Rosiglitazone attenuates hypoxia-induced pulmonary arterial remodeling

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Pulmonary Hypertension (PH) is a disease characterized by increased pulmonary arterial (PA) pressure with concomitant increases in pulmonary vascular resistance (PVR), frequently leading to right ventricular (RV) failure and death (56). The chronic elevation of PVR has been attributed to a combination of vasoconstriction and progressive structural remodeling of the pulmonary arterial tree. Characteristic features of this vascular remodeling include vessel wall thickening as a result of resident vessel wall cell proliferation, migration, and excessive deposition of extracellular matrix (55). PH as a result of chronic hypoxia is a major cause of morbidity and mortality, but the pathobiology of the disease remains unknown. Chronic hypoxic PH and the resulting right heart failure are common disease processes because of the high prevalence of chronic obstructive pulmonary diseases such as emphysema and chronic bronchitis, in addition to the increased incidence of sleep-related alveolar hypoventilation disorders (59).

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors including steroid, thyroid, and retinoid hormone receptors (36). Many structurally diverse ligands including long-chain fatty acids, eicosanoids, thiazolidinediones, and fibrates activate PPARs, which form obligate heterodimers with the 9-cis retinoic acid receptor RXR (14). These PPAR-RXR heterodimeric complexes become transcriptionally active at PPAR response elements and alter the expression of target genes.

The expression of each PPAR isoform is restricted to specific tissues, with PPARγ expressed predominantly in adipocytes and activated macrophages, wherein it regulates adipogenesis and fatty acid metabolism and inhibits macrophage activation, respectively (57, 61). PPARγ is activated by a number of natural ligands with 15-deoxy-Δ12,14-prostaglandin J2 being the most potent (46, 61). PPARγ is also stimulated by synthetic ligands, thiazolidinediones (TZDs), like troglitazone and rosiglitazone, which have been used as therapeutic agents to increase insulin sensitivity in type 2 diabetes mellitus (57). PPARγ is also expressed in vascular smooth muscle cells (SMCs) (58) and vascular endothelial cells (ECs) (13, 37, 43). The expression of PPARs in the vascular wall suggests a potential role in normal vascular function and in the development of vascular disease.

The role of PPARγ in many aspects of vascular function is unclear, but numerous reports in the literature indicate that PPARγ agonists are potent regulators of SMC proliferation and migration. Studies by Law and colleagues (26–28, 34, 63) demonstrated that PPARγ agonists blocked mitogenic ERK signaling in response to PDGF or insulin in SMCs of the systemic vasculature and suppressed migration in response to a variety of chemotactants. These results are supported by a number of reports from other groups showing that PPARγ or retinoid X receptor (RXR) agonists inhibit SMC proliferation and migration and the expression of growth and migration-promoting genes (3, 4, 44, 46, 63). The results from in vivo...
studies are even more compelling. Oral administration of synthetic PPARγ ligands inhibits neointimal hyperplasia in balloon-injured rat and rabbit arterial wall (7, 38, 42). PPARγ agonists also reduce the size of atherosclerotic lesions in LDL receptor-deficient (11) and apolipoprotein E-deficient mice (9). In human subjects with type 2 diabetes, TZDs slow the progression of intimal-medial thickening (40, 41). Overall, these data indicate that PPARγ agonists inhibit SMC proliferation and migration and prevent pathological vascular remodeling in the systemic vasculature.

Recently, Matsuda, et al. (45) reported that the TZDs pioglitazone and troglitazone inhibit the development or PH and attenuate PA remodeling in monocrotaline-treated rats. Pioglitazone (10 mg·kg⁻¹·day⁻¹) only modestly reduced PA pressures, whereas troglitazone (200 mg·kg⁻¹·day⁻¹) essentially normalized PA pressures in monocrotaline-treated animals. Both agents blocked RV hypertrophy and PA wall remodeling. These results indicated that PPARγ agonists have the same beneficial impact on the pulmonary arterial bed as they do in the systemic circulation. However, the physiological and biochemical mechanisms underlying these beneficial effects were not explored.

Given these considerations, we hypothesized that PPARγ agonists might also prevent pulmonary arterial (PA) remodeling and the development of PH in response to chronic hypoxia. We found that the PPARγ agonist rosiglitazone (ROSI) had little effect on PH and had moderate effects on RV hypertension following exposure of rats to chronic hypoxia. However, remodeling of the PA wall was almost entirely attenuated by ROSI. ROSI blocked proliferation of cells in the media and adventitia of the vessel wall but did not affect cell death. ROSI also decreased collagen and elastin deposition in the vessel wall and reduced collagen and elastin production by PA SMCs in vitro. ROSI also increased matrix metalloproteinase (MMP)-2 protein levels in cultured SMCs and MMP-2 activity in lung lysates. Finally, we noted that hypoxia induced the appearance of cells expressing the cell surface marker c-Kit, which is found on hematopoietic progenitor cells and cells of the promyelocytic lineage. ROSI blocked the appearance of these cells even in lung tissue from animals exposed to chronic hypoxic conditions. We conclude that although ROSI prevents vascular remodeling in response to hypoxia, it fails to attenuate the development of PH. This may be due to the inability of ROSI to modulate other prohypertensive processes including vasoconstriction, polycythemia, and increases in cardiac output.

**METHODS**

**Materials.** Male and female Wistar-Kyoto rats were purchased from Harlan (Indianapolis, IN). Polyclonal antibodies to c-Kit (CD117) and proliferating cell nuclear antigen (PCNA) were purchased from Dako Cytomation (Carpenteria, CA). Polyclonal antibodies to α-smooth muscle (α-SM) actin, Fasudil (HA-1077), and periodate-Schiff staining reagents were purchased from Sigma (St. Louis, MO). Picrosirius red stain was obtained from Electron Microscopy Sciences (Hatfield, PA). Polyclonal antibodies to total and phosphorylated forms of Akt and p70 S6 kinase were obtained from Cell Signaling (Danvers, MA). Polyclonal antibodies to MMP-2 and -9 were purchased from Chemicon International (Temecula, CA). Alexa Fluor 594 and Alexa Fluor 488-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). VectaShield mount-

medium with 4,6-diamidino-2-phenylindole was purchased from Vector Laboratories (Burlingame, CA). ApoAlert DNA fragmentation (TdT-mediated dUTP nick end labeling (TUNEL)) kits were obtained from BD Bioscience (San Jose, CA). Novex gelatin zymogram minigels and reagents were purchased from Invitrogen (Carlsbad, CA). Diet 5008 (6.5% fat, 3.31 kcal/g) impregnated with 0.005% ROSI (Avandia; Glaxo Smith Kline, Research Triangle Park, NC) was prepared by Test Diet (Richmond, IN).

**Animal care and treatment.** All animal procedures were performed with approval and in accordance with the guidelines of the University of Colorado Health Sciences Center for Laboratory Animal Care. Male and female Wistar-Kyoto rats (6 wk old) were randomized to normoxic (Nx; 1,600 m, 630 mmHg) or hypobaric hypoxia (Hx; 5,500 m, 410 mmHg) and treated with and without the PPARγ agonist ROSI at 5 mg·kg⁻¹·day⁻¹ for 3 wk (n = 6 animals/treatment group). To test whether ROSI could reverse remodeling, some animals were maintained under normoxic and hypoxic conditions for 21 days and received ROSI for the last 7 days. Fresh food [Diet 5008 with or without (control chow) ROSI], water, and clean cages with fresh bedding were provided every other day. Light was maintained on a 12-h cycle, and humidity was 40–45% with a temperature of 25–27°C. The animals were monitored daily, and weight was checked once a week. At the end of 3 wk, the rats were anesthetized and catheterized and the following measurements were obtained: body weight, hematocrit, mean PA pressure, cardiac output, total pulmonary resistance, mean PA pressure in response to inhaled nitric oxide (NO; 80 ppm) or intravenous delivery of Fasudil. After the rats were killed, RV hypertrophy and PA wall morphometry data were collected.

**Hemodynamic measurements and tissue procurement.** Rats received a combination of ketamine (75 mg/kg) and xylazine (6 mg/kg) intraperitoneally as anesthetic. The ventral neck area and the area dorsally between the scapulae were shaved and the areas scrubbed with betadine solution. A 2-cm incision was made over the right ventral neck area to expose the internal jugular vein. A polyvinyl (PV-1) catheter was threaded into the pulmonary artery via the right internal jugular vein. A catheter pressure tracing was transduced and monitored with an oscilloscope, and the location of the tip was identified by the characteristic shapes of the PA pressure waveforms. The catheter was then securely sutured into place. An additional catheter was inserted into the jugular vein for drug infusion and venous return. The right carotid artery was isolated, and a catheter was inserted 4 cm into the artery and securely sutured into place for measurements of blood gases and cardiac output. All catheters were routed, exteriorized, and sutured in place. Rats were placed in a 3-in. wide, 3-in. high, and 7-in. long Plexiglas box with gas delivery ports for the delivery of air or air mixed with NO. Sampling of the gas inside the box over time showed that complete gas exchange within the box occurred within 2 min. The implanted catheters were attached to pressure transducers and/or cardiac output equipment for hemodynamic measurements. Cardiac output was determined using a standard dye dilution method. Total pulmonary resistance was calculated by dividing the mean PA pressure by cardiac output (50). All measurements were made while the animals were anesthetized.

Following hemodynamic measurements, the anesthetized animals were treated with the RhoA/Rho kinase inhibitor Fasudil (10 mg/kg iv) to dilate the vasculature before fixation. The rats were then euthanized by exsanguination. Lungs were fixed with 4% paraformaldehyde in PBS containing 5 mM EDTA by airway inflation at 30 cmH₂O pressure. Pulmonary arteries were perfused with 4% paraformaldehyde-PBS-5 mM EDTA (to maintain vasodilation) at a pressure similar to that measured in vivo. The heart and lungs were then removed en bloc. Wet RV weights were measured and used to calculate the ratio of RV weight to total body weight. Lung tissue was fixed overnight in 4% paraformaldehyde and embedded in paraffin.

**Immunostaining and TUNEL staining.** Five-micrometer sections of paraformaldehyde-fixed, paraffin-embedded lung tissue were depar-
affinized with Hemo-D and rehydrated in a graded ethanol-water series. Sections were subjected to antigen retrieval in citrate buffer in a microwaveable pressure cooker for 20 min. Sections were blocked with PBS containing 5% horse serum for 30 min at room temperature. The sections were incubated overnight in PBS-5% FBS at 4°C with the primary antibodies as indicated. The sections were then washed and incubated with the indicated Alexa Fluor-conjugated secondary antibodies for 1 h at room temperature. TUNEL staining was performed using ApoAlert DNA fragmentation assay kits according to the manufacturer’s directions.

**Microscopy and lung morphometric analysis.** Five-micrometer sections of fixed lung or heart tissue were subjected to hematoxylin and eosin or pentachrome staining by the University of Colorado Health Sciences Center Histology Core Laboratory. Periodic acid-Schiff staining of heart sections or picrosirius red staining of lung sections was performed as directed by the supplier’s instructions. Distal pulmonary vessels (outside diameter 10–50 μm) were assessed by a blinded observer for degrees of circumferential α-SM actin-positive staining indicative of muscularization. Vessels smaller than 10 μm were considered capillaries and excluded from further consideration. Proximal vessels (outside diameter 50–250 μm) were analyzed for medial wall thickness at four points around the vessel circumference and for lumen diameter along two axes.

Microscopy was performed on a Nikon TE2000-U inverted epifluorescent microscope. Bright-field, phase-contrast, and fluorescent digital deconvolution images were captured to a personal computer with either a Spot RT/KE monochrome camera or Spot Insight color camera (Diagnostic Imaging, Sterling Heights, MI). Images were analyzed and processed with MetaMorph 6.1 software (Molecular Devices, Sunnyvale, CA).

**PA SMC isolation and cell culture.** Five-hundred-micrometer PAs were recovered from adult rat lungs. Segments of the PAs were cut open and mechanically stripped of adventitia and endothelium. The segments were then placed lumen side down into individual wells of a six-well plate. Tissue explants were maintained in complete DMEM supplemented with 200 U/ml penicillin, 0.2 mg/ml streptomycin, and 10% FCS. Although these vessels are larger than those evaluated by morphometric analysis (50–250 μm), techniques to isolate cells from smaller vessels have not been developed.

Since our goal was to obtain pure subpopulations of SMCs, we selectively isolated individual cell colonies with a distinct, although uniform, morphological appearance from primary culture by using cloning cylinders. Expression of SMC-specific markers (α-SM actin and SM-myosin heavy chains) in each isolated cell subpopulation was selected for further experimentation. Individual cell colonies growing from tissue explants in primary culture were isolated by placing cloning cylinders (5–10 mm in diameter, greased on the bottom) over each cell colony of interest. Cells within the ring were trypsinized and transferred to a 24-multwell plate for expansion. Cells were passaged in DMEM containing 10% FCS. During experiments, the cells were placed in sealed Plexiglas chambers filled with either normoxic (80% nitrogen, 20% oxygen) or hypoxic (97% nitrogen, 3% oxygen) gas mixtures. The gas mixture in each chamber was replaced every 24 h.

**Fig. 1.** Rosiglitazone (ROSI) attenuates and reverses hypoxia-induced pulmonary arterial (PA) remodeling. Adult male and female rats were fed untreated or ROSI-impregnated chow and maintained under normoxic or hypoxic conditions for 21 days (A). An additional group of animals was maintained under hypoxic conditions without ROSI for 14 days, followed by hypoxic conditions with ROSI for an additional 7 days (B). Representative bright-field photomicrographs of hematoxylin and eosin-stained lung sections show that ROSI inhibited (A) and reversed (B) hypoxia-induced PA remodeling. Bar, 100 μm. C: morphometric analysis confirms that ROSI blocked (solid bars) or reversed (open bar) hypoxia-induced PA wall thickening compared with control (hatched bars). Data are average values obtained from at least 30 vessels with diameters between 50 and 250 μm from 6 animals per group; error bars represent SD. *P ≤ 0.05.
All studies were carried out using cells at passages 1–8. Cell cultures were tested for mycoplasma contamination with the use of a Gen-Probe Mycoplasma T. C. rapid detection system (San Diego, CA).

Lung and SMC lysates, Western blots, and zymograms. For Western blots, lysates from whole lung or heart (39) or from cultured SMCs (24) were prepared as previously described. After correcting for protein concentrations, cell lysates were mixed with an equal volume of Laemmli SDS loading buffer, resolved on 10% polyacrylamide-SDS gels, and transferred to polyvinylidene difluoride membranes. The blots were blocked with PBS containing 5% dry milk and 0.1% Tween 20 and then treated with antibodies that recognize the target proteins indicated overnight at 4°C. The blots were washed and subsequently treated with appropriate secondary antibodies conjugated to horseradish peroxidase. After the blots were washed, specific immune complexes were visualized with SuperSignal West Pico chemiluminescent substrate.

For zymography, portions of whole lung were homogenized in PBS without protease inhibitors. Equal amounts of lysate protein were mixed with an equal volume of SDS loading buffer (without reducing agent) and immediately resolved on 10% polyacrylamide-gelatin Novex Zymogram minigels. MMP activity was detected using matched reagents according to the manufacturer’s directions.

Cardiomyocyte hypertrophy. Five-micrometer sections of paraformaldehyde-fixed, paraffin-embedded hearts were stained with periodate-Schiff reagents.

Fig. 2. ROSI inhibits muscularization of distal pulmonary arterioles. Adult male and female rats were fed untreated or ROSI-impregnated chow and maintained under normoxic or hypoxic conditions for 21 days. Late ROSI indicates animals subjected to hypoxic conditions for 14 days without ROSI, followed by 7 days of persistent hypoxia with ROSI treatment. A: representative bright-field photomicrographs of lung parenchyma immunostained with antibodies to α-smooth muscle (SM) actin show that ROSI prevented or reversed muscularization of distal arterioles. Arrows indicate scored actin-positive arterioles. Cntrl, control animals. B: morphometric analysis of the number of actin-positive vessels per square millimeter in control (hatched bars), ROSI-treated (solid bars), and late ROSI-treated (open bar) male animals. Data confirm that ROSI blocked or reversed neomuscularization. Data are average values obtained from at least 25 fields from 6 animals per group; error bars represent SD. *P ≤ 0.05; **P ≤ 0.01.

Fig. 3. Effect of ROSI and chronic hypoxia on right ventricle (RV) hypertrophy. Untreated (control, hatched bars) or ROSI-treated (solid bars) adult male rats were maintained under normoxic or chronic hypobaric hypoxic conditions for 21 days. A: hypoxia-induced RV hypertrophy (assessed as average RV weight divided by body weight) was attenuated by ROSI. Data are average values obtained from 6 animals per group. *P ≤ 0.05. B: morphometric analysis of periodate-Schiff-stained heart sections shows that ROSI prevented cardiomyocyte hypertrophy in hypoxia-exposed rats. Data are average cardiomyocyte diameters determined from 100 cell measurements per animal; error bars represent SD. *P ≤ 0.05. C: Western blots of lysates from freshly harvested heart tissue from animals subjected to normoxia or hypoxia, with or without ROSI, were probed with antibodies to total or phosphorylated (P) Akt or p70 S6 kinase as indicated. Blots are representative of 3 separate experiments, and bar graphs indicate the ratios of phosphorylated Akt or p70 S6 kinase to total levels of these enzymes, averaged from 3 experiments (3 times n = 1). *P ≤ 0.05.
date-Schiff reagents to highlight cardiomyocyte borders. Bright-field images of regions of heart containing transverse-sectioned cardiomyocytes were captured to a computer as described above. The diameter of approximately round cardiomyocytes containing visible nuclei was determined in two perpendicular directions by using MetaMorph software and was averaged. The diameters of at least 100 cardiomyocytes were measured from each animal.

Statistics. Statistical analysis was performed using the Super ANOVA software program (Abacus Concepts, Berkeley, CA). Comparisons were performed using two-way analysis of variance followed by the Scheffé’s multiple comparison test for individual comparisons within and between groups of data points. Data were considered statistically significant with a $P$ value $<0.05$.

RESULTS

**ROSI attenuates pulmonary arterial remodeling and RV hypertrophy in response to hypoxia.** There was no statistical difference between the normoxic (Nx)-control and Nx-ROSI or hypoxic (Hx)-control and Hx-ROSI-treated groups in body weight at the end of 3 wk, although the ROSI-treated animals tended to be heavier (data not shown). The animals subjected to chronic hypoxia for 3 wk demonstrated the expected polycythemia; however, there was no statistical difference between the hematocrit of Hx-control and Hx-ROSI-treated groups (data not shown).

Examination of hematoxylin and eosin-stained lung tissue sections demonstrated a profound increase in PA wall thickness in Hx-control compared with Nx-control animals (Fig. 1A). However, ROSI treatment for the entire 21-day period completely blocked PA remodeling. We also investigated whether PPARγ agonist treatment could reverse hypoxia-induced PA remodeling. For these experiments, animals were exposed to hypoxic conditions for 14 days in the absence of ROSI. ROSI treatment was then conducted for next 7 days with continued exposure to hypobaric hypoxic conditions. As
demonstrated in Fig. 1B, substantial PA remodeling was already evident after 14 days of hypoxic exposure. However, in the animals treated with ROSI for the last 7 days of hypoxic exposure, remodeling was reduced to near normal levels. Morphometric analysis (vessel wall thickness/lumen radius) confirmed a marked decrease in proximal and distal PA vessel wall (50- to 250-µm-diameter vessels) remodeling compared with untreated Nx- and Hx-controls (Fig. 1C). Morphometric analysis also demonstrated that starting ROSI after the PA remodeling had been established could reverse the PA remodeling to near normal levels. Similar results were obtained with both male and female rats. To ensure that these differences were not due to differences in vascular tone, we treated the animals with the vasorelaxant Fasudil (RhoA/Rho kinase inhibitor) immediately before lung removal and inflated and perfused the lungs with buffers containing 5 mM EDTA. This attenuation also was readily evident in the lack of distal muscularization of the small PA arterioles within the distal lung parenchyma (Fig. 2). Late ROSI treatment also diminished muscularization of distal arterioles but was much less effective than ROSI treatment initiated at the onset of hypoxic exposure.

RV hypertrophy (RV weight divided by total body weight) was significantly reduced in the Hx-ROSI rats compared with Hx-controls, indicating that ROSI treatment attenuated RV hypertrophy in response to PH (Fig. 3A). The inhibition of RV hypertrophy was due, at least in part, to the ability of ROSI to block hypertrophy of individual cardiomyocytes (Fig. 3B). Cardiomyocyte hypertrophy has been linked to increased activation of Akt (16, 47) and p70 S6 kinase (51) signaling pathways in response to various stimuli. We found that hypoxia significantly increased Akt activation (phospho-Akt levels) but had no effect on p70 S6 kinase activity in heart lysates (Fig. 3C). However, cardiac phospho-Akt levels were markedly reduced in Hx-ROSI rats.

ROSI regulates proliferation, but not cell death, in the hypoxic PA wall. To begin to delineate the mechanisms by which ROSI attenuates PA remodeling in response to hypoxia, we examined the impact of ROSI on proliferation and apoptosis in PAs of lung tissue sections. Using immunostaining for PCNA as a marker for proliferating cells, we detected numerous proliferating cells in the thickened PA walls of Hx-control animals (Fig. 4, A and B). These proliferating cells were largely detected in the adventitial layer of the PA wall and did not stain for α-SM actin (Fig. 4C). However, PCNA-positive cells were virtually absent in PA walls from Hx-ROSI animals, suggesting that ROSI blocks proliferation of cells in the PA wall. ROSI might also prevent PA wall thickening by increasing

![Fig. 5](http://ajplung.physiology.org/)

Fig. 5. No significant differences in apoptosis in the PA wall were observed with ROSI and/or hypoxia treatments. Adult male rats were fed a normal diet or chow impregnated with ROSI and maintained under normoxic or hypoxic conditions for 21 days. A: 5-µm sections of paraformaldehyde-fixed lung tissue were subjected to TdT-mediated dUTP nick end labeling (TUNEL) and DAPI (nuclei) staining. Representative fluorescent digital deconvolution photomicrographs show no significant differences in apoptosis among treatment groups. Bars, 100 µm. B: morphometric quantitation of TUNEL-positive cells vs. total cell number (DAPI) in the vessel wall. Data confirm no significant differences in apoptosis in the PA wall under any of the conditions.
apoptosis of vessel wall cells. We assessed apoptosis in lung sections by TUNEL staining and found no significant differences under any condition (Fig. 5, A and B).

**ROS1 regulates extracellular matrix metabolism.** Another potential mechanism by which TZDs might reduce PA wall remodeling could be by inhibiting the synthesis and deposition of extracellular matrix (ECM) or by increasing the breakdown of the ECM. To test this hypothesis, we measured collagen deposition in the PA wall by pentachrome and picrosirius red staining, and elastin deposition by pentachrome staining. We noted substantial amounts of collagen (yellow material in pentachrome-stained sections, red material in picrosirius red-stained sections) in the media and adventitia of vessels from Hx-control animals (Fig. 6A). Likewise, the number of elastin fibers (gray fibers in pentachrome-stained sections) was elevated in Hx-control vessel walls. However, collagen and elastin staining in Hx-ROSI or Hx-late ROSI animals were reduced to levels comparable to those observed in Nx-control animals.

Similar results were obtained with cultured PA SMCs. Pentachrome staining revealed a marked increase in intracellular and extracellular elastin production in cells exposed to hypoxia but not in cells exposed to hypoxia and treated with ROSI (Fig. 6B). Likewise, picrosirius red staining revealed increased collagen synthesis in hypoxic SMCs but not in cells exposed to hypoxia and treated with ROSI (Fig. 6, B and C).

The impact of ROS1 on certain MMPs also was examined. In SMCs, ROSI reduced expression of MMP-9, either alone or in...
SMCs were maintained under normoxic (21% O₃) or hypoxic (3% O₂) conditions with or without exposure to hypoxic conditions (Fig. 7). ROSI decreases MMP-2 levels in whole lung lysates and isolated PA SMCs. Western blots of whole cell lysates were probed with antibodies to either MMP-9 or MMP-2 as indicated. The positions of active, latent, and SMW forms indicated at right of blot. Data show that ROSI stimulated expression of MMP-2 even in cells exposed to hypoxia. ROSI had no effect on MMP-9 levels. Blot is representative of 3 separate experiments. Norm, normoxic; Hypox, hypoxic.

MMP-2 by other proteases. Similar results were noted in whole lung lysates, where ROSI increased SMW forms of MMP-2. Thus the decreased deposition of ECM in ROSI-treated animals may be due, in part, to increased ECM breakdown by MMP-2.

**DISCUSSION**

In this study we have shown that the PPARγ agonist ROSI inhibits remodeling of the PA wall and muscularization of distal arterioles normally observed in chronic hypoxic PH. More impressive was the observation that ROSI reversed remodeling due to prior hypoxic exposure. The decreased remodeling appears to be due to the repression of cell proliferation, ECM deposition, and the inhibition of accumulation of c-Kit-positive cells in the PA wall. Experiments are currently underway to evaluate the overall contribution of each of these processes to PA remodeling and the beneficial impact of ROSI. Despite the ability of ROSI to attenuate vascular remodeling, this agent did not prevent the development of PH.

Our observations in the pulmonary circulation are consistent with the large number of reports showing a beneficial impact of ROSI and other TZDs on systemic vascular function. In the systemic vasculature, TZDs have been shown to block vascular remodeling associated with mechanical damage (7, 38, 42) and to prevent the formation of atherosclerotic lesions in a variety of model systems (9, 11). These agents also inhibit SMC proliferation and the pro-proliferative signaling pathways in cell culture (3, 4, 26–28, 34, 44, 63). In addition, TZDs repress MMP-9 levels (53, 65), increase the expression and/or activity of MMP-2 (15, 32), and alter ECM metabolism in various cell types.

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**Fig. 7.** ROSI decreases matrix metalloproteinase (MMP)-9 levels and increases MMP-2 levels in whole lung lysates and isolated PA SMCs. A: PA SMCs were maintained under normoxic (21% O₂) or hypoxic (3% O₂) conditions with or without ROSI for 72 h. Western blots of whole cell lysates were probed with antibodies to either MMP-9 or MMP-2 as indicated. The position of active, latent, and small molecular weight (SMW) forms of MMP-2 are indicated at right of blot. B: Polyvinylidene difluoride membranes used for MMP Western blotting in A were stained with Coomassie blue to verify equal protein loading. C: Zymogram of MMP activity in whole lung lysates from adult male rats fed a normal diet or chow impregnated with ROSI and maintained under normoxic or hypoxic conditions for 21 days. A representative zymogram is shown, with the positions of MMP-9 and MMP-2 indicated at left of gel and the positions of active, latent, and SMW forms indicated at right. Results indicate that ROSI increased SMW forms of MMP-2 in normoxic or hypoxic animals. Zymogram is representative of 3 separate experiments; n = 1 per treatment.
and tissue types. Our results indicate that ROSI has a similar impact on PA remodeling and cells that make up the PA wall. It is interesting, however, that ROSI had little or no effect on the development of hypoxic PH. TZDs have been long recognized to exhibit antihypertensive effects in the systemic circulation (7, 18, 52). These effects are largely due to the negative impact of TZDs on angiotensin II receptor signaling (22, 30, 60) and RhoA/Rho kinase activation (62), which normally promote vasoconstriction. TZDs also block the production of endothelin-1 and other vasoactive peptides by ECs (23) and decrease the generation of oxidative stress/reactive oxygen species (25). In our studies, Rho kinase-mediated vasoconstriction (20, 48) appeared to be the predominant cause of hypoxia-induced PA pressure elevation, since acute inhibition of Rho kinase with Fasudil normalized PA pressures. The inability of ROSI to repress Rho kinase-mediated vasoconstriction in the pulmonary circulation given that TZDs inhibit RhoA/Rho kinase activation and block vasoconstriction in the systemic vasculature remains a mystery.

One answer may lie in the ability of troglitazone or pioglitazone to decrease PA pressures in monocrotaline-treated rats (45). Monocrotaline-induced vasoconstriction also can be reduced with Fasudil, indicating that monocrotaline, like hypoxia, promotes Rho kinase-mediated vasoconstriction. This suggests that different TZDs may have diverse effects on RhoA/Rho kinase signaling. However, the differences between our results and those of Matsuda et al. (45) may have other explanations. Strain-specific differences (Wistar-Kyoto in our
particular vascular bed and other factors such as the levels of

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Another interesting result was the inability of inhaled NO to reduce elevated PA pressures. NO is frequently used in clinical PH to elicit vasorelaxation. However, reports from other groups suggest that the effectiveness of NO is dependent on the particular vascular bed and other factors such as the levels of phosphodiesterase activity (5, 6, 29). Our data suggest that RhoA/Rho kinase inhibitors such as Fasudil may be more effective than inhaled NO at promoting pulmonary vasodilation.

A related issue is the development of hypoxic PH (elevated PA pressure) in rats treated with ROSI, which represses PA remodeling. This suggests that the remodeling or vessel wall thickening may not be a necessary factor in the development of the hypertension itself. This concept is supported by studies employing methods to prevent or compensate for fixation method-dependent changes in lumen area. These studies showed that when the pulmonary vascular bed was maximally vasodilated during lung fixation, there was no reduction in vessel luminal area associated with the medial and adventitial thickening (33, 35). Other experiments have demonstrated that angiotensin-converting enzyme inhibitors prevent PA remodeling in rats exposed to chronic hypoxia but do not attenuate the development of PH or RV hypertrophy (10). More recent studies indicate that acute inhibition of RhoA/Rho kinase signaling almost completely reverses PH in rats exposed to chronic hypoxia, although the brief exposure to the inhibitors would not be expected to have any effect on the structural thickening of the PA wall (48). If this is the case in human PH, then agents such as ROSI that prevent or reverse remodeling without affecting vasoconstriction may have little benefit. However, the dissociation of vascular remodeling from PH may merely reflect a feature of the hypoxic rodent models that is not relevant to humans or other large animals. For example, studies employing neonatal calves exposed to chronic hypoxia show pronounced decreases in lumen cross-sectional area due to inward encroachment on the vascular lumen (19), and constrictive and obliterative neointimal lesions are characteristic of human severe idiopathic pulmonary arterial hypertension (PAH). Thus ROSI and the other TZDs may prove useful in preventing or reversing the obliterative neointimal lesions of severe PAH (54). We are currently assessing the impact of

Fig. 9. Effect of ROSI and chronic hypoxia on pulmonary hemodynamic parameters. Adult male and female rats were fed a normal diet (control, hatched bars) or chow impregnated with ROSI (solid bars) and maintained under normoxic or hypoxic conditions for 21 days. Average mean PA pressure (A), cardiac output (B; males only), and total pulmonary resistance (C; TPR; males only) were measured as described in MATERIALS AND METHODS. Results show that ROSI failed to normalize PA pressures and TPRs in hypoxic animals. Cardiac output was elevated in both normoxic and hypoxic rats treated with ROSI. Data are averages obtained from n = 6 animals per group; error bars represent SD. *P ≤ 0.05.

Fig. 10. Effect of inhaled NO or intravenous Fasudil on mean PA pressures. Adult male rats were fed a normal diet or chow impregnated with ROSI and maintained under normoxic or hypoxic conditions for 21 days. Mean PA pressures were allowed to return to initial levels between the 2 treatments. Control animals (hatched bars) received ambient air alone. Fasudil normalized PA pressures were measured while the animals were exposed to NO mixed with air (80 ppm; solid bars) followed by intravenous injection of Fasudil (open bars). PA pressures were only normalized in animals treated with troglitazone at 200 mg·kg⁻¹·day⁻¹. In our hands, troglitazone and ROSI both exhibited “toxic” effects including decreased liver function, hepatic steatosis, global edema, and systemic hypotension leading to left heart failure in a significant number of animals. Thus the ability of troglitazone to reduce PA pressures as reported by Matsuda et al. (45) may be due to indirect systemic effects of high-dose treatment, rather than a direct effect of this agent on PA tone.

studies vs. Sprague-Dawley (in the studies by Matsuda et al.) or differences between monocrotaline- and chronic hypoxia-induced PH may be involved. We have examined the impact of ROSI on hypoxia-induced PH in Sprague-Dawley rats and obtained the same results as those reported presently (unpublished data; Crossno JT Jr, Reusch JEB, Morris KG, and Klemm DJ). We are currently testing the impact of ROSI on monocrotaline-induced PH. However, it is worth noting that Matsuda et al. (45) observed only modest decreases in PA pressure when using pioglitazone at 10 mg·kg⁻¹·day⁻¹, a dose comparable to our dose of ROSI at 5 mg·kg⁻¹·day⁻¹. PA pressures were only normalized in animals treated with troglitazone at 200 mg·kg⁻¹·day⁻¹. In our hands, troglitazone and ROSI both exhibited “toxic” effects including decreased liver function, hepatic steatosis, global edema, and systemic hypotension leading to left heart failure in a significant number of animals. Thus the ability of troglitazone to reduce PA pressures as reported by Matsuda et al. (45) may be due to indirect systemic effects of high-dose treatment, rather than a direct effect of this agent on PA tone.
ROSIGLITAZONE INHIBITS HYPOXIC PULMONARY ARTERIAL REMODELING

Our studies have also shown increased deposition of c-Kit-positive cells in the PA wall of animals exposed to chronic hypoxia and their absence in ROSI-treated animals. Such cells were originally reported by Davie et al. (12) in a newborn calf model of hypoxic PH. These investigators proposed that the c-Kit-positive cells may contribute to adventitial neovascularization, which increases local circulation and nutrient availability, which promotes further remodeling. These cells also could take on characteristics of resident vessel wall cells such as SMCs, ECs, and fibroblasts, among others. The proliferation, hypertrophy, or matrix production by these cells would exacerbate arterial wall thickening. Further analysis of these cells by Frid et al. (21) and our laboratory (unpublished data, Crossno JT Jr and Klemm DJ) indicates that they also express cell surface markers characteristic of the monocyte/macrophage lineage (CD14, CD11b, CD45, CD68, ED1, and ED2). Depletion of these cells in the circulation with clodronate-liposomes or gadolinium chloride (21) or via treatment with a c-Kit-neutralizing antibody (unpublished data, Crossno JT Jr and Klemm DJ) prevented PA remodeling but, once again, not the PH. The origin of these cells is unclear. Hayashida et al. (31) used mice transplanted with bone marrow from green fluorescent protein (GFP)-expressing donor mice. Their data indicate that bone marrow-derived cells contribute to PA remodeling in hypoxia-induced PH. We have employed mice transplanted with GFP-expressing bone marrow to determine whether these c-Kit-positive cells come from bone marrow or circulating sources. In preliminary experiments we have found GFP-positive/c-Kit-positive cells in the PA wall of animals exposed to chronic hypoxia (unpublished data, Crossno JT Jr and Klemm DJ). These results suggest that the c-Kit-positive cells reported in this study and previous studies arise from the bone marrow.

Finally, we observed a decrease in RV hypertrophy in ROSI-treated animals with elevated PA pressures. How is this accomplished? Cardiomycocyte hypertrophy is in part mediated by increased Akt and/or p70 S6 kinase signaling in response to certain stimuli (16, 47, 51). Our data show that hypoxia stimulates Akt activity but has no affect on p70 S6 kinase activation. ROSI almost completely blocks the activation of Akt by hypoxia. Thus it appears that TZDs decouple cardiac hypertrophy or remodeling from pressure overload via the downregulation of Akt signaling. This observation is consistent with previous reports showing that TZDs decrease cardiac hypertrophy in response to systemic pressure overload or mitral regurgitation (1, 17, 49). TZDs and PPARγ activation also have been shown to blunt cardiomyocyte hypertrophy in vitro (2, 64). Although this may seem to be a beneficial outcome, such inappropriate remodeling could lead to damage or premature failure of the ventricular wall in the face of elevated PA pressures.

In conclusion, we have demonstrated that ROSI inhibits and reverses PA wall remodeling and RV hypertrophy in response to chronic hypoxia. The blockade of PA remodeling is associated with decreased cell proliferation and ECM deposition in the vessel wall and with the decreased appearance of c-Kit-positive cells in the adventitia. Despite the lack of remodeling observed with ROSI treatment, hypoxic exposure still leads to increased PA pressure, which appears to be primarily due to RhoA/Rho kinase-mediated vasoconstriction. Our results are consistent with the ability of ROSI and other TZDs to block vascular remodeling in the systemic vasculature but also highlight an important difference in the ability of ROSI to affect vasoconstriction in the pulmonary versus systemic vasculature.

REFERENCES


ROSIGLITAZONE INHIBITS HYPOXIC PULMONARY ARTERIAL REMODELING


