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

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Summary

Hydrogen sulfide (H_2S), an endogenous "gasotransmitter", exists in the central nervous system. However, the central cardiovascular effects of endogenous H_2S 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

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Introduction

Hydrogen sulfide (H_2S), 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_2S 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_2S is mostly generated by CSE, while the central H_2S 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_2S 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_2S 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_2S contributes to cardiovascular regulation. For example, intravenous injection of H_2S 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_2S 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_2S induces a hyperpolarization and reduces an input resistance of CA1 neurons or dorsal raphe neurons in K_{ATP} channels-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_2S 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_2S 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_2PO_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 dimethysulfoxide (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 % Pontamine 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_2S, was

injected into LCV in 19 rats to observe the dose-dependent effects of central H_2S. In another 7 rats, the cardiovascular functions of central NaHS were determined by increased the concentration of endogenous H_2S 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_2S central cardiovascular responses after CBS was inhibited. Furthermore, to determine whether the cardiovascular effects of central H_2S 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 $P<0.05$.

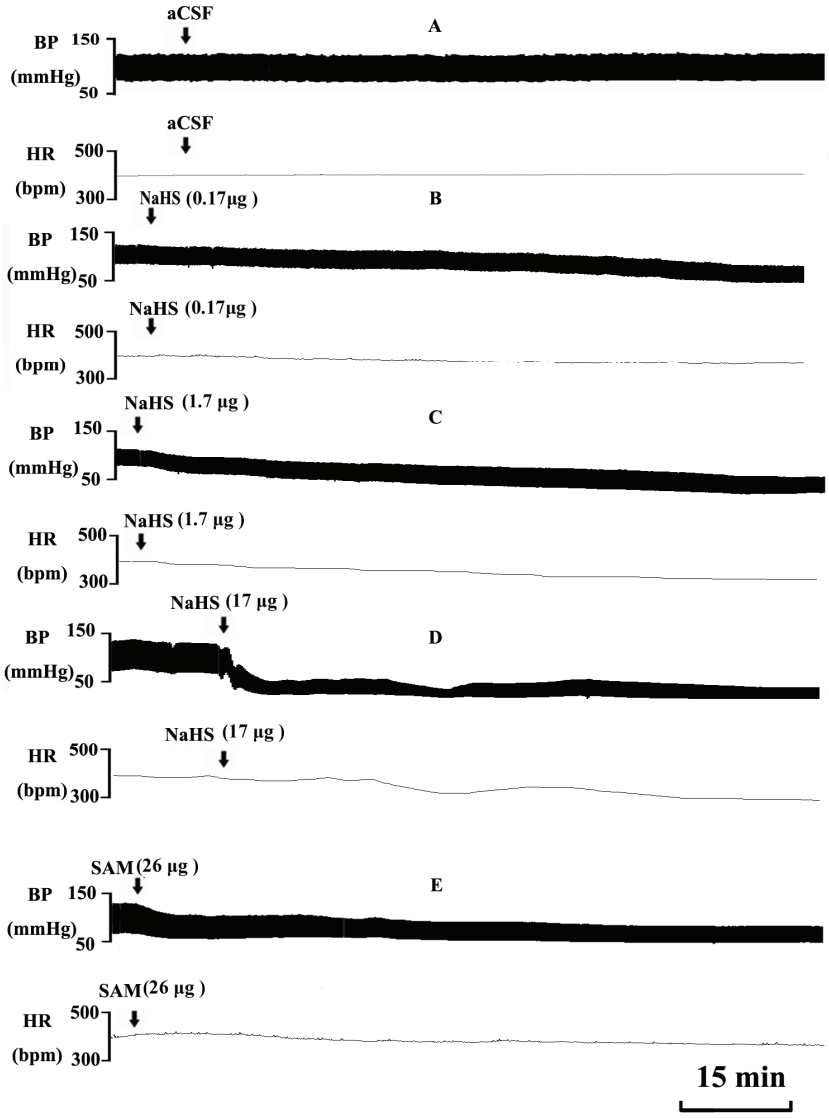

Fig. 1. The representative tracings showing the effects of injection (icv) of artificial cerebrospinal fluid (aCSF, 5 μl, **A**), hydrogen sulfide (NaHS, 0.17~17 μg, **B-D**) or S-adenosyl-L-methionine (SAM, 26 μg, **E**) on the blood pressure (BP) and heart rate (HR) response. The arrow point indicated 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 H_2S 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 bmp, 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.

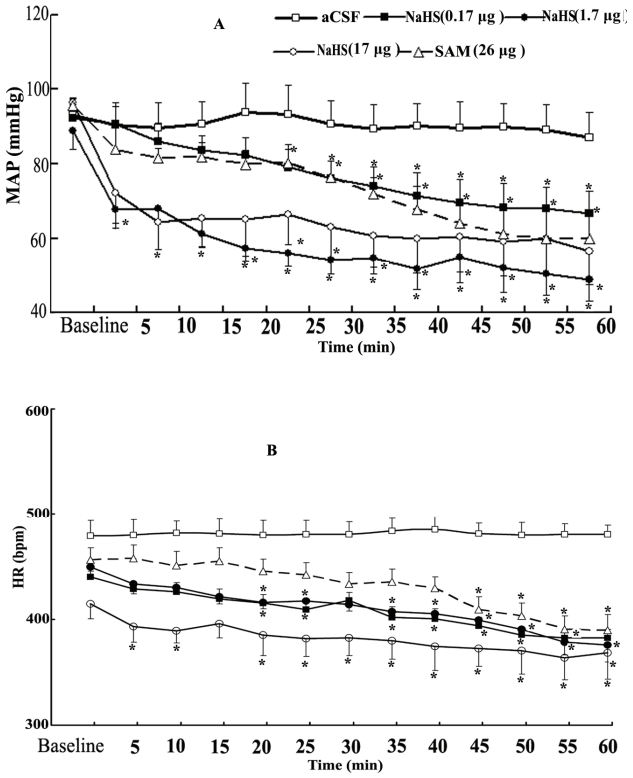

Fig. 2. The effects of icv injection of artificial cerebrospinal fluid (aCSF, 5 μl), hydrogen sulfide (NaHS, 0.17~17 μg) or S-adenosyl-L-methionine (SAM, 26 μg) on blood pressure (**A**) and heart rate (**B**) of rats (n=4-7). * $P<0.05$, compared with preinjection of aCSF, NaHS, or SAM by Student's *t*-test statistical test.

Effects of pretreatment with the K_{ATP} 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 K_{ATP} 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,

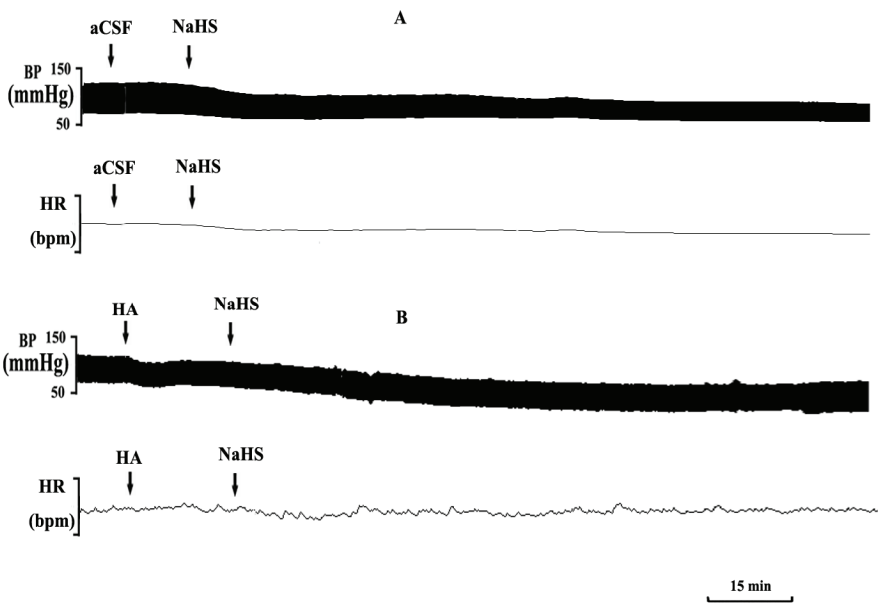

Fig. 3. The representative tracings showing the effects of prior administration of vehicle (aCSF, 5 μl, **A**) or hydroxylamine (HA, 0.7 mg, **B**) on blood pressure (BP) and heart rate (HR) response to icv application of hydrogen sulfide (NaHS, 1.7 μg) in rats. The arrow indicated the time point of prior injection of vehicle (aCSF) or HA. NaHS was injected after 10 min.

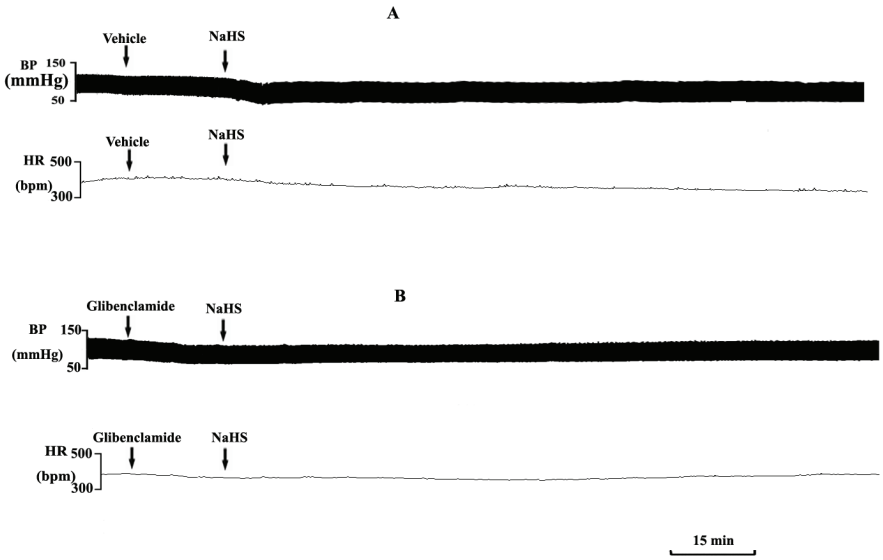

Fig. 4. The representative tracings showing the effects of prior administration of vehicle (aCSF, 5 μl, **A**) or glibenclamide (0.5 μg, **B**) on blood pressure (BP) and heart rate (HR) response to icv application of hydrogen sulfide (NaHS, 1.7 μg) in rats. The arrow indicated the time point of prior injection of vehicle (aCSF) or glibenclamide. NaHS was injected after 10 min.

P<0.05) of icv application of NaHS (1.7 μg) on BP. 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 H_2S donor NaHS or the activator of CBS SAM produced hypotension and bradycardia; and 2. the central cardiovascular effects of endogenous H_2S 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 H_2S 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 H_2S in the brain (Warenycia *et al.* 1989).

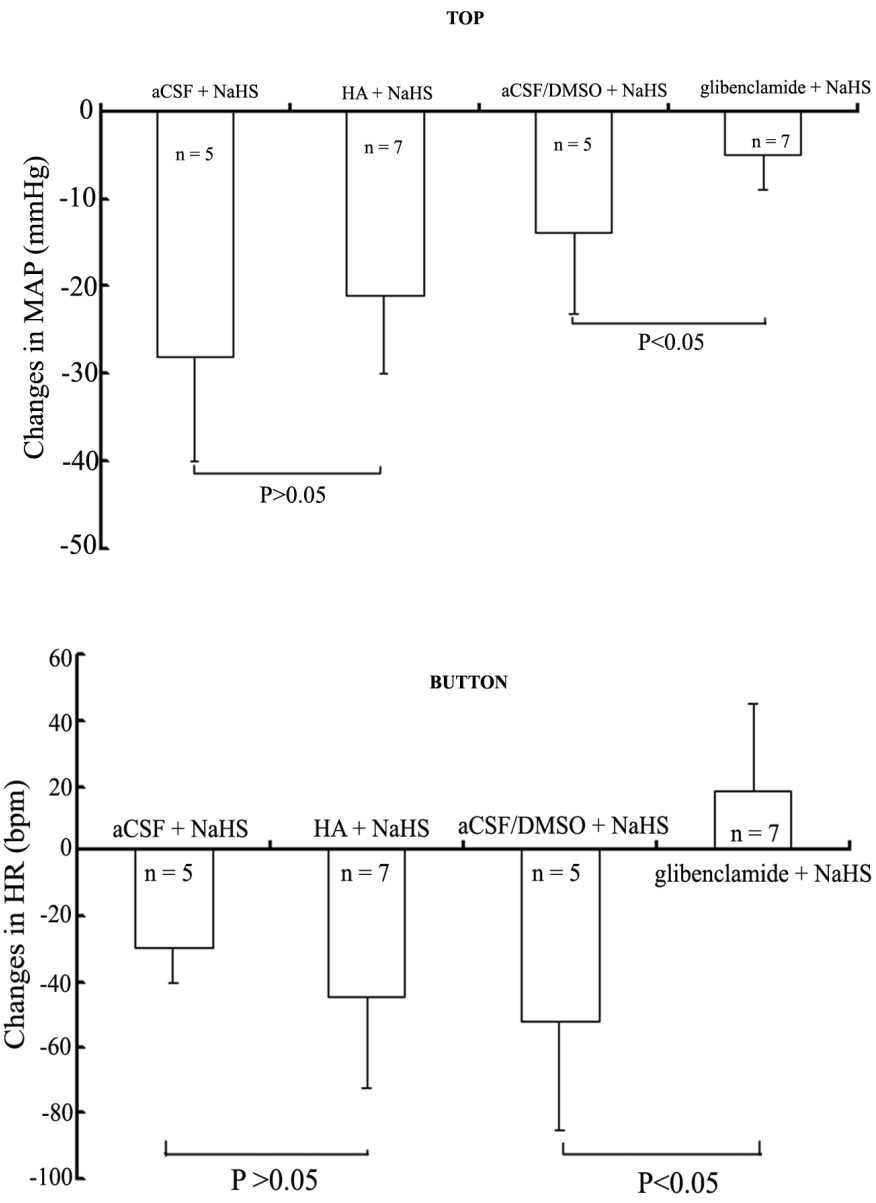

Fig. 5. Bar graphs showing the effects of pretreatment with hydroxylamine (HA, 0.7 mg) or glibenclamide (0.5 μg) on blood pressure (BP) and heart rate (HR) response to icv injection of NaHS (1.7 μg). aCSF + NaHS: pretreatment with aCSF; HA + NaHS: pretreatment with hydroxylamine (HA); aCSF/DMSO + NaHS: pretreatment with mix solution of aCSF and DMSO (the concentration of DMSO was not more than 1 %); glibenclamide + NaHS: pretreatment with glibenclamide. $P<0.05$, compared with pretreatment vehicle (aCSF, ANOVA test).

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 H_2S might serve as an endogenously gaseous messenger in very low concentration. H_2S dissociates to H^+ and HS^- in solution. In physiologic conditions (37 °C, pH 7.4), only a little of H_2S (less than one fifth) exists as the undissociated form (H_2S), and the remaining four fifths exist as HS^- plus a trace of S^{2-} at equilibrium with H_2S (Dombkowski *et al.* 2004, Webb *et al.* 2008). Although which active form of H_2S (H_2S, HS^-, or S^{2-}, the mix of free inorganic sulfides) has not been determined, Ondrias *et al.* (2008) assumed that HS^- (but not H_2S or S^{2-}) is probably the active form of 'H_2S' 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 H_2S (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 H_2S probably produced different responses in BP of rats, low concentration mainly produced hypertension while high dose produced hypotension. Our study didn't found 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 H_2S 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 H_2S 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 H_2S 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 'H_2S' 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 H_2S.

To address the question whether the cardiovascular effects of central H_2S are mediated by K_{ATP} channels activation, the blocker of K_{ATP} glibenclamide was applied to observe whether the cardiovascular effects of central H_2S is effectively attenuated by blocking of K_{ATP} 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 K_{ATP} 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 K_{ATP} 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 K_{ATP} channel activation in mediating central cardiovascular effects of H_2S. In CNS, K_{ATP} channels consist of the Kir6.x 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.x 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 K_{ATP} 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 K_{ATP} channels in brainstem has been determined by previous studies (Ferreira *et al.* 2001, Dallaporta *et al.* 2000), the signaling pathway of K_{ATP} involved in regulation of cardiovascular effects is not clear.

It has been well known that activation of K_{ATP} 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 H_2S 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 H_2S. 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_{ATP} channels is involved in the hypotension of central H_2S. The exact cardiovascular mechanism of central H_2S needs to be further determined.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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