

Serotonin Signaling Through the 5-HT_{1B} Receptor and NADPH Oxidase 1 in Pulmonary Arterial Hypertension

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Objective—Serotonin can induce human pulmonary artery smooth muscle cell (hPASMC) proliferation through reactive oxygen species (ROS), influencing the development of pulmonary arterial hypertension (PAH). We hypothesize that in PASMCs, serotonin induces oxidative stress through NADPH-oxidase–derived ROS generation and reduced Nrf-2 (nuclear factor [erythroid-derived 2]-like 2) antioxidant systems, promoting vascular injury.

Approach and Results—HPASMCs from controls and PAH patients, and PASMCs from Nox1^{-/-} mice, were stimulated with serotonin in the absence/presence of inhibitors of Src kinase, the 5-HT_{1B} receptor, and NADPH oxidase 1 (Nox1). Markers of fibrosis were also determined. The pathophysiological significance of our findings was examined in vivo in serotonin transporter overexpressing female mice, a model of pulmonary hypertension. We confirmed that serotonin increased superoxide and hydrogen peroxide production in these cells. For the first time, we show that serotonin increased oxidized protein tyrosine phosphatases and hyperoxidized peroxiredoxin and decreased Nrf-2 and catalase activity in hPASMCs. ROS generation was exaggerated and dependent on cellular Src-related kinase, 5-HT_{1B} receptor, and the serotonin transporter in human pulmonary artery smooth muscle cells from PAH subjects. Proliferation and extracellular matrix remodeling were exaggerated in human pulmonary artery smooth muscle cells from PAH subjects and dependent on 5-HT_{1B} receptor signaling and Nox1, confirmed in PASMCs from Nox1^{-/-} mice. In serotonin transporter overexpressing mice, SB216641, a 5-HT_{1B} receptor antagonist, prevented development of pulmonary hypertension in a ROS-dependent manner.

Conclusions—Serotonin can induce cellular Src-related kinase–regulated Nox1-induced ROS and Nrf-2 dysregulation, contributing to increased post-translational oxidative modification of proteins and activation of redox-sensitive signaling pathways in hPASMCs, associated with mitogenic responses. 5-HT_{1B} receptors contribute to experimental pulmonary hypertension by inducing lung ROS production. Our results suggest that 5-HT_{1B} receptor–dependent cellular Src-related kinase–Nox1–pathways contribute to vascular remodeling in PAH.

Visual Overview—An online [visual overview](#) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2017;37:1361-1370. DOI: 10.1161/ATVBAHA.116.308929.)

Key Words: hypertension, pulmonary ■ models, animal ■ NADPH oxidase
■ receptor, serotonin, 5-HT_{1B} ■ serotonin

Serotonin has been implicated in the pathogenesis of pulmonary arterial hypertension (PAH)^{1–3} and has been recognized as a potent naturally occurring pulmonary vasoconstrictor⁴ and smooth muscle cell mitogen.² Serotonin promotes pulmonary artery (PA) remodeling and proliferation of human PA smooth muscle cells (hPASMCs) via the 5-HT_{1B} receptor (5-HT_{1B}R) and the serotonin transporter (SERT).^{5–8} Serotonin can also cause constriction of human and rodent PAs via the 5-HT_{1B}R.^{4,9}

Reactive oxygen species (ROS), produced primarily by the NADPH oxidase (Nox) family of enzymes in the vasculature, induce oxidative stress and play a critical role in oxidative

damage to proteins, lipids, and DNA.¹⁰ Altered redox signaling and increased ROS bioavailability have been implicated in chronic diseases, including PAH.^{11,12} Excessive amounts of ROS in PAs can oxidize and inactivate signaling molecules, such as protein tyrosine phosphatases (PTPs), or can drive irreversible protein modification through addition of carbonyl groups on protein side chains, a marker for oxidative stress.^{12,13}

Intracellular ROS levels are regulated by the balance between ROS-generating enzymes, such as Noxs, and antioxidant enzymes that include superoxide dismutases, catalase, and the peroxiredoxin systems,¹⁴ which are regulated by a key transcription factor Nrf-2 (nuclear factor [erythroid-derived

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Nonstandard Abbreviations and Acronyms

5-HT_{1B}R	5-HT _{1B} receptor
c-Src	cellular Src-related kinase
H₂O₂	hydrogen peroxide
hPASCs	human pulmonary artery smooth muscle cells
MMPs	matrix metalloproteinases
mPASCs	mouse pulmonary artery smooth muscle cells
PA	pulmonary artery
PAH	pulmonary arterial hypertension
PAH-hPASCs	human pulmonary artery smooth muscle cells from PAH subjects
PDGFR-β	platelet-derived growth factor receptor beta
PH	pulmonary hypertension
PTP	protein tyrosine phosphatase
ROCK	rho-kinase
ROS	reactive oxygen species
RVH	right ventricular hypertrophy
RVSP	right ventricular systolic pressure
SERT	serotonin transporter
SERT⁺	serotonin transporter overexpressing mice
WT	wild type

2]-like 2). Nrf-2 activators attenuate experimental pulmonary hypertension (PH).¹⁵ Increased expression of Nox isoforms 1 and 4 in PAs has been demonstrated in experimental models of PH and in PASCs from PAH patients.¹² Cellular Src-related kinase (c-Src) is the predominant nonreceptor tyrosine kinase in the vasculature, which is required for regulation of Nox activity,¹⁶ and this may be dysregulated in PAs of PAH patients and experimental PH.¹⁷

Serotonin-induced ROS has been implicated in the proliferative response of proximal bovine and murine PASCs.^{18,19} However, it is unclear whether serotonin influences ROS in hPASC and is the focus of our study.

Although studies have shown that serotonin promotes PA remodeling mainly through SERT and 5-HT_{1B}R,^{4,7,20} the role of Nox isoforms in serotonin-dependent ROS production, antioxidant regulation, and redox-sensitive processes downstream of ROS production has yet to be investigated. It is important to investigate this in the distal hPASCs that contribute to the pathophysiology of PAH. For the first time, we investigate the role of serotonin in Nox-derived ROS in hPASCs, specifically, Nox1-derived ROS in serotonin-induced Nrf-2 dysfunction, protein carbonylation, and oxidation of antioxidant and signaling molecules, peroxiredoxin, and PTPs.

Materials and Methods

Materials and Methods are available in the [online-only Data Supplement](#).

Results

Serotonin Increases ROS Production

Basal ROS production was higher in human pulmonary artery smooth muscle cells from PAH subjects (PAH-hPASCs) compared with hPASCs. In hPASCs, serotonin increased $\cdot\text{O}_2^-$ production at 1, 4, and 24 hours of stimulation, whereas

in PAH-hPASCs serotonin increased $\cdot\text{O}_2^-$ generation more rapidly at 30 minutes and 1 hour (Figure 1A). In control hPASCs, serotonin-induced $\cdot\text{O}_2^-$ generation was blocked by 5-HT_{1B}R antagonist, SB224289, but not a SERT antagonist (citalopram) or a 5-HT_{1D/2A}R inhibitor (ketanserin). In PAH-hPASCs, both the 5-HT_{1B}R and the SERT mediate $\cdot\text{O}_2^-$ generation as SB224289 and citalopram blocked the effects of serotonin (Figure 1B). No effects were observed with serotonin receptor antagonists alone (data not shown).

The Nox1 inhibitor ML171, and the radical scavenger tempol, inhibited serotonin-induced $\cdot\text{O}_2^-$ formation in control and PAH-hPASCs (Figure 1C). To control for non-specific effects of ML171, we examined ROS production in mouse PASCs (mPASCs) derived from wild-type (WT) and Nox1^{-/-} mice. Serotonin-induced ROS production was observed in WT mPASCs but absent in Nox1^{-/-} mPASCs at 30 minutes' stimulation (Figure 1D).

c-Src may be involved in transactivation and phosphorylation of subunits required for Nox assembly²¹ and is a proximal regulator of vascular Nox activation.¹⁶ Exposure of PAH-hPASCs to a Src kinase inhibitor, PP2, inhibited serotonin-induced ROS in PAH-hPASCs while having no effect in control hPASCs (Figure 2A).

Hydrogen peroxide (H₂O₂) is a common substrate for the peroxidation reaction of peroxiredoxins.²² Basal intracellular H₂O₂ levels, assessed by Amplex red, were increased in PAH-hPASCs versus controls. Serotonin caused H₂O₂ production at 5 and 30 minutes in control hPASCs, whereas H₂O₂ production in PAH-hPASCs was more sustained from 30 minutes to 4 hours (Figure 1A in the [online-only Data Supplement](#)). In control hPASCs, serotonin reduced catalase activity to similar levels observed in the PAH-hPASCs, whereas in PAH-hPASCs, serotonin further reduced activity of catalase after 5 minutes (Figure 1B in the [online-only Data Supplement](#)). In line with accumulation of H₂O₂, there was a reduction in basal catalase activity in PAH versus control hPASCs (Figure 1B in the [online-only Data Supplement](#)). In addition, catalase activity was reduced by serotonin treatment (30 minutes to 2 hours) in control hPASCs. In PAH-hPASCs, the already lowered basal catalase activity was further attenuated by serotonin more rapidly than in the control cells, at 5 minutes' stimulation (Figure 1B in the [online-only Data Supplement](#)). In support, glutathione mRNA levels were significantly attenuated in PAH-hPASCs versus control hPASCs (Figure 1C in the [online-only Data Supplement](#)).

Regulation of Nox Isoforms by Serotonin in hPASCs

Basal gene expression of Nox1 was increased in PAH-hPASCs compared with controls (Figure 2B). Serotonin increased Nox1 mRNA expression in control hPASCs to levels observed in PAH-hPASCs. In PAH-hPASCs, serotonin also increased mRNA expression of Nox1 (Figure 2B).

Regulation of Nrf-2 and Antioxidant Systems by Serotonin

Serotonin increased Nrf-2 activity in control hPASCs; yet, in PAH-hPASCs, a statistically significant increase

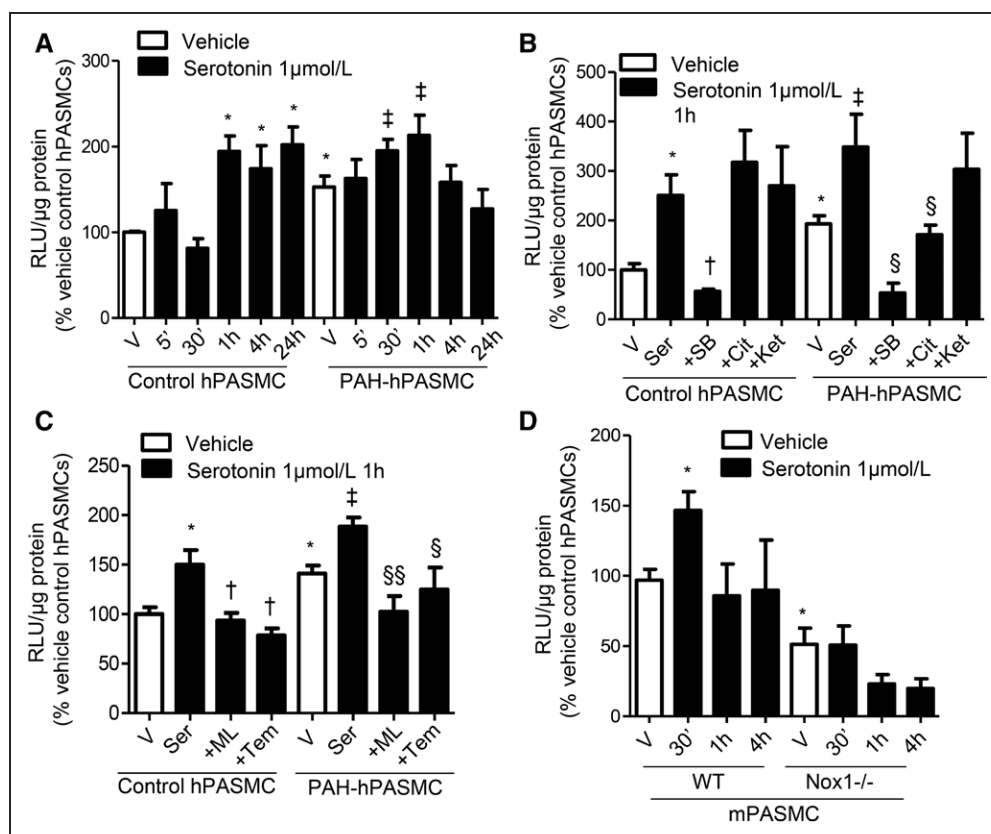


Figure 1. Serotonin increases reactive oxygen species (ROS) production through Nox-dependent mechanisms. Time-dependent increase of ROS production by serotonin (1 $\mu\text{mol/L}$) in control human pulmonary artery smooth muscle cells (hPASMCs) and pulmonary arterial hypertension (PAH)-hPASMCs assessed by lucigenin-enhanced chemiluminescence (A). hPASMCs and human pulmonary artery smooth muscle cells from PAH subjects (PAH-hPASMCs) were exposed to serotonin for the time of peak ROS production (1 h) previously shown in (A), in the presence or absence of inhibitors of Nox1 (ML171, 1 $\mu\text{mol/L}$) and superoxide dismutase mimetic Tempol (10 $\mu\text{mol/L}$; B). Serotonin-induced ROS production in wild-type (WT) and Nox1^{-/-} mouse pulmonary artery smooth muscle cells (mPASMCs; C). ROS production by serotonin (1 $\mu\text{mol/L}$) in control hPASMCs and PAH-hPASMCs at 1 h stimulation, in the presence or absence of 5-HT_{1B}R antagonist, SB224289, SERT inhibitor, citalopram (Cit), or 5-HT_{2A/1D}R antagonist (D). Data are expressed as relative light units (RLU)/ μg protein, expressed as percentage of vehicle control conditions. Results are mean \pm SEM of 5 experiments, in triplicate. * P <0.05 vs vehicle control hPASMC or vehicle WT mPASMCs; † P <0.05 vs treated control hPASMC; ‡ P <0.05 vs vehicle PAH-hPASMCs; § P <0.05, §§ P <0.01 vs treated PAH-hPASMCs determined by ANOVA with Tukey post hoc test. Ket indicates ketanserin; ML, ML171; SB, SB224289; Ser, serotonin; Tem, Tempol; and V, vehicle.

in Nrf-2 activity was not observed with serotonin treatment (Figure 2C). Peroxiredoxin is hyperoxidized and inactivated through sulfenylation. Basal levels of hyperoxidized peroxiredoxin in PAH-hPASMCs were elevated compared with control hPASMCs. Serotonin increased protein expression of hyperoxidized peroxiredoxin in control hPASMCs at 1-hour stimulation, and these effects were blocked by 5-HT_{1B}R antagonist, SB224289 (Figure 2D).

Serotonin Influences Redox Signaling in hPASMCs

Serotonin increased irreversible PTP oxidation exclusively in PAH-hPASMCs, inhibited by 5-HT_{1B}R inhibitor, SB224289, and the Nox1 inhibitor, ML171 (Figure 3A; Figure IIA in the online-only Data Supplement). Consistent with this, serotonin increased irreversible oxidation of PTPs in WT mPASMCs but had no effect in Nox1^{-/-} mPASMCs (Figure 3B; Figure IIB in the online-only Data Supplement). Carbonylation of proteins is a hallmark of oxidative stress. Basal protein carbonylation was increased in PAH-hPASMCs versus control hPASMCs, with no significant effects of serotonin (Figure 3C). There were increased levels of carbonylation in WT, but not Nox1^{-/-},

mPASMCs on serotonin treatment (Figure 3D). Basal Rho-kinase (ROCK) activity was increased in PAH-hPASMCs. Serotonin increased activity in PAH-hPASMCs, an effect that was inhibited by SB224289 and ML171 (Figure III in the online-only Data Supplement).

Serotonin-Induced Proliferation Involves Nox

Serotonin-induced proliferation was increased in PAH-hPASMCs versus control hPASMCs (Figure 4A). In line with ROS production, these effects were attenuated by the 5-HT_{1B}R inhibitor and the Nox1 inhibitor in hPASMCs, with additional inhibitory effects of SERT inhibitor, citalopram, in PAH-hPASMCs (Figure 4A). Consistent with this, proliferation was reduced in Nox1^{-/-} mouse-derived mPASMCs compared with WT mPASMCs when treated with serotonin (Figure 4B).

Serotonin-Induced Alterations in Markers of Fibrosis and Extracellular Matrix Remodeling Involve Nox1-Derived ROS

Extracellular matrix remodeling is a key process involved in the pathobiology of PAH.^{23,24} Loss of balance between matrix

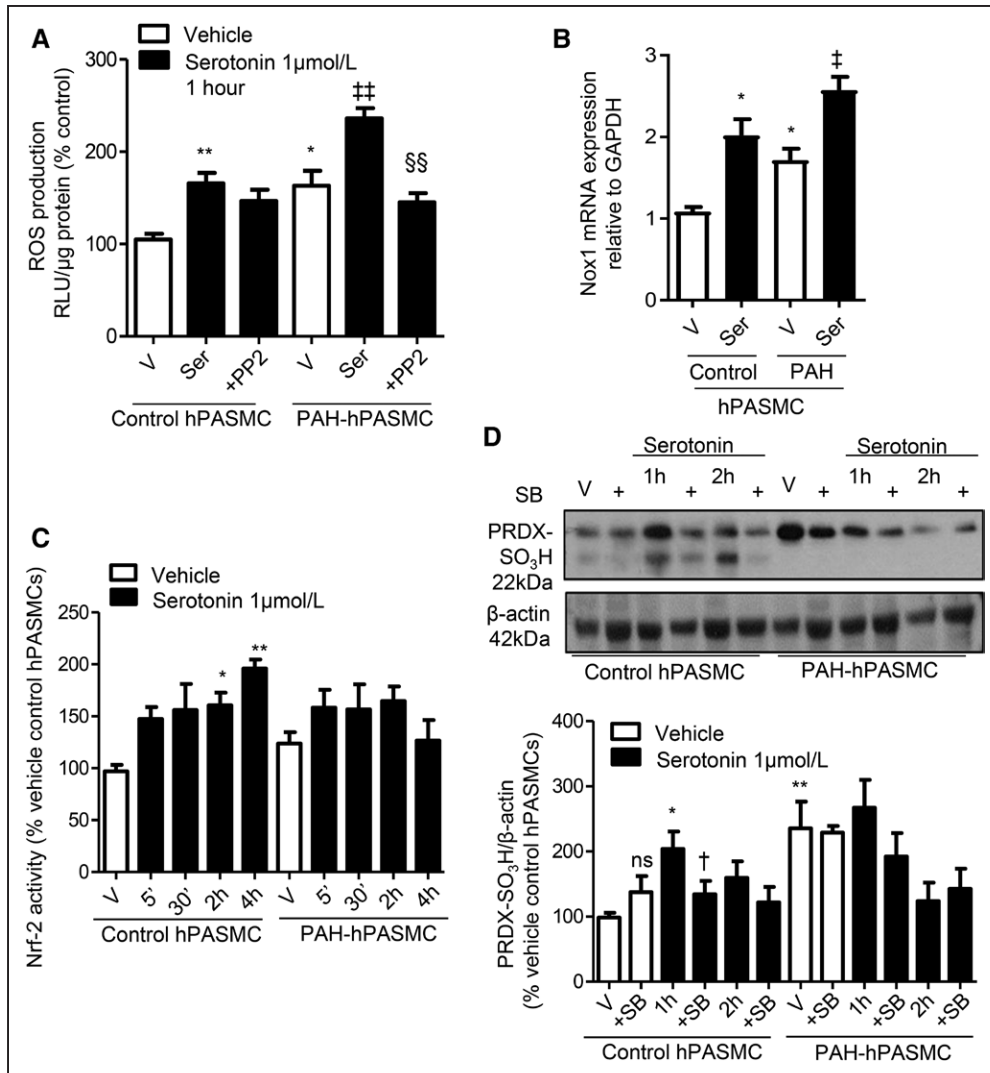


Figure 2. Effect of serotonin on Nrf-2 activation and oxidation of peroxiredoxin. Serotonin-induced reactive oxygen species (ROS) production in the presence or absence of Src kinase inhibitor, PP2 (10 $\mu\text{mol/L}$) in control human pulmonary artery smooth muscle cell (hPASC) and pulmonary arterial hypertension (PAH)-hPASCs. Data are expressed as relative light units (RLU)/ μg protein, expressed as percentage of vehicle control conditions (A). Transcript levels of Nox1 (B) in response to serotonin. Nuclear translocation of Nrf-2 by serotonin at 5 min to 4 h was assessed as an indicator of Nrf-2 activity. Data are expressed as RLU/ μg protein expressed as percentage of vehicle control conditions (C). Effects of serotonin (1–2 h) in the presence or absence of the 5-HT_{1B}R antagonist, SB224289, on protein expression of hyperoxidized peroxiredoxin, hyperoxidized peroxiredoxin (PRDX-SO₃H) in hPASCs (D). Results are mean \pm SEM of 3 to 6 experiments. Graph represents mRNA expression relative to GAPDH. * P <0.05, ** P <0.01 vs vehicle control hPASC or wild-type (WT) vehicle mouse pulmonary artery smooth muscle cells (mPASCs); † P <0.05 vs serotonin 1 h control hPASCs; vehicle human pulmonary artery smooth muscle cells from PAH subjects (PAH-hPASCs; Nox1); ‡ P <0.05, †† P <0.01 vs vehicle PAH-hPASCs; §§ P <0.01 vs serotonin-treated PAH-hPASCs determined by ANOVA with Tukey post hoc test. ns indicates not significant; SB, SB224289; Ser, serotonin; and V, vehicle.

metalloproteinases (MMPs) and TIMPs (tissue inhibitor of matrix metalloproteinases) initiates extracellular matrix and vascular remodeling. Basal MMP2 mRNA was elevated in PAH-hPASCs and further increased by serotonin; these effects were 5-HT_{1B}R- and Nox1-dependent, through the use of pharmacological inhibitors, SB224289 and ML171, respectively (Figure 4C). Basal MMP9 transcript was elevated in PAH-hPASCs, and serotonin further stimulated MMP9 in both control and PAH-hPASCs, effects attenuated in the presence of 5-HT_{1B}R antagonist, SB224289, and Nox1 inhibitor, ML171 (Figure 4D). Serotonin had no effect on secretion of platelet-derived growth factor subunit B (homodimer) from hPASCs

(Figure IVA in the [online-only Data Supplement](#)). Protein expression of platelet-derived growth factor receptor beta (PDGFR- β) was increased under basal conditions in PAH-hPASCs versus control hPASCs, whereas in control hPASCs serotonin increased expression of PDGFR- β where this increase was attenuated in the presence of 5-HT_{1B}R antagonist SB224289 (Figure IVB in the [online-only Data Supplement](#)).

5-HT_{1B}R and Nox1 Staining in Human Lung Tissue Sections

In human lung sections from control subjects, 5-HT_{1B}R staining was absent. In contrast, 5-HT_{1B}R staining was evident in

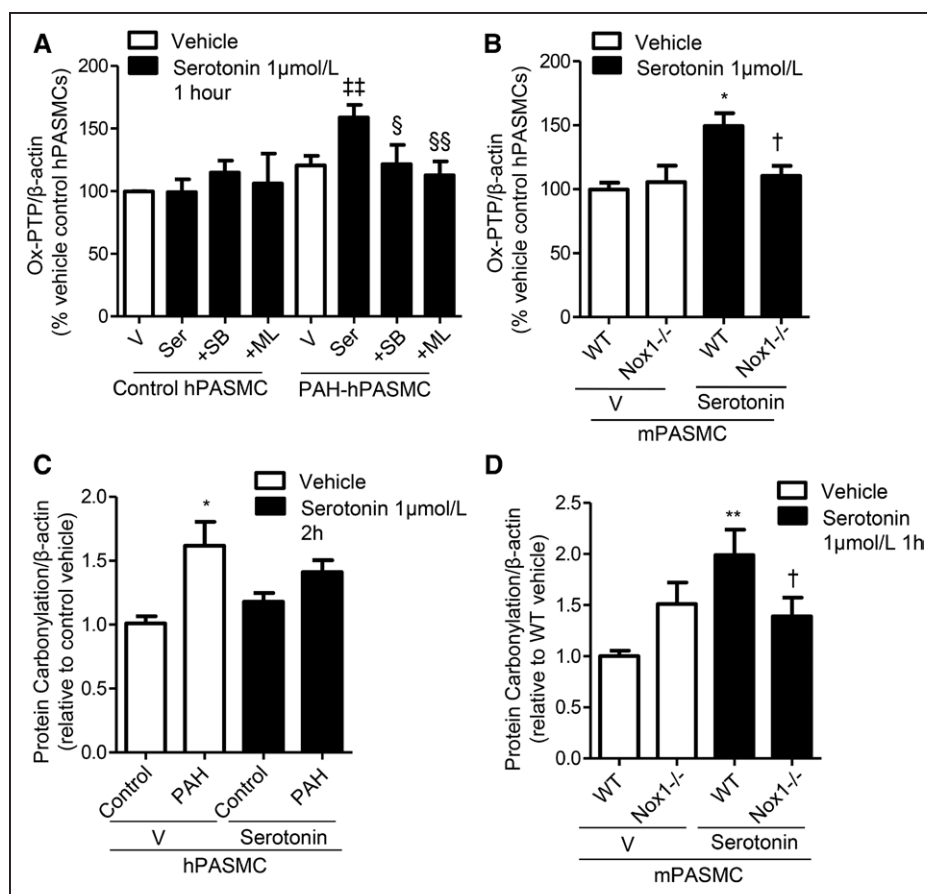


Figure 3. Effects of serotonin on oxidation of protein tyrosine phosphatases (PTPs) and global protein carbonylation. Irreversible oxidation of PTPs using the oxPTP antibody, in response to serotonin in human pulmonary artery smooth muscle cells (hPASCs) in the presence or absence of SB224289 or Nox1 inhibitor, ML171 (**A**). PTP oxidation in wild-type (WT) and Nox1^{-/-} mouse pulmonary artery smooth muscle cells (mPASCs) treated with serotonin (**B**). Total protein carbonylation in response to serotonin in hPASCs (2 h; **C**) and in Nox1^{-/-} mPASCs (1 h; **D**). Results are representative of 3 to 5 experiments where protein expression is relative to β-actin. **P*<0.05, ***P*<0.01, vs vehicle control hPASCs or WT vehicle mPASCs; †*P*<0.05 vs treated WT mPASCs; ††*P*<0.01 vs vehicle pulmonary arterial hypertension (PAH)-hPASCs; §*P*<0.05, §§*P*<0.01 vs serotonin-treated human pulmonary artery smooth muscle cells from PAH subjects (PAH-hPASCs) determined by ANOVA with Tukey post hoc test. ML indicates ML171; SB, SB224289; Ser, serotonin; and V, vehicle.

both the endothelium and the smooth muscle of pulmonary arteries in human lung sections from patients with PAH. Nox1 staining was present in smooth muscle of pulmonary arteries of human lung sections derived from both controls and patients with PAH (Figure V in the [online-only Data Supplement](#)).

5-HT_{1B}R Antagonism In Vivo Attenuates Development of PH in the SERT⁺ Mouse and Chronic Hypoxic Mouse

Female mice that overexpress the gene for human SERT demonstrate PH.⁶ Lungs from serotonin transporter overexpressing (SERT⁺) mice demonstrated increased oxidative stress.²⁵ The 5-HT_{1B}R antagonist, SB216641, reduced the increase in right ventricular systolic pressure (RVSP) in SERT⁺ mice (Figure 5A), and as shown before,⁶ SERT⁺ mice show no change in right ventricular hypertrophy (RVH; Figure 5B). PA remodeling was increased in SERT⁺ mice, and this was reduced by SB216641 (Figure 5C). There was no change in mean arterial pressure (Figure VIA in the [online-only Data Supplement](#)) or heart rate (Figure VIB in the [online-only Data Supplement](#)) by SB216641 in WT or

SERT⁺ mice. In addition, oral administration of SB216641 (15 mg/kg/d) protected against elevations in RVSP, RVH, and the development of pulmonary vascular lesions/remodeling in 8- to 10-week-old female C57BL/6J mice exposed to 15 days chronic hypoxia. Age-matched female mice maintained under normoxic conditions were used as controls (Figure VII in the [online-only Data Supplement](#)). PA remodeling was also increased in hypoxic mice compared with their normoxic counterparts, and this remodeling was ameliorated by SB216641 (Figure VIII in the [online-only Data Supplement](#)). No effects were observed in normoxic control mice treated with SB216641 (Figures VII and VIII in the [online-only Data Supplement](#)).

We investigated whether oxidative stress was regulated by serotonin (and the 5-HT_{1B}R) in SERT⁺ mice by immunohistochemical analysis of 8-OHG (8-hydroxyguanosine), a fluorescent ROS probe, which specifically detects oxidative damage to nucleic acids. Female SERT⁺ mice had increased levels of 8-OHG compared with WT mice, and SB216641 treatment reduced 8-OHG levels (Figure IX in the [online-only Data Supplement](#)). 8-OHG staining was observed in both the

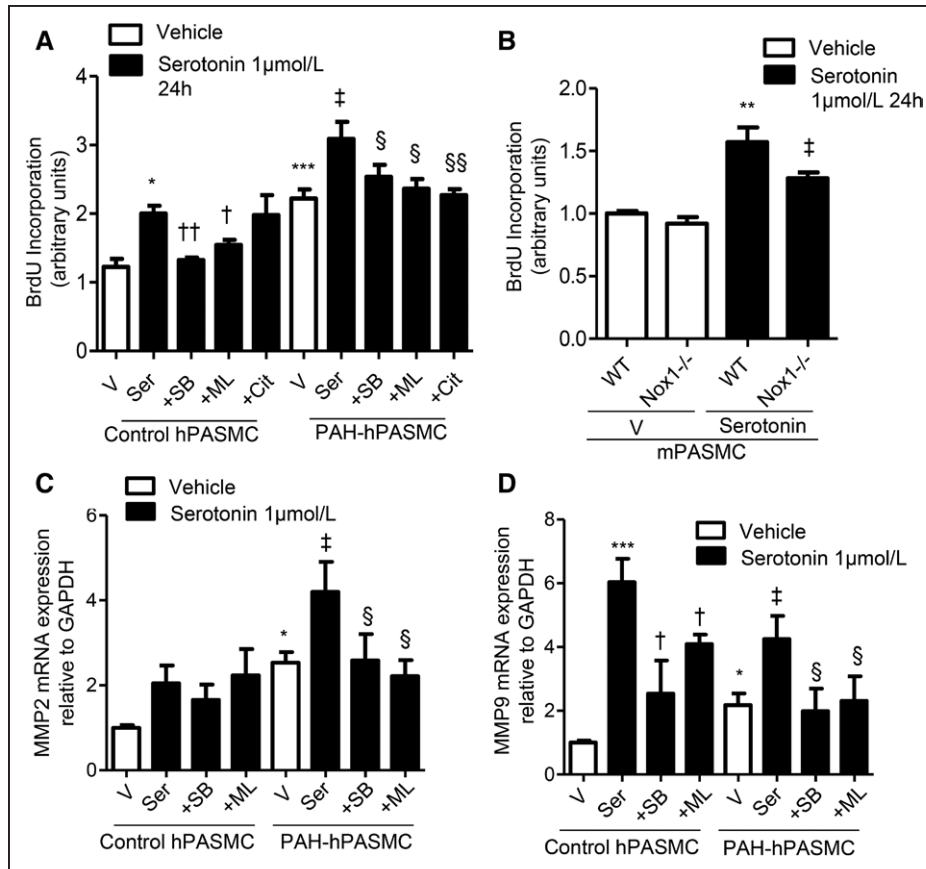


Figure 4. Role of 5-HT_{1B}R and Nox1 in serotonin-mediated cell proliferation and extracellular matrix remodeling. To test whether serotonin regulates proliferation in a Nox/reactive oxygen species (ROS)-dependent manner, BrdU (5-Bromo-2'-deoxyuridine) incorporation was assessed in human pulmonary artery smooth muscle cells (hPASCs; **A**) and wild-type (WT) and Nox1^{-/-} mouse pulmonary artery smooth muscle cells (mPASCs) in response to serotonin (**B**). Transcript expression of MMP2 (**C**) and MMP9 (**D**) after treatment with serotonin with or without SB224289 or ML171. Results are representative of 5 experiments per group where mRNA expression is normalized to GAPDH. **P*<0.05, ***P*<0.01, ****P*<0.001 vs vehicle control hPASCs or WT vehicle mPASCs; †*P*<0.05, ††*P*<0.01 vs serotonin-treated control hPASCs; ‡*P*<0.05 vs vehicle pulmonary arterial hypertension (PAH)-hPASCs or serotonin-treated mPASCs; §*P*<0.05, §§*P*<0.01 vs serotonin-treated PAH-hPASCs determined by ANOVA with Tukey post hoc test. Cit indicates citalopram; ML, ML171; MMP, matrix metalloproteinase; SB, SB224289; Ser, serotonin; and V, vehicle.

cytoplasm and the nucleus, indicating increased RNA and DNA oxidation.

Discussion

Our novel findings demonstrate that in hPASCs serotonin stimulates ROS through Nox, downregulates protective antioxidant mechanisms, stimulates redox signaling and irreversible protein oxidation and carbonylation, and promotes molecular and cellular processes associated with extracellular matrix remodeling and cell growth. These serotonin effects are amplified in hPASCs from patients with PAH and involve c-Src-regulated Nox1 and 5-HT_{1B}R and Nrf-2 dysfunction (summarized in Figure X in the [online-only Data Supplement](#)). Using SERT⁺ mice, we confirm, in vivo, our cell-based findings.

The 5-HT_{1B}R receptor is highly expressed in human PAs, with increased expression in PAH patients,⁴ and mediates serotonin-induced vasoconstriction and PA remodeling. The 5-HT_{2A}R mediates these effects systemically and so the 5-HT_{1B}R effects are pulmonary specific, making it a favorable target for PAH. Serotonin-dependent ROS production in

hPASCs was dependent on 5-HT_{1B}R in control hPASCs, whereas in PAH-hPASCs this was mediated by both the SERT and the 5-HT_{1B}R. Consistently, there is an increase in SERT expression in PAs and hPASCs from PAH patients.²⁶ Female mice overexpressing SERT have a PH phenotype.⁶ There is co-operation between SERT and 5-HT_{1B}R in human and experimental PAH,^{5,20} and dual blockade of the 5-HT_{1B}R and SERT is a plausible therapeutic approach.

Growing evidence implicates a role for Noxs, particularly Nox1 and Nox4, in the pathogenesis of experimental and human PAH.^{12,27} In addition to the constitutive expression of Nox1 in a variety of tissues, Nox1 expression is increased after inflammation, growth factor stimulation, and hypoxia.²⁸ Nox1 also plays a critical role in physiological turnover of PASCs by regulation of intracellular potassium.²⁹

We observed that serotonin induces ROS production through Nox1 activation in hPASCs. Although peak ROS levels induced by serotonin are similar between control and PAH-hPASCs, we suggest that PAH-hPASCs are already primed to ROS, as basal levels are increased in PAH-hPASCs versus control hPASCs. We note that although

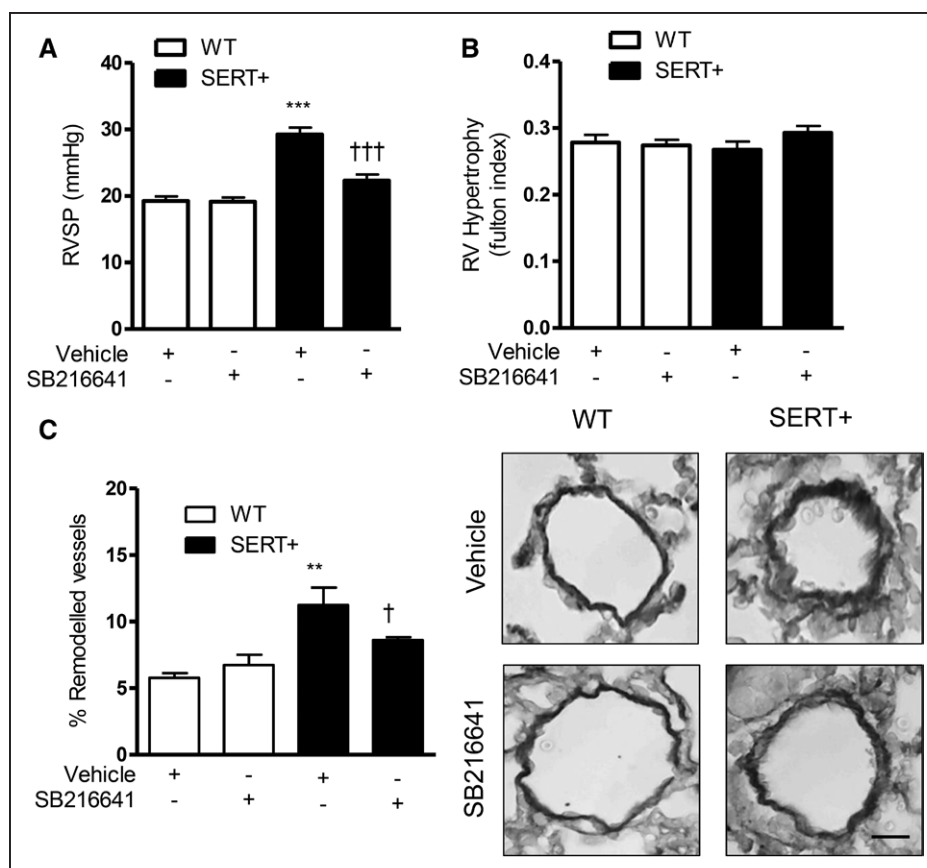


Figure 5. Hemodynamic assessment of pulmonary hypertension in female wild-type (WT) and serotonin transporter overexpressing (SERT⁺) mice treated with SB216641. Right ventricular systolic pressure (RVSP; **A**), right ventricular (RV) hypertrophy (**B**). Effects of SB216641 on percentage of pulmonary vascular remodeling in distal pulmonary arteries in female WT and SERT⁺ mice with representative images (**right**) of pulmonary arteries (Elastin Van Gieson stain; scale bars=50 μ m; **C**). Results are mean \pm SEM, n=8 to 10 per group. *** P <0.001 vs WT vehicle, ††† P <0.001 vs SERT⁺ vehicle, determined by 2-way ANOVA with Tukey post hoc test. Data are mean \pm SEM, n=8 to 10 per group. ** P <0.01 vs WT vehicle; † P <0.05 vs SERT⁺ vehicle, determined by 2-way ANOVA with Tukey post hoc test.

ROS levels were sustained in control hPASMCs, they were quickly recovered in PAH-hPASMCs. Although glutathione transcript level is reduced in PAH versus control hPASMCs, there is no further impairment after serotonin stimulation. Similarly, although catalase activity is downregulated by serotonin in control hPASMCs up to 2 hours, in PAH-hPASMCs, catalase activity recovers more rapidly than in control hPASMCs. Moreover, we found that this effect is mediated via c-Src because PP2, which inhibits Src family kinases, attenuated serotonin-induced Nox1 activation. c-Src plays an important role in activation of MAPKs associated with cell growth, apoptosis, and collagen deposition.³⁰ Previous studies demonstrated that c-Src is both a redox-sensitive downstream target of Nox and an upstream signaling molecule for Nox activation³¹ and can increase protein abundance subunits and adaptor proteins required for regulation of Nox activity.¹⁶ Importantly, PP2 reduced serotonin-stimulated ROS formation exclusively in PAH-hPASMCs, suggesting a role for c-Src in serotonin-mediated Nox activation in PAH.

High concentrations of ROS trigger the oxidation of PTPs, resulting in their loss of function as a phosphate acceptor.³² In association with excessive ROS production by serotonin, we found an increase in irreversibly oxidized PTPs. Our findings suggest that PTP inhibition may play a role in PAH and

that serotonin-induced Nox1-derived ROS may be important in this process.

Other molecular processes that are redox-sensitive relate to total protein carbonyl content, an important index of whole cell protein oxidation.³³ Accumulation of protein carbonyls has been observed in several human pathologies, including diseases of the lung.³⁴ We found increased protein carbonylation in basal conditions, in PAH-hPASMCs compared with control cells, along with increased carbonyl content in response to serotonin in WT mPASMCs, an effect that was absent in mPASMCs from Nox1^{-/-} mice. This suggests a role for Nox1-derived ROS in the regulation of protein carbonylation in hPASMCs. It has been reported that an alternative strategy to antioxidant intervention is to detoxify oxidative-derived carbonyl reaction products.³⁵ Thus, strategies to regulate carbonyl content may have clinical value.

Peroxiredoxin is an antioxidant enzyme important in the degradation of H₂O₂, which can modulate various receptor signaling pathways and protects cells from oxidation-induced death. The active site cysteine (Cys) of peroxiredoxin is oxidized to the sulfenic acid intermediate (Cys-SOH) when a peroxide is reduced. Yet, because Cys-SOH is unstable, it forms a disulfide with Cys-SH. However, under oxidative stress conditions, the sulfenic intermediate (Cys-SOH) can

be easily overoxidized to cysteine sulfinic or sulfonic (Cys-SO₂H/-SO₃H) acids, referred to as hyperoxidation. H₂O₂ is a common substrate for the peroxidation reaction of peroxiredoxins and also a well-known cause for their hyperoxidation.²² In our study, serotonin caused excessive production of H₂O₂ in PAH-hPASMCs, as well as decreased activity of H₂O₂ reducing enzymes, catalase, and glutathione, in PAH-hPASMCs versus control hPASMCs. These effects were further reduced in serotonin-treated PAH-hPASMCs, indicative of increased production and accumulation of H₂O₂ in PAH, potentiated by serotonin. In control hPASMCs, serotonin stimulated ROS production and increased Nrf-2 activity, perhaps as a compensatory mechanism to maintain redox balance. However, in PAH-hPASMCs, serotonin increased ROS production, yet did not induce activity of Nrf-2 as an antioxidant defense mechanism, suggesting overall ROS accumulation and oxidative damage. Of clinical significance, Nrf-2 activators are in human trials for PAH and can attenuate experimental PH.¹⁵

To better understand the functional significance of serotonin-induced oxidative stress, we studied effects on molecular regulators of vascular contraction and proliferation, hallmarks of vascular remodeling in PAH. In human PAH, ROCK inhibitors cause modest reductions in pulmonary vascular resistance.³⁶ As such, ROCK has been implicated in both PA contraction and remodeling.³⁷ Serotonin-induced PASMC proliferation, contraction, and migration involve ERK1/2 MAPK and ROCK. Serotonin-mediated signaling of ROCK occurs through 5-HT_{1B}R activation in a lung fibroblast cell line.⁸ Here, we show in hPASMCs that serotonin-mediated activation of ROCK is mediated via the 5-HT_{1B}R and Nox1-derived ROS. Serotonin stimulated cell proliferation, which was exaggerated in PAH-hPASMCs and dependent on the 5-HT_{1B}R and Nox1 because these effects were absent in the presence of inhibitors of this receptor and oxidase, and in Nox1^{-/-} mice. Apoptosis is another key factor in vascular remodeling characteristic of PAH, as such, it has been previously shown that serotonin inhibits PASMC apoptosis through 5-HT_{1B}R or SERT.³⁸ It would be interesting to understand the role of serotonin and the 5-HT_{1B}R in other vascular cell components, such as human PA endothelial cells, primary cultures of such cells are not readily available from control subjects and patients with PAH, and therefore are difficult to study in relation to the human disease.

Structural remodeling of pulmonary arteries through smooth muscle cell migration, proliferation, and oversecretion of extracellular matrix are the most notable pathological changes in PAH.³⁹ The MMPs, particularly gelatinases MMP2 and MMP9, are involved in extracellular matrix turnover and hence, in smooth muscle cell migration and proliferation. Of note, promoters of functionally related MMPs, such as MMP2/MMP9, are clearly distinct, pointing to different mechanisms of activation.⁴⁰ As evidenced here and as shown previously, concomitant increments in both MMPs are not always observed. For example, in a rat model of pancreatitis-induced lung injury, MMP9 increased in lung tissue, whereas MMP2 remained unchanged.⁴¹ However, the promoter for MMP2 does not harbor a TATA box, and expectedly, transcription from these promoters starts at multiple sites. Such that, expression of MMP2 is mainly determined by the

ubiquitous Sp1 family of transcription factors, where expression of MMP2 in the main part is constitutive, with only modest sensitivity to induction by growth factors or cytokines.⁴² This is in line with our data showing that MMP2 transcript is induced by serotonin solely in PAH-hPASMCs, whereas MMP9 mRNA expression is sensitive to serotonin stimulation in both control and PAH-hPASMCs. Vascular remodeling in both experimental and human PAH has been shown to involve increased expression of profibrotic proteins. In the lung of PAH patients, accelerated turnover of the extracellular matrix because of increased MMP activity occurs.²³ Loss of balance between MMPs and TIMPs initiates extracellular matrix and vascular remodeling and is involved in a variety of pathologies.⁴³ Serotonin increased expression of the profibrotic signaling proteins MMP2 and MMP9 in hPASMCs. These effects were absent in hPASMCs pretreated with inhibitors of the 5-HT_{1B}R or Nox1, suggesting an important role for Nox1 in the profibrotic effects of serotonin.

The rationale for platelet-derived growth factor involvement in the pathogenesis of PAH is strong, with increased expression of ligand and phosphorylated receptor in patient lung tissue.⁴⁴ However, we did not observe any change in the platelet-derived growth factor subunit B (homodimer) levels in the spent culture medium from serotonin-stimulated control and PAH-hPASMCs. Because both serotonin and PDGFR-β have been associated with clinical and experimental PH, and patients with PA hypertension have enhanced activation of PDGFR-β in their lungs,⁴⁴ we investigated whether there is a relationship between these molecules in hPASMCs. Expression of PDGFR-β was increased under basal conditions in PAH-hPASMCs versus control hPASMCs, whereas in control hPASMCs, serotonin increased expression of PDGFR-β, where this increase was absent in the presence of 5-HT_{1B}R antagonist SB224289. A previous study in bovine-derived PASMCs observed serotonin-stimulated production of ROS serving as an intermediate in the transactivation of PDGFR-β by serotonin, where oxidation of PTPs was suggested as the mechanism involving SERT rather than the 5-HT receptors.⁴⁵ In line, we observed that in female human PASMCs, PDGFR-β expression is increased in PAH-hPASMCs versus control hPASMCs. In control hPASMCs, expression of PDGFR-β was elevated by serotonin in a 5-HT_{1B}R-dependent manner. This is consistent with recognized cross talk between platelet-derived growth factor and serotonin signaling in pulmonary arteries.⁴⁶

To test the pathophysiological significance of our findings, we performed *in vivo* studies assessing the effects of the 5-HT_{1B}R antagonist, SB216641, in 2 mouse models of PH, the chronic hypoxic mouse, and in a serotonin-dependent mouse model of PH, the SERT⁺ mouse. Female C57BL/6/J mice exposed to hypoxic conditions developed elevated RVSP, RVH, and evidence of pulmonary vascular remodeling parameters, which were prevented in those mice treated with the 5-HT_{1B}R antagonist, SB216641. Similarly, female SERT⁺ mice developed increased RVSP and PA remodeling at 20 weeks of age; protective effects of SB216641 treatment was observed. RVH remained unaffected, in line with previous work where our group and others have shown a dissociation between RVSP and RVH in the SERT⁺ mouse.⁶ We previously

demonstrated increased ROS expression in the SERT⁺ mouse lung²⁵; lung ROS was reduced by the 5-HT_{1B}R antagonist, suggesting that the 5-HT_{1B}R/ROS axis is important both in vitro in cultured PSMCs and in vivo in the mouse lung. In addition, in support of the role of 5-HT_{1B}R in human PAH, we observed marked 5-HT_{1B}R staining in the endothelium and smooth muscle cell layers of the vasculature in human lung sections from PAH subjects, staining that was absent in lung sections from control subjects.

In conclusion, we show for the first time in hPSMCs from PAH patients that serotonin increases Nox1-dependent ROS generation and decreases Nrf-2-antioxidant systems through c-Src-dependent processes that contribute to oxidation of proteins, DNA damage, and redox-sensitive proliferation and fibrosis of hPSMCs, processes involved in PAH. In line, we observed ROS-dependent protective effects of 5-HT_{1B}R antagonism in the SERT⁺ mouse model of PH. We identify the 5-HT_{1B}R and both 5-HT_{1B}R and SERT in PAH-derived PSMCs, as being particularly important in Nox1-derived ROS production and in serotonin-mediated vascular effects in PAH. Our study provides new molecular insights through c-Src/Nox1/ROS and Nrf-2 antioxidant mechanisms, whereby serotonin influences hPSMC function, which when upregulated may contribute to vascular injury in PAH (summarized in Figure X in the [online-only Data Supplement](#)).

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Disclosures

None.

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Highlights

- Serotonin-induced proliferation of vascular cells, via the serotonin transporter and the 5-HT_{1B} receptor, has been associated with the development of pulmonary arterial hypertension.
- Serotonin stimulates production of reactive oxygen species but mechanisms underlying this, and the significance of serotonin-induced reactive oxygen species in the development of pulmonary arterial hypertension, remain unclear. Here, we show that redox-sensitive effects of serotonin are associated with Nrf-2 dysregulation, contributing to oxidative stress, protein oxidation, proliferation, and extracellular matrix remodeling in pulmonary artery smooth muscle cells from pulmonary arterial hypertension patients.
- We demonstrate in vivo that this may contribute to the pathobiology of pulmonary hypertension.
- Therefore, inhibition of the serotonin-mediated redox system or activation of Nrf-2 could be novel therapeutic strategies in pulmonary arterial hypertension.

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Materials and Methods

Serotonin Signaling Through the 5-HT_{1B} Receptor and NADPH Oxidase 1 in Pulmonary Arterial Hypertension.

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Cell Culture

In vitro studies were performed using primary hPASMCM from distal arteries from PAH patients (PAH-hPASMCMs). Cells were explanted as described¹, and were provided by N.W. Morrell, University of Cambridge, UK. PASMCMs from non-PAH subjects (control hPASMCMs) were also studied (patient details in Supp. Table I) where PAH was not suspected on clinical or radiological grounds in these subjects and cells were derived from macroscopically normal tissue. Experimental procedures using hPASMCMs were approved by Cambridgeshire 1 Research Ethics Committee (reference: 08/H0304/56). Additional *in vitro* studies were performed in primary cultured mouse PASMCMs (mPASMCMs) from the intralobar pulmonary arteries of female Nox1^{-/-} mice and WT littermates (C57BL/6J background), used between passage 2 and 4, as described previously².

Cell Protocols

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, UK) supplemented with antibiotic antimycotic solution (containing 0.25µg/ml amphotericin B; 100U/ml penicillin; 100µg/ml streptomycin; Sigma-Aldrich, UK) and 10% (v/v) fetal bovine serum (Sera Laboratories International, West Sussex, UK). Cells were grown to 70% confluence before serum deprivation for 24 hours in 0.5% fetal bovine serum in phenol-red free DMEM prior to experiments to render them quiescent. Cells were stimulated with serotonin (1µmol/L; 5 minutes-24 hours). In some protocols, cells were pre-treated (30 minutes) with pharmacological inhibitors Acetylphenothiazine (ML171; Nox1 inhibitor, 1µmol/L); 1'-Methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4'-piperidinehydrochloride (SB224289; 5-HT_{1B}R antagonist, 0.1µmol/L); 3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1*H*,3*H*]-quinazolinedione tartrate (ketanserin; 5-HT_{2A/1D}R antagonist, 0.1µmol/L); 1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitrile hydrobromide (citalopram; SERT inhibitor, 0.1µmol/L); 4-Amino-3-(4-chlorophenyl)-1-(*t*-butyl)-1*H*-pyrazolo[3,4-*d*] pyrimidine (PP2; c-Src inhibitor, 0.1µmol/L); 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol; SOD mimetic, 10µmol/L). Doses were based on preliminary experiments and published data.

Reagents and Antibodies

The following antibodies were used: anti-PRDX-SO₂/SO₃H (Abcam, ab16830); anti-PCNA (Santa Cruz, sc-56); anti-Nox1 (Abcam, ab55831); anti-Ox-PTP (R&D systems, MAB2844) and anti-β-actin (Abcam, ab8229). Serotonin (Cat.#3547), PP2, a Src kinase inhibitor (Cat.#P0042) and SB224289, (5-HT_{1B}R antagonist; Cat.#S201); ML171 (Nox1 inhibitor; Cat.#4653); Tempol (SOD mimetic; Cat.#3082); Ketanserin (5-HT_{2A/1D}R antagonist; Cat.#0908) and Citalopram (SERT inhibitor; Cat.#1427) were purchased from Tocris, UK. Doses were based on preliminary experiments and published data.

Lucigenin-Enhanced Chemiluminescence

Lucigenin-enhanced chemiluminescence was used to determine ROS generation as described previously³. ROS production was determined in control hPASMCM, PAH-hPASMCMs and mPASMCMs stimulated with serotonin for 5 minutes to 48 hours. Inhibitor studies (30 minutes' pre-incubation) were carried out at the peak time points for ROS production (1 hour). After stimulation, cells were washed with ice-cold PBS and harvested in lysis buffer (20mmol/L KH₂PO₄, 1 mmol/L EGTA, 1µg/mL aprotinin, 1µg/mL leupeptin, 1µg/mL pepstatin, and 1mmol/L PMSF). 50µl of sample was added to a suspension containing 175µl of assay buffer (50mmol/L KH₂PO₄, 1mmol/L EGTA, 150mmol/L sucrose) and lucigenin (5µmol/L). NADPH (100µmol/L) was added to the suspension containing lucigenin (5µmol/L). Luminescence was measured with a luminometer (AutoLumat LB 953, Berthold)

before and after stimulation with NADPH. A buffer blank was subtracted from each reading. Superoxide anion production was inhibited by tempol and was expressed as relative luminescence units (RLU)/ μg protein, relative to vehicle conditions. For analysis, basal relative light units (RLU) values were subtracted from NADPH-stimulated values. RLU were corrected to sample protein concentration ($\mu\text{g}/\text{ml}$), as assessed by DC assay (Bio-Rad). Results in RLU/ μg protein were then normalised to their respective vehicle control hPASCs data. Data is expressed as relative percentage (%) of vehicle control hPASCs.

Amplex Red Assays

H_2O_2 production and catalase activity was assessed with Amplex Red Hydrogen Peroxide/Peroxidase Assay, or Amplex Red Catalase Assay, (Thermo Fisher, UK) according to instructions, as described previously⁴.

Real-Time PCR

Quantitative real-time PCR was used to analyse mRNA expression using SYBR Green I as previously described⁴. Primers used were designed using the Primer 3 software online (Supp. Table II).

Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) Activity Assay

Nrf-2 activity was determined with the TransAM Nrf-2 assay (Active Motif, USA) following manufacturer's instructions. Briefly, to assess Nrf-2 nuclear accumulation, samples were prepared according to the manufacturer's protocol using a nuclear extract kit (Active Motif, USA). Nuclear preparations ($10\mu\text{g}$) were used for the TransAM Nrf2 ELISA kit (Active Motif, USA) to measure DNA binding of activated Nrf2 nuclear protein, as determined by absorbance measurements at 450nm , as described⁴.

Measurement of Rho Kinase Activity

Rho kinase (ROCK) activity was assessed using the ROCK Activity Assay (Cell Biolabs Inc) following manufacturer's instructions⁵.

Measurement of PDGF-BB levels

PDGF-BB levels in spent medium from control and PAH-hPASCs, was assessed using the PDGF-BB ELISA (R&D Systems, cat# DBB00) according to manufacturer's instructions.

Immunoblotting

Proteins were extracted from hPASCs and protein expression of PDGFR- β (Abcam; cat# ab32570) and hyper-oxidized peroxiredoxin (PRDX-SO₃H) was examined by immunoblotting as described previously⁶. All antibodies were used at 1:1000 dilution unless otherwise stated. Washed membranes were incubated with horseradish peroxidase - conjugated secondary antibodies and probed for immunoreactive proteins by chemiluminescence (WestPico, Pierce).

Protein tyrosine phosphatase oxidation

Irreversible oxidation of PTPs was assessed using anti-Ox-PTP antibody that recognizes the sulfonic acid form of PTP cysteine residues. Briefly, irreversible oxidation of protein tyrosine phosphatases (PTPs) was assessed by immunoblotting using an antibody (anti-Ox-PTP; 1:2000 dilution, R&D Systems) that specifically recognizes the sulfonic acid form of PTP cysteine residues. Following treatment, the sample is alkylated using N-ethylmaleimide (NEM; $1\text{mmol}/\text{L}$) to protect reduced cysteines. Subsequently, the samples are treated with dithiothreitol (DTT; $1\text{mmol}/\text{L}$) and pervanadate ($100\mu\text{mol}/\text{L}$) to convert reduced cysteines to

the sulfonic acid form. Finally, the sulfonic acid form is detected using the Ox-PTP antibody by immunoblotting as previously described⁷.

OxyBlot

Protein carbonylation was assessed by OxyBlot Protein Oxidation Detection Kit (Millipore) following manufacturer's instructions⁸.

5-Bromo-2'-deoxyuridine (BrdU) Incorporation Assay

Proliferation was measured using the BrdU Incorporation assay (Millipore) following manufacturer's instructions as described⁶. Briefly, cells were seeded onto a 96-well plate and starved overnight before stimulation. Cells were incubated with BrdU for 24 hours.

Absorbance was obtained at dual wavelength (450nm and 595nm) with a spectrophotometer (Spectra Max; Molecular Devices, Sunnyvale, CA, USA). The results were normalized as percent of control vehicle conditions

Ethical Considerations: SERT+ mice and C57BL/6J mice

The investigation conforms with the UK Animal Procedures Act (1986) and ARRIVE Guidelines. All mice used were 20-week-old females. Mice were housed in controlled environmental conditions with a 12-hour light/dark cycle with free access to food and water.

SERT+ Mice

SERT+ mice (background: C57BL/6JxCBA) have been previously described⁹. 20-week old female SERT+ mice underwent 14-days treatment with 5-HT_{1B}R antagonist (SB216641; 15mg/kg/day, oral gavage) or vehicle (dH₂O). Age-matched littermates were studied as controls.

Chronic Hypoxia Model of Pulmonary Hypertension

Female C57BL/6J mice (aged 8-10 weeks) were exposed to hypoxic conditions for 15 days using a hypobaric chamber. Age-matched normoxic female mice were assessed as controls. From days 1 to 15, mice underwent treatment with either 5-HT_{1B}R antagonist (SB216641; 15mg/kg/day, oral gavage) or vehicle (dH₂O).

***In vivo* Haemodynamic Measurements**

Pressure measurements were conducted and analysed as described previously¹⁰. Inhalation anaesthesia was used throughout the procedure (1–3% isoflurane/O₂). Depth of anaesthesia was assessed by loss of pain and corneal reflexes. Briefly, right ventricular systolic pressure (RVSP) was measured via a 25-gauge needle advanced into the right ventricle (RV) using a trans-diaphragmatic approach. Mean systemic arterial pressure (mSAP) was obtained via a micro-cannula inserted into the carotid artery. Mice were euthanized by cervical dislocation and heart and lungs removed for subsequent analysis as described below. Eight to 10 mice per group were studied.

Measurement of Right Ventricular Hypertrophy

Right ventricular hypertrophy (RVH) was assessed as weight of the RV free wall/ weight of the left ventricle with the septum.

Human Lung Immunohistochemistry

Formalin-fixed, paraffin embedded tissue sections from human control lung and human PAH lung, were stained using anti-5-HT_{1B}R antibody (Abcam ab13896 1:400), and anti-Nox1 antibody (Sigma, SAB4200097; 1:200). An anti-rabbit IgG secondary antibody was used for

each primary antibody (5µg/ml; Vector Laboratories IMMpress kit) and protein immunolocalisation was visualized with the alkaline phosphatase substrate kit (Vector labs UK; sk-5100).

Lung Immunohistochemistry to assess PA remodeling

At harvest, the lung was perfusion-fixed via the trachea, processed into paraffin where sections (3µm) were cut and stained for elastin and collagen using Millers Elastin and Picro-Sirius red for identification of vascular thickening. Remodeling of pulmonary arteries distal to the airways, was defined by the presence of a double elastic lamina and/or by an increase in the vessel wall diameter in more than 50% of the arterial wall. The total number of remodelled vessels was expressed over the total number of vessels present in a lung section as assessed by a blinded investigator, where at least 100 pulmonary arteries of <80µm external diameter per lung were scored. Lung sections from six mice from each group were studied. Analysis was carried out in a blinded fashion as described previously¹¹.

Reactive Oxygen Species Determination in Lung Sections by Immunofluorescence

Immunohistochemistry of the ROS marker 8-hydroxyguanosine (8-OHG) was determined in the lungs of wild-type (WT) and SERT⁺ mice treated with 5-HT_{1B}R antagonist, SB216641, or vehicle, as previously described¹². Briefly, sections were deparaffinised in a xylene-ethanol-water gradient. Antigen retrieval was performed by boiling slides in EDTA (pH 8.0) for 15 minutes before a 1-hour incubation in a humidified chamber at room temperature with 10% donkey serum/1% bovine serum albumin in 1× TBS-T to block non-specific binding. Sections were incubated with anti- 8-OHG overnight at 4°C (5 µg/mL, ab10802, Abcam). For identification of 8-OHG-positive cells, sections were incubated with Alexa-fluor-488-conjugated donkey anti-goat secondary antibody (A-11055, Molecular Probes, Life Technologies, UK) for 45 minutes at room temperature in the dark. Lipofuscin-mediated auto-fluorescence was removed with 0.1% Sudan Black B (Sigma-Aldrich) in methanol w/v for 10 minutes. Slides were mounted with Pro-Long Gold anti-fade mounting media containing DAPI (4',6-diamidino-2-phenylindole; P-36931, Molecular Probes, Life Technologies). Fluorescence imaging was measured in an Axiovert 200M microscope with a laser-scanning module LSM 510 (Carl Zeiss, Germany). DAPI was excited at 405nm and Alexa-Fluor 488 at 488nm. Images were captured with the LSM 510 Zen evaluation software (Zeiss, UK).

Statistical Analysis

Mean values ± SEM were calculated and statistical comparisons made using Graph Pad Prism 7.02 software, with 1-way or 2-way ANOVA followed by Tukey's post hoc test or two-tailed Student's *t*-test where appropriate. P<0.05 was considered statistically significant (*).

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Supplemental Material

Serotonin Signaling Through the 5-HT_{1B} Receptor and NADPH Oxidase 1 in Pulmonary Arterial Hypertension.

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Table I. PAH Patient and non-PAH patient information.

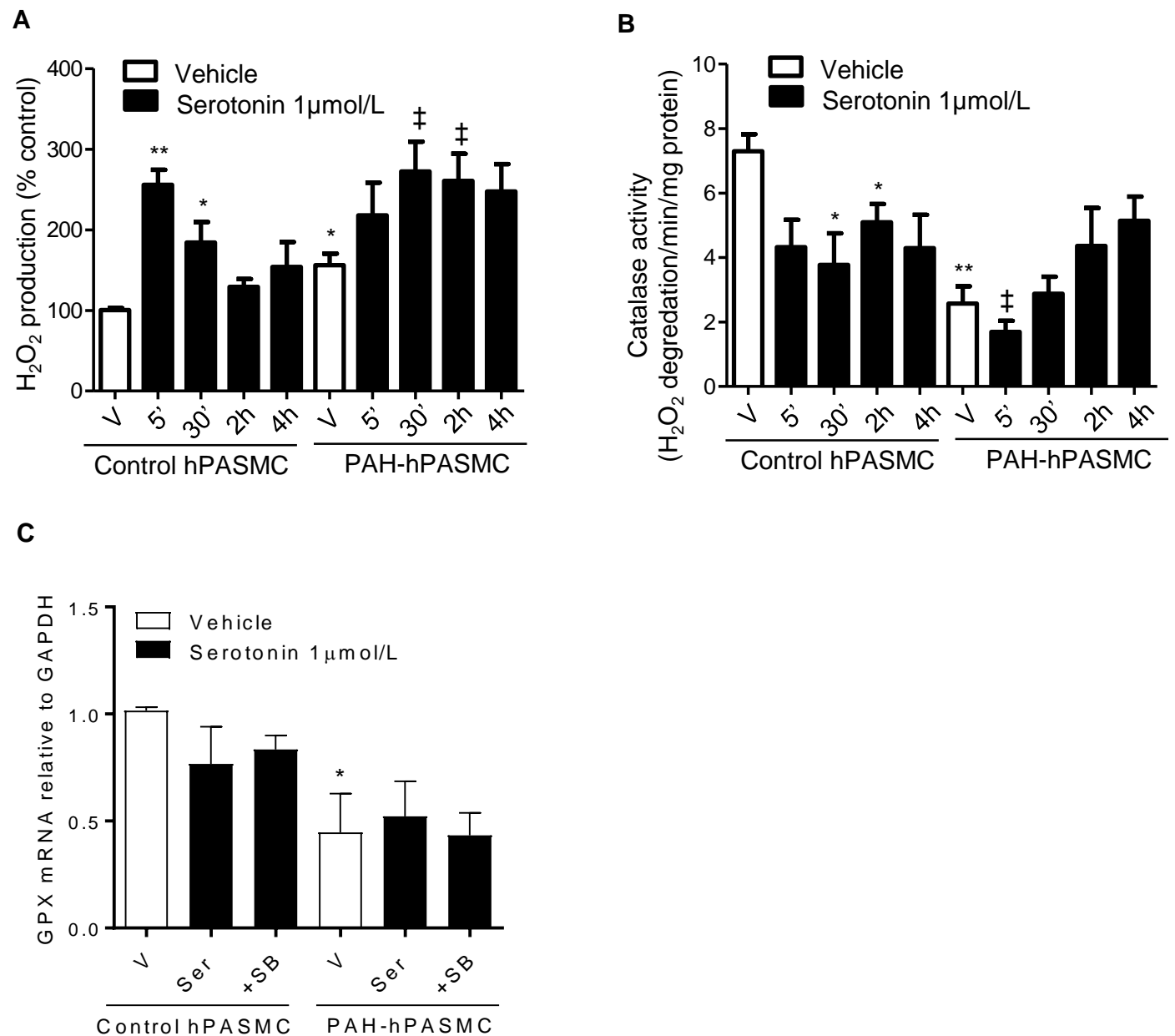
Patient Group	Sex	Age (years)	Disease Status
Non-PAH	Female	57	COPD
		58	Mild emphysema
		59	Squamous cell carcinoma
		64	Lung carcinoma
		70	Lobectomy
PAH	Female	30	HPAH (R899X)
		33	IPAH
		39	IPAH
		41	HPAH (N903S)
		53	IPAH

Pulmonary artery smooth muscle cell subject origin and characteristics. Known characteristics of subjects from whom cells were derived. COPD, chronic obstructive pulmonary disease; HPAH, heritable pulmonary arterial hypertension (gene mutation in parenthesis); IPAH, idiopathic pulmonary arterial hypertension.

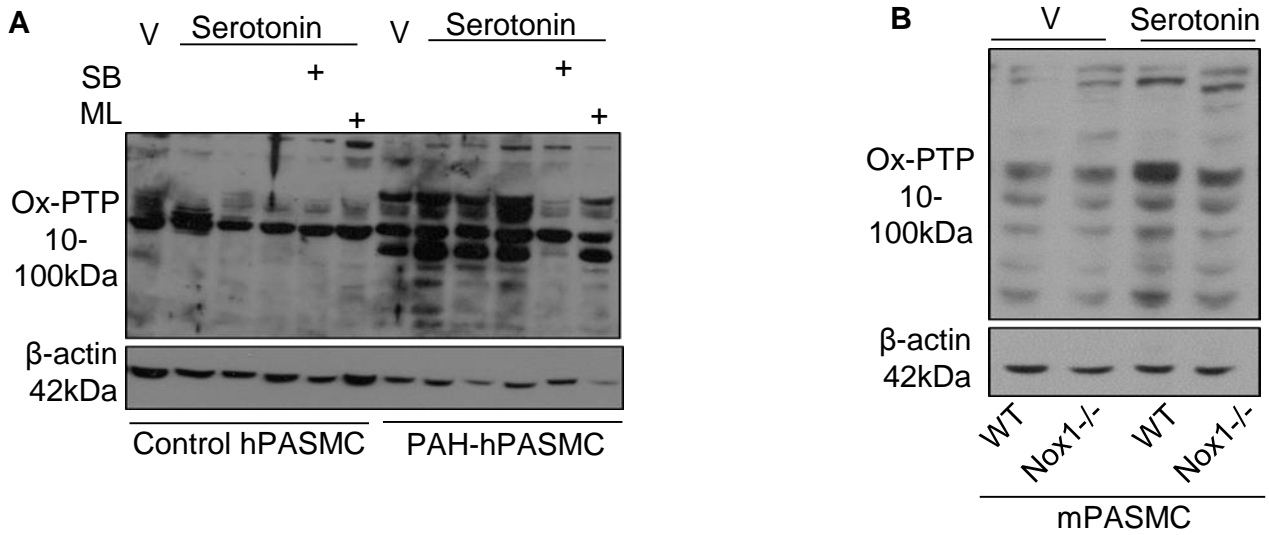
Table II. Human primers for real time PCR analysis.

Human Gene	Forward Primer	Reverse Primer
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
MMP2	TCTCCTGACATTGACCTTGGC	CAAGGTGCTGGCTGAGTAGATC
MMP9	TTGACAGCGACAAGAAGTGG	GCCATTCACGTCGTCCTTAT
Nox1	TCACCAATTCCCAGGATTGA	TGTGGTCTGCACACTGGAAT
GPx	AGTCGGTGTATGCCTTCTCG	TTGAGACAGCAGGGCTCGAT

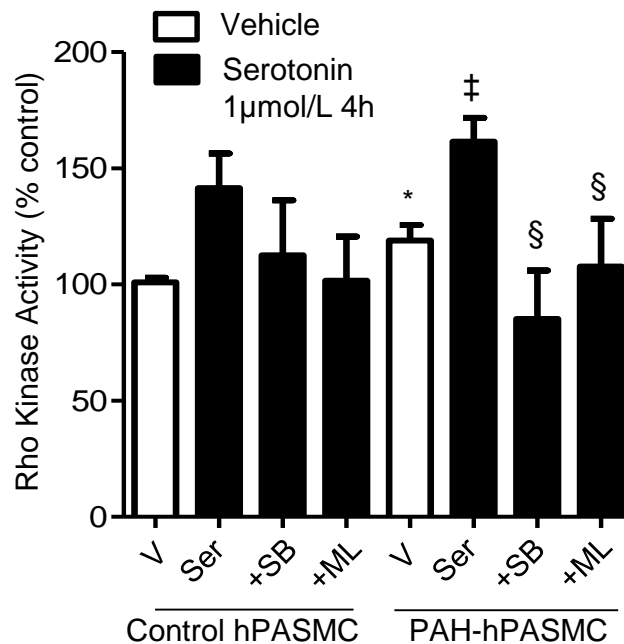
Human primers targeted to the above genes were designed using Primer 3 software, and were used to assess gene expression in control hPASMCs and PAH-hPASMCs.



Supplemental Figure I: Effect of serotonin on H₂O₂ and antioxidants, catalase and glutathione. H₂O₂ production (A) and catalase activity (B) in hPASCs was measured by Amplex Red assay in cells exposed to serotonin for 5 minutes to 4 hours. Data are expressed as RLU/μg protein corrected to standard curve and expressed as percentage of vehicle conditions. Glutathione mRNA expression (C) in hPASCs. mRNA expression is relative to GAPDH. Results are mean ± SEM of 5-6 experiments, in triplicate. *p<0.05, **p<0.01 vs. vehicle control hPASC; ‡p<0.05 vs. vehicle PAH-hPASCs determined by ANOVA with Tukey's post-hoc test. V= vehicle; Ser= serotonin; SB= SB224289.

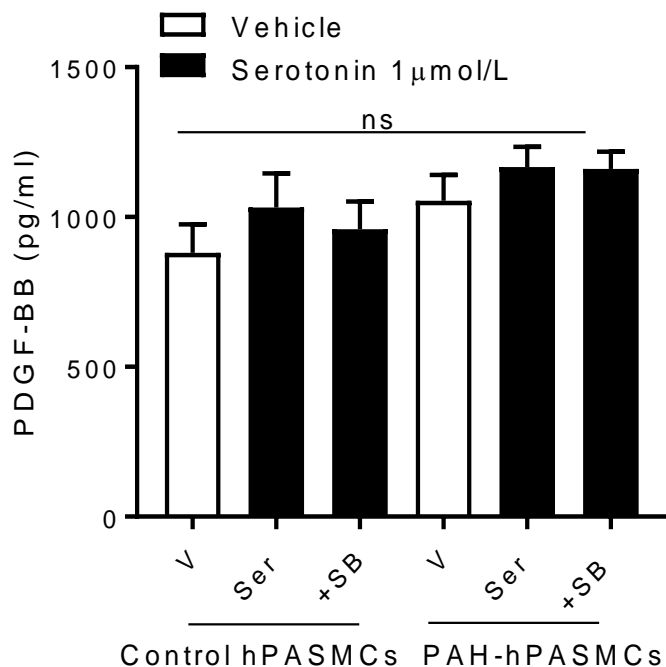


Supplemental Figure II: Serotonin promotes oxidation of PTPs. Irreversible oxidation of PTPs using the oxPTP antibody, in response to serotonin in the presence or absence of 5-HT_{1B}R antagonist SB224289 or Nox1 inhibitor, ML171 in hPASCs (A) and in WT and Nox1^{-/-} mPASCs (B). Values are mean \pm SEM of 4 experiments. Protein expression is relative to β -actin. Representative blots corresponding to Figure 3A and 3B. V= vehicle; SB= SB224289; ML= ML171; WT= wild-type.

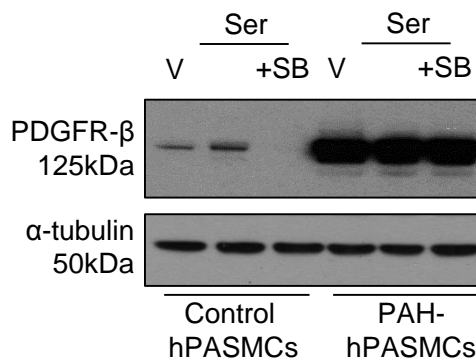
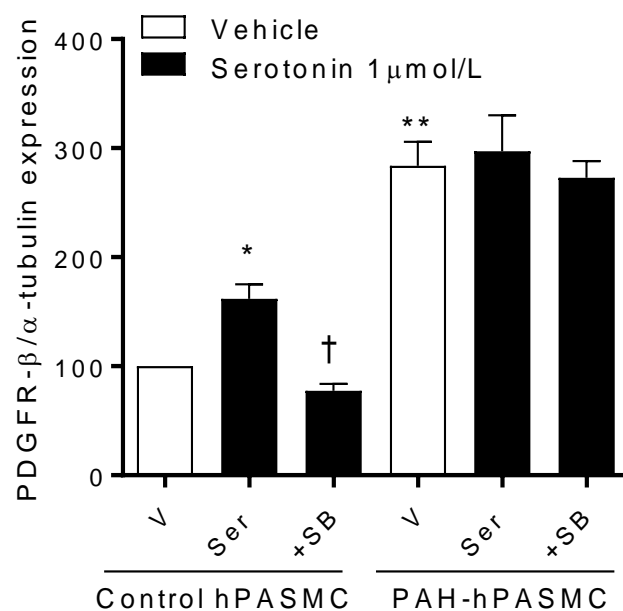


Supplemental Figure III: Role of 5-HT_{1B}R and Nox1 in serotonin-mediated vascular contraction. Rho Kinase activity assay was used to assess serotonin-induced Rho kinase activity in hPASCs. Results are expressed as mean±SEM of 5 experiments relative to protein concentration. *p<0.05 vs vehicle control hPASCs; ‡p<0.05 vs vehicle PAH-hPASCs; §p<0.05 vs treated PAH-hPASCs determined by ANOVA with Tukey's post-hoc test. V= vehicle; Ser= serotonin; SB= SB224289; ML= ML171.

A

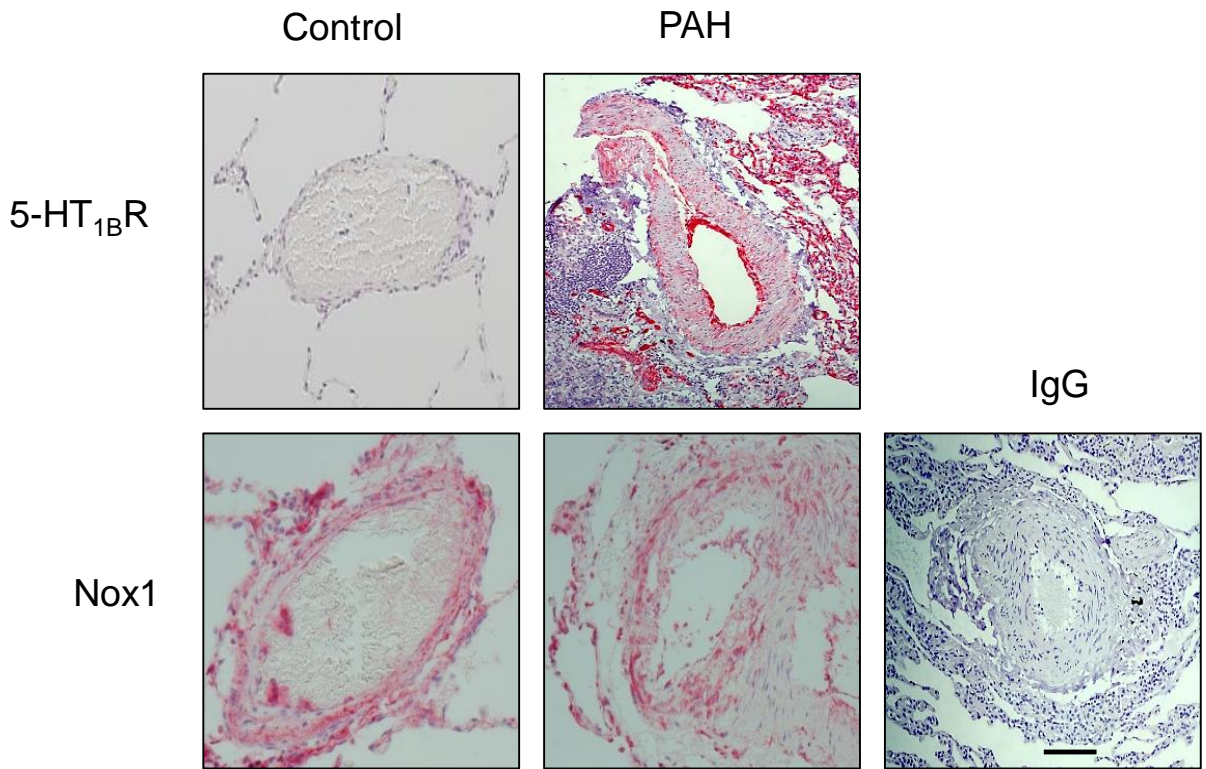


B

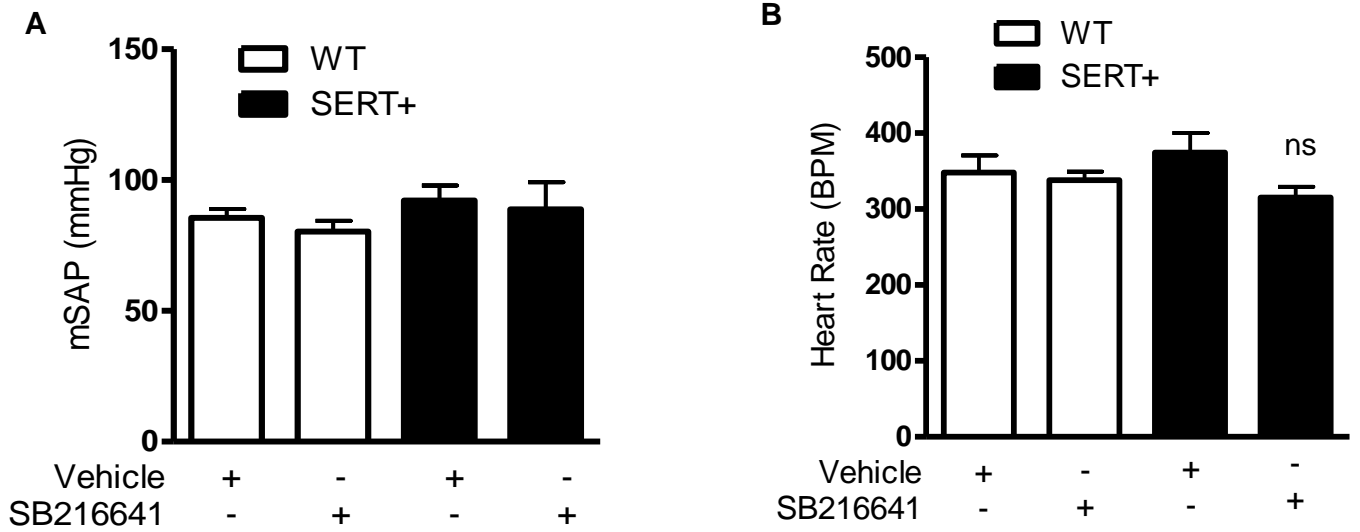


Supplemental Figure IV: Effect of serotonin on secretion of PDGF-BB and PDGFR-β expression. Secretion of PDGF-BB into hPASMC culture supernatant assessed by ELISA (A). Expression of PDGFRB in response to serotonin in hPASMCs (B). Results are mean ±SEM, n=6 per group. ns: not significant. *p<0.05; **p<0.01 vs vehicle control hPASMCs; †p<0.05 vs serotonin-treated control hPASMCs. V= vehicle; Ser= serotonin; SB= SB224289.

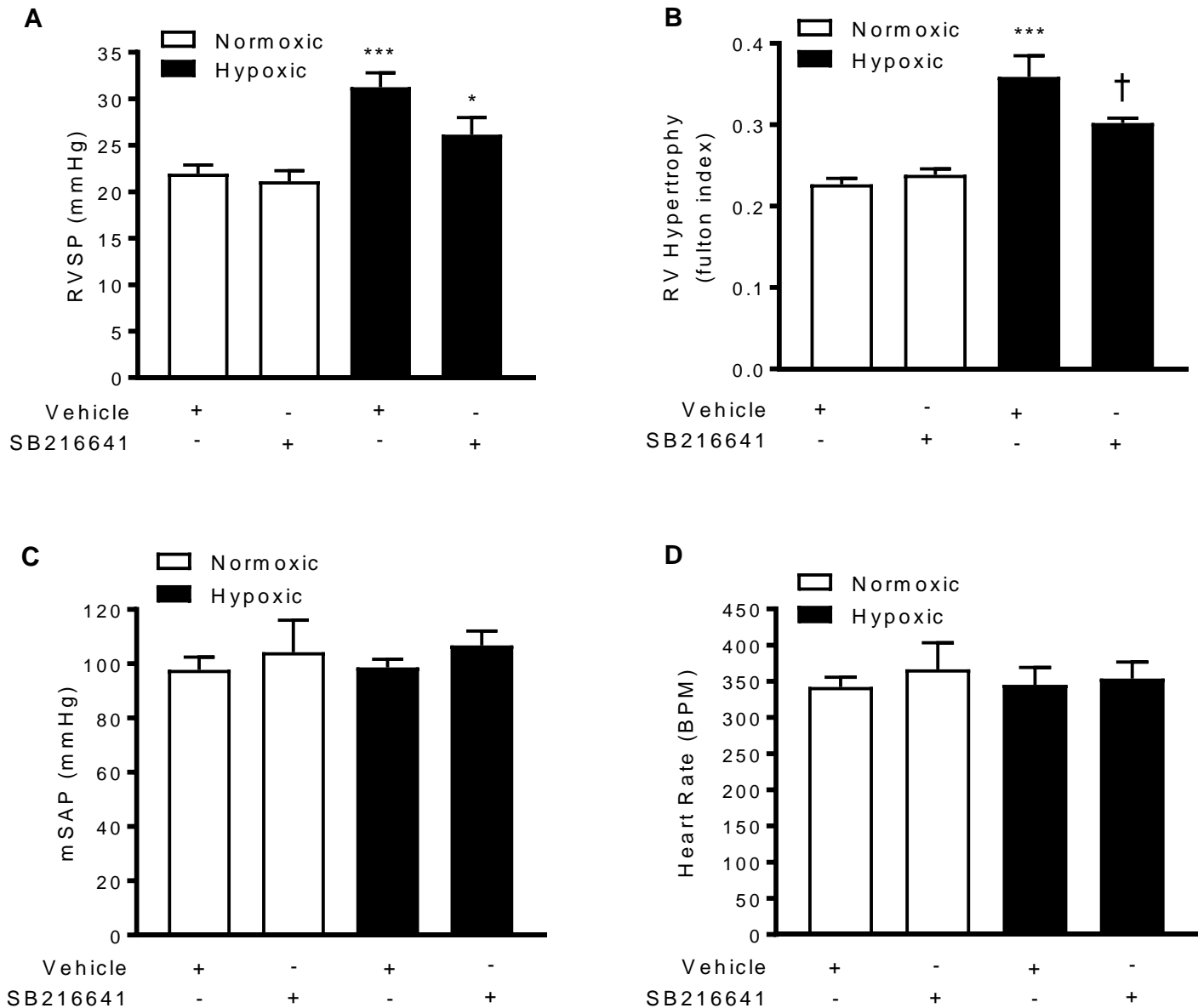
Supplemental Figure V



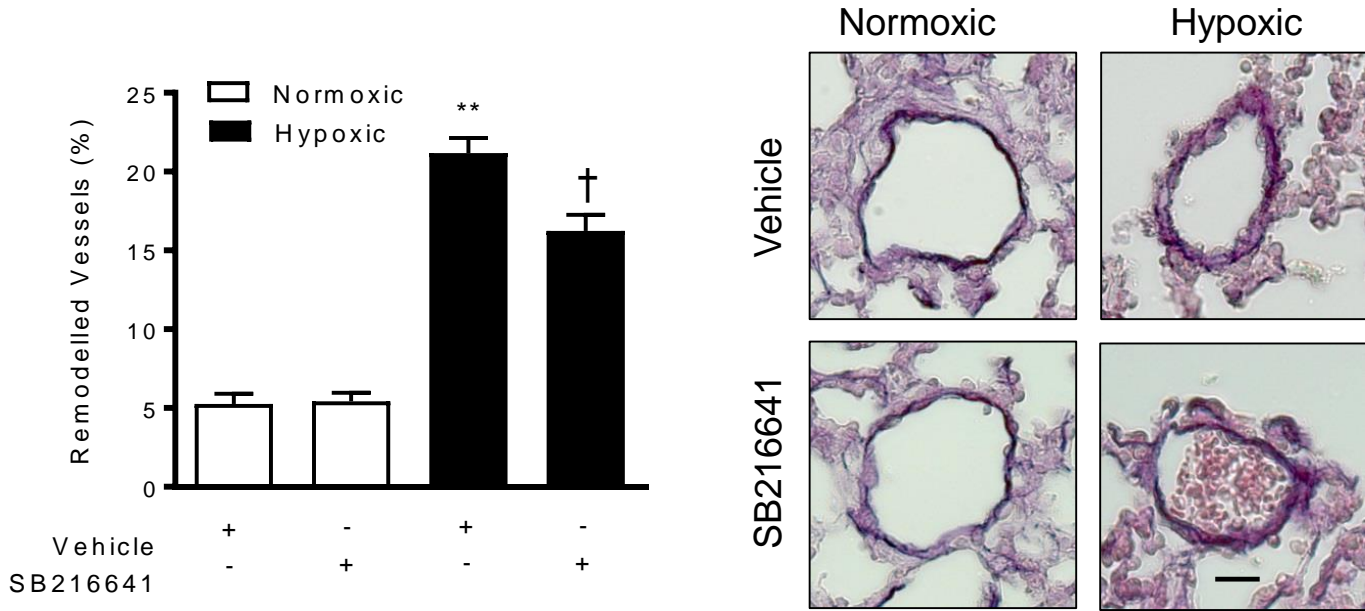
Supplemental Figure V: 5-HT_{1B} receptor and Nox1 staining in human control and PAH lung sections. 5-HT_{1B} receptor and Nox1 staining in human pulmonary arteries in lung sections from controls and PAH patients, counterstained with haematoxylin. Scale bars = 100 microns. n=2-5 per group.



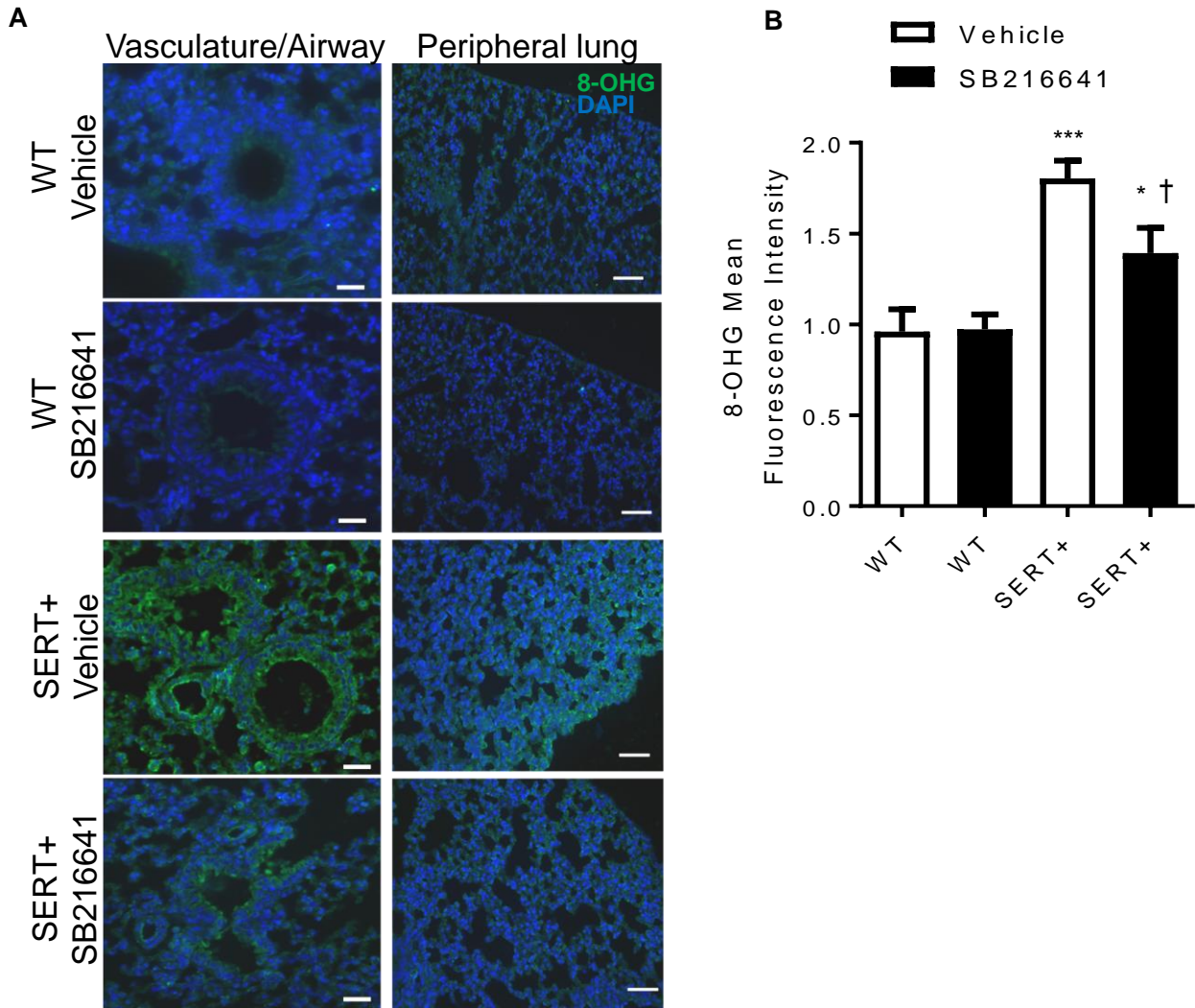
Supplemental Figure VI: Haemodynamic assessment of pulmonary hypertension in female WT and SERT+ mice treated with SB216641. Mean systemic arterial pressure (mSAP) (A) and heart rate (HR) (B). Results are mean \pm SEM, n=8-10 per group. ns: not significant. WT = wild-type, BPM = beats per minute.



Supplemental Figure VII: Haemodynamic assessment of pulmonary hypertension in female normoxic and hypoxic mice treated with SB216641. Right ventricular systolic pressure (RVSP) (A), right ventricular hypertrophy (B), mean systemic arterial pressure (mSAP) (C) and heart rate (HR) (D). Results are mean \pm SEM, n=8-10 per group. * p <0.05; *** p <0.001 versus normoxic vehicle treated mice; † p <0.05 versus hypoxic vehicle treated mice. BPM = beats per minute.

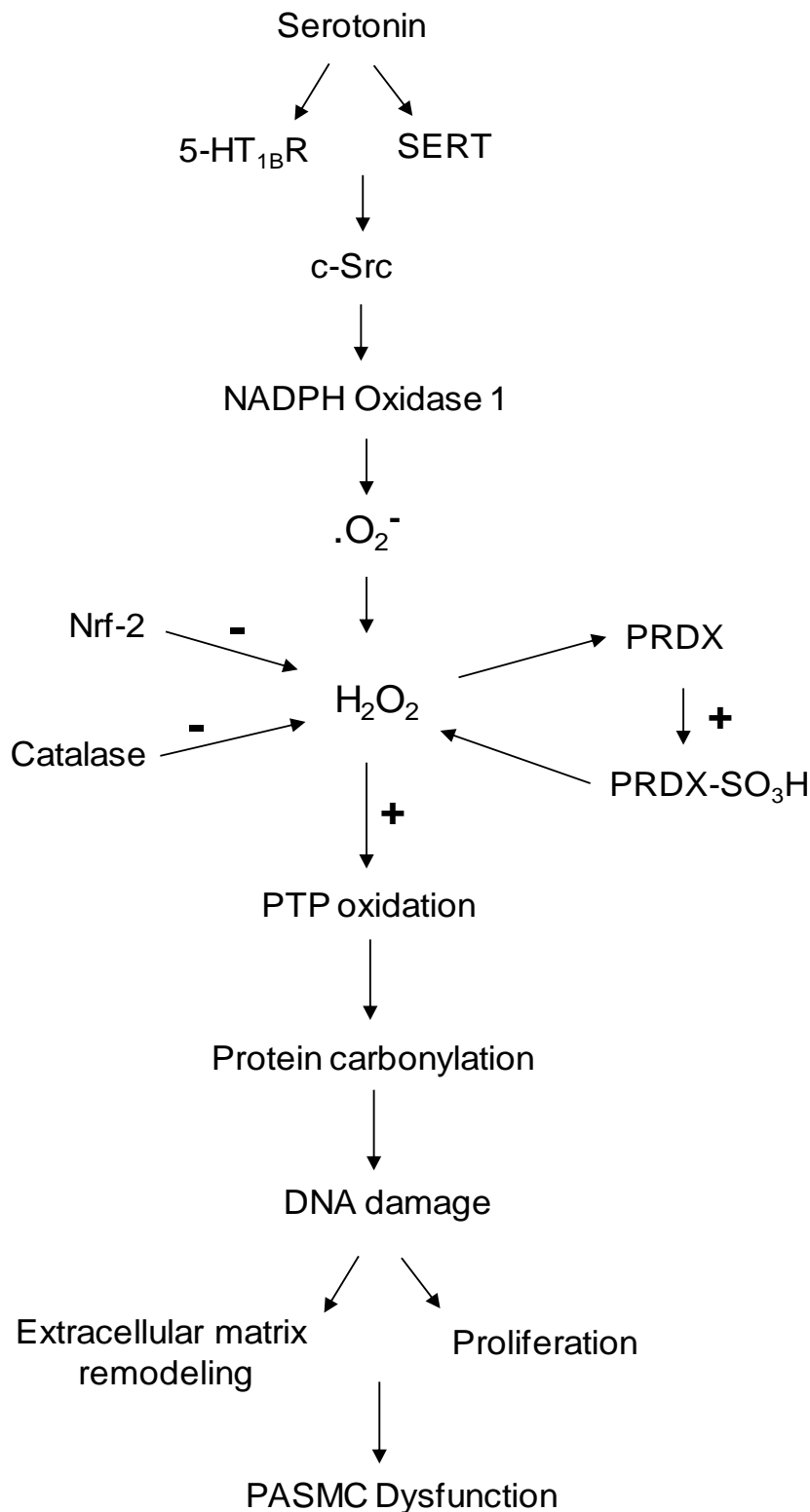


Supplemental Figure VIII: Pulmonary vascular remodelling in female normoxic and hypoxic mice treated with SB216641. Effects of SB216641 on percentage of pulmonary vascular remodeling in distal pulmonary arteries in female normoxic and hypoxic mice with representative images (right) of pulmonary arteries (Elastin Van Gieson stain; scale bars = 50 microns). Results are mean \pm SEM, n=8 per group. **p<0.01 vs Normoxic vehicle, †p<0.05 versus hypoxic vehicle-treated mice, determined by 2-way ANOVA with Tukey's post-hoc test.



Supplemental Figure IX: Oxidative stress marker, 8-hydroxyguanosine, is increased in the lungs of female SERT+ mice. Lung sections obtained from wild-type (WT) and SERT+ mice treated with the 5-HT_{1B}R antagonist (SB216641) or vehicle, were immunostained for the oxidative-stress marker 8-hydroxyguanosine (8-OHG). Sections were counterstained with the nuclear stain DAPI (4',6-diamidino-2-phenylindole). Scale bar: 50 μ m (A). 8-OHG mean fluorescence intensity was measured in lung sections using ImageJ software; n = 4 (B). *p<0.05; ***p<0.01 vs WT vehicle; †p<0.05 vs SERT+ vehicle, determined by 2-way ANOVA with Tukey's post-hoc test. WT = wild-type, SERT+ = serotonin transporter overexpression. SB216641 = 5-HT_{1B}R antagonist.

Supplemental Figure X



Supplemental Figure X: Schematic of putative role of serotonin in hPASMCs. Actions of serotonin in PASMCs are mediated not only via the 5-HT_{1B}R and SERT, but also involve the activation of Noxs, particularly Nox1, which leads to $\cdot\text{O}_2^-$ and H_2O_2 production. Excessive ROS production coupled with impaired Nrf2-mediated antioxidant mechanisms in response to serotonin may promote oxidation of PTPs and total protein carbonylation and proteins involved in mitogenic and fibrotic effects, leading to deleterious oxidative stress and pulmonary vascular remodeling. + activation; - inhibition.