Exogenous H$_2$S Ameliorates High Salt-Induced Hypertension by Alleviating Oxidative Stress and Inflammation in the Paraventricular Nucleus in Dahl S Rats

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Abstract
Hydrogen sulfide (H$_2$S) is an important gaseous signaling molecule that regulates cardiovascular activity in animals. The hypothalamic paraventricular nucleus (PVN) is a major integrative region involved in blood pressure (BP) regulation. We explored whether exogenous H$_2$S application by intraperitoneal injection of sodium hydrosulfide (NaHS) alleviates BP increase induced by a high salt diet (HSD) and the role of PVN in Dahl salt-sensitive (Dahl S) rats. Dahl S rats were divided into four groups according to diet regime (normal salt diet [NSD] and HSD) and treatment method (daily intraperitoneal NaHS or saline injection). We monitored BP, food and water intake, and body weight for 8 weeks. Plasma, kidney, and brain tissues were collected at the end of the experiment. We found that exogenous H$_2$S not only delayed BP elevation but also attenuated the increase in the levels of norepinephrine, cystatin C, and blood urea nitrogen in the plasma of Dahl S rats with an HSD. Furthermore, H$_2$S enhanced the total antioxidant capacity, superoxide dismutase, and glutathione peroxidase in the PVN. Exogenous H$_2$S attenuated the protein expression of the nuclear factor-κB pathway and proinflammatory cytokines, which were significantly higher in the PVN in rats with an HSD than in rats with an NSD. Additionally, exogenous H$_2$S relieved PVN neuronal apoptosis induced by an HSD. These findings suggest that exogenous H$_2$S attenuates hypertension caused by an HSD by ameliorating oxidative stress, inflammation, and apoptosis in the PVN. This study provides evidence of the benefits of peripheral H$_2$S therapy for hypertension.

Keywords H$_2$S · PVN · Oxidative stress · Inflammation · NF-κB · Apoptosis

Introduction
Hypertension is a type of cardiovascular and cerebrovascular disease that is characterized by a continuous elevation of blood pressure (BP) that results in associated serious medical illness [1]. According to the report on “Nutrition and Chronic Disease Status of Chinese Residents (2020)” in China in 2019, the death rate due to chronic diseases, such as cerebrovascular and cardiovascular diseases, was as high as 80.7%. In addition, hypertension is considered the primary risk factor for stroke in China [1, 2]. Accumulating evidence indicates that nutritional lifestyles, such as a high salt diet (HSD), are one of the most important factors that contribute to the development of hypertension.

It is well established that the hypothalamic paraventricular nucleus (PVN) is an important nucleus for the central regulation of cardiovascular activity. It contains rich autonomic neurons that participate in the regulation of sympathetic nerve activity and BP [3, 4]. Neuronal activation of the PVN can increase the neuro-excitatory response, raise BP, and increase heart rate under conditions of stress [3, 4].

Over the past several years, hydrogen sulfide (H$_2$S) has been recognized as a toxic and hazardous gas for the human body owing to the discovery of several features, such as...
olfactory paralysis, sudden loss of consciousness, pulmonary edema, mucosal irritation, and keratoconjunctivitis in people exposed to high concentrations of H$_2$S [5, 6]. In the past few decades, increasing evidence has shown that H$_2$S plays a positive role in the regulation of crucial physiological functions, such as hippocampal activity and muscle relaxation. H$_2$S in low concentrations plays a protective role against many diseases [7]. Like carbon monoxide (CO) and nitric oxide (NO), H$_2$S plays an important physiological role as a signaling molecule and provides therapeutic effects in the treatment of a wide range of diseases [8, 9]. Endogenous H$_2$S is mainly present in mammals and is catalyzed by three enzymes, cystathionine-β-synthase (CBS), cystathionine-γ-lyase, and 3-mercaptoppyruvate sulfur transferase, of which CBS is the primary enzyme involved in neumodulation [10]. A recent study found that microinjection of CBS inhibitors, hydroxylamine or amino-oxyacetate, into the rostral ventrolateral medulla produces an increase in renal sympathetic nerve activity, mean arterial pressure (MAP), and heart rate [11].

A study has shown that a high-fat diet (HFD) can lower the biosynthesis of H$_2$S in the liver, kidneys, and lung and increase plasma interleukin (IL)-6, IL12p40, and granulocyte colony-stimulating factor (G-CSF) levels in mice [12]. In another study, Wu et al. [13] found that exogenous H$_2$S application by intraperitoneal injection of sodium hydro-sulfide (NaHS), a donor of H$_2$S, reduced not only the degree of kidney fibrosis but also the expression level of tumor necrosis factor-α (TNF-α), IL-6, monocyte chemoattractant protein-1, and nuclear factor-kB (NF-kB) subunits p50, p65, and phosphorylated p65 (p-p65) in the kidneys of HFD-induced obese mice. That study suggested that H$_2$S mitigates renal injury via the reduction of kidney inflammation in HFD-induced obese mice. H$_2$S also plays an important role in alleviating hypertension and kidney damage induced by an HSD in Dahl salt-sensitive (Dahl S) rats. Exogenous H$_2$S application by intraperitoneal injection of NaHS inhibits myocardial hypertrophy in HSD-stimulated Dahl S rats by enhancing antioxidant capacity and inhibiting oxidative stress in myocardial tissues. It also protects against HSD-induced renal damage by enhancing antioxidant capacity and inhibiting renal oxidative stress [14, 15]. In contrast, HSD increases MAP and induces an inflammatory state in the PVN of Dahl S rats [16]. Endogenous or exogenous H$_2$S changes by microinjection of hydroxylamine hydrochloride or GYY4137 into the PVN attenuates sympathetic activity and hypertensive responses partly by decreasing reactive oxygen species (ROS) and proinflammatory cytokines (PICs) within the PVN in rats with HSD [17]. However, further investigation of the effects of intraperitoneally injected NaHS on BP increase induced by HSD is needed to determine the central mechanism of PVN neuronal activity underlying BP changes.

This study aimed to explore whether exogenous H$_2$S exposure via intraperitoneal NaHS injection influences BP increase induced by an HSD and determine the role of PVN in such BP changes using adult Dahl S rats. Findings from these experiments contribute to our understanding of the role of H$_2$S treatment in peripheral tissue for hypertension associated with lifestyle-based eating habits, such as high salt intake.

**Materials and Methods**

**Subjects**

Eight-week-old male Dahl S rats were purchased from the Charles River Laboratory Animal Technology Co. Ltd. (Beijing, China) and allowed to acclimate for 1 week before the experiment. They were fed in the Animal Center in the School of Life Sciences, Henan University. All rats were maintained on a 12-h light/dark cycle and had free access to food and water before the experiment. The experimental protocols were approved by the Animal Care and Use Committee of the College of Medicine, Henan University (Ethical Clearance No. HUSOM2020-299). The design of the experiment conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

**Experimental Design**

Twenty-four Dahl S rats were divided into four groups according to diet type (normal salt diet [NSD] or HSD) and treatment (intraperitoneal saline or NaHS injection): (1) NSD group: rats were fed with food containing 0.3% sodium chloride (NaCl) and intraperitoneally injected with saline daily; (2) NSD + NaHS group: rats were fed with food containing 0.3% NaCl and injected intraperitoneally with NaHS daily; (3) HSD group: rats were fed with food containing 8% NaCl and intraperitoneally injected with saline daily; (4) HSD + NaHS group: rats were fed with food containing 8% NaCl and intraperitoneally injected with NaHS daily. Rats in the NSD + NaHS and HSD + NaHS groups were given 90 µmol/kg/day NaHS by intraperitoneal injection daily. The amount of the NaHS application was administered according to the methods of Huang et al. [14]. The rats were treated for 8 weeks, and food and water intake and body weight (BW) were monitored weekly.

**Blood Pressure Measurements and Tissue Collection**

BP and heart rate of all rats were measured while conscious using the tail-cuff method on a non-invasive BP system from Zhongshi Technology (Beijing, China) following...
manufacturer. Each rat was acclimatized for restraint in the tail-cuff tube for 15 min/day over 3 consecutive days. After adaptation training, BP and heart rate of each rat were measured using the tail-cuff method for another 3 consecutive days. Average BP and heart rate values were used as a baseline for all rats. Subsequently, rats were given different diets and treatments. BP and heart rate were measured three times each week, and these measurements were repeated for 8 weeks. The average of the three BP and heart rate values each week were considered the BP value of each rat for that week. On the last day of the experiment, 24-h food intake, water intake, and BW were measured. Rats were then euthanized, and tissue samples of the blood, heart, kidneys, and brain were collected. The weights of the heart and kidneys of each rat were measured after collection. Trunk blood was collected in chilled tubes containing ethylenediaminetetraacetic acid (2 mg/ml) from the abdominal aorta of rats and centrifuged for 30 min at 4 °C. The plasma was transferred to a 2 ml centrifuge tube and stored in a – 80 °C freezer to measure the level of circulating norepinephrine (NE), blood urea nitrogen (BUN), and cystatin C (Cys-C) using the enzyme-linked immunosorbent assay (ELISA) kit following manufacturer guidelines. For the PVN tissue collection, the methods were referenced from Larson et al. [18]. In brief, male Dahl S rats were euthanized at the end of the experiment. The brains were removed quickly, and the hypothalamic PVN was punched out using a 1-gauge needle (1.5 mm inner diameter). To identify the PVN tissue, the optic tract was identified, and an approximately 1-mm-thick brain section was taken from the rostral endpoint of the optic tract. Samples were frozen in liquid nitrogen and stored at – 80 °C until used for further molecular analysis.

**Hematoxylin–Eosin and Masson Staining of the Renal Structure**

The kidney was fixed in 4% paraformaldehyde solution and then underwent paraffin embedding. An 8-µm tissue specimen was taken from a transverse kidney section for hematoxylin–eosin (HE) and Masson staining in strict accordance with the experimental steps. A Leica image processing and analysis system was used for image acquisition. The images were qualitatively analyzed to determine structural changes in the kidneys.

**Immunohistochemical and Immunofluorescent Studies of PVN**

The rats were anesthetized with sodium pentobarbital and transcardially perfused with saline, followed by 4% paraformaldehyde solution. The brains were removed and immersed immediately in 4% paraformaldehyde for 24 h and subsequently transferred to 30% sucrose solution until the brain was deposited in the bottom of the bottle. We collected 20-µm coronal sections containing the PVN. ROS in the PVN were detected using fluorescent-labeled dihydroethidium staining and visualized using a confocal laser-scanning microscope (Leica, Wetzlar, Germany). Fluorescence intensity for DHE in the PVN was analyzed and quantified using the Image J software (NIH, Bethesda, USA). For the immunofluorescence staining of PICs, including TNF-α, IL-6, and IL-1β, in the PVN, brain coronal sections (20 µm) containing the PVN were first washed in phosphate-buffered saline (PBS) three times for 10 min each. Sections were incubated with 5% horse serum in PBS for 1 h, and then incubated with mouse anti-TNF-α antibodies (1:100 dilution, Santa Cruz Biotechnology, Dallas TX, USA), mouse anti-IL-6 antibodies (1:100 dilution, Abcam), or mouse anti-IL-1β antibodies (1:100 dilution, Santa Cruz Biotechnology) in PBS containing 0.5% Triton X-100 and 5% horse serum for 72 h at 4 °C. Subsequently, sections were washed with PBS three times for 10 min each, followed by incubation with a secondary antibody: Alexa Fluor® 488 donkey anti-mouse immunoglobulin G (IgG; 1:1000) or Alexa Fluor® 594 donkey anti-mouse IgG (1:1000) overnight at 4 °C. The sections were mounted on microslides, and images were acquired using a Leica microscope and quantified using the Image J software. The method of quantification of immunoreactivity of PICs within the PVN is described in a previous study [16].

**The ELISA Measurement of Glutathione Peroxidase, Total Antioxidant Capacities, Superoxide Dismutase, and Malondialdehyde in the PVN**

The activity of glutathione peroxidase (GSH-Px), total antioxidant capacities (T-AOC), superoxide dismutase (SOD), and malondialdehyde (MDA) in the PVN was measured using commercial ELISA kits (Nanjing Jiancheng, Nanjing China) according to manufacturer instructions.

**Western-Blotting Measurement of the NF-κB Pathway- and Apoptosis-Associated Proteins in the PVN**

The PVN tissues were collected quickly, submerged in liquid nitrogen, and stored at – 80 °C. The total protein was extracted from the PVN tissues, and western-blotting was performed to detect the target proteins. The volume of protein was loaded according to the results from the preliminary experiment of protein detection following the western-blotting procedure after protein quantification with bicinchoninic acid (BCA) protein assay kit. The sample loading volume of the protein involved was 80 µg in the NF-κB pathway and 60 µg in the apoptosis pathway. The primary antibodies, including anti-p50, anti-p65, anti-p-p65, anti-Bax, anti-Bcl-2, anti-cleaved-caspase3, and anti-cleaved...
poly ADP-ribose polymerase (PARP) were incubated at 4 °C overnight. Secondary antibodies were incubated at room temperature for 1 h to detect the proteins involved in NF-kB and apoptosis signaling. The final gel concentration chosen in this study was 12% according to the protein molecular weight and the band separation results. The species in which each primary antibody was raised was the rabbit, and the species in which the primary antibody was raised for incubating the reference protein β-actin was the mouse. The results were normalized to the level of β-actin. The reaction was detected with an enhanced chemiluminescence system (Thermo Scientific, MA, USA), and the band was semi-quantified using the Image J software.

**Transferase‐Mediated dUTP Nick End Labeling Staining of the Neurons in the PVN**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was applied to evaluate neuronal apoptosis using a cell death detection kit (Servicebio, Wuhan, China) following manufacturer instructions. Briefly, 8-μm brain slices containing PVN were fixed on slides for 30 min with 4% paraformaldehyde. They were then permeabilized with 0.1% Triton X-100 and incubated with a 50-μl TUNEL reaction mixture for 60 min at 37 °C in darkness. The slices were then rinsed three times with PBS. After counterstaining with 5 mg/ml 4′,6-diamidino-2-phenylindole for 5 min at room temperature, the brain slices were photographed using a fluorescent microscope (Leica DMIL, German). The percentages of TUNEL-positive cells were calculated using the formula: apoptotic index = (positively stained apoptotic cells)/(total number of cells) × 100%.

**Reagents and Antibodies**

The reagents and antibodies used in the current study are outlined in Table 1. Anti-TNF-α and anti-IL-1β antibodies were purchased from Santa Cruz Biotechnology.

| Reagent | Company | Catalog # | Source | Dilutions for WB | Dilutions for IF |
|---------|---------|-----------|--------|------------------|------------------|
| Primary antibodies | | | | | |
| Anti-TNF-α | Santa Cruz | Sc-52746 | Mouse | / | 1:100 |
| Anti-IL-6 | Abcam | Ab9324 | Mouse | / | 1:100 |
| Anti IL-1β | Santa Cruz | Sc-52012 | Mouse | / | 1:100 |
| Anti-p65 | Absin | Abs131170 | Rabbit | 1:500 | / |
| Phospho-p65 | Absin | Abs130624 | Rabbit | 1:500 | / |
| Anti-p50 | Absin | Abs146717 | Rabbit | 1:500 | / |
| Anti-β-actin | Abcam | Ab8224 | Mouse | 1:10,000 | / |
| Anti-Bax | Absin | Abs130057 | Rabbit | 1:500 | / |
| Anti-Bcl-2 | Absin | Abs131701 | Rabbit | 1:500 | / |
| Anti-cleaved-caspase3 | Absin | Abs132005 | Rabbit | 1:500 | / |
| Anti-cleaved-PARP | Absin | Abs132006 | Rabbit | 1:500 | / |
| Secondary antibodies | | | | | |
| Anti-mouse 488 | Abcam | Ab150159 | Donkey | 1:1000 | / |
| Anti-mouse 594 | Jackson | NC0322938 | Donkey | 1:1000 | / |
| Anti-rabbit IgG (H&L) | Novusbio | NB7160 | Goat | 1:5000 | / |
| Other kits | | | | | |
| BCA (bicinchoninic acid) protein assay kit | Epizyme | ZJ101 | / | / | / |
| Reactive oxygen species (ROS) assay kit | Nanjing Jiancheng | E004-1-1 | / | / | / |
| Total antioxidant capacity assay kit | Nanjing Jiancheng | A015-1-2 | / | / | / |
| Superoxide dismutase (SOD) assay kit | Nanjing Jiancheng | A001-3-2 | / | / | / |
| Glutathione peroxidase (GSH-PX) assay kit | Nanjing Jiancheng | A005-1-2 | / | / | / |
| Malondialdehyde (MDA) assay kit | Nanjing Jiancheng | A003-1-2 | / | / | / |
| Sodium hydrosulfite hydrate (NaHS) | Sigma | 207683-19-0 | / | / | / |
| Tunnel cell apoptosis detection kit | Servicebio | G1501 | / | / | / |
| Rat noradrenaline (NA) ELISA kit | CUSABIO | CSB-E07022r | / | / | / |
| Blood urea nitrogen (BUN) test kit | Rayto | S03036 | / | / | / |
| Rat cystatin C (Cys-C) ELISA kit | CUSABIO | CSB-E08385r | / | / | / |

*WB* western blotting, *IF* immunofluorescence
Anti-IL-6 and anti-β-actin antibodies and Alexa Fluor® 488 donkey anti-mouse IgG were purchased from Abcam (Cambridge, UK). Alexa Fluor® 594 donkey anti-mouse IgG were purchased from Jackson ImmunoResearch Labs (West Grove, USA). Anti-p65, p-p65 (Ser536), anti-p50, anti-Bax, anti-Bcl-2, anti-cleaved-caspase3, and anti-cleaved-PARP antibodies were purchased from Absin (Shanghai, China). Goat anti-rabbit IgG (H&L) secondary antibodies were purchased from Bio-Techne China (St. Louis, MO, USA). The TUNEL cell apoptosis detection kit was purchased from Sigma-Aldrich LLC (St. Louis, MO, USA). The rat noradrenaline (NA) ELISA Kit and Cys-C ELISA kit were purchased from CUSABIO Technology LLC (Houston, USA). The BUN test kit was purchased from Rayto (Shenzhen, China).

Statistical Analysis

All data are presented as means ± standard errors of the mean. For the BP data analysis, a two-way repeated-measures analysis of variance (ANOVA) was used, followed by Tukey’s post hoc tests. For other data analyses, differences between groups were determined using two-tailed Student’s t-tests using the GraphPad Prism 8.0 software (San Diego, CA, USA). A P < 0.05 was considered statistically significant.

Results

Exogenous H2S Delayed BP Increase Induced by an HSD in Dahl S Rats

We first analyzed the BP data of the four groups of rats by performing a two-way repeated-measures ANOVA. Results showed a significant main effect of group (F3,16 = 121.7, P < 0.001) and time (F8,128 = 122.4, P < 0.001), and a significant group × time interaction (F24,128 = 20.4, P < 0.001). Post hoc analysis was conducted for BP across the four groups for each week. Results showed that an HSD significantly increased BP in Dahl S rats from the second week and resulted in hypertension from week 5 in rats with HSD but not in rats with NSD. The intraperitoneal injection of NaHS reduced the BP increase induced by the HSD from the 4th week significantly more in HSD + NaHS rats than in rats in the HSD group. However, intraperitoneal NaHS injection did not affect the BP of rats in the NSD + NaHS group compared with rats in the NSD groups (NSD + NaHS vs. NSD, all P > 0.05 for all weeks; Fig. 1A). As shown in Fig. 1A, the BP elevation in the HSD + NaHS group slowed from week 3, and a significant difference in BP was observed in comparison with that of the HSD group from the week 4. In addition, heart rate was significantly faster in the HSD group rats than that in rats in the NSD group on the week 8 of testing. Intraperitoneal NaHS injection significantly reduced heart rate in the HSD + NaHS rats (Fig. 1B). Exogenous H2S application delayed HSD-induced BP elevation and increased heart rate, although rats could still develop hypertension over an extended period.

Fig. 1 H2S slowed down the blood pressure elevation and reduced the heart rate increase induced by high salt diet in Dahl S rats. A The change in mean arterial pressure (MAP) of rats in 4 groups during the 8-week experiment. B The change of heart rate in 4 groups of rats in the 8th week. Values are expressed as means ± SEM (n=5). **P < .01, ***P < .001 compared with group NSD; #P < .05, ###P < .001 compared with group HSD. NSD normal salt diet, HSD high salt diet, NaHS sodium hydrosulfide, BL baseline.
Exogenous H₂S Alleviated Kidney Damage Induced by HSD in Dahl S Rats

A previous study showed that an HSD could induce renal oxidative stress and kidney damage in Dahl S rats [15]. In this experiment, we measured relative physiological indicators, such as heart weight, kidney weight, BW, water intake, and food intake on completion of the experiment on week 8 (Table 1). We found that an HSD significantly increased heart weight, kidney weight, heart weight/BW, kidney weight/BW, and water intake in rats in the HSD group compared with those in rats in the NSD group. Intraperitoneal H₂S application attenuated the increase in these indicators in HSD + NaHS rats significantly more than that in rats in the HSD group. No difference was observed for heart weight, kidney weight, BW, heart weight/BW, kidney weight/BW, water intake, or food intake between rats in the NSD + NaHS and NSD groups, which indicated that intraperitoneal H₂S application did not affect the above indicators in rats with an NSD. In addition, there were no differences in BW or food intake among the four groups. This suggested that H₂S application did not affect the growth of rats with different diets during the 8 weeks (Table 2).

The concentration of plasma NE is an important indicator that indirectly reflects the activity of the sympathetic nerves. The volume of BUN and Cys-C in the plasma are additional indicators for evaluating renal function. We tested the concentration of plasma BUN and Cys-C at the end of the experiment in all four groups. The levels of plasma NE, BUN, and Cys-C were significantly higher in HSD rats than in the NSD group, and these increases induced by the HSD were significantly inhibited with treatment with NaHS. In addition, there were no differences in the levels of plasma NE, BUN, and Cys-C between the NSD and NSD + NaHS groups (Fig. 2). Given that the increased excitation of the renal sympathetic nerve, as indicated by the higher plasma NE concentration, may damage renal function, the increase in plasma NE, BUN, and Cys-C in HSD rats suggested that HSD caused kidney damage. Exogenous H₂S application alleviated the kidney damage induced by the HSD. These findings were supported by the evaluation of renal structures using the HE and Masson staining method. HE staining

Table 2 Changes in the relative physiological indicators at the end of week 8 of the experiment

| Group              | NSD            | NSD + NaHS     | HSD            | HSD + NaHS     |
|--------------------|----------------|----------------|----------------|----------------|
| Heart weight (g)   | 1.31 ± 0.04    | 1.38 ± 0.11    | 1.64 ± 0.05*** | 1.49 ± 0.04**  |
| Kidney weight (g)  | 2.95 ± 0.21    | 3.26 ± 0.21    | 3.83 ± 0.23*** | 3.48 ± 0.11    |
| Body weight (BW) (g)| 331.60 ± 2.87 | 328.40 ± 26.06 | 325.04 ± 14.02 | 321.20 ± 11.99 |
| Heart weight/BW (%)| 0.38 ± 0.02    | 0.36 ± 0.01    | 0.55 ± 0.04**  | 0.47 ± 0.02    |
| Kidney weight/BW (%)| 0.87 ± 0.07    | 0.88 ± 0.07    | 1.15 ± 0.03*** | 1.08 ± 0.01**  |
| Water intake (g)   | 39.44 ± 4.43   | 34.18 ± 2.97   | 129.96 ± 11.36*** | 111.44 ± 7.36** |
| Food intake (g)    | 22.80 ± 2.32   | 25.06 ± 0.65   | 24.16 ± 1.07   | 23.54 ± 0.74   |

BW body weight, NSD normal salt diet, HSD high salt diet, NaHS sodium hydrosulfide hydrate

**P < .01, ***P < .001 compared with the NSD group

#P < .05, ##P < .01 compared with the HSD group

Fig. 2 Effects of NaHS intraperitoneal injection on the level of plasma NE, BUN and Cys-C at the end of the experiment in Dahl S rats. A The changes in plasma NE level (an indirect indicator of sympathetic activity) in 4 groups of rats. B The changes in plasma BUN level (an indicator of kidney injury) in 4 groups of rats. C The changes in plasma Cys-C level (an indicator of kidney injury) in 4 groups of rats. Values are expressed as means±SEM (n=3). **P < .01, *P < .05, ##P < .01. NE norepinephrine, BUN blood urea nitrogen, Cys-C cystatin C
showed that the glomerular structure was more disorganized, and the borders of the glomeruli were more obscure in the rats in the HSD group compared with those of NSD rats. This renal structure disorder was reversed in rats in the HS + NaHS group, and the borders of the glomeruli became visible again. Masson staining also showed greater relief from renal fibrosis in the rats in the HSD + NaHS group compared with that in the rats in the HSD group (Fig. 3).

H$_2$S Reduced Oxidative Stress and Enhanced Anti-oxidative Capacity in the PVN in Dahl S Rats

Changes in ROS in PVN tissues were first detected by DHE immunofluorescence staining (Fig. 4A). Results showed that exogenous H$_2$S significantly reduced the increase in ROS in the PVN induced by an HSD in Dahl S rats (Fig. 4B). MDA is used as an indicator of lipid peroxidation of membranes. The MDA level in the PVN tissue was increased significantly more in HSD rats than in rats in the NSD group. NaHS treatment inhibited the MDA increase caused by the HSD in the rats in the HSD + NaHS group (Fig. 4C). SOD and GSH-Px are also key anti-oxidases. The levels of enzyme-driven and non-enzymatic antioxidants, such as T-AOC, SOD, and GSH-Px, in the PVN were decreased in rats in rats with HSD, but not in rats with NSD. NaHS treatment improved these antioxidants in PVN tissue in HSD + NaHS rats (Fig. 4D–F). These findings suggest that H$_2$S enhances the antioxidant capacity of the PVN tissue of Dahl S rats with a long-term HSD.

H$_2$S Relieved the Inflammatory Response Induced by an HSD in the PVN of Dahl S Rats

A previous study showed that an HSD induces an inflammatory response in the PVN of Dahl S rats [16]. In this study, we first tested whether exogenous H$_2$S application ameliorates the inflammatory response induced by the HSD in Dahl S rats. The NF-κB signal pathway in the PVN plays an important role in developing HSD-induced hypertension [19]. Thus, we evaluated the expression level of NF-κB pathway-related proteins in the PVN using western-blotting analysis. Results showed that the expressions of p50, p65, and p-p65 were significantly upregulated in the HSD group. Exogenous H$_2$S treatment significantly attenuated the increase in protein expressions of p50, p65, and p-p65 induced by the HSD in the PVN (Fig. 5). These findings suggest that the effect of H$_2$S on attenuation of hypertension is due to the inhibition of the activity of the NF-κB signaling pathway in the PVN.
Because the activated NF-κB protein may enter the cell nucleus to promote the production and expression of PICs, we assessed the expressions of TNF-α, IL-6, and L-1β in PVN tissues in the four groups of rats using immunofluorescence staining. We found that exogenous H$_2$S attenuated the increased expressions of TNF-α, IL-6, and L-1β induced by the HSD in PVN tissue of Dahl S rats (Fig. 6). This suggested that H$_2$S reduces the inflammatory response in the PVN caused by a long-term HSD in Dahl S rats and that exogenous H$_2$S plays a protective role in the PVN inflammatory response.

**Exogenous H$_2$S Alleviated Cell Apoptosis Induced by an HSD in Dahl S Rats**

We evaluated the effect of exogenous H$_2$S on cell apoptosis in the PVN. An HSD significantly increased the apoptotic index and the expression of Bax/Bcl-2, cleaved-caspase3, and cleaved-PARP in the PVN of rats, which suggested that an HSD induces cell apoptosis in the PVN. However, exogenous H$_2$S significantly attenuated the expression levels of these apoptotic proteins (Fig. 7), which suggests that exogenous H$_2$S has a protective effect on neurons in the PVN.

**Discussion**

The present study produced three key findings: (i) exogenous H$_2$S application by intraperitoneal injection delayed the HSD-induced BP increase in Dahl S rats by alleviating peripheral tissue damage in the heart and kidneys; (ii) exogenous H$_2$S application attenuated oxidative stress, protein expression of the NF-κB pathway, and production of PICs in the PVN of Dahl S rats; and (iii) exogenous H$_2$S application reduced cell apoptosis of the PVN induced by an HSD in Dahl S rats. During the development of hypertension induced by an HSD in Dahl S rats, the kidneys and PVN are the two main target organs that are significantly impacted. Similar to other bio-gaseous molecules, such as CO and NO, H$_2$S has been considered as a novel molecule that can be used to treat high BP and kidney damage caused by an HFD [13, 20]. Exogenous H$_2$S application plays a protective role
in the development of hypertension by reducing oxidative stress and attenuating the inflammatory response and cell apoptosis, which have an impact on the kidneys and PVN function.

Previous study have shown that exogenous H$_2$S application decreases elevated BP by inhibiting HSD-induced excessive oxidative stress and kidney damage in Dahl S rats [15]. However, it is not known whether this method of H$_2$S application delays the increase in BP and the development of hypertension via a central mechanism by affecting PVN neuronal activity. In this study, we focused on the PVN because it is a key brain nucleus in the hypothalamus for regulating sympathetic outflow, heart rate, BP, food intake, and water intake [3, 16, 21]. PVN neurons integrate signals from circumventricular organs and other cardiovascular-relevant brain regions, convey information to the rostral ventrolateral medulla or directly to the spinal cord, control the sympathetic activities, and finally regulate the function of the heart and kidneys. It should be noted that PVN neuronal activity can also be modulated by multiple factors, such as angiotensin II, glutamate, PICs, and other gas signal molecules, including NO, CO, and H$_2$S. Numerous studies have shown that PVN plays an important role in the development of salt-sensitive hypertension. Liang et al. investigated the effects of direct microinjection of GYY4137, a donor of H$_2$S, into the PVN on BP changes in Dahl S rats on an HSD. They found that the application of H$_2$S into the PVN attenuated BP elevation by regulating oxidative stress and the inflammatory response [17]. We have further answered the above question, whereby exogenous intraperitoneal H$_2$S application delays hypertension development by protecting peripheral organs, such as the kidneys, and the central brain tissue of the PVN, and by reducing oxidative stress, expression of the NF-$\kappa$B pathway, PIC activity, and neuronal apoptosis.

This study confirmed findings of the protective role of H$_2$S for heart and kidney damage in Dahl S rats and other animals. We found that exogenous H$_2$S application reduced the increase in heart weight, heart weight/BW, and heart rate caused by an HSD. This findings are consistent with

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**Fig. 5** Effects of NaHS intraperitoneal injection on NF-$\kappa$B signaling in PVN of Dahl S rats. A The representative immunoblots of P-P65, P65 and P50 in the PVN. It shows that NaHS intraperitoneal injection reduced the increase in the protein expression of P-P65 (B), P65 (C), P-P65/P65 (D) and P50 (E) induced by HSD in the PVN of the Dahl S rats. Values are expressed as means ± SEM ($n=3$). *$P<.05$, **$P<.01$; $\#$$P<.05$.
findings that exogenous H$_2$S application inhibits myocardial hypertrophy in HSD-induced Dahl S rats [15]. The kidneys were also damaged in rats with HSD, with increased renal fibrosis as reported in this and other studies [22, 23]. An HSD-induced glomeruli structure disorder and renal fibrosis and caused disordered renal function as evidenced by the increase in plasma NE, BUN, and Cys-C. The level of NE in the plasma is an indirect indicator of sympathetic nerve activity, and previous findings have shown that an HSD increases sympathetic nervous activity, as measured by circulating NE [17, 24]. H$_2$S treatment reversed the increase in these indicators. Therefore, these findings strengthen previous reports of the protective role of H$_2$S against HSD-induced renal damage [14, 25–28].

To date, the mechanisms underlying the protective role of H$_2$S against HSD-induced hypertension have remained unclear. H$_2$S has a powerful antioxidant effect, mainly owing to its ability to directly remove ROS and downregulate enzymes that produce ROS [29]. It is primarily used as an antioxidant and anti-neuritis drug and plays a protective
role in the nervous system by preventing neuronal damage caused by hypoxia [30–32]. In the brain, the PVN is a key area that controls sympathetic outflow, BP, and salt-sensing mechanisms. H₂S in the PVN delays the occurrence of hypertension in response to an HSD and protects cell growth under conditions of oxidative stress [17]. The main characteristics of oxidative stress are an increase in the production of oxidants and impaired antioxidant defense capabilities. Increased oxidative stress in the PVN plays a vital role in regulating heart function and maintaining sympathetic nerve activity in spontaneously hypertensive rats [33]. H₂S inhibits oxidative stress and inflammation caused by HSD in the PVN. It shows that NaHS intraperitoneal injection attenuated the increase in the protein expression of Bax/Bcl-2 (D), cleaved-caspase3 (E) and cleaved PARP (F) in the PVN of the Dahl S rats. Values are expressed as means±SEM (n=3). *P < .05, **P < .01, ***P < .001; #P < .05, ##P < .01.
by hypertension. Moreover, it improves endothelial function and reduces hypertension [34]. In our study, we found that exogenous H$_2$S treatment increased the production of T-AOC, SOD, and GSH-Px and reduced the accumulation level of MDA to protect Dahl S rats.

We also evaluated the expression of NF-κB pathway-associated proteins, including p50, p65, and p-p65 using western-blotting methods. NF-κB is considered a key pro-inflammatory transcription factor that is involved in the expression of various genes, including cytokines [35]. Under normal conditions, NF-κB is sequestered in the cytoplasm via its inhibitory proteins, IκBs [36]. A series of kinases are activated via one or several signaling pathways once cells receive extracellular stimulation. This leads to the phosphorylation or degradation of IκBs, which allows NF-κB to be released from the compound. Then, NF-κB translocates from the cytoplasm into the nucleus and specifically binds to the κB site of associated genes to regulate the transcription of related genes, such as various genes of the cytokines. In this study, we showed that NaHS treatment can significantly inhibit the activation of NF-κB induced by the HSD in the PVN of Dahl S rats. This is consistent with the finding that H$_2$S exerts anti-inflammatory effects by inhibiting NF-κB signaling in high glucose-induced inflammation [37]. Other studies have also confirmed that NF-κB activation promotes the expression of PICs in the central nervous system [19, 38]. Activation of NF-κB is highly dependent on the p50/p65 protein heterodimer, and the level of expression of these proteins (p50, p65, and p-p65) in the signaling pathway is often related to local or systemic inflammation [39]. Our exploration of H$_2$S treatment in rats with a long-term HSD indicated that peripheral application of H$_2$S significantly downregulates the expression of p50, p65, p-p65, and p-p65/p65. H$_2$S may relieve the inflammatory response in brain tissue through the mediating the downregulation of the expression of NF-κB pathway-related proteins in the PVN. As neuro-modulators of the central nervous system, PICs are involved in regulating neuronal activity [16]. NF-κB proteins in the cytoplasm translocate into the nucleus following activation to promote the transcription of PICs genes, such as TNF-α, IL-6, and IL-1β. Thus, it seems logical that H$_2$S reduces protein expression of the NF-κB, which eventually results in the decreased expression of PICs in the PVN. The increased PICs in the PVN regulate sympathetic nerve activity in the Dahl S rats, which ultimately contributes to the development of hypertension [40, 41]. We postulate that the HSD-induced hypertension in Dahl S rats partially by enhancing the activity of the signaling pathway of ROS/NF-κB/PICs in the PVN. The exogenous H$_2$S application then attenuated the activity of this pathway in the PVN neurons, reduced the hyperactivity of the cardiovascular activity-related neurons in the PVN and peripheral sympathetic activity, which ultimately delayed the increase in BP in the Dahl S rats on an HSD. However, these signaling pathways and the role of H$_2$S need to be further investigated, specifically using inhibitors of H$_2$S-generating enzymes, such as cystathionine β-synthase, in central and peripheral tissue.

Apoptosis, or programmed cell death, is a highly regulated mechanism of cell death that plays a critical role in the normal development and maintenance of tissue homeostasis in multicellular organisms [42]. Apoptosis is regulated by apoptosis family proteins, which include pro-apoptotic protein Bax and antiapoptotic proteins Bcl-2, cleaved-caspase3, and cleaved-PARP [43]. Our results indicated that H$_2$S decreases the apoptotic index, Bax/Bcl-2 ratios, and protein expression levels of cleaved-caspase3 and cleaved-PARP in PVN tissues that had increased under an HSD. Treatment with exogenous H$_2$S reversed the damage caused by the HSD and protected Dahl S rats by decreasing the cell apoptotic level in the PVN.

This study confirmed the role of exogenous H$_2$S administered by intraperitoneal injection in alleviating HSD-induced hypertension in Dahl S rats. We showed that H$_2$S alleviates hypertension not only by relieving the injury of peripheral organs, such as kidneys and heart, caused by an HSD but also by reducing ROS/NF-κB/PIC signaling and cell apoptosis in the PVN (Fig. 8). Our findings provide a basis for initiating H$_2$S-based therapy for hypertension associated with

![Fig. 8 A proposed mechanism that exogenous hydrogen sulfide alleviates the hypertension induced by HSD in Dahl S rats. In one side, a long-term high salt diet can damage the peripheral organ such as heart and kidneys of the rats, in another side, it also can induce the oxidative stress, inflammatory response and cell apoptosis in PVN of the Dahl S rats. exogenous hydrogen sulfide released from the NaHS which was injected intraperitoneally can alleviate the hypertension induced by HSD through reducing the changes in the above indicators. Dahl S Dahl Salt sensitive, HSD high salt diet; i.p. intraperitoneal injection, NaHS sodium hydrosulfide, ROS reactive oxygen species, PVN paraventricular nucleus, PICs proinflammatory cytokines.](image-url)
lifestyle-based eating habits, such as high salt intake. As a new gaseous neural mediator, H$_2$S has been known to participate in a variety of physiological and pathophysiological processes in the body. The concentration of H$_2$S or NaHS is a crucial factor that needs to be considered in this study. The relationship between biosafety and the dose of H$_2$S application in the prevention or treatment of various diseases is needed for confirmation in future clinical research. With the accumulating evidence of H$_2$S functions and its target effects in both animal and human studies, we anticipate that H$_2$S will be valuable for the treatment in a variety of diseases because of its significant antioxidant, anti-inflammatory, anti-apoptotic, and neuroprotective properties.

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Author contributions All authors contributed to the study conception and design. Writing the first draft: YL; interpreted results of the experiments: EJ and DW; material preparation, data collection and analysis: YL, YF, QH, YL, and EJ; edited and revised the manuscript: YF, EJ and DW. All authors read and approved the final manuscript. Contribution to the Field: Exogenous H$_2$S application can protect high salt diet-induced renal injury through enhanced antioxidant capacity and inhibiting renal oxidative stress in Dahl rats (see Huang et al. [14]). It also attenuates sympathetic activity and hypertensive response by directly injection of donor of H$_2$S into PVN partly due to decrease of ROS and PICs within PVN in high salt-induced hypertension. Here, we show that exogenous H$_2$S application by intraperitoneal injection of NaHS can ameliorate high salt-induced hypertension through not only protecting the renal and other physiological function, but also delay the BP elevation by inhibiting ROS/NF-κB/PICs signals and cell apoptosis in PVN of hypothalamus. This study provides an evidence and possibility for treatment the high salt-sensitive hypertension from the donor of H$_2$S.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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