Rosiglitazone Attenuates Chronic Hypoxia-Induced Pulmonary Hypertension in a Mouse Model

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Chronic hypoxia contributes to pulmonary hypertension through complex mechanisms that include enhanced NADPH oxidase expression and reactive oxygen species (ROS) generation in the lung. Stimulation of peroxisome proliferator-activated receptor γ (PPAR γ) reduces the expression and activity of NADPH oxidase. Therefore, we hypothesized that activating PPAR γ with rosiglitazone would attenuate chronic hypoxia-induced pulmonary hypertension, in part, through suppressing NADPH oxidase-derived ROS that stimulate proliferative signaling pathways. Male C57Bl/6 mice were exposed to chronic hypoxia (CH, FIO₂ 10%) or room air for 3 or 5 weeks. During the last 10 days of exposure, each animal was treated daily by gavage with either the PPAR γ ligand, rosiglitazone (10 mg/kg/d) or with an equal volume of vehicle. CH increased: (1) right ventricular systolic pressure (RVSP), (2) right ventricle weight, (3) thickness of the walls of small pulmonary vessels, (4) superoxide production and Nox4 expression in the lung, and (5) platelet-derived growth factor receptor β (PDGFR β) expression and activity and reduced phosphatase and tensin homolog deleted on chromosome 10 (PTEN) expression. Treatment with rosiglitazone prevented the development of pulmonary hypertension at 3 weeks; reversed established pulmonary hypertension at 5 weeks; and attenuated CH-stimulated Nox4 expression and superoxide production, PDGFR β activation, and reductions in PTEN expression. Rosiglitazone also attenuated hypoxia-induced increases in Nox4 expression in pulmonary endothelial cells *in vitro* despite hypoxia-induced reductions in PPAR γ expression. Collectively, these findings indicate that PPAR γ ligands attenuated hypoxia-induced pulmonary vascular remodeling and hypertension by suppressing oxidative and proliferative signals providing novel insights for mechanisms underlying therapeutic effects of PPAR γ activation in pulmonary hypertension.

Keywords: hypoxia; PPAR γ ; rosiglitazone; pulmonary hypertension

Pulmonary hypertension, a devastating disease that causes significant morbidity and mortality, is defined as an elevation of mean pulmonary artery pressure above 25 mm Hg at rest or above 30 mm Hg with exercise. In 2002, 15,668 deaths and 260,000 hospital visits were attributed to patients with pulmonary hypertension (1). Pulmonary hypertension is most commonly caused by diverse clinical conditions that produce chronic alveolar hypoxia, including chronic obstructive pulmonary disease, obstructive sleep apnea, and high-altitude living. These conditions promote pulmonary vasoconstriction, vascular remodeling, and endothelial dysfunction leading to the development of pulmonary hypertension. However, the underlying pathogenesis of this disorder remains to be fully elucidated.

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Chronic hypoxia has been shown to cause pulmonary hypertension in epidemiologic studies in human subjects and in various animal models. Alterations in nitric oxide (NO) bioavailability and in the production of reactive oxygen species (ROS) have been implicated as important mechanisms by which hypoxia induces pulmonary vascular dysfunction. In addition to evidence that depletion of NADPH oxidase prevented hypoxia-induced pulmonary hypertension in the mouse (2, 3), hypoxia caused increased Nox4 expression in the mouse lung (4). Together, these reports indicate that NADPH oxidase-derived superoxide is an important mediator in pulmonary hypertension caused by hypoxia. These observations led us to further investigate the efficacy of strategies that reduce hypoxia-stimulated, oxidant-mediated signals in the treatment of experimental pulmonary hypertension.

Several studies have suggested a potential role for PPAR γ in the regulation of vascular nitroso-redox balance. PPAR γ is a ligand-activated transcription factor that belongs to the nuclear hormone receptor superfamily. Structurally diverse ligands activate PPAR γ including thiazolidinediones, a class of anti-diabetic medications. PPAR γ is expressed in many tissues and is an important regulator of genes involved in cellular differentiation and growth, inflammation, apoptosis, and angiogenesis (5–8). Our lab has shown that PPAR γ ligands stimulate NO release from endothelial cells through PPAR γ -dependent signaling pathways (9, 10). This enhanced endothelial NO release was not related to increased endothelial nitric oxide synthase (eNOS) expression but was mediated, in part, by alterations in the post-translational regulation of eNOS activity (10). PPAR γ ligands also produced coordinate reductions in endothelial NADPH oxidase expression and activity and increased Cu/Zn superoxide dismutase expression and activity (11). Collectively, these studies suggest that activation of the PPAR γ receptor has potential to simultaneously reduce superoxide generation and enhance NO production in vascular cells.

PPAR γ activation may also attenuate signaling pathways associated with vascular smooth muscle cell proliferation and remodeling. For example, recent evidence indicates that PPAR γ ligands, through inhibition of platelet-derived growth factor (PDGF) signaling, attenuate pulmonary hypertension in ApoE-deficient mice fed a high-fat diet (12). Moreover, several reports have demonstrated that PPAR γ ligands inhibit PDGF-stimulated vascular smooth muscle cell migration *in vitro* (13, 14). PDGF receptor activation promotes cell proliferation, migration, and survival through complex downstream signaling cascades, including the phosphatidylinositol-3-kinase (PI3-kinase) pathway (15). The dual specificity phosphatase, phosphatase tensin homolog deleted on chromosome 10 (PTEN), has the capacity to dephosphorylate and thereby inactivate the PDGF receptor (16, 17) as well as dephosphorylate PIP₃ to PIP₂, thereby attenuating Akt activation (16). In addition, PTEN is inhibited by ROS (18, 19), and its expression can be stimulated by PPAR γ ligands (20, 21). On the other hand, smooth muscle-targeted depletion of either PTEN (22) or PPAR γ (23) caused pulmonary hypertension in mice. Collectively, these findings prompted our examination of PPAR γ

and its ability to regulate PDGF and PTEN signaling pathways in the lung during hypoxia.

Several reports indicate that ligand-induced PPAR γ activation attenuates pulmonary vascular dysfunction in animal models of pulmonary hypertension, although the mechanisms for these effects have not been completely defined. For example, PPAR γ activation with either pioglitazone or troglitazone significantly reduced pulmonary hypertension and pulmonary artery wall thickening in a rat model of monocrotaline-induced pulmonary hypertension (24). Similarly, treatment with rosiglitazone reduced hypobaric hypoxia-induced right ventricular hypertrophy and pulmonary artery remodeling in Wistar-Kyoto rats (25). Studies have also shown that PPAR γ activation reduced proliferation of vascular smooth muscle cells and promoted apoptosis in several cell lines *in vitro* (26). PPAR γ is abundantly expressed in pulmonary vascular endothelium in normal patients, but its expression was reduced in the characteristic plexiform lesions of patients with pulmonary hypertension (27). Therefore, the purpose of the current study was to explore PPAR γ as a therapeutic target in hypoxia-induced pulmonary hypertension in the mouse model and to examine whether PPAR γ activation modulates oxidative signaling pathways implicated in pulmonary vascular remodeling.

MATERIALS AND METHODS

Mouse Model of Chronic Hypoxia and Thiazolidinedione Treatment

Male C57Bl/6 mice (age 8–10 wk) were purchased from the Jackson Laboratory (Bar Harbor, ME) and exposed to chronic hypoxia (CH, FiO₂ 10%) or room air (Control) for 3 or 5 weeks. All animals had access to standard mouse chow and water, *ad libitum*, during both CH and normoxic conditions. Each mouse was weighed weekly and housed socially in groups of five mice per cage. During the last 10 days of exposure, each animal was gavaged daily with either the PPAR γ ligand, rosiglitazone (10 mg/kg/d) or with an equal volume of vehicle (100 μ l 0.5% methyl cellulose). All animal studies were approved by the Institutional Animal Care and Use Committee of the Atlanta VA Medical Center.

Measurement of Right Ventricular Systolic Pressure and Cardiac Chamber Size

Right ventricular systolic pressure (RVSP) was measured in mice lightly anesthetized with isoflurane. Before mice were killed, the right internal jugular vein was surgically exposed and cannulated with a 0.8-F microtip pressure transducer (Millar Instruments, Houston, TX) (28). The transducer was advanced into the right ventricle, and the right ventricular pressure was continuously monitored for 10 minutes. Heart rates (300–450 beats/min were deemed acceptable) and pressure waveforms were monitored to ensure the validity of the pressure measurements. Data were analyzed using a Powerlab system (AD Instruments, Denver, CO). At the end of the treatment period, mice were killed with isoflurane, and the hearts were removed. The free wall of the right ventricle (RV) was then carefully dissected from the left ventricle (LV) and septum (S), and each were individually weighed to permit calculation of the RV:LV + S weight ratio as an index of right ventricular hypertrophy.

Immunohistochemical and Morphometric Analysis

To reduce the contribution of vasoconstriction to the analysis of pulmonary vascular morphometry, lungs from Control and CH-exposed animals were perfused at pressures comparable to the RVSP for that group. Despite this preparation, the pulmonary vasculature may not have been maximally dilated before fixation, and the relative contributions of smooth cell contraction and increased tissue mass to the medial thickness cannot be determined with certainty. Lungs were then filled with optimal cutting temperature (OCT) compound via tracheostomy and frozen at -80°C . Sections (6 μm) from the lungs were fixed in 4% formaldehyde, washed three times (5 min each) in PBS, and

endogenous peroxidase activity was quenched with 3% H₂O₂ in PBS. Sections were permeabilized with 0.05% Tween-20 in PBS, blocked with 5% donkey serum, and incubated overnight at 4°C with anti- α -smooth muscle actin (α -SMA) antibody (LabVision Corporation, Fremont, CA). Sections were incubated with biotinylated donkey anti-rabbit 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), and sections were counterstained with hematoxylin and coverslipped. Rabbit IgG was used to control for nonspecific antibody binding. Multiple high-power photomicrographs were obtained using a Leica DM4000B microscope (Wetzlar, Germany). In each section, the number of α -SMA-positive blood vessels associated with intra-acinar airways was counted per high-power field, and those blood vessels with luminal diameters less than 100 μm were subjected to digital morphometric analyses. Luminal circumference (LC) and outer vessel circumference (OC) were determined from α -SMA-positive vessels using Scion Image Software (Scion Image Beta 4.0.2; Scion Corporation, Frederick, MD). The following formulae were used: lumen cross-sectional area = $(\text{LC})^2/4\pi$, and muscular wall thickness = $[(\text{OC}/2\pi) - (\text{LC}/2\pi)]$.

NADPH Oxidase Expression and Superoxide Production in Lung Tissue after Hypoxia

Real-time PCR was performed to quantify mRNA levels of Nox4. Briefly, isolation of total RNA from whole lung homogenates was performed according to the manufacturer's protocol (RNeasy Mini Kit; Qiagen, Valencia, CA). Total RNA was reverse transcribed using random primers and a SuperScript II kit (Invitrogen, Carlsbad, CA). The first-strand cDNA was purified using a microbiospin 30 column (Bio-Rad Laboratories, Hercules, CA) in Tris buffer and then stored at -80°C until used. Lung cDNA was amplified using a 96-well iCycler real-time thermocycler (Bio-Rad Laboratories). The mRNA copy numbers were calculated as fold-change compared with control. None of the treatment conditions had a significant effect on 18S or ribosomal protein S9 expression.

To examine Nox4 protein levels in lung tissue, lungs were pressure-perfused, then embedded in OCT and frozen at -80°C . Frozen lung sections (5 μm) were then prepared and stained with primary antibody to Nox4 (1:50; gift from Dr. David Lambeth, Emory University) followed by secondary rhodamine red-labeled antibody. Sections from each treatment group were examined by fluorescence microscopy, and images were acquired at $\times 40$ magnification using identical instrument settings.

Superoxide generation in the lung was examined using several complementary approaches. Frozen sections from mouse lung were subjected to estimation of superoxide generation using dihydroethidium (DHE). Lungs were perfused blood-free, then perfused with and embedded in optimal cutting temperature (OCT) compound and frozen at -80°C . Sections (30 μm) were then prepared and stained with DHE (10 μM) by covering the section with 30 μl of DHE and a coverslip followed by incubation at 37°C in a humidified, 5% CO₂ atmosphere for 30 minutes. Sections from each treatment group were examined by fluorescence microscopy, and images were acquired at $\times 20$ magnification using identical instrument settings. Alternatively, superoxide production was measured in lung samples based on previously published protocols with modifications for lung tissue. In brief, lungs were perfused with 5 ml PBS and excised. Three 3.5-mm biopsy punches were taken from each lung and incubated in 50 μM DHE Krebs-HEPES buffer for 30 minutes at 37°C . The tissue was then homogenized in 100% methanol, filtered, and subjected to high-performance liquid chromatography (HPLC) analysis to detect oxy-ethidium. Oxy-ethidium values were normalized to the protein concentration of the same sample (29). In separate experiments, superoxide production was measured using electron spin resonance (ESR) spectroscopy and the spin probe, 1-hydroxy-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine hydrochloride (CMH; Noxygen Science, Elzach, Germany), as we have previously reported (11, 30).

Western Blot Analysis of PDGFR β and PTEN

Immediately after mice were killed, homogenates of peripheral lung tissue were prepared and their protein concentrations determined as

previously reported (31). Whole lung homogenates (45 μ g protein/lane) were subjected to SDS-PAGE (4%-12% gradient gels) (Invitrogen) followed by electroblotting of proteins onto polyvinylidene fluoride (PVDF) or nitrocellulose membranes. After appropriate blocking, the blots were probed with primary antibodies (1:1,000) specific to platelet-derived growth factor receptor β (PDGFR β), phosphorylated-PDGFR β (P-PDGFR β), PTEN, or β -actin (Santa Cruz Biotechnology, Santa Cruz, CA) in 5% powdered nonfat dry milk on a rocking platform overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Levels of proteins were normalized to the β -actin content of the same sample. For all blots, immunodetection was performed using a chemiluminescence method (SuperSignal; Pierce Biotechnology, Rockford, IL), and relative immunoreactive levels of proteins were quantified using the ChemiDoc XRS imaging system and Quantity One software (version 4.5; Bio-Rad Laboratories).

In Vitro Hypoxia Exposure Studies

To more precisely define the impact of hypoxia on specific cell types in the pulmonary vascular wall, human pulmonary artery endothelial cells (HPAEC) and smooth muscle cells (HPASMC) were propagated in culture according to the manufacturer's (Lonza, Allendale, NJ) protocols. Confluent monolayers of HPAEC or HPASMC were then exposed to hypoxic conditions (1% O₂/5% CO₂) in a hypoxia exposure chamber (Biospherix, Lacona, NY) or to normoxic conditions (21% O₂/5% CO₂) for 72 hours. Rosiglitazone (10 μ M) or an equivalent volume of methyl cellulose vehicle was added to the media of each dish of cells for the final 24 hours of exposure to normoxic or hypoxic conditions. Hypoxia-exposed cells were collected in a glove box in a hypoxic (1% O₂) atmosphere to prevent artifactual loss of hypoxia-stimulated signals during cell processing. Cell homogenates were collected and subjected to Western blotting using antibodies directed against Nox4 (gift from Dr. David Lambeth, Emory University), PPAR γ (Bethyl Laboratories, Montgomery, TX), and CDK4 (Santa Cruz) followed by laser densitometry.

Statistical Analysis

In all experiments data were analyzed by one-way ANOVA followed by *post hoc* analysis with the Bonferroni correction to examine

differences between specific groups. The level of statistical significance was taken as $P < 0.05$.

RESULTS

CH-Induced Pulmonary Hypertension and Vascular Remodeling

As illustrated in Figure 1A, exposure to CH for 3 or 5 weeks caused pulmonary hypertension measured as significant increases in RVSP. Average heart rate for all animals after anesthesia was 385 ± 7.9 ($n = 31$), and neither CH exposure nor rosiglitazone treatment had a significant effect on heart rates measured at the time of RVSP measurements (data not shown). Treatment with rosiglitazone for the final 10 days (Days 12–21) of the 3-week CH regimen prevented CH-induced pulmonary hypertension. Furthermore, treatment with rosiglitazone for the final 10 days (Days 26–35) of the 5-week regimen reversed established pulmonary hypertension in CH-exposed mice. Compared with CH exposure for 3 weeks, CH exposure for 5 weeks produced an additional, but statistically insignificant, increase in RVSP.

To assess the impact of alterations in systemic or pulmonary pressures on cardiac mass, measurements of the weights of the right ventricle (RV) and left ventricle (LV) plus septum (S) were recorded. Compared with Control conditions, exposure to CH for 3 or 5 weeks significantly increased the ratio of RV:LV+S weight, an index of right ventricular hypertrophy (Figure 1B). These findings occurred in the absence of significant increases in the size or weight of the left ventricle (data not shown). Treatment with rosiglitazone significantly reduced elevations in the RV:LV+S ratio caused by exposure to CH for 3 or 5 weeks.

To examine the impact of CH on the distribution of muscular layers in small pulmonary vessels, the number of α -SMA-positive vessels less than 100 μ m was counted per microscopic field (Figure 2). Exposure to CH for 3 weeks increased the number of α -SMA-positive profiles observed per microscopic field con-

Figure 1. Rosiglitazone attenuates chronic hypoxia (CH)-induced pulmonary hypertension and right ventricular hypertrophy. Mice were exposed to room air (Control, C) or chronic hypoxia (CH, 10% oxygen) for 3 or 5 weeks. Ten days before killing, each mouse was gavaged with the peroxisome proliferator-activated receptor γ (PPAR γ) ligand, rosiglitazone (R, 10 mg/kg/d) or vehicle. In A, before killing, mice were anesthetized, and the right internal jugular vein of each mouse was surgically exposed and cannulated with a pressure transducer that was advanced to record right ventricular pressure. Each bar represents the mean right ventricular systolic pressure (RVSP) in mm Hg $\pm$ SEM from six to eight animals. * $P < 0.05$ versus C; ** $P < 0.05$ versus CH 3 or 5 weeks. In B, hearts were dissected and weighed at the time of killing. Each bar represents the mean $\pm$ SEM right ventricular: left ventricular + septum (RV:LV+S) weight ratio from six to eight animals. * $P < 0.001$ CH versus C and versus C + R; ** $P < 0.01$ CH versus CH + R.

sistent with enhanced muscularization of distal pulmonary vessels. Morphometric analyses of vascular profiles ($< 100 \mu\text{m}$ luminal diameter) from α -SMA-stained lung sections demonstrated that CH reduced the luminal cross-sectional area of α -SMA-positive vessels, and increased the degree of vessel muscularization measured as the thickness of the vascular wall. Treatment with rosiglitazone reduced CH-mediated increases in vessel wall thickness.

CH Increased Nox4 Expression and Superoxide Generation in the Lung

Recent reports have demonstrated that CH stimulates up-regulation of Nox4 in the lung (4) and that CH-induced pulmonary hypertension is abrogated in NADPH oxidase knockout mice (2). Our results confirm that exposure to CH for 3 weeks increased levels of Nox4 mRNA in the lung, and provide new evidence that treatment with rosiglitazone prevented CH-mediated Nox4 induction (Figure 3A). Furthermore, increased Nox4 mRNA levels were associated with corresponding increases in Nox4 protein staining in vascular profiles from lung tissue of CH-exposed mice (Figure 3B). The representative images shown in Figure 3B illustrate increased intensity of staining with the Nox4 antibody in lungs from CH-exposed animals and attenuation by treatment with rosiglitazone. Labeling of airway epithelium (Figure 3B, *double arrowhead*) and alveolar structures (Figure 3B, *arrowhead*) was also detected as previously reported (4). Substitution of an irrelevant IgG for the Nox4 primary antibodies produced little background labeling (data not shown). Hypoxia was also sufficient to increase the expression of Nox4 in HPAEC (Figure 3C), and hypoxia-induced increases in Nox4 were reduced by treatment with rosiglitazone. These findings provide additional evidence that hypoxia significantly increases Nox4 expression in cells composing the pulmonary vascular wall.

CH-Induced Increases in Nox4 NADPH Oxidase Subunit Expression Were Associated with Increased Superoxide Generation in Lung Tissue

As shown in Figure 4A, in frozen tissue sections treated with DHE, the intensity of fluorescence, which is proportional to the amount of superoxide produced (32), is increased in lungs from CH-exposed animals, and treatment with rosiglitazone attenuated CH-induced increases in superoxide production. More quantitative assessments of lung superoxide generation using ESR spectroscopy with the spin probe, CMH (Figure 4B), and HPLC analysis of DHE oxidation (Figure 4C) provide additional evidence that hypoxia increased lung superoxide production and that these increases are attenuated in animals treated with rosiglitazone. Although CH did not alter PPAR γ expression when analyzed using RT-PCR of whole lung homogenates (data not shown), *in vitro* hypoxia exposure significantly reduced PPAR γ protein levels in isolated HPAEC and HPASMC (Figure 5), and treatment with rosiglitazone did not attenuate effects of hypoxia on PPAR γ expression.

CH-Induced Alterations in PDGFR β and PTEN

Because PDGF has been implicated in the pathogenesis of pulmonary hypertension (33–35), we examined the effect of CH exposure on PDGFR β expression and activity as well as its effects on the downstream inhibitory phosphatase, PTEN. Exposure to CH for 3 weeks increased PDGFR β protein expression and levels of the active phosphorylated-PDGFR β (Figures 6A and 6B). Treatment with rosiglitazone prevented CH-mediated increases in PDGFR β activation, reflected as P-PDGFR β .

Since CH stimulated increases in both PDGFR β expression and activity (Figure 6), but rosiglitazone attenuated only activation of the PDGFR β , we examined the ability of PPAR γ to regulate PTEN after CH exposure because PTEN can directly dephosphorylate and deactivate the PDGF receptor (17). As illustrated in Figure 7, exposure to CH for 3 weeks caused significant reductions in PTEN protein expression, and treatment with rosiglitazone attenuated these reductions in PTEN expression. To our knowledge, this is the first report to demonstrate alteration of hypoxia-induced reductions in PTEN expression in the lung by a PPAR γ ligand.

DISCUSSION

Pulmonary hypertension is a complex disorder. Although recent evidence suggests that existing treatment strategies (including prostacyclin and its analogs, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors) have improved mortality in patients with pulmonary hypertension, mortality rates remain high (36, 37). These findings emphasize the urgent need for additional studies to enhance our understanding and treatment of this disorder. In an attempt to better understand mechanisms of hypoxia-associated pulmonary hypertension and the potential role of PPAR γ as a novel target that regulates signaling events leading to pulmonary vascular remodeling and hypertension, the current study employed a well-established mouse model of chronic hypoxia.

Exposure to normobaric, chronic hypoxia for 3 or 5 weeks produced significant pulmonary hypertension consistent with previous reports. Treatment with rosiglitazone during the last 10 days of exposure both prevented and reversed established pulmonary hypertension. Although shorter rosiglitazone treatment durations might provide similar efficacy, those regimens were not examined in the current study. These observations are consistent with recent reports demonstrating that treatment with rosiglitazone reduced hypoxia-induced right ventricular hypertrophy and pulmonary artery remodeling in Wistar-Kyoto rats (25) and attenuated pulmonary hypertension and pulmonary artery wall thickening in monocrotaline-treated rats (24). In the current study, treatment with rosiglitazone also reduced CH-mediated increases in RVSP, an effect not observed in the Wistar-Kyoto rat model (25). We postulate that these discrepancies may relate to differences in rosiglitazone administration between the previous study (5 mg/kg/d mixed in chow) versus the 10 mg/kg/day dose supplied by gavage in the current study. Another study reported reduced PPAR γ expression within vascular lesions in patients with severe pulmonary hypertension (27). In the present study CH did not reduce levels of PPAR γ mRNA or protein in whole lung homogenates of mice (data not shown). While the loss of PPAR γ expression may be confined only to advanced plexiform lesions in the late stages of pulmonary hypertension and therefore not seen in the current model with more moderate pulmonary hypertension, our *in vitro* studies provide new evidence that hypoxia is sufficient to reduce PPAR γ expression in cells composing the pulmonary vascular wall. It is important to emphasize that the *in vitro* use of 21% oxygen for control and 1% oxygen for hypoxic conditions likely over- and underestimates, respectively, the true mixed venous oxygen concentrations sensed by cells of the pulmonary arterial wall in health and disease. These findings suggest that PPAR γ expression elsewhere in the lung may mask targeted hypoxia-induced reductions in PPAR γ expression in the vascular wall when analyzed at the level of the whole lung. Additional studies will be required to further determine the role of reduced PPAR γ expression in pulmonary hypertension pathogenesis as well as the mechanisms of hypoxia-induced

Figure 2. Rosiglitazone attenuates CH-induced vascular remodeling. Mice were exposed to Control (C) or chronic hypoxia (CH) for 3 weeks $\pm$ treatment with rosiglitazone (R) as described in Figure 1. The lungs were isolated, pressure perfused, fixed, sectioned, and stained with antibodies to α -smooth muscle actin (α -SMA). (A) Quantification of the number of α -SMA-positive acinar blood vessels less than 100 μm diameter. Each bar represents the mean $\pm$ SEM number of α -SMA-positive acinar blood vessels counted per microscopic field in two to four sections from each of five animals per group. * $P < 0.01$ CH versus C and CR. (B) Wall thickness of α -SMA-positive acinar blood vessels. Each bar represents the mean $\pm$ SEM wall thickness of α -SMA-positive acinar blood vessels in μm . * $P < 0.01$ CH versus C; ** $P < 0.01$ CH versus CH + R. (C) Lung sections (6 μm) were prepared and incubated with α -SMA primary antibody followed by secondary anti-rabbit antibody. Scale bar in each image = 50 μm .

alterations in PPAR γ expression. Nonetheless, our *in vitro* observations suggest that despite significant hypoxia-induced reductions in PPAR γ expression, pulmonary vascular wall cells remain capable of responding to rosiglitazone, as illustrated by

rosiglitazone-mediated attenuation of hypoxia-induced Nox4 expression.

Mounting evidence suggests that CH-induced activation of NADPH oxidase, an important enzymatic source of superoxide,

Figure 3. Rosiglitazone attenuates hypoxia-induced increases in Nox4 expression. Mice were exposed to Control (C) or chronic hypoxia (CH) for 3 weeks $\pm$ treatment with rosiglitazone (R) as described in Figure 1. In A, RNA was extracted from lung tissue, and real-time PCR performed for Nox4 and ribosomal protein S9. Each bar represents the mean $\pm$ SEM Nox4/S9 expressed as fold-change compared with control animals. * $P < 0.05$ CH versus C; ** $P < 0.001$ CH versus CH + R. In B, 5- μm frozen lung sections were prepared and stained with primary antibodies to Nox4 followed by secondary rhodamine red-labeled antibody. Representative images ($\times 40$) of small arterioles (arrows) in each treatment group are shown. Labeling of airway epithelium (double arrowhead) and alveolar structures (single arrowhead) are shown. Scale bar in each image = 50 μm . In C, human pulmonary artery endothelial cells (HPAEC) were exposed to control conditions (C, room air/5% CO_2) or chronic hypoxia conditions (CH, 1% O_2 /5% CO_2) for 72 hours $\pm$ treatment with rosiglitazone (R, 10 μM) for the final 24 hours of exposure. Cells were collected for Western blotting using antibodies directed against Nox4 and CDK4. Each bar represents the mean $\pm$ SEM Nox4 level relative to CDK4 as % control ($n = 6$). * $P < 0.01$ versus C. A representative immunoblot is presented below the bar graph.

Figure 4. Rosiglitazone attenuates CH-induced increases in superoxide production in lung tissue. Male C57Bl/6 mice were exposed to room air (C) or chronic hypoxia (CH) for 3 weeks $\pm$ rosiglitazone (R) during the last 10 days of exposure as described in Figure 1. In A, frozen lung sections (30 μ m) were prepared and treated with dihydroethidium (DHE, 10 μ M). Representative images ($\times 20$) from each treatment group are depicted. In B, lung tissue from each group was collected and subjected to electron spin resonance spectroscopy for measurement of superoxide production. Each bar represents the mean $\pm$ SEM superoxide production per mg dry weight expressed as % Control from seven to nine animals in each group. * $P < 0.05$ versus C. In C, lung tissue from each group was collected and incubated with DHE, and superoxide-mediated oxidation of DHE was measured using high-performance liquid chromatography. Each bar represents the mean $\pm$ SEM oxidized DHE relative to the protein content of the sample from four to five animals in each group. * $P < 0.05$ versus C.

plays a critical role in pulmonary hypertension in the mouse (2, 3, 38–42). Our findings not only demonstrate that CH-induced pulmonary hypertension was associated with increased Nox4 expression in the lungs (Figure 3), but also provide novel evidence that hypoxia stimulates Nox4 expression in HPAEC *in vitro*. These findings are consistent with previous reports that hypoxia specifically increased the expression of the Nox4 NADPH oxidase subunit in the lungs of C57Bl/6 mice without increasing the expression of other NADPH oxidase subunits (4). Our findings extend these reports to demonstrate that the PPAR γ ligand, rosiglitazone, attenuated CH-induced increases

in Nox4 expression *in vitro* and *in vivo* and ROS production in the lung. These current findings provide additional evidence that PPAR γ ligands regulate NADPH oxidase expression *in vivo* (30). While Nox4 knockout mice would be an ideal model to further support the mechanistic connection between PPAR γ , Nox4, and hypoxia-induced pulmonary hypertension, these mice are currently unavailable.

Several reports have emphasized the importance of PDGF in pulmonary hypertension (33). When activated, PDGF receptors stimulate cell migration, proliferation, and survival through subsequent activation of several downstream signaling path-

Figure 5. Hypoxia reduces PPAR γ expression in HPAEC and human pulmonary artery smooth muscle cells (HPASMC). (A) HPAEC and (B) HPASMC were exposed to control (C, 21% O $_2$) or hypoxic (CH, 1% O $_2$) conditions for 72 hours $\pm$ treatment with rosiglitazone (R, 10 μ M). Whole cell lysates were then prepared and subjected to Western blotting for PPAR γ or CDK4 followed by laser densitometric analysis. Each bar represents the mean $\pm$ SEM PPAR γ relative to β -actin level as % control ($n = 6$). * $P < 0.01$ versus Control. Representative immunoblots are presented below each bar graph.

Figure 6. Rosiglitazone attenuated CH-induced increases in PDGFR β activation. Male C57Bl/6 mice were exposed to room air (C) or CH for 3 weeks $\pm$ rosiglitazone (R) during the last 10 days of exposure as described in Figure 1. In A, PDGFR β expression levels were measured in lung homogenates by Western blotting and calculated relative to β -actin in each sample. Each bar represents the mean PDGFR β $\pm$ SEM as % Control from six animals. * P < 0.05 CH versus C and versus C + R. In B, phosphorylated PDGFR β expression levels were measured in lung homogenates by Western blotting and calculated relative to PDGFR β in each sample. Each bar represents the mean levels of P-PDGFR β relative to PDGFR β $\pm$ SEM as % Control from five animals. A representative blot is depicted. * P < 0.05 CH versus C, and ** P < 0.05 CH versus CH + R.

ways, including Src, phosphatidylinositol 3 kinase (PI3K), phospholipase C γ , and Ras. In patients with pulmonary hypertension, PDGFR β expression was increased in the lungs, and the PDGF receptor antagonist, imatinib, reversed monocrotaline-induced pulmonary hypertension in rats and hypoxia-induced pulmonary hypertension in mice by inhibiting PDGFR β phosphorylation (35). Furthermore, several reports have suggested that PPAR γ ligands inhibit PDGF-stimulated vascular smooth muscle cell migration *in vitro* (13, 14, 35, 43). Our studies demonstrate that CH for 3 weeks increased PDGFR β expression and activity in the lung, and rosiglitazone therapy attenuated PDGFR β activation.

To further explore mechanisms by which CH activated the PDGFR β , we examined PTEN, a dual specificity phosphatase that can dephosphorylate both lipid and protein substrates, including the PDGF receptor (16), thereby inhibiting Akt and PDGF-stimulated proliferative signaling cascades. The rationale for these studies was further supported by several lines of evidence: (1) NADPH oxidase-derived ROS facilitate PDGF signaling by inhibiting PTEN (44), (2) PPAR γ ligands stimulate PTEN expression potentially through stimulation of PPAR

Figure 7. CH-mediated reductions in PTEN are attenuated by rosiglitazone. Male C57Bl/6 mice were exposed to room air (C) or CH for 3 weeks $\pm$ rosiglitazone (R) during the last 10 days of exposure as described in Figure 1. PTEN expression levels were measured in lung homogenates by Western blotting and calculated relative to β -actin in each sample. Each bar represents mean PTEN levels $\pm$ SEM as % Control from 12 to 14 animals per group. A representative blot is depicted. * P < 0.05 versus all other groups.

response elements in the PTEN promoter (20, 21), and (3) PTEN overexpression reduced vascular smooth muscle cell proliferation and migration and inhibited injury-induced vascular remodeling *in vivo* (45, 46). Our findings demonstrate that CH causes significant and previously unreported reductions in lung PTEN expression that are attenuated by treatment with rosiglitazone. Rosiglitazone failed to significantly increase PTEN expression in normoxic mice, suggesting that rosiglitazone-mediated attenuation of hypoxia-induced reductions in PTEN expression may be more complicated than simple stimulation of the PTEN promoter. The potential importance of reduced PTEN in pulmonary hypertension is further supported by evidence that genetic interruption of PTEN in vascular smooth muscle caused development of pulmonary hypertension and vascular remodeling in mice exposed to normoxia (22). These reports, along with the current study, suggest that CH stimulates PDGF signaling not only through enhanced PDGFR β expression in the lung, but also by inhibiting the expression of the regulatory phosphatase, PTEN. Thus we postulate that chronic hypoxia causes pulmonary hypertension and vascular remodeling, in part, through the generation of NADPH oxidase-derived ROS that stimulate PDGF and inhibit PTEN signaling pathways. The ability of PPAR γ ligands to attenuate hypoxia-induced alterations in PTEN expression and to lower oxidative stress-induced PTEN inactivation provides a unique strategy to lower PDGFR β phosphorylation and activation and downstream proliferative signals. We postulate that these integrated effects contribute to the ability of PPAR γ to attenuate pulmonary vascular smooth muscle cell proliferation and hypertrophy as well as vascular remodeling caused by chronic hypoxia.

There are several important limitations to the current study that deserve additional consideration. First and foremost, there may be species-dependent differences in vascular responses to hypoxia and modulation by thiazolidinediones that limit the ability to translate observations made with mouse models to

human studies (47). It is well recognized that murine models of hypoxia-induced pulmonary hypertension are characterized more by medial thickening of the pulmonary vasculature rather than the plexiform lesions composed of proliferative intraluminal endothelial cells that characterize many forms of severe human pulmonary hypertension. However, recent evidence that Nox4 is upregulated in lungs of hypoxia-exposed mice and in lung tissue from patients with pulmonary hypertension (4) provides some assurance that the mouse model accurately mimics alterations in NADPH oxidase observed in human disease. Furthermore, our *in vitro* data provides novel evidence that hypoxia increases Nox4 expression in human pulmonary vascular cells and that rosiglitazone attenuates hypoxia-induced increases in Nox4 in lung cells. Although the precise mechanisms by which chronic hypoxia leads to pulmonary hypertension in animal models (48) remains controversial, the current study focuses on the PPAR γ receptor as a novel therapeutic target in pulmonary hypertension and explores downstream targets that participate in regulation of nitroso-redox balance in the pulmonary circulation. While our findings indicate that alterations in Nox4 and PTEN are involved in PPAR γ -regulated responses to chronic hypoxia, there are other mediators implicated in pulmonary hypertension pathogenesis (49) that could be modulated by PPAR γ ligands. Our studies do not exclude the potential involvement of other mediators in PPAR γ effects on CH-induced pulmonary hypertension, and explorations of additional pathways remain areas of active interest in our laboratories. For example, Rho-kinase-mediated vasoconstriction plays a major role in hypoxia-induced pulmonary hypertension (25), and PPAR γ ligands inhibit Rho-kinase in the systemic circulation (50, 51), suggesting that PPAR γ activation could inhibit CH-induced pulmonary hypertension by modulating Rho-mediated vasoconstriction. Although recent evidence in a rat model of hypoxia-induced pulmonary hypertension demonstrated that rosiglitazone was unable to inhibit sustained Rho-kinase-mediated arterial vasoconstriction (25), the modulation of Rho-kinase and other mediators involved in pulmonary hypertension pathogenesis deserve additional consideration as targets of PPAR γ in the pulmonary vasculature.

Our findings indicate that PPAR γ activation provides a unique strategy for attenuating CH-induced proliferative signaling pathways, in part, by suppressing NADPH oxidase-derived ROS that stimulate PDGF and inhibit PTEN. To our knowledge, this is the first report to demonstrate involvement of PTEN in CH-induced pulmonary hypertension and to attenuate these alterations with a PPAR γ ligand. This report thereby provides novel evidence for a mechanistic connection between CH-induced alterations in NADPH oxidase-derived ROS and its contribution to pulmonary vascular remodeling and hypertension.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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