Department of Clinical Laboratory[1], SanAiTang Hospital, Lanzhou; Department of General Surgery[2], First Hospital of Lanzhou University, Lanzhou; Department of Physiology[3], Second Military Medical University, Shanghai, China

The cardiovascular inhibition functions of hydrogen sulfide within the nucleus tractus solitarii are mediated by the activation of K_{ATP} channels and glutamate receptors mechanisms

Wei Qiao[2], Lei Yang[2], Xiao-Yun Li[1], Nong Cao[2], Wei-Zhong Wang[3], Chen Chai[1], Yan Lu[1]

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Yan Lu, Department of Clinical Laboratory, SanAiTang Hospital, 74 Jing-Ning Road, Lanzhou 730030, Gansu Province, China
lu73free@yahoo.com.cn
Chen Chai, Department of General surgery, First Hospital of Lanzhou University, 1 DongGang West Road, Lanzhou 730000, Gansu Province, China
chasechai@gmail.com

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Hydrogen sulfide (H_2S), the colorless gas with the smell of rotten eggs, has been regarded as a novel gaseous signaling molecule. Although H_2S has been proved been involved into the cardiovascular functions, the cardiovascular functions of H_2S within the nucleus tractus solitarii (NTS) are not clear. Unilateral microinjection of NaHS (2 to 200 pmol), a H_2S donor, into the NTS caused transient and dose-dependent hypotension and bradycardia ($P < 0.01$). Microinjection of CBS allosteric activator S-ademetionine (SAM) into the NTS also produced significant decreases in BP (from 101 ± 8 to 82 ± 7 mmHg, $P < 0.01$) and HR (from 469 ± 16 to 449 ± 14 bpm, $P < 0.01$), which was very similar to those of NaHS. Pretreatment with hydroxylamine, a CBS inhibitor, failed to affect the cardiovascular functions of intra-NTS NaHS. However, pretreatment with glibenclamide (10 nmol), a K_{ATP} channel blocker, eliminated the on BP (from -23 ± 4 to -5 ± 1 mmHg, $P < 0.01$) and HR (from -24 ± 2 to -5 ± 1 bpm, $P < 0.01$) by 78% and 79%, respectively, of intra-NTS NaHS (20 pmol). Likewise, pretreatment with kynurenic acid (Kyn, 5 nmol) also attenuated the effects of NaHS on BP (from -29 ± 3 to -12 ± 3 mmHg, $P < 0.01$) and HR (from -19 ± 2 to -9 ± 2 bpm, $P < 0.01$) by 59% and 53%, respectively, of intra-NTS NaHS (20 pmol). These data support the hypothesis that endogenous H_2S produces cardiovascular inhibition functions in the NTS, mainly mediated by K_{ATP} channels regulation or/and glutamate receptors.

1. Introduction

Hydrogen sulphide (H_2S), well-known as a toxic gas with the smell of rotten eggs (Beauchamp et al. 1984; Reiffenstein et al. 1992), is a novel candidate of gasotransmitters besides nitric oxide (NO) and carbon monoxide (CO) (Wang 2002). H_2S has been found in most of mammalian tissues and produces various functions on nervous system, vascular (Beltowski 2004; Tang et al. 2005), and gastrointestinal smooth muscles (Gallego et al. 2008; Teague et al. 2002). Endogenous H_2S is enzymatically synthesized from L-cysteine or homocysteine through three key enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) or 3-mercaptopyruvate sulfurtransferase (3MST) along with cysteine aminotransferase (CAT) (Kamoun 2004; Stipanuk et al. 1982). Notably, the vascular H_2S is mostly generated by CSE, while the central H_2S is mainly produced by CBS from cysteine (Abe et al. 1996; Hosoki et al. 1997). Actually, the higher concentration of H_2S (50∼160 μM) in the brain (Goodwin et al. 1989; Savage et al. 1990; Warenycia et al. 1989) than that in plasma (∼46 μM) (Li et al. 2005), suggests that it might be produced and exert its effect locally in the brain. Recently, accumulating evidence has shown that H_2S is an important regulator of brain function. H_2S induces hippocampal long-term potentiation (LTP) (Harris et al. 1984), regulates neuroendocrine responses (Dello Russo et al. 2000) and inhibits synaptic transmission in hippocampus (Nagai et al., 2004).

H_2S has been demonstrated contribute to cardiovascular regulations. For example, intravenous injection of H_2S induced a transient hypotension in anesthetized rats, which could be effectively antagonized by the K_{ATP} channel blocker glibenclamide. *In vitro*, NaHS, the donor of H_2S could dilate vessel, inhibit myocardial function. In the CNS, H_2S induces 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 showed that microinjection of H_2S donor NaHS into the hypothalamus reduces BP and HR in rats, which could be effectively antagonized by prior administration of gliclazide (Dawe et al. 2008). In conscious Wistar-Kyoto rats, intracerebroventricular (ICV) infusion of NaHS produces pressor effects (Ufnal et al. 2008). Additionally, NMDA receptor activity, which has been demonstrated to play vital roles in baroreflex in medulla oblongata, can be enhanced by H_2S to facilitate the induction of LTP (Harris et al. 1984).

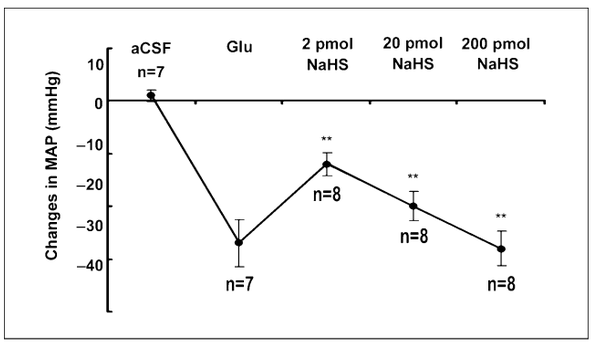
It is well known that the nucleus tractus solitarii (NTS), which is situated at the dorsomedial part of medulla oblongata, serves as a putative gateway of various visceral sensory (Guyenet 2006;

Zhang et al. 1995) and plays a pivotal role in arterial baroreflex in the cardiovascular system (Reis 1984; Seagard et al. 2000). Arterial baroreceptor and chemoreceptor signals reach the NTS along the vagus and glossopharyngeal nerves. The NTS at first integrates afferent signals and sends an excitatory transmitter, glutamate, to the caudal ventrolateral medulla (CVLM) neurons in medulla oblongata. Then, CVLM GABAergic neurons monosynaptically project to and directly inhibit sympathoexcitatory neurons in the rostral ventrolateral medulla (RVLM)(Aicher et al. 2000) that are the principal origin of excitatory stimulus to the sympathetic preganglionic neurons in the thoracic spinal cord and regulate vascular tone, cardiac output and heart rate via autonomic nervous system (Schreihofer et al. 2000). Relatively higher concentration of endogenous H2S (50∼160 μmol/L) in the brain than it in serum led us to hypothesize that endogenous H2S may influence cardiovascular functions of rats in the NTS level. The aim of the present study was to test the hypothesis in anesthetized rats.

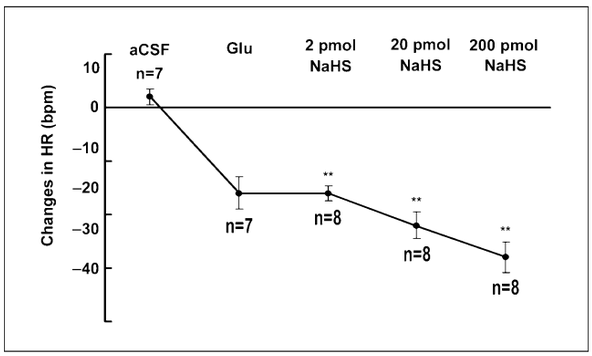
2. Investigation and results

2.1. Cardiovascular effects of unilateral microinjection of NaHS or CBS allosteric activator SAM into the NTS

Figure 1 shows the representative initial tracing of BP and HR responses of injection NaHS (2∼200 pmol), a donor of H_2S, into the NTS in anesthetized rats. Microinjection of NaHS (2, 20, and 200 pmol) into the NTS produced a dose-dependently decrease in BP and HR, and the lowering of the values was especially significant at the highest dose (200 pmol) of NaHS (change in MAP: -28 ± 2 mmHg; change in HR: -28 ± 3 bpm, $P < 0.01$) (Fig. 2). Typically, hypotension and bradycardia occured 15 s. after the administration of NaHS, reached nadir in 50 s, and persisted for 1 to 2 min. Figure 3 (top) shows the representative



(A)



(B)

Fig. 2: Line graph showing the effects of microinjection of NaHS (2–200 pmol), glutamate (Glu, 50 mmol/L) or vehicle (aCSF) into NTS on the MAP (*A*) and HR (*B*) of rats. n = 7–8 for each group. $^{**}P < 0.01$ vs. vehicle (aCSF)

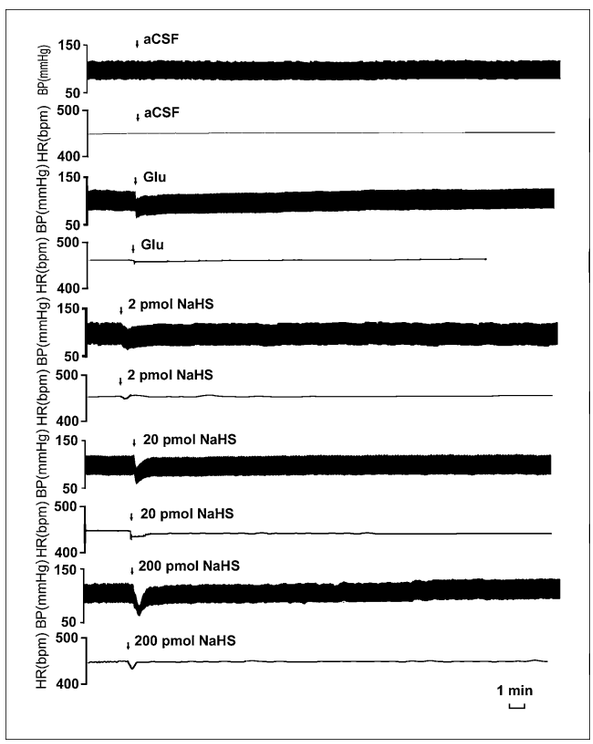


Fig. 1: TOP: Representative tracings showing BP and HR responses to unilateral microinjection of aCSF, Glu or NaHS (2, 20, 200 pmol) into the NTS in anaesthetized and paralyzed rats (*A*). BUTTON: The functional region of the NTS was identified by the depressor effects of L-Glu (5 nmol)

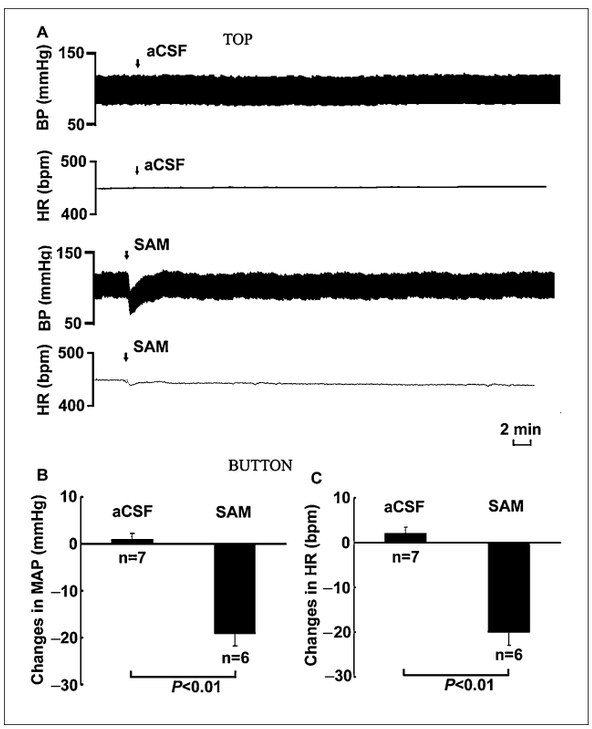


Fig. 3: TOP: Representative tracings showing BP and HR responses to unilaterally injection of vehicle (aCSF, 100 nl) or SAM (100 pmol) into the NTS (*A*). BUTTON: Bar showing the effects of intra-NTS SAM (0.1 nmol) on changes in blood pressure (BP, B) and heart rate (HR, C). n = 6–7 for each group. $^{**}P < 0.01$ vs. vehicle (aCSF)

original tracing of cardiovascular function of unilateral microinjection of SAM into NTS in rats. Unilateral intra-NTS SAM (0.1 nmol) produced significantly hypotension and bradycardia (changes in MAP = –19 ± 3 mmHg; changes in HR = –20 ± 3 bpm, $P < 0.01$). In the control tests (n = 7), microinjection of vehicle (aCSF, 100 nl) into the NTS produced no significant effects on BP (89 ± 6 vs. 89 ± 6 mmHg, $P > 0.05$) and HR (436 ± 20 vs. 438 ± 20 bpm, $P > 0.05$). The effects of intra-NTS application of NaHS (2∼200 pmol) or SAM (0.1 nmol) are summarized in Fig. 2 and Fig. 3 (bottom).

2.2. Effects of pretreatment with HA, the inhibitor of CBS, on the cardiovascular functions of intra-NTS NaHS

Fig. 4 (top) shows the representative initial tracing of pretreatment with HA (10 nmol), the inhibitor of CBS, on the cardiovascular functions of intra-NTS NaHS. Miroinjection of HA (10 nmol) into the NTS transiently increased the basal BP (from 99 ± 6 to 107 ± 6 mmHg, $P < 0.01$) and HR (from 475 ± 21 to 482 ± 21 bpm, $P < 0.01$). However, pretreatment with HA failed to modify hypotension (–24 ± 3 with HA vs. –29 ± 3 mmHg with vehicle, $P > 0.05$) and bradycardia (–16 ± 2 with HA vs. –19 ± 2 bpm with vehicle, $P > 0.05$) of intra-NTS NaHS (20 pmol) on (Fig. 4, button), suggests that HA neither influenced the cardiovascular functions of intra-NTS NaHS nor influenced the conversion between NaHS and H_2S. Pretreatment with vehicle (aCSF in 100 nl, n = 7) did not influence the cardiovascular responses of intra-NTS NaHS (20 pmol). The effects of pretreatment with HA (10 nmol) on cardiovascular responses to intra-NTS NaHS (20 pmol) are summarized in Fig. 4 (button).

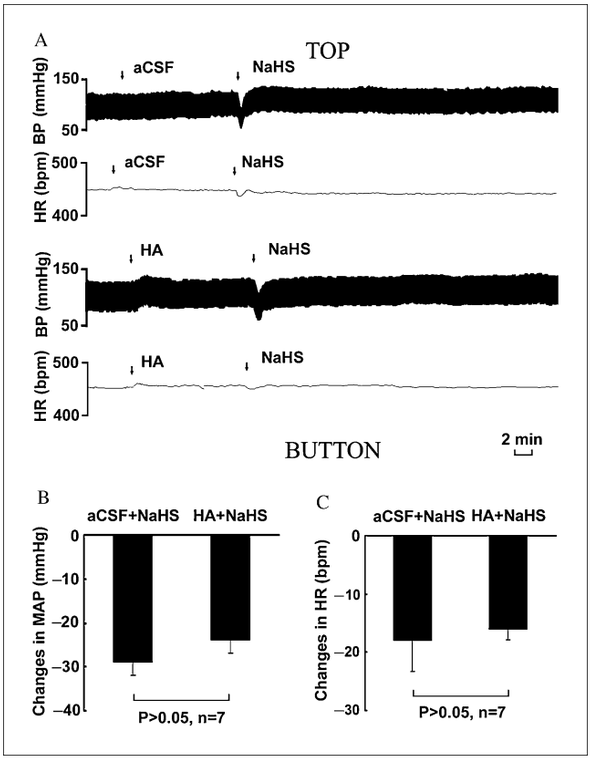


Fig. 4: TOP: Representative tracings showing the effects of unilaterally prior microinjection of HA (10 nmol) on decreases in BP and HR induced by NaHS (20 pmol) within the NTS (*A*). BUTTON: Effects of microinjection of HA into the NTS on the blood pressure (BP, B) and heart rate (HR, C) responses induced by intra-NTS NaHS (20 pmol). n = 7 for each group. $^{**}P < 0.01$ vs. vehicle (aCSF)

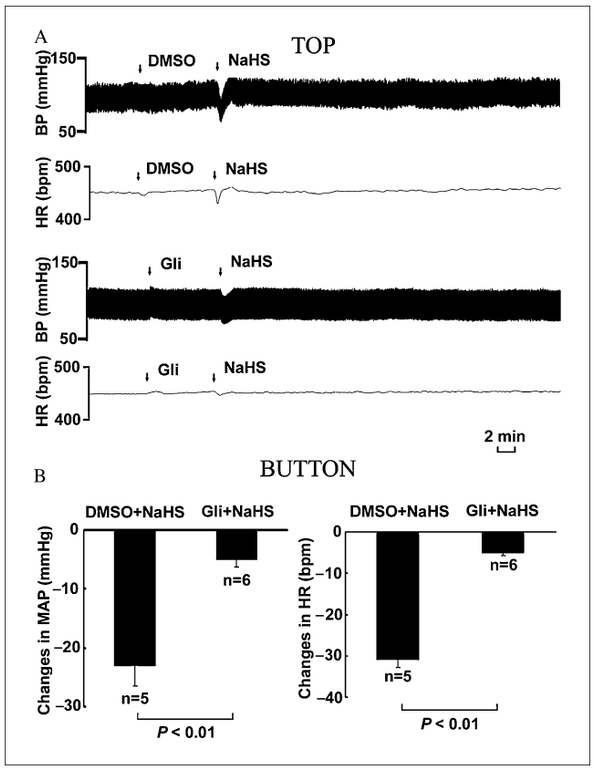


Fig. 5: TOP: Representative tracings showing the effects of pretreatment with Gli (10 nmol) on BP and HR responses to intra-NTS NaHS (20 pmol) in anaesthetized and paralyzed rats (*A*). BUTTON: Effects of microinjection of Gli into the NTS on the blood pressure (BP, B) and heart rate (HR, C) responses induced by intra-NTS NaHS (20 pmol). N = 5–6 in each group. $^{**}P < 0.01$ vs. vehicle (1% DMSO)

2.3. Effects of pretreatment with K_{ATP} channel blocker glibenclamide or non-selective glutamate receptor antagonist kynureuic acid on the cardiovascular functions of intra-NTS NaHS

Figure. 5 (top) and Fig. 6 (top) shows the representative initial tracing of K_{ATP} channel blocker glibenclamide (Gli, 10 nmol, n = 6) or non-selective glutamate receptor antagonist kynureuic acid (Kyn, 5 nmol) on the cardiovascular functions of intra-NTS NaHS. Microinjection of Gli (10 nmol, n = 6) into the NTS significantly raised the basal BP (from 95 ± 7 to 104 ± 7 mmHg, $P < 0.01$) and HR (from 455 ± 17 to 462 ± 17 bpm, $P < 0.05$). Importantly, the hypotension (–5 ± 1 with Gli vs. –23 ± 4 mmHg with vehicle, $P < 0.01$) and bradycardia (–5 ± 1 with Gli vs. 24 ± 2 bpm with vehicle, $P < 0.01$) induced by intra-NTS NaHS were decreased about 78% and 79%, respectively, by pretreatment with Gli, compared with pretreatment with vehicle. In the control group (n = 5), microinjection of vehicle (1% DMSO, 100 nl) into the NTS produced no significantly influence on the basal BP (100 ± 7 vs. 99 ± 7 mmHg, $P > 0.05$) and HR (479 ± 8 vs. 478 ± 7 bpm, $P > 0.05$). Besides, prior application of the vehicle failed to modify hypotension (–23 ± 4 with vehicle vs. –29 ± 3 mmHg with aCSF, $P > 0.05$) and bradycardia (–24 ± 2 with vehicle vs. –19 ± 2 bpm with aCSF, $P > 0.05$) of intra-NTS NaHS (20 pmol). The effects of pretreatment with Gli (10 nmol) on BP and HR responses to intra-NTS NaHS (20 pmol) are summarized in Fig. 5 (buttom). Microinjection of Kyn (5 nmol) into NTS elicited significant increase in the basal BP and HR (from 103 ± 8 to 109 ± 8 mmHg in MAP, $P < 0.05$; 449 ± 16 to 464 ± 17 bpm in HR, $P < 0.01$). Prior application of Kyn also significantly decreased the transient hypotension and

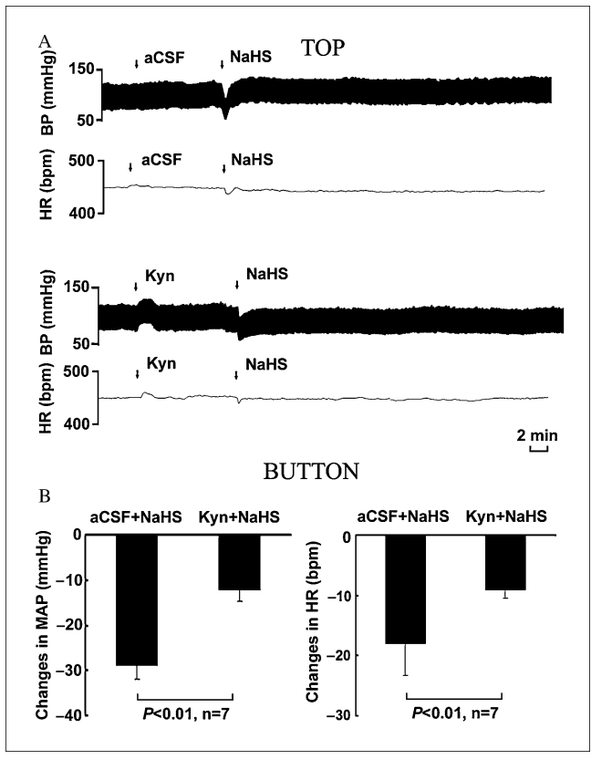


Fig. 6: TOP: Representative tracings showing the effects of pretreatment with Kyn (5 nmol) on BP and HR responses to microinjection of NaHS into the NTS (A). BUTTON: Effects of prior microinjection of Kyn into the NTS on the blood pressure (BP, B) and heart rate (HR, C) responses induced by intra-NTS NaHS (20 pmol). N = 7 for each group. $^{**}P < 0.01$ vs. vehicle (aCSF)

bradycardia of NaHS (20 pmol) within the NTS (-12 ± 3 with Kyn vs. -29 ± 3 mmHg with vehicle in MAP; -9 ± 2 with Kyn vs. -19 ± 2 bpm with vehicle in HR, $P < 0.01$) by 59% and 53%, respectively. In the control experiments ($n = 7$), prior injection of 100 nl aCSF into the NTS did not alter the cardiovascular functions of intra-NaHS. The effects of prior injection of Kyn (5 nmol) on cardiovascular responses to intra-NTS NaHS (20 pmol) are summarized in Fig. 6 (buttom).

3. Discussion

In our present study, our most important findings are: topical application of NaHS into the NTS produced dose-dependant hypotension and bradycardia in rats. The hypotension and bradycardia of intra-NTS NaHS are probably mediated by the activation of K_{ATP} channel and/or glutamate receptors.

H_2S, which has been ever considered as a kind of toxic gas by respiratory inhibition, is suggested as a novel candidate of gasotransmitters (Wang 2002). Previous studies show that H_2S facilitates hippocampal long-term potentiation (LTP) by the activated NMDA receptors and regulates the release of CRH from hypothalamus (Abe et al. 1996; Dello Russo et al. 2000). Recently, several studies have investigated the central control of H_2S on cardiovascular actions. For example, Ufnal et al. (2008) demonstrated that infusion of NaHS, into lateral cerebral ventricle (LCV) in the rats elicited hypertension and tachycardia responses. In hypothalamus, microinjection of NaHS or CBS activator H_2S produces cardiovascular inhibition and the effects are mediated by K_{ATP} channels (Dawe et al. 2008).

The present study clearly indicated that microinjection of NaHS (2∼200 pmol) into the NTS produced does-dependent cardiovascular inhibition effects. Our results are further confirmed by microinjection of the allostic activator SAM into the NTS. It has been demonstrated that SAM acts as an allosteric activator CBS and enhances its activity approximately two-fold (Abe et al. 1996; Eto et al. 2002). Our study showed that microinjection of SAM produced significantly hypotension and bradycardia, very similar to those of microinjection of NaHS.

HA, as an inhibitor of CBS, was also prior applied into the NTS to observe the influences of inhibition in CBS activities on the cardiovascular function of intra-NTS. HA is a putative intermediate in the transformation of L-arginine to NO, it effectively inhibits the activity of NO synthase. Additionally, as an allosteric inhibitor of CBS, HA effectively inhibits the production of endogenous H_2S (Abe et al. 1996; Han et al., 2005). However, our results showed that pretreatment with HA produced no inhibitory effect on the cardiovascular functions of intra-NTS NaHS. This indicates that HA, as an inhibitor of CBS, affects neither the cardiovascular functions of intra-NTS NaHS nor the conversion between NaHS and H_2S.

Based on previous studies (Dawe et al. 2008; Harris et al. 1984), we speculated that K_{ATP} channels opening or glutamate receptors activation might mediate the cardiovascular functions of intra-NTS NaHS: (1) the expression of K_{ATP} channels in the NTS has been pharmacologically identified (Dallaporta et al. 2000; Ferreira et al. 2001); (2) the vasodilatation and hypotension within hypothalamus are mediated by K_{ATP} channels opening mechanisms. (3) Activation of K_{ATP} channels in NTS produces significantly hypotension and bradycardia, similar to those of intra-NTS NaHS. To test this hypothesis, we prior employed K_{ATP} channels blocker glibenclamide into the NTS to observe the effects of the block of K_{ATP} on the cardiovascular functions of intra-NTS NaHS. Expectedly, pretreatment with glibenclamide effectively reduced cardiovascular inhibition of H_2S within NTS, suggesting the cardiovascular functions of H_2S in the NTS may be partly mediated by K_{ATP} channels activation mechanisms. K_{ATP} channel consists of four sulfonylurea receptors (SUR1 or SUR2) and four inward rectifier K^+ (Kir6.1 or Kir6.2) (Wheeler et al. 2008). In CNS, K_{ATP} channels consist of the Kir6.x potassium channel subunits and the sulfonylurea receptor subunits (Babenko et al. 1998; Kang et al. 2004), similar to those in heart and muscle (Liss et al. 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 et al. 1999). Previous studies show that the central K_{ATP} channels, which play vital role in glucose homeostasis, might be independent of cytosolic second messengers (Minami et al. 2004, 2003). Although the existence of K_{ATP} channels in brainstem has been determined by previous studies (Dallaporta et al. 2000; Ferreira et al. 2001), the signaling pathway of K_{ATP} involving into regulating cardiovascular effects is not clear.

Glutamate receptors comprise the subtypes of ionotropic receptors (NMDA, AMPA, Kainate) (Frigero et al. 2000; Leone et al. 1989; Seagard et al. 2000) and metabotropic receptors (mGluRs) (Simms et al. 2006) in modulating the baroreceptor reflex in the rat NTS (Abe et al. 1996). Therefore, we hypothesized that H_2S probably produces hypotension and bradycardia by increasing the sensitivity of glutamate receptors. To test the hypothesis, the non-selective antagonist Kyn was prior applied into NTS to observe the block of glutamate on cardiovascular functions of H_2S within the NTS. Our results showed that the cardiovascular functions elicited by intra-NTS NaHS were effectively decreased by pretreatment with Kyn, indicating that glutamate receptors probably mediate the cardiovascular functions of H_2S within the NTS. However, it remains to be determined which subtype(s) of glutamate receptors are involved in the mechanism of H_2S-stimulated cardiovascular effects.

In conclusion, endogenous H_2S in the NTS produces inhibition of cardiovascular functions, which might be mediated by the activation of K_{ATP} channels and glutamate receptors.

4. Experimental

4.1. Chemicals and drugs

NaHS, L-glutamate (Glu), K_{ATP} channels blocker (glibenclamide, Gli), non-selective ionotropic glutamate receptor antagonist (Kynurenic acid, Kyn), SAM, HA were purchased from Sigma chemical (St. Louis, MO).

4.2. General procedure

Adult male Sprague-Dawley (SD) rats (weighing 220 to 300 g), purchased from the Laboratory Animal Center of Lanzhou University, were used in the present experiments. All of animals shared humane treatment according to institutional guidelines for animal handling. They were housed in controlled conditions, including a 12-h light-dark cycle with access to standard rat chow and tap water. All surgical and experimental procedures were carried out on the basis of institutional animal care guidelines. The methods for animal preparation, microinjection and histological procedures were performed as described previously (Wang et al. 2008).

Briefly, after 3-days accommodation, the rats were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneal administration). Then the right femoral artery and vein were cannulated with polyethylene catheters for the measurement of arterial blood pressure (BP) and drug administration, respectively. The catheters were prior filled with heparinized saline (50 U/mL) or 0.9% NaCl. The signals of BP from the transducer was continuously collected and analyzed by a computerized system with professional software (XJH-2007, China), and heart rate (HR) was indirectly computed from the electrocardiogram waveforms and displayed on another channel. A trachea was cannulated and connected to an animal ventilator (DW-2000, China). The rats were mechanically ventilated with oxygen-enriched room air and then paralyzed with triethiodide (10 mg/kg initially and a half every 30 min, i.v.). Anesthesia condition was assessed by the stability of basal BP, and BP response to pain stimulus on hind paw. Whenever necessary, urethane (0.4 g/kg) was intravenously supplemented to achieve a stable and adequate level of anesthesia. Subsequently, the rats were fixed in the stereotaxic apparatus (MP-8003, China). The dorsal surface of medulla oblongata was exposed by removing the partial occipital and cerebellum. In this process, an infrared heating lamp was used to keep rat body temperature at 37 °C.

4.3. Microinjection of drugs into the NTS

According to stereotaxic coordinates, multibarrel micropipette (tip diameter 20–30 μm) was lowered into the NTS (0.5–0.8 mm rostral to the obex, 0.5–1.0 mm lateral to the midline, and 0.2–0.5 mm below the dorsal surface of the medulla). The micropipette was filled with Glu, NaHS solution (2, 20 or 200 pmol), Gli, SAM, Kyn or HA by a microsyringes. Gli was initially dissolved in dimethyl sulfoxide (DMSO) and finally diluted with 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 $NaHCO_3$, 3.4 glucose, with pH 7.4). The final concentration of DMSO in aCSF was not more than 1%, which produced little effect on BP and HR of rats in preliminary experiment. The other drugs were dissolved into aCSF. The dose of NaHS, SAM, HA, Gli and Kyn were based on previous studies (Mandel et al. 2008; Nishimura et al. 1995). All drugs were slowly (over 30 s) injected into the NTS in a volume of 100 nl by a syringe under the guidance of an operating microscope. Functional identification of the NTS depended on the depressor response produced by prior injection of 5 nmol L-glutamate. At the end of the experiments, an overdose of urethane (0.4 g/kg, i.v.) was applied to deeply anaesthetize the rats and then 20 nl of 2% pontamine sky blue was microinjected into the drug site to confirm the marked location at the histological construction. Intracardiac perfusion with 0.9% NaCl (250 mL) and, subsequently, with 4% phosphate-buffered paraformaldehyde solution (250 mL) was carried out. Then the whole brain was removed and transferred to both 20% and 40% sucrose for fixation until its sinking. Subsequently, the frozen brain was sectioned (50 μm) in coronal view and stained with neutral red. The histological site of drug injection is illustrated in Fig. 7.

4.4. Experimental protocols

To test the dosage effects of endogenous H_2S within NTS, 31 anesthetized and paralyzed rats, which were randomized into 4 groups, received topically application of the H_2S donor NaHS (2, 20 and 200 pmol). The cardiovascular functions of endogenous H_2S were further determined by increasing the content of endogenous H_2S in NTS by microinjection of SAM, the allosteric activator of CBS into NTS in 6 rats. As control, the vehicle (aCSF, 100 nl) was injected into NTS in another 7 rats. The original tracings of the effects of intra-NTS NaHS, SAM or aCSF persisted for at least 60 min after the drug injection. Another 14 rats were initially pretreated with HA (n = 7) or the vehicle (aCSF, n = 7) 10 min before microinjection of 20 pmol NaHS into the NTS to observe the inhibitor of CBS on the cardiovascular function of intra-NTS NaHS. Furthermore, to identify whether the cardiovascular responses to intra-NTS NaHS were mediated by K_{ATP} channel activation, glibenclamide, a K_{ATP} channel blocker (n = 6), or the vehicle (1% DMSO, n = 5) was prior injected into the NTS 10 min before NaHS (20 pmol) was topically applied into the same site. Likewise, to determine whether the cardiovascular responses to intra-NTS NaHS were mediated by glutamate receptor, kynurenic acid, a non-selective ionotropic glutamate receptor antagonist (n = 7), or the vehicle (aCSF, n = 7) was prior injected into the NTS 10 min before NaHS (20 pmol) was microinjected into the same site. The original tracings of BP and HR responses to intra-NTS NaHS were recorded for at least 60 min.

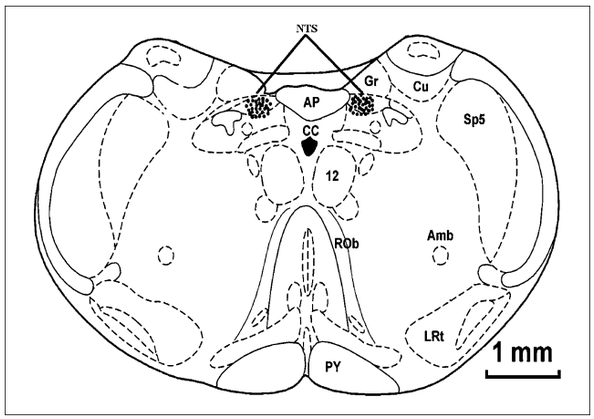


Fig. 7: Location of microinjection sites in the NTS. Coronal view of the rat medulla with the black circles (·) showing the sites of drugs microinjection in the nucleus tractus solitarii (NTS) 0.5–0.8 mm rostral to the obex at the level of oblongata medulla. Amb, nucleus ambiguous; AP, area postrema; CC, central canal; Cu, cuneate nucleus; Gr, gracile nucleus; NTS, nucleus tractus solitarii; PY, pyramidal tract; Rob, raphe obscurus nucleus; LRt, lateral reticular nucleus; Sp5, spinal trigeminal nucleus; and 12, hypoglossal nucleus

4.5. Statistical analysis

Blood pressure (BP) is presented as mean arterial pressure (MAP), calculated from the following formula: diastolic + [(systolic-diastolic)/3]. Data were shown as mean ± SEM and analyzed using Student's *t* test and one-way analysis of variance (ANOVA). *P* values of less than 0.05 were considered to be statistically significant.

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