Hydrogen Sulfide Reduces Renal Ischemia-Reperfusion Injury by Enhancing Autophagy and Reducing Oxidative Stress

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Abstract

Background

Renal ischemia-reperfusion injury (IRI) is a major cause of acute kidney injury. Hydrogen sulfide (H2S) exerts a protective effect in renal IRI. The present study was carried out to investigate the effects of exogenous H2S on renal IRI by regulating autophagy in mice.

Methods

Mice were randomly assigned to control, IRI, and NaHS (28, 56 and 100 µmol/kg) groups. Renal IRI was induced by clamping the bilateral renal pedicles for with non-traumatic arterial clamp for 45 min and then reperfused for 24 h. Mice were administered intraperitoneally with NaHS 20 min prior to renal ischemia. Sham group mice underwent the same procedures without clamping. Serum and kidney tissues were harvested 24 h after reperfusion for functional, histological, oxidative stress, and autophagic determination.

Results

Compared with the control group, the concentrations of serum creatinine (Scr), blood urea nitrogen (BUN), and malondialdehyde (MDA), the protein levels of LC3II/I, Beclin-1, and P62, as well as the number of autophagosomes were significantly increased, but the activity of superoxide dismutase (SOD) was decreased after renal IRI. NaHS pretreatment dramatically attenuated renal IRI-induced renal dysfunction, histological changes, MDA concentration, and p62 expression in a dose-dependent manner. However, NaHS increased the SOD activity and the protein levels of LC3II/I and Beclin-1.

Conclusions

These results indicate that exogenous H2S protects the kidney from IRI through enhancement of autophagy and reduction of oxidative stress. Novel H2S donors could be developed in the treatment of renal IRI.

Background

Ischemia-reperfusion (IR) is a recognized pathophysiological condition characterized by first blocking the blood supply and then restoring blood supply after surgery [1]. Renal IR injury (IRI) is a common pathophysiological process in clinical practice and one of the main mechanisms leading to acute kidney injury (AKI) [2]. Many clinical conditions can lead to renal IRI, including hemorrhagic shock, kidney transplantation, and nephrectomy [2–4]. The pathogenesis of renal IRI involves inflammation, oxidative...
stress, and apoptosis [5]. Studies have shown that autophagy is induced in proximal tubular cells and kidney during AKI [6]. Renal IRI can be developed through shock, partial nephrectomy, and kidney transplantation. IRI is a dynamic process that involves a variety of pathophysiological processes, such as apoptosis, necrosis, autophagy, free radicles, calcium overload, accumulation of reactive oxygen species, energy metabolism disorders, and endothelial dysfunction [1, 7, 8, 9]. IRI has been considered one of the most important causes of clinical AKI [10]. Therefore, the prevention and treatment of renal IRI is indeed an urgent issue to be solved.

Hydrogen sulfide (H$_2$S) is once considered to be a toxic gas that can damage the nervous system and respiratory system [11]. However, in recent years, H$_2$S has been recognized as the third endogenous gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO) [12, 13]. In mammalian tissues, H$_2$S can be endogenously produced from L-cysteine (L-Cys) and catalyzed by three major enzymes: cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST). CSE and CBS are two pyridoxal-5′-phosphate (PLP)-dependent enzymes, while 3-MST is a PLP-independent enzyme [14]. CBS, CSE, and 3-MST play different roles in producing H$_2$S in the kidney [15]. Previous studies have shown that H$_2$S has a protective effect on kidney IRI [16, 17]. However, the detailed mechanism has not been fully illuminated.

Autophagy is another mechanism of programmed cell death, also known as type II programmed cell death [18]. It is a process in which carbohydrates, proteins, and other molecules in the cytoplasm or damaged organelles are encapsulated into vesicles, which then fuse with lysosomes to form autolysosomes under the regulation of autophagy-related genes. Damaged organelles and macromolecules are degraded by the lysosomal enzymes, providing nutrients for cell proliferation and regeneration, and maintaining energy homeostasis [19]. Autophagy is a conservative pathophysiological process that not only meets the requirements of cellular metabolism by generating materials and energy, but also eliminates some mismatched molecules and renews some of the organelles [20]. Recent studies have reported that H$_2$S can protect the liver and heart during IR by inhibiting autophagy [20–22]. H$_2$S also protects the kidney against IRI, but it remains unclear whether autophagy is involved in the protective process.

In the present study, the kidney IR model on mice was established and the effect and mechanism of H$_2$S on protecting against renal IRI were further investigated.

**Methods**

**Animals**

Eight-week-old C57BL/6 healthy male mice weighing 20 ± 2 g were purchased from the Animal Research Center of Nanjing University.
All procedures were in accordance with the requirements of the Animal Protection and Research Ethics Committee of Henan University, which also complied with the 2010 National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80 – 23).

Animal experiments were approved by the Committee of Medical Ethics and Welfare for Experimental Animals of Henan University School of Medicine (HUSOM-2019-075) in compliance with the Experimental Animal Regulations formulated by the National Science and Technology Commission, China.

**Kidney IR procedure**

Forty mice were randomly divided into 5 groups (n = 8 for each group): sham, IRI, IRI + 28 µmol/kg NaHS (an H$_2$S donor), IRI + 56 µmol/kg NaHS, and IRI + 100 µmol/kg NaHS. NaHS (dissolved in saline) was intraperitoneally injected 20 min before ischemia. Sham and IRI groups were injected with the same volume of saline. Mice were anesthetized with an intraperitoneal injection of 1.5 % pentobarbital sodium (75 mg/kg). Renal ischemia was induced by clamping the bilateral renal pedicles with non-traumatic arterial clamp for 45 min. Sham group underwent the same procedures without clamping. The mice were then reperfused for 24 h after ischemia, then the blood and kidney were removed. The mice died after tissue extraction under anesthesia.

**Assessment of renal function**

The renal function was assessed by measuring the levels of serum creatinine (Scr), blood urea nitrogen (BUN), and blood cystatin C (Cys-C). The serum was separated by centrifugation (4 °C, 5000 rpm, 5 min) and stored at -80°C. The levels of Scr and BUN in the serum were measured according to the protocols described in the corresponding kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China), as well as the Cys-C level in the serum was measured according to the instructions (Elabscience, Wuhan, Hubei, China). The levels of Scr, BUN, and Cys-C were quantified using a spectrophotometer at 546, 640, and 405 nm, respectively.

**Determination of oxidative stress**

After reperfusion, the kidney was removed and washed in saline solution, and then stored at -80°C. Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were measured by biochemical analysis. The kidney tissue was homogenized in saline and centrifuged at 3000 rpm/min for 10 min at 4°C, and the supernatant was saved. Protein concentration was measured using the BCA protein assay kit. MDA content and SOD activity were determined using economical kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

**Hematoxylin and eosin (HE) staining and immunohistochemistry (IHC)**

After 24 h of reperfusion, the mice were anesthetized with 1.5 % pentobarbital sodium by intraperitoneal injection and then rapidly perfused with saline. The left kidney was removed and fixed in 4 %
paraformaldehyde for 24 h, then the tissue was dehydrated and embedded in paraffin. 6 µm sections were performed in both HE and immunohistochemical staining. For HE staining, the sections were stained with HE using standard methods. For immunohistochemical staining, p62 antibody was used. The sections were then incubated with the corresponding secondary antibody for 30 min at room temperature. Immunoreactivity was visualized with the 3,3’-diaminobenzidine detection kit, and then the sections were counterstained, dehydrated, and mounted at room temperature. The stained cells were examined under a light microscope.

**Transmission electron microscopy (TEM)**

Half of the left kidney from mice was cut into small pieces (1 mm³) in 4 % paraformaldehyde plus 2.5 % glutaraldehyde. The renal medulla and cortical areas were fixed in 2.5 % glutaraldehyde solution overnight in 4°C refrigerator and fixed in osmic acid. The small pieces were then dehydrated and embedded in the epon. Ultrathin sections were cut with an ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained with uranyl acetate and lead citrate. The ultrathin sections were observed with a JEM100CX-II transmission electron microscope (JEOL Ltd., Tokyo, Japan).

**Western blot analysis**

Kidney tissue was homogenized in RIPA lysis buffer with protease inhibitors at 4°C. The homogenate was then centrifuged (12000 rpm, 10 min, 4°C) and the homogenate supernatant was collected and stored at -80°C. Protein concentration was quantified by the Bradford assay. Samples (40 µg total protein) were separated by 15 % and 10 % SDS-PAGE, respectively, and transferred onto polyvinylidene fluoride membranes (Roche, Palo Alto, CA, USA). After blocking with 5 % bovine serum albumin for 1 h at room temperature, membranes were incubated overnight at 4°C with primary antibodies, including LC3 II/I, Beclin-1 and β-actin (Cell Signaling Technology, Boston, MA, USA). β-actin was used as control. After washing for 3 times (10 min/time) with tris-buffered saline containing Tween-20, membranes were incubated with secondary antibody for 1 h at room temperature. The membranes were visualized using the electrochemiluminescence detection kit and scanned by an ultra-microscanner EPSON Perfection V33 (Seiko Epson Co., Nagano, Japan) and analyzed by Image J software (NIH).

**Statistical analysis**

All data were expressed as mean ± standard error of the mean (SEM). The normal distribution between the two groups was compared by t test. The significance of the difference between the groups was determined by one-way ANOVA. *P* < 0.05 was considered statistically significant.

**Results**

**NaHS pretreatment can improve renal pathology caused by renal IRI**
Renal histological changes induced by renal IRI in mice were assessed by HE staining. No apparent change was observed in the control group. There were significant changes in the IRI group compared to the control group, including the swelling and necrosis of renal tubular epithelial cells, necrotic cells detached from the basal membrane, and the disappeared nucleus. Compared with the IRI group, 28, 56, and 100 µmol/kg NaHS reduced the changes of renal tubular epithelial cells (Fig. 1). Morphological results showed that the renal IRI model was successfully established, and exogenous H₂S had a protective effect on renal IRI.

**NaHS pretreatment can mitigate renal dysfunction caused by renal IRI**

Renal IRI induces kidney dysfunction in mice characterized by increased levels of Scr, BUN, and Cys-C. To investigate whether NaHS pretreatment can improve renal dysfunction caused by renal IRI in mice, we examined the levels of Scr, BUN, and CysC in plasma. Compared with the control group, plasma Scr was significantly increased in the IRI group ($P < 0.01$). Compared with the IRI group, pretreatment with 100 µmol/kg NaHS reduced plasma Scr levels ($P < 0.05$) (Fig. 2A). Compared with the control group, plasma BUN levels were notably increased in the IRI group ($P < 0.01$). Pretreatment with 56 µmol/kg NaHS significantly reduced plasma BUN levels ($P < 0.01$). In addition, 100 µmol/kg NaHS reduced plasma BUN levels ($P < 0.05$) (Fig. 2B). Plasma Cys-C levels were dramatically elevated in the IRI group compared with the control group ($P < 0.01$). Pretreatment with 28, 56, and 100 µmol/kg NaHS reduced plasma Cys-C levels ($P < 0.05$) compared with the IRI group (Fig. 2C).

**Effect of NaHS pretreatment on renal oxidative stress markers**

To investigate whether exogenous H₂S had antioxidant effects during renal IRI, we used biochemical methods to detect both SOD activity and MDA content. As shown in Fig. 3A, mice subjected to renal IRI showed a significant decrease in SOD activity compared to the control group ($P < 0.01$). Pretreatment with 28 and 56 µmol/kg NaHS restored the decrease in SOD activity induced by IRI ($P < 0.05$). Pretreatment with 100 µmol/kg NaHS dramatically increased the SOD activity compared with the IRI group ($P < 0.01$). In addition, mice subjected to renal IRI showed a dramatic increase in MDA content compared to the control group ($P < 0.01$). The increase in MDA content by IRI was alleviated by the pretreatment with 28 and 56 µmol/kg NaHS ($P < 0.05$). Pretreatment with 100 µmol/kg NaHS dramatically decreased the MDA content compared with the IRI group ($P < 0.01$) (Fig. 3B).

**Effect of NaHS pretreatment on renal autophagosomes**

After renal IRI, the ultrastructure and autophagosome of the kidney were observed by TEM. Compared with the control group, the ultrastructure of the kidney in IRI group was severely damaged: the mitochondrial ridge and membrane were merged or disappeared, the nucleus was swollen, the
endoplasmic reticulum was expanded, the particles were released, the peripheral space of the nucleus was enlarged, and the autophagosomes (black arrow) were visible. While in the NaHS treatment group, the perinuclear space was slightly expanded, as well as the mitochondrial ridge and membrane were mildly merged. One autophagosome was found in both 56 and 100 µmol/kg NaHS groups (black arrow) (Fig. 4).

**Effect of NaHS pretreatment on the expressions of LC-3, Beclin-1 and p62**

Compared with the control group, kidney IRI increased the expression levels of Beclin-1 and LC-3 II/I, but there was no significant change ($P > 0.05$; Fig. 5A, B). Compared with the control group, kidney IRI increased the expression levels of p62 ($P < 0.01$; Fig. 5C). The expression levels of Beclin-1 and LC-3 II/I were upregulated in 28 µmol/kg NaHS group, but there was no significant change ($P > 0.05$; Fig. 5A, B). For the 56 µmol/kg NaHS group, the expression level of LC-3 II/I was not increased ($P > 0.05$; Fig. 5B), while the expression level of Beclin-1 was significantly increased ($P < 0.05$; Fig. 5A). The expression levels of LC-3 II/I were increased in 100 µmol/kg NaHS group ($P < 0.05$; Fig. 5B). Compared with the IRI group, the expression level of p62 was downregulated in 28 and 56 µmol/kg NaHS groups, but there was no significant change ($P > 0.05$; Fig. 5C). The expression level of Beclin-1 was dramatically increased in 100 µmol/kg NaHS group ($P < 0.05$; Fig. 5A). Furthermore, the expression level of p62 was dramatically decreased in 100 µmol/kg NaHS group ($P < 0.05$; Fig. 5C).

**Effect of NaHS pretreatment on p62 in kidney tissues**

Renal IRI significantly increased the expression level of p62 when compared to the control group. Compared with the IRI group, the expression level of p62 was decreased in 28, 56, and 100 µmol/kg NaHS groups (Fig. 6).

**Discussion**

$\text{H}_2\text{~S}$ has been recognized as the third endogenous gasotransmitter along with NO and CO [23]. $\text{H}_2\text{~S}$ is known to play important roles in many physiological and pathophysiological processes in various organs. It has been found to protect the heart, liver, kidney, lung, and brain from IRI [24–28]. In the present study, the effect and mechanism of 28, 56, and 100 µmol/kg NaHS on renal IRI were investigated. We demonstrated that induction of 45 min renal ischemia by clamping the bilateral renal pedicles with a non-traumatic arterial clamp could lead to AKI. Intraperitoneal injection with 56 and 100 µmol/kg NaHS 20 min before ischemia improved renal function and histological changes.

Kidney is one of the main metabolic organs of the body. AKI induced by renal IR can cause renal damage, which is characterized by elevated levels of BUN and Scr [29]. Recent studies have found that serum Cys-C concentrations are less influenced by external factors, such as age, gender, and circadian rhythm, which are now used as early markers to predict the renal dysfunction caused by cardiovascular diseases [30].
Cys-C has been considered as a key marker of renal function after renal IRI [17, 31, 32]. Therefore, Cys-C was determined in the present study. To investigate whether the administration of NaHS can mitigate renal dysfunction caused by renal IRI, we examined renal function markers in the blood of mice, including Scr, BUN, and Cys-C. The results showed that BUN, Scr, and Cys-C were significantly increased by kidney IRI, indicating that the kidney function of the mice was severely impaired. Renal IRI-induced increases in Scr, BUN, and Cys-C were reduced by administration of 56 and 100 µmol/kg NaHS, demonstrating that H₂S can improve renal function induced by renal IRI. AKI induced by renal IR can cause renal damage, which is characterized by elevated levels of BUN and Scr.

When the kidney is damaged, renal tubules and glomeruli can undergo pathological changes, and studies have shown that AKI can lead to cell necrosis of renal tubular [31, 33]. In the present study, we found that the necrosis of renal tubular epithelial cells was induced by renal IRI. Administration of NaHS was able to attenuate the severity of the morphological changes. The renal ultrastructure in IRI was severely damaged, while NaHS could significantly improve renal ultrastructural damage. These results indicate that exogenous H₂S could mitigate the morphological structural and ultrastructural damage in the kidney after renal IRI. It is well known that oxidative stress can generate oxygen free radicals, which contribute to tissue injury [34]. Oxidative stress is involved in the pathophysiological process of renal IRI [35]. Many studies have shown that H₂S is a powerful antioxidant. It protects rat cardiac myocytes from hypoxia/ischemia-induced oxidative stress damage by eliminating reactive oxygen species and lipid peroxidation production of MDA [36, 37]. H₂S also exerts antioxidant effects by increasing the levels of glutathione and the activity of SOD [38]. Studies have shown that renal IR can lead to the generation of oxygen free radicals, increased MDA content, decreased SOD activity, and ultimately the oxygen free radical scavenging capacity of the kidney is reduced [39]. In this study, we examined the effects of NaHS on MDA content and SOD activity of the kidney in mice. The results showed that NaHS significantly inhibited the increase of MDA content and the decrease of SOD activity induced by renal IRI, suggesting the anti-oxidant effect of exogenous H₂S on renal IRI in mice. It can be concluded that NaHS pretreatment can significantly improve the oxygen free radical clearance ability and the total tissue antioxidant capacity in the kidney with IRI. Our results indicate that exogenous H₂S can increase the activity of antioxidant enzymes in the tissues and organs, clear the excessive oxygen free radicals produced by renal IRI, and reduce the lipid peroxidation damage in renal tissues, thus play a protective role in the process of renal IRI.

Autophagy is an essential process responsible for maintaining the homeostasis and has been observed in eukaryotic organisms [40]. LC3, a mammalian homologue of yeast Atg8, is the microtubule-associated protein and is a prominent marker for late macroautophagy [41]. There are two subtypes of LC3 in cells: LC3-I and LC3-II. LC3-I is a soluble form in the cytosol that produces LC3-II [41]. Beclin-1 is one of the important proteins that regulate the process of autophagy. A complex of Beclin-1 and class III phosphatidylinositol-3 kinase is involved in autophagolysosome formation [42]. p62, an autophagy receptor, acts as a selective substrate for autophagy. p62 can directly interact with LC3 for autophagosome formation [43]. Considering the degradation of p62 is dependent on autophagy, the level
of p62 can increase in response to the inhibition of autophagy. Therefore, the accumulation of p62 has been used as a marker for inhibition of autophagy or defects in autophagic degradation [44]. It has been shown that H₂S protects the liver and heart during IR by inhibiting autophagy [16, 17]. However, it is unclear whether H₂S protects the kidney by inhibiting or promoting autophagy during renal IRI. Our results showed that the expression levels of LC-3II /I and Beclin-1 were increased after renal IRI. The increase in LC-3II /I protein level caused by renal IRI was further upregulated by 100 µmol/kg NaHS. Furthermore, the increase in Beclin-1 protein level caused by renal IRI was upregulated by 56 and 100 µmol/kg NaHS. The expression of p62 in kidney was further detected by IHC. The results showed that the expression of p62 was increased after renal IRI, and was reduced by 28, 56, and 100 µmol/kg NaHS. Our results demonstrated that renal autophagy occurred during renal IRI, and the autophagic levels were increased after pretreatment with exogenous H₂S. These results together indicate that pretreatment with exogenous H₂S can attenuate renal IRI by enhancing autophagy. It is clear that H₂S could protect the kidney against IRI by promoting autophagy.

**Conclusion**

In conclusion, our results indicate that exogenous H₂S preconditioning can protect the kidney from IRI by promoting autophagy and reducing oxidative stress. Novel H₂S donors could be developed in the treatment of renal IRI.

**Abbreviations**

AKI: Acute Kidney Injury; BUN: Blood Urea Nitrogen; CBS: cystathionine-β-synthase; CO: Carbon Monoxide; CSE: cystathionine-γ-lyase; Cys-C: cystatin C; HE: Hematoxylin and Eosin; H₂S: Hydrogen sulfide; IHC: Immunohistochemistry; IRI: Ischemia-Reperfusion Injury; L-Cys L-cysteine; MDA: Malondialdehyde; 3-MST: 3-mercaptopyruvate sulfurtransferase; NIH: Institutes of Health; NO: Nitric Oxide; Scr: SerumCreatinine; SEM: Standard Error of the Mean; SOD: Superoxide Dismutase; TEM: Transmission Electron Microscopy

**Declarations**

**Acknowledgements**

Not applicable.

**Authors' contributions**

HL: conceptualization, experimental methodology and writing. SWW, SSA, BG, TST: experimental, methodology, analysis and interpretation of the data. DDW, and YZL: conceptualization and writing. All the authors read and approved the final version of the manuscript.

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**Availability of data and materials**

Not applicable.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing Interest**

The authors declare that they have no conflicts of interest related to this work.

**References**


**Figures**
Figure 1

The effect of NaHS on renal morphological changes assessed by HE staining. Control group (A, a), IRI group (B, b), IRI + 28 µmol/kg group (C, c), IRI + 56 µmol/kg group (D, d), and IRI + 100 µmol/kg group (E, e). Scale bars: 50 µm in A, B, C, D, and E, as well as 20 µm in a, b, c, d, and e.
Figure 2

The effect of NaHS on serum creatinine (a), BUN (b), and Cys C (c) levels. *P < 0.05: IRI group vs control group; **P < 0.01: IRI group vs control group; # P < 0.05: IRI + NaHS group vs IRI group; ## P < 0.01: IRI + NaHS group vs IRI group.
Figure 3

The effect of NaHS on renal SOD activity (A) and MDA content (B). *P < 0.05: IRI group vs control group, **P < 0.01: IRI group vs control group, # P < 0.05: IRI + NaHS group vs IRI group, ## P < 0.01: IRI + NaHS group vs IRI group.
Figure 4

Observation of autophagosomes in mouse by transmission electron microscopy. Control group (A), IRI group (B), IRI + 28 μmol/kg group (C), IRI + 56 μmol/kg group (D), and IRI + 100 μmol/kg group (E). The black arrows represent the autophagosomes. Scale bars: 667 nm in A, B, C, D, and E.
Figure 5

The effect of NaHS on the expression levels of LC-3 II/I (A), Beclin-1 (B), and p62 (C) in mouse kidney after renal IRI. *P < 0.05: IRI group vs control group, **P < 0.01: IRI group vs control group, # P < 0.05: IRI + NaHS group vs IRI group, ## P < 0.01: IRI + NaHS group vs IRI group.
Figure 6

The effect of NaHS on autophagy-related protein p62 expression in mouse kidney demonstrated by IHC. Control group (A, a), IRI group (B, b), IRI + 28 µmol/kg group (C, c), IRI + 56 µmol/kg group (D, d), and IRI + 100 µmol/kg group (E, e). Scale bars: 50 µm in A, B, C, D, and E, as well as 20 µm in a, b, c, d, and e.

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