Gene transfer of cystathionine β-synthase into RVLM increases hydrogen sulfide-mediated suppression of sympathetic outflow via $K_{ATP}$ channel in normotensive rats

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Gene transfer of cystathionine β-synthase into the rostral ventrolateral medulla (RVLM) increases hydrogen sulfide-mediated suppression of sympathetic outflow via $K_{ATP}$ channel in normotensive rats. Am J Physiol Heart Circ Physiol 308: H603–H611, 2015. First published January 16, 2015; doi:10.1152/ajpheart.00693.2014.—Hydrogen sulfide has been shown to have a sympathoinhibitory effect in the rostral ventrolateral medulla (RVLM). The present study examined the function of cystathionine β-synthase (CBS)/hydrogen sulfide system in the RVLM, which plays a crucial role in the control of blood pressure and sympathetic nerve activity. Adenovirus vectors encoding CBS (Ad-CBS) or enhanced green fluorescent protein (AdEGFP) were transferred into the RVLM in normotensive rats. Identical microinjection of AdCBS into the RVLM had no effect on systolic blood pressure and heart rate (HR) in conscious rats. Acute experiments were performed at day 7 after gene transfer in anesthetized rats. Microinjection of the CBS inhibitors hydroxylamine (HA) or amino-oxyacetate into the RVLM produced an increase in the renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP), and HR. There was a potentiation of the increases in RSNA, MAP, and HR because of the CBS inhibitors in AdCBS-injected rats compared with AdEGFP-injected rats. Pretreatment with pinacidil, a $K_{ATP}$ channel activator, abolished the effects of HA in two groups. Microinjection of glibenclamide, a $K_{ATP}$ channel blocker, produced increases in RSNA, MAP, and HR in AdCBS-injected rats. No changes in behavior were observed in AdEGFP-injected rats. Furthermore, Western blot analysis indicated an increase in the expression of sulfonylurea receptor 2 and inward rectifier $K^{+}$ in AdCBS-injected rats. These results suggest that the increase in $K_{ATP}$ channels in the RVLM may be responsible for the greater sympathetic outflow and pressor effect of HA in AdCBS-injected rats compared with AdEGFP-injected rats.

cystathionine β-synthase; rostral ventrolateral medulla; renal sympathetic nerve activity; gene transfer; blood pressure

HYDROGEN SULFIDE (H$_2$S) is an endothelium-derived hyperpolarizing factor that enhances relaxation of the peripheral vasculature (2, 28). Recent studies (9, 13, 34) have also reported its physiological functions in the central nervous system. In mammalian tissues, H$_2$S is produced through degradation of l-cysteine mainly by two main enzymes: cystathionine β-synthase (CBS) and cystathionine γ-lyase (15). CBS is primarily found in the brain, whereas CSE is mainly expressed in the peripheral tissues (15). CBS has been found in regions of the brain, such as the paraventricular nucleus (11) and rostral ventrolateral medulla (RVLM) (13), which are responsible for the regulation of sympathetic nerve activity and thus arterial blood pressure.

However, conflicting results have also been reported regarding the role of H$_2$S in regulating sympathetic nerve activity and blood pressure. Previous experiments have demonstrated that bilateral microinjection of sodium hydrosulfide (NaHS), an H$_2$S donor, into the RVLM decreases arterial blood pressure, and the CBS inhibitor hydroxylamine (HA) produces a pressor response (13). Contrary, another study has shown that the microinjection of NaHS into the RVLM has no effect on mean arterial pressure (MAP) (26). Furthermore, conflicting results have been obtained with respect to the effects of H$_2$S in the paraventricular nucleus (11, 26). Although the effect of H$_2$S or CBS inhibitors in the RVLM on cardiovascular function has been extensively investigated, most studies are conducted in acute experimental conditions and with the use of anesthetized animals. The long-term effects of increased H$_2$S production in the central nervous system on the regulation of sympathetic nerve activity and blood pressure in conscious rats remain unclear.

It has been shown that microinfusion of NaHS into the hypothalamus decreases arterial blood pressure by opening ATP-sensitive potassium ($K_{ATP}$) channels (9). Furthermore, we have shown that H$_2$S increases the sensitivity of the carotid sinus baroreflex, an effect that is blocked by $K_{ATP}$ channel blocker (30, 31). These findings indicate that $K_{ATP}$ channel-dependent mechanisms may be involved in H$_2$S-mediated regulation of sympathetic nerve activity and blood pressure in the central nervous system.

Several studies provide evidence that the overexpression of CBS by adenoviral vectors encoding cDNA of CBS leads to a long-term effect of increased endogenous H$_2$S production (16, 25). The aims of the present study were to determine if endogenous H$_2$S production can be increased by CBS gene transfer in the RVLM, if this change contributes to the functional effects on the regulation of sympathetic nerve activity and blood pressure, and if these effects are mediated by a $K_{ATP}$ channel mechanism. For this purpose, we transfected adenovirus encoding constitutive CBS into the RVLM of rats.

METHODS

General procedures and gene transfer into the RVLM in vivo. Experiments were carried out on male Sprague-Dawley (SD) rats
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weighing between 300 and 320 g (provided by the Experiment Animal Center of Hebei Province, China). The procedures were approved by the Experimental Animal Care and Use Committee of HeBe Medical University and complied with the NIH guidelines (Guide for the Care and Use of Laboratory Animals). Standard rodent diet and tap water were available ad libitum to all rats.

Adenoviral vectors carrying CBS gene (AdCBS, GenBank: M88344.1, supplied from GeneSil Biotechnology) or enhanced green fluorescent protein (AdEGFP; as a control vector) were delivered into the RVLM by microinjection bilaterally. The rats were housed in a temperature-controlled room on a 12-h:12-h light-dark cycle with standard chow and tap water ad libitum.

On the day of the RVLM injections, each rat was anesthetized with pentobarbital sodium (50 mg/kg ip). The coordinates of the RVLM, determined using the Paxinos and Watson atlas, were 11.96 mm posterior to bregma, 1.6 mm lateral to the midline, and 10.8 mm ventral to the dura. The RVLM was functionally identified by a pressor response of at least 25 mmHg in response to an injection of L-glutamate (2 nmol). The histological identification was made to verify each microinjection site. An adenoviral suspension (1 × 10⁹ plaque forming units/ml) was injected into the bilateral RVLM within 10 min with a syringe infusion pump (KDS101, KD Scientific, Holliston, MA). The total volume of the injection was 100 nl for each side of the RVLM. After the injection, all rats recovered from the anesthesia and were unrestrained and free to move about their cages.

Seven days after injection, rats were used for functional experiments.

**Measurement of systolic blood pressure in conscious rats.** The systolic blood pressure (SBP) of the tail artery was measured using a noninvasive computerized tail-cuff system (NIBP, ADInstruments, Sydney, Australia) in the conscious state as previously described (32). Briefly, the SBP was consecutively measured every other day between 8:00 AM and 10:00 AM. Before the measurements were taken, the rats were warmed for 5–20 min at 28°C to allow detection of tail artery pulsations and achieve a steady pulse level. The SBP was obtained by averaging six measurements.

**RVLM sample preparation.** The rat was anesthetized with an overdose of pentobarbital sodium (100 mg/kg ip) at the end of experiments, and subsequently the brain of the rat was quickly removed and frozen in liquid nitrogen and stored at −80°C until being sectioned. The tissue containing the injected RVLM sites was obtained through punch-out technique as previously described (36).

**Western blot analysis for measurement of CBS and subunits of KATP channels protein in RVLM.** Western blot analysis for CBS, inward rectifier K⁺ 6.1 and 6.2 (Kir6.1 and Kir6.2), and sulfonylurea receptors 1 and 2 (SUR1 and SUR2) protein from tissue containing the injection sites of the RVLM obtained using the micropunch

![Image](https://example.com/image1.png)

**Fig. 1.** Representative sections showing the sites of the rostral ventrolateral medulla (RVLM) microinjection and representative Western blot analysis of cystathionine β-synthase (CBS) protein and hydrogen sulfide (H₂S) levels in the RVLM of rats. A: plots of RVLM (red) according to Paxinos and Watson’s rat atlas and green fluorescence at day 7 after the RVLM microinjection of adenovirus vectors encoding enhanced green fluorescent protein (AdEGFP). B: representative Western blot analysis of the CBS protein expression in the RVLM before and after CBS gene transfer. *P < 0.05 vs. day 0. Values are means ± SE; n = 6 for each group. C: H₂S levels in the RVLM before and after CBS gene transfer. *P < 0.05 vs. day 0. Values are means ± SE; n = 6 for each group. D: representative Western blot analysis of the CBS protein expression in the RVLM of rats infected with adenovirus vectors encoding CBS (AdCBS) and infected with AdEGFP at day 7 after gene transfer. *P < 0.05 vs. AdEGFP. Values are means ± SE; n = 6 for each group. E: H₂S levels in rats treated AdEGFP and in rats treated with AdCBS. *P < 0.05 vs. AdEGFP-treated rats. Values are means ± SE; n = 6 for each group.

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Measurement of H₂S level in RVLM. H₂S levels in the RVLM were measured using a rat H₂S enzyme-linked immunosorbent assay (ELISA) kit (Cambridge, MA) according to the manufacturer’s instructions as previously described (11). Briefly, a 96-well microplate was coated with an antibody specific for H₂S. Standard and samples were transferred to assay plate, incubated at 37°C, and then washed. Subsequently, horseradish peroxidase-conjugated reagent (Gaithersburg, MD) was added, incubated, and then washed. Chromogenic solution were added and kept in the dark at 37°C. Stop solution was added to stop the reaction. The optical density was measured at 450 nm using a ELISA plate reader (ELX-800, BioTeK, Winooski, VT).

Microinjection into RVLM. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip followed by 20 mg·kg⁻¹·h⁻¹ iv) and instrumented for recording the renal sympathetic nerve activity (RSNA), MAP, and heart rate (HR) at day 7 after the gene transfer. Glibenclamide (Ward Hill, MA) and pinacidil (St. Louis, MO) were dissolved in dimethyl sulfoxide and finally diluted with artificial cerebrospinal fluid (aCSF) consisting of (in mM) 133.3 NaCl, 3.4 KCl, 1.3 CaCl₂, 1.2 MgCl₂, 0.6 NaH₂PO₄, 32.0 NaHCO₃, and 3.4 glucose (pH 7.4). The final concentration of dimethyl sulfoxide in aCSF was <0.05%, which showed no effect on RSNA, MAP, and HR. HA (St. Louis, MO) and amino-oxyacetate (AOA; St. Louis, MO) were dissolved into aCSF. The left femoral artery was cannulated to record MAP with a pressure transducer. The HR was measured by triggering from the pulsatile blood pressure. The RSNA was recorded as previously described (13). The left kidney was exposed through a retroperitoneal incision, 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 ensure thatafferent activity was not recorded. The nerve was placed a pair of silver recording electrodes and was 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 a alternating current amplifier (Quadt, Bridge, Australia) to simultaneously record MAP and RSNA. Background electrical noise was determined after section of the central end of the nerve and was subtracted from the integrated RSNA values (5). The percent change in RSNA from baseline was calculated. The changes in integration of the nerve discharge during the experiment were expressed as a percentage of the basal value.

Experimental protocols. In this study, the following two groups of rats were used. SD rats were randomly subjected to the RVLM microinjection of AdEGFP (AdEGFP-treated rats) or AdCBS (Ad-CBS-treated rats). Acute experiments were carried out at day 7 after the gene transfer.

First, SBP and HR were measured before gene transfer and every other day after the gene transfer in two groups of conscious rats (n = 6 for each group). At the same time, CBS protein expression and H₂S production in the RVLM were measured in two groups (n = 6 for every time) to determine the time period of gene transfer. The H₂S level was determined with ELISA. The maximal effects were observed at day 7 after the CBS gene transfer. Therefore, the following experiments were performed at day 7 after the gene transfer.

Second, the experiments were performed to examine whether the endogenous H₂S-mediated inhibitory effects in the RVLM on sympathetic outflow was enhanced in rats injected with AdCBS compared with rats injected with AdEGFP. After a stable 30-min recording of the MAP, HR, and RSNA was obtained, aCSF, as vehicle solution, was microinjected into the RVLM. Subsequently, the CBS inhibitors HA (10, 25, and 50 mM, 100 nl) or AOA (1 mM, 100 nl), were microinjected into the RVLM, and the RSNA, MAP, and HR were recorded. One dose was tested per rat.

Lastly, to test the possibility that the increased suppression of sympathetic outflow was mediated by KATP channels, the microinjection of HA (50 mM, 100 nl) was carried out after pinacidil (20 μM, 50 nl) pretreatment, and alone microinjected glibenclamide (40 μM, 50 nl) into the RVLM and investigated subunits of K₄.₆.₇ channels protein in the RVLM. In two groups of the rats, the samples were quickly collected for the measurement of the SUR1, SUR2, Kir6.1, and Kir6.2 protein expression in the RVLM at day 7 after the gene transfer (n = 6 for each group).

Statistical analysis. All values are expressed as means ± SE. Responses of RSNA, MAP, and HR to the various doses of drugs were expressed as the percent change over the basal value. Differences between groups were analyzed using one-way ANOVA, followed by post hoc Bonferroni test and unpaired t-test. P < 0.05 were considered to indicate statistical significance.
RESULTS

Fluorescence expression of enhanced green fluorescence protein in the RVLM. To measure the role of H\(_2\)S in the central regulation, we used an adenovirus encoding the CBS gene to overexpress CBS in the RVLM. We targeted the RVLM by microinjection of either AdEGFP alone or AdCBS. Seven days after the microinjection, the specific location of EGFP on brain sections was determined with a fluorescent stereomicroscope (Fig. 1A).

Measurement of the CBS protein and H\(_2\)S production in the RVLM. To confirm effective overexpression of CBS in the RVLM with these viruses, a Western blot analysis of micropunches obtained from the RVLM was performed at days 0, 3, 7, 11, or 14 after the gene transfer. The expression of the CBS protein peaked at day 7 after the CBS overexpression and then declined over time in the RVLM of rats transfected with AdCBS (Fig. 1B). In addition, the H\(_2\)S production was measured in the RVLM. The time course of the H\(_2\)S production was compatible with that of the expression of the CBS protein in the RVLM (Fig. 1C).

As shown in Fig. 1D, CBS protein production was increased by 83% in rats treated with AdCBS compared with rats treated with AdEGFP at day 7 after the gene transfer. In addition, the level of H\(_2\)S was significantly higher in rats transfected with AdCBS than in rats transfected with AdEGFP (114 ± 7.5 vs. 73.1 ± 5.6 pg/mg; \(P < 0.05\)) at day 7 after the gene transfer (Fig. 1E).

\(\text{H}_2\text{S}\)-mediated sympathetic responses. Figure 2 shows the time course of SBP and HR before and after the microinjection of AdEGFP or AdCBS into the RVLM, recorded with a noninvasive computerized tail-cuff system in conscious rats. There were no significant differences in SBP and HR between the two groups in the conscious state. We next examined the effect of microinjection of HA on the RSNA, MAP, and HR at day 7 after the gene transfer in both groups of animals (Fig. 3). The magnitudes of the increases in the RSNA, MAP, and HR evoked by HA were significantly increased in the AdCBS-treated group compared with the AdEGFP-treated group (RSNA, 21.3 ± 3.7 vs. 14.2 ± 3.2%; MAP, 17.0 ± 2.1 vs. 9.7 ± 1.3 mmHg; and HR, 24.6 ± 4.2 vs. 14.7 ± 3.1 beats/min; \(P < 0.05\)). No change was noted in the responses of RSNA, MAP, and HR to microinjection of HA into the RVLM between noninjected normal rats and rats injected with AdEGFP (6; data not shown). In addition, the responses to microinjection of AOA, another CBS inhibitor, were consistent with those of HA (Fig. 4).

To determine if the endogenous H\(_2\)S-mediated inhibitory effect is dependent on K\(_{\text{ATP}}\) channel function, the RSNA, MAP, and HR responses to the RVLM injection of HA were determined, after the pretreatment with pinacidil (K\(_{\text{ATP}}\) chan-
nel activator) in rats. After the initial effect of HA on RSNA, MAP, and HR was tested and these variables returned to baseline level, pinacidil was microinjected into the RVLM followed by HA. Fifteen minutes after pinacidil injection, another dose of HA was injected into the RVLM. Pretreatment with pinacidil completely abolished the increases in response to the administration of HA in two groups (Fig. 5).

To determine if KATP channels are involved in the mechanism of the potentiation of the increases in RSNA, MAP, and HR in response to the administration of HA in AdCBS-injected rats, glibenclamide was injected into the RVLM. Microinjection of glibenclamide into the RVLM at day 7 after the gene transfer elicited a small but significant increases (change in RSNA, 11.4 ± 2.1%; change in MAP, 14.1 ± 2.7 mmHg; and change in HR, 22.7 ± 2.7 beats/min, P < 0.05) in rats transfected with AdCBS. In contrast, microinjection of glibenclamide did not alter RSNA, MAP, and HR (change in RSNA, 4.2 ± 2.1%; change in MAP, 4.5 ± 1.2 mmHg; and change in HR, 6.1 ± 1.3 beats/min, P < 0.05) in rats transfected with AdEGFP (Fig. 6).

CBS gene transfer increased the expression of KATP channels subunits. Western blot analysis showed that Kir6.1 and SUR2 proteins expression was increased, whereas SUR1 and Kir6.2 showed no change in the RVLM in AdCBS-injected rats compared with AdEGFP-injected rats at day 7 after the gene transfer (Fig. 7).

**DISCUSSION**

The major findings of this study are as follows. First, this study showed for the first time that long-term increases in H$_2$S production in the RVLM were evoked by CBS gene transfer into the RVLM in rats. Second, although overexpression of CBS in the RVLM had no effect on SBP and HR in the conscious state, it potentiated the increases in RSNA, MAP, and HR in response to the administration of CBS inhibitor in AdCBS-injected rats compared with AdEGFP-injected rats. These results suggest that the increase in K$_{ATP}$ channels in the RVLM may be responsible for the greater sympathetic outflow and pressor effect of the CBS inhibitors. These findings suggest that the CBS/H$_2$S system is a novel target for cardiovascular gene therapy, which may be useful in certain cardiovascular diseases considered to have with heightened sympathetic outflow.

H$_2$S, like other gaseous signal molecules, such as carbon monoxide and nitric oxide (NO), is considered as an important
endogenous gaseous transmitter in many physiological functions (28, 33). However, most of these experiments were performed in anesthetized state and examined only acute effects of H2S. Recent studies determine the effect CBS overexpression by adenoviral vectors carrying CBS gene produce an increase in H2S production for a much longer period (16, 25). Dumasius et al. (10) observed that the recombinant adenoviruses produce physiologically significant levels of transgene as early as 2–4 h after infection. Consistent with those findings, in the current study, we found the GFP fluorescence and expression appeared as early as 24 h, peaked at day 7, and lasted 2 wk after the gene transfer (n = 4; data not shown). Adenoviruses are proven to allow highly efficient delivery of transfer to the brain and infect both neuronal and glial cell types (8, 27). In the present study, the successful gene transfer into the RVLM was confirmed by several methods. First, the expression of GFP fluorescence was localized in the RVLM without noticeable diffusion. Second, CBS protein expression in the RVLM was confirmed by Western blot analysis. The increase in CBS protein peaked at day 7 after CBS gene transfer in the RVLM. Third, the time course of the H2S production was compatible with the expression of CBS protein in the RVLM in this study.

Fig. 5. Effects of pretreatment with the RVLM microinjection of pinacidil on the RSNA, MAP, and HR responses to the microinjection of HA were investigated in AdEGFP-treated rats and AdCBS-treated rats. Microinjections of HA were carried out 15 min after pinacidil pretreatment. A: original tracing recordings showing the effects of HA (50 mM, 100 nl), pinacidil (20 μM, 50 nl), and HA plus pinacidil microinjection into the RVLM on RSNA, MAP, and HR. B: summary data showing the effect of HA, pinacidil, and HA plus pinacidil microinjection into the RVLM on RSNA, MAP, and HR. *P < 0.05 vs. baseline values. #P < 0.05 vs. AdEGFP. Values are means ± SE; n = 6 for each group.

H2S plays a notable role in the regulation of physiological functions in the central nervous system. It is well known that RVLM plays an important role in maintaining baseline sympathetic vasomotor tone and arterial blood pressure (6, 23). The presence of CBS-positive neurons in the RVLM of the brain stem suggests that H2S may serve as a physiological regulator of the sympathetic nervous system (13). In the current study, we found that the magnitudes of the increases in RSNA, MAP, and HR evoked by HA were larger in the group transfected...
with CBS compared with the control group. Similarly, administration of AOA into the RVLM elicited consistent responses. These data indicate that the overexpression of the CBS gene in the RVLM leads to an increased endogenous H2S production, which in turn plays a functional role in inhibiting baseline sympathetic vasomotor tone in the RVLM. These data suggest that the endogenous H2S-mediated effect in the RVLM is more effective in suppressing RSNA in AdCBS-treated rats than AdEGFP-treated rats.

If this is the case, what is the mechanism of the potentiated increases in RSNA, MAP, and HR in response to the administration of HA into the RVLM of AdCBS-injected group? One of the possible mechanisms is that the increased H2S caused by CBS gene transfer may potentially involve the change of KATP channels in the RVLM, which is supported by the following findings. One experiment has suggested that NaHS evokes a concentration-dependent hyperpolarization and decreases the input resistance of CA1 neurons by opening of KATP channels (24). In addition, microinfusion of NaHS into the RVLM decreases arterial blood pressure by opening KATP channel in rats (13). These results indicate that KATP channels are involved in the action of H2S in the central nervous system. In the current study, we found that pretreatment with pinacidil, a KATP channel activator, abolished the effects of HA on RSNA, MAP, and HR, suggesting that the sympathoinhibitory effects of endogenous H2S in the RVLM is mediated by the KATP channels. Furthermore, microinjection of glibenclamide, a KATP channel blocker, produced increases in RSNA, MAP, and HR in AdCBS-injected rats. No changes in behavior were observed in AdEGFP-injected rats. These data indicate either an increase in the number of KATP channels or an increase in the frequency of openings in the RVLM of rats injected with AdCBS. It is well known that KATP channels consist of four inward rectifier K+/H11001 (Kir6.1 or Kir6.2) and four sulfonylurea receptors (SUR1 or SUR2) (29). In the brain, KATP channels consist of the sulfonylurea receptor subunits and the Kir6.x potassium channel subunits (1, 19). We next further examined the expression of the isoforms of KATP channels. In the current study, the expression of the pore-forming Kir6.1 protein and SUR2, the major binding site for the K+ channel opener, were obviously increased, indicating that the number of KATP channels was increased. These findings suggested that the upregulation of Kir6.1 and SUR2 of KATP channels in the RVLM after CBS gene transfer may be responsible for the potentiation of the increases in

Fig. 6. Effects of glibenclamide (Gli) microinjected into the RVLM on RSNA, MAP, and HR in AdEGFP-treated rats and AdCBS-treated rats. A: original tracing recordings showing the effects of glibenclamide (40 μM, 50 nl) microinjected into the RVLM on RSNA, MAP, and HR. B: summary data showing the effect of glibenclamide microinjected into the RVLM on RSNA, MAP, and HR (n = 6). Data are means ± SE. *P <0.05 vs. baseline values. #P <0.05 vs. AdEGFP. Values are means ± SE; n = 6 for each group.
RSNA, MAP, and HR in response to HA. Whereas whether the frequency of openings of K_{ATP} channels was increased, further studies are needed to address this question.

Recently, the novel endogenous gas H_{2}S has been recognized as another cardiovascular gasotransmitter that exerts important cardiovascular effects, which is similar to many physiological roles of NO. Kishi et al. (20, 21) found that the increase in NO production caused by the overexpression of endothelial NO synthase in the bilateral RVLM decreases MAP, HR, and sympathetic nerve activity in conscious rats. Previous studies have shown that microinjection of NaHS in the RVLM decreased sympathetic nerve activity and blood pressure (13), suggesting that H_{2}S tonically inhibits sympathetic vasomotor tone in the RVLM. However, in the present study, the increase in H_{2}S production caused by the overexpression of CBS did not change blood pressure. Another possibility to consider is that overexpression of CBS did not change blood pressure may be because the depressor effect of H_{2}S is weaker compared with rats injected with AdEGFP-injected rats, when injected HA into the RVLM. This may explain why endogenous H_{2}S-mediated effect in the RVLM of AdCBS-injected rats is more effective in suppressing sympathetic outflow. However, we did not explore the detailed mechanisms involved in the interaction between H_{2}S and K_{ATP} channels in the regulation of sympathetic vasomotor tone in the RVLM requires further investigation.

Fig. 7. Representative Western blot analysis results showing expression of subunits of ATP-sensitive potassium channels in punched RVLM samples. *P < 0.05 vs. AdEGFP. Values are means ± SE; n = 6 for each group. SUR, sulfonylurea receptor; Kir, inward rectifier K⁺.

Furthermore, we propose that the compensatory mechanisms against the sympathoinhibitory effects of H_{2}S would be in a dominant position in AdCBS-injected rats compared with in AdEGFP-injected rats, which may explain why endogenous H_{2}S-mediated effect in the RVLM of AdCBS-injected rats is more effective in suppressing sympathetic outflow. However, we did not explore the defensive mechanisms against the sympathoinhibitory effects of H_{2}S caused by CBS overexpression on RVLM neurons. Further studies are needed to address this question.

Although H_{2}S is considered an endogenous opener of K_{ATP} channels in many different cell types, the molecular mechanism involved in the interaction of H_{2}S and K_{ATP} channel proteins is still not clear. Previous studies have shown that H_{2}S either interacts with Cys6 and Cys26 residues of the extracellular NH2-terminal of the rvSUR1 subunit of the K_{ATP} channel complex or breaks the disulfide bond involving Cys6 and Cys26 and then changes the K_{ATP} channel complex configuration, leading to the opening of the pore-forming Kir6.1 subunit (17). These findings indicate that the molecular mechanisms of H_{2}S interaction with K_{ATP} channels may be potentially involved in sympathetic outflow in the RVLM. However, our present study does not establish how to increase the subunits of K_{ATP} channels and subsequently potentiated the suppression of sympathetic outflow by overexpression of CBS in the RVLM in rats. The identification of more detailed mechanisms involved in the interaction between H_{2}S and K_{ATP} channels in the RVLM requires further investigation.

Previous studies have shown that HA, an allosteric inhibitor of CBS, acts as an NO donor to increase γ-aminobutyric acid (GABA) levels (7) and release NO (12). Sympathetic nerve activity was decreased by infusions of the NO donor sodium nitroprusside and GABA into the RVLM (14, 18). In this study, microinjection of HA into the RVLM induced an increase in the MAP, HR, and RSNA. Therefore, these data suggest that the influence of microinjection of HA in the RVLM was not mediated by NO or GABA.

Contradictory results have been reported by several studies concerning the role of H_{2}S in the regulation of cardiovascular function in the RVLM (13, 26). These inconsistent results are most likely caused by the instability of the H_{2}S donor. NaHS, as an H_{2}S donor, has been used in many previous studies. However, NaHS is an unstable and short-lived donor and does not mimic the slow and continuous production of H_{2}S in vivo (4, 22). Furthermore, NaHS in a water-based solvent may be quickly oxidized by oxygen (35). Although morpholin-4-ium 4 methoxyphenyl (morpholin) phosphorodithioate (GYY4137), another H_{2}S donor, effectively avoids the shortcoming of NaHS, its biological effects only last ~8 min in vivo (11). In this study, we found that gene transfer of CBS in the RVLM using an adenoviral vector augments in vivo H_{2}S production for a much longer time in vivo. These data suggest that CBS gene transfer, as an H_{2}S donor, is a useful tool that may effectively avoid the shortcoming of NaHS in future experiments.

In conclusion, the present study demonstrates a technique of transferring the CBS gene into the RVLM of rats for the first time in vivo. Furthermore, it shows that the endogenous H_{2}S-mediated effect in the RVLM is more effective in suppressing sympathetic nerve activity in rats injected with AdCBS compared with rats injected with AdEGFP, and the effect may be mediated via K_{ATP} channels. Taken together, these findings...
provide new insights into the role of the new gaseous transmitter $\text{H}_2\text{S}$ as a potential target for cardiovascular gene therapy. These findings may be useful for which certain pathological conditions that exhibit heightened sympathetic activity.

**GRANTS**

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

X.-c.d. and Y.-m.w. conception and design of research; X.-c.d., R.G., and Q.G. performed experiments; X.-c.d. and S.J. edited and revised manuscript; R.G., S.-y.l., L.X., and H.-m.X. drafted manuscript; S.-y.l. and S.J. analyzed data; S.-y.l., L.X., and Q.G. prepared figures; L.X., H.-m.X., Q.G., and S.J. interpreted results of experiments; Y.-m.w. approved final version of manuscript.

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