

Peroxisome proliferator-activated receptor gamma (PPAR γ) regulates thrombospondin-1 and Nox4 expression in hypoxia-induced human pulmonary artery smooth muscle cell proliferation

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ABSTRACT

Transforming growth factor- β 1 (TGF- β 1) and thrombospondin-1 (TSP-1) are hypoxia-responsive mitogens that promote vascular smooth muscle cell (SMC) proliferation, a critical event in the pathogenesis of pulmonary hypertension (PH). We previously demonstrated that hypoxia-induced human pulmonary artery smooth muscle (HPASMC) cell proliferation and expression of the NADPH oxidase subunit, Nox4, were attenuated by the peroxisome proliferator-activated receptor γ (PPAR γ) agonist, rosiglitazone. The current study examines the hypothesis that rosiglitazone regulates Nox4 expression and HPASMC proliferation by attenuating TSP-1 signaling. Selected HPASMC were exposed to normoxic or hypoxic (1% O₂) environments or TSP-1 (0-1 μ g/ml) for 72 hours \pm administration of rosiglitazone (10 μ M). Cellular proliferation, Nox4, TSP-1, and TGF- β 1 expression and reactive oxygen species generation were measured. Mice exposed to hypoxia (10% O₂) for three weeks were treated with rosiglitazone (10 mg/kg/day) for the final 10 days, and lung TSP-1 expression was examined. Hypoxia increased TSP-1 and TGF- β 1 expression and HPASMC proliferation, and neutralizing antibodies to TSP-1 or TGF- β 1 attenuated proliferation. Rosiglitazone attenuated hypoxia-induced HPASMC proliferation and increases in mouse lung and HPASMC TSP-1 expression, but failed to reduce increases in TGF- β 1 expression or Nox4 expression and activity caused by direct TSP-1 stimulation. Transfecting HPASMC with siRNA to Nox4 attenuated hypoxia- or TSP-1-stimulated HPASMC proliferation. These findings provide novel evidence that TSP-1-mediated Nox4 expression plays a critical role in hypoxia-induced HPASMC proliferation. PPAR γ activation with exogenous ligands attenuates TSP-1 expression to reduce Nox4 expression. These results clarify mechanisms of hypoxia-induced SMC proliferation and suggest additional pathways by which PPAR γ agonists may regulate critical steps in the pathobiology of PH.

Key Words: hypoxia, Nox4, PPAR γ , rosiglitazone, thrombospondin-1

Pulmonary hypertension (PH) is a progressive disorder with poor survival. Although, advances in the modern drug treatment era have led to improvements in survival, morbidity is unacceptably high, and the disease prognosis remains poor.^[1] The pathogenesis of PH is complex and involves derangements of molecular mediators of vascular tone and patency, as well as enhanced proliferation of cells in the vascular wall. These changes result in pulmonary arterial remodeling, a process that increases pulmonary vascular resistance, right ventricular pressure, and leads to cor pulmonale.^[2-4]

Chronic hypoxia is one of the most common causes of PH, and belongs to the Group III category of the revised World Health Organization classification of PH.^[5] The mechanisms by which chronic hypoxia exposure mediates PH are complex and multifactorial.^[6] Several recent studies have implicated NADPH oxidase-generated reactive oxygen species (ROS) in PH pathogenesis. NADPH oxidase is a

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hypoxia-responsive, multisubunit protein complex which produces ROS which alters pulmonary vascular structure and function and may contribute to the pathogenesis of vascular diseases such as atherosclerosis and pulmonary hypertension.^[7-10] NADPH oxidase was originally described in phagocytic cells where its activation led to the generation of ROS critically involved in host defense.^[11,12] Nox2, also known as gp91^{Phox}, the catalytic moiety of the NADPH oxidase complex, was the first member of the Nox family to be discovered in phagocytic cells.^[8]

Nox4 is a constitutively active homologue of Nox2 expressed in a variety of non-phagocytic cells.^[13-15] For example, Nox4 is abundantly expressed in pulmonary vascular wall cells,^[12,15] and its expression is selectively upregulated in the lungs of hypoxia-exposed mice, as well as in the vascular media of patients with idiopathic pulmonary arterial hypertension (IPAH).^[15] Emerging evidence demonstrates that hypoxic increases in Nox4 expression are mediated in part by increased transforming growth factor β (TGF- β)-1,^[16] a multifunctional matrix protein that contributes to the pathogenesis of many vascular diseases,^[17,18] pulmonary vascular remodeling, and PH.^[19,20] Recently, NF- κ B,^[14] and hypoxia-inducible factor-1 α (HIF1- α)^[21] were shown to modulate the transcriptional expression of Nox4 by binding to its promoter. Upregulation of Nox4 in the hypoxic pulmonary vasculature is associated with increased ROS generation, and vascular smooth muscle cell (VSMC) and endothelial cell (EC) proliferation, which contribute to the development of vascular remodeling and dysfunction in PH.^[15]

The matrix-associated glycoprotein, thrombospondin (TSP)-1, regulates TGF- β 1 activation,^[22] and smooth muscle proliferation (SMC).^[23] Detailed mechanism studies revealed that TSP-1 interacts with the latency associated protein (LAP) of TGF- β 1, inducing a conformational change that allows the active site of TGF- β 1 to bind to its receptor.^[24,25] Although, TSP-1 could plausibly modulate Nox4 expression through its ability to activate latent TGF- β 1, a detailed understanding of its role on redox signaling pathways in PH is lacking. TSP-1 also functions as a potent mitogen and chemoattractant for SMC.^[26,27] While little is known about the role of TSP-1 in the development of PH, its expression is increased in the lungs of patients with sickle cell disease and idiopathic pulmonary arterial hypertension (IPAH).^[28] Hypoxia stimulates TSP-1 expression in the mouse pulmonary artery,^[29] and TSP-1 null mice exposed to chronic hypoxia were protected from the development of pulmonary vascular remodeling and PH.^[30] These studies suggest that hypoxia-induced TSP-1 expression contributes to the development of PH, whereas its absence is protective.

Our lab recently reported that activation of the nuclear hormone receptor peroxisome proliferator-activated

receptor gamma (PPAR γ) attenuated hypoxia-induced Nox4 expression, PH, and pulmonary vascular remodeling.^[31] In a separate report, we confirmed that Nox4 was an essential mediator of human pulmonary artery smooth muscle cell (HPASMC) proliferation caused by hypoxia.^[14] PPAR γ is expressed in the lung and pulmonary vasculature and participates in vascular homeostasis. Available evidence suggests that plexiform lesions in patients with PH have decreased PPAR γ expression, a finding that was confirmed in an in vivo model of severe PH in rats.^[32] Conversely, PPAR γ ligands attenuated or reversed PH in several models including chronic hypoxia-exposed mice,^[31] and Apo E^{-/-} mice fed high fat diets,^[33] as well as in hypoxia-^[34] and monocrotaline-treated^[35] rats. Because PPAR γ activation attenuated TGF- β 1-induced vascular injury,^[36] the current study was performed to further examine the role of TGF- β 1 and TSP-1 in the induction of Nox4 by hypoxia in PASMC. We hypothesized that PPAR γ activation would attenuate hypoxia-induced increases in TSP-1 and TGF- β 1 and thereby attenuate hypoxia-induced increases in HPASMC Nox4 expression.

MATERIALS AND METHODS

Cell culture and in vitro hypoxia studies

Monolayers of HPASMC (Lonza, Walkersville, Md.) were grown and maintained at 37°C in a 5% CO₂ atmosphere in smooth muscle growth medium (Lonza) containing 5% fetal bovine serum, 10 ng/ml human epidermal growth factor, 1.0 mg/ml hydrocortisone, 12 mg/ml bovine brain extract, 50 mg/ml gentamicin, and 50 ng/ml amphotericin. HPASMC were placed in hypoxic (1% O₂, 5% CO₂) or normoxic conditions (21% O₂, 5% CO₂) in a cell culture incubator at 37°C for 72 hours unless otherwise specified, as reported.^[14] The synthetic PPAR γ ligand, rosiglitazone (10 μ M in vehicle) or an equal volume of vehicle 1% dimethyl sulfoxide (DMSO, Fisher Scientific, Fair Lawn, N.J.) was added to the HPASMC culture media during the final 24 hours of exposure to normoxic or hypoxic conditions as indicated. We previously reported that this hypoxia regimen stimulated Nox4 expression and activity and proliferation in HPASMC and that treatment with this rosiglitazone regimen attenuated these hypoxia-induced derangements.^[14]

Assays of HPASMC proliferation

Proliferation of HPASMC was determined using an 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT assay; ATCC, Manassas, Va.) as we recently reported.^[14] In selected studies, neutralizing antibodies to TGF- β 1 (1 μ g/ml, Santa Cruz Biotechnology, Santa Cruz, Calif.) or TSP-1 (1 μ g/ml, Thermo Scientific, Fremont, Calif.) were added to the culture media three hours prior to hypoxia exposure.

TGF- β 1 enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay (ELISA) kit (Promega, Madison, Wisc.) was used to detect bioactive TGF- β 1 protein secreted by HPASMC into the culture media. Cells (5×10^4) were propagated in a 6-well plate to 80% confluence in growth media supplemented with 5% FBS. The cells were then washed with PBS, and placed in fresh media supplemented with 1% FBS. The ELISA was performed according to the manufacturer's protocol. Samples or standards containing known amounts of TGF- β 1 were incubated in a 96-well plate precoated with antibodies to TGF- β 1. Horseradish peroxidase-conjugated secondary antibodies were then added. The amount of TGF- β 1 was determined by measuring the formation of the colored product at 450 nm on a plate reader. In parallel experiments, the media was acid treated prior to performing the ELISA to detect total (bioactive + latent) TGF- β 1.

Smad 3 reporter construct, transfection techniques, and luciferase assays

The canonical TGF- β 1 signaling pathway involves phosphorylation of the Smad 3 transcription factor which leads to binding and activation of the promoter elements of target genes. Purified plasmid containing the Smad 3 binding element with attached luciferase reporter was provided as a gift from Dr. Alan Ramirez (University of Louisville). The plasmid (0.5 μ g) was transfected using the TransIT-2020 Transfection Reagent according to the manufacturer's protocol (Mirus Bio LLC, Madison, Wisc.). After transfection, the cells were allowed to incubate for 24 hours in an incubator at 37°C. To control for transfection efficiency, cells were cotransfected with (0.1 mg/well) Renilla luciferase (Promega, Madison, Wisc.). The cells were then treated with purified recombinant human TGF- β 1 (R and D Systems, Minneapolis, Minn.) for 24 hours. To assess the effect of PPAR γ activation on early TGF- β 1 induced Smad3 DNA binding, rosiglitazone (10 μ M) or vehicle DMSO was added to selected wells after six hours of incubation with TGF- β 1 (2 ng/ml). Cells were then washed with 1x PBS and placed in passive lysis buffer (300 μ l, Promega). Luciferase activities were measured with the Luciferase Assay System (Promega, Madison, Wisc.). Light detection was recorded with a luminometer (PerkinElmer). Relative light units in each sample were normalized to renilla, and all samples were examined in triplicate.

Western blot analysis

After treatment, HPASMC monolayers were washed, collected, and lysed as previously reported.^[37] Protein content of the lysate was determined using a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, Ill.). Equal amounts of protein were then loaded onto 4-12% gradient gels (Invitrogen, Carlsbad, Calif.) and resolved with sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE), followed by electroblotting onto polyvinylidene fluoride (PVDF) or nitrocellulose membranes. The membranes were blocked with 5% powdered nonfat dry milk for one hour and incubated overnight with primary antibodies to TSP-1 (Thermo Scientific, Fremont, Calif.; 1:1000) or Nox4 (Dr. David Lambeth, Emory University; 1:1000). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) raised against the species in which the primary antibodies were developed. Immunodetection was then performed using chemiluminescence (SuperSignal, Pierce Biotechnology, Rockford, Ill.). Relative immunoreactive protein levels were quantified using the Chemidoc XRS imaging system and Quantity One software (Bio-Rad Laboratories). All samples were normalized to their respective content of cyclin-dependent kinase 4 (CDK 4).

Amplex red H₂O₂ assay

TSP-1 (0.1-1 μ g/ml; Athens Research and Technology, Athens, Ga.) was added to the culture media of HPASMC for 24-72 hours, and H₂O₂ production was measured with Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Molecular Probes, Eugene, Oreg.) as previously described.^[14] The assay is based on the detection of H₂O₂ which reacts with 1:1 stoichiometry with Amplex[®] Red reagent in combination with horseradish peroxidase to produce highly fluorescent resorufin red. H₂O₂ released from HPASMC was then quantified by fluorometric detection on a plate reader (λ_{ex} = 590 nm and λ_{em} = 560 nm), followed by plotting sample values against a standard curve containing serial dilutions of known concentrations of H₂O₂.

RNA isolation, reverse transcription, and quantitative PCR

Total RNA was isolated from HPASMC using the RNeasy Mini Kit (Quiagen, Valencia, Calif.) and quantitated using NanoDrop spectrophotometry (Thermo Scientific, Wilmington, Del.). cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, Calif.) and reverse transcribed from 1 μ g of total RNA. Real-time PCR was then performed to characterize the expression of target genes with primers based on human RNA sequences using iQ SYBR Green Supermix and the iCycler Real-Time PCR Detection System (Bio-Rad). Primer sequences were as follows: TGF- β 1, forward primer (CAGAAATACAGCAACAATTCCTGG) and reverse primer (TTGCAGTGTGTTATCCCTGCTGTC); Nox4, forward primer (GCTGACGTTGCATGTTTCAG) and reverse primer (CGGGAGGGTGGGTATCTAA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward primer (AGCCACATCGCTCAGACAC) and reverse primer (GCCCAATACGACCAATCC); 9S, forward primer (CTGACGCTTGATGAGAAGGAC) and reverse primer (CAGCTTCATCTTGCCCTCAT; Eurofins MWG Operon,

Huntsville, Ala.). Expression of target mRNA in each sample was normalized to its GAPDH or 9S content. The relative abundance of target mRNA in each sample was calculated using the $\Delta\Delta C_t$ method.^[38]

RNA interference

Nox4 gene expression was reduced using Nox4 small interfering RNA (siRNA, Qiagen) as described previously.^[14] HPASMC monolayers were transfected with 35 nM siNox4 or si control for 24 hours before treatment with TSP-1 (1 $\mu\text{g}/\text{ml}$) or hypoxia (1% O_2 , 5% CO_2). Real-time PCR was employed to confirm Nox4 knock down by at least 50% in all studies. HPASMC proliferation was measured with MTT assays at the conclusion of each study.

Mouse model of chronic hypoxia exposure

Eight-week-old male C57Bl/6 mice were exposed to normoxic (21% O_2) or hypoxic (10% O_2) conditions for three weeks as we reported.^[31] During the final 10 days of exposure to hypoxic or normoxic conditions, each animal was given rosiglitazone (10 mg/kg/day) or an equal volume of vehicle (methylcellulose) daily by oral gavage. We previously reported that this hypoxia regimen stimulated increased right ventricular systolic pressures, right ventricular hypertrophy, and pulmonary vascular remodeling and that these hypoxic derangements were attenuated by this rosiglitazone regimen.^[31] All animals had access to standard mouse chow and water ad libitum, and all procedures were reviewed and approved by the Atlanta VA Medical Center Institutional Animal Care and Use Committee.

Statistical analysis

For all experiments, statistical analysis was performed by one-way ANOVA followed by a Tukey's post-hoc analysis to detect differences among experimental groups. The level of statistical significance was set at an alpha value of $P \leq 0.05$.

RESULTS

Rosiglitazone or neutralizing antibodies to either TSP-1 or TGF- β 1 attenuated hypoxia-induced HPASMC proliferation

As illustrated in Figure 1, hypoxia increased HPASMC proliferation, and treatment with rosiglitazone or with neutralizing antibodies to either TSP-1 or TGF- β 1 attenuated proliferation in both control and hypoxia-exposed cells. These results suggest that the constitutive expression of TSP-1 and TGF- β 1 contribute to autocrine stimulation of HPASMC proliferation under basal conditions, and that enhanced production of TGF- β 1 and TSP-1 contribute to hypoxic increases in HPASMC proliferation.

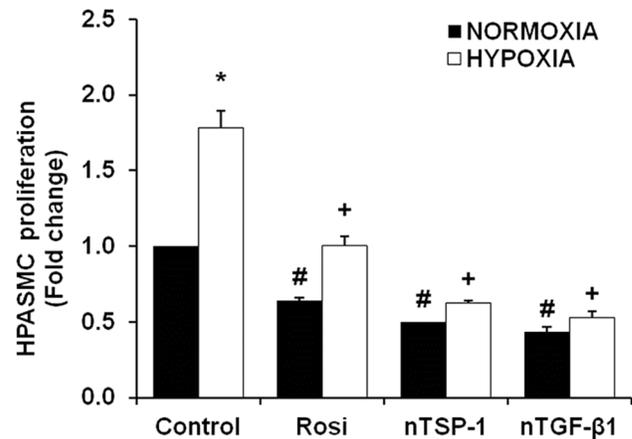


Figure 1: Rosiglitazone or neutralizing antibodies to TSP-1 or TGF- β 1 attenuated hypoxia-induced HPASMC proliferation. HPASMC exposed to normoxic (21% O_2) or hypoxic (1% O_2) conditions for 72 hours were treated with neutralizing antibodies to either TSP-1 (nTSP-1, 1 $\mu\text{g}/\text{ml}$) or TGF- β 1 (nTGF- β 1, 1 $\mu\text{g}/\text{ml}$) 3 hours prior to exposure, or rosiglitazone (Rosi, 10 μM) was administered during the last 24 hours of exposure. After treatment, an MTT assay was performed to measure cellular proliferation ($n = 6$). * $P < 0.05$ vs. Control-Normoxia, + $P < 0.05$ vs. Control-Hypoxia, # $P < 0.05$ vs. Control-Normoxia.

Rosiglitazone failed to regulate either TGF- β 1 expression or mediators of the TGF- β 1 canonical signaling pathway

As shown in Figure 2, hypoxia caused small but significant increases in TGF- β 1 mRNA levels and secretion of bioactive TGF- β 1 from HPASMC. Treatment with rosiglitazone caused small but statistically insignificant increases in TGF- β 1 mRNA (Fig. 2A) and protein (Fig. 2B) in control HPASMC and failed to attenuate the hypoxic-induction of TGF- β 1 mRNA and protein. Exposure to hypoxic conditions for 72 hours also increased bioactive TGF- β 1 secretion that was not reduced by rosiglitazone treatment (data not shown). TGF- β 1 binds to its receptor to stimulate Smad 2/3 recruitment, phosphorylation, and colocalization with Smad 4. This complex translocates into the nucleus where it binds to and activates target DNA sequences.^[39] To evaluate the effect of PPAR γ activation on this canonical TGF- β 1 pathway, Smad 3 DNA binding was assessed following stimulation with TGF- β 1 and treatment with rosiglitazone. As illustrated in Figure 2C, rosiglitazone (10 μM) did not attenuate TGF- β 1-induced increases in Smad 3 DNA binding as measured by a luciferase reporter assay.

Hypoxia increased TSP-1 expression in HPASMC

Because rosiglitazone failed to regulate TGF- β 1 expression and signaling, we examined TSP-1 levels in hypoxia-exposed HPASMC. As shown in Figure 3, compared with control conditions, exposure to hypoxia for 72 hours increased HPASMC TSP-1 protein levels as detected by Western blotting. The hypoxic induction of TSP-1 was attenuated

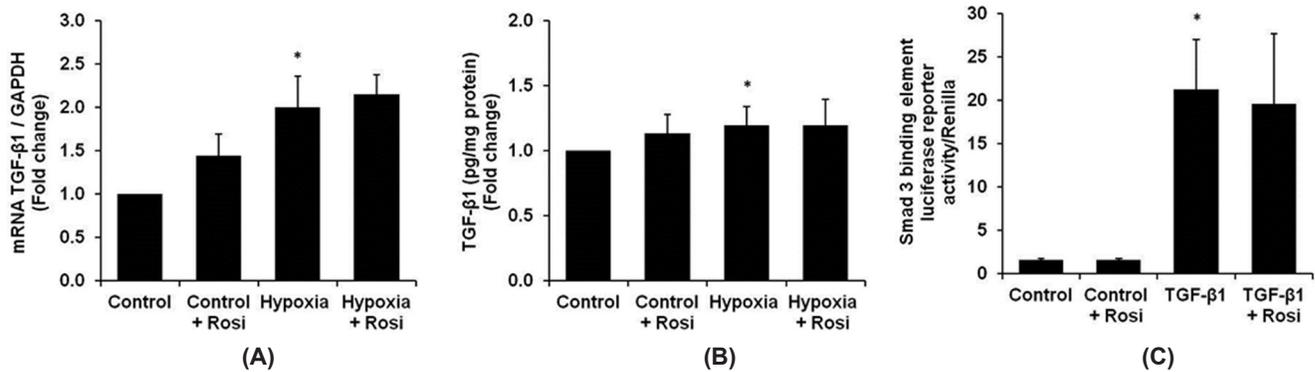


Figure 2: Rosiglitazone treatment did not modulate hypoxia-induced increases in HPASMC TGF- β 1 mRNA, protein or TGF- β 1-induced activation of the canonical TGF- β 1 signaling pathway. HPASMC were exposed to normoxic or hypoxic conditions for 72 hours \pm rosiglitazone (Rosi, 10 μ M). TGF- β 1 expression was measured in cell lysates by (A) qRT-PCR (n = 12), and in the cell media by (B) ELISA (n = 3). (C) Smad 3 binding element luciferase reporter activity was measured in HPASMC treated with TGF- β 1 (2 ng/ml) for 24 hours \pm rosiglitazone (10 μ M) (n = 3). * P < 0.05 vs. Control; ** P < 0.01 vs. Control.

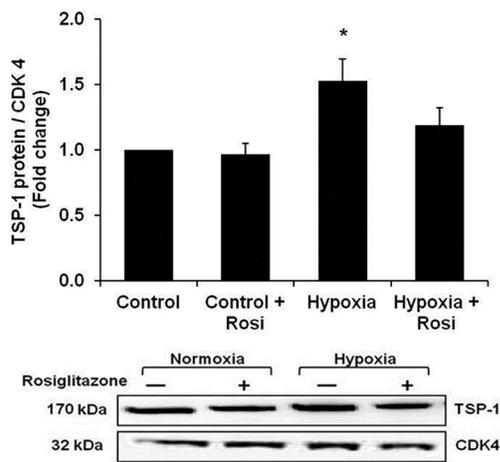


Figure 3: Rosiglitazone attenuated hypoxia-induced increases in TSP-1 protein expression. HPASMC were exposed to normoxic (control) or hypoxic conditions for 72 hours. During the final 24 hours of exposure, HPASMC were treated with rosiglitazone (Rosi 10 μ M). HPASMC were collected, and proteins were isolated for Western blot analysis. Each bar represents the mean \pm SEM TSP-1 band density normalized to CDK 4 and expressed relative to control samples (n=9). * P < 0.05 vs. Control. A representative TSP-1 immunoblot is presented below the bar graph.

by treatment with rosiglitazone during the last 24 hours of hypoxia exposure.

Exogenous administration of TSP-1 increased Nox4 expression and ROS generation

We have previously reported that hypoxia stimulates Nox4 expression and ROS generation in the lung in vivo,^[31] and in HPASMC in vitro.^[14] Since hypoxia increased TSP-1 expression in HPASMC, the ability of TSP-1 to directly stimulate Nox4 expression and augment the generation of ROS was examined. As shown in Figure 4A, TSP-1 (1 μ g/ml) increased HPASMC Nox4 protein expression. Figure 4B demonstrates that TSP-1 increases Nox4 expression after 48 or 72 hours, but not after 24 hours. Amplex

Red assays demonstrate that TSP-1 also stimulates corresponding increases in HPASMC H₂O₂ production (Fig. 4C). Together, these results suggest that TSP-1 is sufficient to stimulate Nox4 expression and activity in HPASMC.

Nox4 siRNA attenuated TSP-1 or hypoxia-induced proliferation of HPASMC

Previous evidence indicated that Nox4 induction was important for hypoxia-mediated increases in HPASMC proliferation.^[14,40] We therefore, assessed the effect of Nox4 inhibition on TSP-1-mediated HPASMC proliferation. We employed our recently published Nox4 siRNA protocol to reduce basal Nox4 mRNA and protein levels and hypoxia-induced H₂O₂ production in HPASMC.^[14] As illustrated in Figure 5, both hypoxia and TSP-1 caused comparable increases in HPASMC proliferation over control conditions, and Nox4 siRNA significantly reduced cellular proliferation in response to both stimuli. These results provide novel evidence that both TSP-1 and hypoxia-induced HPASMC proliferation are Nox4-dependent processes.

Rosiglitazone does not attenuate TSP-1-stimulated Nox4 expression

To further assess the regulation of TSP-1-induced Nox4 expression by PPAR γ Nox4 expression was measured in HPASMC following direct stimulation with TSP-1. As shown in Figure 6, TSP-1 (1 μ g/ml) increased Nox4 mRNA after 72 hours of treatment, and rosiglitazone (10 μ M), administered during the last 24 hours of exposure failed to attenuate TSP-1 stimulated Nox4 expression. Taken along with our previous report,^[14] these findings indicate that hypoxic stimulation of TSP-1 contributes to Nox4 induction and that rosiglitazone attenuates hypoxia-induced increases in TSP-1 expression rather than signaling pathways downstream of TSP-1.

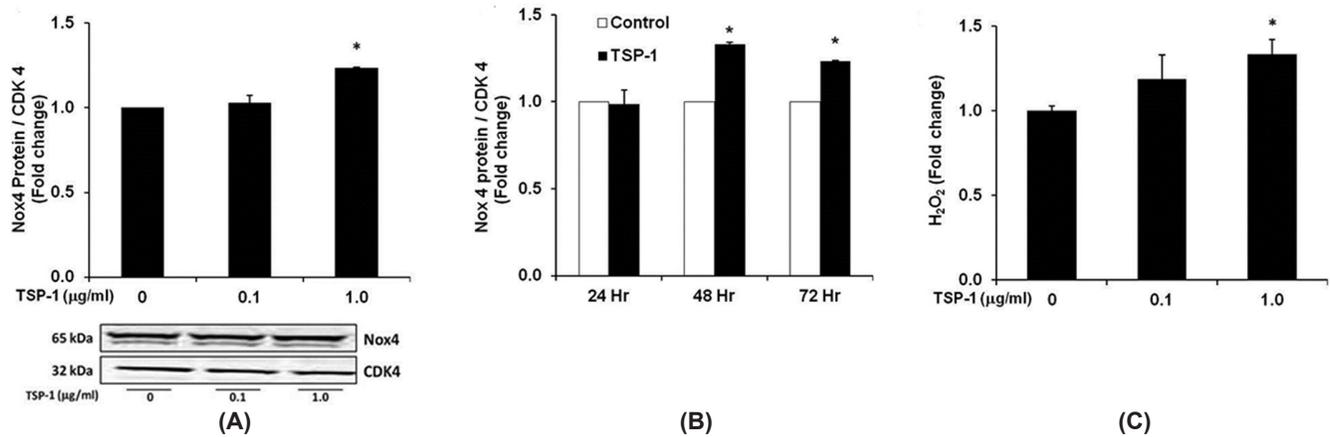


Figure 4: TSP-1 caused dose and time-dependent increases in HPASMC Nox4 expression and ROS generation. Western blot to detect Nox4 protein was performed on protein isolated from HPASMC monolayers treated with (A) graded concentrations of TSP-1 for 72 hours ($n = 3$), or (B) 1 $\mu\text{g/ml}$ of TSP-1 for 24-72 hours ($n = 3$). Nox4 immunoblots are presented below the bar graph. (C) HPASMC monolayers were treated with graded concentrations of TSP-1 for 72 hours and H_2O_2 production was measured by Amplex Red assay ($n = 3$). (A) $*P < 0.01$ vs. Control, (B) $*P < 0.001$ vs. Control, (C) $*P < 0.01$ vs. Control.

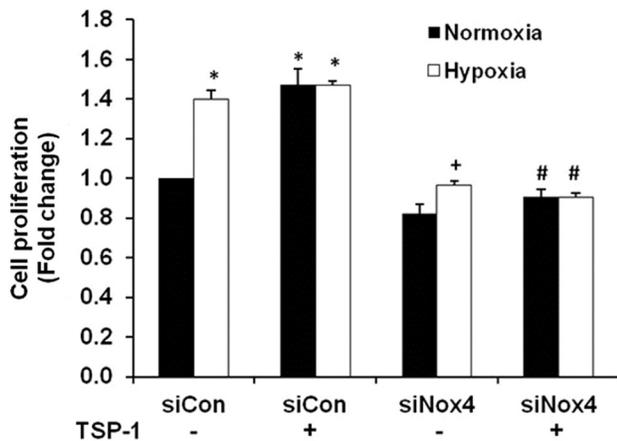


Figure 5: Nox4 siRNA decreased hypoxia or TSP-1-induced HPASMC proliferation. HPASMC were transfected with 35 nM siNox4 or a scrambled control (siCon). Twenty-four hours later, HPASMC were exposed to normoxic or hypoxic conditions for 72 hours \pm TSP-1 (1 $\mu\text{g/ml}$). An MTT assay was then performed. Each bar represents mean \pm SEM HPASMC proliferation expressed relative to control samples ($n = 3$). $*P < 0.05$ vs. Normoxia-siCon, $^+P < 0.05$ vs. Hypoxia-siCon, $\#P < 0.05$ vs. TSP-1.

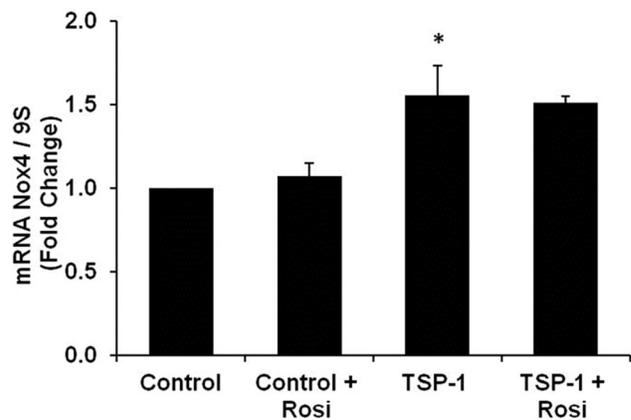


Figure 6: TSP-1 induces Nox4 expression and rosiglitazone fails to attenuate this increase. HPASMC were treated with TSP-1 (1 $\mu\text{g/ml}$) for 72 hours and rosiglitazone (10 μM) was added to the cell culture media during the final 24 hours of treatment. Total RNA was collected and quantitative real-time PCR of Nox4 transcripts was performed. Each bar represents the mean \pm SEM Nox4 transcripts normalized to 9S and expressed as fold change relative to control ($n = 3-6$). $*P < 0.05$ vs. Control.

Rosiglitazone attenuated hypoxia-induced TSP-1 expression in mouse lung in vivo

To confirm that rosiglitazone regulates TSP-1 in vivo, C57Bl/6 mice were exposed to control or hypoxic conditions for three weeks and selected mice were treated with rosiglitazone daily by oral gavage during the final 10 days of exposure as reported.^[31] As shown in Figure 7, hypoxia increased TSP-1 expression in mouse lung, and treatment with rosiglitazone attenuated hypoxic TSP-1 induction.

DISCUSSION

Hypoxia is a clinically important cause of PH that

contributes to alterations in pulmonary vascular function. Current evidence demonstrates that Nox4 is an important mediator of pulmonary vascular dysfunction in hypoxic environments.^[15] We previously reported that rosiglitazone attenuated hypoxia-induced increases in Nox4 expression and activity, pulmonary vascular remodeling, and PH in the mouse lung.^[31] Subsequent reports confirmed that Nox4 was a critical mediator of HPASMC proliferation caused by hypoxia.^[14,40] Because hypoxia stimulates TGF- β 1 signaling^[41] which drives Nox4-mediated ROS generation and HPASMC proliferation,^[40] we first examined the ability of rosiglitazone to regulate hypoxia-induced increases in TGF- β 1 signaling. However, our findings indicate that

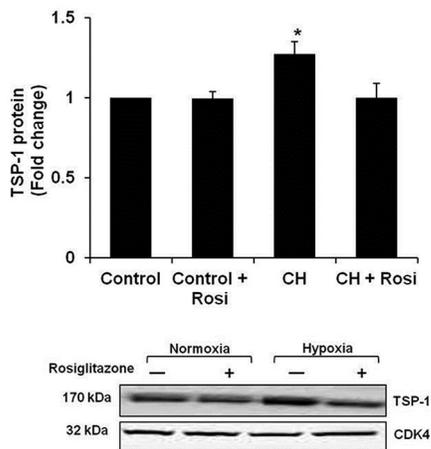


Figure 7: Rosiglitazone attenuates chronic-hypoxia-induced TSP-1 protein expression. C57Bl/6 mice were exposed to normoxic (Control) or chronic hypoxic (CH) (10% O₂) conditions for three weeks. During the last 10 days of treatment, selected animals were gavaged with either rosiglitazone (Rosi, 10 mg/kg/day) or vehicle. Protein was prepared from whole lung homogenate for Western blot analysis of TSP-1 expression (n = 8). **P* < 0.05 vs. Control. A representative TSP-1 immunoblot is presented below the bar graph.

rosiglitazone treatment conditions that successfully inhibited hypoxia-induced HPASMC proliferation failed to attenuate hypoxia-stimulated increases in TGF- β 1 mRNA, protein, or SMAD 3 nuclear binding in HPASMC. However, TGF- β 1 neutralizing antibodies attenuated hypoxia-induced HPASMC proliferation. Taken together, these findings confirm an important role for TGF- β 1 in HPASMC responses to hypoxia, but suggest that PPAR γ regulates hypoxia-induced Nox4 expression in HPASMC through pathways independent of TGF- β 1.

The matricellular protein, TSP-1, is an SMC mitogen^[42] whose expression is also upregulated in the hypoxic pulmonary vasculature.^[29] Therefore, we hypothesized that rosiglitazone-mediated attenuation of hypoxia-induced Nox4 expression and HPASMC proliferation involved TSP-1. The present study provides novel evidence for the role of TSP-1 in mediating hypoxia-induced Nox4 expression and H₂O₂ generation, and further defines the impact of rosiglitazone on these signaling pathways. To our knowledge, this is the first to report to establish that TSP-1 is a proximal mediator of Nox4 expression. This adds to our understanding of mechanisms by which Nox4 is upregulated in hypoxic vasculature.

Our studies focused on Nox4, because it is selectively upregulated by hypoxia.^[14,15,31] Compared to other NADPH oxidase isoforms, Nox4 also displays several unique characteristics including production of H₂O₂ rather than superoxide.^[43,44] Nox4 is potently stimulated by hypoxia,^[21] and unlike other NADPH oxidase isoforms, it remains constitutively active and modulates downstream events through effects of H₂O₂ on redox sensitive targets.^[45] Our lab demonstrated that Nox4 expression and activity were

essential for hypoxia-induced HPASMC proliferation and that rosiglitazone attenuated increased Nox4 expression, in part by preventing NF- κ B-mediated activation of the Nox4 promoter.^[14] Although the precise mechanisms by which Nox4-derived H₂O₂ stimulates HPASMC proliferation remain to be defined, we postulate that activation of kinases and transcription factors participate in the altered expression of growth promoting genes.^[8,46,47]

The current findings provide novel evidence for the involvement of hypoxic increases in TSP-1 mediating Nox4 expression and pulmonary vascular SMC proliferation. Previous reports have established the importance of TSP-1 in vascular cell mitogenic responses.^[48-50] Our findings extend these reports and suggest that TSP-1-mediated regulation of Nox4 plays a critical role in the proliferative responses of pulmonary vascular wall cells to hypoxia.

Few corollary studies supporting the role of TSP-1 in the pathogenesis of hypoxia-induced pulmonary vascular wall proliferation, remodeling, and PH in vivo are available. Existing studies have employed knockout models of TSP-1^[28,30] or its cognate receptor, CD47^[28] to define the role of TSP-1 in hypoxia-induced PH. TSP-1 null mice were protected from the development of chronic hypoxia-induced PH, RVH, and pulmonary vascular remodeling,^[28,30] and displayed decreased responses to the administration of acute pulmonary vasoconstrictors.^[30] These studies demonstrate a clear role for TSP-1 in the pathogenesis of PH. We recently demonstrated that GKT137831, a pharmacological Nox4 inhibitor also attenuates chronic hypoxia-induced RVH and vascular remodeling (manuscript in press). In the present study, we show that TSP-1 regulates Nox4 expression, H₂O₂ production, and hypoxic HPASMC proliferation. Collectively, these lines of evidence suggest the possibility that the TSP-1 knockout mice are protected from chronic hypoxia-induced PH, RVH, and vascular remodeling because of the absence of TSP-1-mediated Nox4 expression and ROS generation. Recent studies confirm the association between TSP-1 and ROS production. In hypoxic environments and in PH, TSP-1-induced CD47 activation led to decreased caveolin-1 (eNOS binding protein) expression, subsequent uncoupling of endothelial nitric oxide synthase (eNOS) and ROS production. These findings mirror other authors conclusions that hypoxia enhances TSP-1 expression in the mouse lung in vivo and in pulmonary vascular wall cells,^[29] and suggest that hypoxia-induced PH may develop in response to TSP-1-driven ROS generation from sources that include NADPH oxidases and eNOS.

A major implication of our findings relates to the potential ability to activate the PPAR γ receptor with ligands such as rosiglitazone in order to modulate the enhanced production of a variety of mediators involved in pulmonary vascular cell proliferation and PH. For example, we

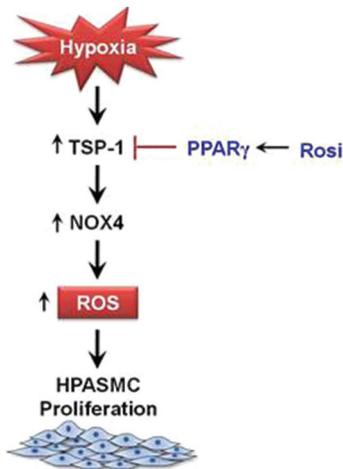


Figure 8: Putative mechanisms by which PPAR γ ligands attenuate hypoxia-induced HPASMC proliferation. The current schema depicts relationships that exist between TSP-1, Nox4, and ROS generation in hypoxia-induced HPASMC proliferation. Hypoxia enhances Nox4 expression and ROS generation in a TSP-1-mediated manner. Activation of PPAR γ with rosiglitazone attenuates hypoxia-induced HPASMC proliferation through inhibitory effects on TSP-1 expression and signaling.

have shown that PPAR γ ligands not only attenuate the enhanced expression and activity of Nox4,^[31] but also favorably and therapeutically attenuate increases in endothelin signaling in the hypoxic lung.^[51] The molecular mechanisms for these effects remain to be completely defined. While we demonstrated that rosiglitazone attenuated hypoxia-induced increases in Nox4 expression by reducing NF- κ B binding to the Nox4 promoter,^[14] PPAR γ regulation of the TSP-1 promoter remains to be established and constitutes an area of active investigation in our laboratory. The presence of NF- κ B binding elements in the TSP-1 promoter^[25] suggests that rosiglitazone-mediated reductions in TSP-1 expression may also occur through a similar mechanism. Alternatively, rosiglitazone decreased hypoxia-induced PDGFR β activation,^[31] and because PDGF stimulates TSP-1 expression, these findings suggest several candidate pathways for PPAR γ -mediated regulation of TSP-1 expression. The presence of a PPAR response element in the TSP-1 promoter further suggests PPAR γ could regulate TSP-1 expression through direct interactions with the TSP-1 promoter.

The current study has several important limitations. The examination of hypoxia-induced mechanisms in cultured cells may inadequately model hypoxia-induced PH in vivo. Furthermore, although the hypoxia regimen (1% O₂) employed in the current study represents a severe degree of hypoxemia rarely encountered by pulmonary vascular wall cells in common clinical scenarios, recent reports suggest that these levels of hypoxemia may indeed occur in the cells characterizing the pathological lesions in the pulmonary circulation of IPAH patients. Lastly, recent reports have drawn attention to the increased risk of

cardiovascular disease in diabetics taking rosiglitazone.^[52] Importantly, studies have not demonstrated adverse cardiovascular effects in diabetics taking another synthetic PPAR γ agonist, pioglitazone. These observations suggest that the observed cardiovascular complications may be attributable to the specific drug rather than to the TZD class and that only through more detailed mechanistic studies can the true therapeutic potential of targeting this receptor be realized.

In conclusion, our results provide novel evidence that TSP-1 is a proximal mediator of hypoxia-induced Nox4 expression and HPASMC proliferation. In addition, the synthetic thiazolidinedione PPAR γ ligand, rosiglitazone, attenuated hypoxic induction of TSP-1 expression and HPASMC proliferation. These findings further clarify mechanisms by which hypoxia stimulates proliferation of pulmonary vascular wall cells contributing to vascular remodeling in PH. Evidence that PPAR γ ligands attenuated these hypoxia-induced proliferative signals adds to the literature supporting PPAR γ as a therapeutic target in PH and provides additional insights into mechanisms by which PPAR γ mediates therapeutic effects (Fig. 8).

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