Cystathionine-β-Synthase Gene Transfer Into Rostral Ventrolateral Medulla Exacerbates Hypertension via Nitric Oxide in Spontaneously Hypertensive Rats

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BACKGROUND
Rostral ventrolateral medulla (RVLM) plays a crucial role in the central regulation of cardiovascular functions. Cystathionine-β-synthase (CBS) is a major hydrogen sulfide (H2S)-generating enzyme that has been identified mainly in the brain. The present study was designed to examine CBS expression and determine its roles and mechanisms of regulating sympathetic outflow and blood pressure (BP) in the RVLM in spontaneously hypertensive rats (SHR).

METHODS AND RESULTS
CBS expression was decreased in the RVLM in SHR compared to Wistar-Kyoto (WKY) rats. Accumulating evidences suggest that H2S interacts with nitric oxide (NO) to regulate cardiovascular function. Therefore, we hypothesize that the decrease in CBS expression in the RVLM may be involved in the disorder of l-arginine/NO pathway, which subsequently affects BP in SHR. Overexpression of CBS in the RVLM caused significant increases in BP, heart rate, and urinary norepinephrine excretion in SHR but not in WKY. Acute experiments were carried out at day 7 after gene transfer. NO metabolite levels, neuronal NO synthase, and γ-amino butyric acid were decreased in SHR after CBS gene transfer. Furthermore, pressor responses to microinjection of NG-nomomethyl-l-arginine into RVLM were blunt in SHR transfected with AdCBS compared to SHR transfected with AdEGFP.

CONCLUSIONS
Overexpression of CBS in the RVLM elicits enhanced pressor responses in SHR, but not in WKY, and the NO system is involved in these effects. The results suggest that alterations of H2S signaling in the brain may be associated with the development of hypertension.

Keywords: blood pressure; cystathionine-β-synthase; hydrogen sulfide; hypertension; nitric oxide; overexpression; rostral ventrolateral medulla.

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Hydrogen sulfide (H2S) is an endothelium-derived hypopolarizing factor that enhances the relaxation of peripheral vasculature,1–3 recent studies4–6 have also reported its physiological functions in the central nervous system. In mammalian tissues, H2S is produced through degradation of l-cysteine mainly by 2 main enzymes: cystathionine-β-synthase (CBS) and cystathionine-γ-lyase. CBS is primarily found in the brain, whereas cystathionine-γ-lyase is mainly expressed in the peripheral tissues.7 The role of H2S in cardiovascular regulation has been investigated in specific areas of the central nervous system, including the nucleus tractus solitarii,8 paraventricular nucleus of the hypothalamus,5 and rostral ventrolateral medulla (RVLM).6,9 It is well known that the RVLM is an important central site responsible for maintaining baseline sympathetic vasomotor tone and arterial blood pressure (ABP);10,11 abnormalities in this brain region may be directly relevant to the pathophysiology of heart failure12,13 and hypertension.14,15 Although the precise function of H2S in cardiovascular regulation in the RVLM remains unclear, its role is very interesting due to the importance of the RVLM in the pathogenesis of hypertension in spontaneously hypertensive rats (SHR).

Previous studies have shown that the inhibitory system (nitric oxide, NO) in the RVLM is diminished in SHR14,15 Moreover, accumulating evidence suggests that cross-talk occurs between the H2S and NO signaling pathways, which could induce synergistic16,17 or negatively related regulatory effects.18–20 In this study, the expression of the CBS protein and H2S production were decreased in the RVLM of SHR compared to the normotensive Wistar-Kyoto (WKY) rats. Therefore, we hypothesize that the decrease in CBS in the RVLM is involved in the disorder of the NO/nitric oxide synthase (NOS) pathway in SHR and affects blood pressure (BP). Therefore, we tested this hypothesis by overexpressing CBS via recombinant adeno-virus vectors encoding CBS gene transfected into the RVLM in vivo.
METHODOLOGY

Animals

Adult (16–20 weeks old) male WKY and SHR (provided by Vital River Laboratory Animal Technology), weighing 260–300 g, were used. The procedures were approved by the Experimental Animal Care and Use Committee of HeBei 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.

Gene transfer into the RVLM in vivo

Adenovirus encoding enhanced CBS gene (AdCBS, GenBank: M88344.1, supplied from GeneSil Biotechnology Company) or adenovirus encoding enhanced green fluorescent protein (AdEGFP; as a control vector) were delivered into the RVLM by microinjection bilaterally. The following 4 groups of rats were used in this study: AdEGFP-treated WKY, AdEGFP-treated SHR, AdCBS-treated WKY, and AdCBS-treated SHR.

On the day of the RVLM injections, each rat was anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal). The coordinates for the RVLM, determined using the Paxinos and Watson atlas, were 11.96 mm posterior to bregma, 1.66 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 mm Hg in response to an injection of L-glutamate (2 nmol). An adenoviral suspension (1 × 10^9 plaque forming units/ml) was injected into the bilateral RVLM within 10 minutes 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. At the end of experiments, the brain of the rat was quickly removed, frozen in liquid nitrogen, and stored at −80 °C until being sectioned.

Western blotting for measurement of CBS and 3 isoforms of recombinant NOS protein in the RVLM

Western blot analysis was used to determine CBS, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) protein expression in the RVLM in the 4 groups of animals. The primary antibodies to CBS, nNOS, eNOS, and iNOS (Santa Cruz Biotechnology) were diluted in blocking buffer and incubated with the membrane for 12 hours at room temperature. GAPDH (Bioworld Technology) was used as a loading control. Densitometric analysis was conducted on the protein bands for quantitative comparison as described previously.

Measurement of systolic BP

The systolic blood pressure (SBP) of the tail artery was measured using a noninvasive computerized tail-cuff system (NIBP; AD Instruments, Sydney, Australia) in the conscious state as described previously. Briefly, the SBP was measured consecutively every other day between 8:00 AM and 10:00 AM. Before the measurements were taken, the rats were warmed for 5–20 minutes at 28 °C to allow detection of tail artery pulsations and achieve a steady pulse level. The SBP was obtained by averaging 6 measurements. The hypertensive criterion was a SBP equal to or more than 150 mm Hg.

RVLM microinjection

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg, intraperitoneally followed by 20 mg/kg per hour, IV). N^G^-monomethyl-L-arginine (L-NMMA, 100 nmol; St Louis, MO) was microinjected bilaterally into the RVLM at day 7 after gene transfer in the 4 groups of rats to examine the mechanism by which a decrease in NO production, caused by overexpression of CBS in the RVLM, affects BP. The left femoral artery was cannulated, and the ABP was measured with a pressure transducer. The heart rate (HR) was measured by triggering from the pulsatile BP. The renal sympathetic nerve activity (RSNA) was recorded as previously described. 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 distally clamped to ensure that afferent activity was not recorded. The pressure and nerve discharge signal were transmitted through an amplifier into a PowerLab data acquisition system (8SP; AD Instruments, QUAD, Bridge, Australia) to simultaneously record the ABP and RSNA. After a stable 30-minute recording of the ABP, HR, and RSNA was obtained, artificial cerebrospinal fluid, as vehicle solution, was microinjected into the RVLM. Subsequently, L-NMMA was microinjected into the RVLM, and the RSNA, ABP, and HR were recorded. One dose was used per rat. The changes in integration of the nerve discharge during the experiment were expressed as a percentage of the basal value.

Measurement of H\textsubscript{2}S, NO metabolites (NOx), and urinary norepinephrine excretion levels in the RVLM

H\textsubscript{2}S levels in the RVLM were measured using a rat H\textsubscript{2}S ELISA kit (NovaTeinBio, Cambridge, MA) according to the manufacturer’s instructions as described previously. NO metabolite (NOx) levels were used as an index of the NO level. NO production in the RVLM was evaluated based on the concentrations of stable nitrate/nitrite metabolites using a Nitrate/Nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI).

Measurement of urinary norepinephrine excretion and GABA in the RVLM

Urinary norepinephrine, a marker of sympathetic nerve activity, was measured for 24 hours after urine obtained by the metabolic cage method. The urinary norepinephrine
concentration was measured by high-performance liquid chromatography before the gene transfer and at day 7 after the gene transfer. The concentration of γ-aminobutyric acid (GABA) in the brain was measured by high-performance liquid chromatography, as described previously. The content of GABA was quantified by comparison of the area with known standards.

**Statistical analysis**

All data are expressed as the means ± SE. One- or 2-way analysis of variance followed by the Bonferroni test for post hoc analysis was used when multiple comparisons were performed. A paired t-test was used to compare the urinary norepinephrine excretion before and after the gene transfer. Differences were considered statistically significant when \( P < 0.05 \).

**RESULTS**

**Characterization of AdCBS**

CBS expression peaked at day 7 after CBS gene transfer and declined over time, as indicated by green fluorescence. Localization of CBS gene expression in the RVLM was indicated by enhanced green fluorescence protein expression (Figure 1a).

Western blot analysis revealed that CBS protein expression was lower in the RVLM in SHR transfected with AdEGFP compared to WKY transfected with AdEGFP. In addition, the expression of the CBS protein was significantly increased in the RVLM in AdCBS-treated rats compared to AdEGFP-treated and nontreated rats at day 7 after gene transfer. However, the increase in the CBS protein caused by overexpression of CBS was not different between SHR and WKY. We also measured the production of \( \text{H}_2\text{S} \) in the RVLM at day 7 after the gene transfer. The level of \( \text{H}_2\text{S} \) was significantly higher in rats transfected with AdCBS than in AdEGFP-treated rats. The basal level of \( \text{H}_2\text{S} \) was lower in the SHR treated with AdEGFP than that in the AdEGFP-treated WKY. There was no difference in the \( \text{H}_2\text{S} \) concentration in the groups treated with AdCBS (Figure 1b,c,d).

**CBS overexpression increases the SBP, HR, and urinary norepinephrine excretion in SHR**

Figure 2 shows the changes in the SBP and HR before and after the microinjection of AdEGFP or AdCBS into the RVLM, recorded with the noninvasive computerized tail-cuff system in conscious rats. The SBP and HR were significantly increased between day 5 to day 10 in SHR, but not in WKY, after overexpression of CBS in the RVLM. Baseline SBPs and HRs did not differ between the AdEGFP-transfected rats and nontreated rats (\( n = 6 \); data not shown).

The 24-hour urinary norepinephrine excretion was significantly higher in the SHR groups than those in the WKY groups before gene transfer. At day 7 after the gene transfer, the urinary norepinephrine excretion was much higher in the AdCBS-treated SHR than the AdEGFP-treated SHR, but this effect was not observed in the WKY groups. However, urinary norepinephrine excretion in both the SHR and WKY groups showed no change before and after gene transfer in the AdEGFP-treated rats (Figure 3c).

**NOS protein expression and NOx level in the RVLM**

\( \text{nNOS} \) protein expression in the RVLM showed no significant difference between the SHR and WKY groups. However, \( \text{nNOS} \) protein expression in the RVLM was significantly

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*Figure 1.* Overexpression of CBS in the RVLM. (a) Schematic showing delivery of adenovirus to the RVLM. Arrows indicate the RVLM and green fluorescence at day 7 after microinjection of AdCBS. (b) CBS protein expression in the RVLM tissue at day 7 after gene transfer. (c) Relative values of CBS protein in the RVLM at day 7 after gene transfer. (d) Hydrogen sulfide (\( \text{H}_2\text{S} \)) level in the RVLM at day 7 after gene transfer. Values are mean ± SE. *\( P < 0.05 \) vs. AdEGFP-transfected WKY rats; \( \# P < 0.05 \) vs. AdEGFP-transfected SHR. \( n = 6 \) for each group. Abbreviations: AdCBS, adenovirus encoding enhanced CBS gene; AdEGFP, adenovirus encoding enhanced green fluorescent protein; CBS, cystathionine-β-synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RVLM, rostral ventrolateral medulla; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto.
lower in SHR transfected with AdCBS than the SHR transfected with AdEGFP, but this effect was not observed in the WKY groups. The expression of eNOS and iNOS protein did not differ among the groups. The NOx level was decreased in SHR transfected with AdCBS compared to SHR transfected with AdEGFP. Moreover, our present data showed a significant decrease in GABA release in SHR transfected with AdCBS compared to WKY treated with AdEGFP, but this effect was not observed in the WKY groups (Figure 3a,b).

GABA level in the RVLM

As indicated in Figure 3d, the release of GABA in the RVLM was decreased in AdEGFP-treated SHR compared to AdEGFP-treated WKY. Moreover, our present data showed a significant decrease in GABA release in SHR transfected with AdCBS compared to SHR transfected with AdEGFP. There was no difference in GABA concentration in the WKY groups.

RVLM microinjection

Bilateral microinjection of L-NMMA into the RVLM produced increases in the RSNA, ABP, and HR at day 7 after gene transfer in all four groups. The pressor response evoked by microinjection of L-NMMA into the RVLM was blunt in SHR transfected with AdEGFP compared to WKY transfected with AdEGFP. Furthermore, pressor responses were markedly smaller in AdCBS-treated SHR than those in AdEGFP-treated SHR, but no significant differences were observed in the WKY groups. Microinjection of the vehicle control (100 nl, artificial cerebrospinal fluid) into the RVLM showed no effects on the RSNA, ABP, and HR (n = 6; data not shown) (Figure 4a,b).

DISCUSSION

The major findings of this study are as follows: (i) Overexpression of CBS in the RVLM elicited increases in the SBP, HR, and urinary norepinephrine excretion in SHR. The results indicate that CBS/H₂S system plays an important role responsible in maintaining baseline sympathetic vasomotor tone and ABP; and the decreased CBS in the RVLM of SHR may have a suppressing influence on hypertension as a compensatory mechanism. (ii) Overexpression of CBS in the RVLM reduced NOS expression, NO production, and GABA concentration. Furthermore, blockade of NO production in the RVLM resulted in a blunted responsiveness in the SHR transfected with CBS, suggesting that in the CBS overexpressed SHRs, decreased NO/NO system may mediate enhanced increases in BP and HR. Taken together, the results indicate that excessive central sympathetic activation in SHR involves the interactions between the H₂S/CBS and NO/nNOS systems.

H₂S, similar to other gaseous signaling molecules, such as carbon monoxide and NO, is considered an important endogenous gaseous transmitter in many physiological functions. However, most of these experiments were performed in an anesthetized state and examined only the acute effects of H₂S. Sodium hydrosulfide (NaHS), as an unstable and short-lived donor, does not mimic the slow and continuous process of H₂S production in vivo. In addition, NaHS in a water-based solvent may be quickly oxidized by oxygen. Recent studies have determined the effects that CBS overexpression produces a sustained increase in CBS, resulting in a longer period of increased H₂S production. Adenoviruses have been shown to allow highly efficient delivery of transfers to the brain and infect both neuronal and glial cell types. In the present study, the profound effect of overexpression of CBS, which produces a longer period of increased H₂S levels in rats transfected with AdCBS, was demonstrated. The increase in the CBS protein and H₂S production peaked at day 7 after CBS gene transfer into the RVLM. These results proved the effectiveness of CBS overexpression in the RVLM in rats transfected with AdCBS.

In the neurogenic hypertension state, many central and peripheral factors are significantly altered. In this study, CBS protein expression was decreased within the RVLM occurred only in SHR. Specific overexpression of CBS in the RVLM caused profound elevations in the SBP, HR, and urinary norepinephrine excretion in SHR but did not affect these variables in the physiological state in normotensive WKY. Therefore, we infer that decreased CBS in the RVLM of SHR may be a compensatory mechanism of hypertension and has a beneficial effect of suppressing further BP elevation. H₂S modulates neurotransmission by influencing N-methyl-D-aspartic acid receptor (NMDA) receptors and second messenger systems including intracellular Ca²⁺ concentration and intracellular caused a profound elevation levels and so forth. H₂S activates adenylyl cyclase and increases cAMP production, which may boost the activity of protein kinase
A and consequently NMDA phosphorylation. The NMDA receptor channel contributes to the late component of the excitatory postsynaptic potential and controls a cation channel. Furthermore, some studies have shown that NMDA receptors are involved in BP regulation and suggested that the decreased NMDA receptor subunits in the RVLM of SHR could be a protective response driven by a homeostatic mechanism aimed at decreasing the sensitivity to excitatory inputs to the RVLM. Thus, we propose that downregulation of the CBS/H₂S system and reducing NMDA receptor subunits within the RVLM, in an attempt to restore the cardiovascular homeostasis and inhibit even higher BP development, may enhance compensatory activation of the RVLM.

Then, how is the RVLM CBS involved in BP regulation? One of the possible mechanisms may involve an interaction with the NO/NOS system. It has been shown that the inhibitory mechanism of sympathetic regulation via NO within the RVLM was reduced in SHR. In the present study, the expression of the nNOS protein in the RVLM was not different between WKY and SHR, whereas NOx was decreased in SHR. Furthermore, the pressor response to the microinjection of L-NMMA into the RVLM was blunt in SHR compared to WKY. These results suggest that the NO in the RVLM was reduced in SHR. Recent studies demonstrate a common signaling pathway in which NO–H₂S crosstalk mediates the effects on vascular functions such as vasodilation, vascular remodeling (migration and proliferation), and angiogenesis. Therefore, H₂S signaling in the RVLM may be directly involved in the dysfunctional l-arginine/NO pathway in SHR. Evidence has accumulated that indicates H₂S and NO compete and are antagonists to each other. This notion is supported by the findings that H₂S can downregulate expression or inhibit the activity of eNOS and subsequent NO production by altered l-arginine/BH4, increased HO-1/CO, and other unknown mechanisms.

Consistent with those findings, in the current study, we found that the nNOS protein and NOx level in the RVLM were lower in SHR transfected with AdCBS than SHR transfected with AdEGFP. These data suggest that the increase in H₂S production after CBS gene transfer within the RVLM might have caused the decreases in nNOS protein expression and NOx levels in SHR. Furthermore, we found that the pressor responses to L-NMMA were notably smaller in AdCBS-treated SHR compared to in AdEGFP-treated SHR. These results suggest that the endogenous NO-mediated effect of suppressing RSNA is reduced in AdCBS-treated SHR compared with in AdEGFP-treated SHR. These observations support the speculation that downregulation of
nNOS, caused by overexpression of CBS, may be responsible for the reduced suppression of sympathetic outflow and the exacerbation of hypertension in SHR. Therefore, we propose that the decreased expression of the CBS in the RVLM of SHR may serve as a counter hypertensive mechanism involving a decrease in the NO/NOS system and an attempt to decrease sympathetic nerve activity. In contrast to the results obtained in CBS-transfected SHR, overexpression of CBS in the RVLM in WKY did not affect the SBP and HR. It has been reported that injection of NaHS into the RVLM attenuates the ABP, HR, and RSNA in anesthetized normotensive Sprague-Dawley rats. In contrast, another study has shown that microinjection of NaHS into the RVLM has no effect on mean arterial pressure. These inconsistent results are most
likely due to the instability of NaHS.\textsuperscript{26,27} Furthermore, modifications that are made between the time that a solution is prepared and the time that the biological effect is measured can dramatically affect the results.\textsuperscript{28} In the present study, overexpression of CBS in the RVLM did not downregulate the NO system and GABA concentration in WKY transfected with AdCBS. In addition, our study clearly indicated that overexpression of CBS in the RVLM produces a longer period of endogenous H\textsubscript{2}S elevation, which is more likely to induce other mechanisms to maintain homeostasis in the physiological state. These findings could likely explain why overexpression of CBS did not change BP in WKY. However, we did not explore the reasons why overexpression of CBS did not alter the NO system and GABA concentration in the RVLM. Further studies are needed to address this question.

Overexpression of CBS in the RVLM downregulated the NO/nNOS system. In turn, the decreased NO/nNOS system may directly reduce GABA release and subsequently contribute to BP elevation. In SHR, a disinhibition of the GABA-mediated inhibition of neuronal activity in the RVLM has been reported.\textsuperscript{39} This notion is supported by the finding that the pressor response evoked by bicuculline, a GABA receptor antagonist, microinjection into the RVLM of WKY was greater than that of stroke-prone spontaneously hypertensive rats (SHRSP).\textsuperscript{14} Furthermore, the increase in NO production caused by the overexpression of eNOS in the RVLM of SHRSP increases the release of GABA in the RVLM, and the increased NO production in the RVLM reduces the disinhibition of the RVLM of SHRSP through this increase in GABA release.\textsuperscript{14}

In the present study, overexpression of CBS in the RVLM downregulated the NO system and GABA concentration in SHR. These results suggested that the decrease in NO caused by the overexpression of CBS may lead to the decrease in GABA release which contribute to BP elevation in SHR. This study did not investigate the molecular mechanism regarding the interaction between the NO/nNOS system and the H\textsubscript{2}S/CBS system in the RVLM. Determining the molecular mechanism will be a key focus for future studies.

In summary, the present study demonstrates that overexpression of CBS in the RVLM increases the SBP, HR, and urinary norepinephrine excretion in the conscious state in SHR. These effects may be mediated by the NO/nNOS system within the RVLM in SHR. These findings suggest that an interaction between the H\textsubscript{2}S/CBS and NO/nNOS systems is involved in excessive central sympathetic activation and that the decreased CBS expression in the RVLM may be an important compensatory mechanism to counter the down-regulation of the NO/nNOS system in SHR. These results provide new insights into the central mechanisms of BP regulation in hypertension within the RVLM of SHR.

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DISCLOSURE

The authors declared no conflict of interest.

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