H_2S inhibition of chemical hypoxia-induced proliferation of HPASMCs is mediated by the upregulation of COX-2/PGI_2

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Abstract. The hypoxia-induced proliferation of pulmonary artery smooth muscle cells (PASMCs) is the main cause of pulmonary arterial hypertension (PAH), in which oxidative stress, cyclooxygenase (COX)-2 and hydrogen sulfide (H_2S) all play an important role. In the present study, we aimed to examine the effects of H_2S on the hypoxia-induced proliferation of human PASMCs (HPASMCs) and to elucidate the underlying mechanisms. The HPASMCs were treated with cobalt chloride ($CoCl_2$), a hypoxia-mimicking agent, to establish a cellular model of hypoxic PAH. Prior to treatment with $CoCl_2$, the cells were pre-conditioned with sodium hydrosulfide (NaHS), a donor of H_2S. Cell proliferation, reactive oxygen species (ROS) production, COX-2 expression, prostacyclin (also known as prostaglandin I2 or PGI_2) secretion and H_2S levels were detected in the cells. The exposure of the HPASMCs to $CoCl_2$ markedly increased cell proliferation, accompanied by a decrease in COX-2 expression, PGI_2 secretion and H_2S levels; however, the levels of ROS were not altered. Although the exogenous ROS donor, H_2O_2, triggered similar degrees of proliferation to $CoCl_2$, the ROS scavenger, N-acetyl-L-cysteine (NAC), markedly abolished the H_2O_2-induced cell proliferation, as opposed to the $CoCl_2$-induced proliferation. The $CoCl_2$-induced proliferation of HPASMCs was suppressed by exogenously applied PGI_2. The addition of H_2S (NaHS) attenuated the $CoCl_2$-induced cell proliferation through the increase in the intercellular content of H_2S. Importantly, the exposure of the cells to H_2S suppressed the $CoCl_2$-induced downregulation in COX-2 expression and PGI_2 secretion from the HPASMCs. In conclusion, the results from the current study suggest that H_2S inhibits hypoxia-induced cell proliferation through the upregulation of COX-2/PGI_2, as opposed to ROS.

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Introduction

Pulmonary arterial hypertension (PAH) is a severe and frequently fatal disease. The hallmark of PAH is the development of gradually increased pulmonary vascular resistance, which eventually enhances the afterload of the right ventricle and leads to right heart failure (1). The etiopathogenesis of PAH is commonly associated with chronic hypoxemia in disorders, such as chronic obstructive pulmonary disease and interstitial lung disease (2). Increasing evidence indicates that apart from vasoconstriction, smooth muscle cell proliferation and hypertrophy, usually leading to pulmonary vascular remodeling and increased resistance, play a crucial role in the development of PAH (3). Therefore, it would be of great importance to elucidate the molecular mechanisms underlying hypoxia-induced smooth muscle cell proliferation, which may in turn lead to the discovery of novel therapeutic agents/targets.

Oxidative stress, characterized by increased reactive oxygen species (ROS) production, is involved in cell proliferation induced by a number of stimuli in a variety of cell models (4-7). Reportedly, in human pulmonary artery smooth muscle cells (HPASMCs), NADPH oxidase (NOX)4 mediates cell proliferation triggered by transforming growth factor-β1 (TGF-β1) (3). The inhibition of oxidative stress with nitrite or superoxide dismutase (SOD) has been shown to ameliorate PAH (8,9). Of note, prostacyclin (also known as prostaglandin I2 or PGI_2), an effective but expensive clinical drug, has been used for the treatment of PAH via vasodilatation (10). The endogenous

formation of PGI_2 is mainly attributed to normal cyclooxygenase-2 (COX-2) expression; however, COX-2 expression is reportedly downregulated in hypoxia-induced PAH (2).

Hydrogen sulfide (H_2S) has been recognized as an important cellular signaling molecule, alongside nitric oxide (NO) and carbon monoxide (CO), playing a number of physiological and pathological roles in mammals (11-13). H_2S has been shown to exert various protective effects on the cardiovascular system, including myocardial preservation, the improvement of endothelial function, inhibition of proliferation and/or induction of the apoptosis of smooth muscle cells (14). Many of these effects elicited by H_2S are mediated by the upregulation of COX-2 (15). However, plasma H_2S levels in Wistar rats have been shown to be reduced in hypoxia-induced pulmonary vascular structural remodeling (16). We therefore hypothesized that H_2S can abolish the hypoxia-induced proliferation of pulmonary artery smooth muscle cells (PASMCs) and may thus consequently ameliorate PAH through the upregulation of COX-2/PGI_2.

To confirm our hypothesis, in the present study, we carried out the following experiments: The chemical hypoxia agent, cobalt chloride ($CoCl_2$), was employed to treat HPASMCs in order to establish a cellular model of hypoxic PAH. Hypoxia-induced changes, such as oxidative stress, a decrease in COX-2/PGI_2 expression, as well as endogenous H_2S levels were observed. In addition, we aimed to determined whether the exogenous administration of H_2S in the form of sodium hydrosulfide (NaHS) affects the chemical hypoxia-induced proliferation of HPASMCs, as well as to elucidate the mechanisms involved.

Materials and methods

Materials. $CoCl_2$, NaHS, N-acetyl-L-cysteine (NAC), PGI_2 and 2',7'-dichlorofluorescein-diacetate (DCFH-DA) were all purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kyushu, Japan). Specific monoclonal antibodies against COX-2, hypoxia-inducible factor-1α (HIF-1α) or β-actin were obtained from Epitomics, an Abcam Company (San Francisco, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) high glucose medium and fetal bovine serum (FBS) were both supplied by Gibco-BRL (Grand Island, NY, USA).

Cell culture. HPASMCs, derived from human pulmonary arterial tissue, were supplied by ScienCell Research Laboratories (Carlsbad, CA, USA), and maintained in DMEM high glucose medium supplemented with 15% FBS at 37˚C under an atmosphere of 5% CO_2 and 95% air. The cells were passaged approximately every 2 days.

Determination of cell proliferation. Cell viability was assessed according to the instructions provided with the CCK-8. A viability >100% indicated cell proliferation, whereas a viability of <100% indicated cell damage, as previously described (17). The HPASMCs were plated in 96-well plates at a density of 5,000 cells/well. When the cells were grown to approximately 60-70% confluence, the indicated treatments were applied. CCK-8 solution (10 μl) at a 1:10 dilution with FBS-free DMEM high glucose medium (100 μl) was added to each well followed by another 3 h of incubation at 37˚C. The absorbance (A) was measured at 450 nm on a microplate reader (Molecular Devices, LLC, Silicon Valley, CA USA). The experiments were performed 4 times, as previously described (18).

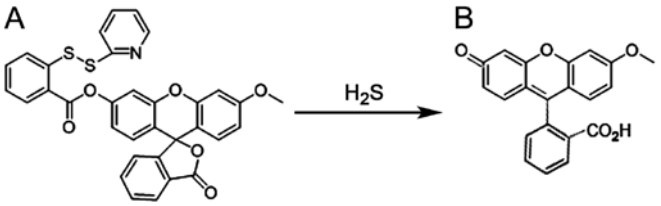

Figure 1. Hydrogen sulfide (H_2S) reacts with its probe. (A) WSP-1 is a synthesized H_2S probe. The probe is not fluorescent under normal conditions. (B) When it reacts with H_2S, the probe turns into a fluorescent compound.

Observation of intracellular ROS content. Intracellular ROS was determined by the oxidative conversion of cell permeable DCFH-DA to fluorescent 2',7'-dichlorofluorescein (DCF) (18). At the end of the indicated treatments, the HPASMCs were washed and incubated with 10 μmol/l DCFH-DA solution at 37˚C for 20 min in the dark. Intercellular DCF fluorescence was observed over the entire field of vision using a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus, Tokyo, Japan). The mean fluorescence intensity (MFI) of DCF from 4 random fields was analyzed using ImageJ 1.47 software.

Measurement of intracellular H_2S content. Intracellular free H_2S levels were determined using the H_2S fluorescent probe (WSP-1; kindly provided by Professor Ming Xian at the Department of Chemistry, Washington State University, Pullman, WA, USA), as previously described (19,20) and the chemical equation is presented in Fig. 1. After the indicated treatments, the HPASMCs were washed with phosphate-buffered saline (PBS) twice and incubated with 100 μmol/l WSP-1 combined with the surfactant, cetyltrimethylammonium bromide (CTAB; 50 μmol/l), at 37˚C for 20 min in the dark. H_2S-derived fluorescence was measured over the entire field of vision under a fluorescent microscope connected to an imaging system (BX50-FLA; Olympus). The MFI of 4 random fields was analyzed using ImageJ 1.47 software.

Western blot analysis of protein expression. Following heat-induced denaturation at 100˚C for 5 min, equal amounts of protein from the indicated groups were loaded. The total proteins were separated in 12% SDS-PAGE by electrophoresis, and then transferred into PVDF membranes. After blocking with 5% BSA in TBS-T, the membranes were incubated with primary antibodies against HIF-1α, COX-2 or β-actin at 4˚C overnight. After 3 washes with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. The membranes were washed again and developed with an enhanced chemiluminescence system (Applygen Technologies, Beijing, China), and the signals were then exposed to X-ray film. The integrated optical density of the protein bands was calculated using ImageJ 1.47 software.

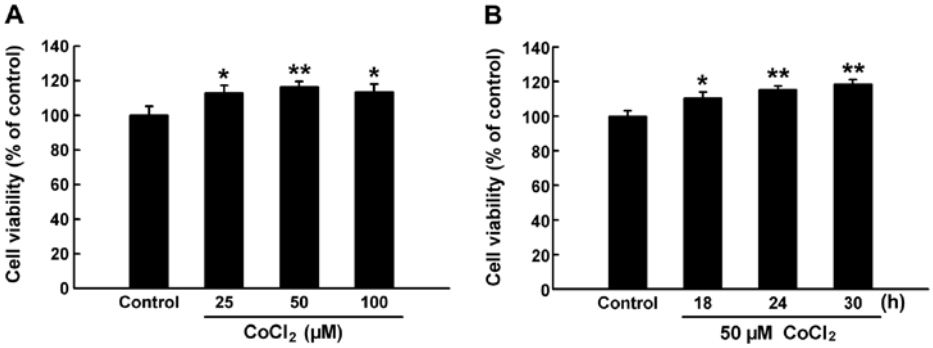

Figure 2. Effect of cobalt chloride ($CoCl_2$) on the proliferation of human pulmonary arterial smooth muscle cells (HPASMCs). After the indicated treatments, cell viability was measured using the Cell Counting Kit-8 (CCK-8) to assess cell proliferation. (A) HPASMCs were treated with increasing concentrations (25-100 μM) of $CoCl_2$ for 24 h. (B) HPASMCs were treated with 50 μM $CoCl_2$ for 18-30 h. Data are presented as the means ± SD, n=4. *P<0.05, **P<0.01, compared with the control (untreated) group.

Measurement of PGI_2 by enzyme-linked immunosorbent assay (ELISA). The HPASMCs were plated in 96-well plates. After the indicated treatments, the levels of PGI_2 in the culture medium were determined by ELISA according to the manufacturer's instructions (Boster Biotechnology Co., Ltd., Wuhan, China). The amount of PGI_2 in the culture medium was normalized to the cell number measured using the CCK-8. The experiment was performed at least 4 times with similar outcomes.

Statistical analysis. All the data are expressed as the means ± SD, and analyzed using SPSS 13.0 software. The significance of inter-group differences was evaluated by one-way analyses of variance (ANOVA). Differences were considered to be significant if the two-sided probability was P<0.05.

Results

Chemically-induced hypoxia enhances the proliferation of HPASMCs. In order to determine whether chemical hypoxia induces cell proliferation, we first exposed the HPASMCs to the chemical hypoxia agent, $CoCl_2$, at the indicated concentrations and exposure times. We then assessed the cell viability using the CCK-8. As shown in Fig. 2A, treatment with $CoCl_2$ at concentrations ranging from 25 to 100 μM significantly increased cell viability to >100% (P<0.05 and P<0.01 compared with the control group), indicating the induction of cell proliferation by chemical hypoxia. In addition, our results indicated that 50 μM $CoCl_2$ had the most prominent effect on the proliferation of the HPASMCs. Subsequently, we exposed the HPASMCs to 50 μM $CoCl_2$ for the indicated periods of time in order to observe the time course of chemical hypoxia-induced proliferation. As shown in Fig. 2B, during the time period of 18-30 h, exposure of the cells to 50 μM $CoCl_2$ enhanced the proliferation of the HPASMCs in a time-dependent manner. In addition, treatment with 50 μM $CoCl_2$ for 24 h significantly increased HIF-1α expression in the HPASMCs, indicating a hypoxic state (P<0.05) (Fig. 3). These results suggest that $CoCl_2$ promotes the proliferation of HPASMCs through the induction of hypoxia.

ROS may not be involved in chemical hypoxia-induced proliferation of HPASMCs. A growing body of evidence indicates that ROS play a critical role in hypoxia-induced cell proliferation (3,9). In this study, to examine the role of ROS in the proliferation of HPASMCs, the cells were exposed to the exogenous ROS donor, H_2O_2. As shown in Fig. 4A, H_2O_2 had similar effects to $CoCl_2$ in the induction of cell proliferation; i.e., at concentrations ranging from 6 to 50 μM, exposure of the HPASMCs to H_2O_2 for 24 h induced marked proliferation, and the most prominent effects on cell proliferation were induced at 25 μM; however, treatment with 25 μM H_2O_2 did not alter HIF-1α expression in the HPASMCs (Fig. 3). Of note, prior to exposure to 50 μM $CoCl_2$ or 25 μM H_2O_2, the cells were pre-conditioned with the ROS scavenger, NAC. The results revealed that pre-treatment with NAC markedly blocked the H_2O_2-induced cell proliferation, but did not alter the effects of $CoCl_2$ (Fig. 4B). Moreover, we observed the effects of exposure to $CoCl_2$ on the intercellular ROS content and found that treatment with 50 μM $CoCl_2$ for 6-24 h did not affect the levels of ROS (Fig. 4C and D). These data suggest that chemical

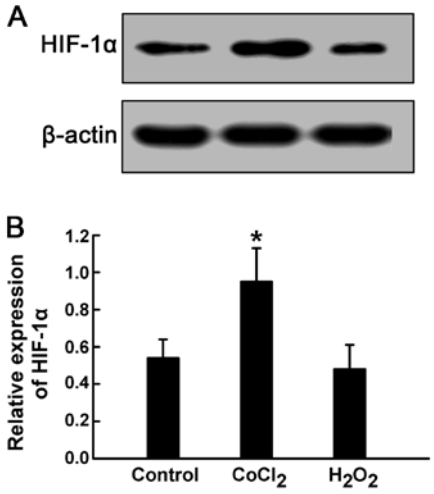

Figure 3. Effect of indicated treatments on hypoxia-inducible factor-1α (HIF-1α) expression in human pulmonary arterial smooth muscle cells (HPASMCs). (A) HPASMCs were treated with 50 μM cobalt chloride ($CoCl_2$) or 25 μM H_2O_2 for 24 h. Western blot analysis was performed to detect HIF-1α expression. (B) Quantitative analysis of HIF-1α expression in (A) using Image J 1.47 software. Data are presented as the means ± SD, n=4. *P<0.05, compared with the control (untreated) group.

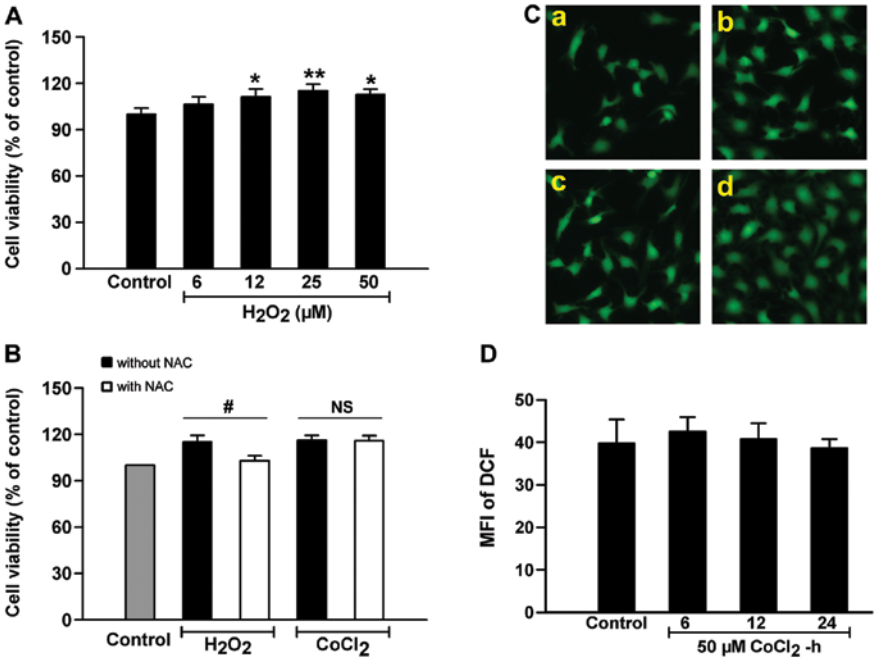

Figure 4. Role of reactive oxygen species (ROS) in the proliferation of human pulmonary arterial smooth muscle cells (HPASMCs) induced by cobalt chloride ($CoCl_2$). (A) The cells were treated with increasing concentrations (6-50 μM) of H_2O_2 for 24 h and then cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) . (B) The cells were treated with 25 μM H_2O_2 or 50 μM $CoCl_2$ for 24 h in the absence or presence of pre-conditioning with 1,500 μM N-acetyl-L-cysteine (NAC) for 1 h and then cell proliferation was measured using the CCK-8. (C) HPASMCs were treated with 50 μM $CoCl_2$ for (a) 0 h, (b) 6 h, (c) 12 h and (d) 24 h and 2',7'-dichlorofluorescein (DCF) staining was performed followed by photofluorography to observe intracellular ROS levels. (D) Quantitative analysis of the mean fluorescence intensity (MFI) of DCF in (C) using Image J 1.47 software. Data are presented as the means ± SD, n=4. *P<0.05, **P<0.01 compared with the control (untreated) group. $^{\#}$P<0.05 compared with the group treated with 25 μM H_2O_2 alone group. NS (not significant), P >0.05 compared with the group treated with 50 μM $CoCl_2$ alone.

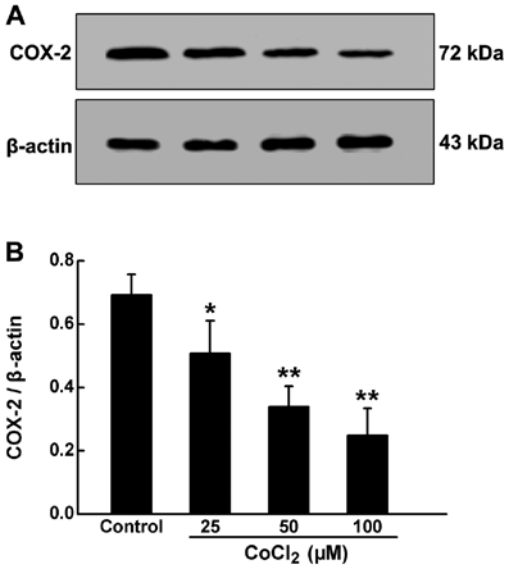

Figure 5. Effect of cobalt chloride ($CoCl_2$) on cyclooxygenase-2 (COX-2) expression in human pulmonary arterial smooth muscle cells (HPASMCs). (A) HPASMCs were treated with increasing concentrations (25-100 μM) of $CoCl_2$ for 24 h. Western blot analysis was performed to detect COX-2 expression. (B) Quantitative analysis of COX-2 expression in (A) using ImageJ 1.47 software. Data are presented as the means ± SD, n=4. *P<0.05, **P<0.01 compared with the control (untreated) group.

hypoxia-induced cell proliferation may not be dependent on ROS, and that other mechanisms are involved.

Chemically-induced hypoxia inhibits COX-2 expression in HPASMCs. COX-2 is responsible for the regulation of proliferation and the hypertrophy of PASMCs. In order to elucidate the roles of COX-2 in the proliferation induced by chemical hypoxia, we measured COX-2 protein expression by western blot analysis. As shown in Fig. 5, following exposure of the HPASMCs to $CoCl_2$ at increasing concentrations for 24 h, the expression of COX-2 was significantly reduced in a dose-dependent manner.

PGI_2 is involved in chemical hypoxia-induced proliferation of HPASMCs. PGI_2 is produced by the catalytic action of COX-2. To determine the downstream pathway of COX-2, we investigated the effects of PGI_2 on the chemical hypoxia-induced proliferation of HPASMCs. Following exposure of the cells to $CoCl_2$ at increasing concentrations for 24 h, we measured the levels of PGI_2 in medium using ELISA. As shown in Fig. 6A, the exposure to $CoCl_2$ markedly suppressed the release of PGI_2 from the HPASMCs. In addition, the exogenous administration of PGI_2 partially abolished the $CoCl_2$-induced proliferation of HPASMCs (Fig. 6B).

Endogenous H_2S deficiency in HPASMCs exposed to chemically-induced hypoxia. In a number of tissues, normal COX-2 expression attributes to basic H_2S levels in cardiomyocytes and gastrointestinal mucosa (11,21). Thus, we hypothesized that the chemical hypoxia-induced COX-2/PGI_2 downregulation may be due to endogenous H_2S deficiency. To confirm our hypothesis, experiments were carried out to detect intercel-

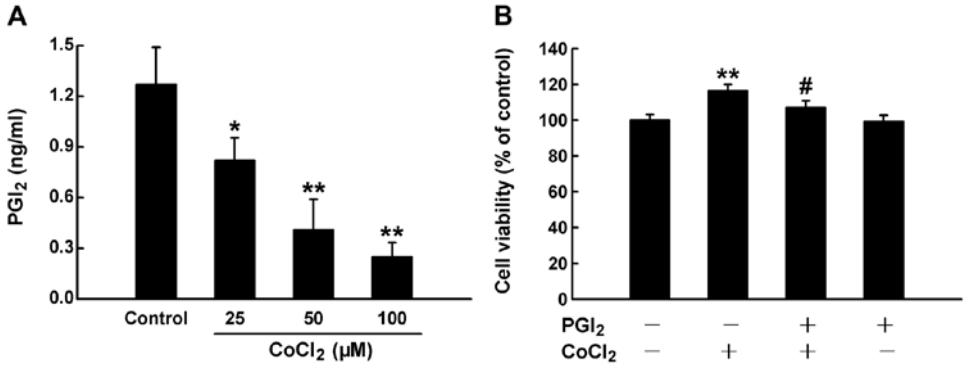

Figure 6. Role of prostacyclin (also known as prostaglandin I2 or PGI_2) in human pulmonary arterial smooth muscle cell (HPASMC) proliferation induced by cobalt chloride ($CoCl_2$). (A) HPASMCs were treated with increasing concentrations (25-100 μM) of $CoCl_2$ for 24 h. ELISA was used to measure PGI_2 expression in the medium. (B) HPASMCs were treated with 50 μM $CoCl_2$ for 24 h in the absence or presence of 2 ng/ml PGI_2 and then cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) . *P<0.05, **P<0.01 compared with the control (untreated) group. $^{\#}$P<0.05 compared with the group treated with 50 μM $CoCl_2$ alone.

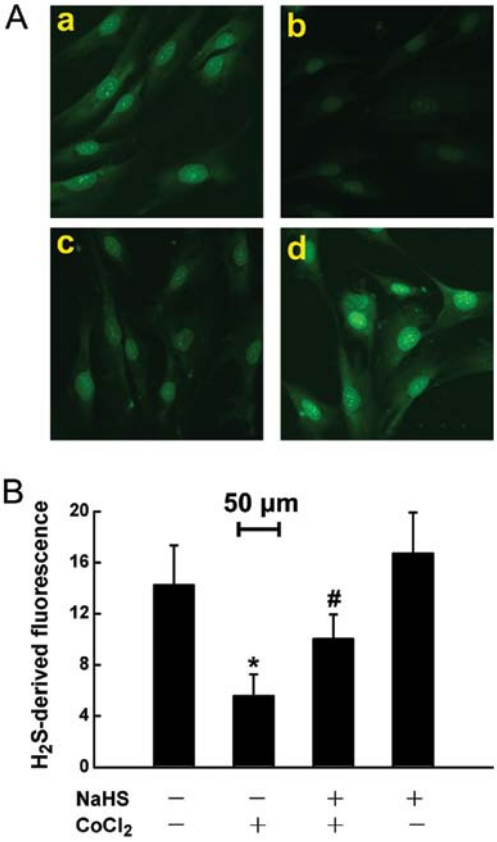

Figure 7. Levels of hydrogen sulfide (H_2S) in human pulmonary arterial smooth muscle cells (HPASMCs) under the indicated experimental conditions. (A) H_2S probe staining followed by photofluorography to observe intracellular free H_2S levels. (a) Normal HPASMCs; (b) HPASMCs treated with 50 μM cobalt chloride ($CoCl_2$) for 24 h; (c) HPASMCs pre-treated with 400 μM sodium hydrosulfide (NaHS) for 30 min and then treated with 50 μM $CoCl_2$ for 24 h; (d) HPASMCs treated with 400 μM NaHS for 30 min followed by a further 24 h of culture. (B) Quantitative analysis of the mean fluorescence intensity (MFI) of H_2S-derived fluorescence in (A) using ImageJ 1.47 software. Data are presented as the means ± SD, n=4. *P<0.05 compared with the control (untreated) group. $^{\#}$P<0.05 compared with the group treated with 50 μM $CoCl_2$ alone.

lular H_2S levels using the fluorescent probe, WSP-1, followed by photofluorography. The results revealed that exposure to 50 μM $CoCl_2$ for 24 h markedly suppressed the generation of H_2S in the HPASMCs (Fig. 7A-b). This inhibitory effect of $CoCl_2$ was significantly reversed, in part by the exogenous administration of NaHS (a donor of H_2S) (Fig. 7A-c), which alone did not alter intercellular H_2S levels (Fig. 7A-d).

Administration of H_2S partly abolishes the chemical hypoxia-induced proliferation of HPASMCs. Although the exogenous administration of H_2S prevented the $CoCl_2$-induced H_2S deficiency to a certain extent, we hypothesized that H_2S may affect the chemical hypoxia-induced proliferation of HPASMCs. The cells were therefore treated with 400 μM NaHS (a donor of H_2S) for 30 min prior to treatment with 50 μM $CoCl_2$ for 24 h followed by the measurement of cell proliferation. The results revealed that pre-treatment with NaHS markedly eliminated the $CoCl_2$-induced proliferation of HPASMCs (Fig. 8A). In addition, cell growth was observed by acquring images using a microscope. The images depicted that the chemical hypoxia-induced changes were mainly characterized by an increased cell number (proliferation), rather than by the cell size (hypertrophy) (Fig. 8B-b). Of note, this effect was suppressed by pre-treatment with H_2S (Fig. 8B-c). These results indicate that insufficient H_2S levels may contribute to the chemical hypoxia-induced proliferation of HPASMCs.

Involvement of COX-2/PGI_2 in the inhibition of cell proliferation by H_2S. Since the chemical hypoxia-induced proliferation was associated with the downregulation of COX-2/PGI_2 and insufficient levels of H_2S in the HPASMCs, we wished to determine the association between endogenous H_2S and COX-2/PGI_2. Thus, prior to the treatment of HPASMCs with 50 μM $CoCl_2$ for 24 h, the cells were pre-conditioned with 400 μM NaHS for 30 min, followed by the measurement of COX-2 expression and PGI_2 secretion. We found that exogenously applied H_2S (NaHS) partially abrogated the downregulation in the expression of COX-2 (Fig. 9A and B) and the reduced secretion of PGI_2, which was induced by $CoCl_2$ (Fig. 9C).

Discussion

The present study demonstrates that chemically-induced hypoxia enhances the proliferation of HPASMCs. During this process, there a marked decrease in COX-2 expression and

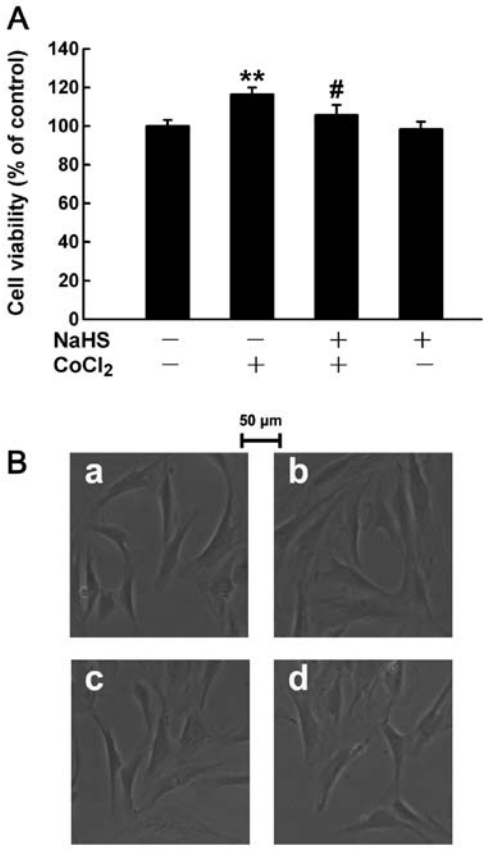

Figure 8. Effect of hydrogen sulfide (H_2S) on cobalt chloride ($CoCl_2$)-induced proliferation of human pulmonary arterial smooth muscle cells (HPASMCs). (A) The cells were treated with 50 μM $CoCl_2$ for 24 h with or without pretreatment with 400 μM sodium hydrosulfide (NaHS) for 30 min. After the treatments, cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) . Data are presented as the means ± SD, n=4. **P<0.01 compared with the control (untreated) group. $^{\#}$P<0.05 compared with the group treated with 50 μM $CoCl_2$ alone. (B Images of cell growth (a-d) in the indicated groups were acquired. (a) Normal HPASMCs; (b) HPASMCs treated with 50 μM $CoCl_2$ for 24 h; (c) HPASMCs treated with 50 μM $CoCl_2$ for 24 h combined with 400 μM NaHS pre-treatment for 30 min; (d) HPASMCs treated with 400 μM NaHS for 30 min followed by a further 24 h of culture.

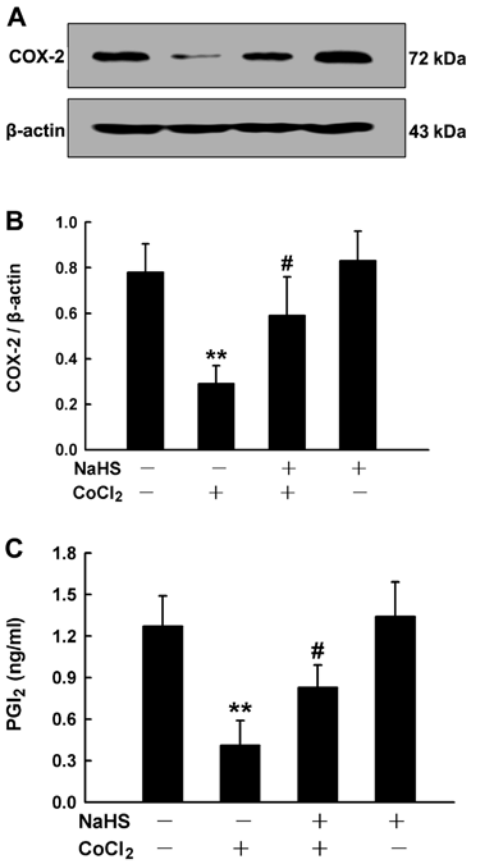

Figure 9. Role of cyclooxygenase-2 (COX-2)/prostacyclin (also known as prostaglandin I2 or PGI_2) in the inhibition of the proliferation of human pulmonary arterial smooth muscle cells (HPASMCs) by hydrogen sulfide (H_2S). The cells were treated with 50 μM cobalt chloride ($CoCl_2$) for 24 h with or without pre-treatment with 400 μM sodium hydrosulfide (NaHS) for 30 min. (A and B) At the end of the treatments, cyclooxygenase-2 (COX-2) expression in the cells was detected by (A) western blot analysis and (B) quantitative analysis using ImageJ 1.47 software. (C) After the treatments PGI_2 secretion from the cells was measured by ELISA. Data were presented as the means ± SD, n=4. **P<0.01 compared with the control (untreated) group. $^{\#}$P<0.05 compared with the group treated with 50 μM $CoCl_2$ alone.

PGI_2 secretion from the cells occurs; however, as shown by our results, the levels of oxidative stress were not significantly altered. Of note, our findings also indicated that the production of H_2S, a novel endogenous gaseous molecule, was reduced, whose donor, NaHS, blocked the chemical hypoxia-induced proliferation of HPASMCs. Our results also revealed that the inhibition of cell proliferation by H_2S was attributed to the upregulation of COX-2/PGI_2.

In this study, we first created a simple cellular model of hypoxic PAH. PAH is a disease of the pulmonary circulation, which can be defined as a mean pulmonary arterial pressure of >25 mmHg at rest or >30 mmHg during exercise, accompanied by a pulmonary capillary wedge pressure of <15 mmHg (22). Sustained PAH usually leads to an increase in pulmonary vascular resistance, which in turn enhances right ventricular load, and ultimately induces right ventricular failure and death (23). On the other hand, pulmonary vascular remodeling and the resultant increases in pulmonary vascular resistance play important roles in the development of PAH. Through the inhibition of cell proliferation or the hypertrophy of PASMCs and the protection of endothelial function, the development of PAH can be effectively attenuated. Since hypoxia is one of the main mechanisms involved in the proliferation of PASMCs (24), we created a cellular model of hypoxia-induced PAH by the exposure of HPASMCs to the chemical hypoxia agent, $CoCl_2$. Hypoxic conditions can be induced either by physical hypoxia, such as 1% O_2, 5% CO_2 and 94% N_2 in a modular incubator chamber, or by chemical hypoxia, using hypoxia-mimicking agents, such as $CoCl_2$, manganese chloride ($MnCl_2$) and sodium thiosulfate (18). Due to the advantages of simple operation and stable hypoxic effects, we selected the latter in the current study. As shown by our results, the exposure of HPASMCs to $CoCl_2$ markedly elicited the upregulation of HIF-1α expression, indicating the hypoxic condition of the cells. Of note, exposure to $CoCl_2$ induced marked cell proliferation characterized by an increase in cell number without an obvious increase in cell size. To measure cell proliferation, we used the CCK-8 assay, as previously used in the study by Wu *et al* to examine the proliferation of T lymphocytes (17). We therefore believed that our cellular model of hypoxic PAH was functional.

A novel finding of the present study was that oxidative stress may not play a critical role in the chemical hypoxia-induced proliferation of HPASMCs. Oxidative stress is considered a key regulator of the balance between cell proliferation and the onset of differentiation through ROS (7). Abnormal ROS accumulation often leads to delayed differentiation and/or uncontrollable cell growth, namely proliferation. The development of hypertension and atherosclerosis is closely associated with the overproduction of ROS (6,25,26). Similar studies on hypoxia-induced PAH are also available (3) and, moreover, the ROS scavenger, SOD, has been shown to ameliorate PAH (8). In the current study, we also found that the ROS donor, H_2O_2, promoted HPASMC proliferation, which was suppressed by another ROS scavenger, NAC. However, NAC did not suppress $CoCl_2$-induced proliferation and treatment with $CoCl_2$ did not enhance the levels of ROS in the HPASMCs, indicating that ROS may not be involved in the chemical hypoxia-induced proliferation of HPASMCs. The differences in the results between some of the above mentioned studies and our findings, may be due to the different experimental models used; for instance, differences between *in vivo* and *in vitro* studies, or between physically- and chemically-induced hypoxia.

Another important finding of the current study was that the downregulation of COX-2/PGI_2 participated in the chemical hypoxia-induced proliferation of HPASMCs. COX-2 is a multifunctional protein, whose roles are complex and ambivalent in different models, mediating either neuronal toxicity or cardiac protection (27,28). For example, in the cardiovascular system, COX-2 usually exerts beneficial effects. As previously demonstrated, the cardioprotective effects of estrogen, zileuton and atorvastatin are mediated by COX-2 (27,29,30). In accordance with a previous study (2), our data demonstrated that exposure to $CoCl_2$ markedly reduced COX-2 expression in the HPASMCs. Our data further indicated that PGI_2, a product of COX-2 from cells was also markedly reduced. The exogenous administration of PGI_2 markedly suppressed the $CoCl_2$-induced proliferation of HPASMCs, suggesting that COX-2/PGI_2 are not only involved in classical vasodilatation, but also in the proliferation of smooth muscle cells; this fact enhances our knowledge of the mechanisms of action of PGI_2. Currently, PGI_2 and its analogues have been widely used in the clinical management of patients with PAH through effective vasodilatation. However, the mortality rate of patients with this disorder has not been significantly reduced over the past years. In addition, treatment costs, namely the inhalation of aerosolized PGI_2 are extremely high, approximately several hundred dollars per day (10).

In chemical hypoxia-induced proliferation, the deficiency of H_2S was observed in the HPASMCs. Similar to NO and CO, H_2S, as a gaseous signaling molecule, has a number of physiological and pathological roles, such as cardioprotection (15,31), vasodilatation (32-34) and dermal protection (13,35). The endogenous formation of H_2S is attributed to enzymes, such as cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (MPST) (12,19). These enzymes convert L-cysteine or its derivatives to H_2S in various tissues and organs. However, in human pulmonary arterial tissues, the function of H_2S has not yet been fully elucidated. In the present study, we found that the levels of H_2S were markedly decreased by treatment with $CoCl_2$, whcn compared to the control (untreated) HPASMCs. These findings are in accordance with those presented in the study by Zhang *et al* on lung tissue and pulmonary arteries of Wistar rats exposed to physical hypoxia (36). We also found that exogenously applied NaHS (donor of H_2S) partially recovered the deficiency in H_2S in the HPASMCs exposed to $CoCl_2$ and, more importantly, NaHS suppressed the $CoCl_2$-induced cell proliferation. This was perhaps one of the mechanisms underlying the H_2S inhibition of pulmonary arterial remodeling *in vivo*. As previously demonstrated, H_2S can also inhibit arterial remodeling by endothelial protection (37) and the induction of the apoptosis of smooth muscle cells (38). In the current study, we mainly investigated the effects of H_2S on cell proliferation and the underlying mechanisms. Certain studies have suggested that H_2S reduces the proliferation of smooth muscle cells through the downregulation of the mitrogen-activated protein kinase (MAPK) pathway (39). Others studies have indicated that the inhibition of cell proliferation by H_2S is involved in the stabilization of p53 coupled with the induction of downstream molecules, including p21 and Bax (40). Notably, a recent study demonstrated that H_2S inhibits both HIF-1α translation by enhancing the phosphorylation of eukaryotic translation initiation factor 2α (41); another study demonstrated that H_2S inhibits the activation of HIF-1α in a von Hippel-Lindau protein- and mitochondrial-dependent manner (42). In this study, treatment with H_2S markedly upregulated COX-2 expression and enhanced PGI_2 secretion from $CoCl_2$-stimulated HPASMCs, which may be associated with the inhibition of HIF-1α. Taking the above mentioned data into consideration, we therefore suggested that the H_2S inhibition of the proliferation of HPASMCs induced by chemical hypoxia may be associated with the upregulation of COX-2/PGI_2. In the future, the H_2S donor or its precursor, L-cysteine, may be applied to the treatment of patients with PAH through the enhancement of PGI_2 production, thus effectively reducing the treatment costs.

In conclusion, the present study demonstrates that during the chemical hypoxia-induced proliferation of HPASMCs, the expression of COX-2/PGI_2 and the levels of H_2S are markedly suppressed. The exogenous administration of PGI_2 or H_2S markedly eliminated the chemical hypoxia-induced cell proliferation. In addition, our data demonstrated that the H_2S-induced inhibition of cell proliferation was mediated by the upregulation of COX-2/PGI_2. The data from the present study provide novel insight into the role of H_2S in ameliorating the chemical hypoxia-induced proliferation of HPASMCs. The modulation of endogenous H_2S production or the exogenous administration of the H_2S donor, NaHS, may be a novel therapeutic strategy for PAH.

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