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Biochemical and Biophysical Research Communications 313 (2004) 22–27
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The possible role of hydrogen sulfide on the pathogenesis of spontaneous hypertension in rats☆

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Received 5 November 2003

Abstract

Hydrogen sulfide (H_2S) is a newly found modulator in vascular system. This work showed that gene expression of cystathionine γ-lyase (CSE), a H_2S generating enzyme, and the activity of CSE in thoracic aorta were suppressed in hypertension rats. The plasma level of H_2S also decreased in those rats. Exogenous administration of H_2S could increase the plasma level of H_2S and enhance the CSE activity of aorta. Exogenous administration of H_2S also attenuated the elevation of pressure and lessened the aorta structural remodeling during the development of hypertension. In WKY rats, the gene expression and activity of CSE also decreased when the endogenous production of H_2S was deprived by administration of DL-propargylglycine (specific inhibitor of CSE), accompanying the elevated pressure and the development of vascular remodeling. The results showed that endogenous H_2S system was involved in both the maintenance of basal blood pressure and the development of hypertension. Exogenous H_2S could exert beneficial effect on the pathogenesis of spontaneous hypertension.

Keywords: Hypertension; Hydrogen sulfide; Thoracic aorta

Endogenous hydrogen sulfide (H_2S) is formed from cysteine by pyridoxal-5′-phosphate-dependent enzymes, including cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) [1]. The expressions of these enzymes have been detected in various tissues. CBS is highly expressed in brain and the physiological concentrations of H_2S may facilitate the induction of LTP in the hippocampus [2]. The decrease in endogenous H_2S has been suggested to be involved in some aspects of the cognitive decline in Alzheimer's disease [3]. CSE has been demonstrated to be expressed in thoracic aorta, tail artery, mesenteric artery, and pulmonary artery, where the expression of CBS was not detectable [4]. The endogenous production of H_2S from rat vascular tissues has been determined to be ~46 μM. These observations suggested the potential physiological functions of H_2S in the cardiovascular system. The cardiovascular effects of both endogenous and exogenous H_2S have been studied recently. It has been demonstrated that H_2S is an important vasoactive factor relaxing rat aortic tissues both in vitro and in vivo [4,5]. By analogy to other endogenous gaseous molecules, such as nitric oxide (NO) and carbon monoxide (CO), H_2S was suggested as a new gaseous transmitter fulfilling a physiological role in regulating cardiovascular functions, however, the pathophysiological role of H_2S in cardiovascular diseases has not been explored. Hypertension is one of the most popular cardiovascular diseases with abnormal vasoconstriction and vascular structural remodeling [6]. To date, the mechanisms of hypertension have not been fully understood yet. NO and CO have been found to play important roles in the pathogenesis of hypertension [7,8]. Then does the production of endogenous H_2S also change the development of spontaneous hypertension? Does the changes of H_2S have any potential role in the pathogenesis of hypertension? The present study was therefore designed to observe the differences of CSE/H_2S

☆ This study was supported by Special fund for promotion of education, Ministry of Education PRC and The Major Basic Research Program of China (G2000056905).
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doi:10.1016/j.bbrc.2003.11.081

system between the hypertensive rats and rats with normal blood pressure, and further evaluate the effects of H_2S donor and CSE inhibitor on the hypertensive process.

Materials and methods

Animals and groups. The study was approved by the Animal Research Committee of Peking University. Twenty-four male WKY rats (45–55 g) at the age of 4 weeks were randomly divided into WKY control group ($n = 8$), WKY + NaHS (H_2S donor) group ($n = 8$), and WKY + DL-propargylglycine (PPG) group ($n = 8$). Another 24 male SHR rats (45–55 g) were also randomly divided into SHR control group ($n = 8$), SHR + NaHS group ($n = 8$), and SHR + PPG group ($n = 8$), respectively. Rats of WKY + NaHS group and SHR + NaHS group were injected with NaHS 56 μmol/kg. Rats of WKY + PPG group and SHR + PPG group were injected with PPG (37.5 mg/kg). SHR control and WKY control rats were injected with the same volume of physiological saline water. All of the drugs were injected intraperitoneally at the same time each day for 5 weeks. NaHS was newly prepared everyday.

Measurement of hemodynamic parameters and sample preparation. Systolic blood pressure was determined in conscious and quiet rats by means of the standard tail-cuff method after acclimatization from initiation of the treatment for 4-week-old rats to the termination of the treatment. Animals were anesthetized with urethane (1 g/kg body weight) intra-peritoneally after the blood pressures were detected. A silicone catheter (outer diameter 0.9 mm) was introduced into the right carotid to get blood. The plasma was collected from the blood by centrifugation and stored at −70 °C for the measurement of plasma H_2S concentration. After the chest was opened, the thoracic aorta was separated and the medial part of artery was rapidly removed to liquid nitrogen for quick freezing and then stored at −70 °C. The heart was removed and the left ventricle (LV) plus septum (SP) was dissected free. These tissues were blotted and weighed using an electronic scale. The wet weight ratio of LV + SP against whole heart was calculated as an indicator of right ventricular hypertrophy.

Measurement of H_2S concentration in the plasma. As much as 0.1 ml of plasma was added into a test tube containing 0.5 ml of 1% zinc acetate and 2.5 ml distilled water. Then 0.5 ml of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl and 0.4 ml of 30 mM $FeCl_3$ in 1.2 M HCl were also added to the test tube for 20 min of incubation at room temperature. The protein in the plasma was removed by adding 1 ml of 10% trichloroacetic acid to the reaction mixture and centrifugation. The optical absorbance of the resulting solution at 670 nm was measured with a spectrometer (Shimadzu UV 2100, Japan). The H_2S concentration in the solution was calculated against the calibration curve of the standard H_2S solution.

Measurement of H_2S production in thoracic aorta. Tissue H_2S production rate was measured as described previously [1]. Briefly, vascular tissues were homogenized in ice-cold 50 mM potassium phosphate buffer (pH 6.8). Reactions were performed in 25 ml Erlenmeyer flasks. The reaction mixture contained: 10 mM L-cysteine, 2 mM pyridoxal 5′-phosphate, 100 mM potassium phosphate buffer (pH 7.4), and 10% (W/V) homogenates. Cryovial test tubes (2 ml) were used as the center wells each contained 0.5 ml of 1% zinc acetate as trapping solution. A filter paper of $2 \times 2.5\,cm^2$ was put into the central well of the flask to increase the air/liquid contacting surface. The flasks were then flushed with N_2 before being sealed with a double layer of parafilm. The catalytic reaction was initiated by transferring the flasks from an ice bath to a 37 °C shaking water bath. After incubating at 37 °C for 90 min, 0.5 ml of 50% trichloroacetic acid was added into the reaction mixture to stop the reaction. The flasks were sealed again and incubated in the shaking water bath for an additional hour at 37 °C to ensure a complete trapping of H_2S. The contents of the center wells were then transferred to test tubes and mixed with 3.5 ml distilled water and 0.5 ml of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl. To each tube, 0.4 ml of 30 mM $FeCl_3$ in 1.2 M HCl was added immediately. After 20 min of incubation at room temperature, the absorbance of the resulting solution at 670 nm was measured with a spectrometer (Shimadzu UV 2100, Japan). The H_2S concentration in the solution was calculated against the calibration curve of the standard H_2S solution. For each sample, the measurement was done in duplicate. The H_2S production was expressed in a unit of nmol/g wet tissue/min.

Measurement of CSE mRNA in thoracic aorta by quantitative reverse transcription-polymerase chain reaction. Total RNA was extracted from aortic tissues using TRIzol reagent. The cDNA was synthesized using oligo$(dT)_{15}$ primer and M-MLV reverse transcriptase. The polymerase chain reaction (PCR) primers used to amplify the fragment of CSE cDNA were:

CSE-S: 5′-TCCGG ATGGA GAAAC ACTTC

CSE-A: 5′-GCTGC CTTTA AAGCT TGACC

PCR using these two primers yielded a 400 bp fragment of wild-type rat CSE cDNA. The competitive internal standard for the measurement of CSE cDNA had the same sequences as the 400 bp fragment of wild-type rat CSE cDNA, except that a fragment of 39 bp at the down-stream site of CSE-S primer was deleted [9,10]. Quantitative PCR was performed in a 0.2 ml of PCR tube containing 2 μl of rat aorta cDNA, 2 μl of 0.12 fmol/L competitive internal standard, 1 μl of 5 μM/each CSE-S and CSE-A mixture, 1 μl of 2.5 mM/each dNTP mixture, 2 mM $MgCl_2$, 2.5 μl of 10× PCR buffer, and 1.25 U *Taq* DNA polymerase, in a total volume of 25 μl. PCR products were separated in a 2% agarose gel and stained by ethidium bromide. The ratio of optical density of the two DNA bands was measured by a Gel Image Analysis System (AlphaImager, Alpha Innotech, CA, USA) under UV light. A standard curve of the ratio was drawn using the same condition as described above except that rat aortic cDNA was changed to a series of dilutions of the plasmid containing the 400 bp wild-type CSE cDNA fragment. The relative amount of CSE cDNA in samples was then obtained from the standard curve [11]. To calibrate the amount of sample loaded in PCR mixture, β-actin cDNA was measured using the same method after the quantitative PCR. Three microliters of PCR product was amplified again using the rat β-actin primers (β-actin-S: 5′-ATCTG GCACC ACACC TTC, β-actin-A: 5′-AGCCA GGTCC AGACG CA). Relative amount of β-actin cDNA in loaded sample was then obtained from a pre-made standard curve of β-actin cDNA measurement. Standardized relative amount of CSE cDNA was used for further analysis.

Morphological analysis of thoracic aorta. Medial part of the thoracic aorta was excised and immersed in 10% formol. A 0.6-cm specimen was dehydrated in graded ethanol solutions and embedded in paraffin. Four 4-μm-thick sections were cut and stained according to the modified Weigert's method (for elastin) for determination of lumen diameter and medial cross-section area. Morphometric analysis was performed with the Optilab algorithm on LeicaQ550cw cytogenic workstation. Each section was examined for three times in a blinded manner. Medial cross-sectional area (mm^2) was defined as the area between the internal and external elastic lamina. Medial stress was calculated as follows: (systolic arterial blood pressure × lumen radius)/medial thickness (mmHg) [12].

Chemicals and reagents. NaHS solution was freshly prepared by mixing the stock solution of sodium sulfide and hydrochloric acid. Phosphate 5′-pyridoxal was purchased from Sigma. TRIzol reagent was purchased from Invitrogen. Oligo$(dT)_{15}$ primer, dNTP, and M-MLV reverse transcriptase were purchased from Promega. All the other chemicals were purchased from Beijing Chemical Reagents, PR China.

Data analysis. The results were expressed as means ± SE. Student's *t* test for unpaired samples was used to compare the mean values between the same treated groups. The changes in blood pressure of the same rats were analyzed using paired Student's *t* test. Statistical

significance was set at $P < 0.05$. For comparison of the differences among control and experimental groups, ANOVA followed by a post hoc analysis (Bonferroni test) was used by using SPSS 10.0 statistic analysis software.

Results and discussion

Hypertension, a disease with a high incidence in the population, affects all parts of the cardiovascular system. The complex interactions between vasoconstrictor and vasodilatory molecules play a crucial role in maintaining the homeostasis of the vascular wall, structurally as well as functionally [7].

Gaseous transmitters including NO and CO have been demonstrated playing pivotal roles in the regulation of vascular homeostasis. Endogenous NO and CO act not only as vasodilatory forces that help us to maintain an equilibrium with vasoconstrictor forces and hence regulate the pressure response to different stimuli, but as modulators attenuating the remodeling of target organs [13–15]. It was demonstrated that the relative deficit of NO and CO contributed much to the pathogenesis of hypertension [16,17]. H_2S was suggested to be a newly found gas transmitter because of its similar biological features and vasorelaxant effects to NO and CO [18]. Then does the production of endogenous H_2S also change in the hypertension?

In our study, the systolic blood pressure of rats in SHR control group elevated significantly from 4 weeks old to 9 weeks old (89.38 ± 5.63 vs 183.57 ± 11.8 mmHg, $P < 0.05$). The systolic blood pressure and the left to whole heart ratio of rats in SHR control group were both much higher than those of rats in WKY control group in 9 weeks old (183.57 ± 11.8 vs 107.5 ± 22.68 mmHg, 0.85 ± 0.02 vs 0.82 ± 0.02, $P < 0.05$), suggesting the development of hypertension in rats of SHR control group (see Figs. 1 and 2). Our subsequent study showed that the plasma level of H_2S in SHR control group was much lower than that of WKY control (20.35 ± 9.2 vs 48.40 ± 13.36 μM, $P < 0.05$). The producing rate of H_2S in aorta of SHR control group decreased significantly as compared with that of WKY controls (15.63 ± 2.89 vs 25.31 ± 5.99 nmol/g wet tissue/min, $P < 0.05$). It was demonstrated that the activity of H_2S generating enzymes in tissues could be represented by H_2S production in homogenates of tissues in the presence of 10 mM L-cysteine and 2 mM pyridoxal 5′-phosphate [8]. And CSE is the only H_2S generating enzyme that exists in thoracic aorta. Therefore, it was suggested that the inhibition of CSE activity caused by hypertension in aorta resulted in a decreased circulation level of H_2S (see Table 1).

We also detected the CSE gene expression in aorta by competitive reverse transcription polymerase chain reaction (RT-PCR) (Fig. 5). The relative amount of CSE mRNA in aorta of rats in SHR control group was lower than that in WKY control group (0.08 ± 0.008 vs 0.09 ± 0.007, $P < 0.05$), which indicated that CSE gene expression was down-regulated in the development of hypertension. The above finding suggested that the down-regulation of CSE gene expression resulted in a suppression of H_2S production, which might be responsible for the decreased H_2S level in plasma. The results also showed that the aortic structural remodeling developed in rats of 9-week-old SHR control group. We found that the medial cross-sectional area (μm^2) and the medial stress (mmHg) were increased in rats of SHR control compared with those of WKY control (597545.48 ± 58600.67 vs 475517.96 ± 30981.3 μm^2, 1440.17 ± 84.07 vs 896.87 ± 189.38 mmHg, $P < 0.05$). Taking together the fact that H_2S can relax the arteries and portal vein even in physiological concentration [4,19], we hypothesized that when the circulating and aortic local H_2S diminished because of the lower

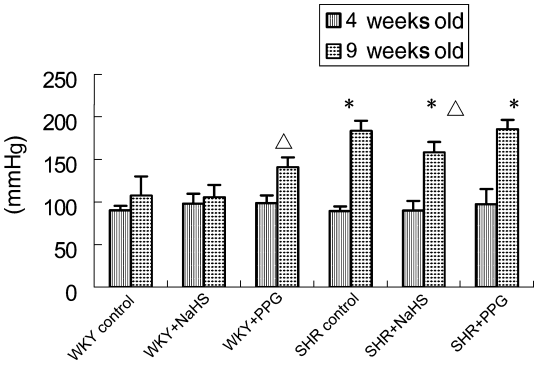

Fig. 1. The blood pressure of rats in both 4 weeks old and 9 weeks old (mmHg). Comparison of the blood pressure among the groups of rats. WKY control: WKY control group. WKY + NaHS: WKY + NaHS group. WKY + PPG: WKY + PPG group. SHR control: SHR control group. SHR + NaHS: SHR + NaHS group. SHR + PPG: SHR + PPG group. *$P < 0.05$ vs WKY group, $^{\Delta}P < 0.05$ vs control group.

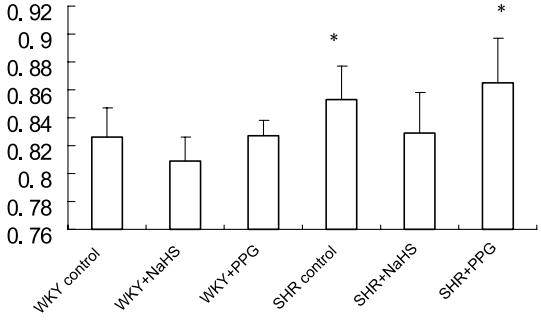

Fig. 2. Wet weight ratio of LV + SP against whole heart. Comparison of the wet ratio of LV + SP against whole heart. LV: left ventricle. SP: septum. WKY control: WKY control group. WKY + NaHS: WKY + NaHS group. WKY + PPG: WKY + PPG group. SHR control: SHR control group. SHR + NaHS: SHR + NaHS group. SHR + PPG: SHR + PPG group. *$P < 0.05$ vs WKY group.

Table 1
Comparison of the H_2S production and plasma level of H_2S in aorta ($x \pm s$) ($n = 8$)

Groups	H_2S production (nmol/min/g wet tissues)			Plasma level of H_2S (μM)		
	WKY	SHR	t	WKY	SHR	t
Control	25 ± 6	$16 \pm 3^{*}$	4.074	48 ± 13	$20 \pm 9^{*}$	4.89
+NaHS	$53 \pm 16^{\Delta}$	$28 \pm 10^{*,\Delta}$	3.91	57 ± 21	$96 \pm 32^{*,\Delta}$	2.78
+PPG	16 ± 3	$13 \pm 5^{\Delta}$	1.75	$21 \pm 7^{\Delta}$	13 ± 10	1.68
F	26.23	12.73		10.25	40.78	

$^{\Delta}P < 0.05$ vs control group.
$^{*}P < 0.05$ vs WKY group.

transcriptional level of CSE and its activity, the vasorelaxation would be attenuated, and the vasoconstriction would be obvious subsequently.

To test the above hypothesis, we examined what would happen when H_2S was supplied exogenously to the SHR rats. In our study, NaHS, a donor of H_2S, was used intra-peritoneally everyday. NaHS was widely used in the studies of H_2S for the following reasons: (1) NaHS dissociates to Na^+ and HS^-in solution, then HS^- associates with H^+ and produces H_2S. It does not matter whether the H_2S solution is prepared by bubbling H_2S gas or by dissolving NaHS. In physiological saline about one-third of the H_2S exists as the undissociated form (H_2S) and the remaining two-thirds as HS^- at equilibrium with H_2S; (2) the use of NaHS enables us to define the concentrations of H_2S in solution more accurately and reproducibly than bubbling H_2S gas; (3) the influence of 1 mM or less sodium ion on the physiological experiments is negligible; and (4) NaHS at concentrations used in the present study does not change the pH of the medium [20].

After 5 week's injection of NaHS, the plasma level of H_2S, the production rate of H_2S, and the CES expression in aorta of rats in SHR + NaHS group all increased significantly compared to those of SHR control (95.663235 vs 30.35 ± 9.2 μM, 27.63 ± 9.36 vs 15.63 ± 2.89 nmol/g wet tissue/min, and 0.12 ± 0.010 vs 0.09 ± 0.006, $P < 0.05$). Also, the systolic blood pressure decreased much at the same time (158.13 ± 12.52 vs 183.57 ± 11.8 mmHg), the medial cross-section and medial stress were also down-regulated (533838.46 ± 56386.45 vs 597545.48 ± 58600.67 μm^2, 1232.86 ± 131.38 vs 1440.17 ± 84.07 mmHg), suggesting that aortic structural remodeling was attenuated by the increased level of H_2S in SHR rats. Zhao et al. [4] demonstrated that an intravenous bolus injection of H_2S at 2.8 and 14 μmol/kg body weight provoked a transient (29.5 ± 3.6 s) decrease in mean arterial blood pressure of rats by 12.5 ± 2.1 and 29.8 ± 7.6 mmHg, respectively. H_2S might also inhibit the aortic smooth muscle cell proliferation [21]. Our results suggested that the up-regulation of H_2S system was responsible for the alleviation of BP in rats of SHR + NaHS group after the administration of NaHS, and H_2S system might also exert effects on the remolding of aortic structure by inhibiting the proliferation of smooth muscle cells and reducing blood pressure and medial stress.

Upon the above findings, we also assumed that exogenously applied NaHS might exert some sort of positive feedback on the gene expression and the subsequent enzymatic activity of CSE, which was also found in our forgoing studies on pulmonary hypertension published in our journal [22], where we found that the up-regulation of both CSE gene expression and enzymatic activity could result in an increased endogenous H_2S production and thus exert anti-HPH (hypoxia induced pulmonary hypertension) effect effectively in collaboration with exogenously applied H_2S.

However, the effects of exogenous H_2S on WKY rats were much different from those on SHR rats. The results showed that the extra supply of H_2S did not change the circulation level of H_2S although the CSE expression and the producing rate of H_2S in aorta increased (see Table 1, Fig. 5, and Fig. 6). The blood pressure and the aortic structure did not change, either (see Figs. 1, 3, and 4). We supposed that the balance between the production and cleanup of H_2S, and the balance between H_2S and other gaseous transmitters could be responsible for the stabilization

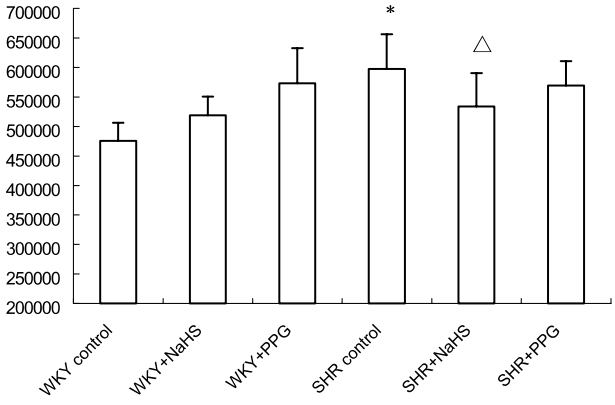

Fig. 3. Medial cross-sectional area of rat aorta (μm^2). Comparison of the Medial cross-sectional area of rat aorta. WKY control: WKY control group. WKY + NaHS: WKY + NaHS group. WKY + PPG: WKY + PPG group. SHR control: SHR control group. SHR + NaHS: SHR + NaHS group. SHR + PPG: SHR + PPG group. $^{*}P < 0.05$ vs WKY group, $^{\Delta}P < 0.05$ vs control group.

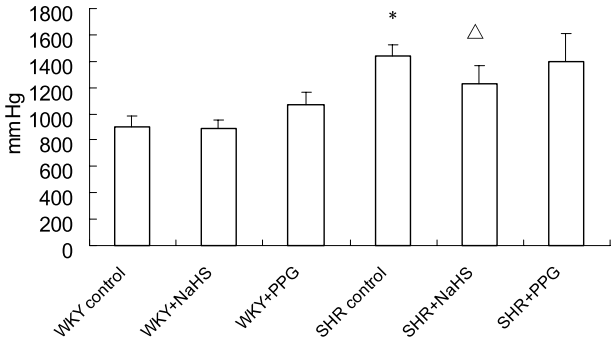

Fig. 4. Medial stress of rat aorta (mmHg). Comparison of the Medial stress of rat aorta. WKY control: WKY control group. WKY + NaHS: WKY + NaHS group. WKY + PPG: WKY + PPG group. SHR control: SHR control group. SHR + NaHS: SHR + NaHS group. SHR + PPG: SHR + PPG group. $^{*}P < 0.05$ vs WKY group, $^{\Delta}P < 0.05$ vs control group.

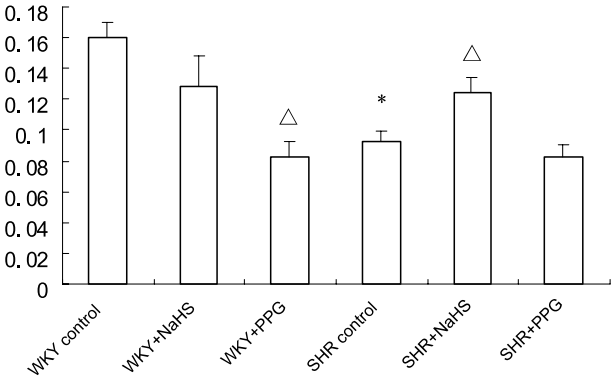

Fig. 5. Relative quantity of CSE expression in aorta (10^{-8} fmol). Comparison of the CSE transcriptional level in aorta. WKY control: WKY control group. WKY + NaHS: WKY + NaHS group. WKY + PPG: WKY + PPG group. SHR control: SHR control group. SHR + NaHS: SHR + NaHS group. SHR + PPG: SHR + PPG group. $^{*}P < 0.05$ vs WKY group, $^{\Delta}P < 0.05$ vs control group.

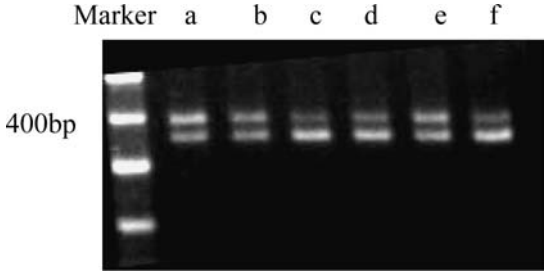

Fig. 6. Electrophoresis of CSE RT-PCR productsin aorta. Electrophoresis of CSE RT-PCR products in agarose gel after competitive PCR. Marker: DNA marker. (a) WKY control; (b) WKY + H_2S; (c) WKY + PPG; (d) SHR control; (e) SHR + H_2S; and (f) SHR + PPG. The two bands in agarose gel stained by ethidium bromide are the CSE wild-type fragment (400 bp) and the CSE competitive internal standard (361 bp), respectively.

of the basal level of H_2S. The mechanisms need further investigation.

To further evaluate the roles of endogenous H_2S in the pathogenesis of hypertension, PPG, a specific inhibitor of CSE, was used in both SHR and WKY rats. The results showed that the plasma level and producing rate of H_2S and the CSE expression in aorta were all similar between SHR + PPG group and SHR group, the blood pressure and medial cross-sectional area of SHR + PPG group were similar to those of SHR control. Interestingly, however, the plasma level and producing rate of H_2S and the CSE expression in aorta were much lower in rats of WKY + PPG group than those of WKY control group, along with the increased blood pressure and medial cross-sectional area of WKY + PPG group as compared with those of WKY controls. It may be deduced that endogenous H_2S is a pivotal factor in maintaining basal blood pressure. Once the hypertension formed with obvious inhibition of H_2S/CSE system, the further down-regulation of H_2S would have little effect.

But there are still many questions which need further studies. For example, given that H_2S is a new sort of gaseous transmitter similar to NO and CO, and the three small molecular gases were hypothesized forming a unique interactive network, do the interactions of the three gases play a more important role in the pathogenesis of hypertension other than any one of the three? Published data have shown that the endogenous production of H_2S from rat aortic tissue and the expression level of CSE in cultured vascular SMCs may both be enhanced by NO donor treatment [23]. Hosoki et al. [20] found that the vasorelaxant effect of sodium nitroprusside (SNP), a NO donor, was enhanced by incubating rat aortic tissues with 30 μM NaHS. Is the interaction of NO and H_2S helpful to explain the positive feedback of the production of H_2S in hypertension rats? Additionally, it has been widely accepted that NO and CO exert their vasorelaxation effects through the activation of GMP. However, the updated data showed that H_2S relaxed rat aortic tissues in vitro in a K_{ATP} channel dependent manner which is absolutely different from the way of NO and CO. Whether the cellular mechanisms responsible for this vascular effect of H_2S, as well as its physiological significance, thoroughly differ from those of the traditional vosoactivators remains to be examined.

In conclusion, endogenous H_2S/CSE system is one of the key factors in maintaining basal systolic blood pressure. The deficit of H_2S/CSE system is responsible for the development of spontaneous hypertension accompanying with aorta remodeling. Exogenously supply of H_2S donor exerted protective effect in the pathogenesis of hypertension.

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