The Cardiovascular Effects of Central Hydrogen Sulfide Are Related to $K_{ATP}$ Channels Activation

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Summary
Hydrogen sulfide (H$_2$S), an endogenous "gasotransmitter", exists in the central nervous system. However, the central cardiovascular effects of endogenous H$_2$S are not fully determined. The present study was designed to investigate the central cardiovascular effects and its possible mechanism in anesthetized rats. Intracerebroventricular (icv) injection of NaHS (0.17~17 μg) produced a significant and dose-dependent decrease in blood pressure (BP) and heart rate (HR) ($P<0.05$) compared to control. The higher dose of NaHS (17 μg, $n=6$) decreased BP and HR quickly of rats and 2 of them died of respiratory paralyse. Icv injection of the cystathionine beta-synthetase (CBS) activator s-adenosyl-L-methionine (SAM, 26 μg) also produced a significant hypotension and bradycardia, which were similar to the results of icv injection of NaHS. Furthermore, the hypotension and bradycardia induced by icv NaHS were effectively attenuated by pretreatment with the $K_{ATP}$ channel blocker glibenclamide but not with the CBS inhibitor hydroxylamine. The present study suggests that icv injection of NaHS produces hypotension and bradycardia, which is dependent on the $K_{ATP}$ channel activation.

Key words
Rat • Hydrogen sulfide • Blood pressure • Heart rate • Central

Introduction
Hydrogen sulfide (H$_2$S), which was originally considered as a toxic gas with the smell of rotten eggs (Reiffenstein et al. 1992, Beauchamp et al. 1984), has been found in most of tissues in mammalian and produces profound influences on nervous system (Eto et al. 2002, Kimura 2002), vascular (Beltowski 2004, Tang et al. 2005), and gastrointestinal smooth muscles (Teague et al. 2002, Gallego et al. 2008). It has been demonstrated that endogenous H$_2$S is produced from L-cysteine metabolism mainly by cystathionine beta-synthetase (CBS), cystathionine gamma-lyase (CSE), or 3-mercaptosulfur-transferase (MST) (Lowicka and Beltowski 2007, Yang et al. 2005). The vascular H$_2$S is mostly generated by CSE, while the central H$_2$S including brainstem is mainly produced by CBS from cysteine (Hosoki et al. 1997, Abe and Kimura 1996). The brainstem containing cardiovascular centers displays the greatest uptake of sulfide (Warenycia et al. 1989). Previous studies show that H$_2$S modulates vasodilatation by endothelium-dependent (Distrutti et al. 2006) and endothelium-independent mechanism (Wang 2002), but also regulates neuronal functions in the CNS, including the induction of hippocampal long-term potentiation.
(Hosoki et al. 1997, Abe and Kimura 1996, Eto et al. 2002) and the release of the corticotrophin-releasing hormone from the hypothalamus (Lowicka and Beltowski 2007, Boehning and Snyder 2003, Wang 2002). Therefore, H$_2$S has been proposed to be an endogenous "gasotransmitter" besides nitric oxide (NO) and carbon monoxide (CO) (Wang 2003, Laggner et al. 2007, Chen et al. 2007).

It has been found that H$_2$S contributes to cardiovascular regulation. For example, intravenous injection of H$_2$S induces a transient hypotension in anesthetized rats, which can be mimicked by the K$_{ATP}$ channel opener pinacidil and effectively antagonized by the K$_{ATP}$ channel blocker glibenclamide (Wang 2002, Zhao et al. 2001). In vitro, H$_2$S can relax aortic tissue or hyperpolarize membrane in isolated vascular smooth muscle cells (VSMC) (Tang et al. 2005, Wang 2002). In the central nervous system (CNS), H$_2$S induces a hyperpolarization and reduces an input resistance of CA1 neurons or dorsal raphe neurons in K$_{ATP}$ channel-dependant manner (Reiffenstein et al. 1992). Recently, Dawe et al. (2008) report that microinjection of NaHS into the hypothalamus reduces BP and HR in rats, which could be effectively antagonized by prior application of the K$_{ATP}$ channel blocker gliclazide. In the waked Wistar Kyoto rats, however, intracerebroventricular (icv) injection of NaHS produces a significant pressor effect (Ufnal et al. 2008). It is not clear whether this cardiovascular effect of icv H$_2$S is dependent on the K$_{ATP}$ channel activation. Hence, in the present study, the main aim was to determine the relationship between the central effect of H$_2$S and the functional state of the K$_{ATP}$ channel.

**Materials and Methods**

**General procedure**

Male Sprague-Dawley (SD) rats (weighing 200 to 250 g) were employed in this study. Each animal experimentation was in accordance with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda, or European Guidelines on Laboratory Animal Care. The methods for animal preparation, icv injection and histological procedures were similar to those described previously (Lu et al. 2005, Lu et al. 2007). In brief, rats were anesthetized with urethane (1.3 g/kg, i.p.). For direct measurement of BP, a catheter was inserted into the right femoral artery. BP was sequentially measured and displayed on a channel of a recording system (XJH, 2007, China) by a computer and HR was computed from the BP waveforms and displayed on another channel of the recording system. BP and HR were recorded continuously. Another catheter was inserted into right femoral vein for drug administration. Following tracheotomy, 30 rats (for determination of dose-dependent effects of NaHS or SAM) were spontaneously ventilated. The other rats (pretreatment with hydroxylamine, glibenclamide or vehicle) were paralyzed with triethiodide (10 mg/kg initially and 4 mg/kg every 30 min, i.v.) and artificially ventilated with oxygen-enriched room air. Adequacy of anesthesia was assessed by monitoring the stability of BP, and BP response to noxious stimulation. Body temperature was maintained at about 37 °C with an infrared heating lamp.

**Icv injection**

The rats were fixed on a stereotaxic frame (MP8003, China) and received a limited craniotomy. Icv injection was performed by a microsyringe (5 μl). The stereotaxic coordinates of lateral cerebral ventricle (LCV) were determined according to the Paxinos and Watson rat atlas (1.0 mm lateral to medial line, 1.5 mm caudal to bregma, and 4.5 mm deep from the bone surface). All chemicals were obtained from Sigma Corporation (America). NaHS, hydroxylamine and SAM was dissolved in artificial cerebrospinal fluid (aCSF, in mM: 133.3 NaCl, 3.4 KCl, 1.3 CaCl$_2$, 1.2 MgCl$_2$, 0.6 NaH$_2$PO$_4$, 32.0 NaHCO$_3$, and 3.4 glucose, pH to 7.4 by 0.5 M hydrochloric acid). The NaHS solution was strictly temporary prepared in an enclosed vital before microinjection, which made NaHS solution less dissociated. Glibenclamide was initially dissolved in dimethylsulfoxide (DMSO) and diluted with aCSF to the final concentration (the final percentage of DMSO in aCSF is not more than 1 %). The dose of NaHS, SAM, HA and glibenclamide was based on our preliminary experiment and previous studies (Dawe et al. 2008, Nishimura et al. 1995b, Nishimura et al. 1995a, Lin et al. 1999). The volume of drug injection was 5 μl, and delivered over a period of approximately 30 s. At the end of each experiment, 5 μl of 2 % Pontamin sky blue solution was injected into LCV to identify the injection area. The brain was removed and sectioned to determine the injection area. Histological examination revealed that the dye was correctly injected into the LCV in all experimental rats.

**Experimental protocol**

First, NaHS (0.17-17 μg), a donor of H$_2$S, was
injected into LCV in 19 rats to observe the dose-dependent effects of central H$_2$S. In another 7 rats, the cardiovascular functions of central NaHS were determined by increased the concentration of endogenous H$_2$S by icv application of the allosteric CBS activator SAM (26 μg). ACSF (5 μl) was injected (icv) in 4 rats as control. The responses to icv injection of NaHS, SAM, or aCSF were followed at least 1 hour. HA (n=7), an inhibitor of CBS, was prior respectively applied into LCV of rats, and NaHS (1.7 μg) was injected after 10 min, BP and HR response was followed at least 1 hour after NaHS injection to observe the H$_2$S central cardiovascular responses after CBS was inhibited. Furthermore, to determine whether the cardiovascular effects of central H$_2$S was mediated by K$_{ATP}$ channels (n=7), the K$_{ATP}$ channel blocker glibenclamide was prior icv injected, and NaHS (1.7 μg) was centrally applied after 10 min. The mixed solution of aCSF and DMSO (100:1, n=4) was applied as vehicle group.

**Statistical analysis**

All values are presented as mean ± SE. The magnitudes of the changes in mean arterial pressure (MAP) and HR at the different times after injection of agents were compared with a one-way repeated-measures ANOVA followed with the Newman-Keuls test for post hoc analysis was used when multiple comparisons were made. Pre- vs. post-injection comparisons in same animal were evaluated by Student's t-test. The criterion for statistical significance was set at $P<0.05$.

**Fig. 1.** The representative tracings showing the effects of injection (icv) of artificial cerebrospinal fluid (aCSF, 5 μl, A), hydrogen sulfide (NaHS, 0.17~17 μg, B-D) or S-adenosyl-L-methionine (SAM, 26 μg, E) on the blood pressure (BP) and heart rate (HR) response. The arrow point indicated the time point of icv injection of aCSF, NaHS or SAM.
Results

Effects of icv injection of NaHS or SAM on BP and HR

Fig. 1 presented the representative original tracings of BP and HR in response to icv injection of NaHS (0.17–17 μg), SAM (26 μg) or aCSF. Injection of aCSF did not change MAP (96±5 vs. 94±4 mmHg, P>0.05, n=4) and HR (481±30 vs. 461±43 beats per min (bpm), P>0.05, n=5). Central application of NaHS (0.17–17 μg) produced a significant and dose-dependent decrease in BP (0.17 μg: from 92±4 to 67±7 mmHg, n=7; 1.7 μg: from 89±4 to 49±4 mmHg, P<0.05, n=6) and HR (0.17 μg: from 440±8 to 382±8 bpm, P<0.05, n=7; 1.7 μg: from 449±8 to 376±16 bpm, P<0.05, n=6). The hypotension and bradycardia occurred 5 min after administration of NaHS, followed by a sustained decrease, and reached the nadir after 40 min. BP and HR didn’t return to the baseline levels within 60 min. Icv injection of NaHS (17 μg, n=4) produced rapidly hypotension (from 97±2 to 57±9 mmHg, P<0.05) and bradycardia (from 415±14 to 368±24 bpm, P<0.05, n=5). In 6 rats, 2 of them died of respiratory paralysis within 15 min because of no artificial ventilation promptly. The central cardiovascular effects of endogenous H₂S were further determined by application of SAM, an activator of CBS, into LCV of rats. Icv injection of SAM (26 μg, n=7) elicited a significant decrease in BP and HR, which was similar to those of icv NaHS. The hypotension (from 94±6 to 71±10 mmHg, n=8, P<0.05) and bradycardia (from 444±35 to 385±64 bpm, n=7, P<0.05) induced by icv injection of SAM also occurred 5 min after administration, followed a sustained decrease in BP and HR, and didn’t return to baseline within 60 min. The changes in MAP and HR in response to icv injection of NaHS or SAM were summarized in Fig. 2.

Effects of pretreatment with HA on the cardiovascular effects of icv injection of NaHS

Fig. 3 presented the representative original tracings of the effect of prior application of vehicle (aCSF, 5 μl, n=5) or the CBS inhibitor HA (0.7 mg, n=7) on the BP and HR responses to icv injection of NaHS. Pretreatment with aCSF neither altered the basal BP (92±4 mmHg vs. 97±8 mmHg, P>0.05) and HR (437±37 vs. 446±41 bpm, P>0.05) nor influenced the responses of BP (from 97±7 to 63±13 mmHg, P>0.05) and HR (from 446±41 to 416±36 bpm, P>0.05) to icv injection of NaHS (Fig. 4). Icv injection of HA produced a significant decrease in BP (from 93±3 to 76±5 mmHg, P<0.05) but didn’t influence HR (433±5 vs. 418±9 bpm, P>0.05). Prior icv injection of HA didn’t alter the BP (aCSF pretreatment: –28±12 vs. HA pretreatment: –21±9 mmHg, P>0.05) or HR (aCSF pretreatment: –30±10 vs. HA pretreatment: –45±28 bpm, P>0.05, Fig. 5) responses to icv NaHS. The influences of prior application of HA on the BP or HR response to NaHS were summarized in Fig. 5.

Effects of pretreatment with the Kₐ₅P channels blocker glibenclamide on the cardiovascular response to icv injection of NaHS

Fig. 4 presented the representative original tracings of the effect of prior application of vehicle (aCSF, 5 μl, n=5) or the Kₐ₅P channels blocker glibenclamide (0.5 μg, n=7) on the BP and HR responses to icv injection of NaHS. Icv injection of vehicle didn’t alter the basal BP (93±9 vs. 93±11 mmHg, P>0.05) and HR (465±28 vs. 462±22 mmHg, P>0.05), but also didn’t influence hypotension (from 93±11 to 79±18 mmHg, P>0.05) and bradycardia (from 462±22 vs. 410±49,
Central application of glibenclamide (0.5 μg, n=7) produced no significant influences on the basal BP (from 103±5 to 99±5 mmHg, P>0.05) and HR (437±39 vs. 435±36 bpm, P>0.05), but significantly decreased the hypotension (−14±9 vs. −5±4 mmHg, P<0.05) and bradycardia (−52±33 vs. 18±27 bpm, P<0.05) induced by icv injection of 1.7 μg NaHS (Fig. 5).

**Discussion**

In the present study, our important findings were: 1. central application of the endogenous H₂S donor NaHS or the activator of CBS SAM produced hypotension and bradycardia; and 2. the central cardiovascular effects of endogenous H₂S were dependent on the K_ATP channel activation.

In the present study, we found that icv application of NaHS (0.17~17 μg) produced a sustained and marked hypotension and bradycardia. It is known that the cerebral spinal fluid (CSF) of rat is about 250 μl. The final concentration of H₂S in CSF in present study is about 40-400 μmol/l, does not exceed twice of the physiological concentration level, under the lethal concentration of H₂S in the brain (Warenycia et al. 1989).
More recently, it is reported that the concentration of tissue free hydrogen sulfide is only on the order of 15 nM, which is very lower than the presently accepted values (Furne et al. 2008), implicating that H₂S might serve as an endogenously gaseous messenger in very low concentration. H₂S dissociates to H⁺ and HS⁻ in solution. In physiologic conditions (37 °C, pH 7.4), only a little of H₂S (less than one fifth) exists as the undissociated form (H₂S), and the remaining four fifths exist as HS⁻ plus a trace of S²⁻ at equilibrium with H₂S (Dombkowski et al. 2004, Webb et al. 2008). Although which active form of H₂S (H₂S, HS⁻, or S²⁻, the mix of free inorganic sulfides) has not been determined, Ondrias et al. (2008) assumed that HS⁻ (but not H₂S or S²⁻) is probably the active form of ‘H₂S’ because the effects of NaHS on stimulating NO release from NO donors depend on the pH. The higher dose (17 μg) produced obviously toxic responses because the rats died for respiratory inhibition if not artificial ventilation promptly. It is hypothesized that the hypotension and bradycardia of H₂S (0.17~17 μg) are the physiological responses rather than toxic responses. However, our results are different from the results reported by Ufnal et al. (2008). It may be due to following reasons: 1. In our study, the rats were anaesthetized; 2. NaHS was administrated by an bolus injection (20 mM) in our study, while it was administrated by continuously infusion (100~400 nM of NaHS/h) in Ufnal’s study (Ufnal et al. 2008). 3. The doses of NaHS used in Ufnal’s study (100~400 nM of NaHS/h) were significantly lower than...
those in our present study. In a thesis, Huang et al. reported that electrophoresis NaHS (–60 nA, –90 nA or –120 nA) produced exciting-inhibiting biphasic responses in presympathetic neurons in rats (Huang 2008). Based on their results, we supposed that H2S probably produced different responses in BP of rats, low concentration mainly produced hypertension while high dose produced hypotension. Our study didn’t find significant hypertension in any time point probably because of the differences in the way of administration and anesthetized rats.

Our conclusion is also supported by icv injection of CBS activator SAM. Previous studies have demonstrated that SAM is an allosteric regulator of CBS, which activates CBS by approximately two-fold (Finkelstein 2007, Abe and Kimura 1996). We found that central application of SAM (26 μg) produced such a significant decrease in BP and HR as those of central application of NaHS, strongly supporting that central H2S produces a decrease in BP and HR in anesthetized rats.

Besides, our study shows that HA, an inhibitor of CBS, significantly decreased basal BP but didn’t influence basal HR. It has been reported that HA is a donor of NO. Central application of HA can efficiently decrease BP by increasing the central concentration of NO (Lin et al. 1999). Additionally, HA effectively inhibits the production of endogenous H2S as an allosteric inhibitor of CBS (Abe and Kimura 1996, Han et al. 2005). However, in our study we observed that HA didn’t influence the cardiovascular effects of central application of NaHS, suggesting that HA doesn’t affect the conversion between H2S and NaHS. Previous studies also indicate that the release of NO was stimulated by NaHS not only from NO donors but also from rat brain homogenate and from L1210 cells (Ondrias et al. 2008). This may be supported by numerous reports showing that ‘H2S’ shares many biological effects with NO (Cabrera and Bohr 1995). It is assumed that the hypotension and bradycardia induced by icv injection of NaHS probably be the consequence of increase in the release of NO in central system because icv injection of S-nitrosothiols, a donor of NO, produces the similar hypotension and bradycardia as NaHS. HA as a kind of donor of NO, has been well accepted (Lin et al. 1999). In addition, HA can inhibit nitric oxide synthase (Abe and Kimura 1996, Han et al. 2005). In our present study, however, icv injection of HA, an inhibitor of nitric oxide, didn’t alter the hypotension and bradycardia induced by icv injection of NaHS. The data shown above argue against the opinion that the cardiovascular functions of icv injection of NaHS might be the results of increase in the release of NO by H2S.

To address the question whether the cardiovascular effects of central H2S are mediated by KATP channels activation, the blocker of KATP glibenclamide was applied to observe whether the cardiovascular effects of central H2S is effectively attenuated by blocking of KATP channel. Our data indicates that glibenclamide completely abolishes the hemodynamic effects induced by icv injection of NaHS. Hence, it suggests that the central hemodynamic effects of NaHS are mediated by KATP channels activation. It has been reported that glibenclamide effectively antagonizes the depressor effects within posterior hypothalamus and vasorelaxation of smooth muscles (Geng et al. 2007, Dawe et al. 2008) as a selective blocker of KATP channels. In our present study, we found that the cardiovascular effects of icv injection of NaHS were effectively antagonized by glibenclamide. However, we didn’t know the exact role of KATP channel activation in mediating central cardiovascular effects of H2S. In CNS, KATP channels consist of the Kir6.2 potassium channel subunits and the sulfonylurea receptor subunits (Kang et al. 2004, Babenko et al. 1998), similar to those in heart and muscle (Liss and Roeper 2001). Kir6.2 subunits belong to the inward rectifier potassium channel family, while SUR subunits belong to the ATP-binding cassette protein superfamily (Aguilar-Bryan and Bryan 1999). Previous studies show that the central KATP channels, which play a vital role in glucose homeostasis, might be independent on cytosolic second messengers (Minami et al. 2003, Minami et al. 2004). Although the existence of KATP channels in brainstem has been determined by previous studies (Ferreira et al. 2001, Dallaporta et al. 2000), the signaling pathway of KATP involved in regulation of cardiovascular effects is not clear.

It has been well known that activation of KATP channels is crucial to keep neuronal excitability in chemoreflex pathways in NTS (nucleus tractus solitarii, NTS) level (Zhang et al. 2008). However, whether the hypotension induced by central H2S is dependent on chemoreflex are not clear. Because the cardiovascular responses to application of NaHS or SAM into LCV might be mediated by integrative interactions between different central cardiovascular regions, no evidence is available to determine which regions are involved in mediating the cardiovascular functions of central H2S. Perhaps the reduction of the release of several
neurotransmitters, including excitatory transmitter glutamate (Soundarapandian et al. 2007) and inhibitory transmitter GABA (Avshalumov and Rice 2003) as well as the functions of NMDA receptors by activation of K$_{\text{ATP}}$ channels is involved in the hypotension of central H$_2$S. The exact cardiovascular mechanism of central H$_2$S needs to be further determined.

Conflict of Interest
There is no conflict of interest.

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