Effects of H$_2$S on Myogenic Responses in Rat Cerebral Arterioles

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Background: The potential biological significance of hydrogen sulfide (H$_2$S) has attracted growing interests in recent years, but its role in the myogenic response of rat cerebral arterioles has not been explored.

Methods and Results: Rats were injected with NaHS (an H$_2$S donor, 2–200 μmol·kg$^{-1}$·day$^{-1}$, i.p.) or saline for 3 weeks. MBP was measured with a tail-cuff method. Cerebral arterioles were isolated and cannulated in an organ bath system, and vessel diameters were measured with an image-shearing device. Changes in diameter in response to stepwise increases in intravascular pressure (20–120 mmHg) were investigated under no-flow conditions. After the treatments, plasma H$_2$S increased and MBP decreased significantly. NaHS reduced the myogenic response in a dose-dependent manner. This effect was markedly attenuated by glibenclamide, a K$_{ATP}$ channel blocker. Blockade of nitric oxide (NO) production with NG-nitro-L-arginine methyl ester (L-NAME, a NO synthase inhibitor) enhanced, whereas removal of the endothelium abolished the inhibitory role of NaHS on the myogenic response.

Conclusions: For the first time it has been demonstrated that H$_2$S decreases the myogenic response of cerebral arterioles in vivo, and this effect is endothelium-dependent and partially mediated by K$_{ATP}$ channels. (Circ J 2012; 76: 1012–1019)

Key Words: Cerebral arterioles; Hydrogen sulfide; Myogenic reactivity

The vascular myogenic response is the rapid and maintained constriction of a blood vessel in response to pressure elevation. At physiological pressure levels, the vascular myogenic response produces a tonic arteriolar constriction necessary for the action of vasodilators. The myogenic response participates in local regulation of blood flow and protects dependent capillary beds from the large increases in hydrostatic pressure induced by postural changes. The myogenic responses are generated exclusively by vascular smooth muscle, the tone generated by this mechanism can be modulated by factors released from the endothelium and other tissues.

Hydrogen sulfide (H$_2$S) has been best known for decades as the toxic gas dubbed “rotten egg gas.” Recently, H$_2$S has been recognized as a novel gasotransmitter alongside nitric oxide (NO) and carbon monoxide. In the cardiovascular system of intact rats, H$_2$S regulates vascular tone by relaxing blood vessels in vitro and decreasing blood pressure (BP). Zhao et al also demonstrated that application of NaHS, a H$_2$S donor, induces vasodilation of rat aortic rings in a concentration-dependent manner starting from 60 μmol/L. The latest research has shown that H$_2$S is a dilator of the newborn cerebral circulation and that endogenous cystathionine γ-lyase (CSE) can produce sufficient H$_2$S to decrease vascular tone. Despite the finding that H$_2$S can cause vasorelaxation by activating ATP-sensitive K$^+$ channels (K$_{ATP}$ channels), the precise signaling mechanism for the effect of H$_2$S on vascular myogenic response is still unclear.

Because H$_2$S induces vasodilatation, we hypothesized that prolonged exposure to H$_2$S would alter K$_{ATP}$ channel-dependent responses in cerebral arterioles. To this end, we aimed to study the effect of H$_2$S on the myogenic response and mechanism of cerebral arterioles in rats.

Methods

Animals
Male SD rats (age: 3 months) were used. Approval for the...
experiments was obtained in advance from the Animal Ethics Committee of Xuzhou Medical College. Rats were randomly assigned to 4 groups: Control (rats treated with physiological saline 0.5 ml/kg · day⁻¹ i.p., n=6), NaHS-2 (rats treated with NaHS, a donor of H₂S, 2 μmol · kg⁻¹ · day⁻¹ i.p., n=6), NaHS-20 (rats treated with NaHS 20 μmol · kg⁻¹ · day⁻¹ i.p., n=6), NaHS 200 (rats treated with NaHS 200 μmol · kg⁻¹ · day⁻¹ i.p., n=6). NaHS was dissolved in physiological saline and the entire volume of NaHS was injected intraperitoneally at the same time each day for 3 weeks. The animals were housed in a temperature-controlled (23±2°C) room with a 12:12-h light-dark cycle. Water and rat chow were provided ad libitum. Normal rats were used when to explore the mechanism of H₂S in the effect on myogenic response. NaHS was used as a source of H₂S for the following reasons: it dissociates to Na⁺ and HS⁻ in solution, then HS⁻ associates with H⁺ to produce H₂S. In physiological saline, approximately one-third of the H₂S exists as the undissociated form (H₂S), and the remaining two-thirds exists as HS⁻ at equilibrium with H₂S.⁵,¹⁰

**Measurement of BP**

The mean BP (MBP) was measured by the tail-cuff method using an automatic sphygmomanometer (Softron BP-98A; Softron, Tokyo, Japan). Before each measurement, rats were placed in a cage and subsequently warmed at 37°C for 5 min.

**Isolation of Arterioles**

The rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.) and euthanased by decapitation. The brain was carefully removed and placed in cold (0–4°C) physiological salt solution (MOPS pH 7.4, 4°C) comprising (mmol/L) NaCl, 142; KCl, 5.0; CaCl₂, 2.0; MgSO₄, 1.2; NaH₂PO₄, 1.2; glucose, 5.0; pyruvate, 2.0; EDTA, 0.2; 3-N-morpholino)propanesulfonic acid, 3.0. The cerebral arterioles (branch of middle cerebral artery, 100–200 μm in diameter and 2–3 mm in length) were then isolated. Each vessel was used for only 1 experimental protocol. The vessels were cannulated in a perfusion chamber (3 ml) and perfused with MOPS (pH 7.4, 37°C). All side branches along the vessels were carefully ligated to ensure no leakages. The intravascular pressure was then increased to 80 mmHg, and the diameter of the vessel recorded (passive diameter, PD80) when the vessel achieved their maximum diameter. After equilibration at 37°C for 60 min, the arterioles spontaneously reduced to their resting state diameter, which was recorded as the basal diameter (BD80). Basal tone (BT) was recorded as the basal diameter (BD80) and the diameter of the vessel recorded (passive diameter, PD80) when the vessel achieved their maximum diameter. After equilibration at 37°C for 60 min, the arterioles spontaneously reduced to their resting state diameter, which was recorded as the basal diameter (BD80). Basal tone (BT) was calculated by the formula: BT=(PD80–BD80)/PD80×100%.

**Removal of the Endothelial Cells**

The endothelium of the arterioles was removed by perfusion of the vessels with air, as previously described in detail.⁴³ The arteriole was tied proximally to a pipette, and air (approximately 1 ml) was injected through the pipette into the lumen for 1 min. Then the arteriole was tied to a distal pipette and perfused with MOPS (pH 7.4, 37°C) for 20 min at a pressure of 20 mmHg to clear the debris. The outflow stopcock was then closed and the perfusion pressure raised to 80 mmHg for 30 min to achieve a stable tone. At this pressure the efficacy of endothelial denudation was ascertained by testing the arteriolar responses to acetylcholine (300 μmol/L), an endothelium-dependent vasodilator, after the administration of the air bolus. After removal of the endothelium, the vessels were equilibrated at 37°C for 60 min.

**Pressure-Induced Myogenic Response**

In all protocols, the BD of the arterioles was measured in the presence of 20, 40, 60, 80, 100 and 120 mmHg perfusion pressure and under no-flow conditions. Vessels were equilibrated at 80 mmHg for 60 min. After that, the pressure was returned to 20 mmHg and then increased in 20 mmHg steps to 120 mmHg. Each pressure step was maintained for 5–10 min to allow the vessels to reach a stable diameter. All active and passive diameters at different pressures were normalized by the formula: Di=di/PD80×100%, where di is the internal diameter of arterioles, and PD80 is the diameter in Ca²⁺-free solution at 80 mmHg.

**Mechanism of H₂S Effect on Myogenic Response**

To explore the mechanism of H₂S on the myogenic response, isolated arterioles of normal rats was incubated with NaHS in the presence or absence of glibenclamide (a specific inhibitor of the KATP channel), NG-nitro-L-arginine methyl ester (L-NAME, a specific inhibitor of eNOS), SQ22536 (a specific adenylyl cyclase inhibitor of cAMP), or denudation of the endothelium. After equilibration of the vessel at 80 mmHg for 60 min, vessels were exposed to glibenclamide (10 μmol/L), L-NAME (300 μmol/L) or SQ22536 (100 μmol/L) for 30 min, then NaHS (60 μmol/L) for 5–10 min before changes in diameter in response to stepwise increases in perfusion pressure were assessed; The vessels with endothelial denudation were equilibrated at 37°C for 60 min, subsequently treated with NaHS (60 μmol/L) for 5–10 min directly, then the pressure-diameter relationships were assessed.

**Passive Diameter**

At the end of each experiment, the perfusion solution was changed to a Ca²⁺-free solution containing 1 mmol/L EGTA. The vessels were incubated for 10 min, then the stepwise increase in pressure was repeated, and the PD of the arterioles at each pressure step was obtained.

**Myogenic Tone Index (MTI) and Myogenic Response Index (MRI) in Control and NaHS Groups**

We defined myogenic tone as the difference in vessel diameter in the presence and absence of calcium for a changed pressure. The degree of myogenic tone was quantified using the MTI:¹² MTI=1–Dca/Dcaff, where Dca is the vessel inner diameter in calcium (2.0 mmol/L) MOPS at a particular pressure and Dcaff is the arteriolar inner diameter in calcium-free MOPS at the same pressure. The myogenic response, on the other hand, was defined as the contractile response elicited by a vessel to increasing pressure, which opposes the passive response elicited in the absence of calcium. The relative myogenic responsiveness among arterioles of different groups was estimated by calculating the MRI for each pressure step as described:¹²¹³ MRI=[100×(Di–Df)/Di]/{1/(Pf–Pi)}, where Di is the final inner diameter, Df is the initial inner diameter, Pf is the final pressure and Pi is the initial pressure.

**Measurement of Plasma H₂S Concentration**

A 75-μL sample of plasma from each group was diluted in deionized water (final volume, 500 μL) and added to tubes already containing zinc acetate (1% w/v, 250 μL) to trap H₂S. Subsequently, N, N-dimethyl-p-phenylenediamine sulfate (20 μmol/L; 133 μL) in 7.2 mol/L HCl was added, followed by FeCl₃ (30 μmol/L; 133 μL) in 1.2 mol/L HCl. Thereafter, trichloroacetic acid (10% w/v, 250 μL) was used to precipitate any protein that might be present in the culture media and after centrifugation (10,000×g) the absorbance (670 nm) of aliquots
from the resulting supernatant (300 μl) was determined using a 96-well microplate reader.14,15

Drugs and Chemicals
NaHS, L-NAME, glibenclamide and SQ22536 were purchased from Sigma Chemical Company (St Louis, MO, USA). All chemicals were dissolved in distilled water, except glibenclamide, which was dissolved in DMSO at a final concentration <0.1% (w/v). NaHS was prepared freshly before use.

Statistical Analysis
Data are expressed as means±SE, and n refers to the number of rats from which cerebral arterioles were isolated. Statistical significance was calculated by repeated-measures of ANOVA followed by the Tukey-Kramer post hoc test and Student’s t-test, as appropriate. Significance level was taken at P<0.05.

Results
Characteristics of In Vivo Administration of NaHS
Table shows the changes in body weight, MBP and plasma concentration of H2S after 3 weeks of the treatment. Compared with the Control group, treatment with different concentrations for 3 weeks obviously increased (P<0.05) the plasma level of H2S in the NaHS-20 and NaHS-200 groups, and decreased (P<0.05) the MBP in the NaHS-200 group.

Effect of NaHS on the BT of Rat Cerebral Arterioles
The isolated vessels were cannulated at 80 mmHg intravascular pressure, and the mean diameter of arterioles from the Control, NaHS-2, NaHS-20 and NaHS-200 groups was 173.0±12.8 μm, 149.3±8.9 μm, 179.6±9.2 μm and 170.4±6.7 μm, respectively. After equilibration at 37°C for 60 min, the arterioles came to resting state and the respective BD80 was 116.6±8.0 μm, 109.8±8.3 μm, 138.4±6.0 μm, 129.4±3.7 μm. The BT of isolated cerebral arterioles from the Control and NaHS groups was calculated and is shown in Figure 1. Compared with the BT of the Control group (32.5±1.0%), the NaHS groups (NaHS-2, NaHS-20 and NaHS-200) showed a decrease in BT to 26.8±1.4% (P<0.05), 22.7±2.4% (P<0.05), 23.8±2.3% (P<0.05), respectively. Decreased BT suggests that the vessel’s contractility had deteriorated.

Effect of NaHS on the Myogenic Response in Cerebral Arterioles
Changes in diameter were measured as the transmural pressure was increased between 20 mmHg and 120 mmHg. Figures 2A–D shows the normalized data for the diameters of the isolated cerebral arterioles from the Control and NaHS groups in response to stepwise increases of 20 mmHg in intraluminal pressure, between 20 and 120 mmHg, with calcium and calcium-free solution. There was a significant difference (Figures 2A–D, P<0.05) in vascular diameter in the presence and absence of calcium at all examined pressures, indicating the presence of active tone through the pressure range of 20–120 mmHg. In the Control group (Figure 2A), the pressure-induced tone manifested to the greatest degree between 40 and 100 mmHg. At pressures less than 40 mmHg, vascular diameter tended to increase with increasing pressure to some extent, but the ability to maintain a high degree of intrinsic tone was modest at pressures greater than 100 mmHg, as shown by the gradual increase in vascular diameter with further pressure increments. The myogenic responses of the NaHS-2 and NaHS-20 groups (Figures 2B,C) were similar to those of the Control group. However, the vascular diameters of the NaHS-200 group increased with increasing pressure to some extent between 20 and 120 mmHg.

In the absence of Ca2+, the pressure-PD relationship in the cerebral arterioles was also obtained (Figure 2), and the stepwise increases in intravascular pressure elicited continuous increases in the “PD”. There was a significant difference in vascular diameter in the presence and absence of calcium at all examined pressures, indicating the presence of active tone throughout the pressure range from 20 to 120 mmHg.

**Table.** Effect of NaHS on Body Weight, MBP and Plasma H2S Concentration In Vivo

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (g)</th>
<th>MBP (mmHg)</th>
<th>H2S (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>199.17±1.54</td>
<td>220.83±7.57</td>
<td>107.33±4.14</td>
</tr>
<tr>
<td>NaHS-2</td>
<td>203.33±2.11</td>
<td>219.17±15.99</td>
<td>24.24±3.07</td>
</tr>
<tr>
<td>NaHS-20</td>
<td>197.50±2.50</td>
<td>220.83±10.36</td>
<td>96.67±5.30</td>
</tr>
<tr>
<td>NaHS-200</td>
<td>199.17±2.01</td>
<td>220.83±10.15</td>
<td>46.11±6.74**</td>
</tr>
</tbody>
</table>

Data are means±SE. *P<0.05, compared with Control group; †P<0.05, compared with NaHS-2 group; ‡P<0.05, compared with NaHS-20 group.

MBP, mean blood pressure; H2S, hydrogen sulfide.
Figure 2. Effects of in vivo administration of NaHS on the myogenic response of cerebral arterioles. (A–D) Changes in diameter of cerebral arterioles in response to stepwise increases of intravascular pressure are compared in the Control and NaHS-treated groups. No additional NaHS was added to the perfusion solution. *P<0.05, active diameter vs. passive diameter (PD) at corresponding intravascular pressures. Comparison of the myogenic response (E) and myogenic tone index (F) among the cerebral arterioles of NaHS treated rats at different pressure ranges. †P<0.05, NaHS-200 group vs. Control group; ‡P<0.05, NaHS-200 group vs. NaHS-2 group; §P<0.05, NaHS-200 group vs. NaHS-20 group.
MTI and MRI in Cerebral Arterioles

A comparison of the MRI between the arterioles of the different groups at each pressure step is shown in Figure 2E. The MRI represents the change in diameter per mmHg for the stepwise changes in pressure. Between 20 and 40 mmHg, a positive MRI was displayed in all groups, indicating an absent or less developed myogenic response. At the ranges of 40–60 mmHg and 60–80 mmHg, there were stronger negative MRIs in the Control, NaHS-2 and NaHS-20 groups, suggesting a powerful myogenic response. However, the MRI of NaHS-200 group was positive, and gradually increased in the ranges of 40–60 mmHg, 60–80 mmHg and 80–100 mmHg.

Figure 2F depicts the MTI as a function of pressure for the arterioles from the different groups. The MTI in the NaHS-200 group was decreased significantly compared with that in the Control and NaHS-2 groups (P<0.05) at 80, 100, 120 mmHg, respectively. And the differences between the NaHS-200 and NaHS-20 groups existed only at 100 and 120 mmHg intravascular pressure.

Mechanisms for NaHS-Reduced Inhibition of Myogenic Response

To show the mechanism of NaHS in reducing the myogenic response, the following experiments were carried out. Isolated arterioles of normal rats were cannulated and equilibrated, then incubated with 60 μmol/L NaHS (final concentration in perfusion chamber, which can reduce the myogenic response, Figure 3) in the presence or absence of glibenclamide (a specific inhibitor of K\textsubscript{ATP} channel), L-NAME (a specific inhibitor of eNOS), SQ22536 (an adenylyl synthase inhibitor of cAMP), or denudation of the endothelium.

The myogenic contraction of cerebral arterioles was significantly reduced (P<0.05) when the isolated arterioles were incubated with 60 μmol/L NaHS, in response to stepwise increases of 20 mmHg intraluminal pressure, from 20 to 120 mmHg (Figure 3). At all pressures between 20 and 120 mmHg, vascular diameter tended to increase with increasing pressure to some extent, and did not manifest pressure-induced myogenic tone to the greatest degree from 40 to 100 mmHg.

Figures 4–7 show the NaHS-induced change in the myogenic response in the presence of K\textsubscript{ATP} channel inhibitor glibenclamide, eNOS inhibitor (L-NAME), cAMP synthesis inhibitor (SQ22536), and endothelium denudation, respectively. Incubation of cerebral arterioles with glibenclamide (10 μmol/L) for 30 min significantly reduced the NaHS-induced reduction in myogenic tone when pressure was increased from 20 to 120 mmHg (Figure 4A, P<0.05), whereas inhibiting the K\textsubscript{ATP} channels alone did not change the myogenic response (Figure 4B, P>0.05). These data indicate that H\textsubscript{2}S reduces the myogenic response of cerebral arterioles though opening of the K\textsubscript{ATP} channels.
Interestingly, incubation of cerebral arterioles with L-NAME (300 μmol/L) for 30 min further enhanced the effect of H₂S on the myogenic response (Figure 5A, P<0.05). This result suggests that there may be an interaction between NO and H₂S, and the existence of NO may interfere with the effect of H₂S on the myogenic response.

In contrast, pretreatment of arterioles with SQ22536 (100 μmol/L) for 30 min had no effect on the myogenic response to NaHS (60 μmol/L) when the pressure was increased from 20 to 120 mmHg (Figure 5B).

In addition, removal of the endothelial cells significantly reduced (P<0.05) the NaHS-induced vascular dilatation in the range of 40 to 120 mmHg, indicating that NaHS can attenuate myogenic tone via the endothelial cells (Figure 5C).

**Discussion**

In the present study, we examined the effects of NaHS on the cerebral arterioles’ response to altered intravascular pressure. The main findings of this study are: (1) isolated cerebral arterioles from rats are able to spontaneously decrease their diameter, in the absence of any drug, in response to increases in intravascular pressure; (2) NaHS attenuates the myogenic response; and (3) the effect of NaHS is mediated by K<sub>ATP</sub> channels and NO level in the vascular vessels.

In our experimental system, a change in blood flow in itself can significantly affect the diameter of microvessels, most likely via endothelial factors, such as NO, in response to changes in wall shear stress. To eliminate completely the pos-
sible effects of flow-related changes in vascular diameter, our study was designed to observe the effects of changes in pressure in the absence of flow.

Blood vessels constrict upon transmural pressure elevation and dilate upon reduction of transmural pressure. This behavior, termed the myogenic response, is inherent to smooth muscle and independent of neural, metabolic, and hormonal influences. The myogenic response is a fundamental property of small arteries and has been previously documented in both human and animal studies. In our study, a pressure-dependent active tone (constriction) developed in all cerebral arterioles of rats. This tone is thought to be associated with the membrane potential of vascular smooth muscle, which can be influenced by Ca2+. Lack of extracellular Ca2+ causing a passive dilation of arterioles in response to pressure, indicating an obligatory role for Ca2+ in pressure-dependent tone development of cerebral arterioles.

H2S can be produced from cysteine by pyridoxal-5’-phosphate-dependent enzymes, including cystathionine β-synthase and CSE. Recently, 3-mercaptopruvate sulfurtransferase along with cysteine aminotransferase were found, which can produce H2S from cysteine in the presence of α-ketoglutarate. H2S occurs natively in blood and other tissues. For example, the H2S concentration in rat serum is approximately 460 μmol/L and the amount of H2S produced in the brain has been reported to be as high as 50–160 μmol/L. Intravenous bolus injection of H2S will increase the plasma level of H2S and decrease the mean arterial BP. In the present study, we found that NaHS treatment for 3 weeks at doses of 2, 20, and 200 μmol kg−1 day−1 increased the plasma level of H2S and obviously decreased the mean arterial BP. In addition, NaHS treatments also caused an increase in the myogenic response and a decrease in the myogenic tone of cerebral arterioles.

In vitro, H2S induces a concentration-dependent relaxation of phenylephrine-precontracted rat aortic tissues and also directly increases the KATP channel current and hyperpolarized membrane in isolated vascular smooth muscle cells (SMCs). In our experiment, NaHS induced an immediate reduction in the myogenic response in vitro, which can be explained by the vascular relaxation effect of H2S. Previous studies claimed that H2S relaxes the vasculature through KATP channels and that H2S-induced vasorelaxation can be inhibited by glibenclamide. Our data support this and confirm that the KATP channel is not the only way that induces vasorelaxation simultaneously.

Our experimental results do not support that H2S relaxes the vasculature in synergy with NO. A recent study showed that L-NAME significantly increased myogenic tone. We found that inhibition of eNOS with L-NAME enhanced the NaHS-induced reduction of the myogenic response, indicating an interaction between H2S and NO and that this interaction may generates nitrosothiol, which exhibits little or non vasorelaxant activity.

The NO-induced vasorelaxation is mainly mediated by the cGMP pathway, but not the cAMP pathway. Quoth different from NO, H2S relaxes vascular tissues independent of the activation of the cGMP pathway. Another study reported that H2S facilitates hippocampal long-term potentiation by increasing the sensitivity of NMDA receptors following a rise in intracellular cAMP. But in our experiment, the cAMP pathway was not the mechanism of the H2S-induced decrease in the myogenic response. This experimental result is consistent with the report of Zhao et al., who showed that the H2S-induced relaxation of rat aortic tissues was caused mainly by direct interaction of H2S and SMCs, based on the failure of denervation of vascular tissues in vitro to alter the H2S effect and on the observation that H2S still significantly relaxed vascular tissues after endothelial removal. A previous study showed that the myogenic response can occur independently of the endothelium, but it can be regulated by endothelial substances. We presumed that H2S plays its role in regulating the myogenic response through the endothelium. Our experimental results are consistent with that presumption, because after endothelial cells was removed, the H2S-induced reduction of myogenic tone was attenuated. These results suggest that the endothelium plays an important role in regulating the myogenic response of cerebral arterioles. It well known that endothelium-derived hyperpolarizing factor plays a significant role in the microvasculature, in addition to NO. The possible mechanism explaining our result is that H2S acts as a hyperpolarizing factor, the effect of which is amplified by the endothelium; alternatively, endothelium-derived vasorelaxant factors might have been released by H2S.

Conclusions

Our results demonstrate for the first time that in vivo administration of NaHS decreased the myogenic response of cerebral arterioles, and this effect is endothelium-dependent and partially mediated by KATP channels. We believe that the endothelium plays a critical role in the H2S-induced reduction of the myogenic response, but its mechanism remains to be further elucidated.

Acknowledgment

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References


