Pulmonary Arterial Hypertension

Peroxisome Proliferator-Activated Receptor-γ Ameliorates Pulmonary Arterial Hypertension by Inhibiting 5-Hydroxytryptamine 2B Receptor

Yahan Liu, Xiao Yu Tian, Guangmei Mao, Xi Fang, Man Lung Fung, John Y.-J. Shyy, Yu Huang, Nanping Wang

Abstract—An elevated plasma level of 5-hydroxytryptamine (5-HT) or upregulation of 5-HT receptor signaling or both is implicated in vascular contraction and remodeling in pulmonary arterial hypertension (PAH). Recently, peroxisome proliferator-activated receptor-γ (PPARγ) agonists have been shown to ameliorate PAH. However, their effects on the 5-HT-induced contraction of pulmonary arteries remain unknown. Here, we examined the role of PPARγ in inhibiting 5-HT2B receptor (5-HT2BR) to ameliorate PAH. Pulmonary arteries from PAH rats induced by monocrotaline or chronic hypoxia showed an enhanced vasoconstriction in response to BW723C86, a specific agonist for 5-HT2BR. Expression of 5-HT2BR was also increased in pulmonary arteries from the PAH rats, accompanied by vascular remodeling and right ventricular hypertrophy. Treatment with the PPARγ agonist rosiglitazone in vivo reversed the expression and the vasoconstrictive effect of 5-HT2BR as well as the thickening of pulmonary arteries. In pulmonary artery smooth muscle cells, 5-HT induced the gene expression of 5-HT2BR, which was inhibited by rosiglitazone, pioglitazone, or adenosinemediated overexpression of constitutively activated PPARγ. The pharmacological effect of PPARγ was through the suppression of the 5-HT-induced activator protein-1 activity. These results demonstrated the beneficial effect of PPARγ on 5-HT2BR-mediated vasoconstriction, providing a new mechanism for the potential use of PPARγ agonists in PAH.

(Hypertension. 2012;60:1471-1478.) Online Data Supplement

Key Words: vasoconstriction ■ pulmonary arterial hypertension ■ 5-hydroxytryptamine 2B receptor ■ peroxisome proliferator-activated receptor-γ

Pulmonary arterial hypertension (PAH), characterized by vasoconstriction and vascular remodeling of pulmonary arteries, leads to a progressive increase in pulmonary arterial pressure and right ventricular failure.1 Previous studies demonstrated that 5-hydroxytryptamine (5-HT), also known as serotonin, participates in PAH.2-4 Acting as a potent vasoconstrictor of pulmonary vessels, 5-HT promotes hypertrophy and proliferation of smooth muscle cells (SMCs).5-6 Patients with PAH have increased plasma concentrations of 5-HT, resulting from the reduced uptake of 5-HT.7-11 Interestingly, the appetite-depressing drugs aminorex and fenfluramine, which inhibit 5-HT uptake but enhance its release by platelets, may contribute to pulmonary hypertension.12-17 5-HT receptors primarily expressed in the vessel wall are 5-HT1B, 5-HT2A, and 5-HT2B receptors.18 Belonging to the G-protein-coupled receptor family, these receptors elicit several proliferative pathways such as Ras-extracellular signal-regulated kinase and phosphatidylinositol 3-kinases-Akt.19 A pathophysiological role of 5-HT2B receptor (5-HT2BR) was suggested by the increased binding to 5-HT2BR in lung vascular bed of the hypoxia-induced PAH mouse model and corroborated by the results that genetic or pharmacological inactivation of 5-HT2BR prevented the development of PAH in mice.20-26 5-HT2BR is currently under active investigation as a therapeutic target for PAH. However, there is currently no evidence of 5-HT2BR-mediated constriction of pulmonary arteries in PAH.

Emerging evidence suggests that peroxisome proliferator-activated receptor-γ (PPARγ) agonists might be beneficial in treating PAH.27 Functioning as a transcription factor, PPARγ regulates a myriad of genes involved in glucose and lipid metabolism, as well as vascular inflammation and remodeling.28-30 The expression of PPARγ was reduced in lung tissue from PAH patients, rats with hypoxia-induced
PAH, as well as SMCs and endothelial cells isolated from mouse pulmonary arteries exposed to hypoxia. Similarly, PAH developed in mice with SMC- or endothelial cell-specific deletion of PPARγ. In contrast, PPARγ activation ameliorated PAH. However, the effect of PPARγ agonists on the vasoconstriction in PAH remains unknown.

In the present study, we examined the role of 5-HT2BR in mediating the 5-HT-induced vasocontraction in rat PAH models and tested the hypothesis that the beneficial effects of PPARγ agonist rosiglitazone are mediated through its action on 5-HT2BR.

**Methods**

**Monocrotaline- and Chronic Hypoxia-Induced PAH in Rats**

Rats were given monocrotaline (MCT; 60 mg/kg) or phosphate-buffered saline as control by a subcutaneous injection. Three weeks after the MCT injection, rats were killed for the evaluation of PAH parameters. Starting from 1 week before the MCT injection, rats were given rosiglitazone (10 mg/kg per day, dissolved in H2O) or water via oral gavage. Alternatively, rats were exposed to normobaric hypoxia (10% oxygen) or normoxia (21% oxygen) for 3 weeks, and then treated with rosiglitazone (20 mg/kg per day) or water with oral gavage for 3 days.

**Right Ventricle Hypertrophy and Pulmonary Vascular Remodeling**

Cardiac and pulmonary arterial remodeling was assessed as described in the online-only Data Supplement.

**Isometric Tension Measurement**

Left lungs were removed and placed in oxygenated Krebs-Henseleit solution. Pulmonary arteries were carefully dissected from adjacent connective tissue and cut into several ring segments of ≈2 mm long for measuring isometric force. Organ chambers (Multi Myograph System, Danish Myo Technology A/S) were filled with (37°C) Krebs solution containing (in mmol/L): 119.0 NaCl, 4.7 KCl, 2.5 CaCl2, 1.0 MgCl2, 25.0 NaHCO3, 1.2 KH2PO4, and 11.0 D-glucose. Each ring was suspended between 2 tungsten wires (diameter, 40 μm) in the chamber under optimal resting tension (2.5 mN as previously determined for the pulmonary arteries) and left for 90-minute equilibration. Vasoreactivity was measured to compare contractions in response to 5-HT, the 5-HT2BR agonist BW723C86, and 5-HT2AR agonist TCB-2 with or without the antagonist LY272015 or ketanserin for 5-HT2BR and 5-HT2AR, respectively.

**Adenoviral Infection**

Adenovirus (Ad) expressing constitutively active-PPARγ, Ad-dominant-negative c-Jun, Ad-IκBα, and Ad-green fluorescent protein were described previously. Pulmonary artery SMCs (PASMCs) were infected with the adenoviruses for 2 hours and then maintained in serum-free DMEM.

**Statistical Analysis**

Results represent mean±SEM. Comparisons among groups involved ANOVA followed by unpaired Student t test. P<0.05 was considered statistically significant.

**Results**

**Rosiglitazone Ameliorated Right Ventricle Hypertrophy and Vascular Remodeling in Rats With PAH**

MCT-treated rats developed right ventricle (RV) hypertrophy, a key pathological feature of PAH, as illustrated by increased ratios of RV:body weight and RV:left ventricle plus septum. Treatment with rosiglitazone for 4 weeks ameliorated RV hypertrophy and vascular remodeling in rats with PAH.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Rosiglitazone (RSG) ameliorated right ventricle (RV) hypertrophy and vascular remodeling in rats with pulmonary arterial hypertension (PAH). A and B, Ratios of RV:body weight (BW) and RV:left ventricle (LV) plus septum weight. C, Weigert’s elastic staining revealed medial thickening changes in lungs. D, Vascular remodeling was indicated by medial wall thickness (%) and wall area (%). Bar=50 μm. Results are mean±SEM from 6 to 8 rats. *P<0.05 vs vehicle or normoxia group. †P<0.05 vs monocrotaline (MCT) group. CH indicates chronically hypoxic.
hypertrophy (Figure 1A). Chronic hypoxia resulted in a similar RV hypertrophy. However, a short period of rosiglitazone treatment (3 days) was less effective for RV hypertrophy induced by hypoxia (Figure 1B).

Weigert’s elastic stain revealed prominent vascular remodeling in the pulmonary arterioles from MCT- or chronic hypoxia-treated rats as characterized by medial thickening and increased wall area. Rosiglitazone treatment effectively prevented the MCT-, but not chronic hypoxia-, induced vascular remodeling (Figure 1C and 1D).

Rosiglitazone Inhibited 5-HT2BR Expression in PAH Rats

Because of the involvement of 5-HT2BR in PAH, we then explored the possible relationship between 5-HT2BR and PAH by examining 5-HT2BR expression in the 2 PAH models. Immunostaining showed an increased level of 5-HT2BR in pulmonary arteries of rats with MCT- and chronic hypoxia-induced PAH, primarily in the medial layers (Figure 2A and 2B). Rosiglitazone treatment mitigated the increase in 5-HT2BR expression. Western blotting and quantitative polymerase chain reaction showed that protein and mRNA levels of 5-HT2BR were elevated in MCT and chronic hypoxia-induced PAH, which were inhibited by rosiglitazone treatment (Figure 2C through 2F). In contrast, expression of 5-HT2AR at protein and mRNA levels remained unaltered in pulmonary arteries from the PAH rats.

Rosiglitazone Inhibited 5-HT2BR-Mediated Vasoconstriction in PAH Rats

To functionally correlate the upregulation of 5-HT2BR with vasoconstriction under PAH and the beneficial effect of rosiglitazone, pulmonary arteries were dissected from groups of animals for isometric tension measurement responding to a 5-HT2BR agonist. Vehicle- and normoxia-treated rats showed minimal contractive response in pulmonary arteries in response to 5-HT2BR agonist BW723C86 (Figure 3A and 3D). However, BW723C86 elicited a pronounced contraction
in pulmonary arteries from MCT- or chronic hypoxia-induced PAH. The enhanced response was significantly attenuated in the animals treated with rosiglitazone (Figure 3A, 3B, 3D, and 3E). In contrast, contractions in response to TCB-2, which activates 5-HT2AR, were not different between the arteries from the PAH and normal rats. Rosiglitazone had no effect on vasoconstriction in response to 5-HT2AR agonist (Figure 3C and 3F).

The 5-HT2BR antagonist LY272015 was used to ascertain the role of 5-HT2BR in mediating the enhanced contraction of pulmonary arteries. As shown in Figure 4, LY272015 significantly inhibited the 5-HT-induced contractions of arterial rings from both MCT- and hypoxia-induced PAH but not those from normal rats (Figure 4A, 4B, 4D, and 4E). The suppressive effects of LY272015 on 5-HT-induced contractions were diminished in the arterial rings from rosiglitazone-treated PAH rats (Figure 4C and 4F). Unlike LY272015, ketanserin, a 5-HT2AR antagonist, reduced 5-HT-induced contractions of arterial rings from both the PAH and normal rats. The suppressive effect of ketanserin on 5-HT-induced contractions was not affected by rosiglitazone.
treatment (Figure 4). Thus, it is indicated that the enhanced contraction of pulmonary arteries from PAH rats was primarily mediated through 5-HT2BR, which was inhibited by rosiglitazone.

**PPARγ Activation Inhibits 5-HT2BR Expression in Cultured PASMCs**

To elucidate the mechanism by which the 5-HT2BR-mediated vasoconstriction in response to 5-HT is enhanced in pulmonary arteries from PAH rats, we asked whether 5-HT increased the gene expression of 5-HT2BR. As shown in Figure 5A and 5B, 5-HT increased the expression of 5-HT2BR at both mRNA and protein levels in a time-dependent manner. Rosiglitazone inhibited the upregulation of 5-HT2BR by 5-HT. Pioglitazone, another PPARγ agonist, had a similar effect (Figure 5C). Conversely, the inhibitory effect of rosiglitazone was abolished in the presence of the PPARγ antagonist GW9662 (Figure 5D), suggesting a PPARγ-specific action. Furthermore, the 5-HT-induced expression of 5-HT2BR was suppressed in PASMCs infected with adenovirus expressing a constitutively active form of PPARγ (Figure 5E).

To identify the subtype of 5-HT receptors that mediate the inductive effect of 5-HT, we pretreated the PASMCs with the selective antagonists before the cells were exposed to 5-HT. The results showed that 5-HT2BR antagonist LY272015, but not the 5-HT2AR antagonist ketanserin, attenuated the induction of 5-HT2BR. In addition, the 2B-selective agonist BW723C86 elicited a similar induction whereas TCB-2, a relatively selective agonist at 2A, had little effect (Figure 5F). These studies indicate that 5-HT induction of 5-HT2BR is mediated via 5-HT2BR itself.

**Activator Protein-1 Contributes to the Upregulation of 5-HT2BR in PASMCs**

The transcription factor activator protein-1 (AP-1) and its upstream mitogen-activated protein kinase in hepatic cells are known to be activated by 5-HT stimulation of 5-HT2BR. Using the transcription element search system (http://www.chil.upenn.edu/cgi-bin/tess/tess), we found 2 canonical AP-1-responsive elements in the 5′-flanking region of rat 5-HT2BR gene. Chromatin immunoprecipitation assay showed that 5-HT increased the AP-1/c-Jun binding at both sites, which were inhibited by rosiglitazone (Figure 6A). In line with this result, dominant-negative c-Jun inhibited the induction of 5-HT2BR whereas the nuclear factor-κB inhibitor IκBα had little effect (Figure 6B). In addition, pretreatment with specific inhibitors for c-Jun N-terminal kinase or p38 mitogen-activated protein kinase attenuated the upregulation of 5-HT2BR (Figure 6C).

**Discussion**

In the present study, we demonstrated a functional role of 5-HT2BR in mediating the enhanced vasoconstriction in the
rat models of PAH. In PASMCs, 5-HT stimulated the expression of 5-HT2BR via an AP-1-dependent mechanism. The PPARγ agonist rosiglitazone decreased 5-HT2BR expression in vitro and in vivo, attenuated 5-HT2BR-mediated vasoconstriction, and prevented cardiovascular remodeling associated with PAH.

Despite that 5-HT2BR antagonist PXR08066 has been in a phase II clinical trial for PAH treatment, the vasoconstrictive effect of 5-HT2BR in pulmonary arteries from PAH animals remains largely undefined. Previous studies have shown that 5-HT, a potent vasoconstrictor, induces vasoconstriction in pulmonary arteries, and the effects were mainly mediated by 5-HT1B and 5-HT2AR. Under normal condition, circulating 5-HT level was elevated in MCT-induced PAH rats (Figure S1, available in the online-only Data Supplement). It remains unclear that, in PAH models and patients, the elevation in circulating 5-HT and the upregulation of 5-HT2BR which comes first. In PASMCs, 5-HT can increase 5-HT2BR expression (Figure 5). Conversely, activation of 5-HT2BR with a specific agonist BW723C86 triggered a rapid increase in plasma 5-HT level. Thus, the elevation in circulating 5-HT and the increased expression of 5-HT2B may lead to the hyperreactivity of pulmonary arteries to 5-HT and play a pivotal role in the pathogenesis of PAH. We hypothesized that 5-HT induction of 5-HT2BR may lead to the hyperreactivity of pulmonary arteries to 5-HT and play a pivotal role in the pathogenesis of PAH; rosiglitazone may reverse the vascular hyperreactivity and remodeling in PAH by both inhibiting the 5-HT2BR expression in the arterial walls and reducing 5-HT level in the circulation (Figure S1).

In search of the regulatory mechanisms, we defined a critical role of AP-1 in mediating the induction of 5-HT2BR by 5-HT. The identified AP-1 upregulation was through activator protein-1 (AP-1) activation. A, The diagram depicts the putative AP-1 motifs in the 5′-flanking region of the rat 5-HT2BR gene. Pulmonary artery smooth muscle cells (PASMCs) were treated with 5-HT (1 μmol/L) in the presence or absence of rosiglitazone (RSG, 1 μmol/L). Chromatin immunoprecipitation (ChIP) assays were performed with c-Jun antibody or IgG as a negative control. Immunoprecipitated DNA was eluted and amplified by quantitative polymerase chain reaction using specific primers spanning the AP-1 sites. DNA binding was expressed as fold enrichment above IgG control. B, Western blots show the effect of dominant-negative (DN)-c-Jun, IκBα, or green fluorescent protein (GFP; mock) adenoviruses on 5-HT2BR induction by 5-HT. C, Effects of the inhibitors of c-Jun N-terminal kinase (JNK; SP600125, 10 μmol/L, SP), p38 (SB202190, 10 μmol/L, SB), extracellular signal-regulated kinase (ERK; PD98059, 10 μmol/L, PD), or vehicle (dimethyl sulfoxide [DMSO]) on 5-HT2BR with 5-HT induction. Bar graphs are mean±SEM from 3 to 5 experiments. *P<0.05 vs control. **P<0.05 vs 5-HT group.

Figure 6. 5-Hydroxytryptamine (5-HT) 2B receptor (R) upregulation was through activator protein-1 (AP-1) activation. A, The diagram depicts the putative AP-1 motifs in the 5′-flanking region of the rat 5-HT2BR gene. Pulmonary artery smooth muscle cells (PASMCs) were treated with 5-HT (1 μmol/L) in the presence or absence of rosiglitazone (RSG, 1 μmol/L). Chromatin immunoprecipitation (ChIP) assays were performed with c-Jun antibody or IgG as a negative control. Immunoprecipitated DNA was eluted and amplified by quantitative polymerase chain reaction using specific primers spanning the AP-1 sites. DNA binding was expressed as fold enrichment above IgG control. B, Western blots show the effect of dominant-negative (DN)-c-Jun, IκBα, or green fluorescent protein (GFP; mock) adenoviruses on 5-HT2BR induction by 5-HT. C, Effects of the inhibitors of c-Jun N-terminal kinase (JNK; SP600125, 10 μmol/L, SP), p38 (SB202190, 10 μmol/L, SB), extracellular signal-regulated kinase (ERK; PD98059, 10 μmol/L, PD), or vehicle (dimethyl sulfoxide [DMSO]) on 5-HT2BR with 5-HT induction. Bar graphs are mean±SEM from 3 to 5 experiments. *P<0.05 vs control. **P<0.05 vs 5-HT group.
inflammation. It is postulated that the c-Jun N-terminal kinase/AP-1 pathway may function as a unifying mechanism for the regulation of 5-HT2B by the PAH-related risk factors.

Emerging evidence highlights the potential application of PPARγ agonists in the treatment of PAH.50 Our study further revealed a previously unknown effect of PPARγ on the pulmonary arterial contraction in PAH. We have shown that rosiglitazone decreased the induction of 5-HT2BR by 5-HT in PASMCs and attenuated the 5-HT2BR hyperresponsiveness in response to 5-HT-triggered contraction in the PAH. The action of rosiglitazone is likely PPARγ specific because the PPARγ antagonist GW9662 abolished whereas constitutive activation of PPARγ mimicked the effect. It is worth noting that the beneficial effects of PPARγ agonists on PAH are multifaceted. It has been well established that effects of PPARγ agonists on insulin resistance, endothelial dysfunction, oxidative response, and inflammation may all contribute to the improvement of vascular pathology in PAH.48,49 In hypoxia-induced PAH rats, a 3-day treatment with rosiglitazone was not as effective for the resolution of cardiovascular remodeling. We speculate that the ineffectiveness of rosiglitazone on hypoxia-induced RV hypertrophy was likely because of the relatively short regimen. Previous studies have shown that treatment with rosiglitazone for 7 to 10 days during hypoxia reduced cardiovascular remodeling.33,37 Nevertheless, the inhibitory effect of PPARγ on the 5-HT-triggered vasoconstriction makes it a promising target for the PAH treatment. Other than the serotonin system, endothelin-1/endothelin-1 receptors also play an important target for the PAH treatment. Other than the serotonin system, on the 5-HT-triggered vasoconstriction makes it a promising agonists may play their interventional roles in the diseases associated with pulmonary arterial remodeling such as PAH.

In conclusion, our results suggested that 5-HT2BR is a new target for the beneficial effect of PPARγ on the vascular contraction and remodeling in PAH. Future studies on the interaction between PPARγ agonists and 5-HT pathways should provide new insight into a unique mechanism by which these agonists may play their interventional roles in the diseases associated with pulmonary arterial remodeling such as PAH.

Perspectives
We demonstrate a novel role for the PPARγ agonist in attenuating the 5-HT-induced contraction in pulmonary arteries of PAH rats by inhibiting 5-HT2BR. Future studies are needed to validate this action in pulmonary arteries from the patients with PAH.

Sources of Funding
This work was supported by grants from National Natural Science Foundation of China/Research Grants Council Joint Research Scheme (N_CUHK428/09 and 30931160434), the National Science Foundation of China (30890041), the Ministry of Science and Technology of China (2010CB912500), and the Hong Kong General Research Fund (CUHK46610).

Disclosures
None.

References


50. Sauvaugue S, Thorin E, Caron A, Dupuis J. Endothelin-1-induced pulmonary vasoreactivity is regulated by ET(A) and ET(B) receptor interactions. J Vasc Res. 2007;44:375–381.

Peroxisome Proliferator-Activated Receptor-γ Ameliorates Pulmonary Arterial Hypertension by Inhibiting 5-Hydroxytryptamine 2B Receptor
Yahan Liu, Xiao Yu Tian, Guangmei Mao, Xi Fang, Man Lung Fung, John Y.-J. Shyy, Yu Huang and Nanping Wang

Hypertension. 2012;60:1471-1478; originally published online October 29, 2012; doi: 10.1161/HYPERTENSIONAHA.112.198887

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/60/6/1471

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2012/10/29/HYPERTENSIONAHA.112.198887.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENTS

Peroxisome Proliferator-activated Receptor γ Ameliorates Pulmonary Arterial Hypertension by Inhibiting 5-HT2B Receptor

Yahan Liu, Xiao Yu Tian, Guangmei Mao, Xi Fang, Man Lung Fung, John Y-J Shyy, Yu Huang, Nanping Wang

Institute of Cardiovascular Science (Y.L., G.M., X.F., N.W.), Peking University Health Science Center, Beijing, China, Institute of Vascular Medicine (X.Y.T., Y.H.), Li Ka Shing Institute of Health Sciences, School of Biomedical Sciences, Chinese University of Hong Kong, Hong Kong SAR, China, Department of Physiology (M.L.F.), Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China, and Cardiovascular Research Center (N.W., J.S.), Xi’an Jiaotong University, Xi’an, China.

Running Title: PPARγ inhibits 5-HT2BR in pulmonary hypertension

Correspondence to Nanping Wang, Institute of Cardiovascular Science, Peking University, Beijing 100191, China. Phone: 86-10-82801146; Fax: 86-10-82801159, E-mail: nanpingwang2003@yahoo.com; or Yu Huang, Institute of Vascular Medicine and School of Biomedical Science, The Chinese University of Hong Kong, Hong Kong, China, E-mail: yu-huang@cuhk.edu.hk
Methods

Animals, cell culture and reagents
Male Sprague-Dawley rats were used and the experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals with the approval by the institutional committee. Rat pulmonary artery SMCs (PASMCs) were isolated and grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Monocrotaline (MCT), 5-HT, N\textsubscript{G}-nitro-L-arginine methyl ester (L-NAME), GW9662 were from Sigma-Aldrich (St. Louis, MO). Rosiglitazone malate was from GlaxoSmithKline (BRL-49653-C). BW723C86, TCB-2, LY272015, ketanserin, SP600125, SB202190 and PD98059 were from Tocris Bioscience (Bristol, UK). Pioglitazone was from Cayman Chemical (Ann Arbor, MI). Mouse monoclonal antibody against 5-HT\textsubscript{2BR} was from BD Bioscience Pharmingen (San Diego, CA). Polyclonal rabbit antibody against 5-HT\textsubscript{2AR} was from Abcam (Cambridge, UK). PPAR\textsubscript{γ} antibody was from Cell Signaling Technology (Beverly, MA). Antibody against c-Jun was from Santa Cruz Biotechnology (Santa Cruz, CA). Serotonin ELISA kit was from LDN GmbH & Co. KG (Nordhorn, Germany).

Plasma 5-HT measurement
Rats were killed by CO\textsubscript{2} inhalation and their blood was drawn into a heparin-containing tube by venipuncture from inferior vena cava vein. The collected blood was centrifuged at 3,000 rpm at 4 °C for 10 min. The plasma was separated and stored at -80 °C until being assayed. The 5-HT concentration was measured using an enzyme immunoassay (ELISA) kit (BA E-5900 5-HT EIA, LDN GmbH & Co. KG, Nordhorn, Germany).

Assessments of cardiac and pulmonary vascular remodeling
Rats were killed by CO\textsubscript{2} inhalation and hearts were removed and weighed. The ratios of right ventricle (RV) to left ventricle plus septum (RV/LV+S) and RV to body weight (RV/BW) were calculated to assess RV hypertrophy. Lung tissues were fixed in 4% paraformaldehyde at 4°C overnight dehydrated and embedded in paraffin. Four-micrometer tissue sections were cut. After rehydrated to water, sections were subjected to Weigert’s elastic stain to highlights the internal elastic lamina (IEL) and the outer limit of the media by the external elastic lamina (EEL) with a layer of smooth muscle cells between the two laminae. The medial wall thickness of pulmonary arterioles ranging from 25 to 100 µm under microscope at magnification × 400 was measured. To assess muscularization, the IEL and EEL of each arterial profile were traced with the computer mouse on the screen to record the circumference. Computer calculated actual area occupied by the media (total area) and lumen (luminal area). Then for calculating wall area = total area–luminal area; wall area % = (total area – luminal area) ×100/total area. The medial thickness (MT) was measured under the microscope as the distance between the external and internal elastic laminae. The external diameter (ED) and inner diameter (ID) were measured as the diameter of the EEL and IEL. The MT then calculated by using the followed formula: MT = (ED – ID)/2; medial wall thickness % = 2×MT×100/ED. 10-15 arteries per animal were
evaluated and the measurements were made at several points randomly for each vessel, and an average was calculated. The thrombotic events were the blood cells because that the rats were not perfused with PBS and paraformaldehyde during the preparation.

Immunohistochemistry
Frozen sections (6 µm) from pulmonary arteries were fixed with 4% paraformaldehyde, pretreated with 3% H₂O₂ and then 5% donkey serum for 1 h, incubated with the mouse antibody against 5-HT2BR at 4°C overnight. After washed with PBS, the sections were incubated with biotin-SP conjugated goat anti-mouse IgG antibody (1:500, Jackson Immunoresearch, West Grove, PA), then streptavidin-horseradish peroxidase conjugate (1:500; Zymed laboratory, San Francisco, CA). Positive stain was developed by using DAB chromogen substrate (Vector lab, Burlingame, CA). Sections were counterstained with hematoxylin. Images were captured and analyzed using SPOT Advanced software (Diagnostic Instruments, Sertling Heights, MI).

Western blot analysis
Pulmonary arteries were dissected, frozen in liquid nitrogen, and homogenized in RIPA lysis buffer containing protease inhibitors. Protein lysates were also extracted from PASMCs with RIPA buffer and separated on 12.5% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to PVDF membranes, which were blocked with 5% non-fat milk in Tris-buffered saline-Tween (0.2%) (TBS-T) for 1 h and incubated overnight with primary antibody, then horseradish peroxidase (HRP)-conjugated secondary antibody, and visualized with ECL reagent. Autoradiographs were scanned and quantified for band intensities with the use of QuantityOne (BioRad) with normalization to β-actin level.

Quantitative RT-PCR
Total RNA was isolated from pulmonary arteries and PASMCs with the use of TRIzol reagent (Invitrogen). Total RNA was reverse transcribed with use of iScript cDNA synthesis kit (Bio-Rad) and amplified on IQ5 Real-time PCR detection system (Bio-Rad) using SYBR Premix Ex Taq and specific primers for rat 5-HT2BR, 5-HT2AR or β-actin as an internal control. Gene expression was quantified by the comparative CT method and expressed as fold induction of control.

Chromatin immunoprecipitation (ChIP assay)
PASMCs were treated with rosiglitazone (1 µmol/L) for 24 h and then exposed to 5-HT (1 µmol/L, 24 h). Cells were cross-linked with formaldehyde, sonicated and immunoprecipitated with the c-Jun antibody or control IgG. Eluted DNA or the input control were amplified by qPCR using specific primers flanking the proximal and distal AP-1 sites (-304~294, forward: 5’-GGCTTGGCCTTACTTGGTG-3’, reverse: 5’-ACCCCCTCTGTTGGATAGG-3’; -1475~1465, forward: 5’-AACCAGCTCCCAATTCAGA-3’, reverse: 5’-ATTCTCCCCAACT GGCTGTA-3’).


Table S1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle</th>
<th>MCT</th>
<th>MCT + RSG</th>
<th>Normoxia</th>
<th>CH</th>
<th>CH + RSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (Kg)</td>
<td>0.33 ± 0.01</td>
<td>0.28 ± 0.01*</td>
<td>0.33 ± 0.01†</td>
<td>0.49 ± 0.02</td>
<td>0.41 ± 0.01*</td>
<td>0.42 ± 0.05</td>
</tr>
<tr>
<td>(LV+S)/BW (g/Kg)</td>
<td>2.32 ± 0.04</td>
<td>2.16 ± 0.11</td>
<td>2.29 ± 0.08</td>
<td>2.19 ± 0.03</td>
<td>2.29 ± 0.04</td>
<td>2.48 ± 0.09</td>
</tr>
</tbody>
</table>

Table. 1 Body weight (BW), left ventricle weight (LV) in MCT- or chronic hypoxia-induced PAH rats treated with rosiglitazone (RSG). MCT, monocrotaline. CH, chronic hypoxia. BW, body weight. LV, left ventricle. S, septum. Results are means ± SEM from 6-8 rats. *p<0.05 vs. vehicle or normoxia group. †p<0.05 vs. MCT group.
Supplemental Fig. S1. Rosiglitazone attenuated the increased plasma level of 5-HT in MCT-treated PAH rats. Concentration of 5-HT in plasma were measured in vehicle-, MCT-, or RSG-treated MCT- rats by using serotonin ELISA kit. Data were mean ± SEM from 4-6 rats in each group. *p<0.05 vs. vehicle group. #p <0.05 vs. MCT group.
Supplemental Fig. S2. Rosiglitazone did not affect the contractions of pulmonary arteries in normal rats. (A, B) Representative recordings of BW723C86- and TCB-2-induced contractions in pulmonary arteries from vehicle- and RSG-treated rats. (C) Concentration-dependent contractions to 5-HT after the pretreatment with 5-HT2BR antagonist LY272015 (10 nmol/L, 30 min) or 5-HT2AR antagonist ketanserin (5 nmol/L, 30 min) in pulmonary arteries from normal and RSG-treated rats.
Supplemental Fig. S3. Measurement of RV/BW, LV/BW, RV/LV+S and BW in Normal and RSG treated-rats. Results are mean ± SEM from 3 animals. * p<0.05 vs. vehicle.
Supplemental Fig. S4. Constitutively active PPARγ Suppressed 5-HT2BR in PASMCs. PASMCs were infected with Ad-CA-PPARγ or mock. Expression of 5-HT2BR and PPARγ was detected by using western blotting. Results are mean ± SEM from 3 experiments. *p<0.05 vs. mock. * Ad-CA-PPARγ expressed a fusion protein which has a 78-aa VP16 minimal transactivation domain.