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ORIGINAL ARTICLE

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The possible role of hydrogen sulfide as a smooth muscle cell proliferation inhibitor in rat cultured cells

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Abstract Hydrogen sulfide (H_2S) was recently suggested to be a possible endogenous gasotransmitter in physiological concentration. For the purpose of understanding its possible role in the regulation of the cardiovascular system, we explored the potential effect of H_2S on the proliferation of cultured aortic vascular smooth muscle cells (VSMCs) of rats and mitrogen-activated protein kinase (MAPK) as a signaling transduction pathway. Vascular smooth muscle cells were cultured *in vitro* and the cells were divided into six groups: (1) control group, (2) serum group, (3) endothelin group, (4) NaHS group, (5) serum + NaHS group, and (6) endothelin + NaHS group. VSMC proliferation was measured by [^{3}H]thymidine ([^{3}H]TdR) incorporation and MAPK activity in the VSMCs was determined by radioactivity assay. The results showed that endothelin-1 increased VSMC [^{3}H]TdR incorporation 2.39-fold ($P < 0.01$) and MAPK activity 1.62-fold ($P < 0.01$), as compared with controls. Hydrogen sulfide at 5×10^{-5} mol/l, 1×10^{-4} mol/l, and 5×10^{-4} mol/l decreased VSMC [^{3}H]TdR incorporation by 16.8%, 26.60%, and 37.40%, respectively, and reduced MAPK activity by 7.37% ($P > 0.05$), 23.39%, and 33.57%, respectively ($P < 0.01$). The results demonstrated that H_2S could dose-dependently suppress the proliferation of VSMCs through the MAPK pathway.

Key words Hydrogen sulfide · Endothelin · Aorta · Mitogen-activated protein kinase · Gasotransmitter

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Introduction

In the late 1980s, nitric oxide (NO) was first demonstrated as an endogenous gasotransmitter[1] with a variety of vital functions including vasorelaxation,[2] suppression of cell proliferation,[3] inhibition of platelet aggregation,[4] etc. Such findings led biomedical study into a new stage. Several years later, carbon monoxide (CO) was confirmed as another endogenous gasotransmitter,[5] which also showed great importance in 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 as a toxic gas found in the contaminated environmental atmosphere.[6] In recent years, however, more and more studies have suggested endogenous H_2S to be another gasotransmitter in physiological concentration.[7] H_2S is produced endogenously from cysteine by pyridoxal-5′-phosphate-dependent enzymes, including cystathionine β-synthase (CBS) and/or cystathionine γ-lyase (CSE).[8] The enzymes are found highly expressed in brain, ileum, and vessels. H_2S was proven to be a neuromodulator in the brain as well as a tone regulator in smooth muscle in the late 1990s.[9] Recently, Zhao et al. demonstrated that H_2S exerted cardiovascular effects by activating the K_{ATP} channel and hyperpolarizing the membrane potential of vascular smooth muscle cells (VSMCs), in a manner distinctive from that of NO and CO.[10] Does H_2S inhibit the proliferation of vascular smooth muscle cells similarly to NO and CO or differ from them? What signaling transduction pathway is involved in mediating the potential inhibitory effect? All these questions inspired our interest in further studies. The purpose of the present study was to explore the likely effects of H_2S on inhibition of the proliferation of cultured aortic VSMCs of the rat and the possible role of mitrogen-activated protein kinase (MAPK) as its signaling transduction pathway.

Materials and methods

Materials

Ten Sprague-Dawley (SD) rats were supplied by the Animal Center of Peking University Health Science Center following related guidelines. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, MO, USA). [^{3}H]Thymidine was bought from Beijing Atomic Energy Institute. Endothelin (human) was a gift from Phoenix Pharmaceuticals (Belmont, CA, USA). Antibody against MAPK was obtained from Zymed (San Francisco, CA, USA). [γ-^{32}P]ATP (adenosine triphosphate) was purchased from Amersham (Amersham, UK). The NaHS stock solution (1 M) was freshly prepared on the day of the experiment by the reaction of 1 M HCl and 1 M Na_2S. For the purpose of achieving the final concentration of NaHS of 5×10^{-4} M, 2.5 $\times 10^{-3}$ ml NaHS stock solution (1 M) was added into the culture solution (5 ml of DMEM) of VSMCs. For the purpose of achieving a final concentration of NaHS of 5×10^{-5} M and 10^{-4} M, 2.5×10^{-4} ml and 5×10^{-4} ml NaHS stock solution (1 M) were added into the culture solution (5 ml of DMEM) of VSMCs, respectively. At 37°C, the concentration of H_2S of the solution was very stable below 1 mM.[9,11]

Cell culture

Male SD rats, weighing 200–220 g, were killed by anesthetic overdose (intraperitoneal injection of pentobarbital sodium). The thoracic aorta was stripped of adventitia and removed to culture media in a sterile manner. Vascular smooth muscle cells were isolated and cultured according to the method of Hirata et al.[12] Briefly, the cells were maintained in DMEM containing 5 mmol/l glucose, 10% FBS, and antibiotics in a CO_2 incubator at 37°C. Subcultures of VSMCs from four passages were used in the experiments and the cells were serum-starved for 24 h in serum-free DMEM before each experiment. The cells were divided into six groups according to the different treatments and each group included at least six wells: (1) control group where cells were continuously cultured in serum-free DMEM; (2) serum group where 10% FBS was added into the medium; (3) endothelin group[13] where 10^{-7} mol/l endothelin was added; (4) NaHS groups where 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/l of NaHS were added, respectively; (5) serum + NaHS groups where in the presence of 10% FBS, 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/l of NaHS were added, respectively; and (6) endothelin + NaHS groups, where in the presence of 10^{-7} mol/l endothelin, 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/l of NaHS were added, respectively. The experiments were terminated after incubation for 24 h. Because the concentration of NaHS was far below 1 mmol/l, we considered the concentration of H_2S stable in the culture solution within 24 h.

[^{3}H]Thymidine incorporation

The cells were labeled with [^{3}H]thymidine ([^{3}H]TdR) (1 μCi/ml) 6 h before the completion of incubation. The medium was removed, and the cell minilayers were washed sequentially with ice-cold phosphate-buffered saline (PBS) three times and once with 10% ice-cold trichloroacetic acid (TCA) at the end of the experiments. The cells were then solubilized in a solution of 200 μl of 0.1% SDS/0.1 N NaOH. Each 100 μl of cell solution was added to 5 ml of scintillation fluid, and the incorporation of [^{3}H]TdR into the VSMCs was determined by liquid scintillation spectrometry. Incorporation was expressed as dpm per mg protein.

Measurement of MAPK activity of VSMCs[14]

The cells were rinsed with ice-cold PBS containing 1 mmol/l sodium orthovanadate (a nonspecific tyrosine phosphatase inhibitor) and then frozen quickly in liquid nitrogen after the addition of 300 μl of lysis buffer. The dishes were thawed in ice, sonicated, and centrifuged at 16000 $\times$ *g* at 4°C for 20 min. Then, 50 μl of the resulting supernatant was immunoprecipitated with rabbit polyclonal anti-MAPK antibody (2 μg) for 2 h at 4°C, followed by the addition of 50 μl of protein A Sepharose (50% v/v). The anti-MAPK antibody recognizes both ERK-1 and ERK-2 isoforms of MAPK. The incubation continued for 1 h at 4°C. The immunoprecipitates were washed three times with 1 ml of lysis buffer and twice with the kinase buffer, then suspended in 45 μl of kinase buffer. The reaction lasted for 20 min at 30°C after the addition of 5 μl of [γ-^{32}P]ATP (adenosine triphosphate) (50 μM, 1 μCi/assay). When the reaction stopped, an aliquot of the reaction mixture was transfered to Whatman paper, which was washed five times with phosphoric acid and once with ethanol, and radioactivity incorporated into myelin basic protein was counted. Blank samples were substituted with buffer. Mitogen-activated protein kinase activity was counted with pmol/min per mg protein.

Other assays

Cellular viability was assayed with the trypan blue dye-exclusive test. Proteins in the cells were quantitated by the Bradford technique.

Statistics

The results were expressed as mean ± standard deviation. Analysis of variance was used to compare the mean values in various groups. Post hoc analysis was used to compare the data between different groups. A P value of less than 0.05 was considered statistically significant.

Results

Cell viability

The viability of the VSMCs was good, and there was no difference in viability among the groups ($n = 8$; $P > 0.05$) (Table 1). The concentration of NaHS we used in the experiment was 5×10^{-4}, which is the top concentration we used in this study.

Effect of H_2S on endothelin-induced VSMC proliferation

Different dosages of NaHS (5×10^{-5} mol/l, 1×10^{-4} mol/l, and 5×10^{-4} mol/l) did not change VSMC incorporation of [^{3}H]TdR in the absence of either endothelin-1 or FBS ($n = 6$; $P > 0.05$) (Fig. 1).

Vascular smooth muscle cell incorporation of [^{3}H]TdR in the serum + NaHS groups was markedly reduced compared with that of the serum group. Hydrogen sulfide at 5×10^{-5} mol/l, 1×10^{-4} mol/l, and 5×10^{-4} mol/l decreased VSMC [^{3}H]TdR incorporation significantly by 12.2%, 26.60%, and 35.7%, respectively ($n = 6$; $P < 0.01$). The inhibition of VSMC ^{3}H-TdR incorporation by NaHS in the presence of FBS occurred in a dose-dependent manner (Fig. 2).

Vascular smooth muscle cell incorporation of [^{3}H]TdR in the endothelin + NaHS groups was markedly reduced compared with that of the endothelin group; 5×10^{-5} mol/l, 1×10^{-4} mol/l, and 5×10^{-4} mol/l H_2S decreased VSMC [^{3}H]TdR incorporation by 16.8%, 26.60%, and 37.40%, respectively ($P < 0.05$ or $P < 0.01$). The inhibition of VSMC [^{3}H]TdR incorporation by NaHS was dose-dependent in the presence of endothelin-1 ($n = 6$; $P < 0.05$) (Fig. 3).

Table 1. Cell viability in different groups (%)

Group	Cell viability (%)
Control	97.1 ± 3.0
10% FBS	98.6 ± 3.1
ET (10^{-7} mmol/l)	95.8 ± 3.6
NaHS (5×10^{-4} mmol/l)	96.8 ± 2.9
Serum + NaHS (5×10^{-4} mmol/l)	97.1 ± 3.0
ET + NaHS (5×10^{-4} mmol/l)	98.3 ± 3.3

Values are mean ± standard deviation. No statistical significance was noted among the groups ($n = 6$) ($P > 0.05$)
FBS, fetal bovine serum; ET, endothelin

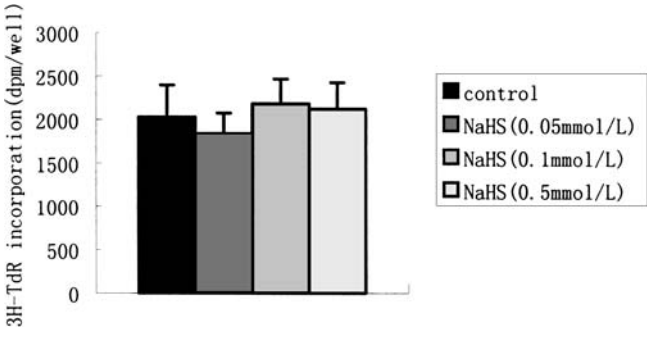

Fig. 1. Effect of H_2S on proliferation of vascular smooth muscle cells (VSMCs) in the absence of endothelin. The values induced by NaHS were not significantly different from those of control. *TdR*, thymidine

Effect of H_2S on VSMC MAPK activities

Different dosages of NaHS (5×10^{-5} mol/l, 1×10^{-4} mol/l, and 5×10^{-4} mol/l) did not change VSMC MAPK activities in the absence of either endothelin-1 or FBS ($n = 6$; $P > 0.05$) (Fig. 4). Cellular MAPK activity in the serum + NaHS groups was markedly reduced compared with that of the serum group; 5×10^{-5} mol/l, 1×10^{-4} mol/l, and 5×10^{-4} mol/l H_2S decreased VSMC MAPK activity significantly by 15.7%, 24.5%, and 34.6%, respectively ($n = 6$; $P < 0.01$). The inhibition of VSMC MAPK activity by NaHS in the presence of FBS was dose-dependent ($n = 6$; $P < 0.01$) (Fig. 5).

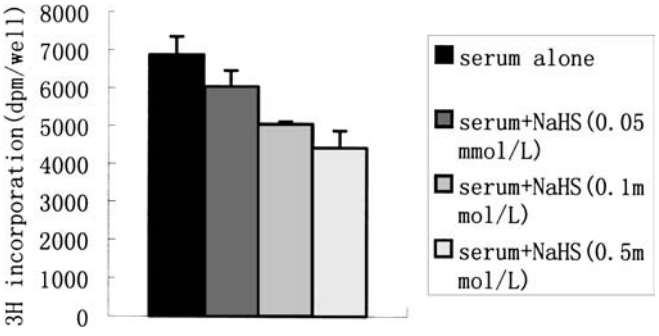

Fig. 2. Effect of H_2S on the proliferation of fetal bovine serum (FBS)-treated VSMCs. The values induced by NaHS were significantly different from those of control (even when the concentration of NaHS was as low as 0.05 mmol/l). The inhibition of VSMC [^{3}H]thymidine ([^{3}H]TdR) incorporation by NaHS in the presence of FBS occurred in a dose-dependent manner ($P < 0.05$)

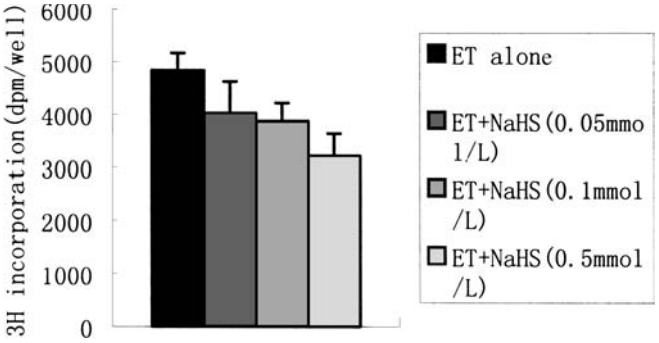

Fig. 3. Effect of H_2S on proliferation of endothelin (*ET*)-treated VSMCs. The values induced by NaHS were significantly different from those of control (even when the concentration of NaHS was as low as 0.05 mmol/l). The inhibition of VSMC [^{3}H-TdR] incorporation by NaHS in the presence of ET occurred in a dose-dependent manner ($P < 0.05$)

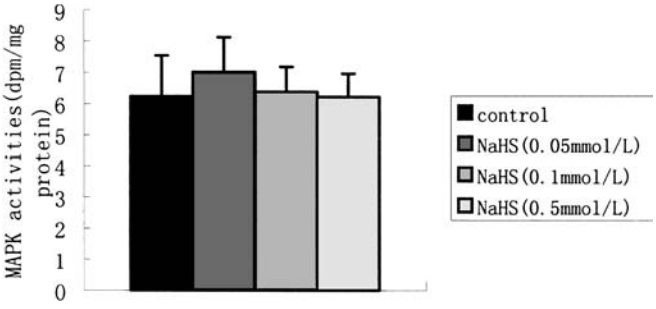

Fig. 4. Effect of H_2S on the activity of mitogen-activated protein kinase (*MAPK*) in the absence of endothelin. The values induced by NaHS were not significantly different from those of the control ($P > 0.05$)

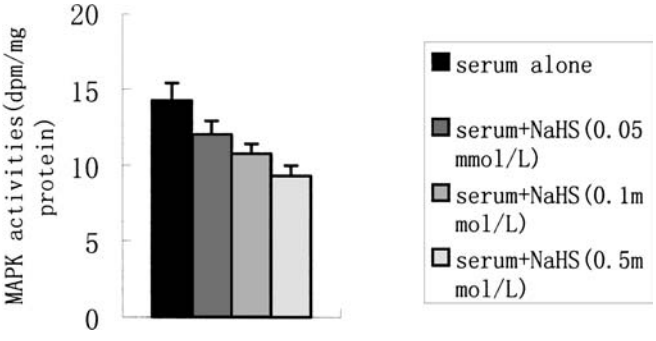

Fig. 5. Effect of H_2S on MAPK activity. The values induced by NaHS were significantly different from those of control (even when the concentration of NaHS was as low as 0.05 mmol/l). The inhibition of VSMC MAPK activity by NaHS in the presence of FBS occurred in a dose-dependent manner ($P < 0.01$)

Cellular MAPK activity in the endothelin + NaHS groups was markedly reduced compared with that of the endothelin group; 5×10^{-5} mol/l, 1×10^{-4} mol/l, and 5×10^{-4} mol/l NaHS decreased cellular MAPK activities by 7.4% ($P > 0.05$), 23.4%, and 33.6%, respectively ($n = 6$; $P < 0.01$). The inhibition of VSMC MAPK activity by NaHS at a concentration of 5×10^{-5} mol/l was not significantly different from that of the FBS control ($P > 0.05$), while the inhibition of VSMC MAPK activity by NaHS at a concentration of 1×10^{-4} mol/l and 5×10^{-4} mol/l in the presence of FBS occurred in a dose-dependent manner ($P < 0.05$) (Fig. 6).

Discussion

Gasotransmitters have led biomedical study into a new stage. NO was first demonstrated as an endogenous gasotransmitter in the late 1980s. It is generated from L-arginine by NO synthase, and has been demonstrated to interfere with many key events, such as vasorelaxation, suppression of cell proliferation, and inhibition of platelet aggregation. Studies by American scientists that confirmed NO as an endogenous gasotransmitter even won a Nobel Prize in 1998. Just a few years later, another sort of gas, CO,

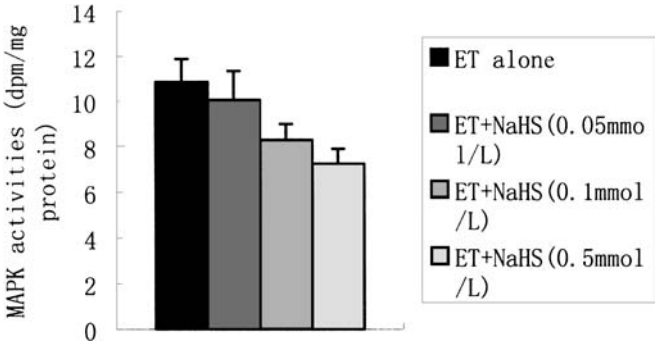

Fig. 6. Effect of H_2S on MAPK activity. The values induced by NaHS (0.05 mmol/l) were not significantly different from those of control, while the values induced by NaHS (0.1 mmol/l) and NaHS (0.5 mmol/l) were significantly different from those of control. The inhibition of VSMC MAPK activities by NaHS in the presence of endothelin (*ET*) occurred in a dose-dependent manner ($P < 0.01$)

which was though to be a toxic gas for many years, was also considered as an endogenous gasotransmitter.[15] Interestingly, CO has similar physiological functions to those of NO in the modulation of vasorelaxation and cell proliferation.[16] Further studies on NO and CO deepened understanding of the underlying mechanisms of a variety of diseases considerably.

In the 1990s, H_2S was found to be a new endogenous gasotransmitter existing in the body as a gas (about one third) and/or NaHS (about two thirds), and a proper balance is kept between the two states.[9] Then, H_2S was first reported as a neuromodulator in the brain because of its essential role in the induction of hippocampal long-term potentiation at physiological concentrations.[17] Hydrogen sulfide was also demonstrated as a vasorelaxant, but it was generated from cysteine in a reaction catalyzed mainly by cystathionine γ-lyase (CSE) in vessels. As we know, vasoconstriction and the structural remodeling of blood vessels are essential processes of many vascular diseases and the proliferation of VSMCs is one of the most important parts of vascular structural remodeling. Nitric oxide and CO have been shown to have great importance in inhibition of vascular structural remodeling.[18] Whether H_2S also interferes with the proliferation of VSMCs similarly to NO and CO is a very important problem to be solved.

Our present study was based on a rat model of cultured thoracic aortic VSMCs in vitro. Vascular smooth muscle cell viability was first assayed with the trypan blue dye-exclusive test and there was no statistical significance among the groups regardless of the various media used.

NaHS was used as a source of H_2S for the following reasons.[9] (1) NaHS dissociates to Na^+ and HS^- in solution, then HS^- associates with H^+ and produces H_2S. It does not matter whether the H_2S solution is prepared by bubbling H_2S gas or by dissolving NaHS. (2) The use of NaHS enables us to define the concentrations of H_2S in solution more accurately and reproducibly than bubbling H_2S gas. The final concentrations of NaHS were from 5×10^{-5} to 5×10^{-4} M, and the concentrations of NaHS were equal to those of NaCl during the reaction of 1 M HCl and 1 M Na_2S. The

increase in concentration of NaCl accompanying the NaHS we added were from 5×10^{-5} to 5×10^{-4} M, which were much lower than the physical concentration of NaCl that the cells were used to. Therefore we think the change in NaCl is too small to be considered. For these reasons, NaHS has been widely used for studies of H_2S.[10,11] The H_2S-saturated solution is 0.09 M. It has been proved that at 37°C, the stability of H_2S in solution varies depending on the initial H_2S concentrations. At the highest concentration tested (1 mM), a drop in the H_2S concentration of around 15% was observed, while at 0.01 mM, no obvious drop occurred.[10,11] Since the concentrations we used were 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/l, we think the concentrations of H_2S in the solution were relatively stable during the 24 h.

Some interesting phenomena happened when further H_2S was added. The proliferative level of the VSMCs was reduced significantly along with the different dosages we used in the serum + NaHS and ET + NaHS groups. Briefly, when three doses of H_2S were added to three groups of cells separately, resulting in three different concentrations of H_2S (5×10^{-5}, 1×10^{-4}, and 5×10^{-4} M) in the culture medium, the incorporation of [^{3}H]TdR, which we used to represent the proliferative level of VSMCs, showed a dose-dependent reduction in both the serum + NaHS and the ET + NaHS groups. The physiological serum concentration of H_2S in rat was determined to be $4.56 \pm 1.42 \times 10^{-5}$ M by Zhao et al.,[10] and the concentration of H_2S in the tissue was thought to be higher than that of serum. We thus suspected that H_2S could suppress the proliferation of VSMCs even at normal concentrations.

In this study, we also explored the effect of H_2S on the possible signaling transduction pathway mediating cellular mechanism of proliferation. The MAPK cascade is one of the most common pathways in cells, and includes three parallel cascades, i.e., the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) cascade, the stress-activated p38-MAPK cascade, and the classical extracellular signal-regulated kinase (ERK)/MAPK cascade. Previous studies have shown that NO activated all three pathways, especially ERK, in the modulation of proliferation. Then, does H_2S also exert an inhibitory effect on VSMC proliferation through ERK? We measured the MAPK (The anti-MAPK antibody recognizes both ERK-1 and ERK-2 isoforms) activity of VSMCs and found that in the absence of endothelin, there was no difference in MAPK activity. However, when the incorporation of [^{3}H]TdR increased steeply in the presence of either serum alone or endothelin alone, the activity of MAPK increased accordingly, which indicates that MAPK is involved in the mediation of endothelin-induced proliferation of these VSMCs. Moreover, when the proliferative level of the VSMCs was inhibited with different dosages of NaHS we used in the serum + NaHS and ET + NaHS groups, MAPK activity decreased accordingly. The MAPK activity was significantly inhibited by the addition of NaHS at concentrations of 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} M ($P < 0.01$), in the serum + NaHS group in a dose-dependant manner. In the ET + NaHS group, the MAPK activity did not decrease remarkably at a concentration of 5×10^{-5} M ($P > 0.05$), while inhibition of MAPK activity by NaHS was significant at concentrations of 1×10^{-4} and 5×10^{-4} M ($P < 0.01$) in a dose-dependent manner. We demonstrated that H_2S could possibly suppress the proliferation of VSMCs through the MAPK pathway.

In conclusion, our observations suggest that endogenous H_2S is not only a vasorelaxant, as reported before, but also a novel inhibitor that dose-dependently suppresses the proliferation of VSMCs through the MAPK pathway. This suggests that H_2S is probably a vital modulator in cardiovascular physiology and pathophysiology. Further studies are needed to fully understand its biological significance and action mechanisms in detail.

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