Original Article

Regulatory Effect of Hydrogen Sulfide on Vascular Collagen Content in Spontaneously Hypertensive Rats

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The present study aimed to examine the regulatory effect of hydrogen sulfide (H_2S) on vascular collagen remodeling in hypertensive rats. After 5 weeks of H_2S donor treatment, tail blood pressure, the endogenous H_2S production rate, levels of hydroxyproline and collagen type I, collagen type I protein expression in the thoracic aorta, [^{3}H]thymidine ([^{3}H]TdR) incorporation, [^{3}H]proline incorporation, and [^{3}H]hydroxyproline secretion in cultured vascular smooth muscle cells (VSMCs) were measured. We also examined the effects of NaHS on angiotensin II–induced mitogen-activated protein kinase (MAPK) activation and angiotensin II type 1 (AT_1) receptor binding affinity. Vascular hydroxyproline and collagen type I levels were high, and collagen type I immunohistochemical staining in the thoracic aorta was strong in SHRs compared to Wistar Kyoto (WKY) rats. [^{3}H]TdR and [^{3}H]proline incorporation and [^{3}H]hydroxyproline secretion were also higher in cultured VSMCs from SHR than those from WKY rats. However, vascular H_2S production was lower in SHR compared with WKY rats. Treatment with NaHS increased vascular H_2S production in SHRs, and partly reversed the changes in [^{3}H]TdR and [^{3}H]proline incorporation and [^{3}H]hydroxyproline secretion. In cultured VSMCs, [^{3}H]TdR and [^{3}H]proline incorporation stimulated by angiotensin II was inhibited by incubation with NaHS. The inhibitory effect of NaHS on VSMC proliferation and collagen generation was stronger in the SHR than in the WKY group. Moreover, NaHS could dose-dependently decrease angiotensin II–induced MAPK activation. NaHS also decreased AT_1 receptor binding as well as the binding affinity of the AT_1 receptor. Thus, in SHRs, which demonstrated vascular remodeling and collagen accumulation, the endogenous H_2S pathway is involved in the regulation of excess vascular collagen. (*Hypertens Res* 2008; 31: 1619–1630)

***Key Words*: hydrogen sulfide, collagen, hypertension, vascular remodeling, spontaneously hypertensive rat**

Introduction

Hypertension is a common cardiovascular disease and one of the leading causes of mortality. However, up to now, the pathogenetic mechanisms responsible for hypertension have not been fully elucidated. Blood pressure is mainly determined both by cardiac output and peripheral vascular resis-

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tance, whereas peripheral vascular resistance depends on blood vessel caliber and blood viscosity. A contributing factor to the pathogenesis of hypertension could be the increased relative thickness of the vascular medium due to structural remodeling (*1*). At the same time, elevated blood pressure promotes blood vessel remodeling. This vascular remodeling includes increased proliferation of vascular smooth muscle cells (VSMCs) and excessive accumulation of extracellular matrix (ECM).

Recently, increasing interest has been focused on vascular remodeling associated with hypertension (*2–4*). The ECM content, such as collagen and elastin in the walls of arteries, is increased in hypertensive patients and in genetic or experimentally-induced hypertension in animals (*5*, *6*). Many researchers have studied the mechanisms of collagen accumulation during hypertension. Angiotensin might participate in this accumulation by mobilizing inflammatory cells, which could release a variety of mediators that act upon fibroblasts and VSMCs to promote the synthesis of collagen (*7*). The use of losartan, an angiotensin II (Ang II) type 1 (AT_1) receptor antagonist, has been shown to decrease the post-transcriptional synthesis of fibril-forming collagen type I molecules in young spontaneously hypertensive rats (SHR) (*8*).

Recent studies have revealed that gaseous molecules such as nitric oxide (NO) and carbon monoxide (CO) play important roles in the pathogenesis of vascular structural remodeling (*4*, *9*, *10*). *In vitro* experimental studies have revealed that relative cell size, total protein content per cell, and the number of polyploid cells were significantly decreased in VSMCs isolated from SHRs after chronic L-arginine treatment (*11*). Another study showed that increased endogenous CO production or exposure of cells to CO led to markedly attenuated VSMC proliferation in response to serum or mitogens (*12*, *13*). Our previous work has demonstrated that both NO and CO participated in abnormal collagen accumulation in blood vessels during pulmonary hypertension induced by high pulmonary blood flow and hypoxia (*14*, *15*). However, the factors studied above could not fully explain the mechanisms of vascular remodeling under pathophysiological conditions, including hypertension.

Hydrogen sulfide (H_2S) is a known toxic gas, although in recent years, it has also been found to be a biological gas that is endogenously generated from cysteine upon catalysis by cystathionine β-synthase (CBS) and/or cystathionine γ-lyase (CSE) (*16*, *17*). Zhao *et al*. revealed the expression of CSE mRNA in endothelium-free rat pulmonary, mesenteric, and tail arteries, as well as the aorta and liver by RT-PCR (*18*). We found that H_2S could exert a variety of cardiovascular functions, such as regulation of the development of hypoxic pulmonary hypertension (*19*), high pulmonary blood flow–induced pulmonary hypertension (*20*), and septic shock (*21*). Notably, we found that H_2S could attenuate hypertension and reduce the aortic structural remodeling of hypertensive rats (*22*). Dysfunction of the vascular H_2S synthase/H_2S pathway was also observed in hypertensive rats induced by N^G-nitro-L-arginine methyl ester (L-NAME) (*23*). However, the mechanisms by which H_2S regulates vascular structural remodeling in hypertensive rats are not fully understood. In a recent study, we found that H_2S could suppress the accumulation of collagen in the walls of pulmonary blood vessels, which might be responsible for the observed reduced hypoxic pulmonary vascular structural remodeling in response to H_2S (*24*). Whether H_2S affects the excess accumulation of collagen in arterial walls in the context of arterial hypertension remains unclear.

We used the SHR model *in vivo* and *in vitro* to measure the production rate of H_2S, to quantify hydroxyproline and collagen type I in the thoracic aorta, and to determine [^{3}H]thymidine ([^{3}H]TdR) and [^{3}H]proline incorporation and [^{3}H]hydroxyproline secretion in order to investigate the regulatory effects of H_2S on vascular collagen remodeling in hypertensive rats.

Methods

Experimental Animals and Grouping

All animal care and experimental protocols complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (documentation 55, 2001) and the Animal Care Committee of the First Hospital, Peking University. Male SHRs (45–55 g), 4 weeks old, were randomly divided into the SHR control group, SHR+NaHS group (each $n=6$), and the SHR+dihydralazine ($n=8$) group. Male Wistar Kyoto (WKY) rats of the same age and body weight were divided into the WKY control group ($n=5$) and the WKY+NaHS group ($n=8$). Rats in the SHR+NaHS and WKY+NaHS groups were injected with 56 μmol/kg/d of NaHS, the H_2S donor. Rats in the SHR control group, WKY control group, and SHR+dihydralazine group were intraperitoneally injected with the same volume of sterile saline. Drugs were injected at the same time each day for 5 weeks. The SHR+dihydralazine group received dihydralazine (15 mg/kg body weight/d) in their drinking water. Other groups were fed a standard diet with free access to drinking water. The NaHS and dihydralazine solution was freshly prepared every day.

Measurement of Systolic Blood Pressure and Sample Preparation

Systolic blood pressure was measured in conscious and sedated rats by means of the standard tail-cuff method. At the end of the experiment, the animals were intraperitoneally anesthetized with urethane (1 g/kg body weight). After the chest was opened, the thoracic aorta was excised and the medial part of the artery was flash frozen in liquid nitrogen and stored at −70°C for further use.

Measurement of H_2S Production in the Thoracic Aorta

According to the methods of Stipanuk and Beck (*16*), the aortic tissues of rats were homogenized in ice-cold potassium phosphate buffered solution (PBS, 50 mmol/L, pH 6.8). Reactions were performed in 25 mL Erlenmeyer flasks. The reaction mixture contained (in mmol/L): 10 L-cysteine, 2.0 pyridoxal 5′-phosphate, 100 PBS (pH 7.4), and 10% (w/v) homogenates. The total volume of the reaction mixture was 1 mL. A small piece of filter paper was put into the central well of the flask, and 0.5 mL of 1% zinc acetate was also added to the central well to trap H_2S in the mixture. 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 incubation for 90 min at 37°C, the reaction was stopped by injecting 0.5 mL of 50% trichloroacetic acid. The flasks were incubated in the shaking water bath for an additional hour at 37°C to complete the H_2S trapping. The contents of the central well were transferred to test tubes and mixed with 3.5 mL of distilled water and 0.5 mL of 20 mmol/L *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 mol/L HCl. Then, 0.4 mL of 30 mmol/L $FeCl_3$ in 1.2 mol/L HCl was immediately added to each tube. After incubating for 20 min at room temperature, the optical absorbance of the resulting solution was spectrophotometrically measured at 670 nm (UV-2100; Shimadzu, Kyoto, Japan). The concentration of H_2S in the solution was calculated based on the calibration curve of the standard H_2S solution. Measurements were performed in duplicate for each sample. The H_2S production is expressed as nmol/mg wet tissue/min.

Determination of Hydroxyproline Content in the Thoracic Aorta

The method used for assaying vascular hydroxyproline was as previously described (*25*). Briefly, after being washed in 0.9% NaCl solution and weighed, isolated aortic tissue was homogenized at a concentration of 100 mg/mL (w/v) using a glass homogenizer. Then, 0.5 mL of homogenate was put into ampoules, followed by addition of 1.5 mL of 6 Eq/L HCl and sealing of the ampoules with a high temperature flame. The samples were hydrolyzed for 8 h at 120°C, and cooled at ambient temperature; 1.2 mL of NaOH solution was then added to neutralize the HCl. The acidity of the sample was then adjusted to pH 5–7 by adding 1 Eq/L HCl or NaOH solution, and the volume was increased to 5 mL with distilled water. The sample was centrifuged for 10 min at 3,000 rpm, and the clear supernatant was removed for further experimentation. Subsequently, citrate buffer solution and chloramine-T were added to the samples, and the mixture was vortexed and incubated for 6 min to complete the reaction. Perchloric acid was then added with mixing for oxidation at ambient temperature for 5 min. Finally, *p*-dimethylaminobenzaldehyde solution was added and incubated at 70°C for 20 min for the color reaction. The solution was then cooled in ice water, and the optical density at 560 nm was measured spectrophotometrically. Hydroxyproline content in the thoracic aorta tissue was calculated using a known equation.

Determination of Vascular Collagen Type I

Aortic rat tissue was washed in 0.9% NaCl solution weighed, and homogenized with a glass homogenizer at 100 mg/mL (w/v). After being centrifuged at 12,000 rpm at 4°C for 10 min, the clear supernatant was removed for further experimentation. A quantitative sandwich immunoassay technique (ELISA kits; R&B, Minneapolis, USA) was used. Samples and standards were loaded in 96-well plates coated with monoclonal antibodies specific for rat collagen type I. After washing to remove unbound protein, an enzyme-linked polyclonal antibody specific for collagen type I was added to the wells. Following a second wash to remove any unbound antibody-enzyme reagent, a solution of H_2O_2 and tetramethylbenzidine was added to the wells. This resulted in the development of blue color in proportion to the amount of target protein bound in the initial step. The reaction was stopped with sulfuric acid, and the optical density of each well was measured at 450 nm. Measurements were performed in duplicate. A standard curve was constructed with rat collagen type I diluted in PBS. The amount of collagen type I in the sample was determined by comparing absorbance values to those of the standard curve.

Immunohistochemical Staining of Vascular Collagen Type I

Paraffin-embedded 4 μm thick tissue sections were placed on 3-aminopropyltriethoxysilane (APES)-coated slides. After dewaxing with dimethylbenzene, thoracic aorta sections were placed in distilled water. Washed sections were treated with 0.3% (v/v) H_2O_2 for 12 min at room temperature, and antigens were then heat processed (92–98°C) for 12 min. After each step, the sections were washed three times with PBS. After treatment with 10% (v/v) normal goat serum for 30 min at room temperature, the sections were incubated with anti–collagen I rabbit polyclonal antibody diluted 1:200 (Boster, Wuhan, China) overnight at 4°C. After washing, the sections were treated with the biotinylated secondary antibody for 20 min at 37°C. Sections were incubated with streptavidin/peroxidase complex (Rabbit SP kit; Zymed, San Francisco, USA) for 30 min at 37°C and washed three times with PBS. The 3,3′-diaminobenzidine tetrahydrochloride substrate (DAB kit; Zymed) was used as a chromogen to visualize the reaction. Finally, the sections were dehydrated with a graded ethanol series, cleared in xylene, and mounted on slides. Brown granules present in aortic smooth muscle cells observed under the microscope were defined as positive signals.

VSMC Culture

Male SHR and WKY rats ($n=5$ each) weighing 200–220 g were intraperitoneally anesthetized with urethane (1 g/kg body weight). The thoracic aorta was stripped of adventitia and removed to culture media in a sterile manner. VSMCs were isolated and cultured according to the method of Hirata *et al*. (*26*). Briefly, the cells were maintained in DMEM (Sigma, St. Louis, USA) containing 5 mmol/L glucose, 10% fetal bovine serum (FBS), and antibiotics in a CO_2 incubator at 37°C. Subcultures of VSMCs from four passages were used, and the cells were serum-starved for 24 h in serum-free DMEM before each experiment. The cells from each SHR and WKY rat were divided into four groups according to treatment, and each group was assessed at least in triplicate: 1) the control group in which cells were continuously cultured in serum-free DMEM; 2) the NaHS groups in which cells were treated with 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/L NaHS; 3) the angiotensin II (Ang II; Sigma) group in which cells were treated with 10^{-7} mol/L Ang II; and 4) the Ang II+NaHS groups in which cells were treated with 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/L of NaHS and 10^{-7} mol/L Ang II. Experiments were terminated after incubation for 24 h. Because the concentration of NaHS was far below 1 mmol/L, we considered the concentration of H_2S to be stable in the culture solution for 24 h. The NaHS stock solution (1 mol/L) was freshly prepared on the day of the experiment.

[^{3}H]TdR Incorporation *In Vitro*

Cultured VSMCs were labeled with [^{3}H]TdR (1 μCi/mL; Beijing Atomic Energy Institute, Beijing, P.R. China) 10 h before completion of the incubation. The medium was removed, and the cell minilayers were sequentially washed three times with ice-cold PBS and once with 10% ice-cold trichloroacetic acid at the end of the experiments. The cells were then solubilized in 200 μL of 0.1% SDS/0.1 Eq/L NaOH. One hundred microliters of each cell solution was added to 5 mL of scintillation fluid, and the incorporation of [^{3}H]TdR into VSMCs was determined by liquid scintillation spectrometry (LS6500, Beckman, Fullerton, USA). Incorporation is expressed as cpm/mL.

Collagen Synthesis and Collagen Secretion *In Vitro*

[^{3}H]Proline is preferentially incorporated into newly synthesized collagen, and collagen is degraded into a high-molecular polypeptide containing hydroxyproline by pepsin at 4°C, whereas other noncollagen proteins are completely degraded (*27*). Therefore, total collagen synthesis and secretion is reflected by [^{3}H]proline incorporation into cell extracts and [^{3}H]hydroxyproline radioactivity in the culture medium. In the present study, the method of Koyano *et al*. (*28*) was slightly modified. Briefly, 10^4 cells were seeded in each well of a 24-well plate (Nunc, Naperville, USA). During the last 12 h of the incubation, 1 μCi of [^{3}H]proline (1 mCi/mL; Amersham-Pharmacia Asia, Hong Kong, P.R. China) was added to each well. At the end of the stimulation, the culture medium was removed, cells were digested for 2 h with 1 mg/mL of pepsin at 4°C, and were finally precipitated in 30% trichloroacetic acid. The precipitated cell pellets were washed twice with ice-cold PBS, and then precipitated in 10% trichloroacetic acid. The same procedure was used to precipitate the culture medium. The precipitates were suspended in 500 μL of 0.5 Eq/L NaOH, 0.1% Triton X-100. Radioactivity was then measured using the liquid scintillation counter.

Western Blotting of Extracellular Signal–Regulated Kinases (ERKs)

Quiescent cells were stimulated with vehicle or Ang II at a final concentration of 10^{-7} mol/L for 10 min. For the NaHS experiment, cells were pretreated with vehicle alone or with various concentrations of NaHS (5×10^{-5}, 1×10^{-4} and 5×10^{-4} mol/L) for 30 min before Ang II addition. After treatment, VSMCs were harvested, and lysed in 150 μL of lysis buffer (20 mmol/L Tris-HCl pH 8, 137 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). The plates were placed on ice for 10 min, and allowed to thaw on the ice. The cells were removed by scraping, transferred to Eppendorf tubes, and sonicated for 5 s. The lysate was centrifuged at 14,000× *g* for 20 min, and the protein supernatant was used for Western blot analysis. Equal amounts of protein were loaded on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane for 1 h at 100 V. Membranes were blocked with buffer containing Tris-buffered saline and 0.1% Tween-20 with 5% w/v nonfat dry milk for 1 h at room temperature, and then incubated with anti-phosphospecific ERK1/ERK2 or anti–total ERK antibody (Santa Cruz Biotechnology, Santa Cruz, USA) diluted 1:500 for 24 h at 4°C. The plates were then washed, incubated with a secondary antibody diluted 1:10,000 for 1 h at room temperature, and washed extensively. Immunoreactive bands were visualized using an ECL kit and exposure of the membrane to X-ray film (Kodak Scientific Imaging Film, X-omat Blue XB-1; Kodak, Rochester, USA). Band intensity was measured using the Image Quant program.

Ang II Binding Experiment

Cells (1×10^7 cells) were frozen in liquid nitrogen, thawed in ice-cold phosphate-buffered saline, and lysed by five cycles of aspiration-expulsion with a 10 mL serological pipette tightly apposed to the bottom of the dish. Lysed cells were centrifuged at 2,500× *g* for 15 min at 4°C and resuspended in binding buffer (25 mmol/L Tris-HCl pH 7.4, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, 0.1% bovine serum albumin, and

Table. 1. Blood Pressure, H_2S Production, and Vascular Hydroxyproline and Collagen Levels in the Thoracic Aorta of Rats

Group	*n*	Blood pressure (mmHg)	Production of H_2S (nmol/mg wet tissue/min)	Vascular hydroxyproline (μg/mg)	Vascular type I collagen (ng/mg wet tissue)
SHR	6	181±10**	15.4±3.4**	8.31±2.78**	1.55±0.39*
SHR+NaHS	6	155±13††	25.7±8.9††	4.86±1.59†	1.21±0.04††
SHR+dihydralazine	8	164±7††	17.8±5.1	7.31±0.96	1.47±0.02
WKY	5	119±9	24.9±7.2	4.45±1.50	1.21±0.04
WKY+NaHS	8	113±10	53.9±16.2**	4.44±0.27	1.19±0.02

*$p<0.05$, compared with WKY group; **$p<0.01$, compared with WKY group; †$p<0.05$, compared with SHR group; ††$p<0.01$, compared with SHR group. Data are means±SD. SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats.

0.01% bacitracin). The samples (20 μg protein in 250 μL) were then incubated for 45 min at room temperature in binding buffer containing increasing concentrations of ^{125}I-Ang II (62.5 pmol/L–4.0 nmol/L) in the presence or absence of NaHS (1×10^{-4} mol/L). Bound and free ligands were separated by filtration through GF/C filters presoaked for 2 h in binding buffer. Nonspecific binding was measured in the presence of 1 μmol/L of unlabeled Ang II.

Additionally, the NaHS inhibition test was performed as follows: lysed cells (20 μg protein/tube) were incubated for 45 min at room temperature in binding buffer containing 2.0 nmol/L of ^{125}I-Ang II in the presence of various concentrations of NaHS (5×10^{-5}, 1×10^{-4} and 5×10^{-4} mol/L). Specific receptor banding capacity was measured as described above.

Statistical Analysis

Data are expressed as the mean±SD. ANOVA was used to compare the mean values in various groups. The Student-Newman-Keuls analysis was used to compare data between different groups. A $p<0.05$ was considered statistically significant.

Results

NaHS Administration Decreases Blood Pressure in SHRs

Systolic blood pressure was higher, by 52%, in the SHR group than in the WKY group ($p<0.01$). However, treatment with NaHS and dihydralazine significantly lowered systolic blood pressure in SHRs ($p<0.01$) (Table 1).

Aortic Production of H_2S Is Decreased in SHRs

H_2S production in thoracic aortic tissue was lower, by 55%, in the SHR group than in the WKY group ($p<0.01$). However, aortic H_2S content was higher, by 67%, in the NaHS-treated SHR group than the untreated SHR group ($p<0.01$). After NaHS treatment, aortic H_2S content was significantly increased in SHRs compared to WKY rats ($p<0.01$). However, dihydralazine treatment had no effect on the aortic production of H_2S (Table 1).

NaHS Treatment Lowers Vascular Hydroxyproline and Collagen Type I Content in SHRs

Quantification of hydroxyproline in the thoracic aorta demonstrated increased content, by 87%, in the SHR group compared to the WKY group ($p<0.01$) (Table 1). However, quantification of aortic hydroxyproline demonstrated decreased content, by 41%, in the NaHS-treated SHR group than the untreated SHR group ($p<0.05$).

The content of collagen type I in the thoracic aorta was 28% higher in the SHRs compared to the WKY group ($p<0.05$) (Table 1). However, collagen type I content was lower, by 23%, in the NaHS-treated SHR group than the untreated SHR group ($p<0.01$).

Representative collagen type I immunohistochemical staining shows intense collagen I granulae in the thoracic aorta of untreated SHRs (Fig. 1A) and dihydralazine-treated SHRs (Fig. 1B), and more collagen type I granulae around the VSMCs. The staining density of collagen type I was quite weak in NaHS-treated SHRs (Fig. 1C) compared to the untreated SHRs. Scant collagen type I staining was observed in the thoracic aorta of WKY (Fig. 1D) and NaHS-treated WKY rats (Fig. 1E).

However, dihydralazine treatment did not markedly impact the quantification of hydroxyproline, content of collagen type I, and representative collagen type I immunohistochemical staining in the thoracic aortas from SHRs.

NaHS Inhibits Ang II–Induced VSMC Proliferation *In Vitro*

In the absence of Ang II, VSMC incorporation of [^{3}H]TdR was 28% higher for SHRs compared to WKY rats ($p<0.05$) (Fig. 2). Incubation with NaHS alone, at 5×10^{-5}, 1×10^{-4}, and 5×10^{-4}mol/L had no effect on VSMC incorporation of [^{3}H]TdR in the WKY or SHR group. The incorporation of [^{3}H]TdR in Ang II (10^{-7} mol/L)–stimulated VSMCs from the SHR control group was markedly higher, by 59.5%, than in the WKY control group ($p<0.05$) (Fig. 2). NaHS at 5×10^{-5},

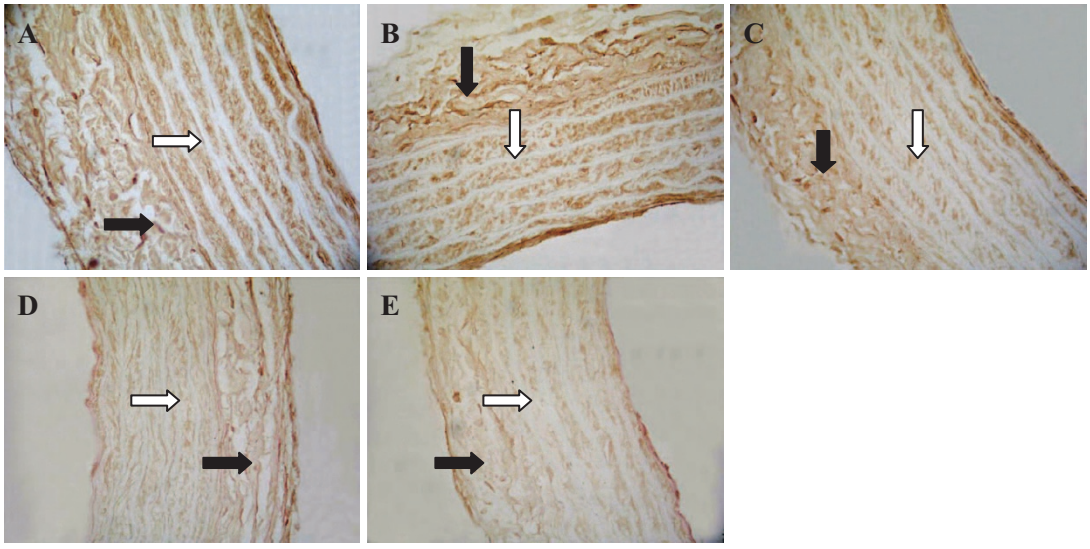

Fig. 1. *Immunohistochemical staining of collagen type I expression in rat aorta sections. The presence of immunoreactive protein is demonstrated by brown staining. Representative collagen type I immunohistochemical staining shows intense signals for collagen type I in the aorta of SHR (A) and dihydralazine-treated SHR (B); few collagen type I granulae were seen in the aortas of WKY rats (D) and NaHS-treated WKY rats (E). More collagen type I granulae were seen around the VSMCs. The staining density of collagen type I significantly decreased in the aortas of NaHS-treated SHR (C) as compared with untreated SHR. The filled arrow indicates the adventitia layer of the aorta, and the open arrow indicates the media layer of the aorta. Magnification: ×600.*

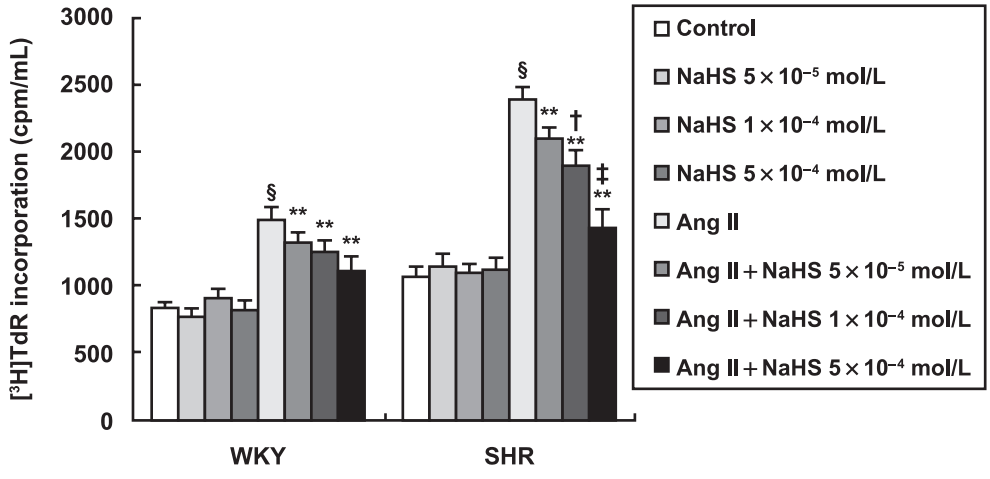

Fig. 2. *Effect of H_2S on proliferation of VSMCs in the absence and presence of Ang II. The values obtained after NaHS treatment were not significantly different from those of the controls in both the WKY and SHR groups in the absence of Ang II. The inhibitory effect of NaHS on Ang II–treated VSMC proliferation was stronger in the SHR than the WKY group. The inhibition of VSMC [^{3}H]TdR incorporation by NaHS in the presence of Ang II was dose dependent. **$p<0.01$ compared with the corresponding control in the presence of Ang II. $^{\dagger}p<0.05$ vs. SHR with Ang II+NaHS (5×10^{-5} mol/L) treatment, $^{\ddagger}p<0.01$ vs. SHR with Ang II+NaHS (10^{-4} mol/L) treatment. $^{\S}p<0.01$ vs. control counterpart.*

1×10^{-4}, and 5×10^{-4}mol/L decreased [^{3}H]TdR incorporation by 11.8%, 16.5%, and 25.7%, respectively ($p<0.05$ or $p<0.01$) in Ang II–treated VSMCs from WKY rats. Meanwhile, NaHS at the above concentrations exerted a more potent inhibitory effect on [^{3}H]TdR incorporation in Ang II–treated VSMCs from the SHR compared to the WKY group.

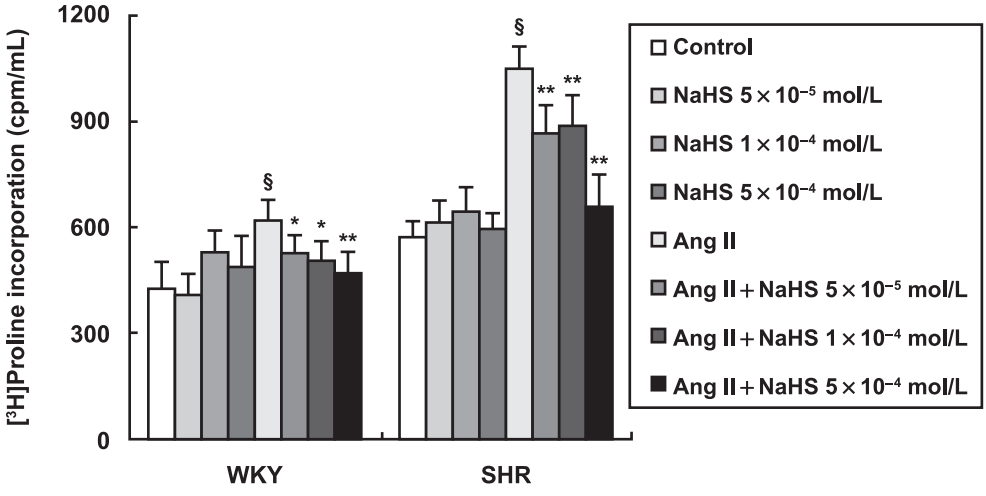

Fig. 3. *Effect of H_2S on collagen synthesis in VSMCs in the absence and presence of Ang II. The values obtained after NaHS treatment were not significantly different from those of the controls in both the WKY and SHR groups. The inhibitory effect of NaHS on Ang II–treated VSMC [^{3}H]proline incorporation was stronger in the SHR than in the WKY group. *$p < 0.05$, **$p < 0.01$ compared with the corresponding control in the presence of Ang II. $^{§}p < 0.01$ vs. control counterpart.*

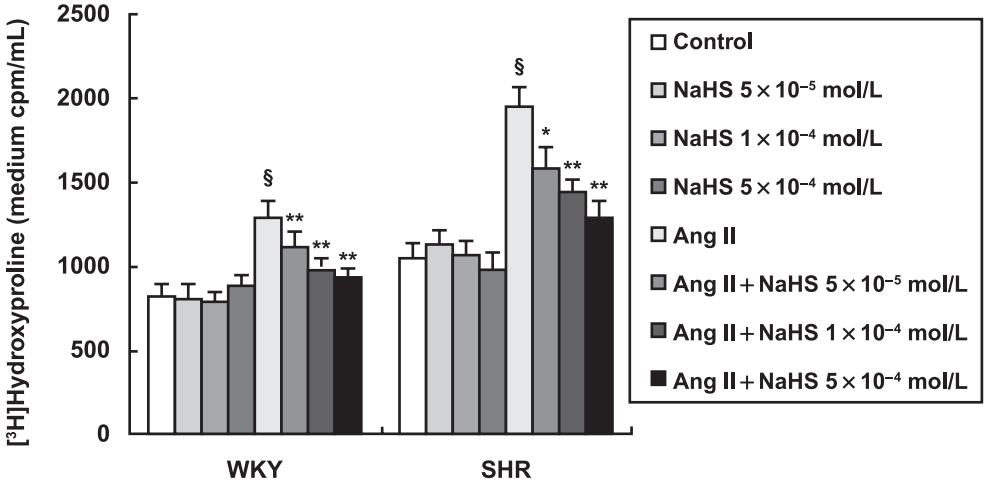

Fig. 4. *Effect of H_2S on collagen secretion by VSMCs in the absence and presence of Ang II. The values obtained after NaHS treatment were not significantly different from those of the controls in both the WKY and SHR groups. The inhibitory effect of NaHS on Ang II–treated VSMC collagen secretion was stronger in the SHR than in the WKY group. The inhibitory effect of NaHS on VSMC [^{3}H]hydroxyproline levels was stronger in the SHR than in the WKY group. *$p < 0.05$, **$p < 0.01$ compared with the corresponding control in the presence of Ang II. $^{§}p < 0.01$ vs. control counterpart.*

NaHS dose-dependently decreased [^{3}H]TdR incorporation by 12.3%, 20.1%, and 40.4%, respectively ($p<0.05$ or $p<0.01$) in the Ang II+NaHS SHR group.

NaHS Attenuates Ang II–Induced Collagen Synthesis and Hydroxyproline Secretion in VSMCs

Compared with the WKY group, the SHR group showed increased VSMC incorporation of [^{3}H]proline, by 34.2%, in the absence of Ang II ($p<0.05$) (Fig. 3). Incubation with NaHS alone (5×10^{-5}, 1×10^{-4} and 5×10^{-4} mol/L) did not change VSMC incorporation of [^{3}H]proline in the WKY or SHR groups in the absence of Ang II ($p>0.05$). Ang II (10^{-7} mol/L) increased [^{3}H]proline incorporation by 70.4% ($p<0.01$) in VSMCs from the SHR group as compared with those from the WKY group. Upon incubation with NaHS at

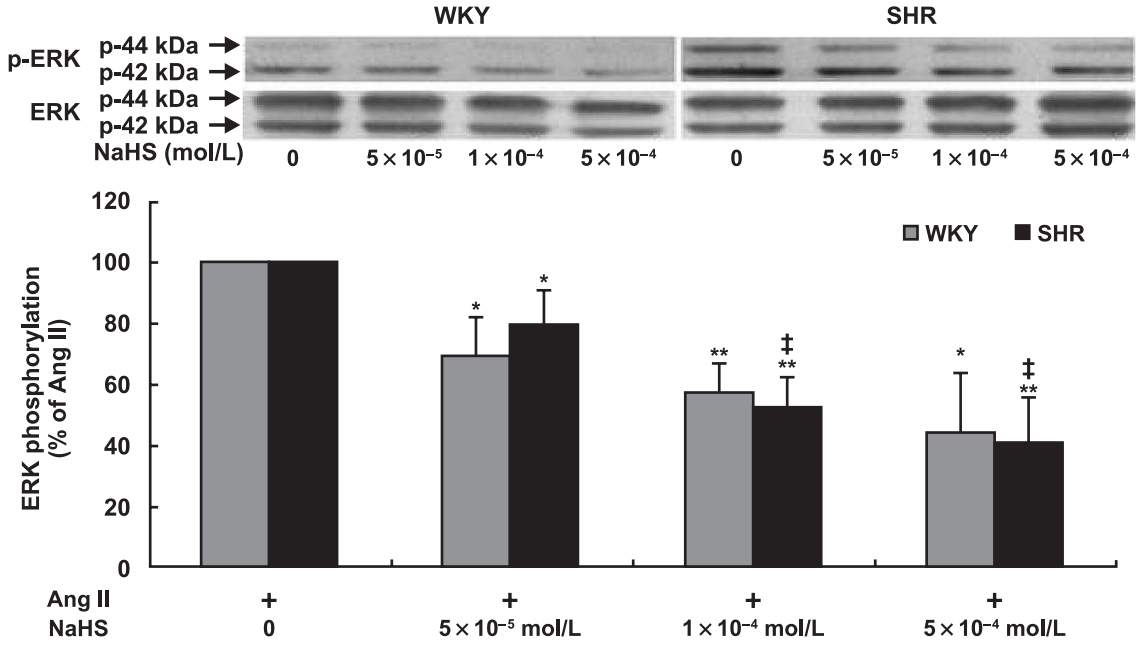

Fig. 5. *Representative Western blot showing the effects of H_2S on Ang II–induced ERK1 and ERK2 phosphorylation in primary cultured VSMCs from WKY (left) and SHR (right). Growth-arrested cells were stimulated with Ang II (10^{-7} mol/L) for 10 min. Cells were pre-exposed to three concentrations of NaHS for 30 min. Cells were harvested as described in the methods. The bar graph demonstrates the percent decrease in ERK phosphorylation in cells from WKY and SHR relative to cells stimulated with Ang II under control conditions. The values induced by NaHS were significantly different from those of Ang II–treated cells alone. Inhibition of ERK1 and ERK2 phosphorylation by NaHS in the presence of Ang II occurred in a dose-dependent manner. *$p<0.05$, **$p<0.01$ compared with the corresponding control in the presence of Ang II. ‡$p<0.01$ vs. SHR with Ang II+NaHS (5×10^{-5} mol/L) treatment.*

5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/L, Ang II–stimulated [^{3}H]proline incorporation decreased by 14%, 17.6%, and 24.4%, respectively ($p<0.05$ or $p<0.01$), compared to VSMCs from WKY rats treated with Ang II alone (Fig. 3). In the SHR group, NaHS at 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/L significantly decreased Ang II–stimulated VSMC [^{3}H]proline incorporation by 17.6%, 15.5%, and 37.5%, respectively (all $p<0.01$), compared with VSMCs treated with Ang II alone.

The secreted [^{3}H]hydroxyproline in the medium from SHR rat VSMCs was 28.9% higher than that obtained from WKY rat VSMCs ($p<0.05$) (Fig. 4). Different concentrations of NaHS (5×10^{-5}, 1×10^{-4} mol/L, and 5×10^{-4} mol/L) did not change [^{3}H]hydroxyproline secretion in the WKY or SHR groups in the absence of Ang II ($p>0.05$) (Fig. 4). Ang II (10^{-7} mol/L) incubation increased [^{3}H]hydroxyproline secretion by 51.8% in the medium from SHR rat VSMCs as compared with that from WKY rat VSMCs ($p<0.01$) (Fig. 4). Upon incubation with NaHS at 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/L, Ang II–stimulated [^{3}H]hydroxyproline secretion was decreased by 13.7%, 24.8%, and 28.2%, respectively (all $p<0.01$), compared to secretion by WKY rat VSMCs treated with Ang II alone. In the SHR group, NaHS at 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} mol/L significantly decreased Ang II–stimulated VSMC [^{3}H]hydroxyproline secretion by 18.7%, 26.4%, and 34.1%, respectively ($p<0.05$ or $p<0.01$), as compared with VSMCs treated with Ang II alone.

NaHS Reduces Ang II–Induced MAPK Activity in VSMCs

ERK activation was determined by Western blot analysis (Fig. 5). Phosphorylated ERK1/2 in the Ang II+NaHS groups was markedly reduced compared with that in the Ang II control group. NaHS at 5×10^{-5}, 1×10^{-4} mol/L, and 5×10^{-4} mol/L significantly decreased VSMC phosphorylated ERK1/2 by 30.3%, 42.3%, and 55.7%, respectively, in the presence of Ang II in the WKY group ($p<0.05$ or $p<0.01$). At the above concentrations, NaHS dose–dependently decreased phosphorylated ERK1/2 by 20.1%, 47.4%, and 59.1%, respectively ($p<0.05$ or $p<0.01$), in Ang II–treated VSMCs from the SHR group (Fig. 5).

NaHS Decreases AT_1 Receptor Binding Affinity

Cells stably expressing the AT_1 receptor were treated with increasing concentrations of NaHS, and their Ang II binding properties were analyzed. Figure 6A shows that NaHS

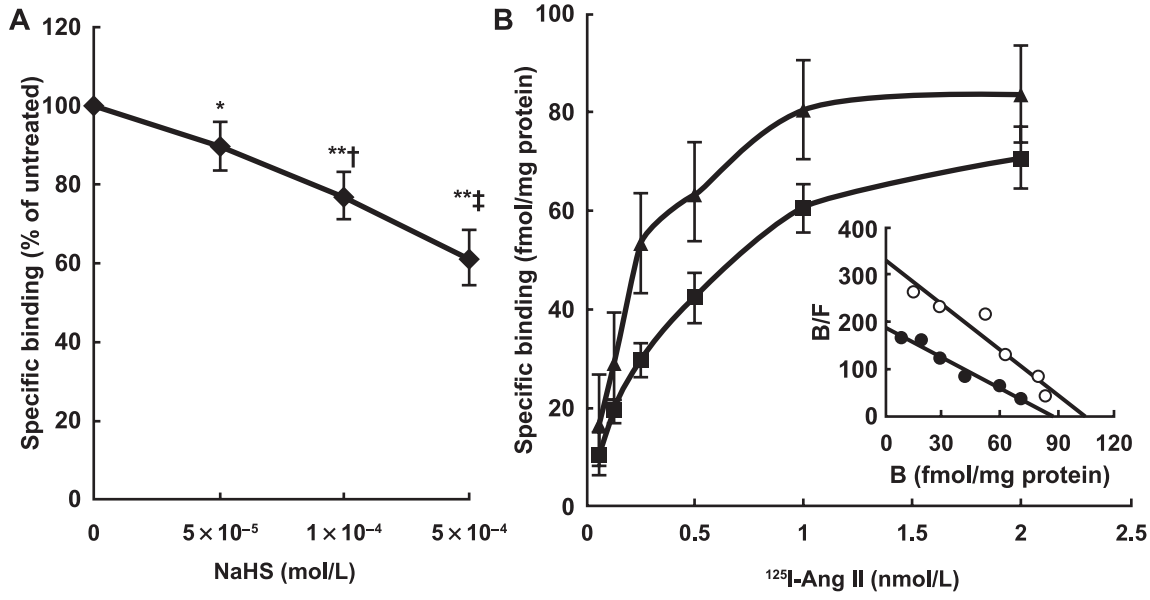

Fig. 6. *H_2S decreases the binding affinity of the AT_1 receptor. A: Concentration-dependent curve showing NaHS inhibition of Ang II binding. Lysed cells stably expressing the AT_1 receptor (20 μg of protein) were incubated with 2 nmol/L of ^{125}I-Ang II for 1 h at room temperature in the presence of increasing concentrations of NaHS (5×10^{-5}, 1×10^{-4} and 5×10^{-4} mol/L). *$p<0.05$, **$p<0.01$ vs. VSMCs in the absence of NaHS. $^{\dagger}p<0.01$ vs. VSMCs with NaHS (5×10^{-5} mol/L) treatment. $^{\ddagger}p<0.01$ vs. VSMCs with NaHS (10^{-4} mol/L) treatment. B: Lysed cells stably expressing the AT_1 receptor (20 μg of protein) were incubated with (■) or without (▲) NaHS (1×10^{-4} mol/L) for 45 min at room temperature in the presence of increasing concentrations of ^{125}I-Ang II. Scatchard analysis of the binding data is shown. Nonspecific binding was assessed in the presence of 1 μmol/L of unlabeled Ang II. Scatchard plot of the effect of NaHS on ^{125}I-Ang II binding in cells incubated with (filled symbols) or without (open symbols) NaHS. B, bound; B/F, bound/free ratio. Each data point represents the mean±SD of quadruple values (representative of four independent experiments).*

decreased the specific binding of ^{125}I-Ang II in a concentration-dependent manner ($p<0.05$ or $p<0.01$). To better define the effect of NaHS, ^{125}I-Ang II saturating binding isotherms were performed in the absence or presence of 1×10^{-4} mol/L of NaHS. Scatchard analysis (Fig. 6B) of the binding data revealed that untreated lysed cells had a high binding affinity (K_d of 0.319±0.041 nmol/L), whereas NaHS-treated lysed cells had a significantly lower K_d of 0.460±0.037 nmol/L ($p<0.01$). Treatment with NaHS significantly affected the maximal binding capacity of the cell lysates (105.17±11.22 fmol/mg of protein *vs*. 85.84±10.17 fmol/mg of protein for the untreated and NaHS-treated cells, $p<0.05$). These results clearly show that NaHS directly decreases AT_1 receptor binding and the binding affinity of the AT_1 receptor.

Discussion

Vascular structural remodeling, including the abnormal proliferation of smooth muscle cells and the excess accumulation of ECM, is an important pathological alteration associated with hypertension pathogenesis. The ECM fibrous components, collagen and elastin, are the main elements responsible for passive vascular mechanical properties, particularly in the aorta and its main branches, where these proteins are abundant (*22*, *29*). Alterations in collagen and elastin have been well-documented in hypertension and are known to contribute to changes in vascular compliance (*23*). Collagen has an important function in maintaining vascular wall integrity. An increase in collagen synthesis and decrease in collagen degradation could result in vascular remodeling. To better understand the mechanisms by which H_2S alleviates vascular structural remodeling, we investigated the regulatory effect of H_2S on the accumulation of collagen in the vessels.

The results showed significantly higher tail blood pressure in SHR compared to WKY rats ($p<0.01$). However, H_2S production in the thoracic aorta of the SHR group was markedly decreased compared to that of the WKY group, suggesting that the endogenous H_2S pathway is downregulated in hypertensive animals. We also found that severe collagen accumulation occurred in the vascular wall of the SHR group, as shown by increased secretion of hydroxyproline, which is an inherent component of collagen. ELISA and immunohistochemistry revealed increased collagen type I content, which is related to anti-stress effects in the vascular wall, in aortic tissues from the SHR group. Thus, we observed collagen accumulation in vascular tissues and vascular remodeling in

hypertensive rats.

To explore the significance of the decreased H_2S generation in vascular remodeling during hypertension, the therapeutic effect was evaluated in SHRs after exogenous H_2S donor administration for 5 weeks. The results showed that exogenous H_2S lowered blood pressure and attenuated collagen accumulation in SHRs. Secretion of hydroxyproline and expression of collagen type I in the thoracic aorta were decreased in NaHS-treated SHRs, whereas dihydralazine, a vasodilator, did not show such an effect despite its ability to reduce blood pressure. These findings suggest that lowered blood pressure is not responsible for the decreased levels of collagen in the thoracic aorta. Although the H_2S concentration increased in the vascular wall, the changes were not obviously different in WKY rats with or without exogenous H_2S treatment. Thus, H_2S could reduce the accumulation of collagen in the vascular walls of SHRs, and down-regulation of H_2S generation might be involved in the vascular structural remodeling observed during hypertension.

In our previous experiments, the level of endogenous H_2S generation was decreased in SHRs, but hypertension could be reversed upon treatment with the H_2S donor NaHS (*22*). This finding suggests that down-regulation of endogenous H_2S generation is involved in the pathogenesis of hypertension. However, until now, the exact mechanisms by which H_2S regulates hypertension have remained unclear. Obviously, the weak and direct vasodilation effect of H_2S is not enough to fully explain the antihypertensive effects of H_2S. Since VSMC proliferation is one of the most important mechanisms associated with vascular structural remodeling, we investigated the direct inhibitory role of H_2S in hypertensive vascular remodeling and observed increased VSMC proliferation and collagen generation in the SHR group compared to the WKY group after NaHS treatment.

Vascular remodeling has an important Ang II–dependent component because interruption of the renin-angiotensin system with angiotensin-converting enzyme (ACE) inhibitors or AT_1 receptor antagonists corrects the vascular structure and endothelial dysfunction in small arteries of hypertensive patients and hypertensive rat models (*30–32*). Increased ERK1/2 signaling by Ang II is associated with VSMC hypertrophy and hyperplasia and inhibition of MEK1/2, the kinase that activates ERK1/2, normalizes exaggerated responses in VSMCs from SHR (*33*). Touyz *et al*. (*34*) also demonstrated that Ang II induced a dose-dependent increase in [^{3}H]proline incorporation and increased Ang II–stimulated DNA and collagen synthesis, which is mediated through ERK1/2-dependent, p38 mitogen-activated protein kinase (MAPK)–independent pathways in SHRs. A possible mechanism underlying Ang II–induced p38 MAPK action in SHR may be oxidative stress–dependent pathways (*35*). In cases of hypertension, Ang II–induced generation of vascular reactive oxygen species is increased (*36*). Disturbances in the regulation of upstream modulators, such as Src, phosphatidyl inositol 3 (PI3) kinase, or other kinases, could contribute to altered growth signaling by Ang II in cells from genetically hypertensive rats. Ang II–induced activation of Src is increased in SHRs, and Src-dependent regulation of p38 MAPK and ERK1/2 is altered in VSMCs from SHRs (*37*). These events may contribute to increased VSMC growth and ECM deposition, which play important pathophysiological roles in vascular remodeling in hypertension. Three different dosages of NaHS cultured with VSMCs produced an obvious reduction in the incorporation of [^{3}H]TdR and [^{3}H]proline, and [^{3}H]hydroxyproline secretion in both the Ang II–treated SHR and WKY groups. The inhibitory effect of NaHS on VSMC proliferation and collagen generation was stronger in the SHRs compared to the WKY group. De Godoy and Rattan reported that SHRs had significantly higher plasma levels of Ang II than WKY rats (50.3 ± 0.9 pg/mL *vs*. 13.2 ± 3.0 pg/mL, $p<0.05$) (*38*). Thus, VSMCs from SHRs might have an intrinsic genetic abnormality in the control of VSMC proliferation. Only by augmenting the proliferative status of VSMCs in the presence of Ang II can we better understand the anti-proliferative effect of NaHS on VSMCs. MAPK signaling pathways have been highly implicated in cell proliferation. In the present study, we examined the effect of NaHS on angiotensin II–induced MAPK activation. The results demonstrate that NaHS significantly inhibited Ang II–induced phosphorylation of ERK1/2 in a dose-dependent manner. Ang II elicits a variety of physiological reactions, including blood pressure regulation, vascular contraction, and cardiovascular cell growth and proliferation. Most of its cardiovascular effects are mediated by the AT_1 receptor. In the present study, we examined the effect of NaHS on the specific binding and the binding affinity of AT_1 receptor. Surprisingly, we found that NaHS could directly inhibit the specific binding and decrease the binding affinity of the AT_1 receptor. These results offered a mechanistic explanation for H_2S inhibition of Ang II–induced cardiovascular cell proliferation and fibrosis.

Therefore, the H_2S donor NaHS suppressed not only total protein synthesis, but also collagen synthesis and secretion, suggesting that H_2S might be involved in the mechanisms of excess accumulation of artery collagen. The mechanisms underlying H_2S anti-proliferative activities have not been fully elucidated. We have previously shown that H_2S could dose-dependently suppress the proliferation of VSMCs *via* the MAPK pathway in the presence of FBS or endothelin (*39*). In the present study, the results demonstrate that H_2S also inhibited Ang II–induced MAPK activity. Inhibition of growth and induction of apoptosis in human aortic smooth muscle cells by CSE overexpression or exogenously applied H_2S were accompanied by marked down-regulation of cyclin D1 and up-regulation of $p21^{Cip/WAK-1}$ (*40*). In the present study, downregulated endogenous H_2S was associated with reduced vascular collagen remodeling and decreased blood pressure in SHRs *in vivo*. Notably, H_2S inhibited the proliferation of VSMCs cultured *in vitro*, and the synthesis and secretion of collagen in the presence of Ang II. Taken together, the above findings suggest that the gaseous transmitter H_2S might be

involved in the regulation of hypertensive vascular collagen remodeling. As a consequence, interventions in the H_2S pathway could represent a new therapeutic strategy for the treatment of hypertension. Further studies are needed to fully understand the mechanisms by which H_2S exerts its effects.

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