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Impact of hydrogen sulfide on carbon monoxide/heme oxygenase pathway in the pathogenesis of hypoxic pulmonary hypertension

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Abstract

Hypoxic pulmonary hypertension (HPH) is an important pathophysiological process of a variety of cardiac and pulmonary diseases. But the mechanisms responsible for HPH are still not fully understood. The discoveries of endogenous gas signal molecules, nitric oxide (NO), and carbon monoxide (CO), have been moving the research of HPH to a new phase. Hydrogen sulfide (H_2S), which is now being considered as the third new gas transmitter, was found to be possibly involved in the pathogenesis of HPH. But whether there exists an interaction between H_2S and CO has not been clear in the pathogenesis of HPH. In this study, we found that H_2S was significantly decreased in the pathogenesis of HPH. However, plasma CO level and the expressions of heme oxygenase (HO-1) protein and HO-1 mRNA were significantly increased. Exogenous supply of H_2S could alleviate the elevation of pulmonary arterial pressure. At the same time, plasma CO level and the expressions of HO-1 protein and mRNA in pulmonary arteries were significantly increased. Whereas, exogenous supply of propargylglycine (PPG), an inhibitor of cystathionine γ-lyase (CSE), decreased the plasma H_2S content and worsened HPH. At the same time, plasma CO level and the expressions of HO-1 protein and mRNA in pulmonary arteries were decreased. The results showed that H_2S could play a regulatory role in the pathogenesis of HPH through up-regulating CO/HO pathway.

Keywords: Hydrogen sulfide; Carbon monoxide; Heme oxygenase; Hypoxia; Hypertension; Pulmonary

Hypoxic pulmonary hypertension (HPH), an important pathophysiological process in the development of a variety of clinical cardiac and pulmonary diseases, has critical influence on the progress and prognosis of the disease. But the mechanisms responsible for HPH are still not fully understood [1]. The discoveries of endogenous gas signal molecules, nitric oxide (NO), and carbon monoxide (CO), have been moving the research of HPH to a very new phase [2,3]. NO is endogenously produced by NO synthase via metabolism of L-arginine and exerts potent pulmonary vasodilation and the inhibition of smooth muscle cell (SMC) proliferation and therefore may regulate vascular remodeling [4,5]. CO, which is also endogenously produced by heme oxygenase via the metabolism of heme to biliverdin, has been found as another gaseous messenger in smooth muscles [6]. Our foregoing experiments indicated that the inhibitor of heme oxygenase-1 (HO-1) could worsen hypoxic pulmonary hypertension and pulmonary vascular structural remodeling, suggesting a significant regulating role of CO in the pathogenesis of hypoxic pulmonary hypertension [3]. NO and CO are simple gases and easy to pass through biological membrane. They are characterized by continuous generation, fast transmission, and extensive action. Therefore, they are of greater special significance in the regulation of pulmonary circulation than that of other organs. Hydrogen sulfide (H_2S) which has been known as a toxic gas for a long time can also be endogenously generated, and it was found to have important

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physiological functions including relaxing aortic arteries of rats in vitro [7–9], Recently, we found H_2S could dose-dependently suppress the proliferation of smooth muscle cells through the mitogen-activated protein kinase pathway [10]. It is now being considered as the third gaseous transmitter [11]. Published data showed that the endogenous production of H_2S from rat aortic tissues was enhanced by NO donor treatment. NO donor also enhanced the expression level of cystathionine γ-lyase (CSE) in cultured vascular SMCs, which indicated that NO could play a regulatory role in H_2S/CSE pathway in vascular system [12]. NO, CO, and H_2S were considered as gasotransmitters, therefore we postulated that NO, CO, and H_2S might possibly interact under physiological and pathological condition, and they might constitute a regulatory network in vascular system. In our recent studies, we also found that endogenous H_2S system was also involved in pathogenesis of HPH [13]. Is there an interaction between H_2S and CO in the pathogenesis of HPH? Would H_2S impact CO/HO pathway in vascular tissue? And what is the mechanism by which H_2S would possibly alleviate HPH? The present study was, therefore, to investigate the effect of H_2S on CO/HO pathway in the development of HPH.

Materials and methods

Hypoxic pulmonary hypertension rat model. The study was approved by the Animal Research Committee of the First Hospital, Peking University. Male Wistar rats weighing from 150 to 180 g were provided by the Animal Department, Health Science Center of Peking University. Twenty-seven rats were randomly divided into four groups: control group ($n = 7$), hypoxia group ($n = 7$), hypoxia + NaHS group ($n = 7$), and hypoxia + propargylglycine (PPG) group ($n = 6$). For hypoxic challenge, hypoxic rats were exposed to normobaric hypoxic chamber with an oxygen concentration of $10.0 \pm 0.5\%$ for 3 weeks and 6 h everyday. Rats in control group were housed in identical cages adjacent to the hypoxic chamber breathing room air. Hypoxia was generated by infusing nitrogen into the chamber. The degree of hypoxia was maintained by the balance between nitrogen infusing and inward leak of air through holes in the chamber [2]. For rats in hypoxia + NaHS group, NaHS dissolved in saline at a dosage of 14 μmol/kg body weight was injected intraperitoneally before hypoxia everyday [13]. For rats in hypoxia + PPG group, PPG was administered intraperitoneally at a dose of 30 mg/kg before hypoxia everyday [14]. An equal volume of normal saline was injected intraperitoneally into rats in the control and hypoxia groups.

Measurement of homodynamic parameters and sample preparation. Three weeks after hypoxic exposure, the rats were anesthetized with urethane (1 g/kg) intraperitoneally. Mean pulmonary artery pressure of each rat was evaluated using a right cardiac catheterization procedure. One lobe was cut out, fixed in 10% formation, and routinely processed into 5 μm paraffin sections for using in immunohistochemistry and in situ hybridization studies. The blood plasma was also prepared for the measurement of plasma H_2S and CO concentrations.

Measurement of H_2S concentration in plasma. In a test tube containing 0.5 ml of 1% zinc acetate and 2.5 ml distilled water, 0.1 ml of plasma was added. Then 0.5 ml of 20 mM *N*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl and 0.4 ml of 30 mM $FeCl_3$ in 1.2 M HCl were also added to the test tube for 20 min of incubation at room temperature. The protein in plasma was removed by adding 1 ml of 10% trichloroacetic acid to solution and centrifuged. The optical absorbance of the resulting solution at 670 nm was measured with a spectrometer (Shimadzu UV 2100, Japan). H_2S concentration in solution was calculated against the calibration curve of the standard H_2S solution [13].

Measurement of CO concentration in plasma. According to Chalmers' method [15], 0.2 ml of plasma was mixed with 1 ml of the hemoglobin solution, which derived from a mixture of 0.25 ml of fresh-packed erythrocytes from rats and 50 ml of 0.25 mol/L ammonia solution. Then 0.1 ml of sodium dithionite was added. The absorbance of the test plasma sample and water blank at 541 and 555 nm was read and the ratio (R) of absorbance at the 541–555 nm readings was calculated. Then the %HbCO from a standard curve derived by mixing different proportions of two Hb solutions containing 100% HbCO and 100% HbO_2 was derived. Then plasma CO concentration was calculated according to the formula as follows: CO (μmol/L) = %HbCO × Hb (mg/L) × 4000/(64,456 × 100 × 0.2).

Immunohistochemistry. The paraffin sections for immunohistochemistry were dewaxed and hydrated, and then processed by 3% H_2O_2 for 15 min, followed by antigen repairing for 10 min (microwave heating method). The slides were washed twice; each for 5 min. HO-1 antibody (Santa Cruz, Canada) was then added at 37 °C for 1 h. The biotinylated anti-rabbit IgG at 37 °C was incubated for 1 h. Horseradish peroxidase streptavidin was added at 37 °C for 1 h. Then DAB was added for 1–10 min and Mayer's hematoxylin for 1 min. The expression of HO-1 by smooth muscle cells in large (exterior diameter of intrapulmonary artery >150 μm), median (exterior diameter of intrapulmonary artery 50–150 μm), and small (exterior diameter of intrapulmonary artery <50 μm) intrapulmonary arteries was observed under microscope. The yellow-brown cytoplasm represented positive signals of HO-1 expression. The mean value of percentage of pulmonary artery smooth muscle cells expressing different abundances of HO-1 (0%, ~50%, and ~100%) was determined [3].

In situ hybridization study. An in situ hybridization method was used to detect HO-1 mRNA by using a commercially available HO-1 mRNA ISH detection kit. Dig-labeled probe to HO-1 (oligo probe) was obtained from TBD Science Technology. The sequences of HO-1 are

(1) 5′-AGAAT GCTGA GTTCA TGAGG AACTT TCAGA-3′
(2) 5′-GCTGC TGGTG GCCCA CGCCT ACACC CGCTA-3′
(3) 5′-TTCCT GCTCA ACATC CAGCT CTTTG AGGAG-3′

Samples of the rat lungs were excised and immediately fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Lung tissues were fixed for at least 24 h and processed for paraffin embedding. Sections (8 μm) were cut and mounted on chrome alum-coated slides. Sections were deparaffinized in toluene, dehydrated in a graded series of ethanol, and washed in 0.01 M PBS. Complex digestive juice was added and digested for 20 min at room temperature. Slides were prehybridized for 2 h at 37 °C. Then hybridization was carried out for 1 h at 37 °C. After hybridization, the slides were washed with 2× SSC for twice, each for 5 min. The positive expression was visualized by incubating sections with DAB for 5–10 min. Sections were then counterstained with hematoxylin, dehydrated in ethanol, mounted with paramount, coverslipped, and examined under a light microscope. The presence of HO-1 mRNA was indicated by the development of a brown color within the cytoplasm. The mean value of percentage of pulmonary arterial smooth muscle cells expressing different abundance of HO-1 mRNA (0%, ~50%, and ~100%) was determined.

Statistical analysis. Results were expressed as means ± SD. The comparison among groups was done using one-way ANOVA followed by Student–Newman–Keuls tests. A value of $P < 0.05$ was considered statistically significant. These data were statistically analyzed by using SPSS 10.0.

Results and discussion

Regulation of pulmonary vascular tone and homeostasis involves a number of vasoconstrictors, relaxing

agents, and growth factors that are released from different cell types in the vasculature and interact through feedback loops. Hypoxia is known to regulate these cellular interactions mainly by modifying gene expression [16]. Previous studies including ours have showed that gaseous transmitters, nitric oxide (NO), and carbon monoxide (CO), played important roles in the pathogenesis of HPH [2,3]. Nitric oxide, identified as an endothelium-derived relaxing factor in 1987, is produced in pulmonary endothelial cells from the amino acid L-arginine and molecular oxygen by endothelial nitric oxide synthase (eNOS) [4]. Nitric oxide diffuses from the endothelium to adjacent smooth muscle cells and exerts potent pulmonary vasodilator effect through the elevation of cyclic guanosine monophosphate by the activation of soluble guanylate cyclase. Aside from a direct vasodilator effect, NO is also involved in the inhibition of smooth muscle cell proliferation and therefore may regulate vascular remodeling [5]. CO, which is also endogenously produced by heme oxygenase via the metabolism of heme to biliverdin, has been found as another gaseous messenger in smooth muscle [6]. We found that the up-regulation of CO/HO played a protective role in the development of hypoxic pulmonary vascular structural remodeling, and exogenous supply of CO could attenuate HPH [3]. However, the mechanisms responsive for vascular pathologic changes have not been fully understood. Gaseous molecules, such as NO and CO, are simple gases and easy to pass through biological membrane, and are characterized by continuous generation, fast transmission, and extensive action. Therefore, they are of greater special significance in the regulation of pulmonary circulation than other organs. Hydrogen sulfide (H_2S) has been best known as a toxic gas. In recent studies, however it was found that H_2S had similar characteristics with NO and CO, and it was considered as the third gas transmitter. Endogenous H_2S can be formed from cysteine by pyridoxal-5′-phosphate-dependent enzymes, including cystathionine β-synthases (CBS) and cystathionine γ-lyase (CSE) [11]. In 1997, Hosoki et al. [9] found that H_2S could relax vascular smooth muscle and decrease mean blood pressure. In 2001, Zhao et al. [12] also found that H_2S could act as a novel endogenous gaseous K_{ATP} channel opener to take vasore-

Table 1
H_2S regulated pulmonary hypertension and endogenous CO/HO-1 system (mean ± SD)

Groups	Rats (n)	mPAP (mm Hg)	Plasma level of H_2S (μmol/L)	Plasma level of CO (μmol/L)	HO-1 protein expression in smooth muscle cells		
					Large PAs	Median PAs	Small PAs
Hypoxia	7	20.49 ± 2.85^{a}	195.54 ± 21.56^{a}	0.35 ± 0.02^{a}	0.79 ± 0.08^{a}	0.77 ± 0.08^{a}	0.76 ± 0.09^{a}
Hypoxia + NaHS	7	$14.43 \pm 5.59^{a,b}$	$323.96 \pm 32.55^{a,b}$	$0.39 \pm 0.03^{a,b}$	$0.88 \pm 0.04^{a,b}$	$0.89 \pm 0.05^{a,b}$	$0.89 \pm 0.06^{a,b}$
Hypoxia + PPG	6	$25.78 \pm 3.13^{a,b}$	$141.80 \pm 45.25^{a,b}$	$0.27 \pm 0.03^{a,b}$	$0.54 \pm 0.06^{a,b}$	$0.56 \pm 0.04^{a,b}$	$0.39 \pm 0.06^{a,b}$
Control	7	14.78 ± 3.61	293.75 ± 25.52	0.31 ± 0.02	0.66 ± 0.08	0.64 ± 0.05	0.54 ± 0.05

mPAP, mean pulmonary arterial pressure; PPG, propargylglycine; and PAs, pulmonary arteries.

Compared with rats in control group, mPAP was significantly increased in hypoxia rats ($p < 0.05$). However, the plasma of H_2S was decreased significantly. The plasma CO and the expression of HO-1 protein in pulmonary arteries of hypoxia group were significantly increased compared to control rats ($p < 0.05$, respectively). Compared with hypoxia group, the plasma H_2S of rats in hypoxia + NaHS group was significantly increased, and mPAP was decreased significantly ($p < 0.05$, respectively). At the same time, compared with control group and hypoxia group, the plasma concentration of CO and the expression of HO-1 protein in pulmonary arteries in hypoxia + NaHS group were significantly increased. However, compared with hypoxia group, the plasma concentration of H_2S was decreased significantly in hypoxia + PPG group ($p < 0.05$) and mPAP was increased significantly ($p < 0.05$). In accordance with a decrease of H_2S in hypoxia + PPG group, the plasma concentration of CO and the expressions of HO-1 protein in pulmonary arteries were significantly decreased ($p < 0.05$, individually) compared to control group.

[a] vs Control group, $p < 0.05$.

[b] vs Hypoxia group, $p < 0.05$.

Table 2
The effect of H_2S on the expression of HO-1 mRNA in smooth muscle cells of hypoxia rat

Group	Rats (n)	Large PAs	Median PAs	Small PAs
Hypoxia	7	0.81 ± 0.05^{a}	0.83 ± 0.04^{a}	0.82 ± 0.03^{a}
Hypoxia + NaHS	7	$0.91 \pm 0.02^{a,b}$	$0.92 \pm 0.02^{a,b}$	$0.93 \pm 0.01^{a,b}$
Hypoxia + PPG	6	$0.42 \pm 0.04^{a,b}$	$0.42 \pm 0.09^{a,b}$	$0.38 \pm 0.05^{a,b}$
Control	7	0.57 ± 0.15	0.53 ± 0.13	0.47 ± 0.10

PPG, propargylglycine and PAs, pulmonary arteries.

Compared with rats in control group, the expression of HO-1 mRNA in pulmonary arteries of hypoxia group was significantly increased ($p < 0.05$). Compared with control group and hypoxia group, the expression of HO-1 mRNA in pulmonary arteries was also significantly increased in hypoxia + NaHS group ($p < 0.05$). However, compared with control group and hypoxia group, the expression of HO-1 mRNA in pulmonary arteries was significantly decreased in hypoxia + PPG group ($p < 0.05$).

[a] vs Control group, $p < 0.05$.

[b] vs Hypoxia group, $p < 0.05$.

laxant effect. And recently we found H_2S could dose-dependently suppress the proliferation of smooth muscle cells through the mitogen-activated protein kinase pathway [7]. We also found that reduced expression and activity of CSE coupled with a decrease in plasma H_2S concentration in lung tissue of rats were associated with the development of experimentally induced HPH and exogenous supply of H_2S could attenuate HPH. It was indicated that H_2S system was involved and exerted beneficial effect on the pathogenesis of HPH [13]. All the above studies discovered that CO and H_2S might play a modulatory role in pathogenesis of HPH. However, published data have shown that the endogenous production of H_2S from rat aortic tissues could be enhanced by NO donor treatment. The NO donor also enhanced the expression level of cystathionine γ-lyase (CSE) in cultured vascular SMCs, which indicated that NO could play a regulatory role in H_2S/CSE pathway in vascular

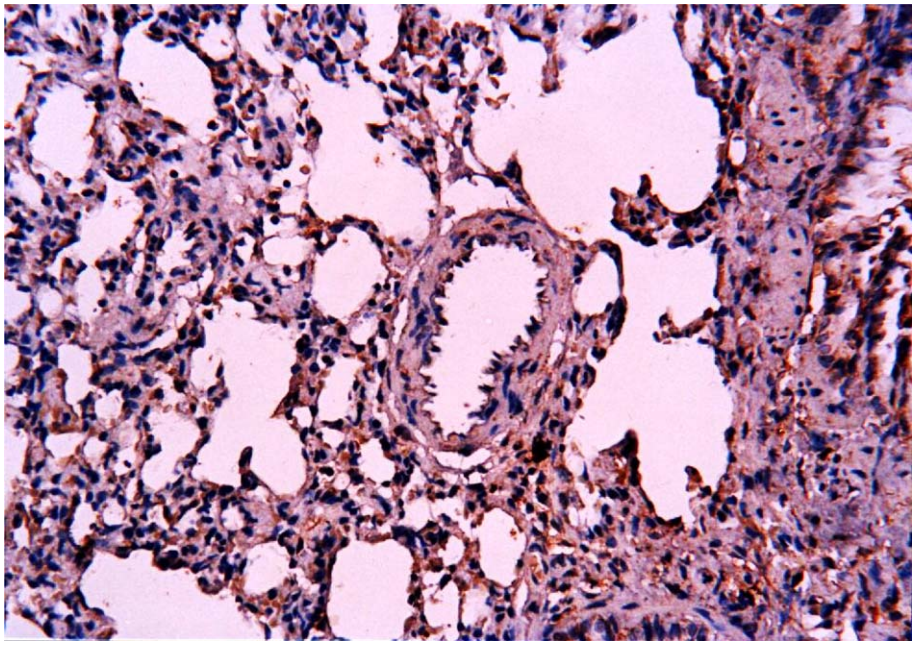

Fig. 1. In control group, HO-I protein expression in smooth muscle cells of small pulmonary arteries. Magnification, × 400.

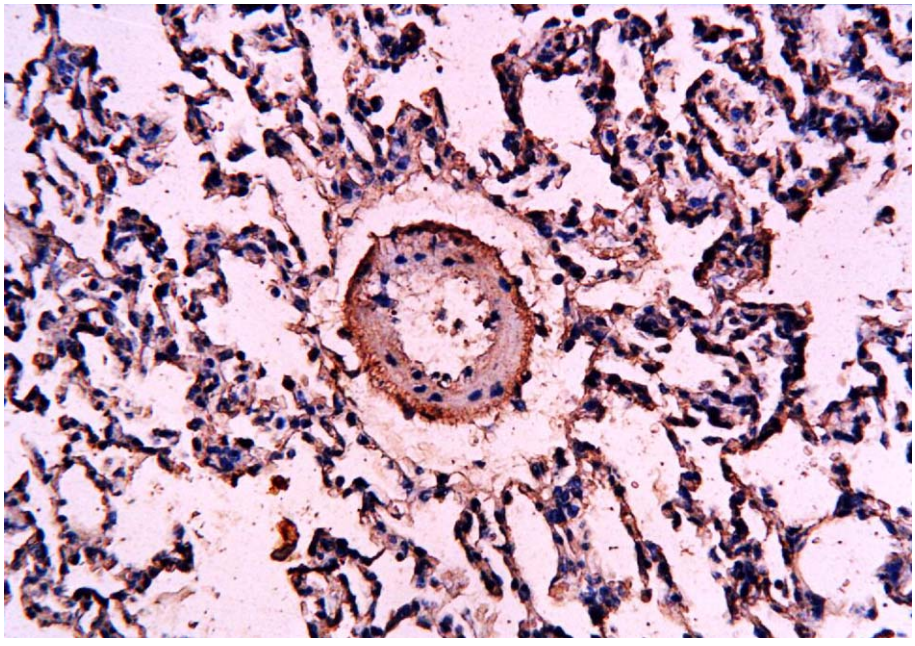

Fig. 2. In hypoxia group, HO-I protein expression in smooth muscle cells of small pulmonary arteries. Magnification, × 400.

system [9]. Since NO, CO, and H_2S were considered as gaseous molecules, we postulated that NO, CO, and H_2S can interact under physiological and pathological condition, and they could possibly constitute a regulatory network in vascular system. However, how H_2S impacts on CO/HO pathway is still unknown in the pathogenesis of HPH. To clarify the interaction between such gaseous molecules is, therefore, very significant for deepening the mechanism by which H_2S regulated HPH.

The result of the present experiment showed that after intermittent exposure to 10% oxygen for 6 h daily for 3 weeks, the rats developed constant chronic pulmonary hypertension. Mean pulmonary artery pressure increased significantly compared with control rats (20.49 ± 2.85 vs 14.78 ± 3.61 mm Hg, $p < 0.05$). Meanwhile, the plasma concentration of H_2S of hypoxia group was significantly decreased compared with control rats (195.54 ± 21.56 vs 293.75 ± 25.52 μmol/L,

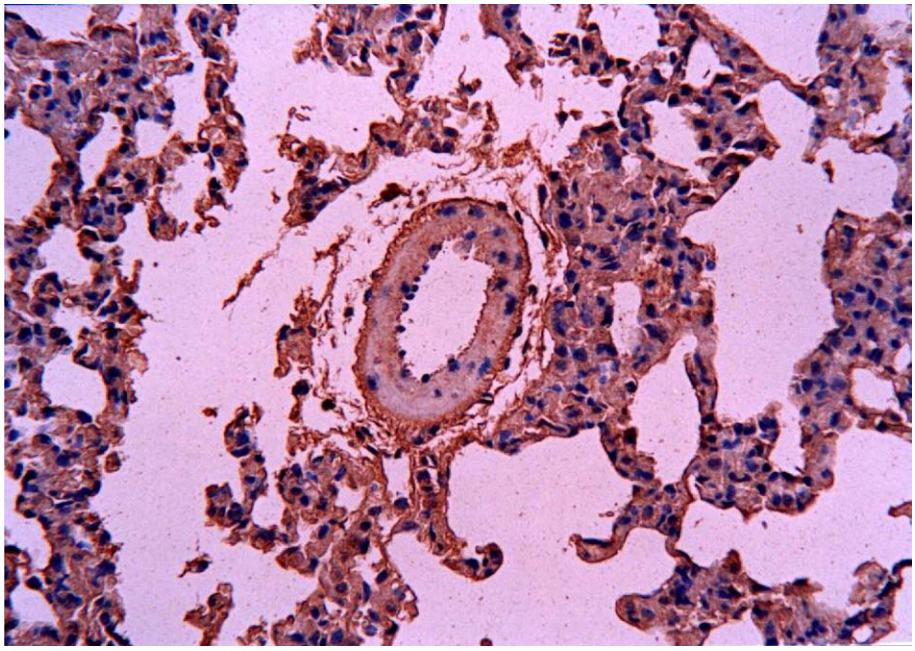

Fig. 3. In hypoxia + NaHS group, HO-I protein expression in smooth muscle cells of small pulmonary arteries. Magnification, × 400.

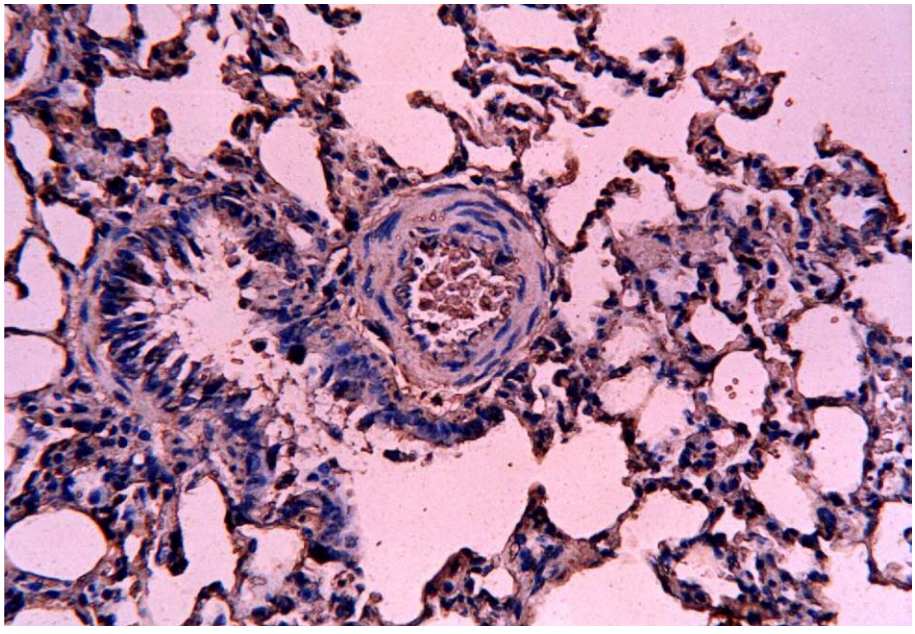

Fig. 4. In hypoxia + PPG group, HO-I protein expression in smooth muscle cells of small pulmonary arteries. Magnification, × 400.

$p < 0.05$). However, the plasma CO level and the expressions of HO-1 protein and mRNA in pulmonary arteries of hypoxia rats were significantly increased compared to control rats ($p < 0.05$, respectively). These results were consistent with our previous study [3–13]. When the hypoxia rats were given NaHS, a donor of H_2S, the mean pulmonary artery pressure was significantly decreased compared with hypoxia rats (14.43 ± 5.59 vs 20.49 ± 2.85, $p < 0.05$). At the same time, the plasma CO level and the expressions of HO-1 protein and mRNA in pulmonary arteries of hypoxia + NaHS group were significantly increased compared to hypoxia rats ($p < 0.05$, respectively). However, when the hypoxia rats were given PPG, an inhibitor of CSE, the mean pulmonary artery pressure was significantly increased compared with hypoxia rats (25.78 ± 3.13 vs 20.49 ± 2.85, $p < 0.05$).

It is very interesting in accordance with a decrease in the plasma level of H_2S in hypoxia + PPG group, the plasma CO level and the expressions of HO-1 protein

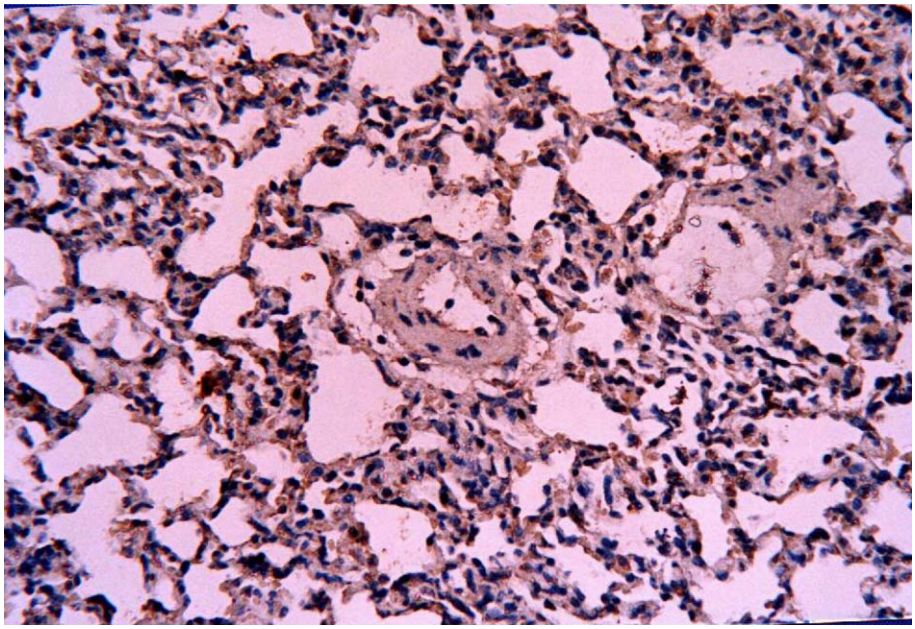

Fig. 5. In control group, HO-I mRNA expression in smooth muscle cells of small pulmonary arteries. Magnification, × 400.

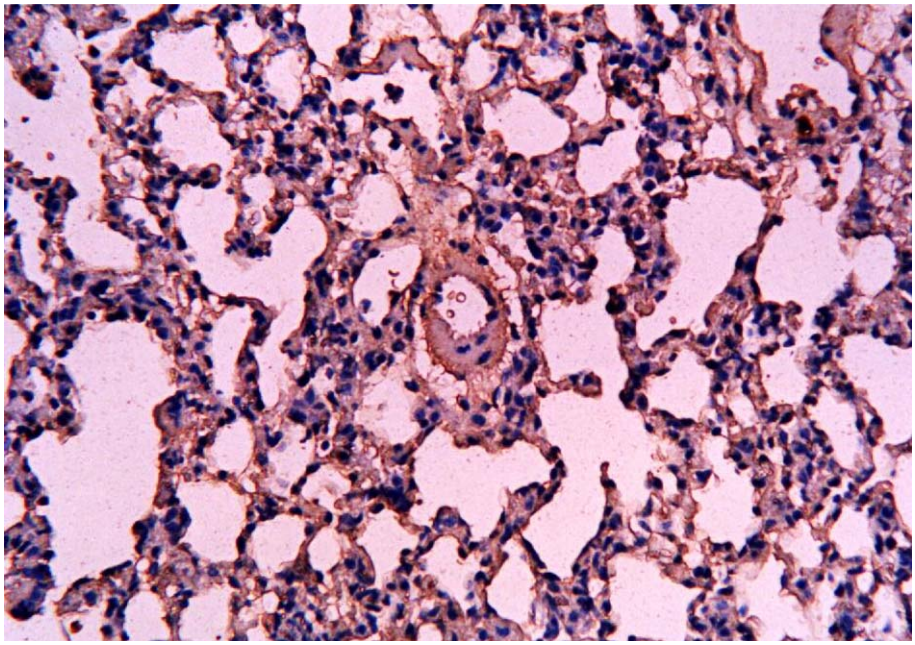

Fig. 6. In hypoxia group, HO-I mRNA expression in smooth muscle cells of small pulmonary arteries. Magnification, × 400.

and mRNA in pulmonary arteries of hypoxia + PPG group were significantly decreased compared to hypoxic and control rats ($p < 0.05$, respectively). The above results suggested that H_2S could up-regulate CO/HO system during the development of HPH. Several lines of investigation provided evidence that CO might be a physiological regulator of cellular interactions in the vasculature, acting as a direct and indirect vasodilator. Directly, CO acts via activation of soluble guanylate cyclase and elevation of cGMP, as in rat aortic and coronary vascular SMC preparations [17,18] as well as in dog femoral, carotid, and coronary arteries. Indirectly, SMC-derived CO may cause SMC relaxation by inhibiting the hypoxic induction of vasoconstrictors, endothelin-1 (ET-1), and platelet-derived growth factor-B (PDGF-B) in adjacent endothelial cells [19]. In addition to its vasodilatory actions, CO was shown to inhibit SMC proliferation by regulating cell cycle-specific

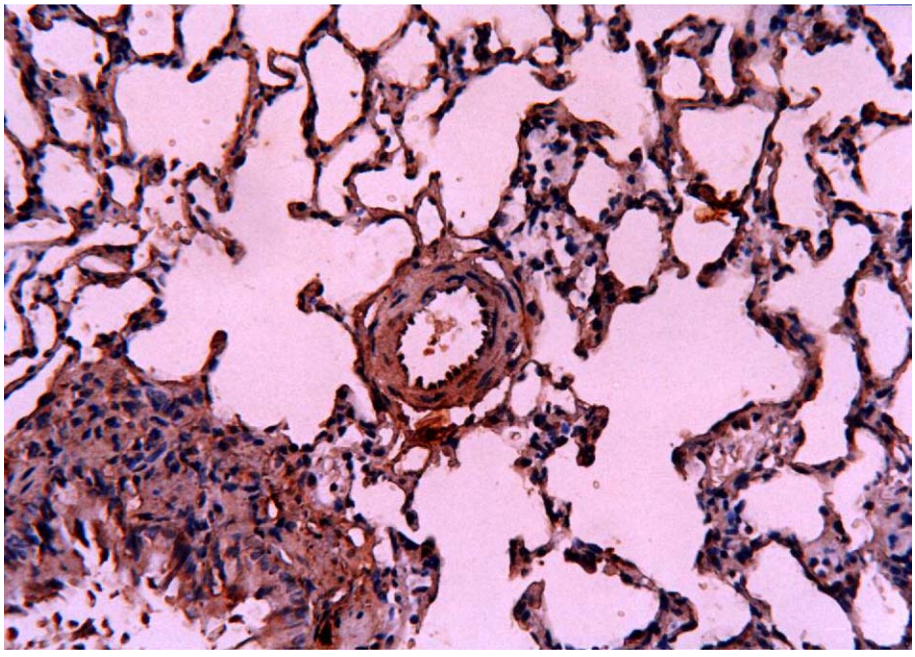

Fig. 7. In hypoxia + NaHS group, HO-I mRNA expression in smooth muscle cells of small pulmonary arteries. Magnification, × 400.

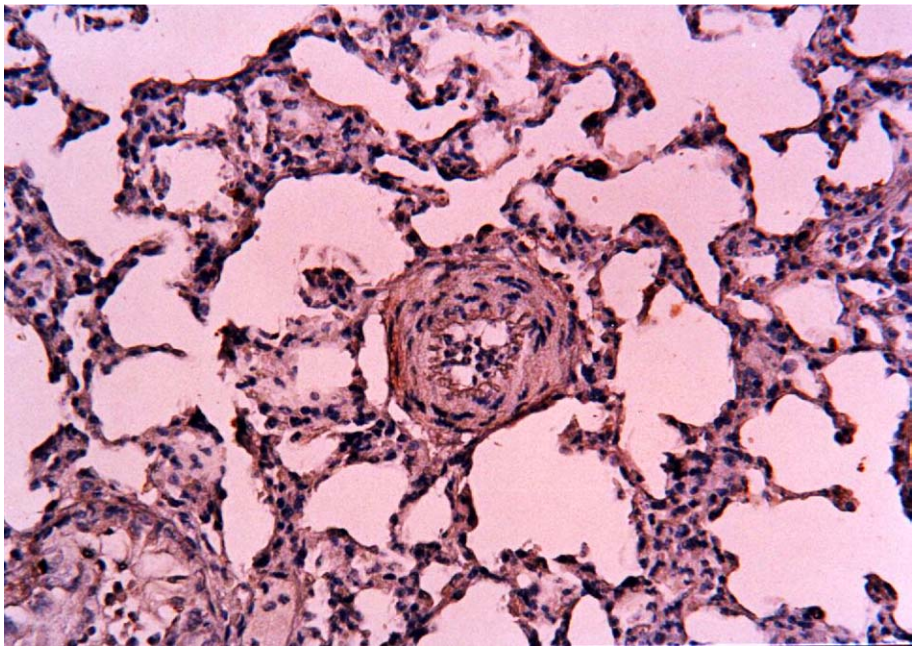

Fig. 8. In hypoxia + PPG group, HO-I mRNA expression in smooth muscle cells of small pulmonary arteries. Magnification, × 400.

transcription factor E2F-1 [20], as well as the expression of the mitogens ET-1 and PDGF-BB in culture [19]. The results of the present experiment indicated that H_2S could play a regulatory role in the pathogenesis of HPH through up-regulating CO/HO pathway (see Tables 1 and 2 and Figs. 1–8).

The mechanisms by which H_2S regulates CO/HO system in pulmonary artery smooth muscle cells are unclear. According to previous studies, H_2S in vivo is metabolized by oxidation in mitochondria or by methylation in cytosol. H_2S can be scavenged by methemoglobin [21], or metallo- or disulfide-containing molecules such as oxidized glutathione [22]. Hemoglobin may be the common "sink" for CO in forming carboxyhemoglobin [23], for H_2S in forming green sulhemoglobin. We speculated that if this sink is filled with increased H_2S, binding of CO would be affected, the release of CO from the sink would increase. In our study, we first found that H_2S could increase the expression of HO-1 gene in pulmonary vascular smooth muscles under hypoxic condition, then the expression of HO-1 protein was also increased. Whether there were other mechanisms involved in the process needs further studies.

Acknowledgments

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