Hydrogen Sulfide Improves the Endothelial Dysfunction in Renovascular Hypertensive Rats

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Short title: Hydrogen sulfide and renovascular hypertension
Summary

As a novel gasotransmitter, hydrogen sulfide (H₂S) has vasodilating and antihypertensive effects in cardiovascular system. Thus, we hypothesized that H₂S might have beneficial effects on thoracic endothelial function in two kidney one clip (2K1C) rats, a model of renovascular hypertension. Sodium hydrosulfide (NaHS, 56 μmol/kg/day) was administrated intraperitoneally from the third day after the 2K1C operation. Along with the development of hypertension, the systolic blood pressure (SBP) was measured before the operation and each week thereafter. The oxidative stress was determined by measurement of malondialdehyde (MDA) concentration, superoxide dismutase (SOD) activity and protein expression of oxidative stress-related proteins (AT₁R, NADPH oxidase subunits). Acetylcholine (ACh)-induced vasorelaxation and angiotensin II (Ang II)-induced vasocontraction were performed on isolated thoracic aorta. The SBP was significantly increased from the first week after operation, and was lowered by NaHS. NaHS supplementation ameliorated endothelial dysfunction. The protein expression of oxidative stress-related proteins were downregulated, while SOD activity upregulated. In conclusion, improvement of endothelial function is involved in the antihypertensive mechanism of H₂S. The protective effect of H₂S is attributable to suppression of vascular oxidative stress that involves inhibition of Ang II-AT₁R action, downregulation of oxidases, as well as upregulation of antioxidant enzyme.

Key words: Hydrogen sulfide • Angiotensin II • Endothelial dysfunction • Oxidative stress
**Introduction**

The novel gasotransmitter hydrogen sulfide (H$_2$S) possessed various physiological and pharmacological functions in cardiovascular system (Wang 2002). Nowadays, H$_2$S has been found to be produced by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and newly determined 3-mercaptopuruvate sulphurtransferase (3-MST) in concert with cysteine aminotransferase (CAT) (Tanizawa 2011). In particular, CSE is the main generating enzyme to be present in vascular bed and additionally located in vascular endothelial cells, which contributes to endothelium-dependent relaxation (Zhao et al. 2001). The vasorelaxing effect of H$_2$S has been appeared in various vascular tissues, such as rat aorta and mesenteric arteries (Wang et al. 2009). Moreover, CSE knockdown increased blood pressure (Yang et al. 2008), which might be eliminated by in vivo administration of H$_2$S (Mancardi et al. 2009). In addition, plasma level of H$_2$S was significantly decreased in spontaneously hypertensive rats (SHRs) (Zhao et al. 2008) and renovascular hypertensive rats (Lu et al. 2010). Thus, we hypothesized that there might be a correlation between H$_2$S and vascular function in hypertensive animal model.

Renovascular hypertension caused by partial renal-artery stenosis always leads to persistent high blood pressure and increased angiotensin II (Ang II) activity (Higashi et al. 2002, Takahashi and Smithies 2004). Ang II, acting via the angiotensin II type 1 receptor (AT$_1$R), increases the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which was proposed as a mechanism underlying the enhanced production of reactive oxygen species (ROS), especially superoxide anion (O$_2^-$) in a rat model of renovascular hypertension (Oliveira-Sales et al. 2009). The association between increased production of O$_2^-$ and endothelial dysfunction has been reported in both experimental and human renovascular hypertension(Cai and Harrison 2000, Dijkhorst-Oei et al. 1999, Taddei et al. 1993).

Vasoactive substances released by endothelium play an important role in regulating vascular tone and blood pressure (Bayraktutan 2002). The imbalance between endothelium-derived relaxing and contracting factors leads to endothelial dysfunction, which is related to cardiovascular mortality (Vanhouette et al. 2009). In a large amount of experimental hypertensive models, such as two-kidney, one-clip (2K1C) renovascular, aortic coarctation, Dahl salt-sensitive, deoxycorticosterone acetate-salt and SHRs, the endothelium-dependent vasorelaxation is severely impaired (Choi et al. 2012). H$_2$S has been reported to be produced from vascular endothelial cells and be considered as a newly endothelial-dependent relaxing factor (EDRF) or endothelial-dependent hyperpolarized factor (EDHF), which plays a pivotal role in regulating endothelial function (Wang 2009). However, no studies show that H$_2$S could improve endothelial dysfunction in 2K1C model.

Sodium hydrosulfide (NaHS) is an H$_2$S donor. Although previous studies reported the antihypertensive
effect of this compound (Ahmad et al. 2014, Ford et al. 2013), the effects of NaHS on vascular function in renovascular hypertension remain poorly understood. In this study, we investigated the effect of NaHS on endothelial function in chronic 2K1C hypertensive rats, which is an ideal animal model of renovascular hypertension, with further elucidation of the underlying mechanisms by focusing on vascular oxidative stress.

Methods

Animals

All the rats used in present study were obtained from our local certified animal facility (Animal Research Center of Hebei Medical University, Hebei, China). 7-week old male Sprague-Dawley rats were randomly divided into 3 groups (n=6): Sham, 2K1C, 2K1C+NaHS. The rats were anesthetized with intraperitoneal injections of pentobarbital sodium (30 mg/kg). In the 2K1C and 2K1C+NaHS groups, a lumbar incision was made to provide access to the left renal artery. Cleared of the connective tissues, the left renal artery was clipped by a rigid U-shaped solid silver clip with an open slit of 0.25mm, resulting in partial occlusion of renal perfusion. The contralateral kidney was left untouched. Sham group underwent the same procedure, except for the clip placement. The rats were kept in cages after surgery, maintained on 12h light/dark cycle with free access to normal chow and tap water. 2K1C+NaHS group received NaHS 56 μmol/kg/day intraperitoneally. The treatment was started from the third day after the surgery and maintained for 4 weeks. Sham and 2K1C groups were received saline as vehicle.

Blood pressure measurement

Systolic blood pressure (SBP) was measured non-invasively by tail-cuff plethysmography (Chengdu Instrument Factory, Sichuan, China) in calm, conscious rats. Briefly, SBP was measured before and further at each week after surgery for 4 weeks. SBP measurement was always conducted between 9:00 and 12:00 AM and was calculated as the average value of 3 to 4 successive measurement.

Isometric force studies

The thoracic aorta was dissected, cleaned of connective tissue and cut into ring segments (-3mm length). The segments were then mounted between stainless steel triangles in a 10 ml organ bath filled with oxygenated (95% O₂ and 5% CO₂) Krebs-Henseleit solution (pH 7.3-7.5) (composition in mmol/l: NaCl 119, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11) at 37°C. An optional baseline tone of 2 g was applied to all rings as a resting tension. After a 60 minute-equilibration, arteries were stimulated by 60 mmol/l KCl. After washing out, rings were pre-contracted by phenylephrine (Phe, 1 μmol/l). When the contraction was steady, acetylcholine (ACh, 10⁻⁸~10⁻⁴ mol/l) or sodium nitroprusside (SNP, 10⁻⁹~10⁻⁶ mol/l) were added respectively in a cumulative
manner to record the endothelial-dependent relaxation or endothelial-independent relaxation response curve. Ang II (10^{-10} - 10^{-6} \text{ mol/l}) was added cumulatively to conduct contraction curve. Further, AT_{1}R inhibitor losartan (10 \mu\text{mol/l}) was applied for 30 minutes before the Ang II-induced contraction was performed.

The extent of relaxation was expressed as percentage of relaxation from a submaximal phenylephrine-induced constriction. Vasocontraction was determined in relative values as the percentage of 60 mmol/l KCl contraction.

**Measurement of plasma H_{2}S level**

H_{2}S concentration in plasma was measured as previous described (Siegel 1965). Briefly, plasma (100 \mu l) were mixed with potassium phosphate buffer (pH 7.4, 2.5 ml) and zinc acetate (1% w/v, 500 \mu l), then further incubated with N,N-dimethyl-p-phenylenediamine sulfate (20 mmol/l, 500 \mu l) in 7.2 mol/l HCl and FeCl_{3} (30 mmol/l, 400 \mu l) in 1.2 mol/l HCl. 10% trichloroacetic acid 1ml was added to terminate the reaction after 20-minute incubation. The absorbance of the mixture was measured at 665 nm. H_{2}S concentration was calculated against a calibration curve of NaHS (0.01 to 100 \mu\text{mol/l}). The results of H_{2}S concentration was expressed as \mu\text{mol/l}.

**Measurement of plasma Ang II level**

Ang II level in plasma was examined with an Ang II radioimmunoassay kit (Beijing Chemclin Biotech Co. Ltd, Beijing, China). Briefly, blood samples were collected in tubes containing 20 \mu l EDTA in ice. After centrifugation at 1000×g for 15 minutes at 4\degree\text{C}, plasma (1 ml) were collected and mixed with enzyme inhibitor 1 (10 \mu l), enzyme inhibitor 2 (20 \mu l) following the instruction of the radioimmunoassay kit. Then aliquots (100 \mu l) were incubated with ^{125}\text{I}-\text{AII} (100 \mu l) and AII antibody (100 \mu l) for 15 hours at 2-8\degree\text{C}, followed by addition of separating reagent (including donkey anti-rabbit IgG serum and polyethylene glycol) 500 \mu l and incubated for 15 minutes at room temperature. The supernatant was discarded after centrifugation at 3,500 r/min for 20 minutes. The radioactive count was determined for 60 seconds. The results were expressed as pg/ml.

**Western blot analysis**

Thoracic aorta were homogenized with ice-cold RIPA lysis buffer and centrifuged at 20,000×g for 20 minutes. The supernatant was collected and analyzed for protein concentration using the bicinchoninic acid (BCA) method (Generay biotechnology, Shanghai, China). Aliquots (100 \mu g protein) were suspended in 6×sample buffer with 5% β-mercaptoethanol and denatured at 99\degree\text{C} for 10 minutes. The protein samples were electrophoresed through 10% SDS-polyacrylamide gel and then transferred onto an immobilon-P polyvinylidene difluoride membrane using wet transfer at 100 V for 90 minutes at 4\degree\text{C}. The membrane was blocked with 0.05% Tween-20
Tris-buffered saline (TBST) containing 5% non-fat milk for 60 minutes at room temperature. The blots were then incubated overnight at 4°C with anti-AT1R (1:800, Abcam, Hongkong), anti-Nox2 (1:1000, Abcam, Hongkong), anti-Nox4 (1:1000, proteintech, Chicago, USA), anti-p67phox (1:1000, EPITOMICS, California, USA) and anti-superoxide dismutase (SOD)-1 (1:1000, proteintech, Chicago, USA) antibodies. All washes were performed in TBST. The membranes were then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:2000, proteintech, Chicago, USA) or HRP-conjugated rabbit anti-goat antibody (1:2000, proteintech, Chicago, USA) for 1 hour. Blots were developed with an enhanced chemiluminescence detection system (Sagecreation, Beijing, China). Densitometry was performed using lane-1 system (Sagecreation, Beijing, China).

*Measurement of malondialdehyde (MDA) concentration*

MDA concentration in aorta was measured by using thiobarbituric acid reactive substances (TBARS) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) as previously described (Costa et al. 2009). Thoracic aorta were dissected out and snapped frozen at -80°C, then homogenized on ice and centrifuged at 10,000×g for 5 minutes to collect the supernatant. The supernatant was mixed with 10% trichloroacetic acid 1 ml and 0.67% thiobarbituric acid 1 ml, followed by 30-minute incubation in a boiling water bath. The absorbance of the mixture (532 nm) was measured. The results were expressed as nmol/mg protein.

*Measurement of SOD activity*

A SOD immunoassay kit was used to determine the total SOD activity in thoracic rings (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Thoracic aorta was perfused with PBS to remove any red blood cells. Homogenized aorta on ice and centrifuged tissue lysate at 10,000×g for 5 minutes. The supernatant contains total SOD was then incubated with working solution for 20 minutes at 37°C. Read the absorbance at 450nm using a microplate reader. The results were expressed as U/mg protein.

**Drugs**

NaHS, Ang II, ACh, SNP were obtained from Sigma (Vienna, Austria). Losartan was obtained from ALEXIS (Switzerland). Phe was obtained from TCI (Japan). NaHS was freshly prepared and used to generate H2S in solution according to previous paper (Qu et al. 2006).

**Statistical Analysis**

Data were expressed as the mean ± SEM. The results were evaluated either by t test or 2-way ANOVA using Graphpad Prism. p<0.05 was considered significant. The dose response curves were analyzed by 2-way ANOVA, followed by vertical contrast with Bonferroni adjustment.

**Results**
Effect of NaHS on plasma H\textsubscript{2}S, Ang II level and hypertension in 2K1C rats

The plasma H\textsubscript{2}S level in 2K1C rats (26.8±4.8 μmol/l) was significantly lower than that in sham rats (50.6±6.9 μmol/l, p<0.05). 4 weeks of NaHS treatment elevated the plasma H\textsubscript{2}S level to 56.4±9.2 μmol/l (p<0.05, Fig. 1A). However, Compared with sham rats, the plasma Ang II level was significantly higher in 2K1C rats (140.2±4.7 pg/ml vs. 101.1±1.9 pg/ml, p<0.01). Treatment with NaHS for 4 weeks lowered the Ang II level to 111.5±3.4 pg/ml (Fig. 1B).

SBP was measured before and weekly after 2K1C surgery. No differences were observed in SBP among the groups before surgery. At the end of the first week, the SBP in 2K1C rats was increased from 105.0±1.2 mmHg to 170.0±4.0 mmHg, and remained elevated throughout the 4-week study period. Sham operation did not affect SBP. Intraperitoneally administrated with NaHS 56 μmol/kg/day from the third day after surgery reduced the elevation of SBP (Fig. 1C) at the second week after surgery and remained lower than that of the non-treated 2K1C rats during the rest of the study period.

Effect of NaHS on Ang II-induced vasocontraction

The Ang II-induced vasocontraction was enhanced in the thoracic aorta of 2K1C rats. Such enhancement of vasocontraction was suppressed by the 4-week treatment of NaHS (Fig. 2A,B). In all the three groups including sham and 2K1C without or with NaHS treatment, the vasocontraction induced by Ang II was almost abolished by a 30-minute preincubation of the aorta with the losartan (10 μmol/l) (Fig. 2C).

Effect of NaHS on endothelium-dependent and-independent vasorelaxation

ACh-induced vasorelaxation was significantly reduced in Phe-precontracted aorta in 2K1C rats (E\textsubscript{max} 50.8±3.7% vs. 100.2±0.3% in sham rats, p<0.05). Treatment with NaHS for 4 weeks improved ACh-induced endothelium-dependent relaxation (64.8±9.4%, Fig. 3A,B). By contrast, the SNP-induced endothelium-independent relaxation showed no differences among sham, 2K1C, and NaHS-treated 2K1C rats. (Fig. 3C,D).

Effect of NaHS on oxidative stress-related proteins

Western blot analysis showed that protein level of AT\textsubscript{1}R, NADPH oxidase isoform p67\textsuperscript{phox} and subunit Nox2, Nox4 was elevated in 2K1C rat thoracic aorta. The over-expression of AT\textsubscript{1}R, p67\textsuperscript{phox}, Nox2 and Nox4 were reversed by chronic treatment with NaHS. (Fig. 4)

Effect of NaHS on MDA level

The MDA level in the thoracic aorta of 2K1C rats was significantly elevated (671.6±94.6 vs. 339.9±77.6 nmol/gprot in sham rats, p<0.05). Treatment with NaHS for 4 weeks normalized the tissue level of MDA (356.9±42.9 nmol/gprot) (Fig. 5).
Effect of NaHS on SOD

The tissue SOD activity in rat thoracic aorta was markedly decreased, whereas NaHS chronic treatment reversed the decreased SOD activity in 2K1C rats (Fig. 6A). The downregulated protein expression of SOD-1 was normalized by NaHS supplementation as well (Fig. 6B).

Discussion

The major findings of this study include: (1) NaHS reduces SBP in 2K1C hypertensive rats; (2) NaHS improves the endothelium-dependent relaxation and suppresses Ang II-induced contraction; (3) This study is the first time to show that H$_2$S downregulates AT$_1$R protein expression and oxidative stress in 2K1C hypertension. Taken together, the results suggest that H$_2$S protect endothelial function by decreasing oxidative stress in 2K1C rats. Figure 7 is a signal transduction diagram depicting the possible mechanisms and assumed pathways.

Previous studies have demonstrated that endothelial function is impaired in 2K1C hypertensive rats (Callera et al. 2000) and NaHS exhibits anti-hypertensive effect in this animal model (Lu et al. 2010), however, the effect of NaHS on endothelial function in renovascular hypertension remains poorly studied. In this study, we demonstrated that NaHS treatment lowered blood pressure in 2K1C hypertensive rats, which is consistent to previous reports. Improvement of endothelium-dependent relaxation is a mechanism underlying the antihypertensive effect of NaHS. Suppression of vascular responsiveness to Ang II is also involved, evidenced by the attenuated contractile response of aorta to Ang II in rats treated with NaHS that is attributable to the lowered plasma level of Ang II and the downregulation of AT$_1$R in vascular cells.

The association between blood pressure elevation and vascular oxidative stress has been extensively reported in the development of hypertension, including essential, renovascular or malignant hypertension (Schulz et al. 2011). As a major source of ROS in vascular cells, NADPH oxidase can be activated by Ang II through acting on AT$_1$R (Drummond et al. 2011). Our results showed that NaHS treatment lowered plasma level of Ang II and downregulated aortic AT$_1$R protein expression in 2K1C hypertensive rats. As a consequence, the inhibition of Ang II-AT$_1$R action likely contributes to the inhibition of NADPH oxidases and the reduction of ROS production.

Nox proteins represent the catalytic subunits of NADPH oxidase. In various animal models of hypertension including Ang II infusion, deoxycorticosterone acetate-salt, and renovascular hypertension as well as SHRs, increased activity of Nox has been observed (Schulz et al. 2011). Ang II was reported to increase expression of several NADPH oxidase subunits, including Nox1 and Nox4 (Seshiah et al. 2002). In this study, we determined the expression of Nox2 and Nox4, the most important Nox isoforms in vascular cells (Brown and Griendling 2009), and found that in 2K1C rats, protein expressions of Nox2 and Nox4 were significantly increased in the
NaHS is commonly used to deliver H$_2$S and increases H$_2$S concentrations rapidly (Li et al. 2008). NaHS incompletely dissociates into H$_2$S in Krebs solution, resulting in about 15-18.5% being present as H$_2$S, with 81.5-85% existing as hydrosulphide, HS$^-$ and a trace of sulphide anion, S$^{2-}$ (Dombkowski et al. 2004). Which form of H$_2$S (H$_2$S, HS$^-$, or S$^{2-}$) is physiologically active, or whether all three forms are active to varying extents are not known, however the active component is commonly termed as the chemical symbol “H$_2$S” (Al-Magableh et al. 2014). The physiological concentration of H$_2$S is difficult to measure and the measurement of H$_2$S not always gives the consistent values, varying from the previous micromolar range to the recent nanomolar range. Current literature reports several H$_2$S measurement methods, such as spectrophotometry, chromatography, ion-selective electrode and nanoparticles. The methylene blue method used in present study is the most common assay for detecting H$_2$S. This method revealed that human and rat serum contains 50-100 μM H$_2$S, which is in consistent with our results. However, this technique detects not only free H$_2$S but also other species such as HS$^-$ and S$^{2-}$, the true plasma concentration of H$_2$S is probably to be lower than tens of micromolar range. Although the methylene method has limitation, the present result is able to indicate that NaHS treatment increased H$_2$S concentration in 2K1C rats.

Previous studies showed that NaHS > 50-100 μM is required to relax blood vessels in organ bath, 10-50 μmol/kg are needed to bring about significant falls in blood pressure. NaHS (56 μmol/kg/day, i.p.) treatment for 4 to 5 weeks decreased blood pressure and oxidative stress, inhibited atherogenesis and reduced atherosclerotic lesion size (Chen et al. 2011, Wang et al. 2009, Yan et al. 2004). Although lower doses of NaHS treatment (e.g. 10 or 30 μmol/kg/day, i.p.) were also effective in reducing blood pressure, a much longer treatment period (3 month) would be needed. Also, treatment with different doses of NaHS (10, 30 and 90 μmol/kg/day) in SHRs showed no obvious dose-related difference in blood pressure reduction (Shi et al. 2007). The present study showed the beneficial effect of NaHS in lowering blood pressure, improving endothelial function, reducing vascular superoxide production were all observed at a dose of 56 μmol/kg/day. However, the present study
demonstrates limitation that we used a fixed dose of NaHS without performing a dose-dependent study, thus may miss the potentially toxic or beneficial effects of NaHS.

In summary, improvement of endothelial function is involved in the antihypertensive mechanism of H$_2$S. The protective effect of H$_2$S is attributable to, at least in part, suppression of vascular oxidative stress that involves inhibition of Ang II-AT$_1$R action, downregulation of NADPH oxidases, as well as upregulation of antioxidant enzyme SOD. H$_2$S may have therapeutic potential for renovascular hypertension.

**Conflict of Interest**

There is no conflict of interest.

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**Abbreviations:**

- ACh: acetylcholine; Ang II: angiotensin II; AT$_1$R: angiotensin II type 1 receptor; BCA: bicinchoninic acid; CBS: cystathionine β-synthase; CSE: cystathionine γ-lyase; CAT: cysteine aminotransferase; EDHF: endothelial-dependent hyperpolarized factor; EDRF: endothelial-dependent relaxing factor; HRP: horseradish peroxidase; H$_2$S: hydrogen sulfide; MDA: malondialdehyde; 3-MST: 3-mercaptoppyruvate sulphurtransferase; NADPH: nicotinamide adenine dinucleotide phosphate; Phe: phenylephrine; ROS: reactive oxygen species; NaHS: sodium hydrosulfide; SNP: sodium nitroprusside; SHRs: spontaneously hypertensive rats; O$_2^-$: superoxide anion; SOD: superoxide dismutase; SBP: systolic blood pressure; TBST: tris-buffered saline; 2K1C: two-kidney, one-clip
References


Figure Legends

**Fig. 1** Administration of NaHS for 4 weeks increased the plasma H$_2$S level (A), reduced the plasma Ang II level (B), and attenuated the development of hypertension (C) in 2K1C rats. Results are expressed as mean±SEM. n=6, *p<0.05, **p<0.01 vs. Sham; #p<0.01 vs. 2K1C.

**Fig. 2** The Ang II-induced contraction was attenuated by NaHS chronic treatment. NaHS chronic treatment ameliorated the contraction to Ang II (A,B). Pretreatment with losartan (10 μmol/l) for 30 minutes almost eliminated the Ang II-induced vasoconstriction in rat thoracic aorta (C). Results are expressed as mean±SEM. n=6, *p<0.05, **p<0.01 vs. Sham; ##p<0.01 vs. 2K1C.

**Fig. 3** Effect of NaHS chronic treatment on endothelium-dependent and endothelium-independent relaxation. NaHS 4-week administration enhanced ACh-induced vasorelaxation in 2K1C rats (A,B). No differences were detected in SNP-induced endothelium-independent relaxation (C,D). Results are expressed as mean±SEM. n=6, **p<0.01 vs. Sham; #p<0.05 vs. 2K1C.

**Fig. 4** NaHS chronic treatment reduces the exaggerated protein expression of AT$_1$R (A), p67phox (B), Nox2 (C) and Nox4 (D) in thoracic aorta of 2K1C rats. Results are expressed as mean±SEM. n=4 or 5, *p<0.05 vs. Sham; #p<0.05 vs. 2K1C.

**Fig. 5** The MDA level in thoracic aorta was reduced by NaHS 4-week treatment. Results are expressed as mean±SEM. n=6, **p<0.01 vs. Sham; ##p<0.01 vs. 2K1C.

**Fig. 6** Treatment with NaHS for 4 weeks remarkably enhanced the tissue activity of SOD (A) and protein expression (B) of SOD-1. Results are expressed as mean±SEM. n=4 or 5, *p<0.05, **p<0.01 vs. Sham; #p<0.05, ##p<0.01 vs. 2K1C.

**Fig. 7** Schematic diagram of mechanisms underlying the effect of NaHS against endothelial dysfunction in 2K1C rats. NaHS prevents endothelial dysfunction in thoracic aorta by inhibition of Ang II-AT$_1$R action and downregulation of NADPH oxidases, as well as upregulation of SOD.
**Fig. 1**

A. Bar graph showing H$_2$S levels (μmol/l) with comparison of Sham, 2K1C, and 2K1C+NaHS conditions. 

B. Bar graph showing Ang II levels (pg/ml) with comparison of Sham, 2K1C, and 2K1C+NaHS conditions. 

C. Line graph showing systolic blood pressure (mmHg) over weeks with comparison of Sham, 2K1C, and 2K1C+NaHS conditions.
Fig. 2

A

Sham  2K1C  2K1C+NaHS

Ang II
$10^{-10}$-$10^{-6}$ mol/l

200s

B

C

% Contraction (% of M KCl)

Sham  2K1C  2K1C+NaHS

Ang II -LogM

% Contraction (% of M KCl)

Sham+losartan  2K1C+losartan  2K1C+NaHS+losartan

Ang II -LogM
Fig. 3

A

Sham

2K1C

2K1C+NaHS

1g

200s

ACh

10^{-8} \text{ to } 10^{-4} \text{ mol/l}

B

% Relaxation

ACh - LogM

-8

-7

-6

-5

-4

Sham

2K1C

2K1C+NaHS

**

# #

C

Phe

10^{-9} \text{ to } 10^{-6} \text{ mol/l}

D

SNP

% Relaxation

SNP - LogM

-9.0

-8.5

-8.0

-7.5

-7.0

-6.5

-6.0

Sham

2K1C

2K1C+NaHS

1g

200s

Phe

SNP

10^{-9} \text{ to } 10^{-6} \text{ mol/l}
Fig. 4

A

AT1R

GAPDH

B

p67phox

GAPDH

C

0.0

0.5

0.0

0.5

1.0

1.5

2.0

2.5

0.0

0.5

1.0

1.5

2.0

2.5

* #

D

Nox2

GAPDH

Nox4

GAPDH

* #

AT1R/GAPDH

p67phox/GAPDH

Nox2/GAPDH

Nox4/GAPDH
Fig. 5

![Graph showing MDA (nmol/gprot) levels for Sham, 2KIC, 2KIC+NadB treatments.](image)
Fig. 6

Panel A: Bar graph showing SOD (U/mgprot) levels in different groups: Sham, 2KIC, and 2KIC+NHS. Significant differences are indicated by ** and #.

Panel B: Western blot analysis of SOD-1 and GAPDH levels. The ratio of SOD-1 to GAPDH is quantified in the same groups as in Panel A, with significant differences indicated by * and ###.
Fig. 7

2K1C \[\rightarrow\] NaHS
\[\rightarrow\]

\[\bigcirc\]

Ang II \[\downarrow\]
+

AT\(_1\)R \[\downarrow\]

MDA \[\downarrow\]  SOD \[\uparrow\]  NADPH oxidase

\{ 
Nox2 \[\downarrow\]
Nox4 \[\downarrow\]
p67phox \[\downarrow\]
\}

\[\rightarrow\]

Endothelial dysfunction

vasorelaxation \[\uparrow\]  vasocontraction \[\downarrow\]