

Original Article

Sildenafil increases connexin 40 in smooth muscle cells through activation of BMP pathways in pulmonary arterial hypertension

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Abstract: Background: Pulmonary arterial hypertension (PAH) is a cardiovascular disorder associated with enhanced proliferation and suppressed apoptosis of pulmonary arterial smooth muscle cells (PASMCs). The sildenafil can regulate the Connexin (Cx) 43 in the PASMCs and thus inhibit the PASMCs proliferation and the remodeling of pulmonary arterial. However, how sildenafil exert regulation in the Cx40 in the PASMCs in PAH remains unclear. Methods and results: Using the rat PAH model induced by the monocrotaline, we demonstrated that the Cx40 in the PASMCs is down-regulated in the PAH. The sildenafil promotes the up-regulation of Cx40 in the PASMCs via bone morphogenetic protein (BMP) signaling, accompanied by an anti-proliferative response in PASMCs. Inhibition of the BMP axis reverses the up-regulation of Cx40 and anti-proliferation of the sildenafil in these cells. In monocrotaline-induced PAH rat models, which display reduced levels of BMP signaling, this study further indicates that the BMP-Cx40 axis is activated in lungs following the sildenafil treatment. Furthermore, we also find in vitro that sildenafil increases the Cx40 expression of PASMCs isolated from MCT-PAH rats and inhibit the proliferation of these cells. These phenomenon are reversed by LDN-193189, the antagonist of type II receptor for bone morphogenetic protein (BMP2) treatment, providing strong evidence for the protect effect of sildenafil and the BMP-Cx40 axis involvement. Conclusions: Taken together, these data suggest the sildenafil activate BMP-Cx40 signaling in the PAH. This axis may be a potential therapeutic target in PAH.

Keywords: Sildenafil, connexin 40, hypertension, pulmonary, bone morphogenetic protein, PASMCs proliferation

Introduction

In the left to right shunt congenital heart disease, the large number of shunt resulted in pulmonary congestion. The pulmonary resistance increased, causing the sustained elevation of pulmonary arterial pressure that leads to the pulmonary arterial remodeling, muscularization, right ventricle failure and death [1]. The muscularization of pulmonary arterial is composed of the proliferation of the pulmonary arterial smooth muscle cells (PASMCs) which are interconnected by gap junction channels [2]. These channels are made of two connexons (also called hemi channels, Cx) whose association at intercellular junctions forms a functional pore enabling the diffusion of ions or intracel-

lular messengers between adjacent cells [3]. The channels are clustered in specific regions named gap junctions providing a low resistance pathway for electronic transmission of vasomotor signals [4]. The Cx family is composed of 20 members named according to their molecular weight and Cx40, Cx43 are expressed in PASMCs [5]. The sildenafil can down-regulate the Cx43 in the PASMCs in the PAH and inhibit the pulmonary arterial remodeling [6]. However, the effect of sildenafil on Cx40 of PASMCs, which has a similar structure to Cx43, and the underlying mechanisms involved have not been well elucidated.

Nitric oxide (NO) is endogenously generated by nitric oxide synthases via the conversion of

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L-arginine into L-citrulline that in turn promotes cyclic guanosine monophosphate (cGMP) generation [7]. Phosphodiesterase type 5 (PDE5) is largely responsible for the hydrolysis of cGMP and is expressed at a high level in the pulmonary circulation compared with systemic vessels [8]. Sildenafil, a specific PDE5 inhibitor, increases intracellular cGMP levels and promotes vasodilatation. It is widely used as an effective treatment for clinical PAH [9]. Recently, Yang J et al. reported that sildenafil modulates bone morphogenetic protein (BMP) signaling in PSMCs. These authors found that Sildenafil enhances canonical BMP signaling via cyclic GMP and cyclic GMP-dependent protein kinase I *in vitro* and *in vivo*, and partly restores deficient BMP signaling in BMPR2 mutant PSMCs [10]. BMP plays an important role in the regulation of Cx [11].

In the present study, we demonstrate that sildenafil enhances the Cx40 expression via a BMP signaling-dependent mechanism. Moreover, the antagonist of type II receptor for bone morphogenetic protein (BMPR2), LDN-193189 reversed the sildenafil effect in the PAH. Furthermore, we also find *in vitro* that sildenafil increases the Cx40 expression of PSMCs isolated from MCT-PAH rats and inhibit the proliferation of these cells. These phenomena are reversed by LDN-193189, the antagonist of BMPR2 treatment. These studies provide further evidence for a central role of sildenafil via BMP signaling in the regulation of Cx40 in the PSMCs in PAH.

Methods

Animals

All experimental protocols and surgical procedures used in this study were approved by The Institutional Animal Care and Use Committee of Nanjing Medical University. Male Sprague-Dawley (SD) rats, 8 weeks of age, weighing 200-220 g, were purchased from Nanjing Medical University animal center. Rats were randomly assigned to one of seven experiment groups (n=6 per group). Rats were housed with free access to food and water under a natural 12/12 h day/night cycle.

Establishment of a PAH model

The Monocrotaline (MCT, Sigma, USA) was administered (60 mg/kg) to rats by subcutane-

ous injection into the back region. The animal's lungs were harvested as described previously [12] at 28th day of the study after hemodynamic assessment. The sildenafil group received daily intragastric administration of sildenafil after the administration of MCT (60 mg/kg; Pfizer, USA). The LDN-193189 group received daily intragastric administration of sildenafil (50 mg/kg; Pfizer, USA) and intraperitoneal injection of LDN-193189 (10 mg/kg; Bi Yun Tian, China). In other groups, the same volume saline was given.

Hemodynamic and right ventricular hypertrophy measurements

Invasive hemodynamic measurements, including pulmonary arterial pressure (PAP) and right ventricular systolic pressure (RVSP) were performed as described previously [12]. After the anesthesia, the rats tracheas were orally intubated with a 16-gauge intravenous catheter and mechanical ventilation commenced using a rodent respirator (tidal volume: 8 mL/kg, respiratory rate: 60 minutes⁻¹). The PAP and RVSP were measured as described previously. Briefly, a PE-50 catheter (Bi Yun Tian, China), angled to 90° over the distal 1 cm and curved slightly at the tip, was introduced into the right external jugular vein, with the angle directed inferiorly, the catheter was inserted proximally, which placed the catheter in the right atrium. The catheter was rotated 90° counterclockwise and inserted further, which placed the catheter in the right ventricle, and then advanced approximately 1.5 cm, into the pulmonary artery. Placement at each stage was confirmed by standard right ventricle or pulmonary arterial pressure trace on the monitor screen connected to the pressure transducer of a BSM-1700 monitor (Nihon Kohden Company, Japan). The data of RVSP, mPAP and mean arterial blood pressure (mPAP) were recorded after 1 minute of stabilization. After the hemodynamic measurement, the RV free wall was dissected from the left ventricle plus septum (LV + S) and weighed separately; RV hypertrophy was expressed as RV weight/(LV + S weights).

Evaluation of pulmonary arterial remodeling

Lung tissue preparation, sectioning, staining with hematoxylin and pulmonary vascular morphometry were performed as described previously [13]. To determine the degree of muscularization of small pulmonary arteries, lung tis-

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sue sections were stained with anti-smooth muscle α -actin. At least 20 arteries with an external diameter smaller than 50 μ m were identified per tissue section. Arteries were scored, as described previously [14].

Western blotting

Lung tissues were homogenized using tissue homogenizer (for tissues only) or lysed in RIPA buffer (Bi Yun-Tian, China) with addition of a protease inhibitor cocktail (Bi Yun-Tian, China) and PMSF. Tissue lysates were equalized with SDS 5 \times sample buffer and electrophoretically separated on 10% polyacrylamide gels and transferred for 1 h on to nitrocellulose membranes. Subsequently membranes were blocked 1 h with 5% non-fat dry milk in Trisbuffered saline/0.1% Tween 20. After blocking, membranes were probed with primary antibodies diluted as follows: Cx40 (1:1000; Abcam, USA), phospho-Smad1/5 (1:1000; Cell Signaling Technology, USA), BMPR2 (1:500; Abcam, USA), β -actin (1:1000, Bi Yun-Tian, China). Samples were normalized to β -actin housekeeping gene and total protein of interest and further quantification was performed. After primary antibody incubation, membranes were incubated with secondary goat anti-rabbit (1:10000, Bi Yun-Tian, China) or rabbit anti-goat (1:10000, Bi Yun-Tian, China) HRP-conjugated antibodies (ZSGB-BIO). After washing, the membrane was visualized by a chemiluminescence reaction using an ECL-detection kit system (Amersham Pharmacia Biotechnology, CA, USA) and further quantified using specific software as Image Lab.

Real-time reverse-transcription polymerase chain reaction

Total RNA of the right lung was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The reverse transcription was then performed in 1 μ g of total RNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). Realtime polymerase chain reaction (RT-PCR) was conducted using ABI PRISM® 7500 Real-time PCR System according to the manufacturer's guidelines. Two-step real-time RT-PCR was used to perform relative quantification of mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an internal housekeeping gene control for the comparative Ct method for the relative quantification of target

genes mRNA expressions. The fluorescent product was detected at the end of each cycle. Product specificity was confirmed by agarose gel electrophoresis and routinely by melting-curve analysis. The primers were designed as Paulo Renato A.V. Correa described using the software Primer3 based on the sequence deposited in the NCBI Nucleotide Bank. The fluorescent product was detected at the end of each cycle. Product specificity was confirmed by agarose gel electrophoresis and routinely by melting-curve analysis. Data were analyzed with the ABI Prism 7500 sequence detection system software (version 1.4) and GAPDH was used as an internal control for input RNA.

Proliferation assay

Rat PASMCS were isolated from small pulmonary arteries, as described previously [15]. Cells were made quiescent for 48 hours and then seeded in 96-well plate. Each well contained either 10% FBS (Invitrogen, USA)/DMEM (Invitrogen, USA) alone or with the sildenafil, or sildenafil + LDN-193189. The cells were incubated for 2 days at 37°C/5% CO₂. At 4th day, 10 μ L of CCK-8 (Dijindo) were added to each well for 4 hrs. The product was then quantified spectrophotometrically at a wavelength of 450 nm. The value of OD in each well was taken as the level of the PASMCS proliferation. Multiple wells were counted and averaged for each condition studied.

Statistical analysis

Data are presented as mean \pm SEM. Statistical analyses were performed by using the IBM SPSS Statistics 19 statistical software. The means among groups were compared using one way ANOVA, followed by Student-Newman-Keuls's post hoc test. Statistical significance was set at $P < 0.05$.

Results

PAH model established

Elevations of RVSP and mPAP were observed in rats 28 days after a subcutaneous injection of MCT compared with the sham (**Figure 1A, 1B**). Likewise, the right ventricular hypertrophy assessed by RV/(LV + S) were significantly increase compared with the sham (**Figure 1C**). HE staining demonstrated a significant PASMCS proliferation and pulmonary vascular remodel-

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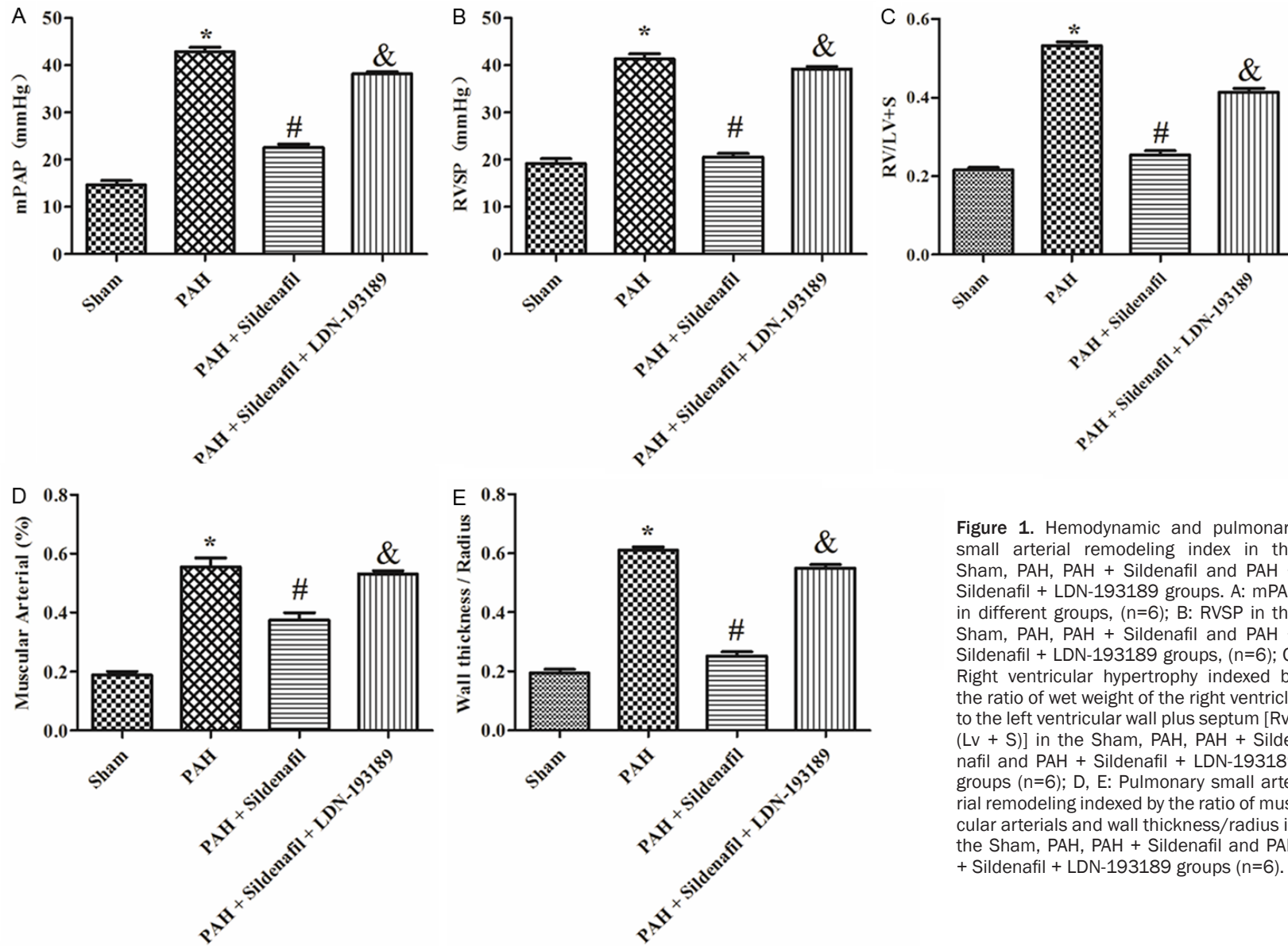


Figure 1. Hemodynamic and pulmonary small arterial remodeling index in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups. A: mPAP in different groups, (n=6); B: RVSP in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups, (n=6); C: Right ventricular hypertrophy indexed by the ratio of wet weight of the right ventricle to the left ventricular wall plus septum [Rv/(Lv + S)] in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups (n=6); D, E: Pulmonary small arterial remodeling indexed by the ratio of muscular arterials and wall thickness/radius in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups (n=6).

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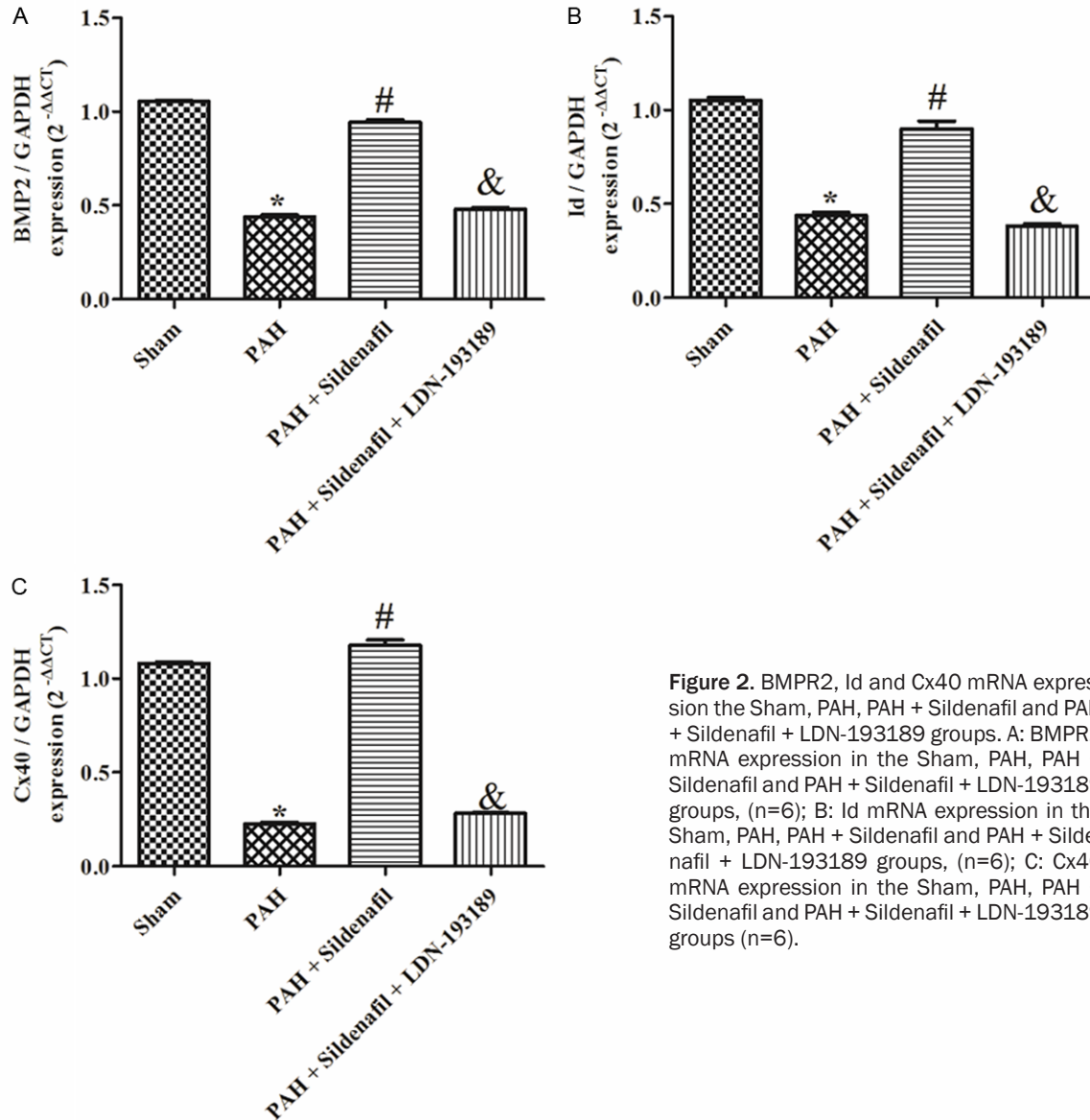


Figure 2. BMPR2, Id and Cx40 mRNA expression in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups. A: BMPR2 mRNA expression in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups, (n=6); B: Id mRNA expression in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups, (n=6); C: Cx40 mRNA expression in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups (n=6).

ing formation, in PAH models compared with the sham (**Figure 3**). Muscular Arterial (%) and Wall thickness/Radius (%) of muscular arteries was significantly increased in PAH groups than those in sham group, respectively (**Figure 1D, 1E**).

BMP signaling and Cx40 expression was altered in MCT-induced PAH

In the PAH group, the expression of BMPR2 and Id mRNA in rat lungs were significantly reduced by day 28 (**Figure 2A, 2B**) compared with the sham. The protein expression of BMPR2 and p-Smad1/5 was also significantly reduced in PAH models compared with the sham (**Figure 4A, 4B**). Furthermore, the gene and protein

expression of Cx40 was significantly decreased by day 28 in the MCT rat lung compared with the sham (**Figures 2C, 4C**).

Sildenafil inhibits the hemodynamic deterioration and arterial remodeling in MCT-induced PAH

After 28 days administration of Sildenafil in the PAH model, it significantly inhibited the elevation of mPAP and RVSP elicited by the MCT compared with the PAH group (**Figure 1A, 1B**); it also significantly inhibited the hypertrophy and the severe vascular remodeling elicited by the MCT indexed by the ratio of muscular arterials and Wall thickness/Radius compared with the PAH group (**Figure 1C-E**). The H&E staining

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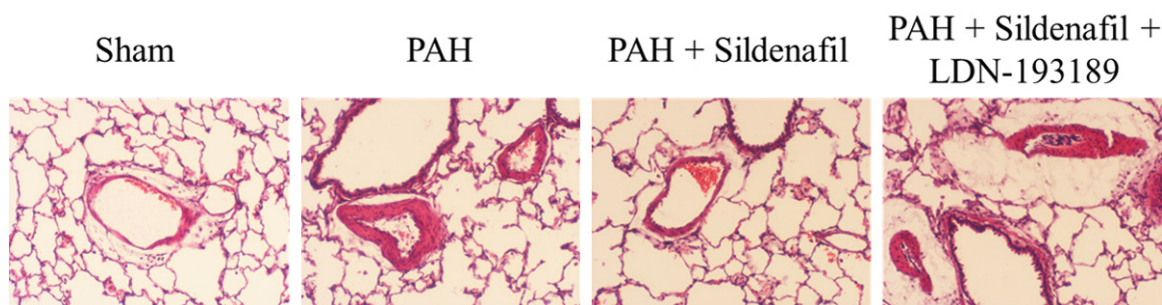


Figure 3. Representative images of HE stained lung sections from the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups, respectively, showing small pulmonary arteries.

also founded that Sildenafil administration inhibited the PSMCs proliferation and remodeling in pulmonary arterial elicited by the MCT (**Figure 3**).

Sildenafil up-regulated the BMP signaling and the Cx40 expression in the MCT-induced PAH

Sildenafil used in the PAH model led to a significant increase in the BMPR2 and Id mRNA in rat lungs compared with the PAH by day 28 (**Figure 2A, 2B**). Western-blotting revealed that BMPR2 and phospho-Smad1/5 expression was also significantly increased at day 28 in the sildenafil group compared with the PAH group (**Figure 4A, 4B**). Furthermore, the expression of Cx40 was significantly increased in the Sildenafil rats compared with the PAH group (**Figures 2C, 4C**).

LDN-193189 reversed sildenafil-stimulated the BMP-Cx40 up-regulation in MCT-induced PAH

LDN-193189 used in the sildenafil rats led to a significant reduction in the BMPR2 and Id mRNA in rat lungs compared with the sildenafil group by day 28 (**Figure 2A, 2B**). Western-blotting revealed that the BMPR2 and phospho-Smad1/5 expression was also significantly decreased in the LDN-193189 + Sildenafil group compared with the sildenafil group at day 28 (**Figure 4A, 4B**). Furthermore, the expression of Cx40 was significantly decreased in the LDN-193189 + Sildenafil rats compared with the sildenafil group (**Figures 2C, 4C**).

LDN-193189 reversed sildenafil inhibition of the development and progression of pulmonary hypertension in MCT-induced PAH

After 28 days administration of LDN-193189 + Sildenafil in the PAH model, the LDN-193189

significantly reversed the sildenafil of the inhibition in the mPAP and RVSP compared with the Sildenafil group (**Figure 1A, 1B**); LDN-193189 also significantly inhibited the anti-hypertrophy and the anti-vascular remodeling effect elicited by the Sildenafil indexed by the ratio of muscular arteries and Wall thickness/Radius (**Figure 1C-E**). The H&E staining also founded that LDN-193189 administration inhibited the Sildenafil effect of inhibition of the PSMCs proliferation and remodeling in pulmonary arterial elicited by the MCT (**Figure 3**).

Sildenafil up-regulate the Cx40 expression of PSMCs isolated from MCT-PAH rats and this effect was reversed by the LDN-193189

Rat PSMCs were provided by small peripheral pulmonary arteries from the PAH rats. The Sildenafil significantly inhibits the proliferation and significantly up-regulate the BMPR2 and Cx40 expression in the PSMCs isolated from the PAH (**Figures 5, 6**). Co-incubation of PSMCs from the PAH rats with sildenafil and LDN-193189 completely inhibited the anti-proliferation and up-regulation of the BMPR2 and Cx40 expression by the sildenafil (**Figures 5, 6**).

Discussion

In this study, we put forward new concepts pertaining to the involvement of cGMP-BMP signaling in regulating the Cx40 expression of PAH. We now report that the PSMCs express the Cx40; the MCT models were associated with significant down-regulations in the expression of BMPR2, Id, p-Smad1/5 and Cx40 mRNA and protein by the end of the 28 day; then, we used the cGMP-specific phosphodiesterase 5 (PDE5) inhibitor, sildenafil, to determine whether the activation of cGMP would show efficacy in the Cx40 expression in PSMCs. We find that the

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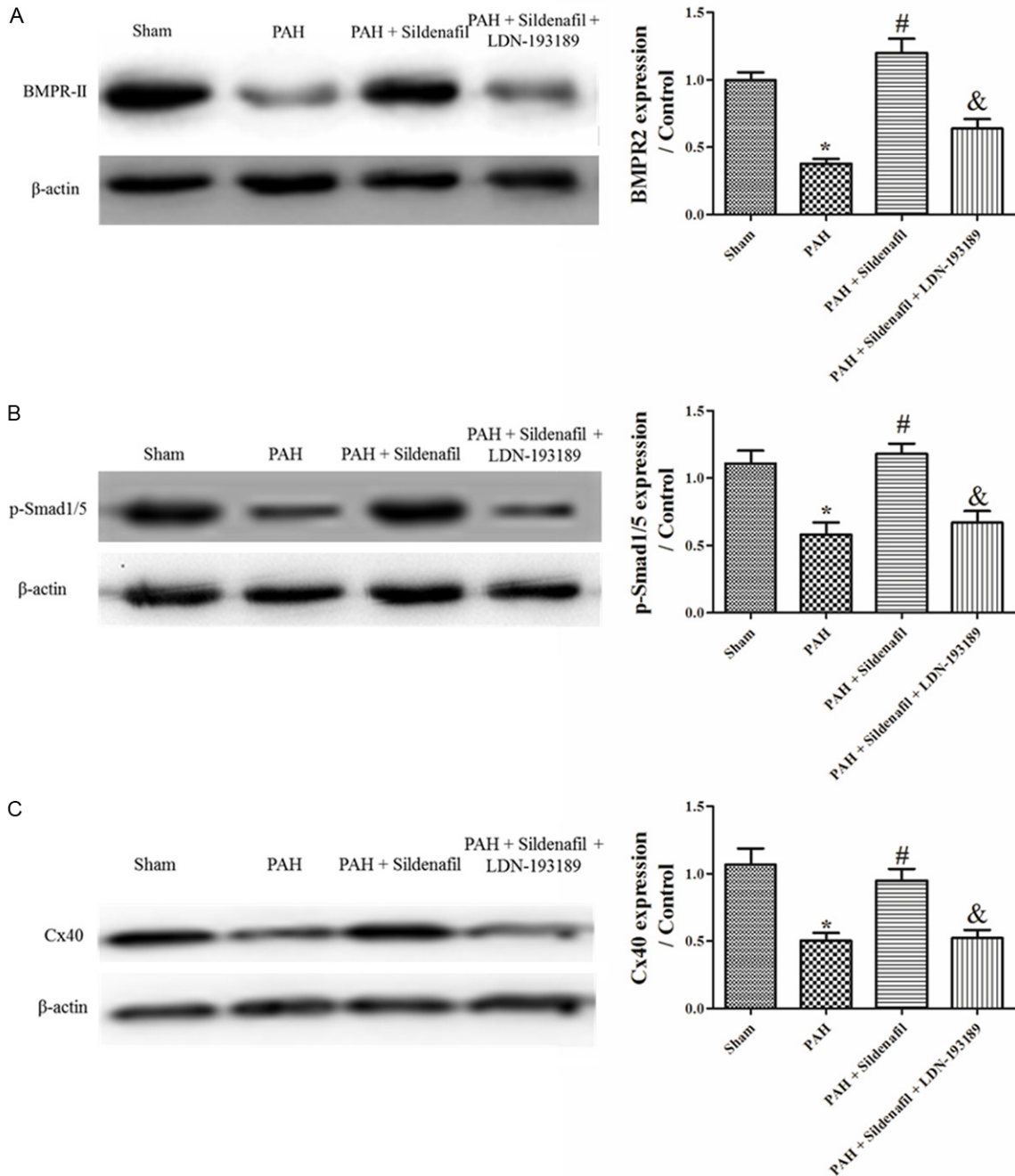


Figure 4. BMPR2, Id and Cx40 protein expression in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups. A: BMPR2 protein expression in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups, (n=3); B: p-Smad1/5 Protein expression in Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 Groups, (n=3); C: Cx40 protein expression in the Sham, PAH, PAH + Sildenafil and PAH + Sildenafil + LDN-193189 groups (n=3).

administration of sildenafil significantly up-regulated the BMPR2, Id, p-Smad1/5 and Cx40 mRNA and protein expression, inhibit the pulmonary arterial muscularization and improve the hemodynamics in lung. Having established that the activation of cGMP could inhibit the up

regulation of the Cx40, we went on to confirm that BMP signaling inhibition could reverse the sildenafil effect in the PAH. Our experiment showed that the BMPR2 inhibitor, LDN-193189 could significantly inhibit the sildenafil-induced up-regulation of Cx40, so we confirmed that the

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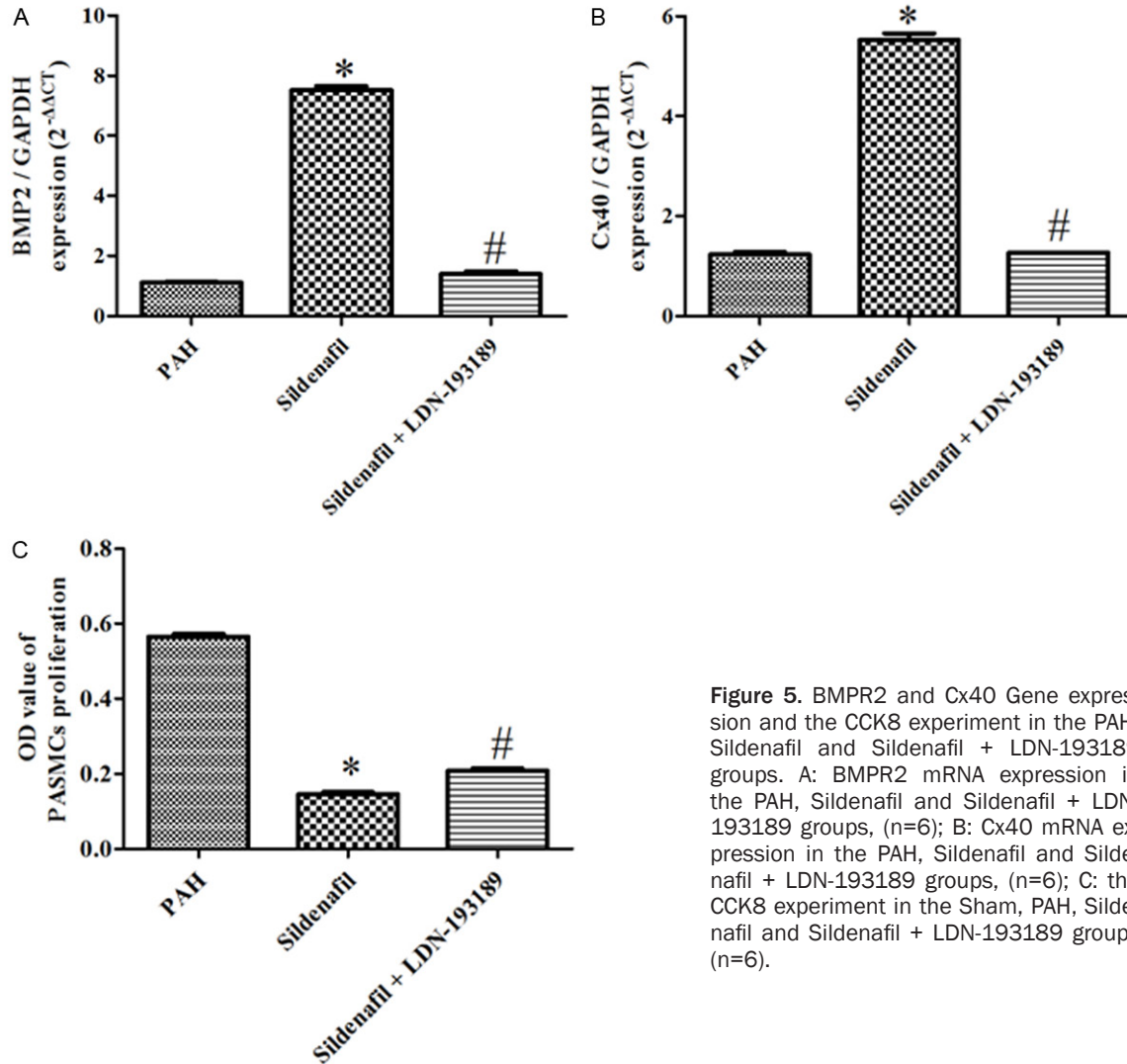


Figure 5. BMPR2 and Cx40 Gene expression and the CCK8 experiment in the PAH, Sildenafil and Sildenafil + LDN-193189 groups. A: BMPR2 mRNA expression in the PAH, Sildenafil and Sildenafil + LDN-193189 groups, (n=6); B: Cx40 mRNA expression in the PAH, Sildenafil and Sildenafil + LDN-193189 groups, (n=6); C: the CCK8 experiment in the Sham, PAH, Sildenafil and Sildenafil + LDN-193189 groups (n=6).

cGMP-BMP signaling way lies in the Cx40 expression of PASMCS in PAH. Moreover, we confirm that sildenafil up-regulate the cx40 expression of PASMCS isolated from MCT-PAH rats, and LDN-193189 could prevent the sildenafil-induced of Cx40 up-regulation *in vitro*.

MCT-induced PAH in the rat has been used extensively to research the pathophysiological changes of PAH. In this animal model, the MCT provokes the pulmonary hypertension and a vascular masculinization of pulmonary arterial in 3-4 weeks [16]. It is demonstrated the pulmonary vasoconstriction severity (pulmonary arteriolar wall thickness, decreased alveolar-sac numbers) were significantly increased in the MCT-treated animals compared to sham. Furthermore, the mPAP, RVSP and RV weight

showed an identical pattern compared to that of vascular remodeling [17]. It is also found that in the MCT model, the increase of mPAP, right ventricular hypertrophy, and small vessel remodeling is seen [18]. In our study, the mPAP, gold standard of the PAH diagnosis, are significantly elevated in the MCT models in 28 days compared to sham, along with the elevation of RVSP and the right ventricle hypertrophy. And there was a significant increase of the vascular remodeling of pulmonary arteries in the MCT model. Moreover, the HE staining showed that the proliferation of PASMCS significantly increased in the MCT model compared to sham. Taken together, the MCT models, better in mimicking pulmonary hemodynamics and vascular muscularization in human PAH, are the ideal models for the study of PAH.

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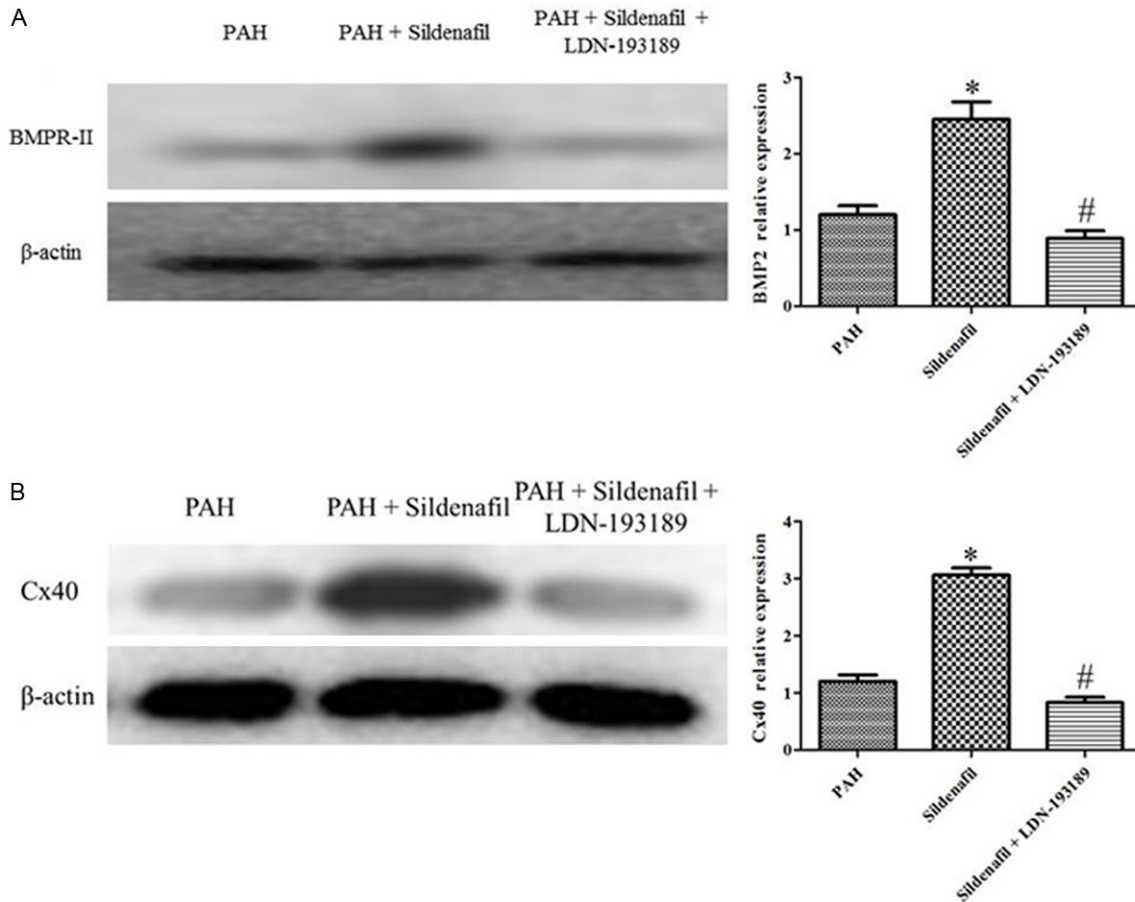


Figure 6. BMP2 and Cx40 protein expression in the PAH, Sildenafil and Sildenafil + LDN-193189 groups. A: BMP2 protein expression in the PAH, Sildenafil and Sildenafil + LDN-193189 groups, (n=6); B: Cx40 protein expression in the PAH, Sildenafil and Sildenafil + LDN-193189 groups, (n=6).

The Cx40 is crucial in vascular physiology. For example, introduction of a mutant form of Cx40 in the endogenous endothelial Cx40 population prevents endothelium-derived hyperpolarization activation during myogenic constriction, enhancing sensitivity to intraluminal pressure and increasing arterial smooth muscle cell proliferation [19-21]. Furthermore, it is suggested that Cx40 (-/-) mice are at significantly greater risk for poor recovery from ischemic insult due to compromised regulation of vascular remodeling, and prolonged inflammatory response [22]. In our research, we found that in PAH, the Cx40 expression level is down-regulated in the pulmonary arterial in PAH. Furthermore, the sildenafil can up-regulate the Cx40 expression in PSMCs of PAH, and this may contribute to the effect of anti-muscularization of sildenafil. Furthermore, we also find *in vitro* that in the PSMCs isolated from the PAH rats, the BMP2 and Cx40 expression of PSMCs decreased,

and this phenomenon is reversed by the sildenafil treatment, providing strong evidence for the protect effect of sildenafil.

Heterozygous non-sense mutations in the gene encoding the BMP2, a member of the TGF β receptor family, underlie the majority of inherited and familial forms of PAH [23]. BMP2 inhibit the proliferation of human and rat smooth muscle cells via the small mothers against SMAD1/5 pathway [24]. In PSMCs isolated from PAH subjects, the anti-proliferative effect of BMPs is greatly reduced [25]. Furthermore, BMP-promoted apoptosis in PAH-PSMCs with reduced BMP2 protein levels is attenuated. Sildenafil up-regulate the BMP signaling, for example, in PAH, sildenafil enhanced BMP-induced phosphorylation of Smad1/5, Smad nuclear localization, and Inhibitor of DNA binding protein 1 gene and protein expression. Furthermore, sildenafil restored the anti-proliferative response to BMP in PSMCs harboring

mutations in BMPR2 [26]. Because the mechanism of action of sildenafil include enhancement of BMP signaling, here, we questioned whether the mechanism of up-regulation of Cx40 with sildenafil in PAH could include it. We demonstrate that in the PAH, the BMPR2 and Id gene is down-regulated; sildenafil increased the level of BMPR2, and enhances BMP-stimulated Id gene expression. We provide evidence that BMP signaling is central to the regulation of Cx40 with sildenafil, because the pharmacological inhibition of BMPR2 with LDN-193189 could abolish the up-regulation of cx40 induced by sildenafil in PSMCs of PAH. To our knowledge, this is the first report that sildenafil regulate the expression of the Cx40 in PSMCs of PAH. Our findings also reveal the critical involvement of BMP signaling during this progress.

In summary, we have provided evidence to confirm that the sildenafil trigger the Cx40 in the PSMCs via the BMP signaling in the MCT models of PAH. Inhibition of BMP signaling reversed the improvement of sildenafil on the Cx40 expression, the hemodynamics and vessel remodeling in the PAH and in vitro. These studies provide proof-of-concept for approaches that manipulate BMP/Cx40 signaling in clinical pulmonary hypertension.

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Disclosure of conflict of interest

None.

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