H₂S inhibition of chemical hypoxia-induced proliferation of HPASMCs is mediated by the upregulation of COX-2/PGI₂

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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 (H₂S) all play an important role. In the present study, we aimed to examine the effects of H₂S on the hypoxia-induced proliferation of human PASMCs (HPASMCs) and to elucidate the underlying mechanisms. The HPASMCs were treated with cobalt chloride (CoCl₂), a hypoxia-mimicking agent, to establish a cellular model of hypoxic PAH. Prior to treatment with CoCl₂, the cells were pre-conditioned with sodium hydrosulfide (NaHS), a donor of H₂S. Cell proliferation, reactive oxygen species (ROS) production, COX-2 expression, prostacyclin (also known as prostaglandin I₂ or PGI₂) secretion and H₂S levels were detected in the cells. The exposure of the HPASMCs to CoCl₂ markedly increased cell proliferation, accompanied by a decrease in COX-2 expression, PGI₂ secretion and H₂S levels; however, the levels of ROS were not altered. Although the exogenous ROS donor, H₂O₂, triggered similar degrees of proliferation to CoCl₂, the ROS scavenger, N-acetyl-L-cysteine (NAC), markedly abolished the H₂O₂-induced cell proliferation, as opposed to the CoCl₂-induced proliferation. The CoCl₂-induced proliferation of HPASMCs was suppressed by exogenously applied PGI₂. The addition of H₂S (NaHS) attenuated the CoCl₂-induced cell proliferation through the increase in the intercellular content of H₂S. Importantly, the exposure of the cells to H₂S suppressed the CoCl₂-induced downregulation in COX-2 expression and PGI₂ secretion from the HPASMCs. In conclusion, the results from the current study suggest that H₂S inhibits hypoxia-induced cell proliferation through the upregulation of COX-2/PGI₂, as opposed to ROS.

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 PGI₂), an effective but expensive clinical drug, has been used for the treatment of PAH via vasodilatation (10). The endogenous...
formation of PGI₂ is mainly attributed to normal cyclooxygenase-2 (COX-2) expression; however, COX-2 expression is reportedly downregulated in hypoxia-induced PAH (2).

Hydrogen sulfide (H₂S) 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). H₂S 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 H₂S are mediated by the upregulation of COX-2 (15). However, plasma H₂S levels in Wistar rats have been shown to be reduced in hypoxia-induced pulmonary vascular structural remodeling (16). We therefore hypothesized that H₂S 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/PGI₂.

To confirm our hypothesis, in the present study, we carried out the following experiments: The chemical hypoxia agent, cobalt chloride (CoCl₂), 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/PGI₂ expression, as well as endogenous H₂S levels were observed. In addition, we aimed to determined whether the exogenous administration of NaHS affects the chemical hypoxia-induced proliferation of HPASMCs, as well as to elucidate the mechanisms involved.

Material and methods

Materials. CoCl₂, NaHS, N-acetyl-L-cysteine (NAC), PGI₂ 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% CO₂ 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).

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 H₂S content. Intracellular free H₂S levels were determined using the H₂S 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. H₂S-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.
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$_2$O$_2$. As shown in Fig. 4A, H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$, the cells were pre-conditioned with the ROS scavenger, NAC. The results revealed that pre-treatment with NAC markedly blocked the H$_2$O$_2$-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...
hypoxia-induced cell proliferation may not be dependent on ROS, and that other mechanisms are involved.
of H₂S in the HPASMCs (Fig. 7A-b). This inhibitory effect of CoCl₂ was significantly reversed, in part by the exogenous administration of NaHS (a donor of H₂S) (Fig. 7A-c), which alone did not alter intercellular H₂S levels (Fig. 7A-d).

Administration of H₂S partly abolishes the chemical hypoxia-induced proliferation of HPASMCs. Although the exogenous administration of H₂S prevented the CoCl₂-induced H₂S deficiency to a certain extent, we hypothesized that H₂S may affect the chemical hypoxia-induced proliferation of HPASMCs. The cells were therefore treated with 400 µM NaHS for 30 min prior to treatment with 50 µM CoCl₂ for 24 h followed by the measurement of cell proliferation. The results revealed that pre-treatment with NaHS markedly eliminated the CoCl₂-induced proliferation of HPASMCs (Fig. 8A). In addition, cell growth was observed by acquiring 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 H₂S (Fig. 8B-c). These results indicate that insufficient H₂S levels may contribute to the chemical hypoxia-induced proliferation of HPASMCs.

Involvement of COX-2/PGI₂ in the inhibition of cell proliferation by H₂S. Since the chemical hypoxia-induced proliferation was associated with the downregulation of COX-2/PGI₂ and insufficient levels of H₂S in the HPASMCs, we wished to determine the association between endogenous H₂S and COX-2/PGI₂. Thus, prior to the treatment of HPASMCs with 50 µM CoCl₂ for 24 h, the cells were pre-conditioned with 400 µM NaHS for 30 min, followed by the measurement of COX-2 expression and PGI₂ secretion. We found that exogenously applied H₂S (NaHS) partially abrogated the downregulation in the expression of COX-2 (Fig. 9A and B) and the reduced secretion of PGI₂, which was induced by CoCl₂ (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
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PGI₂ 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 H₂S, 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 H₂S was attributed to the upregulation of COX-2/PGI₂.

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, CoCl₂. Hypoxic conditions can be induced either by physical hypoxia, such as 1% O₂, 5% CO₂ and 94% N₂ in a modular incubator chamber, or by chemical hypoxia, using hypoxia-mimicking agents, such as CoCl₂, manganese chloride (MnCl₂) 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 CoCl₂ markedly elicited the upregulation of HIF-1α expression, indicating the hypoxic condition of the cells. Of note, exposure to CoCl₂ 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$_2$O$_2$, 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$_2$S was observed in the HPASMCs. Similar to NO and CO, H$_2$S, 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$_2$S is attributed to enzymes, such as cystathionine $\beta$-synthase (CBS), cystathionine $\gamma$-lyase (CSE), and 3-mercaptopropionate sulfurtransferase (MPST) (12,19). These enzymes convert L-cysteine or its derivatives to H$_2$S in various tissues and organs. However, in human pulmonary arterial tissues, the function of H$_2$S has not yet been fully elucidated. In the present study, we found that the levels of H$_2$S were markedly decreased by treatment with CoCl$_2$, when 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$_2$S) partially recovered the deficiency in H$_2$S 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$_2$S inhibition of pulmonary arterial remodeling in vivo. As previously demonstrated, H$_2$S 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$_2$S on cell proliferation and the underlying mechanisms. Certain studies have suggested that H$_2$S reduces the proliferation of smooth muscle cells through the downregulation of the nitrogen-activated protein kinase (MAPK) pathway (39). Others studies have indicated that the inhibition of cell proliferation by H$_2$S 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$_2$S inhibits both HIF-1$\alpha$ translation by enhancing the phosphorylation of eukaryotic translation initiation factor 2$\alpha$ (41); another study demonstrated that H$_2$S inhibits the activation of HIF-1$\alpha$ in a von Hippel-Lindau protein- and mitochondrial-dependent manner (42). In this study, treatment with H$_2$S markedly upregulated COX-2 expression and enhanced PGI$_2$ secretion from CoCl$_2$-stimulated HPASMCs, which may be associated with the inhibition of HIF-1$\alpha$. Taking the above mentioned data into consideration, we therefore suggested that the H$_2$S 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$_2$S 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$_2$S are markedly suppressed. The exogenous administration of PGI$_2$, or H$_2$S markedly eliminated the chemical hypoxia-induced cell proliferation. In addition, our data demonstrated that the H$_2$S-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$_2$S in ameliorating the chemical hypoxia-induced proliferation of HPASMCs. The modulation of endogenous H$_2$S production or the exogenous administration of the H$_2$S donor, NaHS, may be a novel therapeutic strategy for PAH.

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