Bicarbonate-dependent effect of hydrogen sulfide on vascular contractility in rat aortic rings

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Liu YH, Bian JS. Bicarbonate-dependent effect of hydrogen sulfide on vascular contractility in rat aortic rings. Am J Physiol Cell Physiol 299: C866–C872, 2010. First published July 21, 2010; doi:10.1152/ajpcell.00105.2010.—Hydrogen sulfide (H2S), an endogenous gaseous mediator, produces both vasorelaxation and vasoconstriction at different concentrations. We found in the present study that NaHS, an H2S donor, produced stronger vasorelaxant and weaker vasoconstrictive effects in HEPES solution compared with those achieved in Krebs solution. We further screened the buffer components and found that bicarbonate (HCO3−) was the ion to influence the effect of H2S. After examining the vasorelaxant effects of acetylcholine, a vasodilator by releasing nitric oxide, and isoprenaline, a β-adrenoceptor agonist, in HEPES and Krebs buffers, we found the HCO3−-dependent effect was specific to H2S. Blockade of anion exchanger-2 activity with 4,4’-disothiocyanatostilbene-2,2’-disulfonic acid (DIDS) or with HCO3−-free solution abolished the vasoconstrictive effect of NaHS. Moreover, NaHS decreased nitric oxide level in the rat aorta in HCO3−-containing buffer, but this effect was abolished by HCO3−-free buffer or DIDS. In summary, we found for the first time that H2S stimulates anion exchanger to transport extracellular HCO3− in exchange for intracellular superoxide anions, which may further inactivate nitric oxide and induces vasoconstriction.

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HYDROGEN SULFIDE (H2S) is now commonly regarded as the third gaseous transmitter besides nitric oxide (NO) and carbon monoxide. H2S can be synthesized endogenously in mammalian cells largely by the activities of two pyridoxal phosphate-dependent enzymes cystathionine γ-lyase (EC 4.4.1.1), cystathionine β-synthase (EC 4.2.1.22), and a newly discovered enzyme 3-mercapto-sulfurtransferase (EC 2.8.1.2). Expression of cystathionine β-synthase is predominant in the brain and nervous system, whereas cystathionine γ-lyase is mainly expressed in peripheral tissues such as the liver, kidney, heart, and blood vessels. 3-Mercapto-sulfurtransferase was found to contribute toward H2S formation in the brain and in vascular endothelium in the presence of cysteine aminotransferase (2, 21, 22). Rates of sulfide production in tissue homogenates are in the range of 1–10 pmol·s−1·mg protein−1 (5). Extracellular sulfide concentration was found to be in the low micromolar range and varies in different organs and tissues. The roles of H2S as gaseous mediator in nervous and cardiovascular systems have been widely studied in recent decades. The vasoregulatory effect of H2S was first reported by Hosoki et al. in 1997 (8). Subsequent reports showed that at higher concentrations (33–533 μM), H2S elicits vasorelaxation mainly by opening of KATP channels, which increases KATP current density, resulting in hyperpolarization of vascular smooth muscle cells (SMC) (25). Further studies showed that part of the relaxant effect is endothelium dependent, mediated by release of nitric oxide (NO) and/or endothelium-derived hyperpolarizing factor (EDHF) (4, 25). Our lab previously reported that induction of intracellular acidification due to activation of Cl−/HCO3− exchanger contributes to the relaxant response (14).

At lower concentrations (3.3–33 μM), H2S induces vasoconstriction. We found that H2S reduces cAMP level and hence produces vasoconstriction in vascular SMC (16). It has also been suggested that H2S may produce its vasoconstrictive effect by depleting NO, which is a potent vasodilator, to form nitrosothiol (1, 24) or by inhibiting production of NO by endothelial NO synthase (eNOS) directly (13). However, NO may also be inactivated by extracellular superoxide anions (O2·−), a short-lived reactive oxygen species (ROS) (18). We previously reported that H2S stimulates anion exchanger (AE2), which may transport HCO3− in exchange of O2·− (14). The latter may further inactivate NO. The present study was therefore designed to study the role of HCO3−-dependent inactivation of NO in the vasoregulatory effects of H2S.

EXPERIMENTAL PROCEDURES

Drugs and chemicals. Sodium hydrosulfide hydrate (NaHS), phenylephrine hydrochloride (PE), DIDS, isoprenaline (Iso), salbutamol, acetylcholine chloride (ACh), and 4,5-dihydroxy-1,3-benzendisulfonic acid disodium salt monohydrate (Tiron) were purchased from Sigma-Aldrich (St. Louis, MO). DIDS was dissolved in dimethyl sulfoxide (DMSO), salbutamol was dissolved in methanol. All other chemicals were dissolved in deionized water.

NaHS was used as a H2S donor and was freshly prepared on the day of every experiment. In normal physiological solution, one-third of NaHS exists as H2S and the other two-third exists as HS− (1). As used in numerous studies, NaHS has been widely used as a source of exogenous H2S.

Animals. All experiments on animals were approved by the animal ethics committee (IACUC) of National University of Singapore (NUS). Male Sprague-Dawley rats aged 6–8 wk old (250–300 g) were obtained from Centre for Animal Resources (CARE, Singapore). Animals were allowed to obtain food and water ad libitum before the experiment.

Measurement of rat aorta contractility. On the day of experiment, the rats were anesthetized with pentobarbital sodium (50 mg/kg ip) followed by administration of heparin (1,000 units). The rat chest and abdomen were opened through medical sternotomy, and aorta was excised and immediately placed in oxygenated (95% oxygen, 5% carbon dioxide) Krebs solution (composition in mM: 118 NaCl, 4.7 KCl, 1.3 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 1.7 glucose) or HEPES solution (composition in mM: 136 NaCl, 4.7 KCl,
1.3 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 10 HEPES, and 1.7 glucose). Both solutions had their pH adjusted to 7.4.

Connective and adipose tissues were trimmed, and aorta was cut into 1-mm thick rings. Aortic ring segments were mounted on two tungsten wire hooks with one immobilized in organ bath chamber and the other connected to a transducer. The bath chambers were filled with 10 ml of appropriate buffer solutions bubbled with 95% O₂ and 5% CO₂ maintained at 37°C. The segments were allowed to equilibrate for 1 h under a resting tension of 1.5–2.0 g. Any changes in tension were recorded through force transducers connected to a PowerLab system [AD Instruments, Bella Vista (NSW), Australia] running the Chart v5.1 software.

After 1 h equilibration, the vessel response was tested for maximal contraction with 1.68 μM PE. The PE was then washed out allowing the vessel to return to the initial basal tone before each experiment. Subsequently, the rings were precontracted with PE at a concentration of 200 nM or KCl at a concentration of 30 mM, which were found to produce ~70–80% of the maximum contraction (1.68 μM PE) in preliminary experiments (data not shown). KCl (30 mM) was prepared by equimolar reduction of the concentration of NaCl so as to maintain osmolarity of solution.

Two different types of experimental protocols were employed in this study. In the first protocol, KCl or PE precontracted rings were relaxed by NaHS, Iso, or ACh (concentrations were predetermined in preliminary experiments) in a specific buffer with designated buffer contents. When the relaxation response reached a stable state after ~5–10 min, the chambers were washed with another type of buffer with different contents and allowed tension to fall back to baseline. Precontraction and relaxation procedures were then repeated in this new buffer to study the role of buffer components in influencing aortic ring contractility. A third set of precontraction and relaxation procedures may be carried out in the first buffer to ensure good integrity of aortic rings throughout the time-course protocol. Results were shown as percent relaxation of each respective precontraction by KCl or PE.

Fig. 1. Buffer-dependent vasorelaxant effect of H₂S in rat aortic rings precontracted with phenylephrine hydrochloride (PE) (A) or KCl (B) in the absence (HEPES) or presence (Krebs) of HCO₃⁻. A and B: representative tracings and mean data showing percent relaxation. Values are means ± SE, n = 13–17. ***p < 0.001 vs. the respective values in the absence of HCO₃⁻. Vertical bar indicates tension scale (g). Horizontal bar shows time (min).
In the second protocol, PE-precontracted aortic rings were relaxed by 2 μM salbutamol. Upon reaching stable state, NaHS (5–50 μM) was added to the bath solution to study the constrictive effect of NaHS. Procedures were repeated either in varied buffer compositions or after incubation with anion exchanger inhibitor DIDS (100 μM) for 10 min. Results were shown as percentage of additional contraction with respect to relaxation induced by salbutamol.

Effect of ROS scavenger on vascular contractility. Aortic rings were equilibrated in organ bath chamber for 1 h, after which Tiron (10 mM) was added to the chambers before rings were precontracted with PE. Constrictive or relaxant responses induced by NaHS (50 and 200 μM, respectively) were juxtaposed in the presence or absence of Tiron.

NO measurements. After the rat aorta was excised, the adipose and connective tissues were trimmed off. The aorta was cut into 2-mm thick aortic rings and washed repeatedly with respective buffers to remove blood cells and blood clots. The aortic rings were weighed before being placed into the four-port closed chamber (World Precision Instruments, NOCHM-4) containing 1 ml buffer medium. A calibrated carbon fiber NO-selective electrode (ISO-NOP) was placed within the medium, and changes in NO levels were measured as redox current in picoamperes (pA) with the Apollo 4000 Free Radical Analyzer. NO readings were taken 15 min after addition of L-arginine (1 mM). The aortic rings were pretreated with NaHS (30 μM) or DIDS (100 μM) before the addition of L-arginine 10 or 15 min, respectively. NO concentrations were calculated from standard curve.

Statistical analysis. Data are shown as means ± SE with the number of experimental observations indicated in parenthesis. Unless otherwise stated, each experiment used 6 to 20 aortic rings from two to four rats. Statistical analysis was carried out using Student’s t-test for paired samples to compare mean values between two treatments in the same vascular ring; ANOVA followed by post hoc Bonferroni’s test for multiple comparisons unless otherwise stated. Statistical significance was set at $P < 0.05$.

RESULTS

Vasorelaxant effect of NaH$_S$ in rat aortic rings in different buffers. The vasorelaxant effects of NaH$_S$, an H$_2$S donor, in rat aortic rings were first observed and compared in HEPES and Krebs buffers. As shown in Fig. 1, NaH$_S$ induced greater relaxation in rat aortic rings in HEPES buffer than that observed in Krebs buffer. In PE (200 nM)-precontracted rings, NaH$_S$ induced relaxation by almost 100% in HEPES buffer but only by ~56% in Krebs buffer (Fig. 1A). Furthermore, in aortic rings precontracted by KCl (30 mM), NaH$_S$ relaxed aortic rings by nearly 90% in HEPES buffer but only by about 40% in Krebs buffer (Fig. 1B). These data implied that the buffer...
components in these two types of buffers have an influence over NaHS-induced vasorelaxation.

Vasorelaxant effect of NaHS in rat aortic rings in buffers with varying concentration of components. We further screened and investigated which buffer component(s) is/are responsible for the variable vasorelaxation caused by NaHS in different buffers.

To observe the involvement of Na\(^+\) or Cl\(^-\), NaCl concentration was increased from 118 to 136 mM in Krebs buffer [Fig. 2A,a] or decreased from 136 to 118 mM in HEPES solution [Fig. 2A,b]. As shown in Fig. 2A (a and b), there was no significant difference in the vasorelaxation induced by NaHS in varied NaCl concentrations.

The involvement of HEPES was also further examined. HEPES is a zwitterionic organic chemical buffering agent that maintains solution at physiological pH with maximum buffering capacity in the range of 7.2–7.6. As shown in Fig. 2A,c, the presence or absence of HEPES in Krebs buffer did not affect the vasorelaxation effect of NaHS. This suggested that HEPES neither contributed to the buffer-dependent effect of NaHS.

We further observed the effect of NaHS in the presence and absence of HCO\(_3^-\). Similar to HEPES in function, HCO\(_3^-\) maintains pH at physiological range. As shown in Fig. 2A,d and 2B, addition of 25 mM HCO\(_3^-\) into HEPES buffer significantly reduced the vasorelaxation induced by NaHS from ~35% to ~12% in KCl precontracted rings. The data suggested that HCO\(_3^-\), but not NaCl and HEPES, influenced the vasorelaxant effect of NaHS.

Specificity of the HCO\(_3^-\)-dependent vasorelaxant effect of NaHS. To investigate whether the HCO\(_3^-\)-dependent effect is specific to NaHS, we compared the vasorelaxant effects of Iso and ACh in Krebs and HEPES buffers. As shown in Fig. 2, both ACh (200 nM, Fig. 2C) and Iso (5 μM, Fig. 2D) induced vasorelaxation to the similar extent in both PE-precontracted aortic rings in both HEPES and Krebs buffers. These data suggest that the HCO\(_3^-\)-dependent effect is specific to NaHS.

HCO\(_3^-\)-dependent vasoconstrictive effect of NaHS in rat aortic rings. We also observed whether HCO\(_3^-\) can influence the vasoconstrictive effect of NaHS. Salbutamol, a specific β2-adrenergic agonist, reversed PE-induced precontraction by 66.78% and 64.22% in HCO\(_3^-\)-free and HCO\(_3^-\)-containing solutions, respectively (Fig. 3). NaHS (5–50 μM) concentration dependently reversed the vasorelaxant effect of salbutamol in HCO\(_3^-\)-containing buffer, which is consistent with published data (1, 16). However, this vasoconstrictive effect of NaHS was significantly abolished in HCO\(_3^-\)-free solution. These data suggest that HCO\(_3^-\) may enhance NaHS-induced vasoconstriction.

Vasoconstrictive effect of NaHS in the presence and absence of DIDS, an anion exchanger blocker. HCO\(_3^-\) are transported across cell membrane via anion exchangers (AE2). To examine the involvement of AE2, DIDS, an anion exchanger blocker, was employed. As shown in Fig. 4, incubation of aortic rings with DIDS (100 μM) for 20 min abolished the vasoconstrictive effects of NaHS (5–50 μM), which is very similar to what we observed in HEPES buffer where HCO\(_3^-\) are absent. These data
suggest that anion exchangers mediate the HCO₃⁻-dependent vasoregulatory effect of NaHS.

Vascular responses of NaHS in the presence and absence of Tiron, a superoxide scavenger. To further reaffirm the involvement of superoxide in NaHS-induced vascular responses, the effect of NaHS on aortic rings was assessed in the presence or absence of Tiron, a superoxide dismutase (SOD) mimic. As shown in Fig. 5, NaHS induced greater extent of relaxation response (NaHS at 200 μM) and lesser extent of vasoconstrictive response (NaHS at 50 μM) in the presence of Tiron, suggesting that superoxide play important role in mediating vasoregulatory effect of NaHS.

**NaHS regulates NO production in the aortic rings in different buffers.** The level of NO was recorded at real time under different buffer conditions and presence of drugs and inhibitors. As shown in Fig. 6, in the presence of L-arginine, a substrate of eNOS, NO level was significantly higher in HCO₃⁻-free buffer (HEPES) compared with that observed in HCO₃⁻-containing buffer (Krebs). NaHS (30 μM) significantly suppressed NO production in Krebs buffer but not in HEPES buffer. The amount of NO produced in the presence of NaHS was significantly higher in HCO₃⁻-free buffer than that in HCO₃⁻-containing buffer, implying that HCO₃⁻ are involved in lowering NO levels caused by NaHS. Furthermore, incubation of aortic rings with DIDS, an anion exchanger inhibitor, significantly increased NO production. DIDS also significantly reversed the NO-lowering effect offered by NaHS, suggesting that NaHS may decrease NO production via stimulation of HCO₃⁻ anion exchanger activity.

**DISCUSSION**

In the present study, we first observed the different effects of H₂S in HEPES and Krebs solutions. It was found that H₂S induced stronger vasodilatory effect in aortic rings precontracted by either KCl or PE in HEPES buffer than that in Krebs buffer. After screening the buffer components, we found that HCO₃⁻, but not sodium chloride and HEPES compounds, contributes to the buffer dependency effect of H₂S.

Two other types of vasodilators were used to study the specificity of the HCO₃⁻-dependent effect. Iso (β-adrenergic receptor agonist that induces vasodilation via stimulating adenylate cyclase/cAMP/PKA pathway) and ACh (stimulation of NO release via activating guanylyl cyclase/cGMP/PKG pathway) both induced similar extents of vasorelaxation in HCO₃⁻-free and HCO₃⁻-containing buffers, suggesting that the HCO₃⁻-dependent effect is specific to H₂S-induced vascular regulation.

HCO₃⁻ are not permeable to membranes and are therefore mainly transported by HCO₃⁻ transporters and exchangers. There are two main types of HCO₃⁻ transporter proteins, the first being anion exchangers (AE), such as Cl⁻/HCO₃⁻; and second type being Na⁺-dependent HCO₃⁻ transporters, in which Na⁺ ions are crucial for the function of these transport-
ers. As we have shown in this paper that the buffer-dependent effect of H2S was not significantly influenced by difference in NaCl concentration (Fig. 2), the HCO3−/H11002− transporter involved is therefore probably Na+/H11001− independent.

We continued to examine the effect of H2S on anion exchangers. AE2 is the predominant isoform found in aorta and other vascular tissues (3). Its main function is to exchange Cl−/H11002− for HCO3−/H11002−. We previously reported that H2S stimulates AE2 in vascular SMC and induces intracellular acidosis (14). In the present study, we found that blockade of AE2 with DIDS or HCO3−/H11002−-free buffer attenuated H2S-induced vasoconstrictions. These data suggest that AE2 may play important role in mediating effects of H2S.

AE2 was reported to transport extracellular HCO3− in exchange of intracellular O2− in vascular tissue (18). At physiological pH, O2−, as a free radical and a negatively charged species, is transported across biological membranes via anion channels or exchangers (6). Among these transporters, anion exchanger is the primary mechanism for the transport of O2− across cell membrane (10–12, 17, 19). Since H2S stimulates anion exchangers, greater amounts of O2− anions are transported out to the extracellular space where it reacts with NO to produce peroxynitrite (ONOO−) with a rate constant of $7 \times 10^9 \text{M}^{-1}\text{s}^{-1}$ (20). This is about three times faster than its dismutation by SOD in which the reaction has a rate constant of about $2.9 \times 10^9 \text{M}^{-1}\text{s}^{-1}$ (7). The formed ONOO− is further scavenged by H2S (23).

This is further confirmed by our experiments with Tiron, a ROS scavenger, and N-acetyl-l-arginine methyl ester (l-NAME), an NOS inhibitor. Tiron itself alone had no effect on nonprecontracted aortic vascular tension (data not shown). By scavenging superoxide, Tiron attenuated NaHS-induced vasoconstriction (at low concentration) but enhanced its vasorelaxant effects. In addition, we found previously that blocking the production of NO with l-NAME significantly attenuated the vasoconstrictive effects of H2S (16). These data confirmed that H2S may stimulate transportation of superoxide that react with NO to produce vasoconstriction.

Unfortunately, the level of O2− could not be determined in our study because H2S, as a strong reducing agent, interferes the redox reactions in most O2− assays. We therefore determined NO levels amperometrically using NO electrode attached to the free radical analyzer. The electrode allows us to determine NO level as low as 1 nM. It was found that blockade of AE2 with either HCO3−-free buffer or DIDS significantly increased NO level, implying that extracellular O2− is important in regulation of NO level. Consistent with this observation, NaHS decreased NO level, which was abolished by blockade of AE2. This is because NaHS stimulates the activity of AE2, which transports intracellular O2− out to react with NO and form ONOO−. The depletion of intracellular O2− caused by H2S-stimulated AE2 may further inhibit NO uptake in SMC, since NO uptake by SMC is positively dependent on the level of intracellular O2− in SMC (9). Our data confirmed that H2S...
may induce vasoconstriction via transporting O$_2^-$ out to inactivate NO. This is also supported by previous findings that pulmonary vasoconstriction is influenced by inactivation of NO with extracellular O$_2^-$ (18, 19).

In recent years, a lot of attention was given to the possible crosstalk or interactions between NO and H$_2$S. Extensive studies were carried out seeking not only to understand the physiological roles of these gasotransmitters alone but also their effects over other gasotransmitters. Some have found that NO and H$_2$S work in synergy to potentiate the effects of each other (8), whereas other scientists proposed that the presence of one gas attenuates the effect of other (1, 13, 24). More recently, Moore and colleagues (24) proposed a new terminology “gas-eous triumvirate” to address the gaseous transmitters (NO, CO, and H$_2$S). The authors believed that the combination and proportion of these gases is crucial for cellular and physiological functions (15). In the present study, we have showed that H$_2$S depletes NO level only in the presence of HCO$_3^-$ via the activation of AE2 proteins. The significance of this phenomenon is yet to be studied. The current study sheds new light with regard to the relationship between NO and H$_2$S.

In summary, we demonstrated for the first time that H$_2$S induced vascular responses is highly dependent on the presence of HCO$_3^-$. H$_2$S stimulates anion exchangers to transport HCO$_3^-$ in exchange of O$_2^-$ to inactivate NO and therefore induces stronger vasoconstriction and weaker vasodilation in HEPES (HCO$_3^-$-free) solution. Our study added insights to the understanding of vascular action brought about by H$_2$S.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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