Study on the effect of hydrogen sulfide on oxidative stress in renal tubular epithelial cells injured by hypoxia-reoxygenation

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Research article

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Abstract

Background: The present study was designed to determine whether exogenous hydrogen sulfide (H 2 S) inhibits oxidative stress in renal tubular epithelial cells under hypoxia-reoxygenation injury. Material and Methods: NRK-52E cells were divided into 4 group, which were Sham group, HR group, NaHS group and DPI group. Using western blotting to detect the protein expression of NOX2 and NOX4 in the process of hypoxia-reoxygenation. Using real-time quantitative PCR to detect the NOX2 and NOX4 mRNA expression in hypoxia-reoxygenation. The fluorescent probe DCFH-DA was used to detect the content of ROS (reactive oxygen species) in cells. The activity of NADPH oxidase was detected by enzyme-linked immunosorbent assay (ELISA). Results: The protein and mRNA expression of NOX2 and NOX4 were significantly increased in the process of hypoxia-reoxygenation, but decreased with NaHS intervention. ROS content and NADPH oxidase activity were all increased in hypoxia-reoxygenation, but decreased after NaHS intervention. Conclusions: H 2 S can reduce ROS production by inhibiting NOX4 and NOX2 during oxidative stress, and also directly inhibits NADPH oxidase activity.

Introduction

Oxidative stress is an important mechanism of (kidney) ischemia-reperfusion injury [1, 2]. Hydrogen sulfide has a protective effect on renal ischemia-reperfusion injury. Such as, promoting ischemia-reperfusion injury's kidney recovery; promoting the recovery of renal tubular epithelial cells in ischemic injured kidneys. It has been reported that hydrogen sulfide has an inhibitory effect on oxidative stress [3]; however, whether hydrogen sulfide inhibits oxidative stress in renal ischemia-reperfusion injury is unknown. This study was designed to investigate the inhibitory effect of hydrogen sulfide on oxidative stress in renal tubular epithelial cells under hypoxia-reoxygenation injury.

Materials And Methods

Sodium hydrosulfide (NaHS) was purchased from InnoChem Science & Technology(Beijing,China). Diphenyleneiodonium chloride (DPI) was purchased from Sigma-Aldrich (Israel). anti-NADPH oxidase 4 (NOX4), anti-NADPH oxidase 2 (NOX2), and anti-Beta Actin (β-actin) antibodies were purchased from Proteintech Group (Rosemont, IL, USA). Horseradish peroxidase (HRP)-conjugated Affinipure Goat Anti-Rat IgG(H + L) and HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H + L) were purchased from Proteintech Group (Rosemont, IL, USA). Reactive Oxygen Species Assay Kit and Radio Immunoprecipitation Assay (RIPA) Lysis Buffer were purchased from Beyotime Biotechnology (Shanghai, China). The NADPH oxidase activity assay kit was purchased from Ding Biological Technology (Shanghai, China). BCA Protein Assay Kit was purchased from Boster Biological Technology (Wuhan, China). Phenylmethylsulfonyl fluoride (PMSF) and other Western blotting related reagents were purchased from Solarbio Science&Technology (Beijing, China). Rat GAPDH Endogenous Reference Genes Primers was purchased from Sangon Biotech (Shanghai, China). The microRNA was synthesized by Takara Biomedical Technology (Dalian, China). The primers for NOX2, NOX4 and the RNA-related

reagents and reverse transcription kits extracted by Trizol method were purchased from Takara Biomedical Technology (Dalian, China).

Cell culture and Treatments.

The normal rat kidney proximal tubular epithelial cell line, NRK-52E, was purchased from Jennio Biotech (Guangzhou, China).The cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) (Boster, Wuhan, China) containing 10% fetal bovine serum (Tianhang, Zhejiang, China) and were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The cells were randomly divided into 4 groups, which were Sham group, HR group, NaHS group and DPI group. Sham group: cells, cultured in only DMEM/F12, were put in the gaseous environment with 95% air and 5% CO2 at 37˚C for 150 minutes and then were harvested. HR group: cells, cultured in only DMEM/F12, were put in the anoxic gaseous environment with 95% N_2, 1% O_2, 4% CO_2 at 37˚C for 60 minutes; and then placed in the gaseous environment with 95% air, 5% CO_2 at 37˚C for 90 minutes. After that cells were harvested. NaHS group: cells, cultured in DMEM/F12 containing NaHS concentration of 100 μmol/L, were put in the anoxic gaseous environment with 95% N_2, 1% O_2, 4% CO_2 at 37˚C for 60 minutes; and then placed in the gaseous environment with 95% air, 5% CO_2 at 37˚C for 90 minutes. After that cells were harvested. DPI group: cells, cultured in DMEM/F12 containing DPI concentration of 1 μmol/L, were put in the anoxic gaseous environment with 95% N_2, 1% O_2, 4% CO_2 at 37˚C for 60 minutes; and then placed in the gaseous environment with 95% air, 5% CO_2 at 37˚C for 90 minutes. After that cells were harvested.

Western blotting analysis

Cells, being intervened, were lysed with ice-cold RIPA buffer containing 1% protease inhibitor PMSF. The supernatant was gathered after centrifugation and quantified using a BCA Protein Assay Kit. All lysates were diluted with SDS loading buffer and boiled for 5 minutes. Equal amounts of protein (10 μg) were loaded into each lane of a 10% SDS-PAGE gel for electrophoresis (Bio-Rad, Hercules, CA, USA) [4]. Proteins were then transferred onto a PVDF membrane. The membranes were blocked in solution containing 5%non-fat milk powder in Tris-buffered saline containing Tween (TBST, 150 mM NaCl, 20 mM Tris/HCl, pH7.4 and 0.05% Tween20) for 1 h at room temperature and incubated with primary antibodies: NOX2 (1:500), NOX4 (1:500) and β-actin (1:1000) overnight at 4 °C. Membranes were washed with TBST for several times and incubated with HRP-conjugated secondary antibodies (1:5000) for 2 h at room temperature. Membranes were washed with TBST for several times again. The ECL detection kit (Boster, Wuhan, China) was used to detect the signal in the membranes, and images were taken with chemiluminescence apparatus (Bio-rad, Ca, USA). The optical density value of bands was analyzed with Image Lab 6.0 software. Protein levels were normalized to β-actin expression and expressed as fold changes relative to the control group. Repeat three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the cells using RNAiso Plus (Takara, Dalian, China). Total cDNA was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Biotechnology, Dalian, China). Using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China), the RT-PCR was performed with CFX96 Touch Real-Time PCR System (Bio-Rad, CA, USA) according to manufacturer's instructions. Sequences of primers are as follows: NOX2, forward CCTGGAGACCCAGATGCAAGA and reverse CGTGGTGCACAGCAAAGTGA; NOX4, forward ACTGCCTCCATCAAGCCAAGA and reverse GACTTCCAAATGGGCCATCAA; GAPDH, forward TGATTCTACCCACGGCAAGTT and reverse TGATGGGTTTCCCATTGATGA. PCR thermal cycling conditions were as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s [4]. The relative expression of each gene was calculated using $2^{-\Delta\Delta Ct}$ method. Repeat three times.

Detection of Intracellular ROS

Intracellular ROS was detected by means of an oxidation-sensitive fluorescent probe (DCFH-DA) [5]. The operation is strictly according to the instructions of the Reactive Oxygen Species Assay Kit. Cells, seeding in a 6-well plate and after being intervened, were washed twice in phosphate-buffered saline (PBS). Then, they were incubated with 10 μmol/L DCFH-DA at 37ºC for 20 min. Cells were detected and taken photographs by using Leica DMI4000 B automated inverted research microscope. The average optical density of photographs was analyzed with Image-Pro Plus 6.0 software. Repeat three times.

Measurement of NADPH Oxidase Activity

Cells were lysed and the supernatant was gathered and quantified, as mentioned earlier. All lysates were seeded on 24-well plates. The level of NADPH oxidase in the samples was determined by double antibody sandwich method. Operate strictly according to the operating instructions of the NADPH oxidase enzyme-linked immunoassay kit. The standard curve is made by using the standard product in the box. Sample addition: blank hole (blank control hole without sample and enzyme-labeled reagent, other steps of the same operation), standard hole, sample to be tested hole. In the enzyme label coated plate standard accurately add 50 ul sample, sample diluent 40 ul in the sample hole, and then add 10 ul sample to be tested (the sample was eventually diluted 5 times). Add the sample to the bottom of the hole of the enzyme plate, try not to touch the wall of the hole, and gently shake and mix. Incubation: after sealing the plate with membrane, incubate it for 30 minutes at 37℃. Liquid preparation: dilute 30 times concentrated washing solution with distilled water for 30 times and then reserve. Wash: carefully remove the sealing plate film, discard the liquid, shake dry, fill each hole with washing liquid, let stand for 30 seconds, discard, repeat 5 times, pat dry. Enzyme addition: 50 ul enzyme labeled reagent was added to each hole, except for blank holes. Incubation: operations same as before. Wash: operations same as before. Color development: each hole first added color rendering agent A 50 ul, then add color rendering agent B 50 ul, gently shake and mix, 37℃ avoid light color for 15 minutes. Termination: 50 ul termination liquid is added to each hole to terminate the reaction (at this time, blue turns yellow). Measurement: the absorbance (OD value) of each hole was measured sequentially with blank air conditioning zero and 450 nm wavelength. The determination should be done within 15 minutes after the termination liquid is

added. Calculate the sample concentration according to the standard curve, and multiply it by the dilution factor to get the actual concentration of the sample. Repeat three times.

Statistical Analysis

Each experiment was repeated at least three times independently. Statistical analyses were performed by using SPSS 21.0 software. The results are expressed as the mean ± standard error, unless stated otherwise. Statistical analysis was performed via analysis of variance (one-way ANOVA) followed by the LSD-t test for significance. A P-value of less than 0.05 was considered as statistically significant.

Results

NaHS Decreased the Upregulated NOX2 and NOX4 in Hypoxia-reoxygenation.

In the process of hypoxia-reoxygenation, the protein expression of NOX2 and NOX4 was significantly increased in HR group, compared with the Sham group. However, in NaHS group, the protein expression of NOX2 and NOX4 was decreased. The protein expression was almost entirely inhibited in DPI group (Fig. 1).

NaHS Suppressed NOX2 and NOX4 Gene Expression.

The variational trend of NOX2 and NOX4 mRNA is the same as their protein level. Expression of HR group's NOX2 and NOX4 mRNA was increased in hypoxia-reoxygenation. In NaHS group, the expression NOX2 and NOX4 mRNA was decreased (Fig. 2).

ROS and NADPH Oxidase Were Downregulated under Stimulation of NaHS.

ROS is an important product in the process of hypoxia-reoxygenation. The result showed that ROS were markedly increased in HR group but reduced in NaHS group. In DPI group ROS were nearly tally inhibited. (Fig. 3) NOX2 and NOX4 are important components of NADPH oxidase [6, 7]. As illustrated in Figs. 4, NADPH oxidase activity was increased in HR group and also reduced in NaHS group (Fig. 3).

Discussion

AKI is common (8–16% of hospital admissions [8, 9]), serious (fourfold increased hospital mortality [8, 10]), and many aspects of its natural history remain uncertain [11, 12]. Currently, the main reasons cause of AKI we have known include sepsis, major surgery, congestive heart failure, higher age and also comorbidity burden of patients that increase the risk of AKI include CKD, proteinuria, diabetes, obesity,

and the broadening repertoire of medications that either are directly nephrotoxic or may lower the threshold for sustaining AKI [13]. Clinically, about 75% of patients are caused by renal ischemia reperfusion injury (IRI) [14].

Kidney is a highly perfusion organ, which is sensitive to ischemia and ischemia reperfusion injury. Oxidative stress plays an important role in the development of ischemic AKI. Oxidative stress occurs in various harmful stimuli such as ischemia-reperfusion and inflammatory diseases, pathogenic microorganisms produce lipopolysaccharides, and the content of free radicals, reactive oxygen species (ROS) and reactive nitrogen (RNS) in the body increases, and the oxidation degree exceeds the scavenging ability of the body, and tissue damage caused by the imbalance of the oxidation system and antioxidant system [15]. Among them, ROS include superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). RNS include NO, nitrogen dioxide (NO_2) and nitrite peroxide ($ONOO^-$), etc [16]. After oxidative stress in the body, energy metabolism and cell traits will be change, causing lipid peroxidation, protein denaturation, and abnormal gene expression, eventually leading to various diseases and even death in human bodies [17, 18].

The level of oxidative stress is determined by the balance between ROS and antioxidant defense system. Nearly 90% ROS is generated in the mitochondria of human body [19, 20]. Mitochondria produce ROS (mtROS) as byproducts of the ATP generation by the oxidative phosphorylation (oxphos) [20]. ROS are formed when one electron is transferred to molecular oxygen, forming the superoxide anion (O_2^-) [21]. Which means ROS are non-radical and radical reactive molecules [21]. O_2^- can be rapidly dismutated into more stable species such as hydrogen peroxide (H_2O_2), or - in the presence of nitric oxide (NO) - it forms the peroxynitrite anion ($ONOO^-$) [21]. All of these substances can cause a variety of diseases. When ROS production exceeds the antioxidant capacity of the body, oxidative stress and oxidative damage will occur. The increased oxidative stress level after ischemia reperfusion injury is related to the activation of NADPH oxidase, which is the major enzyme that produces ROS during ischemia-reperfusion injury [22].

NADPH oxidases, are multi-subunit complexes, have seven catalytic subunit homologues which are NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2 [23, 24, 25]. All of them contain a peroxidase-like domain [23, 24, 25]. NOX4 is highly expressed in the kidney, and NOX2 and other NOX subunits are also meaningfully expressed in the kidney. They are the source of Reactive oxygen species ROS production in the kidney [26]. ROS produced by NADPH oxidase are involved in mediating many signaling pathways in cells and regulating many physiological activities such as cell growth, cell division, differentiation, migration, apoptosis and aging, which are also closely related to the occurrence and development of diseases [26]. Studies have shown that H_2S has a direct inhibitory effect on NADPH oxidase activity, and micromole H_2S can reduce the expression of NADPH oxidase subunit gp91phox (also known as NOX2) [27].

Recent studies have found that H_2S has many physiological functions, such as relaxing smooth muscle, protecting blood vessels by inhibiting vascular remodeling, participating in shock and inflammatory

reactions, participating in nerve activities, and regulating neuroendocrine functions, etc [28]. H_2S is also considered to be the third gaseous signaling molecule after NO and CO in vivo [28]. Studies have shown that H_2S has a protective effect on renal ischemia-reperfusion injury. In the renal IRI rat model, the expression of CBS/H_2S system was significantly decreased. However, after administration of exogenous H_2S donor NaHS, renal tissue injury and Renal function is improved [29].

Conclusion

In this study, we observed the expression of NOX4 and NOX2, the change of ROS content and the activity of NADPH oxidase, in rat renal tubular epithelial cells pretreated with exogenous hydrogen sulfide at the time of occurring oxidative stress during hypoxia-reoxygenation, and compared with DPI. It is found that: 1. NOX4 and NOX2 expression and ROS content increased significantly, in renal tubular epithelial cells pretreated without H_2S after oxidative stress. 2. After pretreatment with H_2S, the expression of NOX4 and NOX2 and the ROS content were slightly higher than those of normal cells, but there was a significant decrease compared with untreated cells, and the change of NADPH oxidase activity was also the same. In conclusion, H_2S can reduce ROS production by inhibiting NOX4 and NOX2 during oxidative stress, and also directly inhibits NADPH oxidase activity.

Declarations

Funding

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Availability of data and materials

Data sharing is not applicable to this article, as no data sets were generated or analyzed during the current study.

Authors' contributions

Zhicheng Tan, Wenli Liu and Guangyuan Li designed the theme of the review. Di Li performed experiments, acquired, analyzed and interpreted the data, drafted the manuscript. Tianyu Xia and Xiaolin Liu retrieved the relevant literature. All authors critically revised the manuscript, and read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

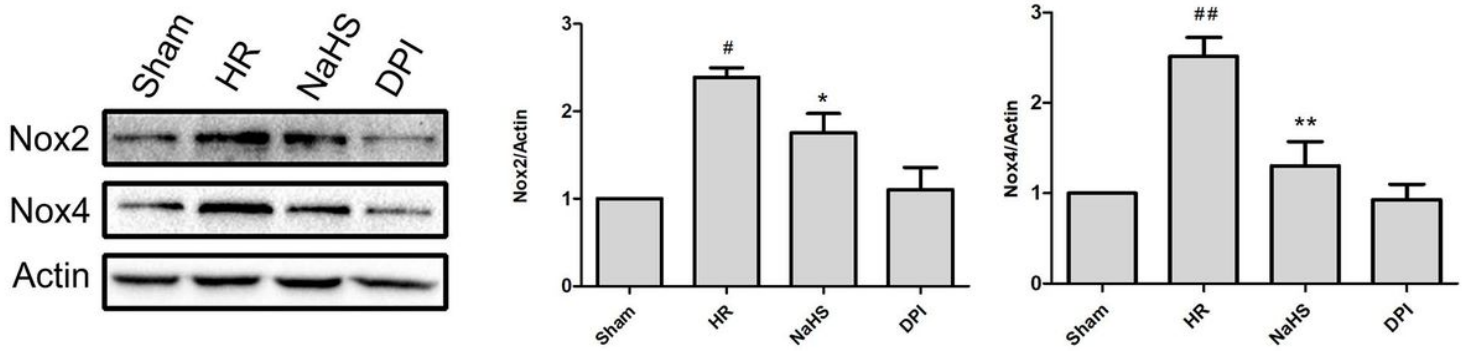

Figure 1

The protein expression of NOX2 and NOX4 in the HR group was significantly increased compared with the Sham group (# P<0.01, ## P<0.01). Compared with the HR group, the protein expression of NOX2 and NOX4 in the NaHS group was significantly decreased (* P<0.05, ** P<0.01). Data are expressed as means± S.E.M. n=3.

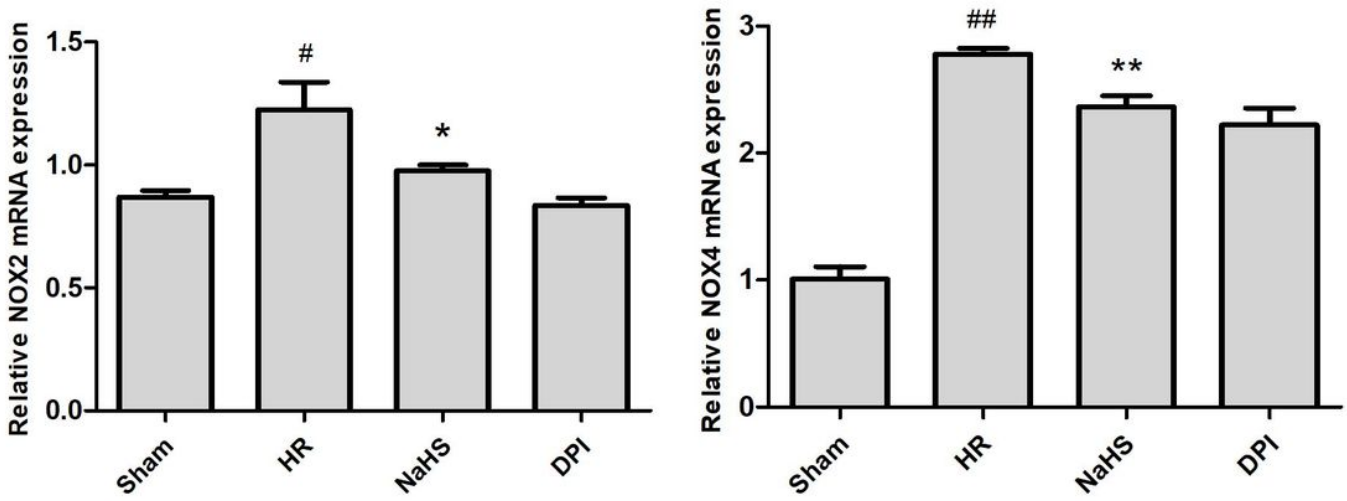

Figure 2

The mRNA expression of NOX2 and NOX4 in the HR group was increased than that in the Sham group (# P<0.01, ## P<0.01, both compared with the Sham group). The expression of NOX2 mRNA and NOX4 mRNA in NaHS group was decreased than that in HR group (* P<0.05, ** P P<0.01, both compared with HR group). Data are expressed as means ± S.E.M. n=3.

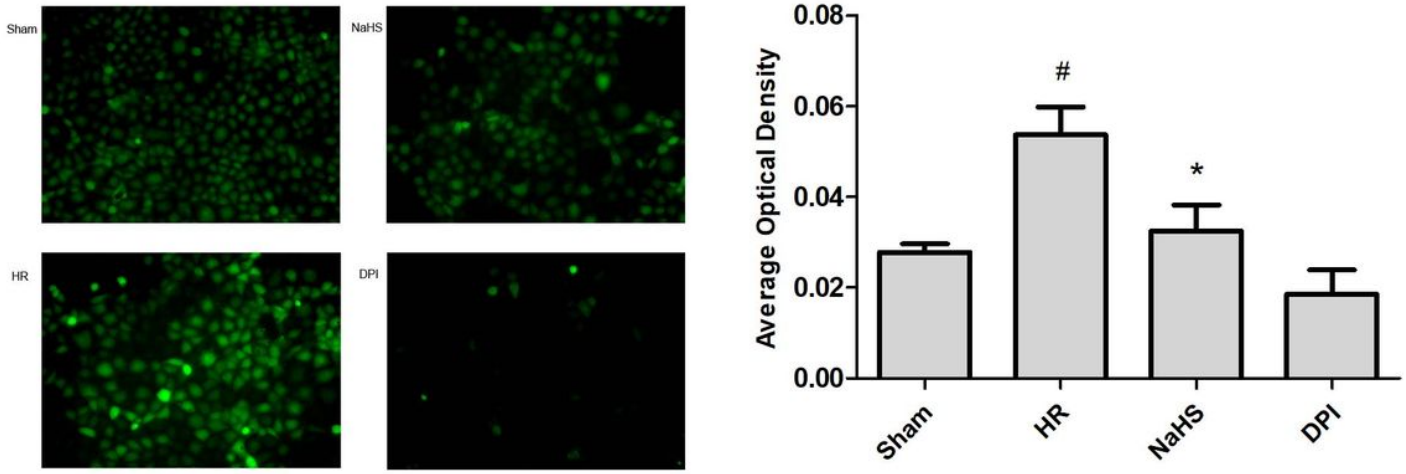

Figure 3

Compared with the Sham group, the average optical density value of ROS in the HR group was enhanced (# P<0.01). The average optical density value of ROS in the NaHS group was reduced than that in the HR group (* P<0.05). Data are expressed as means ± S.E.M. n=3.

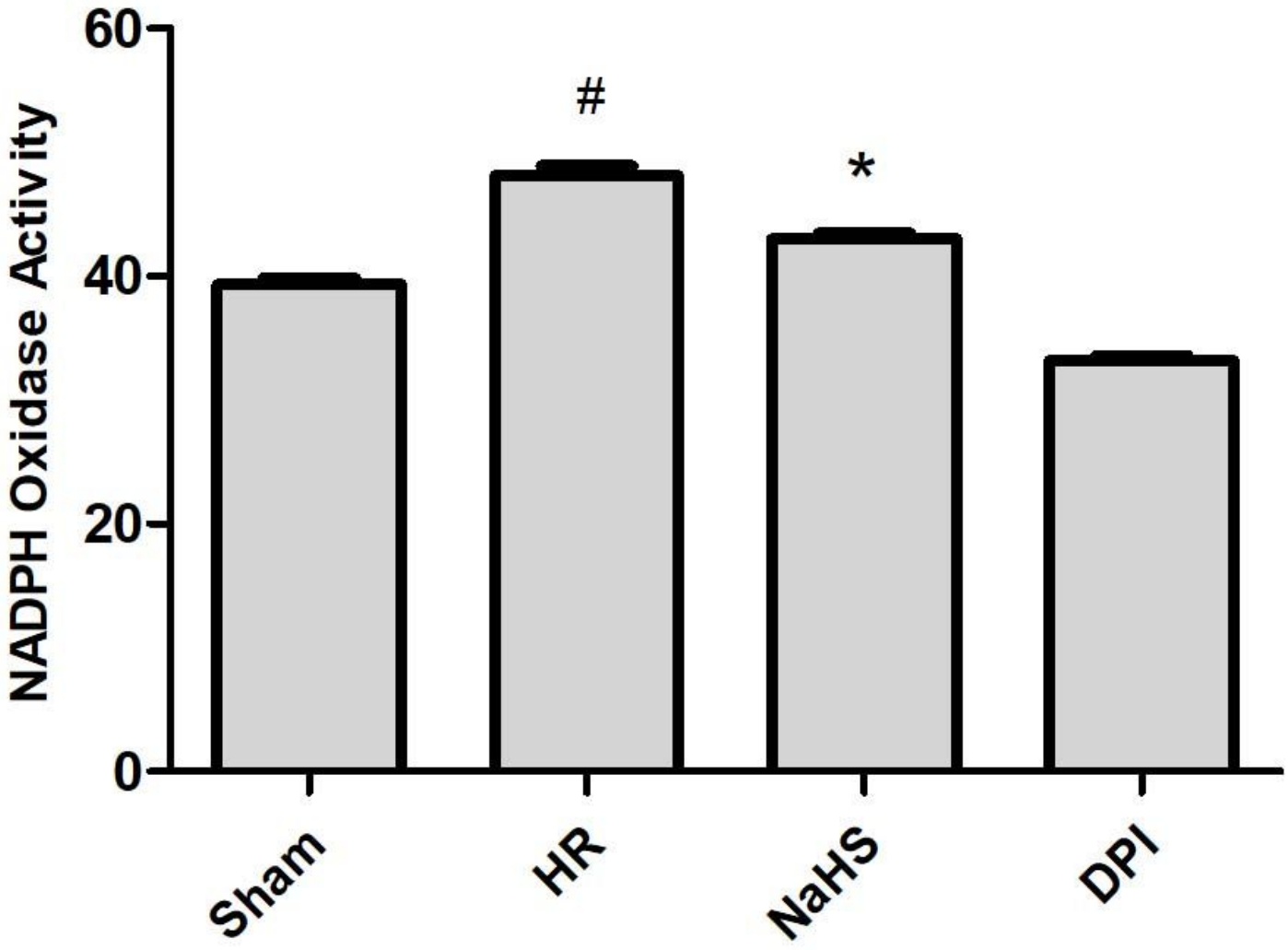

Figure 4

Compared with the Sham group, the NADPH oxidase activity in the HR group was increased (# P<0.01), while the NaHS group was decreased (* P<0.01). Data are expressed as means ± S.E.M. n=3.