control from three experiments performed in triplicate. * $p < 0.05$ vs C. **(E)** HEK 293 cells were transiently transfected with (κ B)x6-luciferase and control renilla luciferase vectors. Selected cells were treated with the synthetic PPAR γ ligand, rosiglitazone (Rosi, 1 μ M) or an equivalent volume of vehicle for 48h. Cell lysates were subjected to luciferase activity assays. Each bar represents the mean $\pm$ SEM luciferase activity relative to renilla in each sample, expressed as fold-change versus control from three experiments performed in triplicate. * $p < 0.05$ vs C.

FIG. 7. Schematic representation of hypoxia-induced PPAR γ inhibition through ERK1/2, Nox4 and NF- κ B p65-mediated mechanisms

The findings in the current study support a signaling pathway wherein hypoxia activates ERK1/2 and increases NF- κ B p65 levels and activity. Our previous work demonstrated that hypoxia stimulated p65 binding to and activation of the Nox4 promoter [25]. The current findings demonstrate that hypoxia-induced Nox4 activity contributes to reductions in PPAR γ and activation of NF- κ B. Hypoxia-induced inhibition of PPAR γ expression may further enhance NF- κ B p65 activation leading to a feed forward cycle of increased Nox4 and decreased PPAR γ expression and activity. ERK1/2 and NF- κ B p65 may also directly inhibit PPAR γ expression and activity. As a consequence of hypoxia exposure, the balance between NF- κ B and PPAR γ signaling becomes dysregulated resulting in the generation of H₂O₂ which promotes pathological pulmonary vascular wall cell proliferation, vascular remodeling, and PH. Interventions that interrupted this hypoxic signaling cascade are depicted in italicized text.