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Title Page

Hydrogen Sulfide in the Rostral Ventrolateral Medulla Inhibits Sympathetic Vasomotor Tone through ATP-sensitive K^+ Channels

Qi Guo, Sheng Jin, Xiu-Li Wang, Ru Wang, Lin Xiao, Rui-rong He, and Yu-ming Wu *

Department of Physiology, Institute of Basic Medicine (Q.G., S.J., R.W., L.X., R.H., Y.W.) and Department of Anesthesiology, the Third Hospital (X.W.), Hebei Medical University, Shijiazhuang 050017, China

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Running Title Page

Running title: Hydrogen sulfide and sympathetic activity

Correspondence to: Yu-ming Wu, MD, PhD, Department of Physiology, Institute of Basic Medicine, Hebei Medical University, 361 Zhongshan East Road, Shijiazhuang 050017, China. Telephone: +86-311-8626-6407, Fax: 86-311-8626-6407, E-mail: wuyum@yahoo.com

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Abbreviations: CBS, cystathionine *β*-synthase; CSE, cystathionine *γ*-lyase; DMSO, dimathyl sulfoxide; *L*-NAME, N_{ω}-nitro-*L*-arginine methyl ester; NaHS, sodium hydrogen sulfide; NMDA, N-methyl-D-asparate.

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Abstract

Hydrogen sulfide (H_2S) acts as an endogenous gaseous transmitter in the central nervous system and plays important roles in regulating cardiovascular function. The rostral ventrolateral medulla (RVLM) is a putative critical central region in the control of sympathetic vasomotor tone and plays an important role in the baroreflex by integrating the inputs from a variety of visceral and somatic stimuli. In this study, we tested the hypothesis that H_2S decreases sympathetic vasomotor tone through K_{ATP} in the RVLM. The arterial blood pressure (ABP), heart rate (HR), and renal sympathetic nerve activity (RSNA) of anesthetized rats were recorded. Bilateral microinjections of sodium hydrosulfide (NaHS, 4, 8, and 16 mM, 50 nl), an H_2S donor, into the RVLM decreased ABP, HR and RSNA in a dose-dependent manner. Preinjection of glibenclamide (40 μM, 50 nl), a K_{ATP} channel blocker, abolished the sympathoinhibitory effects of NaHS (8 mM, 50 nl). Preinjection of a nitric oxide synthase inhibitor, N_ω-nitro-*L*-arginine methyl ester (*L*-NAME, 200 μM, 50 nl) partially inhibited the sympathoinhibitory effects of NaHS. Prior microinjection of Bay K8644 (1 μM, 50 nl), an agonist of Ca^{2+} channels, did not alter the effects of NaHS. Infusion of hydroxylamine (HA, 30 mM, 50 nl), a cystathionine *β*-synthase (CBS) inhibitor, increased BP, HR and RSNA. Taken together, these findings suggest that exogenous H_2S in the RVLM inhibits sympathetic vasomotor tone by opening K_{ATP} channels. Nitric oxide signaling may partially be involved in the sympathoinhibitory effect of H_2S in the RVLM.

Introduction

Recent studies have suggested that hydrogen sulfide (H_2S), in addition to nitric oxide (NO) and carbon monoxide (CO), is a third gaseous signal molecule in the regulation of blood pressure (Wang, 2002). In mammals, H_2S can be produced endogenously in mammals from L-cysteine by pyridoxal-5'-phosphate-dependent enzymes such as cystathionine *β*-synthase (CBS) and cystathionine *γ*-lyase (CSE). The expression of these two enzymes is tissue specific (Wang, 2002) with CBS highly expressed in the brain. In addition, 3-mercaptopyruvate sulfurtransferase has been demonstrated as a H_2S producing enzyme in the brain as well as in the vascular endothelium (Shibuya et al., 2009a; Shibuya et al., 2009b). The endogenous levels of H_2S in the central nervous system, which have been measured in rats, humans, and bovines (Goodwin et al., 1989; Warenycia et al., 1989; Savage and Gould, 1990), play important roles in the hippocampal long-term potentiation and regulation of N-methyl-D-aspartate (NMDA) receptors (Abe and Kimura, 1996). Sodium hydrosulfide (NaHS) is commonly used as an H_2S donor since it dissociates to Na^+ and HS^-, the latter of which then partially binds H^+ to form undissociated H_2S (Lowicka and Beltowski, 2007).

H_2S has an important role in the regulation of physiological functions including learning and memory (Kimura, 2002) and cardiovascular functions (Hosoki et al., 1997; Zhao et al., 2001; Zhao and Wang, 2002). For example, the vasodilator effect

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of H_2S is mediated by K_{ATP} channels (Hosoki et al., 1997; Zhao et al., 2001). Furthermore, the H_2S signaling pathway is involved in vascular collagen remodeling in spontaneously hypertensive rats (SHRs) (Zhao et al., 2008). In previous studies, we have shown that H_2S increases the sensitivity of carotid sinus baroreflex by opening K_{ATP} channels and closing of the L-Ca^{2+} channels (Xiao et al., 2006; Xiao et al., 2007). These findings indicate that H_2S regulates the cardiovascular homodynamic through its central effect on the baroreceptor reflex (Lowicka and Beltowski, 2007). Furthermore, the physiological concentration of H_2S has been shown to facilitate long-term potentiation in the hippocampus, likely through cyclic adenosine 3', 5'-monophosphate-mediated interactions with NMDA receptors (Abe and Kimura, 1996; Kimura, 2000). However, the role of H_2S in the autonomic centers regulating sympathetic outflow remains unknown.

Several studies' findings suggest that H_2S interacts with NO to regulate cardiovascular function. For example, the inhibition of endothelial NO synthase (NOS) activity has been found to reverse the cardioprotective effects of H_2S preconditioning in isolated rat cardiomyocytes by inhibiting cyclooxygenase-2 activity (Hu et al., 2008). In addition, NOS inhibitior-induced hypertension has been found to reduce plasma H_2S concentration, vascular CSE activity, and mRNA expression in rats (Zhong et al., 2003). The rostral ventrolateral medulla (RVLM) is a putative critical central region in the control of sympathetic vasomotor tone and plays an important role in the baroreflex by integrating the inputs from a variety of visceral and somatic stimuli

(Terui et al., 1986; Verberne et al., 1999; Dampney et al., 2003). Although previous studies indicate that the RVLM is a potential target for H_2S to exert its role in the regulation of sympathetic vasomotor tone, no studies have determined the direct effects of H_2S on this important cardiovascular center. Thus, the purpose of the current study was to investigate effect of microinjection of the H_2S donor NaHS into the RVLM on sympathetic vasomotor tone and identify the possible underlying mechanisms.

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Methods

Animal

The experiments were performed on 48 male Sprague-Dawley rats (300 ± 5 g) obtained from the Experimental Animal Center of Hebei Province, China. All animal procedures were carried out in accordance with the National Institute of Health Guidelines for animal research (1996). All protocols and procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Hebei Medical University.

Recording of Arterial blood pressure, Heart rate and Renal Sympathetic Nerve Activity Rats were anesthetized with urethane (1.0 g/ kg i.p.) and their trachea was cannulated for ventilation. A thermostatic bed was used to maintain body temperature in the range of 37-38°C. The left femoral artery was cannulated to record arterial blood pressure (ABP) with a pressure transducer. The heart rate (HR) was measured by triggering from the pulsatile blood pressure. The left kidney was exposed via a retroperitoneal approach and a small branch of the left renal sympathetic nerve around the renal vessels was carefully isolated from the surrounding tissue and clamped distally to eliminate the nerve's afferent activity. The nerve was placed on a bipolar platinum electrode for potential recording and immersed in warm (37°C) mineral oil. The nerve signal was amplified (gain of

20,000–30,000) and band-pass filtered (100–3,000 Hz) by an alternating current amplifier (model P511; Grass Instruments, West Warwick, RI, USA). The pressure and nerve discharge signal were amplifier-fed into a PowerLab/8sp (AD Instruments, QUAD, Bridge, Australia) data acquisition system to simultaneously record ABP and renal sympathetic nerve activity (RSNA). A customized computer program was used to integrate the RSNA; the integrating time was 0.16 sec. Background electrical noise was determined by a completely suppressing RSNA with phenylephrine (20 µg/kg, i.v.) administered before and 5 min after the rats were euthanized by an overdose of sodium pentobarbital (200 mg/kg, i.v.). The electrical noise levels measured with these two methods were similar and were subtracted from the integrated RSNA values and the percent change in RSNA from baseline was calculated.

Microinjection into the RVLM After the rat was anesthetized with urethane (1.0 g/kg i.p.), the rat was placed in a supine position, and the head of the animal was fixed on a stereotaxic frame. The trachea and esophagus were exposed through a midline incision and transected at the lower neck level and reflected rostrally. After retraction of the bilateral longus capitis muscles, the basilar portion of the occipital bone was removed to expose the ventral surface of the medulla with incision of the dura and arachnoid. We then coated the exposed medulla was covered with warm mineral oil and used a strip of thinly twisted cotton to constantly drain cerebrospinal fluid. The coordinates for RVLM were 1.9-2.3 mm lateral to the midline, 2.6-3.3 mm

caudal to interaural line, and 0.3-0.9 mm from the ventral surface. A glass micropipette (tip outer diameter 10 to 30 μm) was inserted into the RVLM (Paxinos et.al, 2005) for microinjection. Chicago sky blue (2%) filled in the micropipette was delivered into the left RVLM unilaterally in 1 min by a nanoliter injector (A203XVZ, World Precision Instruments, Sarasota, Florida, USA).

Protocols After a stable 30 min recording of ABP, HR, and RSNA was obtained, the vehicle solutions were microinjected into the RVLM. Subsequently, NaHS (4, 8, or 16 mmol/l, 50 nl) were microinjected into the RVLM, and ABP, HR, and RSNA were recorded. One dose was tested per rat.

We then investigated the effect of glibenclamide (40 μM, 50 nl) on the responses of ABP, HR, and RSNA to NaHS. We first determined whether a microinjection of NaHS (8 mM, 50 nl) into the RVLM produced a reproducible effect on the sympathetic vasomotor tone by performing identical microinjections of NaHS (8 mM, 50 nl) into the RVLM at 45 min intervals. ABP, HR, and RSNA returned to baseline levels within 45 min following the initial NaHS injection. After an initial microinjection of NaHS (8 mM, 50 nl), glibenclamide (40 μM, 50 nl) was microinjected into the RVLM. Ten minutes after glibenclamide injection, another dose of NaHS (8 mM, 50 nl) was injected into the RVLM. To determine whether NO was involved in the effect of NaHS on the sympathetic vasomotor tone, *L*-NAME (200 μM, 50 nl) was microinjected into the RVLM to inhibit NO synthesis. To determine whether Ca^{2+} was involved in the

effect of NaHS on the sympathetic vasomotor tone, we compared ABP, HR, and RSNA recorded during the administration of NaHS plus Bay K8644 (1 μM, 50 nl), an agonist of calcium channels.

Multiple injections into the RVLM may cause mechanical tissue damage and consequently decrease sympathetic vasomotor tone. To rule out this possibility, we microinjected 6 times vehicle solution in the amount of 50 nl into the RVLM, ABP, HR, and RSNA did not change significantly during and after the microinjection of vehicles (*n*=6).

Immunohistochemistry Immunohistochemical staining was used to determine the expression of CBS in the RVLM. Under deep anesthesia, rats were perfused through the heart with 0.9% saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. The rats' brains were removed, post-fixed for 4 h, equilibrated sequentially with 30% sucrose at 4°C for 24 h and finally frozen in liquid nitrogen for further analysis. Histological verification was carried out with reference to Paxinos and Watson′s coordinates. The brainstem was sectioned into 30 μm-thick coronal slides at the RVLM level on a freezing microtome and collected free floating in PBS. The slides were then incubated with the primary antibody (rabbit anti-CBS polyclonal antibody, 1:300 dilutions, Santa Cruz Biotech, Santa Cruz, CA, USA) in PBS containing 1% goat blood serum for 48 h at 4°C. Subsequently, goat anti-rabbit immunoglobulin G (Zhongshanjinqiao Biotech, Peking,

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China), 1:100) was incubated for 2 h at room temperature. The sections were then incubated for 60 min at room temperature with horseradish peroxidase-conjugated streptavidin (Zymed Labs Inc., South San Francisco, CA, USA). After being washed with 0.1M PBS, the sections were treated for 5 min with 0.005% 3, 3-diaminobenzidine-tetrachloride (Zymed Labs Inc., USA) and 0.001% H_2O_2 in 0.05M Tris-Hcl buffer (6.055g Tris, 1000 ml H_2O, and suitable HCl to obtain a PH of 7.6). All immunohistochemical staining was performed with negative controls using 0.1M PBS to replace primary antibodies.

Drugs

NaHS, *L*-NAME (N_ω-nitro-*L*-arginine methyl ester, $C_6H_{13}N_5O_4$), and hydroxylamine (HA, H_2NON) were purchased from Sigma Aldrich (St. Louis, MO, USA) and dissolved in saline according to the manufacturer's instructions. Bay K8644 (1, 4-Dihydro-2, 6-dimethyl-5-nitro-4- (2-[trifluoromethyl] phenyl) pyridine-3-carboxylic acid methyl ester, $C_{16}H_{15}F_3N_2O_4$) was obtained from Sigma and dissolved in 99% ethyl alcohol. Glibenclamide (5-Chloro-N-[4-(cyclohexylureidosulfonyl) phenethyl]-2-methoxybenzamide, $C_{23}H_{28}ClN_3O_5S$) was obtained from Alfa Aesar (Ward Hill, MA, USA) and dissolved in dimethyl sulphoxide (DMSO, $(CH_3)_2SO$). The final concentration of ethyl alcohol in the BayK8644 injection and the final concentration of DMSO in the glibenclamide injection were both less than 0.05%.

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Statistical Analysis Data were expressed as means ± standard error (SE). Differences between groups were analyzed using one-way analysis of variacnce (ANOVA), the Student-Newman-Kuels test, and Dunnett's t-test. *P* values < 0.05 were considered statistically significant.

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Results

A total of 48 male rats were used in the present study. The distribution of microinjection sites in the RVLM is shown in Figure 1.

Effects of NaHS on ABP, HR, and RSNA Microinjection of NaHS (4, 8, and 16 mM, 50 nl) into the RVLM decreased ABP, HR, and RSNA in a dose-dependent manner (Fig. 2). Following the NaHS microinjection (4, 8 and 16 mM, 50 nl), ABP decreased significantly from 107.05 ± 3.72 to 95.39 ± 3.03 mmHg ($P < 0.01$), from 101.80 ± 3.83 to 82.90 ± 9.59 mmHg ($P < 0.01$), and from 104.86 ± 5.02 to 75.53 ± 4.91 mmHg ($P < 0.01$), respectively. HR decreased from 416.50 ± 14.98 to 404.83 ± 16.04 bpm ($P < 0.01$), from 398.83 ± 25.10 to 376.67 ± 26.92 bpm ($P < 0.01$), and from 400.0 ± 20.78 to 368.83 ± 19.38 bpm ($P < 0.01$), respectively, and RSNA decreased significantly from 100% to 82.82 ± 1.90 % ($P < 0.01$), 71.09 ± 2.20 % ($P < 0.01$), and 54.42 ± 5.23 % ($P < 0.01$), respectively (Figure 2). The responses of ABP, HR, and RSNA to the NaHS injection started within 5 min, reached their peaks in 20 to 25 min, and lasted for 30 to 40 min. Because NaHS at the concentration of 8 mM (50 nl) produced reproducible effect on ABP, HR, and RSNA, we used this concentration of NaHS for the mechanistic evaluation. The homodynamic variables returned to baseline levels within 35 min after injection.

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Effects of glibenclamide on NaHS-induced cardiovascular response We then determined the role of K_{ATP} in the effect of NaHS (8 mM, 50 nl) on ABP, HR and RSNA. After tested the initial inhibitory effect of NaHS on ABP, HR and RSNA and these variables returned to baseline level, glibenclamide (40 μM, 50 nl) was microinjected into the RVLM followed by NaHS (8 mM, 50 nl) 10 min later in 6 rats. Glibenclamide alone had no significant effect on ABP, HR, and RSNA but eliminated the inhibitory effect of NaHS on these cardiovascular variables (Figure 3)**.** The vehicle used to dissolve glibenclamide (0.02 % DMSO in saline) showed no effect on ABP, HR, and RSNA.

Effect of *L*-NAME on the responses of ABP, HR, RSNA to NaHS. To determine if nitric oxide synthesis is involved in the effects of NaHS on sympathetic vasomotor tone, *L*-NAME was microinjected in to the RVLM. The initial NaHS microinjection into the RVLM significantly decreased the ABP from 100.35 mmHg to 82.98 mmHg ($P < 0.01$), HR from 423.5 bpm to 402.3 bpm ($P < 0.01$), and RSNA from 100 % to 66.49 % ($P < 0.01$). When *L*-NAME (200 μM, 50 nl) was microinjected into RVLM after ABP, HR, and RSNA returned to baseline levels, these variables showed no significant changes (Ma *et al.*, 2008). Subsequently, NaHS (8 mM, 50 nl) was microinjected again and ABP decreased from 99.19 to 92.56 mmHg ($P < 0.01$), HR from 426.33 bpm to 416.83bpm ($P < 0.01$), and RSNA from 100.00 % to 83.17% ($P < 0.01$) (Figure 4).

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Effects of Bay K8644 on NaHS responses To determine if Ca^{2+} is involved in the responses of ABP, HR, and RSNA to NaHS mincroinjection into the RVLM, Bay K8644 was used. In 6 rats, after the initial effect of NaHS microinjection into the RVLM on the ABP, HR, and RSNA was determined, Bay K8644 (1 μM, 50 nl) was microinjected into the RVLM. ABP, HR, and RSNA did not change in response to Bay K8644 injection. Following the subsequent NaHS (8 mM, 50 nl) microinjection, ABP decreased from 103.89 to 92.4mmHg ($P < 0.01$), HR decreased from 418.5 bpm to 395.33 bpm ($P < 0.05$), and RSNA decreased from 100 % to 72.69 % ($P < 0.01$). And there is no significance was detected with the initial effect of NaHS microinjection into the RVLM ($P > 0.05$). (Figure 5).

Effects of HA on ABP, HR and RSNA In addition, we determined the effect of blocking endogenous H_2S synthesis on sympathetic vasomotor tone by microinjection of H_2S synthesis inhibitor, HA (Abe and Kimura, 1996). After microinjection of HA (30 mM, 50 nl) into the RVLM, ABP increased significantly from 96.96 ± 4.69 to 104.40 ± 5.05 mmHg ($P < 0.01$), HR increased significantly from 378.83 ± 18.30 to 387.17 ± 17.51 bpm ($P < 0.01$), and RSNA increased significantly from 100 ± 0 % to 120.98 ± 4.42 % ($P < 0.01$). The effect of HA started within 1 min, reached its peak within 5 min, and lasted for 10 to15 min (Figure 6).

Expression of CBS in the RVLM We performed immunohistochemistry staining to show the expression of CBS in the RVLM. The CBS was immunostained by a specific anti-CBS antibody and displayed by DAB method. As shown in Figure 7, the yellow-brown color represents CBS-positive signals. The CBS-positive neurons distributes evenly in the RVLM.

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Discussion

To our knowledge, this is the first study to assess the role of H_2S in the RVLM in the regulation of sympathetic vasomotor tone. We found that microinjection of NaHS into the RVLM decreased ABP, HR and RSNA in a dose-dependent manner. Blocking K_{ATP} channels with glibenclamide abolished the sympathoinhibitory effects of NaHS; and that preinjection of *L*-NAME to inhibit NO synthesis attenuated the sympathoinhibitory effects of NaHS. Furthermore, a Ca^{2+} channel opener did not change NaHS-induced decreases in ABP, HR, and RSNA. Inhibition of H_2S synthesis with hydroxylamine increased ABP, HR and RSNA, suggesting that endogenous H_2S exert tonically inhibits sympathetic vasomotor tone. In addition, immunohistochemistry staining revealed that CBS was expressed in the RVLM. Our findings suggest that H_2S in the RVLM inhibits sympathetic vasomotor tone by opening K_{ATP} channels and that NO synthesis is involved in this process.

H_2S plays a physiological role in regulating cardiovascular functions (Xu et al., 2007; Ji et al., 2008; Xu et al., 2008). For example, intravenous injection of H_2S has been found to produce a transient but significant decrease in mean arterial blood pressure that is due to the relaxation of vascular smooth muscle cells (SMCs) resulting from the opening of K_{ATP} channels and the subsequent hyperpolarizing cell membranes of the SMCs of peripheral vessels (Zhao et al., 2001). It is well known that the RVLM, which contains vasomotor neurons that project directly to sympathetic

preganglionic neurons in intermediolateral cell column in the spinal cord and play a pivotal role in the tonic and phasic regulation of sympathetic vasomotor tone (Dampney et al., 2002). The inhibition of vasomotor neurons in this region leads to reduction of systemic arterial blood pressure and sympathetic outputs. In the current study, microinjection of the H_2S donor, NaHS, into the RVLM decreased the ABP, HR, and RSNA in a dose-dependent manner, suggesting that H_2S decreases the activity of vasomotor neurons in the RVLM. It has been shown that NaHS induces a concentration-dependent hyperpolarization and reduces input resistance of CA1 neurons and dorsal raphe neurons through activation of K_{ATP} channels (Reiffenstein et al., 1992). Furthermore, microinfusion of NaHS into the hypothalamus decreases arterial blood pressure in conscious rats, an effect that is blocked by K_{ATP} channel blocker (Dawe et al., 2008). These findings indicate that K_{ATP} channels are importantly involved in the action of H_2S in the central nervous system. Consistent with those findings, in the current study, we found that pretreatment with glibenclamide, a K_{ATP} channel blocker, abolished the effects of H_2S on ABP, HR, and RSNA, which suggests that the sympathoinhibitory effects of H_2S in the RVLM occur via the opening of K_{ATP} channels.

Although H_2S is an endogenous opener of K_{ATP} channels in many different cell types, the signaling pathways involved in the interaction between H_2S and K_{ATP} channel proteins remain unclear. Jiang et al. found that H_2S interacts with Cys6 and Cys26 residues of the extracellular N terminal of the rvSUR1 subunit of the K_{ATP}

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channel complex (Jiang et al.). Furthermore, direct chemical modification of the rvSUR1 subunit protein constitutes a molecular mechanism for the activation of K_{ATP} channels by H_2S (Jiang et al.). Therefore, the H_2S signaling pathways that interact with K_{ATP} channels are potentially involve the modulation of the rvSUR1 subunit of K_{ATP} channels in the sympathetic vasomotor neurons in the RVLM.

Previous studies have shown that NO donor treatment enhances the endogenous production of H_2S in rat aortic tissues by increasing the activity and expression of CSE (Zhao et al., 2001), suggesting that H_2S and NO interact to facilitate relaxation of vascular smooth muscle (Hosoki et al., 1997). In this regard, pretreatment with the NOS inhibitor *L*-NAME attenuates NaHS-induced cardioprotection following metabolic inhibition preconditioning (Pan et al., 2006). Consistent with those findings, in the current study, we found that pretreatment with *L*-NAME attenuated the inhibitory effects of H_2S on sympathetic vasomotor tone. These data suggest that it is likely that NO synthesis is involved in the sympathoinhititory action of H_2S in the RVLM. This notion is supported by the findings that NO can activates K_{ATP} channels in large dorsal root ganglion neurons via direct S-nitrosylation of cysteine residues in the SUR1 subunit (Kawano et al., 2009). Furthermore, NOS is expressed in the RVLM (Chan et al., 2004).

It has been shown that H_2S increases the sensitivity of carotid sinus baroreflex by opening K_{ATP} channels and closing of the L-type Ca^{2+} channels (Xiao et al., 2006; Xiao et al., 2007). And L-type Ca^{2+} channels have been identified in RVLM

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(Li et al., 1998). In the current study, however, we found that an L-type Ca^{2+} channel opener, Bay K8644, failed to change the inhibitory effects of H_2S on ABP, HR and RSNA, which suggests that L-type Ca^{2+} channels may not be involved in the sympathoinhibitory effect of H_2S in the RVLM.

Because H_2S production from cysteine by brain homogenates was strongly inhibited by the CBS inhibitors HA and aminooxyacetate (Abe and Kimura, 1996), we determined the role of endogenous H_2S in the regulation of basal sympathetic vasomotor tone. We found that microinjection of HA into the RVLM significantly increased ABP, HR, and RSNA. These data suggest that endogenous H_2S tonically inhibits sympathetic vasomotor tone. Previous studies have shown that HA acts as an NO donor to release NO (Gerova et al., 1995) and increase γ-aminobutyric acid (GABA) level (Czajka, 1978). However, infusions of NO donor sodium nitroprusside and GABA into the RVLM have both been shown to decrease sympathetic nerve activity (Yang et al., 1996; Heesch et al., 2006). In the current study, however, microinjection of H_2S into the RVLM induced an increase in ABP, HR and RSNA. Therefore, it is unlikely that the effect of HA in the RVLM was not mediated by NO and GABA. In addition, we found that CBS immunoreactivity-positive neurons were evenly distributed in the RVLM. Although we did not identify the subpopulation of neurons in the heterogeneous RVLM region, it is expected that at least a portion of CBS-positive neurons were vasomotor neurons. Moreover, because H_2S is a small gaseous molecule that can diffuse freely at a certain distance

(Reiffenstein et al., 1992). Its action on the vasomotor neurons can occur without CBS expression. Thus, the expression of CBS in the RVLM provides additional evidence that endogenous H_2S tonically exerts an inhibitory effect on sympathetic outflow.

In summary, findings from the current study provide strong evidence that H_2S inhibits sympathetic vasomotor tone in the RVLM and that endogenous H_2S is involved in the regulation of basal sympathetic outflow. These data suggest that H_2S is a novel therapeutic target that can be manipulated to reduce the sympathetic outflow in certain cardiovascular diseases that have with heightened sympathetic activity.

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Authorship Contributions:

Participated in research design: Yu-ming Wu, Qi Guo, Rui-rong He

Conducted experiments: Qi Guo, Sheng Jin, Ru Wang, Xiu-Li Wang, Lin Xiao, Yu-ming Wu

Performed data analysis: Qi Guo, Sheng Jin, Ru Wang, Lin Xiao, Xiu-Li Wang.

Wrote or contributed to the writing of the manuscript: Qi Guo, Yu-ming Wu, Rui-rong He

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Footnotes:

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Address correspondence to: Dr. Yuming Wu, Department of Physiology, Institute of Basic Medicine, Hebei Medical University, 361 Zhongshan East Road, Shijiazhuang 050017, China. E-mail: wuyum@yahoo.com

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Legends for Figures:

Figure1: Identification of microinjection sites in the RVLM. A, Representative photomicrograph depicting the RVLM injection site in a coronal brain slice. B, Schematic showing the center of microinjection sites in the RVLM. The distance posterior to the interaural line is shown below the diagram in each panel. 4 V, fourth ventricle.

Figure 2: Effects of NaHS microinjected into the RVLM on ABP, RSNA, and HR. A, Original tracing recordings showing the effects of 8 mM (50 nl) NaHS microinjected into the RVLM on ABP, RSNA, and HR. B, C, and D, Summary data showing the effect of NaHS microinjected into the RVLM on ABP (B), RSNA (C), and HR (D) (*n*=6). Data are means ± SE. **: *P* <0.01 compared with control value; ↓, microinjection of NaHS.

Figure 3: Effect of glibenclamide (40 μM) on the responses of ABP, RSNA, and HR to 8mM NaHS.

A, Original tracing recordings showing the effects of NaHS (8 mM), glibenclamide, and NaHS plus glibenclamide microinjection into the RVLM on ABP, RSNA, and HR. B, C, and D, Summary data showing the effect of NaHS, glibenclamide, and NaHS plus glibenclamide microinjection into the RVLM on ABP (B), RSNA (C), and HR (D)

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(*n*=6). Data are means ± SE. **: *P* < 0.01 compared with control value; #: *P* < 0.05, ##: *P* < 0.01 compared with NaHS (8mmol/L); ↓, microinjection of NaHS; †, microinjection of glibenclamide.

Figure 4: Effect of *L*-NAME (200 μM) on the responses of ABP, RSNA, and HR to 8mM NaHS.

A, Original tracing recordings showing the effects of NaHS (8 mM), *L*-NAME, and NaHS plus *L*-NAME microinjection into the RVLM on ABP, RSNA, and HR. B, C, and D, Summary data showing the effect of NaHS, *L*-NAME, and NaHS plus *L*-NAME microinjection into the RVLM on ABP (B), RSNA (C), and HR (D) (*n*=6). Data are means ± SE, **: *P* <0.01 compared with control value; #: *P* < 0.05, ##: *P* < 0.01 compared with NaHS (8mmol/L), ↓, microinjection of NaHS; †, microinjection of *L*-NAME.

Figure 5: Effect of Bay K8644 (1 μM) on the responses of ABP, RSNA, and HR to 8mM NaHS.

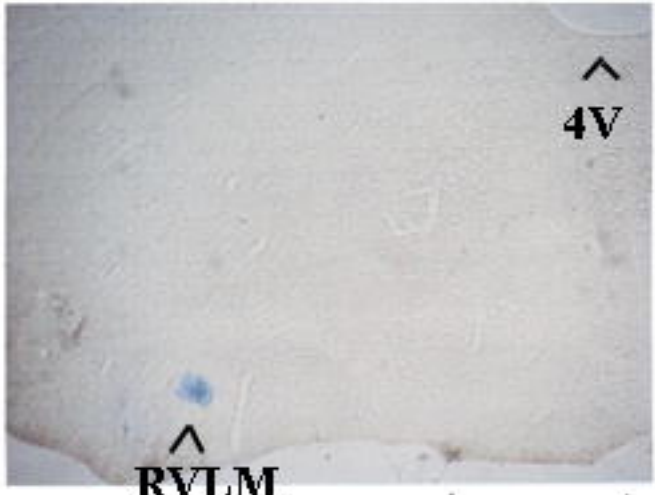
A, Original tracing recordings showing the effects of NaHS (8 mM), Bay K8644, and NaHS plus Bay K8644 microinjection into the RVLM on ABP, RSNA, and HR. B, C, and D, Summary data showing the effect of NaHS, Bay K8644, and NaHS plus Bay K8644 microinjection into the RVLM on ABP (B), RSNA (C), and HR (D) (*n*=6). Data are means ± SE. *: *P* < 0.05, **: *P* < 0.01 compared with control value; ↓,

microinjection of NaHS; †, microinjection of Bay K8644.

Figure 6: Effect of HA microinjected into the RVLM on ABP, RSNA, and HR.

A, Original tracing recordings showing the effects of HA (30 mM) microinjected into the RVLM on ABP, RSNA, and HR. B, C, and D, Summary data showing the effect of HA microinjected into the RVLM on ABP (B), RSNA (C), and HR (D) (*n*=6). Data are means ± SE. **: $P < 0.01$ compared with control value; ↓, microinjection of HA.

Figure 7: The expression of CBS in the RVLM. The yellow-brown cytoplasm represents positive signals of CBS expression: A (magnification ×40), B (magnification×100), C (magnification×400).



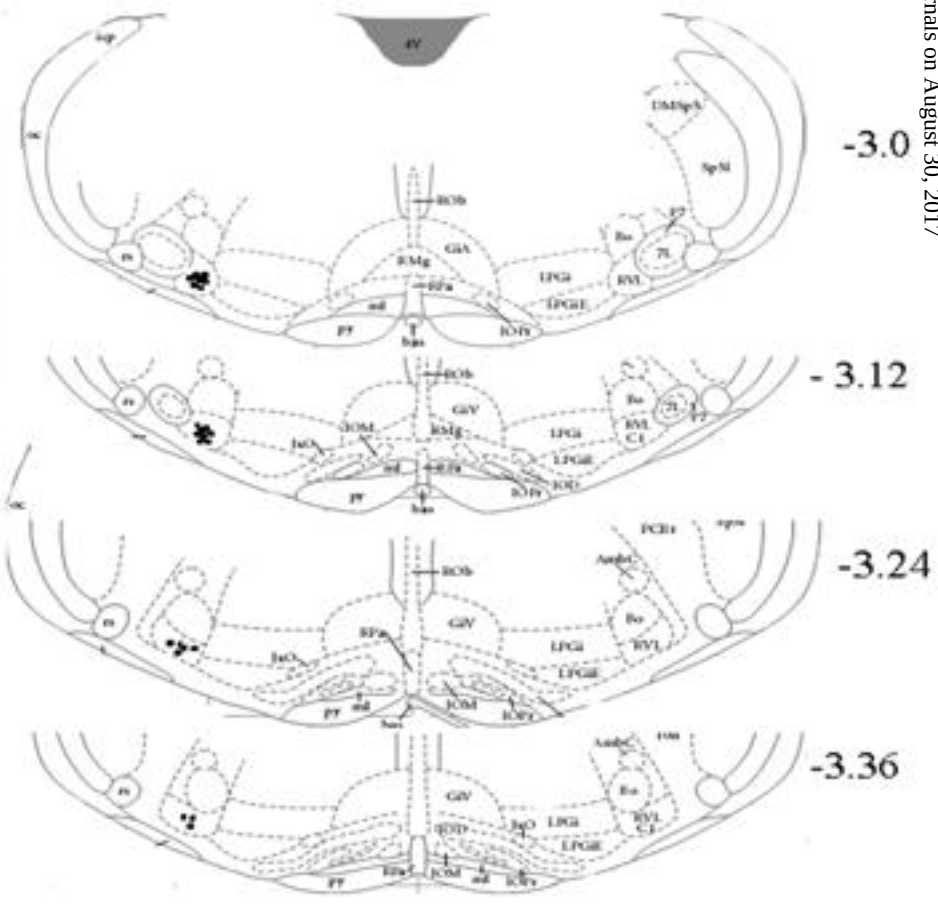


Figure 1

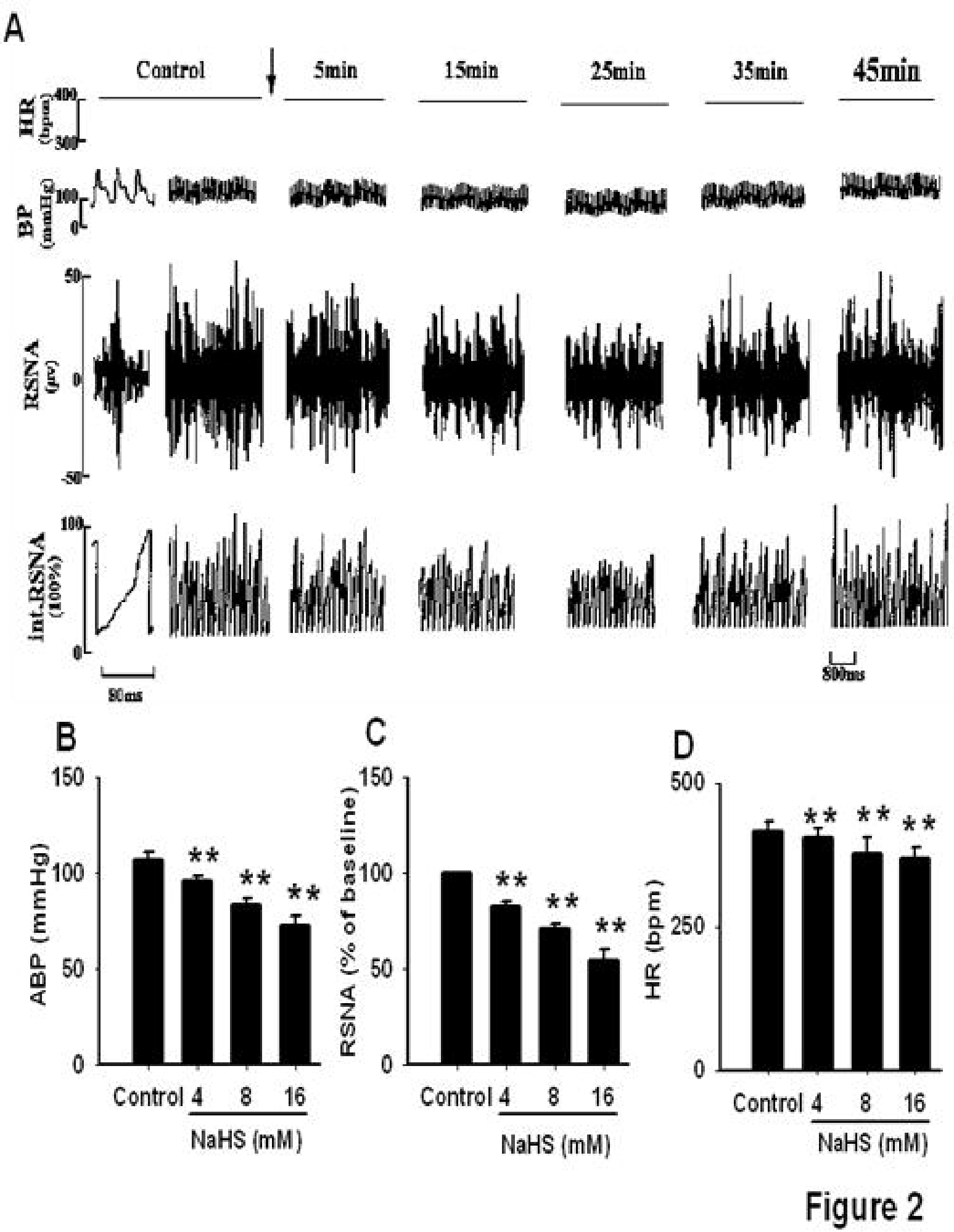

A
Control
5min
15min
25min
35min
45min
HR (bpm)
400
300
BP (mmHg)
100
0
RSNA (μv)
50
0
-50
Int.RSNA (100%)
100
0
80ms
500ms
B
ABP (mmHg)
150
100
50
0
**
Control
4
8
16
NaHS (mM)
C
RSNA (% of baseline)
150
100
50
0
**
Control
4
8
16
NaHS (mM)
D
HR (bpm)
500
250
0
**
Control
4
8
16
NaHS (mM)

Figure 2

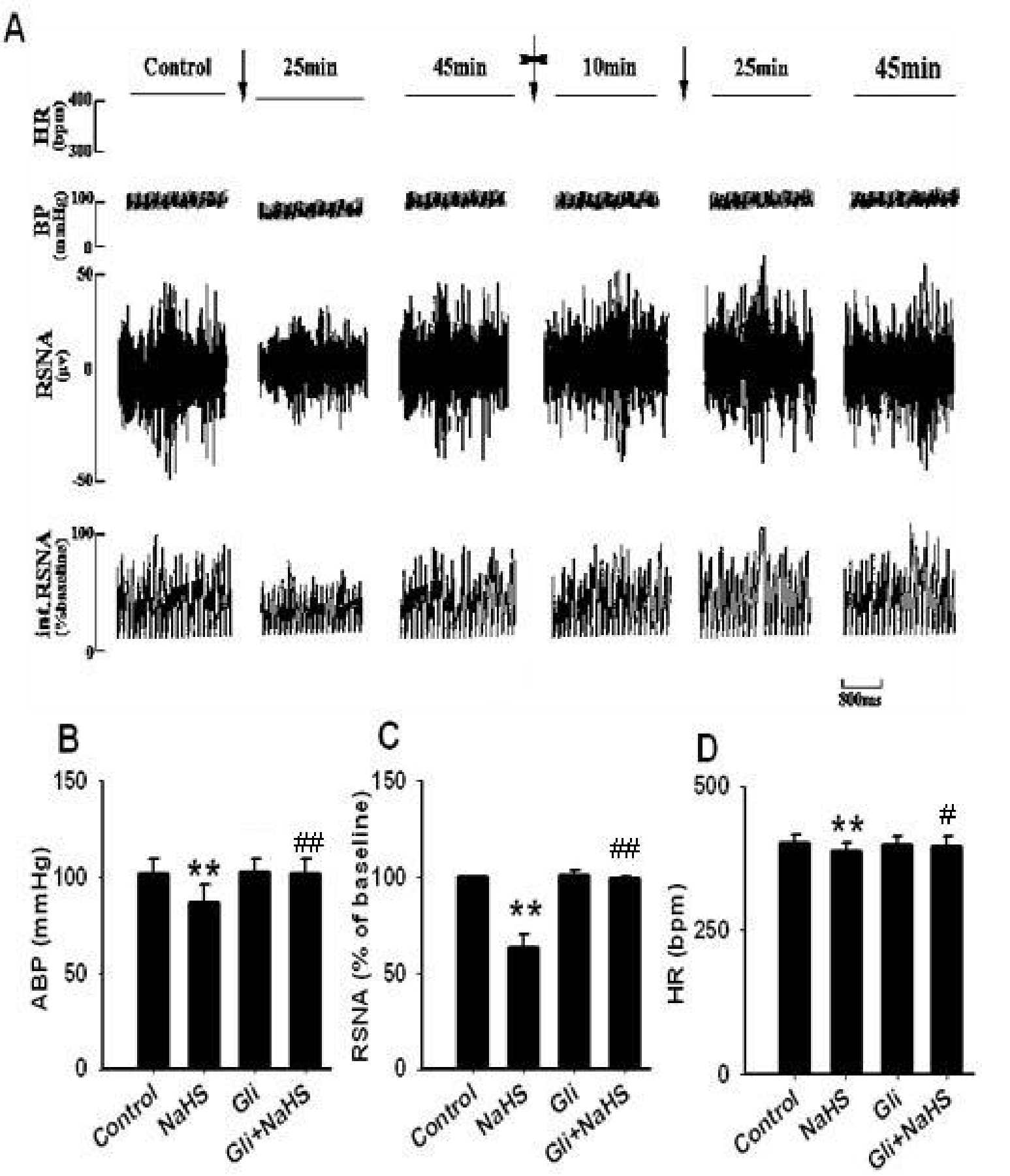

A
Control
25min
45min
10min
25min
45min
HR (bpm)
400
300
BP (mmHg)
100
0
RSNA (μv)
50
0
-50
Int.RSNA (%baseline)
100
0
500ms
B
ABP (mmHg)
150
100
50
0
**
##
Control
NaHS
Gli
Gli+NaHS
C
RSNA (% of baseline)
150
100
50
0
**
##
Control
NaHS
Gli
Gli+NaHS
D
HR (bpm)
500
250
0
**
#
Control
NaHS
Gli
Gli+NaHS

Figure 3

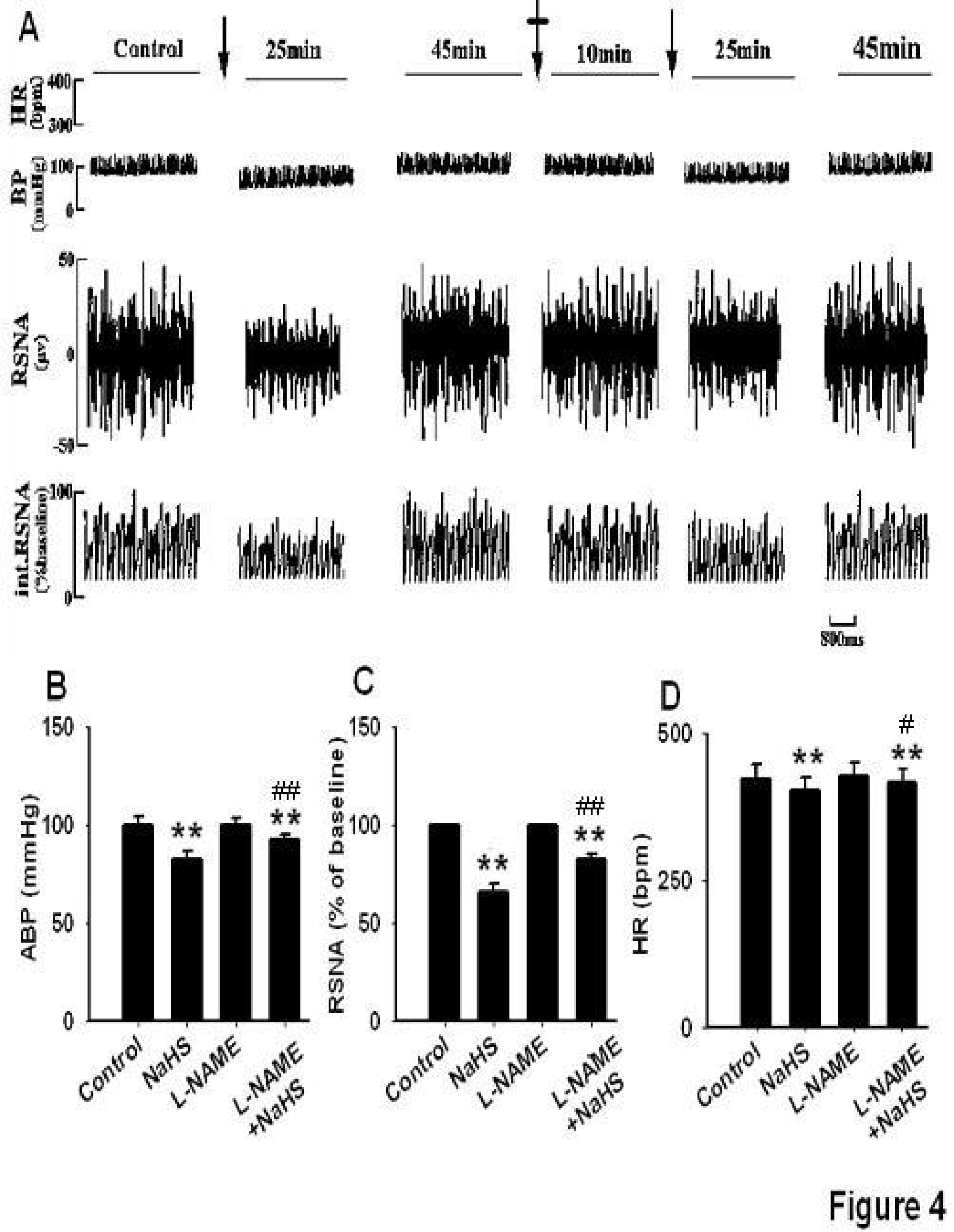

A
Control
25min
45min
10min
25min
45min
HR (bpm)
400
300
BP (mmHg)
100
0
RSNA (μv)
50
0
-50
intRSNA (%baseline)
100
0
100ms
B
ABP (mmHg)
150
100
50
0
**
##
**
Control
NaHS
L-NAME
L-NAME +NaHS
C
RSNA (% of baseline)
150
100
50
0
**
##
**
Control
NaHS
L-NAME
L-NAME +NaHS
D
HR (bpm)
500
250
0
**
#
**
Control
NaHS
L-NAME
L-NAME +NaHS

Figure 4

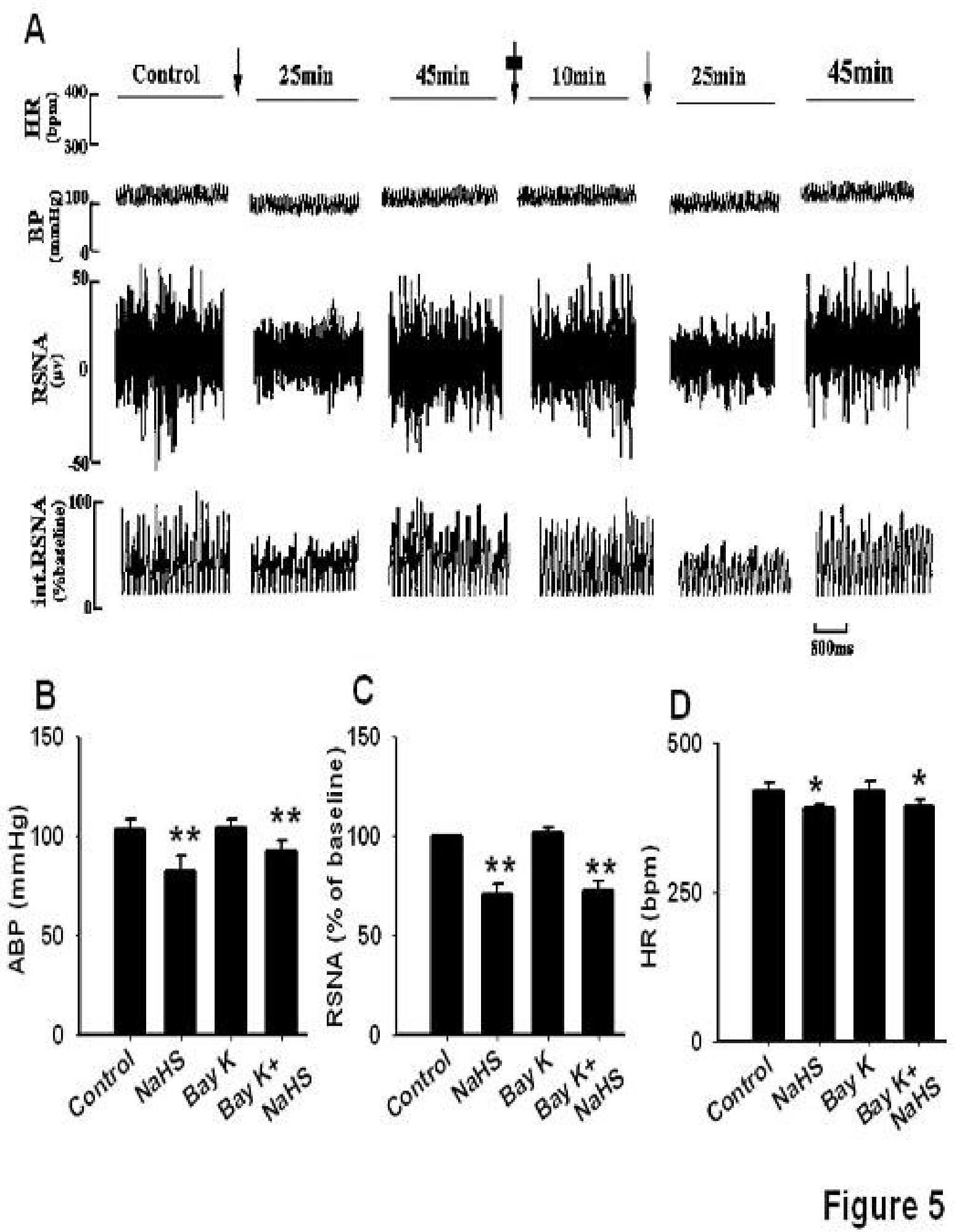

A
Control
25min
45min
10min
25min
45min
HR (bpm)
400
300
BP (mmHg)
100
0
RSNA (μv)
50
0
-50
intRSNA (%baseline)
100
0
500ms
B
ABP (mmHg)
150
100
50
0
**
**
Control
NaHS
Bay K
Bay K+ NaHS
C
RSNA (% of baseline)
150
100
50
0
**
**
Control
NaHS
Bay K
Bay K+ NaHS
D
HR (bpm)
500
250
0
*
*
Control
NaHS
Bay K
Bay K+ NaHS

Figure 5

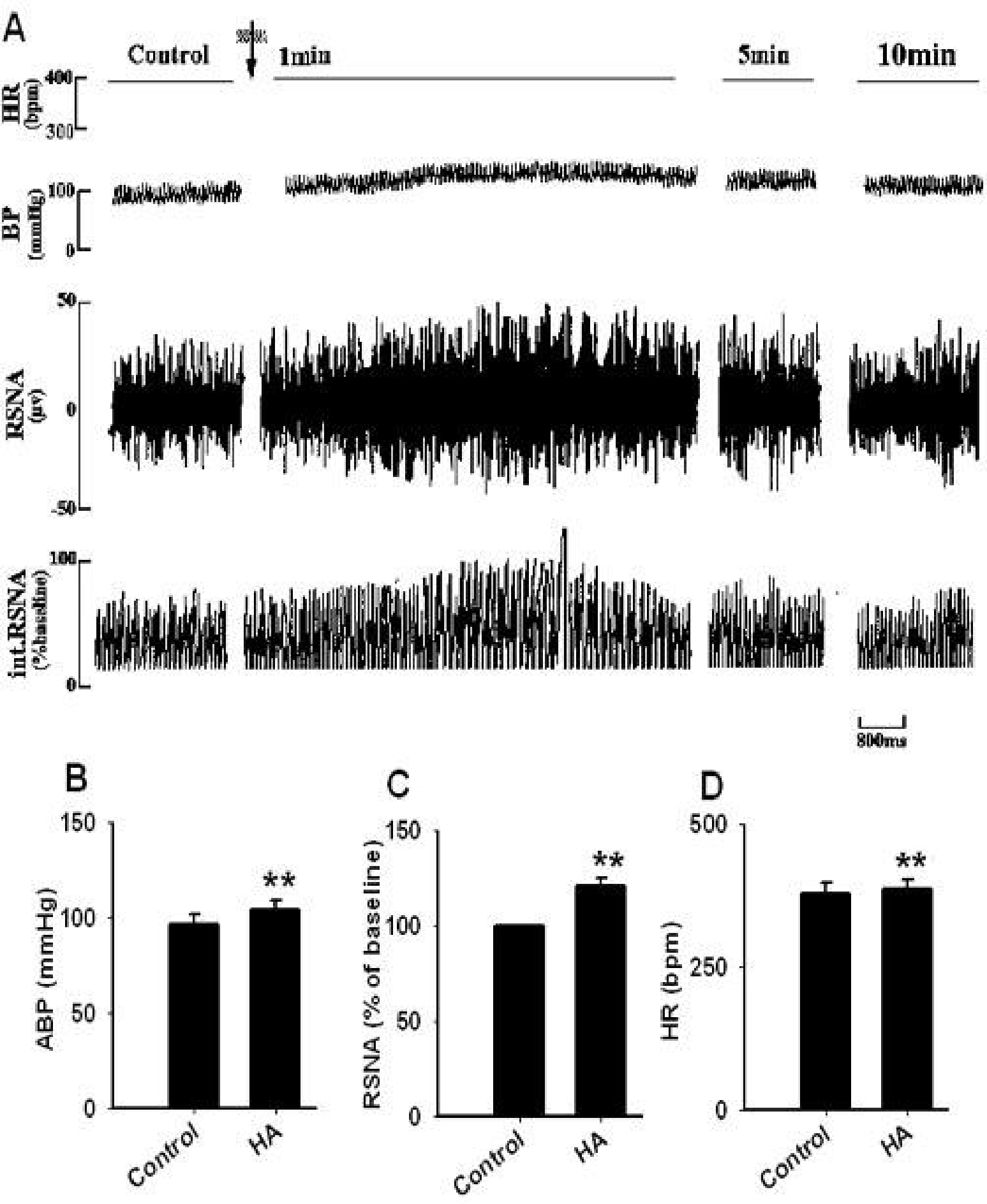
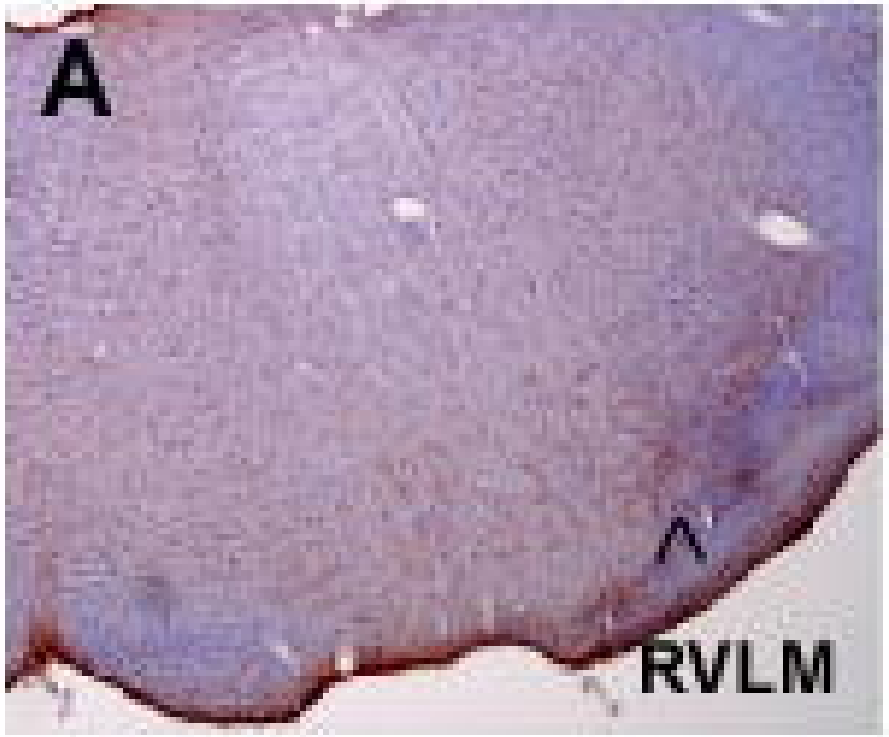

A
Control
1min
5min
10min
HR (bpm)
400
300
BP (mmHg)
100
0
RSNA (μv)
50
0
-50
IntRSNA (%baseline)
100
0
800ms
B
ABP (mmHg)
150
100
50
0
**
Control
HA
C
RSNA (% of baseline)
150
100
50
0
**
Control
HA
D
HR (bpm)
500
250
0
**
Control
HA

Figure 6



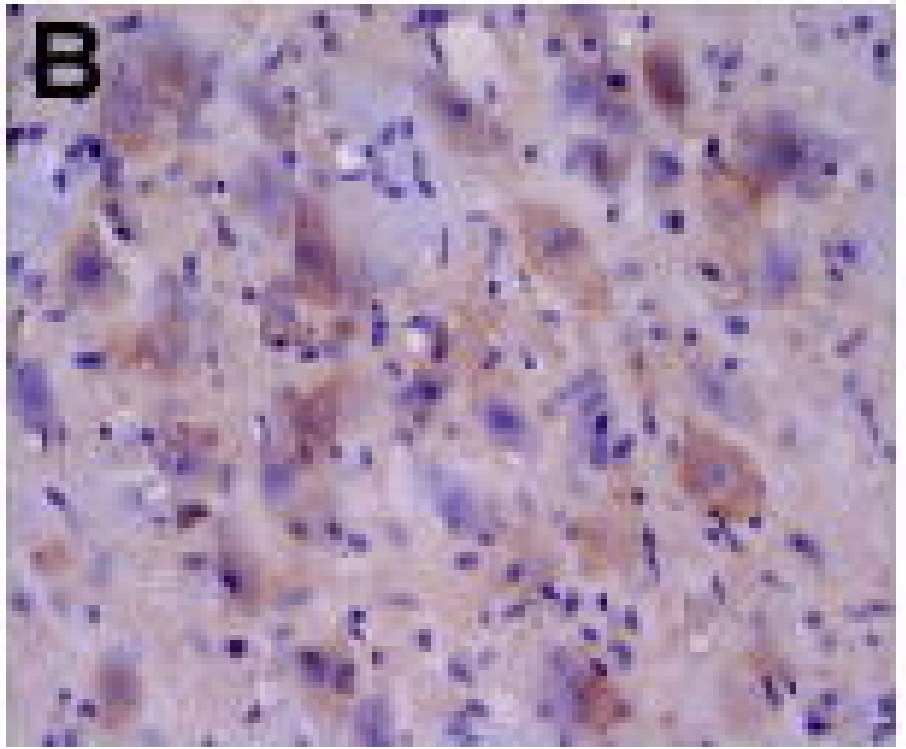


Figure 7