Endogenous Hydrogen Sulfide Regulates Pulmonary Artery Collagen Remodeling in Rats with High Pulmonary Blood Flow

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The mechanisms responsible for the structural remodeling of pulmonary vasculature induced by increased pulmonary blood flow are not fully understood. This study explores the effect of endogenous hydrogen sulfide (H_2S), a novel gasotransmitter, on collagen remodeling of the pulmonary artery in rats with high pulmonary blood flow. Thirty-two Sprague-Dawley rats were randomly divided into sham, shunt, sham+PPG (D,L-propargylglycine, an inhibitor of cystathionine-γ-lyase), and shunt+PPG groups. After 4 weeks of shunting, the relative medial thickness (RMT) of pulmonary arteries and H_2S concentration in lung tissues were investigated. Collagen I and collagen III were evaluated by hydroxyproline assay, sirius-red staining, and immunohistochemistry. Pulmonary artery matrix metalloproteinase-13 (MMP-13), tissue inhibitor of metalloproteinase-1 (TIMP-1), and connective tissue growth factor (CTGF) were evaluated by immunohistochemistry. After 4 weeks of aortocaval shunting, resulting in an elevation of lung tissue H_2S to 116.4%, rats exhibited collagen remodeling and increased CTGF expression in the pulmonary arteries. Compared with those of the shunt group, lung tissue H_2S production was lowered by 23.4%, RMT of the pulmonary artery further increased by 39.5%, pulmonary artery collagen accumulation became obvious, and pulmonary artery CTGF expression elevated ($P < 0.01$) in the shunted rats treated with PPG. However, pulmonary artery MMP-13 and TIMP-1 expressions decreased significantly in rats of shunt+PPG group ($P < 0.01$). This study suggests that endogenous H_2S exerts an important regulatory effect on pulmonary collagen remodeling induced by high pulmonary blood flow. Exp Biol Med 234:504–512, 2009

Key words: hydrogen sulfide; collagen; pulmonary artery; inhibitor of metalloproteinase-1 (TIMP-1); matrix metalloproteinase-13 (MMP-13)

Introduction

Congenital heart disease (CHD) is the most common heart disease in childhood. It is often characterized by increased pulmonary blood flow in those with a left-to-right shunt. This increased blood flow results in vascular injury and pulmonary hypertension during the course of the disease. The mechanisms responsible for structural remodeling induced by increased pulmonary blood flow are not fully understood. Collagen accumulation is one of the important factors in the development of vascular remodeling and pulmonary hypertension. The regulation of pulmonary hypertension has also been indicated to be associated with the role of a novel gasotransmitter, hydrogen sulfide (H_2S), which plays a part similar to nitric oxide (NO) and carbon monoxide (CO) in the relaxation of blood vessels (1–5). Based on the above evidence, we performed the present study to investigate the possible changes and roles of endogenous H_2S in collagen remodeling in rats with high pulmonary blood flow.

Materials and Methods

Animal Model of High Pulmonary Blood Flow. Experiments were conducted in accordance with the Guide to The Care and Use of Experimental Animals issued by the Ministry of Health of the People's Republic of China. Male Sprague-Dawley rats were provided by the Animal Research Center of Peking University First Hospital. The rats were

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kept in a temperature-controlled room with a 12 hour light-dark cycle. Tap water and rat chow were provided *ad libitum*. The animal model of the left-to-right shunt was created according to the method described by Garcia and Diebold (6) with minor modifications (7). Briefly, 32 male Sprague-Dawley rats, weighing 120–140 g, were randomly divided into shunt ($n = 8$), shunt+propargylglycine (PPG, an inhibitor of cystathionine-γ-lyase) ($n = 8$), sham ($n = 8$), and sham+PPG ($n = 8$) groups. We anesthetized rats in the shunt and shunt+PPG groups with 0.25% pentobarbital sodium (40 mg/kg, intraperitoneal injection). We exposed the abdominal aorta and inferior vena cava, and then placed a bulldog vascular clamp across the aorta caudal to the left renal artery. We punctured the aorta at the union of the segment two-thirds caudal to the renal artery and one-third cephalic to the aortic bifurcation with an 18-gauge disposable needle. Then, the needle was slowly withdrawn and a 9–0 silk thread was used to stitch the puncture of the abdominal wall. In the sham and sham+PPG groups, rats underwent the same experimental protocol as mentioned above except for the shunting procedure. We injected rats in the shunt+PPG and sham+PPG groups intraperitoneally with PPG at 37.5 mg/kg/d for 4 weeks (8). We injected rats in the shunt and sham groups with the same volume of physiological saline.

Measurement of Oxygen Saturation. At 4 weeks of the experiment, we weighed and anesthetized the animals with pentobarbital sodium (40 mg/kg, intraperitoneal injection). We analyzed blood samples (0.5 ml) obtained from the pulmonary artery, external carotid artery, and jugular vein using a GASTAT-3 Blood Gas Analysis Apparatus. The ratio Qp/Qs was calculated as an indicator of pulmonary and systemic blood flow. Qp/Qs was calculated by the formula: Qp/Qs = [oxygen saturation of aorta (%) − oxygen saturation of jugular vena cava (%)] / [oxygen saturation of pulmonary vein (%) − oxygen saturation of pulmonary artery (%)]. When the oxygen saturation of the aorta was >95%, we regarded the oxygen saturation of the pulmonary vein as 100%. When the oxygen saturation of the aorta was <95%, we regarded the oxygen saturation of the pulmonary vein as 95%.

The right side of the lung tissue was removed and quickly frozen in liquid nitrogen, then stored at –80°C for homogenate. The left lower part of lung tissue was removed and post-fixed in 10% (wt/vol) paraformaldehyde.

Measurement of H_2S in Lung Tissue. We homogenized lung tissue in a 10-fold volume (w/v) of an ice-cold potassium phosphate buffer (pH = 6.8). The reaction was performed in a 25-ml Erlenmeyer Pyrex flask. We used cryovial test tubes (2 ml) as the center wells, each containing 1 M NaOH of 0.5 ml. The reaction mixture contained lung tissue homogenate and 1 M HCL in a ratio of 1:5. We flushed the flasks containing reaction mixture and central wells with N_2 for 30 seconds before sealing with a double layer of parafilm. The reaction was initiated by transferring the flasks from ice to a shaking water bath at 37°C. After incubation at 37°C for 4 h, we transferred the contents of the central wells to 10-ml beakers, each containing 0.5 ml of antioxidant solution. Subsequently, the solution was measured with a sulfide-sensitive electrode (PXS-270, Shanghai, China) to calculate the lung tissue H_2S against a standard curve.

Morphological Analysis of Small Pulmonary Arteries. We mounted fixed lung tissue in paraffin, and sectioned it at 4 μm thickness. We then stained the elastic fiber according to the modified Weigert's method and counterstained with Van Gieson solution. We performed morphological analysis using a Video-Linked Microscope Digitizing Board System (Leica Q550CW, Germany). Only vessels showing clearly defined external and internal elastic lamina were used in the analysis. The relative medial thickness (RMT) was calculated according to Barth's methods (9).

Hydroxyproline Assay of Lung Tissue. Lung tissue homogenate was dehydrated in 0.2 ml of 6 nmol/L HCl (14 hours). The pH of the samples was adjusted with 6 nmol/L NaOH to 6.0, and then centrifuged at 2000 rpm for 10 min. We performed the hydroxyproline assay according to instructions provided with a commercially available kit (Nanjing Jiancheng Bioengineering Institute, China).

Sirius-Red Staining Analysis of Collagen I and Collagen III. Lung sections were processed by sirius-red staining for 1 min, following dewaxing and dehydration. Polarized light microscopy was used to distinguish type I and type III collagen fibers (Leica, Germany).

Immunohistochemical Analysis. Lung sections were pretreated by 3% H_2O_2 for 15 min, followed by the appropriate antigen repairing treatment: digestion with gastric enzyme for 30 min at 37°C for collagen I and collagen III; heating with microwave for 15 min at 99°C for tissue inhibitor of metalloproteinase 1 (TIMP-1), matrix metalloproteinase-13 (MMP-13), and connective tissue growth factor (CTGF). The slides were blocked with normal bovine serum albumin (BSA) for 30 min at room temperature. Collagen I or III (Boster Bioengineering Ltd., China), MMP-13, TIMP-1 (Neomarkers, USA) and CTGF (Boster Bioengineering Ltd., China) antibodies were then added at 37°C for 2 h respectively in different experiment. We used biotinylated anti-rabbit IgG and horseradish peroxidase streptavidin (Santa Cruz, Canada) sequentially at 37°C for 30 min. To develop a color product, diaminobenzidine (DAB) was added for 1–10 min and Mayer's hematoxylin for 1 min. We observed labeling of the smooth muscle cells in intrapulmonary arteries using light microscopy. The mean percentage of intensity of antibody labeling (0%, ~50%, and ~100%) was determined (9).

Statistical Analysis. All data are expressed as means ± SD. We analyzed data using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls tests for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

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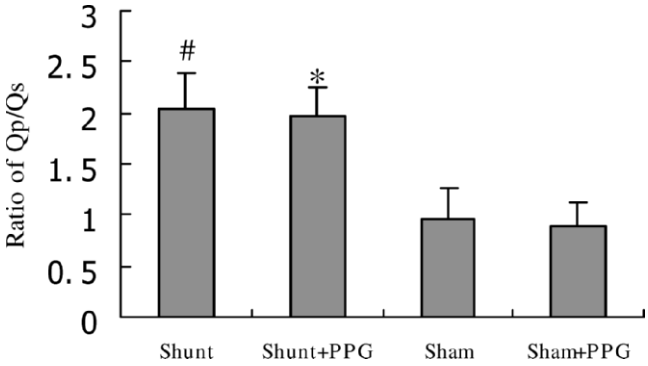

Figure 1. Ratio of Qp/Qs in rats of different groups. #$P < 0.01$ vs. sham; *$P < 0.01$ vs. sham+PPG.

Results

Changes in Oxygen Saturation and H_2S Content. In the present rat model of abdominal aorta-inferior vena cava shunt, Qp/Qs in the shunt group and shunt+PPG group increased significantly as compared with that of sham and sham+PPG group ($P < 0.01$). The shunt and shunt+PPG groups did not differ significantly (Fig. 1).

Compared with the sham group, the lung tissue H_2S content in the shunt group increased significantly ($P < 0.01$). In the shunt+PPG group, lung tissue H_2S content decreased significantly ($P < 0.01$). The sham and sham+PPG groups did not differ significantly in lung tissue H_2S content (Fig. 2).

RMT in Pulmonary Arteries. In contrast to the sham group, RMT of intra-acinar pulmonary arteries in the shunt group increased significantly (16.87 ± 1.86% vs 12.67 ± 1.26%, $P < 0.01$). Compared with shunt group, RMT of intra-acinar pulmonary arteries in the shunt+PPG group increased significantly (23.54 ± 3.07% vs 16.87 ± 1.86%, $P < 0.01$). RMT in sham and sham+PPG groups did not differ significantly (12.67 ± 1.26% vs 13.00 ± 3.20%, $P > 0.05$) (Fig. 3).

Hydroxyproline Concentration and Collagen Content of Lung Tissue. Hydroxyproline concentration in lung tissue in the shunt group was significantly greater than that of the sham group ($P < 0.01$). In the shunt+PPG group, hydroxyproline concentration was significantly greater than that of the shunt group ($P < 0.01$). There were no significant differences between the sham and sham+PPG groups ($P > 0.05$) (Fig. 4).

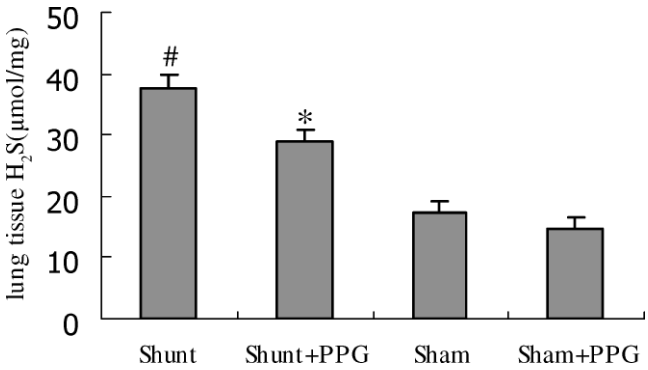

Figure 2. Changes of lung tissue H_2S content in rats of different groups. #$P < 0.01$ vs. sham; *$P < 0.01$ vs. shunt.

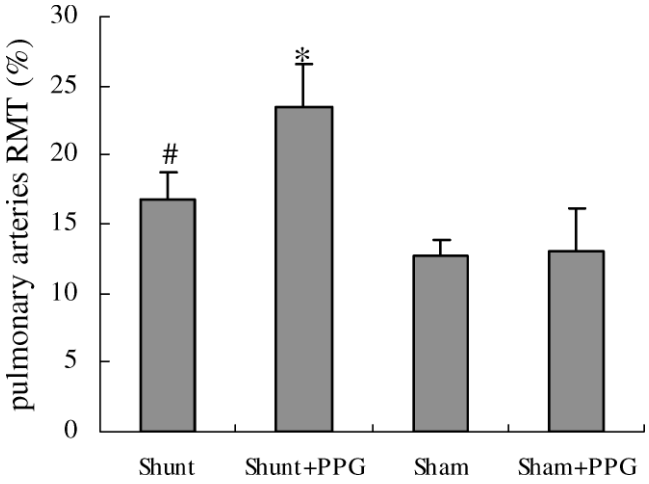

Figure 3. RMT of pulmonary arteries in rats of different groups. #$P < 0.01$ vs. sham; *$P < 0.01$ vs. shunt.

Sirius-red staining showed that collagen I and collagen III were more intensely stained in the pulmonary arteries of the shunt group than in the sham group. The shunt+PPG group showed more predominant collagen I and collagen III than the shunt group (Fig. 5).

Pulmonary Artery Collagen I and Collagen III. Collagen I and collagen III protein expressions of intra-acinar pulmonary arteries in the shunt group were significantly greater than the sham group ($P < 0.01$). In the shunt+PPG group, collagen I and collagen III protein expressions of intra-acinar pulmonary arteries were significantly greater than in the shunt group ($P < 0.01$). There was no significant difference in collagen I and collagen III protein expressions of intra-acinar pulmonary arteries between the sham and sham+PPG groups ($P > 0.05$) (Figs. 6, 7). Using sirius-red staining, collagen I and collagen III in rat pulmonary artery accumulation were also more obvious

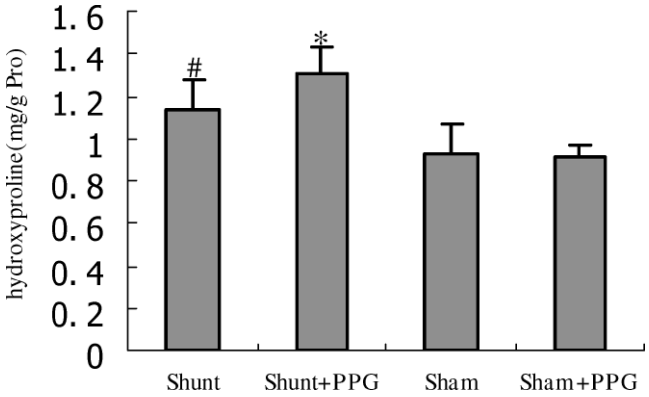

Figure 4. Changes of hydroxyproline concentration in lung tissue in rats of different groups. #$P < 0.01$ vs. sham; *$P < 0.01$ vs. shunt.

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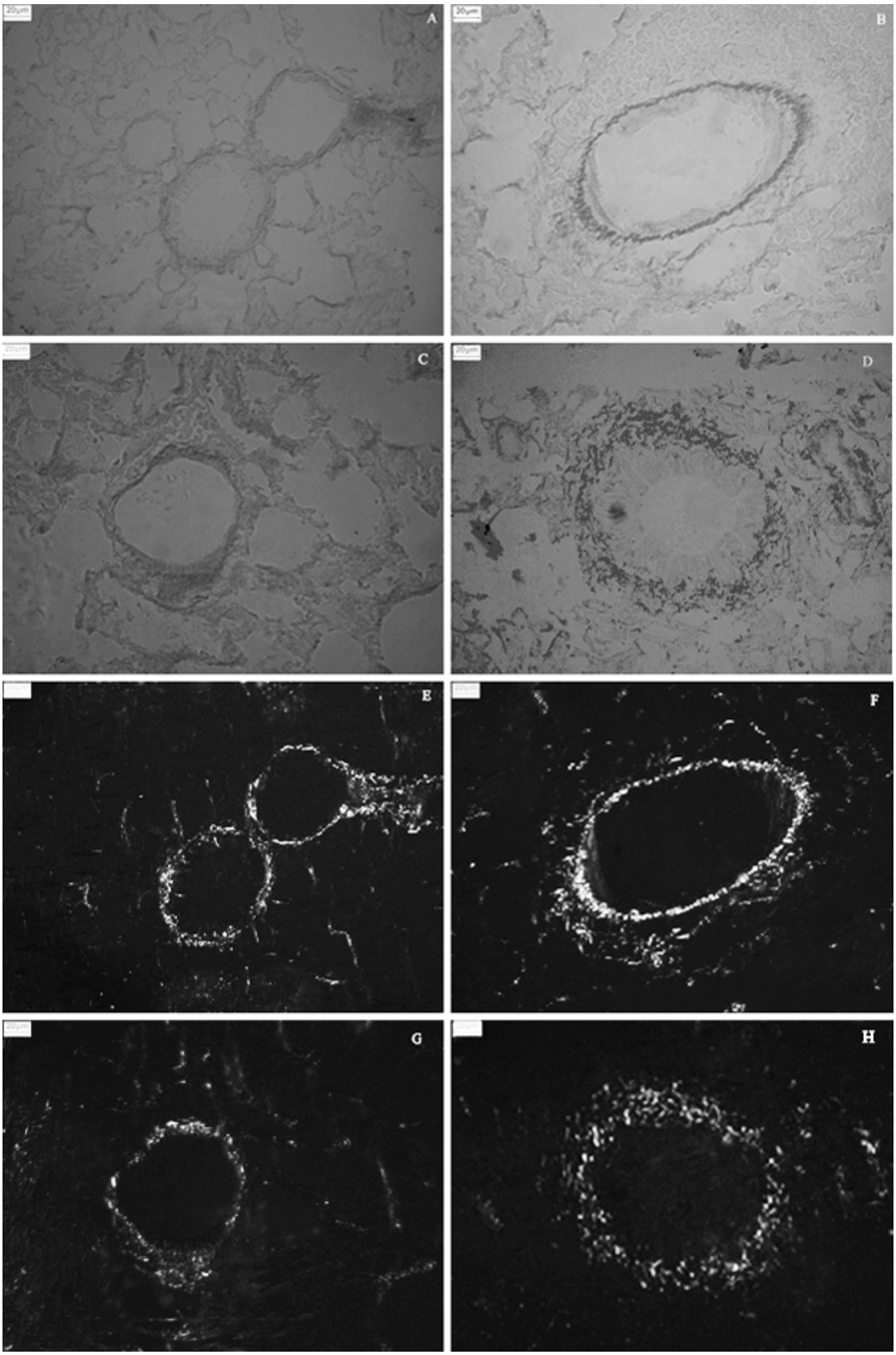

Figure 5. Sirius-red staining analysis of collagen I and collagen III in rat pulmonary artery under microscope (×200). A, sham, B, sham+PPG, C, shunt, D, shunt+PPG. Sirius-red staining analysis of collagen I and collagen III in rat pulmonary artery under polarizing microscope (×200). E, sham, F, sham+PPG, G, shunt, H, shunt+PPG.

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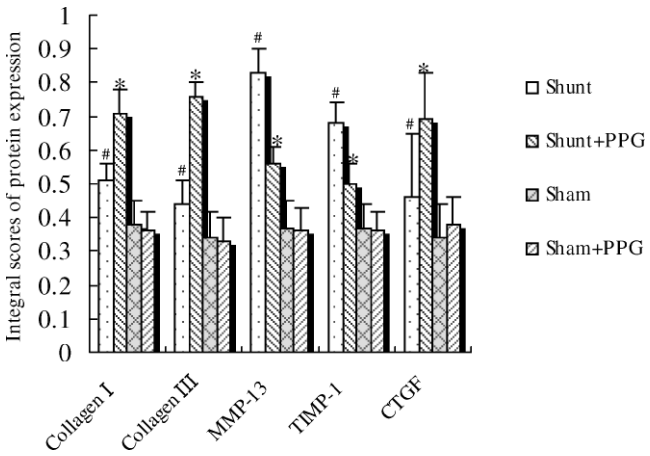

Figure 6. Changes in integral scores of collagen I, collagen III, MMP-13, TIMP-1, and CTGF protein expressions in the pulmonary artery of different groups. #$P < 0.01$ vs. sham; *$P < 0.01$ vs. shunt.

in the shunt group than in the sham group, and in the shunt+PPG group versus the shunt group (Fig. 5).

MMP-13 and TIMP-1 in Pulmonary Arteries. MMP-13 and TIMP-1 protein expressions of intra-acinar pulmonary arteries as well as the ratio of MMP-13/TIMP-1 in the shunt group were significantly greater than the sham group ($P < 0.01$). In the shunt+PPG group, MMP-13 and TIMP-1 and the ratio of MMP-13/TIMP-1 were significantly decreased relative to the shunt group ($P < 0.01$). The sham and sham+PPG groups did not differ significantly (Fig. 6).

CTGF Protein Expression of Intra-Acinar Pulmonary Arteries. In the shunt group, CTGF protein expression of intra-acinar pulmonary arteries was significantly greater than the sham group ($P < 0.01$). In the shunt+PPG group, CTGF was significantly greater than that of the shunt group ($P < 0.01$). There was no significant difference in CTGF between the sham and sham+PPG groups ($P > 0.05$) (Figs. 6, 8).

Discussion

Pulmonary vascular structural remodeling and hypertension due to high blood flow are common complications of congenital heart disease observed with a left-to-right shunt. However, the mechanisms responsible for the remodeling induced by increased pulmonary blood flow have been unclear. In the present study, we created a rat model of high pulmonary blood flow by an abdominal aorta-inferior vena cava shunting operation. We found that in the shunt group, the ratio of pulmonary blood flow and systemic blood flow (Qp/Qs) was significantly greater than in the sham group, which suggests that pulmonary blood flow increased as a result of the shunt. RMT, an indicator of pulmonary vascular structural remodeling, was significantly greater in the shunt group, which suggested that pulmonary vascular structure remodeled due to high pulmonary blood flow.

H_2S is produced endogenously in mammalian tissues from cystathionine metabolism mainly by 3 enzymes: cystathionine-β-synthetase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptosulfurtransferase (MST) (10, 11). The expression of these enzymes is tissue-specific, and CSE mainly catalyzes H_2S production in the cardiovascular system. Our previous studies revealed that H_2S plays an important role in the pathophysiological process of some cardio-pulmonary diseases such as spontaneous hypertension (8), nitric oxide (NO) synthase inhibitor-induced hypertension (12), hypoxia-induced pulmonary hypertension (13), septic and endotoxic shock (14), and isoproterenol-induced myocardial injury (15). These findings suggest that endogenous H_2S could be a novel gasotransmitter in the cardiovascular system (16). In this study, we observed markedly increased H_2S production in a rat model of high pulmonary blood flow.

To explore whether the CSE-H_2S pathway contributes to the pathogenesis of pulmonary vascular structural remodeling induced by high pulmonary blood flow, we applied D,L-propargylglycine (PPG), an irreversible inhibitor of CSE, in this experiment. CSE is a key enzyme in the pathway of cystathionine metabolism to produce endogenous H_2S in rats. PPG was first reported by Abeles and Walsh to inactivate rat liver cystathionase in 1973 (17). After that, further studies showed that CSE enzyme is an alpha 2 beta 2 tetramer where the subunits are distinguishable by charge but not by size and that each subunit of a CSE tetramer became modified by PPG in an inactive CSE (18, 19).

Although the accurate half life of PPG injected intraperitoneally has not been reported, we found that the content of PPG in serum reached its maximum at about 2 h after intraperitoneal injection. Approximately 21.2% of administered PPG was excreted into the urine within 6 h and could not be detected in the urine by about 12 h after administration (20). Moreover, we found that the activity of CSE decreased to about 4% in rats 24 h after PPG injection daily (21). Based on the above data and our previous study (8), PPG was injected intraperitoneally once a day in this experiment.

In the present study, we found that there was no statistical difference in lung tissue H_2S production between sham and sham+PPG group as shown on Figure 2. As we know, the basal endogenous lung tissue H_2S production was in a low level (17.35 ± 1.76 μM) in sham group, because of a low level of the activity of CSE at this state (22). However, in shunt group, the endogenous lung tissue H_2S production was increased (37.56 ± 2.13 μM). It is likely that PPG plays a more obviously inhibitory role in endogenous lung tissue H_2S production in the shunt group where the basal H_2S level is relatively high compared with the sham group, which has a low basal H_2S level. Our study found that in shunted animals administrated PPG for 4 weeks, the H_2S content of lung tissue was significantly lower than in those shunted rats without PPG treatment.

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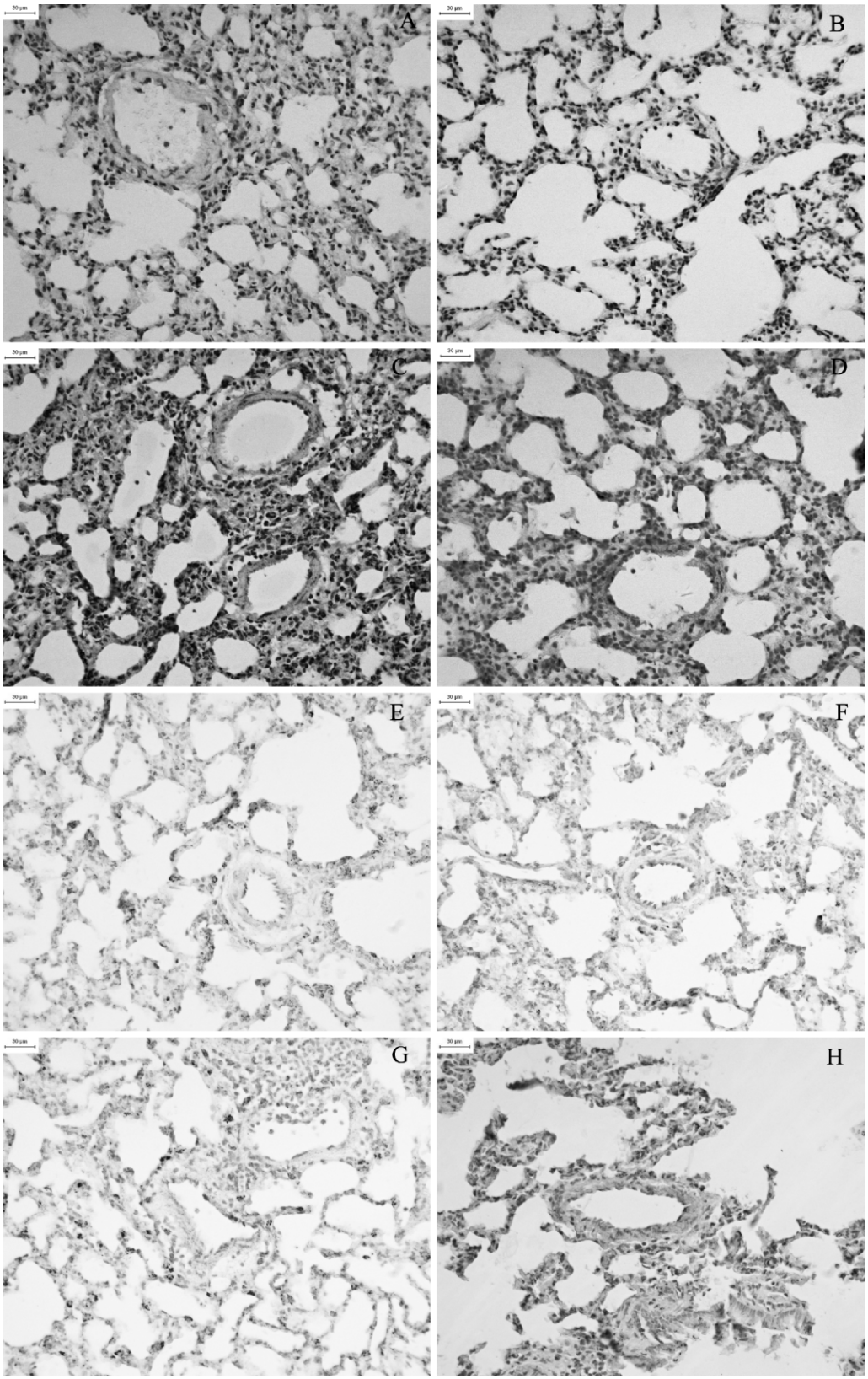

Figure 7. Immunohistochemical analysis of collagen I protein expression in rat pulmonary artery of different groups (DAB, ×200). A, sham, B, sham+PPG, C, shunt, D, shunt+PPG. Immunohistochemical analysis of collagen III protein expression in rat pulmonary artery of different groups (DAB, ×200). E, sham, F, sham+PPG, G, shunt, H, shunt+PPG.

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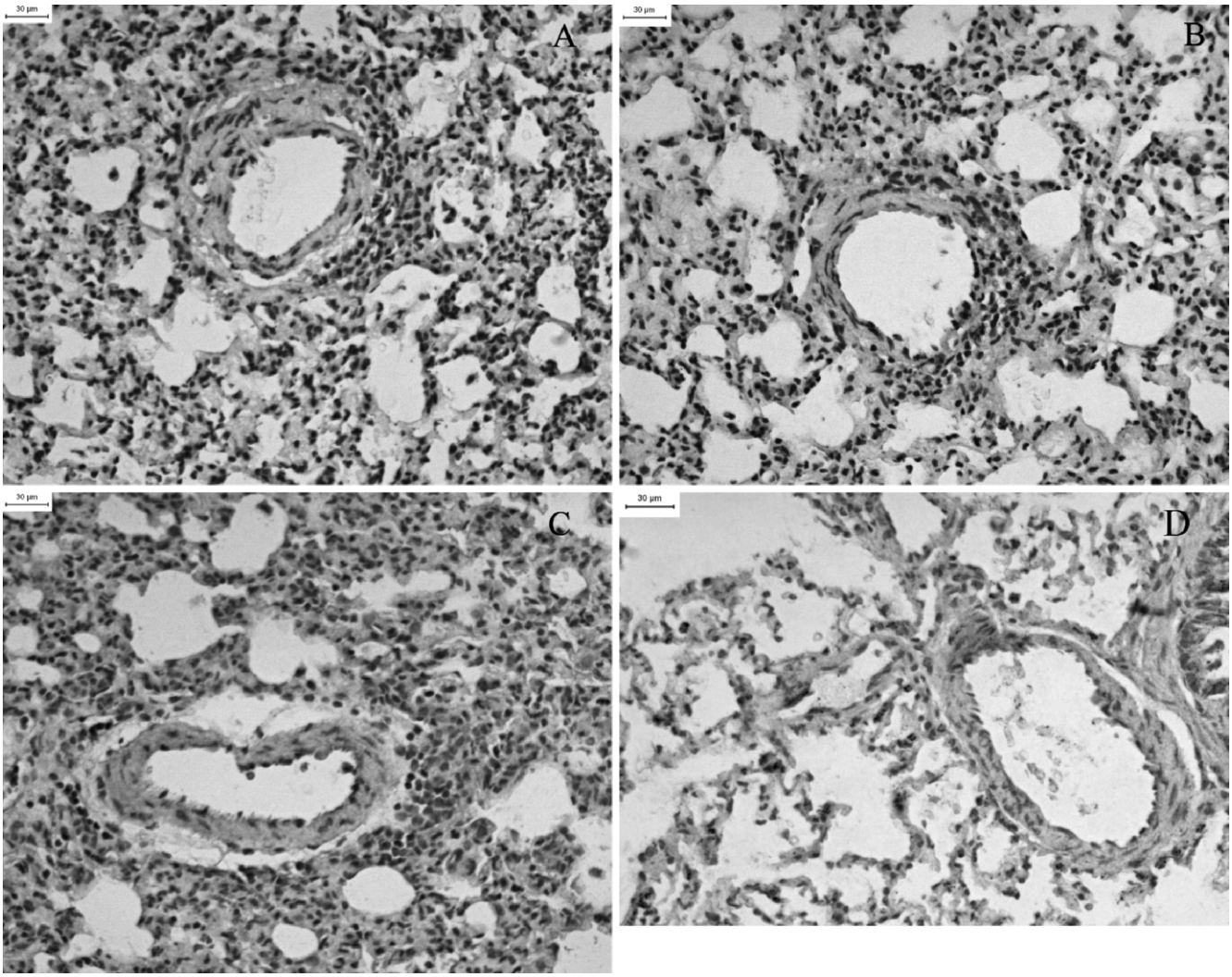

Figure 8. Immunohistochemical analysis of CTGF protein expression in rat pulmonary artery of different groups (DAB, ×200). A, sham. B, sham+PPG, C, shunt, D, shunt+PPG.

However, the pulmonary vascular structure remodeled to an even greater degree than in shunted animals without PPG, which suggests that endogenous H_2S possibly participates in pulmonary vascular structural remodeling induced by high pulmonary blood flow.

The present study aimed to explore the possible effect of endogenous H_2S on collagen remodeling of the pulmonary artery in rats with high pulmonary blood flow. Previous studies have shown that extracellular matrix contributes a great deal to pulmonary vascular structural remodeling. Collagen I and collagen III are the most abundant components of the extracellular matrix of the vascular wall. Changes in the absolute or relative contents of collagen I and collagen III would likely result in structural remodeling. Our previous studies showed that H_2S reduced the collagen remodeling of pulmonary artery under hypoxia (23). However, it is unclear whether H_2S exerts any regulatory effect on the collagen accumulation in pulmonary artery induced by high flow. Since collagen protein contains hydroxyproline up to 10–14% by weight, hydroxyproline concentration was measured as an index of collagen content in this experiment (24). It was found that hydroxyproline concentration of rat lung tissue and the expression of collagen I and collagen III increased in the shunted animals. Interestingly, these factors increased even more in the PPG treated-shunt group where the production of endogenous H_2S was inhibited. This suggests that endogenous H_2S probably plays an important role in the regulation of collagen remodeling induced by high pulmonary blood flow.

Matrix metalloproteinases (MMPs; also called collagenases or matrixins) are key enzymes in extracellular matrix degradation. MMPs involve a family of zinc-dependent endopeptidases. Collagenase MMP-13 degrades mainly fibrillar collagens, which include collagen I and collagen III in the vascular wall. MMP activity is regulated at different levels, including transcriptional control, extracellular activation of proenzymes, and active enzyme, tissue

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inhibitor of metalloproteinases (TIMPs). TIMPs bind to active and alternative sites of the activated MMP to inhibit the activity of MMP. The major member is TIMP-1, a 30-kDa glycoprotein that is synthesized by most cells (25). It was reported that an imbalance between gene expression of interstitial collagenases and gene expression of TIMP-1 contributed to extracellular matrix accumulation (26). Connective tissue growth factor (CTGF), a potent stimulator of collagen synthesis (27), consists of 349 amino acids with four distinct domains and induced by mechanical shear stress *in vitro* (28, 29). As mentioned above, the balance of MMP and TIMP-1 is involved in the regulation of collagen degradation and CTGF contributed to collagen synthesis. So, we included MMP, TIMP-1, CTGF as well as collagen in the present study to investigate the role of H_2S in the regulation of pulmonary collagen accumulation.

In the present study, it was found that the expression of MMP-13 and TIMP-1 is greater in the pulmonary artery after 4 weeks of left-to-right shunt in rat. When PPG was used to inhibit the production of endogenous H_2S in shunted animals, the expression of MMP-13 and TIMP-1 was significantly down-regulated, which suggests that endogenous H_2S probably reduced collagen accumulation through increasing its degradation in the shunted group. Our present study indicated that CTGF expression up-regulated in animals with 4 weeks of shunting. When PPG was used to inhibit the production of endogenous H_2S, CTGF up-regulation was further increased. These results suggest that endogenous H_2S regulates the expression of CTGF, which might be associated with the regulation of collagen remodeling in pulmonary artery induced by high pulmonary blood flow.

In conclusion, this study provides the first evidence that endogenous H_2S exerts regulatory effects on pulmonary artery remodeling induced by high pulmonary blood flow. Endogenous H_2S might participate in regulating collagen metabolism by affecting the degradation and synthesis of collagen. However, the molecular mechanisms by which H_2S regulates pulmonary artery structural remodeling will require further study.

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