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# Time-dependent PPARγ Modulation of HIF-1α Signaling in Hypoxic Pulmonary Artery Smooth Muscle Cells

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## Abstract

**Background—**Pathogenesis of pulmonary hypertension is complex and involves activation of the transcription factor, hypoxia-inducible factor-1 (HIF-1) that shifts cellular metabolism from aerobic respiration to glycolysis, in part, by increasing the expression of its downstream target pyruvate dehydrogenase kinase-1 (PDK-1), thereby promoting a proliferative, apoptosis-resistant phenotype in pulmonary vascular cells. Activation of the nuclear hormone transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ), attenuates pulmonary hypertension and pulmonary artery smooth muscle cell (PASMC) proliferation. In the current study, we determined whether PPARγ inhibits HIF-1α and PDK-1 expression in human PASMCs.

**Methods—**HPASMCs were exposed to normoxia (21% $O_2$) or hypoxia (1% $O_2$) for 2–72 hours ± treatment with the PPARγ-ligand, rosiglitazone (RSG, 10 μM).

**Results—**Compared to normoxia, HIF-1α mRNA levels were elevated in HPASMC at 2 hours hypoxia and reduced to baseline levels by 24–72 hours. HIF-1α protein levels increased following 4 and 8 hours of hypoxia and returned to baseline levels by 24 and 72 hours. PDK-1 protein levels increased following 24 hours hypoxia and remained elevated by 72 hours. RSG treatment at the onset of hypoxia attenuated HIF-1α protein and PDK-1 mRNA and protein levels at 4, 8 and 24 hours of hypoxia, respectively. However, RSG treatment during final 24 hours of 72-hour hypoxia, an intervention that inhibits HPASMC proliferation, failed to prevent hypoxia-induced PDK-1 expression.

**Conclusion—**Hypoxia causes transient activation of HPASMC HIF-1α that is attenuated by RSG treatment initiated at hypoxia onset. These findings provide novel evidence that PPARγ modulates fundamental and acute cellular responses to hypoxia through both HIF-1-dependent and HIF-1-independent mechanisms.

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**Keywords**

**Key Indexing Terms:** PPARγ; HIF-1α; PDK-1; Vascular smooth muscle cell; Pulmonary hypertension

## INTRODUCTION

Pulmonary hypertension (PH), defined as mean pulmonary artery pressure >25 mm Hg at rest, is a complex disorder associated with significant morbidity and mortality. Increases in pulmonary artery pressure and pulmonary vascular resistance lead to right ventricular hypertrophy, and if untreated, right ventricular failure and premature death. Even with current therapies, PH has a poor long-term prognosis. As a result, new treatment options are urgently needed to manage patients with PH and improve outcomes. One novel therapeutic strategy targets the ligand-activated transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ).[1,2] Loss of PPARγ function is associated with PH, and PPARγ expression is reduced in the lungs and pulmonary vascular tissue of patients with PH and in experimental models of PH.[3–11] In contrast, PPARγ activation attenuates PH and vascular remodeling in experimental animal models.[6,10,12–16] Although these reports provide evidence that PPARγ regulates pathways contributing to PH pathogenesis, the precise mechanisms underlying these therapeutic effects remain to be completely defined.

The current study examines the interaction between PPARγ and hypoxia-inducible factor-1 (HIF-1), the master regulator of the cell's transcriptional response to low oxygen levels.[17–23] Hypoxia is a common stimulus of PH that activates genes responsible for angiogenesis, vasoconstriction and proliferation in pulmonary vascular cells. These hypoxia-induced processes, while adaptive in the short term, lead to pathological pulmonary vascular remodeling with chronic exposure.[24,25] HIF-1 is composed of a constitutively expressed β subunit and an oxygen-regulated α subunit.[26] In normoxia, HIF-1α undergoes hydroxylation by prolyl hydroxylase enzymes that target it for ubiquitination and proteosomal degradation. However, because oxygen is a requisite cofactor for prolyl hydroxylase activity, under hypoxic conditions, the prolyl hydroxylases are inhibited, and HIF-1α is stabilized. Upon stabilization, HIF-1α translocates from the cytoplasm to the nucleus where it forms a dimer with HIF-1β and regulates the transcription of over 100 target genes.

HIF-1 promotes cell survival during hypoxia by shifting cellular metabolism and ATP generation from aerobic respiration to glycolysis.[27] This metabolic shift is caused in part through HIF-1-mediated increases in the expression of its downstream target, the pyruvate dehydrogenase kinase-1 (PDK-1). The PDK-1 phosphorylates and inhibits pyruvate dehydrogenase (PDH), preventing the conversion of pyruvate to acetyl-coenzyme A and thereby inhibiting mitochondrial aerobic respiration.[27,28] This slows pyruvate metabolism through the tricarboxylic acid cycle.[21,29] Reduced tricarboxylic acid activity shunts pyruvate away from the mitochondrial electron transport chain thereby attenuating mitochondrial aerobic respiration and stimulating glycolysis. Substantial evidence supports a critical role for HIF-1-mediated, PDK-1-induced metabolic alterations in cell responses to hypoxia. For example, HIF-1α −/− mouse embryonic fibroblasts (MEFs) exposed to hypoxia had higher

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levels of mitochondrial reactive oxygen species (ROS) and underwent hypoxia-induced apoptosis, whereas wild-type MEFs, and HIF-1α −/− MEFs with forced expression of PDK-1, did not undergo hypoxia-induced apoptosis and instead exhibited enhanced survival.[27] These results suggest that without the HIF-1/PDK-1-induced metabolic shifts during hypoxia, toxic levels of mitochondrial ROS accumulate and cells undergo apoptosis instead of proliferation. Although this glycolytic shift allows pulmonary vascular cells to adapt to hypoxia, it also contributes to the proliferative, apoptosis-resistant phenotype that contributes to pulmonary vascular remodeling and PH pathogenesis.

Recent evidence suggests that the activation of PPARγ may inhibit HIF-1α.[30–32] Mitochondrial ROS generation stabilizes HIF-1α, and several reports indicate that activation of PPARγ using agonists such as rosiglitazone (RSG) or pioglitazone decreases cellular ROS and HIF-1α activation.[30–33] Consistent with the critical role of ROS enabling HIF-1 signaling, the administration of antioxidants also decreased HIF-1α protein levels.[32] In addition to inhibiting redox-mediated HIF-1 activation, PPARγ activation can also inhibit other proinflammatory transcription factors, such as NF-κB, through more direct and inhibitory transrepression mechanisms.[34] These reports suggest multiple mechanistic pathways by which PPARγ activation might inhibit HIF-1 signaling. The current study sought to explore this interaction using *in vitro* models of hypoxia-induced pulmonary vascular cell proliferation, as previous reports demonstrate that hypoxia reduces PPARγ expression in these models.[3,4,7,10] We hypothesized that PPARγ activation would attenuate HIF-1α activation and thereby decrease transcription of its downstream target PDK-1 in human pulmonary artery smooth muscle cells (HPASMCs). HPASMCs were exposed to hypoxia, and selected groups were treated with the PPARγ-agonist, RSG. Our findings indicate that PPARγ activation can prevent hypoxia-induced HIF-1α signaling but that intervening with PPARγ activation following the onset of hypoxia exposure fails to inhibit HIF-1 and its downstream targets. These studies provide additional insights into the mechanisms underlying the therapeutic effects of PPARγ activation in PH pathogenesis.

## METHODS

### Cell Culture

HPASMCs were purchased from Lonza (Walkersville, MD) and cell monolayers (passages 3–4) were grown at 37°C in a 5% $CO_2$ atmostphere in culture media (SmGM-2, Lonza) containing 2% fetal calf serum, growth factors and antibiotics as prevoiusly reported.[7]

### Hypoxia Exposure

HPASMCs (passage 4–7) were exposed to hypoxia in a Biospherix (Lacona, NY) exposure chamber. For normoxic conditions, HPASMCs were placed into a standard cell incubator maintained at 37°C with 21% $O_2$ and 5% $CO_2$. For hypoxic conditions, HPASMCs were placed in a hypoxia chamber maintained at 37°C, 1% $O_2$ and 5% $CO_2$ levels. HPASMCs were cultured in normoxic or hypoxic conditions for up to 72 hours as indicated. To investigate the effects of PPARγ activation, RSG (10 μM) or an equivalent volume of vehicle was added to the culture media for the duration of the experiment or for the final 24 hours of the 72-hour exposure period. All manipulations of cells exposed to hypoxia were

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performed in a glove box that maintains a hypoxic environment (1% $O_2$) to avoid the reoxygenation effects during sample processing.

## Western Blotting of Total Cell and Nuclear Protein Extracts

To analyze HIF-1α activation and nuclear translocation, nuclear extracts were prepared from HPASMC. HPASMCs were washed with ice-cold phosphate-buffered saline and resuspended in 400 μl of buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol and a protease and phosphatase inhibitor cocktail (Complete Mini, EDTA-free and PhosSTOP, Roche). After 15 minutes, NP-40 was added to a final concentration of 0.6%, and the samples were centrifuged to collect the supernatants containing the cytoplasmic proteins. The pelleted nuclei were resuspended in 50 μl of buffer B (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, protease and phosphatase inhibitors). After 30 minutes incubation in ice, lysates were centrifuged, and supernatants containing the nuclear proteins were collected.

Nuclear extracts were immunoblotted to determine HIF-1α nuclear translocation. An equivalent amount of protein was loaded per lane and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (4–12% gradient gels, Invitrogen) followed by transfer of proteins onto nitrocellulose membranes. After appropriate blocking, the blots were probed with primary antibodies (1:1,000) specific to human HIF-1α (Cell Signaling), fibrillarin (Cell Signaling), alpha-tubulin (Cell Signaling), PDK-1 (Enzo Life Sciences), CDK4 or β-actin (Santa Cruz) in 5% bovine serum albumin (for HIF-1a and fibrillarin) or 5% nonfat dry milk in tris-buffered saline containing 0.1% tween 20 (TBST) on a rocking platform overnight at 4°C. After washing 3 times with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). After washing as before, proteins were visualized with fluorescent anti-mouse or antirabbit secondary antibodies using the Licor System (Licor, Lincoln, NE). Bands for proteins of interest were quantified by densitometry. Nuclear extracts were normalized to fibrillarin levels within the same lane. The purity of the nuclear extract was determined by quantification of the alpha-tubulin level (a cytoplasmic marker) in the same lane. Cell lysates were normalized to CDK4 or β-actin levels in the same lane.

To analyze PDK-1 expression, HPASMC lysates were suspended in lysis buffer (20 mM Tris pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate, 100mM NaCl, 10mM NaF and 1 mM $Na_3VO_4$) containing protease and phosphatase inhibitors (Complete Mini, EDTA-free and Phos-STOP, Roche). HPASMC suspensions were sonicated and clarified by centrifugation.

## siRNA-Mediated Knockdown of HIF-1α in HPASMC

Subconfluent (50–60%) HPASMCs were transfected with 50 nM of nontargeting control siRNA or siGENOME smart pool siRNA targeting human HIF-1α (Dharmacon Inc., Lafayette, CO) for 12 hours after which the transfection cocktail was aspirated, and the cells were washed with serum-free media and allowed to grow in regular growth media for 12 hours. After replacing with fresh growth media, HPASMCs were incubated in normoxia or

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hypoxia for 72 hours, and cell lysates were analyzed for PDK-1 and HIF-1α protein expression.

### Quantitative Real-time Polymerase Chain Reaction Analysis

RNA was extracted from HPASMCs using an RNeasy kit (Quiagen, Valencia, CA) according to the manufacturer's protocol. The concentration of mRNA was quantified using an ND-1000 Spectrophotometer (NanoDrop Technology, Wilmington, DE). Further, 1 μg of RNA was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on samples using a Bio-Rad iCycler and analyzed by its software. The qRT-PCR specific primers were designed using Probe-Finder web-based software (Roche, Indianapolis, IN). PDK-1 and HIF-1α mRNA levels were normalized to 9S rRNA reference gene (forward: 5′CTGACGCTTGATGAGAAG GAC 3′; reverse: 3′ CCGCCGCCATAAGGAGAAC5′) or to GAPDH reference gene (forward: 5′GCCCAATACGACCAAATCC3′; reverse: 3′AGCCACATCGCTCAGACAC5′). The comparative threshold cycles ($C_t$) values were normalized to the 9S rRNA or GAPDH reference gene using the $2^{-\Delta\Delta C_t}$ method.[35]

### Statistical Analysis

In all experiments, data were analyzed by one-way analysis of variance followed by post hoc analysis with the Student Neuman Keuls test to detect differences between individual groups. Statistical significance was defined as $P < 0.05$, and all graphs are expressed as mean ± standard error of the mean. All statistical analyses were performed using GraphPad Prism software (La Jolla, CA).

## RESULTS

### Hypoxia Causes Transient HPASMC HIF-1α Activation

In previous reports, we demonstrated that hypoxia (1% $O_2$ for 72 hours) caused significant HPASMC proliferation.[31,36] Using this identical model, HPASMCs were exposed to normoxia (21% $O_2$) or hypoxia (1 % $O_2$) for 2–72 hours. HIF-1α mRNA levels were elevated following 2 hours of hypoxia exposure, but similar to normoxic levels at 8 hours of hypoxia exposure, and significantly decreased compared with normoxic levels at both 24 and 72 hours of hypoxia exposure (Figure 1A). In contrast, HIF-1α nuclear protein levels were elevated 4-fold after 4 hours of hypoxia exposure and almost 3-fold after 8 hours of hypoxia exposure (Figure 1B) indicating HIF-1α activation. However, following 72 hours of hypoxia exposure, nuclear levels of HIF-1α protein were not significantly different from baseline (Figure 1B). These results are consistent with previous studies using *in vivo* and *in vitro* models demonstrating that HIF-1α is transiently activated during sustained hypoxia.[37]

### RSG Attenuates Hypoxia-induced HPASMC HIF-1α Activation

Having confirmed the time course of hypoxia-induced HIF-1a activation in HPASMCs, the effect of PPARg activation on hypoxic HIF-1a signaling was examined. To address whether PPARg activation down-regulates hypoxia-induced HIF-1a mRNA levels, HPASMC were pretreated with RSG and exposed to normoxia or hypoxia for 2 hours (to coincide with peak hypoxia-induced increases in HIF-1a mRNA levels, Figure 1A). As illustrated by Figure 2A,

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treatment with RSG attenuated hypoxia-induced HIF-1a mRNA levels. To address the effect of PPARg activation on hypoxia-induced HIF-1a nuclear protein levels, HPASMCs were exposed to hypoxia for 4 hours to coincide with peak nuclear hypoxic HIF-1a translocation (Figure 1B), and selected cells were simultaneously treated with the PPARg agonist, RSG (10 μM). Consistent with down-regulation of HIF-1a mRNA levels, treatment with RSG attenuated HIF-1α nuclear protein levels in HPASMCs exposed to hypoxia for 4 hours (Figure 2B) suggesting that PPARg activation attenuates transcriptional and post-transcriptional HIF-1a activation pathways." Please also accordingly replace "a" with the Greek letter "alpha" in HIF-1a and "g" with the Greek letter "gamma" in PPARg.

## RSG Attenuated Hypoxia-induced HPASMC PDK-1 Expression at Early Time Points

Because RSG attenuated hypoxic PASMC HIF-1α activation, the regulation of the HIF-1α target gene, PDK-1, by PPARγ activation was examined in HPASMCs exposed to hypoxia and treated with ±RSG (10 μM). As illustrated in Figure 3A, treatment with hypoxia caused progressive increases in HPASMC PDK-1 mRNA levels over 24 hours. Treatment with RSG attenuated increases in PDK-1 mRNA levels at 8 hours of hypoxia exposure (Figure 3B). Consistent with mRNA levels, PDK-1 protein levels were also elevated following 24 hours of hypoxia exposure compared with the normoxic control, and with RSG, PDK-1 protein levels decreased (Figure 3C).

## RSG Fails to Attenuate Hypoxia-induced HPASMC PDK-1 Expression at 72 Hours

We previously reported that the HPASMC proliferation caused by exposure to 1% $O_2$ for 72 hours was attenuated by treatment with PPARγ ligands limited to the final 24 hours of hypoxia exposure.[7,8] Therefore, to determine if these antiproliferative effects of PPARγ ligands were mediated by alterations in HIF-1 and its target genes, namely PDK-1 and GLUT1, HPASMCs were exposed to normoxia or hypoxia for 72 hours, and selected cells were treated with RSG during the final 24 hours of exposure. As illustrated in Figure 4A and B, exposure to hypoxia for 72 hours increased HPASMC PDK-1 and GLUT1 protein levels. However, treatment with RSG during the final 24 hours of hypoxia failed to attenuate the increased expression of these proteins. Because Figure 1 demonstrated that hypoxia-induced increases in nuclear HIF-1α levels were no longer present at 72 hours, yet the HIF-1 targets, PDK-1 and GLUT1, were increased at 72 hours, we investigated activation of HIF-2α isoform. As shown in Figure 4C, HPASMC HIF-2α protein levels were increased following 72 hours hypoxia exposure. However, RSG treatment during the final 24 hours of hypoxia failed to attenuate HIF-2α expression. These findings are consistent with previous reports of cellular switching from HIF-1α to HIF-2α signaling during responses to chronic hypoxia.[40]

## siRNA-mediated HIF-1α Depletion Attenuates Chronic Hypoxia-induced PDK-1 Protein Expression in HPASMC

To determine if early hypoxic HIF-1α activation contributes to induction of PDK-1 expression during chronic hypoxia, HPASMCs were transfected with control siRNA or HIF-1α siRNA and then subjected to normoxia or hypoxia for 72 hours. Immunoblotting confirmed substantial knockdown of HIF-1α in siRNA-treated HPASMCs (Figure 5). Densitometric analysis of cell lysates revealed significant attenuation of hypoxia-induced PDK-1 protein expression in cells depleted of HIF-1α. These observations suggest that even

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though HIF-1α activation is not sustained during chronic hypoxia, HIF-1α is necessary for induction of metabolic responses to hypoxia.

## DISCUSSION

PH is an important clinical problem resulting from complex derangements in pulmonary vascular wall cells that lead to structural and functional changes in the pulmonary circulation. Existing therapies are not optimally effective, in part, because they promote vasodilation but fail to address the vascular remodeling and the proliferative vascular cell phenotypes that are central to PH pathogenesis resulting in significant residual morbidity and mortality.[41] Evolving evidence suggests that metabolic derangements such as reduced oxidative phosphorylation, enhanced glycolysis and resistance to apoptosis are major contributing factors to the proliferative pulmonary vascular cell phenotype seen in PH,[17,23,42] and these metabolic derangements are associated with activation of HIF-1α signaling pathways.

In previous studies, we showed that stimulating the metabolic regulator, PPARγ attenuates PH.[10,31,36] Mechanisms for these protective effects involve alterations in the transcriptional and posttranscriptional regulation of important mediators in PH pathogenesis such as endothelin-1, Nox4 and thrombospondin.[31,36,43,44] Reductions in PPARγ expression are associated with PH[3–11] whereas stimulating PPARγ with exogenous ligands reduces PH in multiple experimental models.[6,10,12–16] Because activation of HIF-1α has been associated with PH[17,23,42] and because PPARγ has been reported to inhibit HIF-1α in other systems,[30–33] we hypothesized that PPARγ would inhibit HIF-1α and that this would contribute to its therapeutic effects in PH. The goal of the current study was to determine if PPARγ activation reduced HIF-1α signaling in hypoxia-exposed pulmonary vascular wall cells. Our study provides novel evidence that the activation of PPARγ attenuates HIF-1α signaling in HPASMCs exposed to acute hypoxia but that alternative and likely HIF-1α-independent mechanisms account for the antiproliferative effects of PPARγ activation following more chronic hypoxia exposure.

Consistent with a previous report,[37] we found that HPASMC HIF-1α is activated rapidly after the onset of hypoxia, as evidenced by increases in HIF-1α mRNA in HPASMCs exposed to 2 hours of hypoxia, as well as 4- and 3-fold increases in HIF-1α nuclear protein levels in HPASMCs exposed to 4 and 8 hours of hypoxia, respectively. Although reductions in HIF-1α mRNA and protein suggest transient HIF-1α activation and signaling despite continued hypoxia exposure, reports that HIF-1α −/− mice were protected from hypoxic PH[45] indicate that HIF-1α signaling plays a key role in PH pathogenesis. Our results demonstrate that activating PPARγ coincident with the onset of hypoxia exposure attenuated HPASMC HIF-1α activation. To our knowledge, this is the first article to examine the effect of PPARγ activation on HIF-1α signaling in HPASMCs. PPARγ activation reduced expression of HIF-1α in cancer stem cells[46] and increased HIF-1α in breast cancer cells,[47] whereas both transcription factors were upregulated in cardiac hypertrophy.[48] We previously reported that RSG attenuated HIF-1α activation in hypoxic endothelial cells,[31] and pioglitazone similarly attenuated HIF-1α in skeletal muscle through effects on mitochondrial function.[30] Taken together, these reports along with the current findings

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suggest that tissue- or cell-specific mechanisms may determine the effect of PPARγ activation on HIF-1α signaling.

Not surprisingly, our findings also demonstrate that the relative timing of the activation of these 2 transcription factors plays a critical role in their ultimate effect on pulmonary vascular wall cells. In the current study, treating HPASMCs with the PPARγ ligand, RSG, at the outset and for the duration of hypoxia exposure not only attenuated HIF-1α activation (Figure 2) but also attenuated hypoxic induction of the HIF-1α target gene, PDK-1 (Figure 3). We focused on PDK-1 as well as GLUT1 in the current study because these HIF-1-regulated targets are believed to be critical mediators of the glycolytic and hyperproliferative cell phenotypes that cause pulmonary vascular remodeling in PH[38,39] Interestingly, HPASMC PDK-1 and GLUT1 levels were elevated following 72 hours of hypoxia exposure (Figure 4), a time at which HIF-1α activation was no longer detected (Figure 1). Even though HIF-1α levels were no longer elevated following 72 hours of hypoxia exposure, activation of HIF-2α was observed (Figure 4C) consistent with previous reports of shifting from HIF-1α to HIF-2α during chronic cellular responses to hypoxia.[40] The lack of HIF-1α activation following 72 hours of hypoxia exposure and the inability of PPARγ activation to attenuate hypoxia-induced increases in HIF-2α, PDK-1 or GLUT1 levels at 72 hours (Figure 4) indicate that the antiproliferative effects of PPARγ activation initiated 48 hours after the onset of hypoxia are likely HIF-1-independent.

The results in Figure 5 using siRNA to prevent HIF-1α activation emphasize that both basal and hypoxia-induced HPASMC PDK-1 expression is HIF-1α-dependent. We previously reported that PPARγ activation at 48 hours in this identical hypoxia-exposed HPASMC model successfully reversed hypoxia-induced activation of ERK1/2 and NF-κB and upregulation of Nox4, TSP-1 and ET-1.[7,31,36,43,44] Taken together, these observations indicate that although PPARγ activation can prevent HIF-1α activation in HPASMC, mechanisms for the favorable effects of PPARγ intervention during chronic hypoxia are more attributable to HIF-1α-independent signaling mechanisms.

Although this study clarifies effects of RSG on HIF-1α, the mechanism by which PPARγ activation attenuates HIF-1α remains uncertain. We show that RSG attenuates HIF-1α protein in acute hypoxia; however, RSG has little effect on HIF-1α mRNA during this time period. These results suggest that activation of PPARγ regulates HIF-1α via posttranscriptional or posttransla-tional mechanisms. Additional studies would be required to determine if this regulation is mediated by direct binding of PPARγ to HIF-1α that might prevent its nuclear translocation, or if the suppression is through indirect mechanisms such as increased proteasomal degradation or decreased stabilization of HIF-1α by ROS. Further definition of the molecular mechanisms regulating interactions between PPARγ and HIF-1α can facilitate the optimal application and development of pharmacological targeting of the PPARγ receptor.

Our study has a number of important limitations. The level of hypoxia employed in our *in vitro* HPASMC model is severe and may exaggerate levels of hypoxia to which pulmonary vascular wall cells are exposed under pathophysiological conditions. Further, examining pulmonary vascular cells in isolation *in vitro* fails to accurately reproduce the many

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additional and important factors modulating cell function in the pulmonary vascular wall *in vivo* including cell-cell and cell-matrix interactions, the influence of physical forces, for example, pressure and flow and effect of circulating factors on the vascular wall. In addition, the concentration of RSG used in our *in vitro* studies likely exceeds that found in the serum of patients taking thiazolidinediones. Finally, as illustrated by our time-course studies, hypoxia activates dynamic signaling mechanisms in pulmonary vascular wall cells that change over time. The contributions of pathways prominent in determination of acute cellular responses to hypoxia may diminish over time in response to chronic hypoxia exposure.

Despite these limitations, our findings provide several new and important findings. First, they confirm that the activation of PPARγ with RSG in HPASMC can suppress hypoxia-induced HIF-1α activation and expression of its targets, for example, PDK-1 that contributes to the hypoxia-induced metabolic switch from aerobic to anaerobic respiration. However, based on the timing of PPARγ activation, the current findings also clarify that previously described beneficial effects of PPARγ activation in hypoxia-exposed HPASMCs are likely not attributable to HIF-1α suppression. These results emphasize that the mechanisms by which PPARγ modulates pulmonary vascular cell biology vary with time. Because the most effective therapies for PH would likely be those that target multiple pathologic pathways, we believe that these observations support the continued investigation of PPARγ as a novel therapeutic target in PH with potential to modulate vasoconstriction and smooth muscle cell proliferation.

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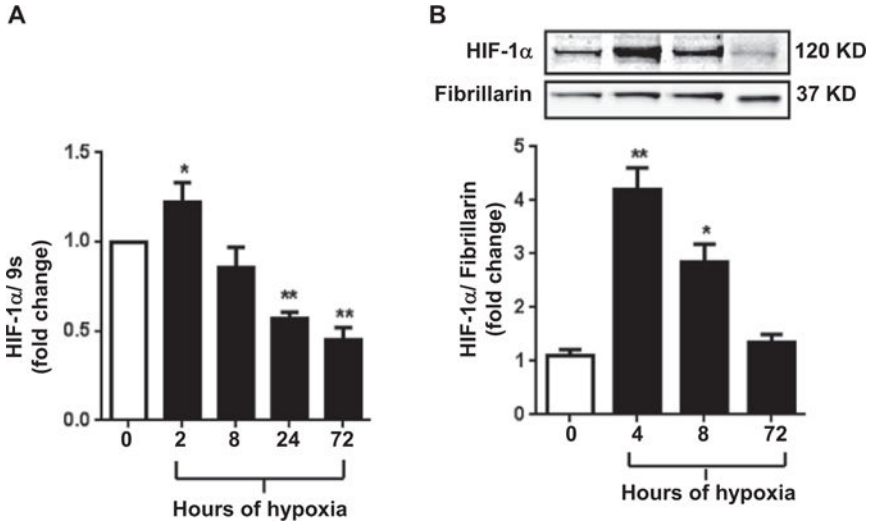


**FIGURE 1.**

Time course of hypoxia-induced HIF-1α activation in HPASMCs. (A) HPASMC HIF-1α mRNA levels were measured with qRT-PCR following exposure to hypoxia (1% $O_2$) for 2, 8, 24 or 72 hours. Data are expressed relative to ribosomal 9S and displayed as fold change versus (vs.) normoxic (21% $O_2$) control ± SE at the same time point ($n = 4–16$). $^{*}P < 0.05$ vs. normoxia and $^{**}P < 0.01$ vs. normoxia. (B) Quantitative densitometric analysis of Western blots for HIF-1α in nuclear protein extracts of HPASMCs exposed to normoxia (*N*, 21% $O_2$) or hypoxia (1% $O_2$) for 4 and 8 hours. In separate experiments, cells were exposed to normoxia or hypoxia for 72 hours. Each bar represents mean ± SE HIF-1α nuclear protein relative to fibrillarin levels in the same sample expressed as fold change over normoxia ($n = 3–7$). $^{*}P < 0.05$ vs. normoxia and $^{**}P < 0.01$ vs. normoxia. SE, standard error.

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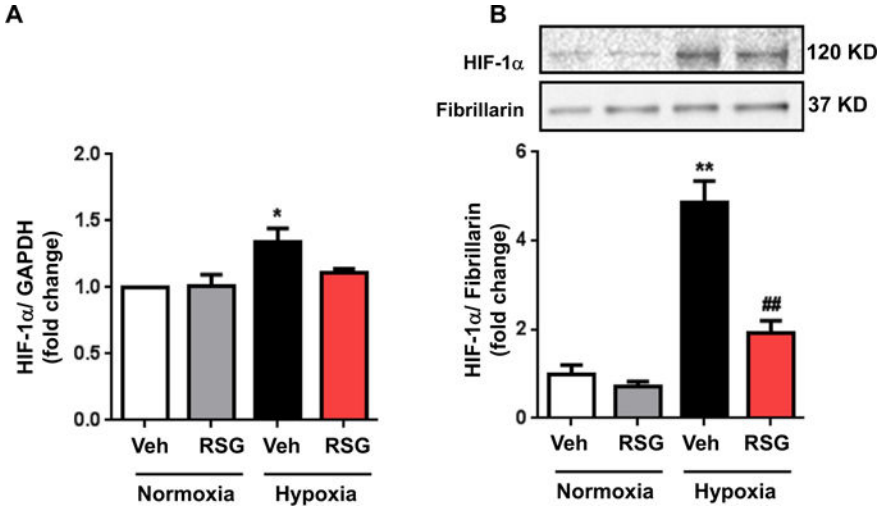


**FIGURE 2.**

Rosiglitazone attenuates early HIF-1α expression in HPASMCs exposed to hypoxia. HPASMCs were exposed to normoxia (21% $O_2$) or hypoxia (1% $O_2$) and simultaneously treated with DMSO (Veh) or rosiglitazone (RSG) (10 μM) for 2–4 hours. (A) HPASMC HIF-1α mRNA levels following treatment for 2 hours. Each bar graph represents mean ± SE HIF-1α mRNA normalized to GAPDH in the same sample expressed as fold change over normoxia ($n = 3$). SE, standard error. (B) Representative immunoblots and averaged desitometric analysis of Western blotting for HIF-1α levels in HPASMC nuclear protein extracts treated for 4 hours. Each bar represents mean ± SE nuclear HPASMC HIF-1α protein levels relative to fibrillarin in the same sample expressed as fold change over normoxia ($n = 3$). $^{**}P < 0.05$ versus (vs.) Normoxia-Veh and $^{\#\#}P < 0.001$ vs. Hypoxia-Veh.

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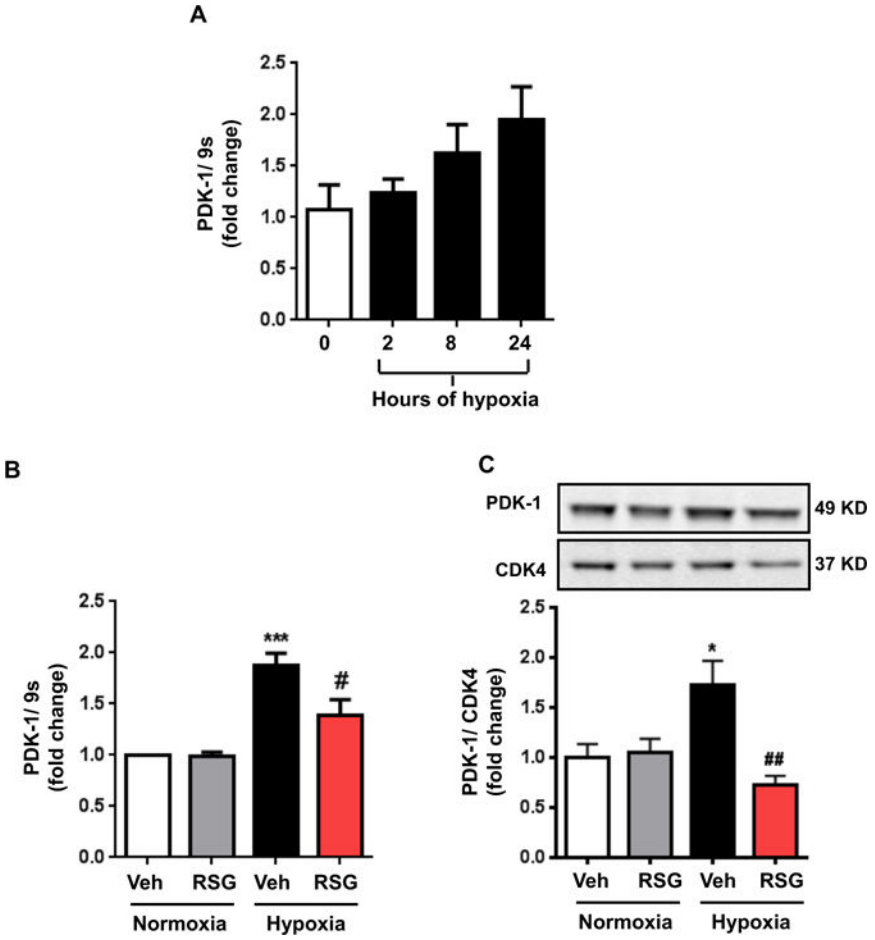


**FIGURE 3.**

Hypoxia-induced PDK-1 expression in HPASMCs is attenuated by rosiglitazone. (A) HPASMCs PDK-1 mRNA levels were measured with qRT-PCR following exposure to hypoxia (1% $O_2$) for 0, 2, 8 or 24 hours. Each bar represents the mean ± SE PDK-1 mRNA relative to ribosomal 9S expressed as fold change versus (vs.) normoxic control ($n = 4$). HPASMCs were then exposed to normoxia (21% $O_2$) or hypoxia (1% $O_2$) for 8 or 24 hours and simultaneously treated with DMSO (Veh) or rosiglitazone (RSG, 10 μM). (B) HPASMC PDK-1 mRNA levels were determined with qRT-PCR following exposure to hypoxia for 8 hours. Each bar represents mean ± SE PDK-1 mRNA relative to ribosomal 9S expressed as fold change vs. normoxic control ($n = 4$). $^{***}P < 0.001$ vs. Normoxia-Veh and $^{\#}P < 0.05$ vs. Hypoxia-Veh. (C) PDK-1 protein levels were examined in HPASMC lysates by Western blot analysis following exposure to hypoxia for 24 hours. Each bar represents mean ± SE PDK-1 relative to CDK4 levels in the same sample expressed as fold change over normoxia ($n = 7$). $^{*}P < 0.05$ vs. normoxia. $^{\#\#}P < 0.01$ vs. Hypoxia-Veh. SE, standard error.

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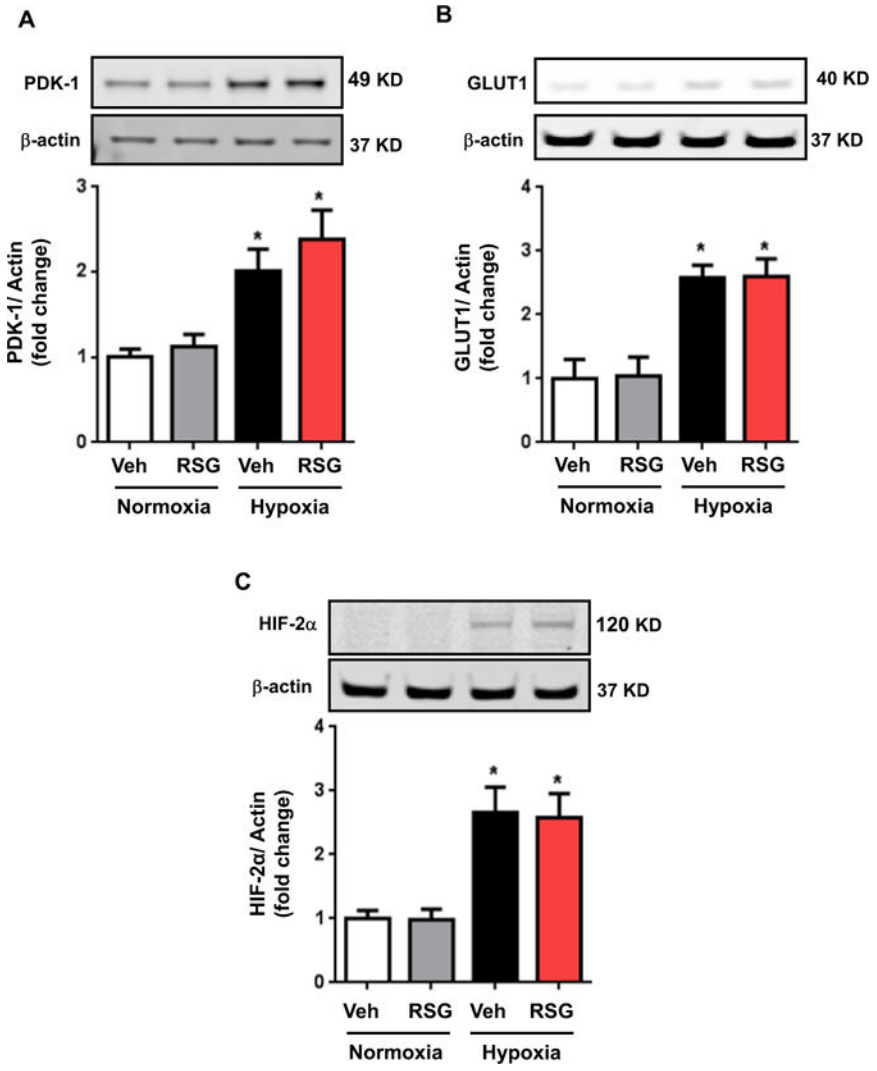


**FIGURE 4.**

Rosiglitazone fails to attenuate chronic hypoxia-induced PDK-1 and GLUT1 protein expression in HPASMC. HPASMCs were exposed to normoxia (21% $O_2$) or hypoxia (1% $O_2$) for 72 hours. During the final 24 hours of exposure, HPASMCs were treated with DMSO (Veh) or rosiglitazone (RSG, 10 μM). Western blotting was performed to determine PDK-1 (A), GLUT1 (B) or HIF-2α (C) levels in HPASMC. Each bar represents the mean ± SE PDK-1, GLUT1, or HIF-2α levels relative to β-actin in the same sample expressed as fold change over normoxia ($n = 6$). $^{*}P < 0.05$ versus Normoxia. SE, standard error.

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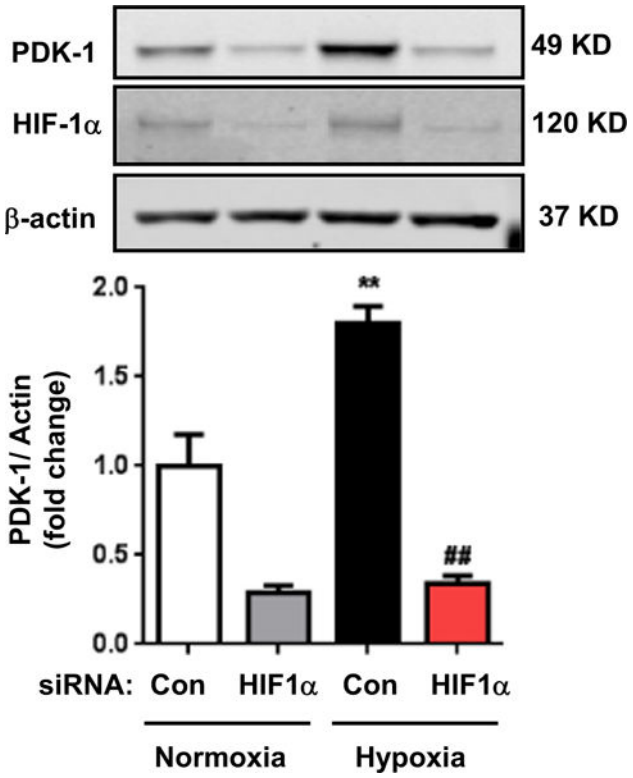


**FIGURE 5.**

Depletion of HIF-1α attenuates chronic hypoxia-induced PDK-1 expression in HPASMC. HPASMCs were transfected with control siRNA or HIF-1α siRNA (to deplete HIF-1α) and then exposed to normoxia or hypoxia for 72 hours. Western blotting was performed to determine PDK-1 levels. Depletion of HIF-1α was verified by Western blotting using an antibody against HIF-1α. Each bar represents mean ± SE PDK-1 level relative to β-actin in the same sample expressed as fold change over normoxia ($n = 3$). $^{**}P < 0.01$ versus (vs.) Normoxia; $^{\#\#}P < 0.01$ vs. hypoxia. SE, standard error.