Acta Pharmacologica Sinica 2006 Dec; 27 (12): 1561–1566

Blackwell Publishing

Full-length article

Interaction between hydrogen sulfide/cystathionine γ-lyase and carbon monoxide/heme oxygenase pathways in aortic smooth muscle cells[1]

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Key words

hydrogen sulfide; cystathionine γ-lyase; carbon monoxide; heme oxygenase

[1] Project supported by the National Natural Science Fundation for Distinguished Young Scholars (No 30425010), National Natural Science Foundation of China (No 30571971) and the State Major Basic Key Research Program (No 2006CB503807).
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Received 2006-04-26
Accepted 2006-06-13

doi: 10.1111/j.1745-7245.2006.00425.x

Abstract

Aim: To investigate the interaction between hydrogen sulfide (H_2S)/cystathionine γ-lyase (CSE) and carbon monoxide (CO)/heme oxygenase (HO) pathways in aortic smooth muscle cells (ASMC). **Methods:** The ASMCs were divided into the following groups: (1) the control group; (2) the zinc protoporphyrin (ZnPP) 20 μmol/L group; (3) the propargylglycine (PPG) 2 mmol/L, 4 mmol/L and 10 mmol/L groups; and (4) the sodium hydrosulfide (NaHS) 1×10^{-5} mol/L, 1×10^{-4} mol/L and 1×10^{-3} mol/L groups. Each of the groups was further divided into 6 h, 12 h, 18 h and 24 h subgroups. The CO level, represented by carboxyhemoglobin (HbCO) content was measured using a spectrophotometric method and H_2S content was detected by a sensitive electrode method. CSE and HO-1 expressions were detected by Western blotting. **Results:** The H_2S content in the medium and CSE expression by ASMC were markedly increased by ZnPP compared with the control group. HbCO content in the medium and HO-1 expression by the ASMC started strengthening following 24 h treatment with PPG at 2 mmol/L, but were further strengthened following 18 h and 24 h treatment with PPG at 4 mmol/L compared with the controls ($P<0.01$). PPG at 10 mmol/L increased the HbCO level in the medium following 18 h treatment and increased HO-1 expression by the ASMC following 12 h treatment. Moreover, NaHS at 1×10^{-5} mol/L and 1×10^{-4} mol/L decreased the HbCO level in the medium and HO-1 expression by the ASMC after 6 h and 12 h treatment, while NaHS at 1×10^{-3} mol/L decreased them at all time points of the treatments. **Conclusion:** The results suggested that endogenous CO/HO and H_2S/CSE pathways inhibited each other in ASMC under physiological conditions.

Introduction

In the late 1980s, nitric oxide (NO) was first demonstrated as an endogenous gasotransmitter[1] with a variety of vital functions including vasorelaxation, suppression of cell proliferation and inhibition of platelet aggregation[2–4]. Such findings led the biomedical study into a new stage. Several years later, carbon monoxide (CO) catalyzed by heme oxygenase (HO) was confirmed as another endogenous gasotransmitter[5] which also showed great importance in the modulation of vascular structure and functions. The studies on NO and CO have made it easier to explain the mechanisms and pathogenesis of diseases. However, the regulation of functions, as well as structures of systems under physiological or pathophysiological conditions, remains unclear. The most hopeful approach is to look for new gasotransmitters. Hydrogen sulfide (H_2S) has been generally considered a toxic gas found in the contaminated environmental atmosphere[6]. In recent years, however, more and more studies have suggested that endogenous H_2S is another gasotransmitter[7]. H_2S is produced endogenously from cysteine by pyridoxal-5'-phosphate-dependent enzymes, including cystathionine β-synthase and/or cystathionine γ-lyase (CSE)[8]. The enzymes were found highly expressed in

the brain, ileum and vessels. In the late 1990s, H_2S was proven to be a neuromodulator in the brain, as well as a tone regulator in smooth muscles[9]. Endogenous H_2S is responsive to neuronal excitation by the Ca^{2+}/calmodulin-mediated pathway, enhancing the NMDA receptor-mediated responses by induced cyclic adenosine monophosphate (cAMP), modifying long-term potentiation (LTP, a synaptic model of memory). More interestingly, Hosoki *et al* found that H_2S could be produced in the portal vein, thoracic aorta, tail artery, mesenteric artery and pulmonary artery[10]. Recently, H_2S was found to exert an inhibitory effect on vascular smooth muscle cell proliferation[11] and promote its apoptosis[12]. H_2S was also found to play a role in the regulation of cardiac function and vasorelaxation by activating the K_{ATP} channel, a distinctive way that is different from NO and CO[13–14].

However, gasotransmitters in vascular tissues might not exist independently, whereas they might have interactions[15]. NO was found to be derived from vascular endothelial cells, H_2S is endogenously generated from vascular smooth muscle cells, while HO exists in both vascular endothelial cells and smooth muscle cells. This suggests that NO, CO and H_2S might represent the most rapid communicative pathway between vascular endothelial cells and smooth muscle cells, being involved in regulating the biological actions. Vascular endothelial cells and smooth muscle cells are the main place for the production of gasotransmitters. At the same time, they are the target cells of gasotransmitters. The above facts provide the basis for the likelihood of interactions among gasotransmitters. Our previous study demonstrated that exogenous supplements of H_2S could alleviate hypoxia-induced elevation of pulmonary arterial pressure. At the same time, H_2S might impact the increased CO production in pulmonary arteries on a chronic hypoxic animal model. However, the above results were derived from hypoxic pulmonary hypertensive rats; whether there are any impacts of H_2S on the CO pathway and vice versa in aortic smooth muscle cells (ASMC) under physiological conditions and its mechanisms remains unclear. Therefore, the present study was undertaken to examine the possible interaction between H_2S/CSE and CO/HO pathways in ASMC.

Materials and methods

Smooth muscle cell culture Male Sprague-Dawley rats, weighing 200–220 g, were killed by anesthetic overdose (intraperitoneal injection of pentobarbital sodium). The thoracic aorta were stripped of adventitia and removed to culture media sterilely. The ASMCs were isolated and cultured according to the method used by Hirata *et al*[16]. Briefly, cells were maintained in DMEM containing 5 mmol/L glucose, 10% FBS, and antibiotics in a CO_2 incubator at 37 ºC. The subcultures of ASMC from passage 4 were used in the experiments and cells were cultured for 24 h in serum-free DMEM before each experiment. The cells were divided into 4 groups according to different treatments, and each group included at least 6 wells. (1) the control group, where cells were continuously cultured in serum-free DMEM; (2) the zinc protoporphyrin (ZnPP, an inhibitor of HO) group, where 20 mmol/L ZnPP was added; (3) the propargylglycine (PPG, an inhibitor of CSE) groups, where 2 mmol/L, 4 mmol/L and 10 mmol/L of PPG were added, respectively; and (4) the sodium hydrosulfide (NaHS, a H_2S donor) groups, where 1×10^{-5} mol/L, 1×10^{-4} mol/L and 1×10^{-3} mol/L of NaHS were added, respectively. Each of the above groups was further divided into 6 h, 12 h, 18 h and 24 h subgroups. ZnPP (Sigma, St Louis, MO,USA) was dissolved in 50 mmol/L sodium carbonate. PPG (Sigma, St Louis, MO, USA) and NaHS (Sigma, St Louis, MO, USA) were dissolved in sterile water.

Measurement of carboxyhemoglobin (HbCO) in conditioned medium To examine the relative amount of CO being released from the ASMC into the medium, hemoglobin (50 μmol/L) was added to the cells during the last hour of incubation, and HbCO was measured by a spectrophotometric method[17]. Medium 0.5 mL was mixed with 1 mL of the hemoglobin solution, which was 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 the sodium dithionite was added. The difference of absorbance at 568 and 581 nm was read via the double-wavelength spectrophotometer. Both the wavelengths were selected according to the absorbance spectrum of HbCO and oxygenated hemoglobin (HbO_2), at which the difference of absorbance of HbCO was largest and the difference of absorbance of HbO_2 was 0. The percentage of HbCO was then calculated from a standard curve derived by mixing different proportions of 2 hemoglobin solutions containing 100% HbCO and 100% HbO_2. We obtained these solutions by gassing hemoglobin solutions with pure oxygen and CO, respectively. For our spectrophotometer, the following relationship was obtained from the standard curve: $\%HBCO=[(OD_{568}- OD_{581})-0.03858]/0.0498$.

Measurement of H_2S in conditioned medium H_2S was measured with a sulfide sensitive electrode (Model 9616; Orion Research; Beverly, MA, USA) as described previously[18]. In brief, sulfide antioxidant buffer was added into standards or samples at a ration of 1:1 and then stirred thoroughly. Electrodes were rinsed in distilled water, blotted dry and

placed into standards and samples. When a stable reading was displayed, the millivolt value was recorded. The H_2S concentration was calculated against the calibration curve of the standard H_2S solution.

Western blotting Cultured cells (3×10^6) were harvested and lysed in a lysis buffer (0.5 mol/L EDTA, 1 mol/L Tris-HCl, pH 7.4, 0.3 mol/L sucrose, 1 μg/mL antipain hydrochloride, 1 mmol/L benzamidine hydrochloride hydrate, 1 μg/mL leupeptin hemisulfate, 1 mmol/L 1,10-phenanthroline monohydrate, 1 μmol/L pepstatin A, 0.1 mmol/L phenylmethylsulfonyl fluoride and 1 mmol/L iodoacetamide). The extracts were clarified by centrifugation at 14 000×*g* for 15 min at 4 ºC. SDS-PAGE and Western blot analysis were performed according to experimental protocol. The primary antibody dilutions were as follows: 1:2000 for HO-1 (Sigma, St Louis, MO, USA), 1:50 for CSE and 1:1000 for β-actin (TBD, Tianjin, China). HRP-conjugated secondary antibody was used at 1:5000. The immunoreactions were visualized by electrochemiluminescence (ECL) and exposed to X-ray film (Kodak Scientific Imaging film, X-Omat Blue XB-1, USA). Membranes were stripped by incubation in a buffer containing 100 mmol/L β-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl, pH 6.8.

Statistical analysis The results were expressed as Mean±SD. ANOVA was used to compare the mean values, including HbCO level in the cultured ASMC medium and HO-1 expression by the ASMC in the various groups. *Post-hoc* analysis was then used to compare the data between the different groups. Independent *t*-test was used to estimate the differences in H_2S content in the cultured ASMC medium and CSE expression by the ASMC between the control group and the ZnPP group. $P<0.05$ was considered statistically significant.

Results

Impact of endogenous CO on the H_2S/CSE pathway To examine the impact of endogenous CO on H_2S release by the ASMCs in the medium, the ASMCs were treated with ZnPP at a concentration of 20 μmol/L. After ZnPP treatment for 6 h, 12 h,18 h and 24 h, the H_2S content in the medium markedly increased by 11.81%, 18.79%, 41.58% and 12.19%, respectively, as compared with that in the medium without ZnPP treatment (all $P<0.01$) as seen in Table 1. The results also showed that the peak time point of the augmentation was at 18 h after the treatment of ZnPP.

After the treatment of ZnPP at a concentration of 20 μmol/L with cultured ASMC for 6 h, 12 h,18 h, and 24 h, CSE expression by ASMC markedly increased by 35.73%, 31.94%, 58.22% and 16.78%, respectively, compared with the controls (all $P<0.01$). The peak time point of the augmentation was also at 18 h after the the ZnPP treatment (Figure 1).

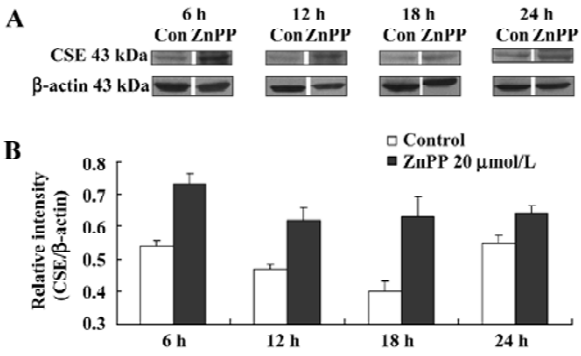

Figure 1. Effect of ZnPP on CSE protein expression by ASMC. After the treatment of ZnPP at a concentration of 20 μmol/L with cultured ASMC for 6 h, 12 h, 18 h, and 24 h, CSE expression by ASMC markedly increased. (A) Representative immunoblots of CSE in the ASMC extracts from the control group and the ZnPP treatment group; and (B) CSE expression by ASMC normalized with β-actin expression. $^bP<0.05$ *vs* control group.

Impact of endogenous H_2S on the CO/HO pathway To examine the impact of endogenous H_2S on CO release by medium of cultured ASMC, the ASMCs were treated with PPG at a concentration of 2 mmol/L, 4 mmol/L and 10 mmol/L, respectively. After PPG treatment at each concentration for 6 h and 12 h, there were no significant changes in CO release by cultured ASMC as seen in Table 2 ($P>0.05$). The CO release started to be augmented by 4.02% following the 24 h

Table 1. H_2S contents (μmol/L) in the medium of ASMC cultured in the presence or absence of ZnPP. $n=8$. Mean±SD. $^bP<0.05$ *vs* control group.

Group	6 h	12 h	18 h	24 h
Control	17.55±0.58	13.76±0.67	10.89±0.33	11.03±0.47
ZnPP 20 μmol/L	19.63±0.94^b	16.34±0.78^b	15.42±0.90^b	12.38±0.85^b
Statistical value	$t=5.33$, $P<0.01$	$t=7.199$, $P<0.01$	$t=13.392$, $P<0.01$	$t=3.897$, $P<0.01$

treatment with PPG at 2 mmol/L, but was clearly augmented by 7.11% and 7.78% following 18 h and 24 h treatment with PPG at 4 mmol/L, respectively (P<0.01). Also, in the presence of PPG at 10 mmol/L, CO release markedly increased by 5.38% and 15.57% compared with the controls at 18 h and 24 h, respectively (all P<0.01; Table 2).

After PPG treatment at each concentration for 6 h, there were no significant changes in HO-1 expression by cultured ASMC (P>0.05). The HO-1 expression by cultured ASMC started to be strengthened by 34.95% following 24 h treatment with PPG at a concentration of 2 mmol/L, but was evidently strengthened by 169.19% and 54.42% after 18 h and 24 h treatment with PPG at 4 mmol/L, respectively(P<0.01). In the presence of PPG at 10 mmol/L, HO-1 expression by cultured ASMC markedly increased by 60.29%, 102.31% and 243.36% compared with the controls at 12, 18 h and 24 h, respectively (all P<0.01). The results also showed that PPG increased HO-1 expression by cultured ASMC at 24 h in a dose-dependent manner (P<0.01; Figure 2).

Impact of H_2S donor on the CO/HO pathway To examine the impact of H_2S on CO release by cultured ASMC, the ASMCs were treated with NaHS, a H_2S donor, at a concentration of 1×10^{-5} mol/L, 1×10^{-4} mol/L, and 1×10^{-3} mol/L, respectively (Table 3). After treatment for 6 h, NaHS at 1×10^{-4} mol/L and 1×10^{-3} mol/L decreased HbCO content in the supernatant of cultured ASMC by 3% and 5.09% (all P<0.05), respectively. After treatment for 12 h, NaHS at each concentration decreased HbCO content in the supernatant of cultured ASMC by 2.74%, 5.47%, and 8% (all P<0.05) in a dose-dependent manner. After treatment for 18 h and 24 h, NaHS at 1×10^{-5} mol/L and 1×10^{-4} mol/L did not change the HbCO content in the supernatant (all P>0.05), while NaHS at 1×10^{-3} mol/L reduced the HbCO content in the supernatant significantly by 5.37% and 8.22% (all P<0.05), respectively.

After NaHS treatment at each concentration for 6 h and

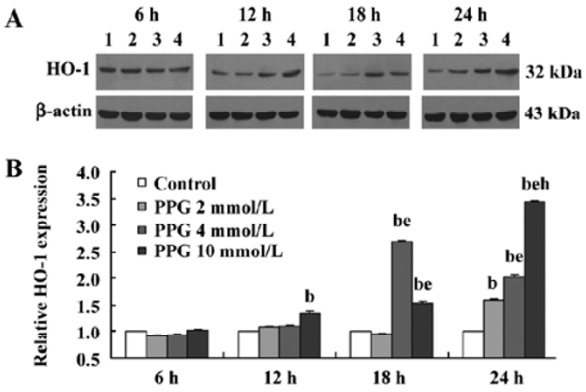

Figure 2. Effect of PPG on HO-1 expression by ASMC. After the treatment of PPG at different concentrations with ASMC for 6 h, 12 h, 18 h, and 24 h, HO-1 expression by ASMC increased, respectively. (A) Representative immunoblots of HO-1 in the ASMC extracts from the control group and the PPG treatment group. Lane 1: control group; lane 2: PPG 2 mmol/L group; lane 3: PPG 4 mmol/L group; lane 4: PPG 10 mmol/L group. (B) HO-1 expression relative to control, with control taken as 1. ^bP<0.05 *vs* control group. ^eP<0.05 *vs* PPG 2 mmol/L group. ^hP<0.05 *vs* PPG 4 mmol/L group.

Table 2. Impact of PPG on HbCO content (%) in the supernatant of cultured ASMC. n=8. Mean±SD. ^bP<0.05 *vs* control group. ^eP<0.05 *vs* PPG 2 mmol/L group. ^hP<0.05 *vs* PPG 4 mmol/L group.

Time (h)	Control	PPG 2 mmol/L	PPG 4 mmol/L	PPG 10 mmol/L	Statistical value
6	3.56±0.07	3.60±0.09	3.59±0.06	3.60±0.05	F=0.031 P=0.992
12	3.68±0.02	3.63±0.05	3.69±0.05	3.64±0.07	F=0.866 P=0.497
18	3.48±0.07	3.49±0.04	3.72±0.04^{be}	3.67±0.10^{be}	F=9.607 P=0.005
24	3.66±0.04	3.81±0.06^b	3.94±0.06^{be}	4.23±0.09^{beh}	F=38.796 P<0.001

Table 3. Impact of NaHS on HbCO content (%) in the supernatant of cultured ASMC. n=8. Mean±SD. ^bP<0.05 *vs* control group. ^eP<0.05 *vs* NaHS 1×10^{-5} mol/L group. ^hP<0.05 *vs* NaHS 1×10^{-4} mol/L group.

Time (h)	Control	NaHS 1×10^{-5} mol/L	NaHS 1×10^{-4} mol/L	NaHS 1×10^{-3} mol/L	Statistical value
6	3.56±0.07	3.49±0.06	3.47±0.03^b	3.38±0.04^{be}	F=6.503, P=0.015
12	3.68±0.02	3.58±0.03^b	3.48±0.03^{be}	3.38±0.02^{beh}	F=96.023, P<0.001
18	3.48±0.07	3.47±0.07	3.44±0.07	3.29±0.05^{beh}	F=4.996, P=0.031
24	3.66±0.04	3.62±0.04	3.61±0.06	3.36±0.13^{beh}	F=11.120, P=0.003

12 h, HO-1 expressions by cultured ASMC decreased in a dose-dependent manner (all $P<0.05$). The results also showed that NaHS at 1×10^{-3} mol/L inhibited HO-1 expression by cultured ASMC at all time points of the treatments, while NaHS at 1×10^{-5} mol/L and 1×10^{-4} mol/L inhibited HO-1 expression by cultured ASMC at 6 h and 12 h of the treatment (Figure 3).

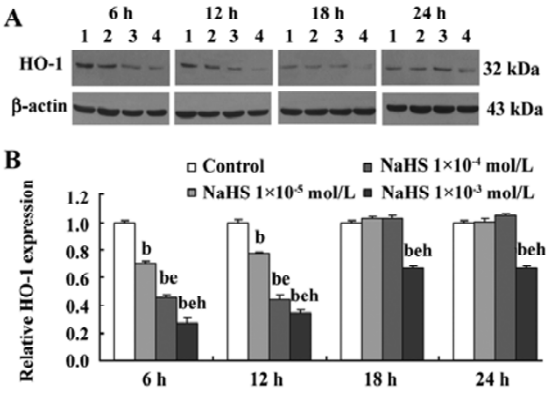

Figure 3. Effect of NaHS on HO-1 expression by ASMC. After the treatment of NaHS at different concentration with cultured ASMC for 6 h, 12 h, 18 h, and 24 h, HO-1 expression by ASMC decreased, respectively. (A) Representative immunoblots of HO-1 in the ASMC extracts from the control group and the NaHS treatment group. Lane 1: control group; lane 2: NaHS 1×10^{-5} mol/L group; lane 3: NaHS 1×10^{-4} mol/L group; lane 4: NaHS 1×10^{-3} mol/L group. (B) HO-1 expression relative to control, with control taken as 1. $^{b}P<0.05$ *vs* control group. $^{e}P<0.05$ *vs* NaHS 1×10^{-5} mol/L group. $^{h}P<0.05$ *vs* NaHS 1×10^{-4} mol/L group.

Discussion

Hydrogen sulfide (H_2S) has been recognized as a toxic gas in water pollution and industrial air pollution with a strong odor for long time. More interestingly, Hosoki *et al*[10] found that H_2S could be produced in the portal vein, thoracic aorta, tail artery, mesenteric artery and pulmonary artery. The vascular smooth muscle was relaxed by the treatment of H_2S in a dose-dependent manner, including intact endothelia and removed endothelia, and in synergy with NO. Endogenous H_2S in rat vascular tissues, as a vascular relaxant factor, could maintain the basal blood pressure balance under physiological conditions[9]. It could also suppress the proliferation of cultured vascular smooth muscle cells (vascular smooth muscle cells) through the mitogen-activated protein kinase pathway in a dose-dependant manner *in vitro*[11]. Based on its endogenous metabolism and physiological functions, H_2S is well positioned in the novel family of endogenous gaseous transmitters. More and more evidence is proving that H_2S might be the third endogenous signaling gasotransmitter besides NO and CO[7]. CO acts as a second gasotransmitter that shares some of the physiochemical properties of NO[15]. Like NO, CO can bind to the iron atom of the heme moiety associated with soluble guanylyl cyclase, thereby activating the enzyme and increasing intracellular cGMP production. CO is produced endogenously by various cell types as a byproduct of heme catabolism, in which HO catalyzes the degradation of heme into equimolar amounts of biliverdin, iron and CO[5].

Recently, the rapid development of systems biology makes us aware that only a single molecule or its pathway cannot regulate the cardiovascular functions independently[19]. It was discovered that H_2S is formed in VSMC, while CO is generated mainly in VSMC, but also in vascular endothelial cells, acting on vascular cells in an autocrine/paracrine manner. Previous studies have also demonstrated that gasotransmitters always interact. For instance, previous studies have indicated that endogenous CO/HO and NO/nitric oxide synthase (NOS) interacted with each other, as did NO/NOS and H_2S/CSE pathways, therefore playing important roles in regulating the homeostasis of the cardiovascular system[20,21]. However, how CO/HO and H_2S/CSE pathways interact is still not clear.

For the purpose of understanding if there are any interactions between CO on the H_2S pathway in ASMC under physiological conditions, we performed the above studies culturing ASMC treated with the CO/HO inhibitor, H_2S/CSE inhibitors and H_2S donor, respectively. We noticed that there was an impact of endogenous CO on the H_2S/CSE pathway in ASMC. We observed that after the ASMC were treated with ZnPP, an inhibitor of HO[22], the H_2S content in the medium markedly increased compared to the controls. The results demonstrated that endogenous CO could down-regulate H_2S production under physiological conditions. The mechanisms, however, were not fully understood. As we know, CSE was the main enzyme in ASMC catalyzing the endogenous production of H_2S. Therefore, we speculated that the induced ASMC CSE expression by ZnPP treatment might be involved in the mechanism by which endogenous CO regulated the H_2S/CSE pathway. In our study, we found that CSE expression by ASMC treated with ZnPP markedly increased compared with that untreated with ZnPP. However, further studies should be conducted to understand the impact of endogenous CO on the CSE gene expression and modulations.

To understand if there are any impacts of H_2S on the CO pathway in ASMC under physiological condition, we performed the present study and found that after treatment with PPG[23], an inhibitor of CSE in cultured ASMC, CO release by

ASMC was augmented. Moreover, NaHS, a H_2S donor, decreased HbCO content in the supernatant of cultured ASMC. The above results indicated that H_2S could markedly inhibit CO release by ASMC under physiological conditions. To examine the possible mechanisms by which H_2S regulated endogenous CO production, we employed Western blot to detect HO-1 expression by ASMC. Our data showed that PPG strengthened the HO-1 expression by cultured ASMC, while NaHS inhibited the HO-1 expression by cultured ASMC. The results revealed that the mechanisms by which H_2S regulated endogenous CO production involved at least the inhibition of HO-1 expression by cultured ASMC. However, further studies dealing with the influence of endogenous H_2S on HO-1 transcription in addition to its translation should be done.

The interaction between CO and H_2S may have potential physiological significance. Both CO and H_2S were produced in VSMC and have distinct characteristics, including fast production, rapid diffusion, extensive action and a shorter life time. They also share similar biological functions. For vascular regulation, both were demonstrated to relax the vessels and inhibit the VSMC proliferation. However, they are different in many aspects, including their signaling pathways. The coordinated regulation of these species suggests that they all are used in cell signaling. These reactions establish "cross-talk" between 2 VSMC-derived gasotransmitters. Such adaptive mechanisms are thought to be of evolutionary importance[24]. However, investigations on the interactions among gasotransmitters and other vasoactive substances need to be further conducted.

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