Original Paper

Roles of the Exogenous H₂S-Mediated SR-A Signaling Pathway in Renal Ischemia/Reperfusion Injury in Regulating Endoplasmic Reticulum Stress-Induced Autophagy in a Rat Model

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Key Words

Exogenous hydrogen sulfide • SR-A signaling pathway • renal ischemia/reperfusion injury • Endoplasmic reticulum stress • Autophagy • Regulation • Gene knockout

Abstract

Objective: This study aims to explore the effect of the exogenous hydrogen sulfide (H₂S)-mediated scavenger receptor A (SR-A) signaling pathway on renal ischemia/reperfusion injury (IRI) by regulating endoplasmic reticulum (ER) stress-induced autophagy in rats. **Methods:** A total of 48 normal Sprague-Dawley (SD) rats and SR-A knockout rats were selected and divided into six groups (n = 8): wild-type (WT) + sham, WT + ischemia-reperfusion (I/R), WT + I/R + NaHS, SR-A^{-/-} + sham, SR-A^{-/-} + I/R, and SR-A^{-/-} + I/R + NaHS. The concentrations of urinary protein, blood urea nitrogen (BUN), serum creatinine (SCR), malondialdehyde (MDA) and H₂S in renal tissue were detected. RT-PCR and Western blotting were used to detect the mRNA and protein levels of IL-6, TGF-β, SR-A, LC3I, LC3II, P62, PERK, ATF6 and IRE1 pathway-related genes. A TUNEL assay was used to detect cell apoptosis. Electron microscopy was applied to observe the number of renal autophagosomes. **Results:** Compared with the WT + sham group, in the renal tissue of the WT + I/R group, the urine volume, urinary protein, BUN, SCR and MDA concentrations, the mRNA and protein expression of IL-6, TGF-β, LC3II/I, and ER stress pathway-related genes, the cell apoptosis index, and the number of autophagosomes were significantly increased 24 h after I/R, while P62 and SR-A protein expression and SOD and H₂S concentrations were significantly decreased (all P < 0.05). The levels of renal injury, autophagy and stress pathway-related genes were decreased in the WT + I/R + NaHS group but were increased in the SR-A^{-/-} + I/R group relative to the WT + I/R group. No significant differences were observed in the urine volume; the concentrations of urinary protein, BUN, SCR and MDA; the SOD activity; the mRNA and protein expression of IL-6, TGF-β, SR-A, GRP78, SR-A, GPR94, ATF4, IRE1, XBP1, ATF6, and eIF2α; the cell apoptosis index; or the number of autophagosomes in rats of the SR-A^{-/-} + I/R and SR-A^{-/-} + I/R + NaHS groups (all P > 0.05). **Conclusion:** These results demonstrate that the exogenous H₂S-mediated SR-A signaling pathway reduces renal IRI injury by up-regulating ER stress-induced autophagy in rats.

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Introduction

Renal ischemic-reperfusion injury (IRI) is a typical complication of organ transplantation, leading to adenosine triphosphate (ATP) depletion, accumulation of toxic metabolites, and consequent tissue damage [1]. During the process of renal transplantation, transient cessation in renal blood flow leads to acute ischemic insult, and the reperfusion further deepens the functional and structural damage to the human kidney [2]. There are two basic mechanisms that underlie IRI, namely, the systemic imbalance of oxidative stress and the immune-inflammatory response caused by restoration of metabolic processes [3]. For IRI, delayed graft function (DGF) and initial poor graft function (IPGF) are both common events, which are reported to influence approximately 25% of the renal tissue from deceased donors [4]. Currently, effective therapeutic regimens for the prevention of renal IRI are urgently needed. It has been proven that propofol, COX metabolites and MiR-155 can mediate renal IRI [5-8], but researchers have focused their attention on the attenuation of endoplasmic reticulum (ER) stress and autophagy for their therapeutic potential [9-11].

The ER plays a pivotal role in protein folding and synthesis and in calcium (Ca²⁺) storage and signaling [12]. ER stress occurs if the ER environment (homeostasis) and ER function (glycosylation) is disturbed, which inevitably leads to the overregulation of PERK kinase (or cell eIF2α kinase) and GRP78 [13]. The ER resident PKR-like kinase (PERK) plays an important role in Akt activation in response to ER stress [14]. Researchers have also demonstrated that hepatic eukaryotic initiation factor 2 (eIF2α) is critical to the insulin-induced ER stress [15]. GRP78 (glucose-regulated protein of 78 kDa) is an ER chaperone, facilitating Ca²⁺ binding and regulating ER stress signaling, protein quality control, and protein folding and assembly [16]. IRI can induce ER stress by perturbing Ca²⁺ homeostasis, resulting in the accumulation of misfolded proteins. ER stress also enhances apoptosis and aggravates cell damage [17]. Moreover, ER stress leads to the potentiation of autophagy, which determines the fate of cells [18]. Inflammation is a direct result of tissue injury and can also lead to ER stress via the production of inflammatory factors and the generation of reactive oxygen species (ROS) [19]. The cluster of differentiation 36 (CD36) macrophage scavenger receptor (SR-A) is a prototypic member of the scavenger receptor (SR) family, a collection of membrane receptors [20]. In recent years, several studies have revealed that SR-A has an essential influence on inflammation, ERS and cell apoptosis [21, 22]. Hydrogen sulfide (H₂S), synthesized in the metabolic pathway that regulates the tissue concentration of sulfur-containing amino acids, is an endogenous gas that acts as a mediator that exerts effects in the central nervous system and the cardiovascular system. A study by Ji et al. has demonstrated that exogenous H₂S post-conditioning can successfully prevent the rat heart from experiencing IRI [24]. In addition, H₂S is regarded as an endogenous signaling molecule which exerts cardio-protection against MIRI in cardiac myocytes and also known as a powerful neuro-protective agent in the central nervous system [25, 26]. Therefore, the present study was conducted to explore the effects of the exogenous H₂S-mediated SR-A signaling pathway on renal IRI by regulating ER stress-induced autophagy in rats.

Materials and Methods

Ethics statement

The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Tongji Hospital, Tongji Medical College, Huazhong University of Science & Technology.

Subjects and grouping

A total of 48 rats (5 to 6 months of age, weighing from 200 to 240 g), including healthy Sprague-Dawley (SD) rats and rats with SR-A gene knockout, were provided by Shanghai Research Center for Southern Model Organisms. The Center also confirmed the genotypes of rats with SR-A gene knockout. The experimental rats were given free access to conventional pellets and drinking water and were kept under a regular 12-h

circadian rhythm at 18 ~ 22°C with good ventilation. Before the experiment, the rats were kept for one week to adapt to the environment and were then divided into six groups (n = 8): the wild-type and sham-operation group (the WT + sham group); the wild-type and ischemia-reperfusion group (the WT + I/R group); the wild-type and ischemia-reperfusion with NaHS therapy group (the WT + I/R + NaHS group); the SR-A gene knockout and sham-operation group (the SR-A^{-/-} + sham group); the SR-A gene knockout and ischemia-reperfusion group (the SR-A^{-/-} + I/R group); and the SR-A gene knockout and ischemia-reperfusion with NaHS therapy group (SR-A^{-/-} + I/R + NaHS group).

Renal IRI

The rats were anesthetized by intraperitoneal injection of 6% chloral hydrate (0.5 ml/100 g, Sigma Chemical Reagent Co., Ltd., Shanghai, China) and placed on the operating table, where their backs were disinfected with 75% ethyl alcohol. A 2 cm incision was made on both sides of the spine at the end of the twelfth rib. Then, the muscle and fascia were separated, the kidney was exposed, and blunt dissection was conducted on the renal pedicle. The renal artery was blocked by a non-invasive vascular clamp. After 5 min, the clamp was removed, and the time of reperfusion was recorded. The rats were put back in their cages and fed as usual, with the incisions sutured and sterilized. The status of the rats was observed. For rats in the WT + sham and SR-A^{-/-} + sham groups, the bilateral renal artery was sutured without the vascular clamp, but the other experimental steps were conducted as described above. For rats in the WT + I/R + NaHS and SR-A^{-/-} + I/R + NaHS groups, after 50 min of ischemia, intraperitoneal injection of 100 µg/kg NaHS (Sigma Chemical Co., Ltd, St. Louis, MO, USA) was carried out. In this experiment, urine was collected before surgery (0 h) and reperfusion for 24 h. Blood and renal tissues were removed 24 h after reperfusion [1].

Collection of urine, plasma and renal tissues

Before surgery, the rats in the 6 groups were put into the metabolic cages; 24 h later, urine was collected and designated 0 h. Then, the rats were put into the metabolic cages after I/R; 24 h later, urine was collected, and the urine volume was recorded. After the rats were weighed separately, intraperitoneal injection of 6% chloral hydrate was performed for anesthesia, and the peritoneal cavity was exposed to allow removal of a certain amount of heparin and aorta blood from the abdomen; this material was then centrifuged at the rate of 5000 rpm and 4°C for 10 min. After the serum was aspirated, the plasma was put into 4 Eppendorf (EP) tubes and kept at -80°C. Twenty-four hours after I/R, the bilateral renal tissues were separated; one was immersed in 4% formalin for fixation overnight, and the other was put into an EP tube with liquid nitrogen and saved at -80°C. There were ten rats in each group.

Urine protein content

Using a quantitative protein detection kit (Nanjing Jiancheng Bioengineering Institute (Jiangsu Province, China), Bradford method was applied to determine urine protein content. The mixtures of samples and reagents were incubated for 5 min, and the absorbance values in all tubes were detected at the wavelength of 595 nm. The concentration of urine protein (mg/L) = (the absorbance value in the testing tube/the absorbance value in the standard tube) × the standard concentration of protein. The 24-hour protein excretion was calculated as the concentration of urine protein × the 24-hour urine volume. There were ten rats in each group.

Concentration of blood urea nitrogen (BUN)

In accordance with the specifications of the BUN determination kit (Rongsheng Biotech, Shanghai, China), the BUN concentration in the serum was analyzed using the urease continuous monitoring assay. The mixtures of samples and reagents were colorimetrically assessed using a spectrophotometer. The value at 30 s (A1) and the value at 90 s (A2) were recorded. The concentration of BUN (mmol/L) = (the absorbance at A2 of the sample tube - the absorbance at A1 of the sample tube)/(the absorbance at A2 of the calibration solution - the absorbance at A1 of the calibration solution) × the concentration of standard solution.

Concentration of serum creatinine (SCR)

Following the specifications of the SCR kit (Rongsheng Biotech, Shanghai, China), the concentration of SCR was analyzed by the picric acid method. The specimens were mixed with the reagent and placed in a water bath at 37°C for 30 s. The initial absorbance (A1) at 0 s in a blank tube and the terminal absorbance

(A2) at 90 s were recorded at a wavelength of 505 nm. The concentration of SCR ($\mu\text{mol/L}$) = (the absorbance at A2 of the sample tube - the absorbance at A1 of the sample tube)/(the absorbance at A2 of the calibration solution - the absorbance at A1 of the calibration solution) $\times$ the concentration of the standard solution. There were ten rats in each group.

Content of renal malondialdehyde (MDA)

The MDA content in the rat kidneys was measured using an MDA kit (Nanjing Jiancheng Bioengineering Institute) and the thiobarbituric acid (TBA) method [27]. After mixing with the reagent, the renal protein samples were placed in a water bath at 95°C for 40 min, cooled with running water and centrifuged at the rate of 3500 rpm for 10 min. The absorbance values in all tubes were detected at the wavelength of 532 nm. The concentration of MDA (nmol/ml) = (the absorbance in the testing tube - the absorbance in the blank tube)/(the absorbance in the standard tube - the absorbance in the standard blank tube) $\times$ standard concentration (10 nmol/ml) $\times$ the dilution ratio of the sample before the test. There were ten rats in each group.

Content of superoxide dismutase (SOD)

The renal SOD content in rats was detected using a SOD kit (Nanjing Jiancheng Bioengineering Institute). The renal protein samples were mixed with the reagent. After 10 min incubation at 37°C, the absorbance at the wavelength of 450 nm was measured. In this reaction system, a single SOD activity unit (U) equals the enzyme quantity at the moment when the SOD inhibition rate reached 50%. SOD inhibition rate = [(control A - blank control A) - (testing A - blank testing A)] $\times$ 100 / (control A - blank control A). SOD activity (U/ml) = SOD inhibition rate/50% $\times$ dilution ratio in reaction system/the concentration of sample protein to be tested. There were ten rats in each group.

Concentration of hydrogen sulfide (H₂S)

The content of H₂S in renal tissues was measured by spectrophotometry. After mixing with 1 ml phosphate-buffer saline (PBS) using an electrophoretometer, 10 mg of rat renal tissues that had undergone I/R 24 h prior was placed into a 2 ml tube, and 0.1 ml acetic acid solution (0.125 ml) was added. Then, 0.15 ml of double distilled water, 0.1 ml of 1% sodium acetate and standards, 20 mM phenylenediamine phthalate solution (0.067 ml) and 30 mM chloroform solution (0.067 ml) were added, and the tissue was immersed in a water bath at 25°C for 20 min and centrifuged at 6000 rpm for 5 min. Absorbance at the wave length of 670 nm was measured using a microplate reader, and the protein concentration was determined by the BCA kit (Boster Biotechnology Co., Ltd., Wuhan, China). The concentration of H₂S in the renal tissue ($\mu\text{mol/gpr}$) = the concentration of H₂S in all specimens/protein concentration. There were ten rats in each group.

Western blot

A BCA kit (Boster Biotechnology Co., Ltd., Wuhan, China) was used to test the protein concentration in rat kidneys (50 mg) that had undergone I/R. The extracted protein was boiled with loading buffer at 95°C for 5 min, and 10 μg was loaded into each well. Then, the protein underwent 10% polyacrylamide gel electrophoresis at 80 V and then at 120 V, followed by wet transfer onto polyvinylidene fluoride (PVDF) at 4°C for 2 h and 150 mV for 70 min. After blocking at room temperature with 5% bovine serum albumin (BSA) for 1 h, primary antibodies against interleukin-6 (IL-6, 1:1000, Cell Signaling Technologies, Beverly, MA, USA), transforming growth factor-beta (TGF- β , 1:1000, CST), SR-A (1:1000, Abcam, Cambridge, MA, USA), LC3II (1:500, Abcam, Cambridge, MA, USA), microtubule associated protein 1 light chain 3 alpha (LC3II, 1:500, Abcam, Cambridge, MA, USA) or P62 (1:1500, Abcam, Cambridge, MA, USA) were added, and then the membranes were incubated overnight at 4°C. After rinsing with Tris-buffered saline with Tween (TBST) three times for 5 min per wash, the corresponding secondary antibody was added and incubated for 1 h. Then, the membrane was washed three times for 5 min per wash and developed by chemiluminescence reagents. Phosphoglyceraldehyde dehydrogenase (GAPDH) was used as an internal reference (1:5000, Kangcheng Biology Engineering Co., Ltd., Shanghai, China). Each group was repeated three times. A Bio-Rad Gel Doc EZ imager (Gel doc ez imager, Bio-Rad, California, USA) was used for developing. The gray values of the target bands were analyzed with Image J software. These procedures were repeated five times for all groups.

Table 1. The primer design of qRT-PCR. Notes RT-qPCR, real-time quantitative polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; IL-6, interleukin-6; TGF- β , transforming growth factor- β ; SR-A, scavenger receptor A; GRP78, heat shock protein family a member 5; eIF2 α , eukaryotic translation initiation factor 2 subunit alpha; LC3 II, microtubule associated protein 1 light chain 3 alpha II

PCR primers sequences	Forward (5'-3')	Reverse (5'-3')
GAPDH	CCATTTGCAGTGGCAAAG	CACCCCATTTGATGTTAGTG
IL-6	ATGAACTCCCTCTTCAC	CTACTTCATCCGAATAGCTC
TGF- β	ATGCCGCCTTCGGGGCTGCGGCTGCTGC	TCAGCTGCACTTGCAGGAGTTCACGA
SR-A	CTCCTGAAGTGGGAACGAAG	GAGGTTGGCTTCCATGTCTTA
GRP78	ATCACGCCGTCCTATGTCCG	TCTCCCCCTCCCTCTTATCC
GPR94	CTTCGCTTCCAGTCTTCCCATC	AGAAGGCGTTCAACATCTTG
IRE1	TGAGGGCAATGAGAAATAAGAAGC	TGTAGGAGCAGGTGATGAGGC
ATF4	TTGATGCCCTGTTAGGTATGGAA	GGTATGAGTGGAGCTCTCTCT
XBP1	GCGAGTCTACGGATGTGAAGGA	TGTGGAGGTTCTTGAATGC
ATF6	GATTGTGGGCGTCACTTCTCG	TGGGATGCCAATGAGCCTG
eIF2 α	TTTAAGGGAGTGGCTTGATTT	AACTGATTGTTCTCTCTC

Quantitative real-time fluorescence PCR (qRT-PCR)

As shown in Table 1, total RNA from 24 h I/R rat kidneys (approximately 30 mg) was extracted using an RNA extraction kit (Promega Co., Madison, WI, USA). The OD_{260/280} values of all RNA samples were tested using an ultraviolet spectrophotometer, and the concentration of RNA was calculated. The RNA was then preserved at -80°C for further use. Reverse transcription was conducted according to the experimental procedures of the RNA RT-PCR Kit (Promega Co., Madison, WI, USA). A two-step reaction sequence was adopted for PCR: initial denaturation at 95°C for 15 min, then 40 cycles of the following conditions: denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR reaction included SYBR Green mix 12.5 μ l, forward primer 1 μ l, reverse primer 1 μ l, cDNA template 2 μ l, and ddH₂O 8.5 μ l (Promega, Madison, WI, USA). With GAPDH as an internal reference, an amplification curve was used to evaluate the reliability of PCR. The C_t values were determined, and the relative gene expression was calculated as $2^{-\Delta\Delta C_t}$. These procedures were repeated five times for all groups.

Terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) assay

After the slices were washed and fixed with 0.1% trypsin for 3 min, they were incubated in 0.3% H₂O₂ methanol solution for 10 min, treated with 0.1% Triton X-100 for 2 min, and then placed in a humid box. TUNEL reagent was added and incubated at 37°C for 1 h after two 5-min PBS washes. Then, the slices were put into a humid box and incubated with diaminobenzidine tetrahydrochloride (DAB) and horseradish peroxidase (HRP)-POD at 37°C for 30 min and underwent DAB development at room temperature for 10 min after three 5-min PBS washes. This was followed by hematoxylin counterstaining, distilled water rinsing, dehydration, cover slipping and slice sealing.

After photomicrographing under a microscope, cells with brown granules in the nucleus were deemed positive. Under a light microscope at 200 $\times$ magnification, 5 fields were randomly selected to count the number of apoptotic cells. The percentage of apoptosis-positive cells in the renal tubule epithelia relative to the total number of renal tubule cells was taken as the apoptotic index (AI) of the renal tubule.

Renal ultra-microstructure

Approximately 1 mm³ of renal tissue sample was double fixed in 4% glutaraldehyde and 1% osmic acid. After gradient dehydration in acetone, a mixed resin/acetone liquor was added; 2 h later, the tissue was embedded in resin and baked at 65°C for 24 h. After ultrathin slicing at a thickness of 50 nm, uranyl acetate and lead citrate were used for double staining. A transmission electron microscope (TEM, HITACHI H-600) was employed to observe and photograph the ultra-microstructure.

Statistical analysis

All data in the experiment were analyzed by SPSS 21.0 integrated software (SPSS Inc. IBM, Chicago, IL, USA). Data are presented as the means $\pm$ standard deviation (SD). The *t* test was used for comparison between two groups whose data followed the normal distribution. One-way analysis of variance (ANOVA) was used for comparisons among groups, with *P* < 0.05 indicating statistical significance.

Results

Comparisons of urine volume and urine protein content after 24 h among the six groups

After measuring the urine volume of rats in each group, no significant difference was found in the urine volume of rats in any group at 0 h (all $P > 0.05$). No significant difference in urine volume was observed 24 h after I/R between the SR-A^{-/-} + sham group and the WT + sham group ($P > 0.05$). Urine volume increased significantly 24 h after I/R in the WT + I/R group compared with the WT + sham group ($P < 0.01$). The urine volume 24 h after I/R in the WT + I/R group was markedly higher than that in the WT + I/R + NaHS, the SR-A^{-/-} + I/R, and the SR-A^{-/-} + I/R + NaHS groups (all $P < 0.01$), but the urine volume 24 h after I/R in the WT + I/R + NaHS and the SR-A^{-/-} + I/R groups was still evidently higher than that in the WT + sham group (both $P < 0.05$). There was no significant difference in urine volume 24 h after I/R between the SR-A^{-/-} + I/R + NaHS and the WT + sham groups ($P > 0.05$) (Table 2).

Based on the results of urinary protein detection, no significant difference was observed in the urine protein content of rats in any group at 0 h (all $P > 0.05$). No significant difference in the urine protein content was observed 24 h after I/R between the SR-A^{-/-} + sham and the WT + sham group ($P > 0.05$). Urine protein content increased significantly 24 h after I/R in the WT + I/R group compared with that in the WT + sham group ($P < 0.01$). Urine protein content in the WT + I/R group 24 h after I/R was markedly higher than that in the WT + I/R + NaHS, the SR-A^{-/-} + I/R and the SR-A^{-/-} + I/R + NaHS groups (all $P < 0.01$), but urine protein content 24 h after I/R in the WT + I/R + NaHS and the SR-A^{-/-} + I/R groups was still evidently higher than that in the WT + sham group (both $P < 0.05$). There was no significant difference in the urine protein content between the SR-A^{-/-} + I/R + NaHS and the WT + sham groups ($P > 0.05$) (Table 3).

Comparisons of concentrations of BUN, CR, and SOD in rats among the six groups

There was a remarkable increase in BUN and CR concentrations in the WT + I/R group, the WT + I/R + NaHS group, the SR-A^{-/-} + I/R group, and the SR-A^{-/-} + I/R + NaHS group when compared with the WT + sham group 24 h after I/R (all $P < 0.05$). The BUN and CR concentrations were reduced significantly in the WT + I/R + NaHS group and the SR-A^{-/-} + sham group compared with those in the WT + I/R group 24 h after I/R, while the concentrations in the SR-A^{-/-} + I/R group and the SR-A^{-/-} + I/R + NaHS group

Table 2. The urine volume of rats at 24 h after I/R in different groups (mean $\pm$ standard deviation, n = 10). Notes: 0 h, before treatment; 24 h, 24 h after ischemia/reperfusion. Compared with the WT + sham group, * $P < 0.05$, ** $P < 0.01$; compared with the WT + I/R group, ** $P < 0.01$.

	0 h	24 h
WT + sham (ml)	14.31 $\pm$ 4.25	13.01 $\pm$ 3.19
WT + I/R(ml)	12.15 $\pm$ 2.91	24.41 $\pm$ 4.12##
WT + I/R + NaHS (ml)	12.54 $\pm$ 1.72	18.98 $\pm$ 3.19***
SR-A ^{-/-} + sham (ml)	13.92 $\pm$ 3.11	12.74 $\pm$ 2.78**
SR-A ^{-/-} + I/R (ml)	15.78 $\pm$ 5.33	19.24 $\pm$ 4.20***
SR-A ^{-/-} + I/R + NaHS (ml)	14.31 $\pm$ 2.10	16.05 $\pm$ 3.98**

Table 3. The discharge of urinary of rats at 24 h after I/R in different groups (mean $\pm$ standard deviation, n = 10). Notes: 0 h, before treatment; 24 h, at 24 h after ischemia/reperfusion. Compared with the WT + sham group, * $P < 0.05$, *** $P < 0.01$; compared with the WT + I/R group, ** $P < 0.01$.

	0 h (ml)	24 h (ml)
WT + sham	17.54 $\pm$ 1.5	19.89 $\pm$ 1.01
WT + I/R	18.33 $\pm$ 1.98	29.07 $\pm$ 4.32##
WT + I/R + NaHS	18.52 $\pm$ 2.63	24.42 $\pm$ 3.05***
SR-A ^{-/-} + sham	17.25 $\pm$ 1.94	17.84 $\pm$ 1.90**
SR-A ^{-/-} + I/R	19.75 $\pm$ 2.17	25.12 $\pm$ 4.73***
SR-A ^{-/-} + I/R + NaHS	19.48 $\pm$ 1.99	20.97 $\pm$ 1.68**

Fig. 1. Detection of serum BUN, SCR, MDA, and SOD in rats of different groups 24 h after ischemia/reperfusion (n = 8). Notes: A, The urease continuous monitoring assay was used to detect the BUN concentration (mmol/L) in rats from each group 24 h after ischemia/reperfusion. B, The picric acid method was used to detect SCR concentration (mmol/L) in rats from each group 24 h after ischemia/reperfusion. C, The thiobarbituric acid method was used to detect MDA concentration (nmol/mg) in rats from each group 24 h after ischemia/reperfusion. D, Serum SOD activity (U/mL) was determined in rats from each group 24 h after ischemia/reperfusion. **P* < 0.05, ***P* < 0.01 compared with the WT + sham group; #*P* < 0.05, ##*P* < 0.01 compared with the WT + I/R group; &*P* < 0.05 compared with the SR-A^{-/-} + I/R group.

were apparently elevated (both *P* < 0.05). The BUN and SCR concentrations in the WT + I/R + NaHS group were markedly decreased compared with the SR-A^{-/-} + I/R group 24 h after I/R (all *P* < 0.05). No significant differences in BUN and SCR concentrations were observed between the SR-A^{-/-} + I/R + NaHS group and the SR-A^{-/-} + I/R group (*P* > 0.05) (Fig. 1A~B).

The MDA concentration and SOD activity were significantly increased in the WT + I/R group, the WT + I/R + NaHS group, the SR-A^{-/-} + sham group, the SR-A^{-/-} + I/R group, and the SR-A^{-/-} + I/R + NaHS group compared with those in the WT + sham group 24 h after I/R, while the SOD activity was obviously decreased (both *P* < 0.01). The MDA concentration was significantly decreased and the SOD activity was markedly elevated in the WT + I/R + NaHS group and the SR-A^{-/-} + sham group compared with those in the WT + I/R group (both *P* < 0.05). The MDA concentration was markedly decreased but the SOD activity was significantly increased in the SR-A^{-/-} + I/R + NaHS and SR-A^{-/-} + I/R groups compared with those in the WT + I/R group (both *P* < 0.05). The MDA concentration in the WT + I/R + NaHS group was apparently higher, and the SOD activity was lower when compared with the SR-A^{-/-} + I/R group, while there was no significant difference in SOD activity between the SR-A^{-/-} + I/R + NaHS group and the SR-A^{-/-} + I/R group (Fig. 1C~D).

Comparison of H₂S concentration in renal tissue among the six groups

The H₂S concentration was significantly decreased in the WT + I/R group, the WT + I/R + NaHS group, the SR-A^{-/-} + sham group, the SR-A^{-/-} + I/R group, and SR-A^{-/-} + I/R + NaHS group 24 h after I/R in comparison with the WT + sham group (*P* < 0.05). The H₂S concentrations in the WT + I/R + NaHS group and the SR-A^{-/-} + sham group were significantly elevated compared with the WT + I/R group 24 h after I/R but were remarkably decreased in the SR-A^{-/-} + I/R group and the SR-A^{-/-} + I/R + NaHS (both *P* < 0.05). H₂S concentrations in WT + I/R + NaHS were remarkably elevated compared with the SR-A^{-/-} + I/R group (all *P* < 0.05). No significant difference was found between the SR-A^{-/-} + I/R + NaHS group and the SR-A^{-/-} + I/R group (*P* > 0.05) (Fig. 2).

Comparison of the levels of the inflammatory factors IL-6 and TGF-β in renal tissues among the six groups

The qRT-PCR and Western blotting results showed no significant differences in IL-6 or TGF-β mRNA or protein expression between the SR-A^{-/-} + sham group and the WT + sham

Fig. 2. H₂S concentration in the renal tissues of rats in different groups 24 h after ischemia/reperfusion (n = 8). Notes: *P < 0.05 compared with the WT + sham group; #P < 0.05 compared with the WT + I/R group; &P < 0.05 compared with the SR-A^{-/-} + I/R group.

Fig. 3. Detection of mRNA and protein levels of IL-6 and TGF-β in rats of different groups 24 h after ischemia/reperfusion (n = 8). Notes: A, qRT-PCR was used to detect the mRNA expression of IL-6 in rats from different groups 24 h after ischemia/reperfusion. B, qRT-PCR was used to detect the mRNA expression of TGF-β in rats from different groups 24 h after ischemia/reperfusion. C, Densitometry for IL-6 protein was determined in rats of different groups 24 h after ischemia/reperfusion. D, Densitometry for TGF-β protein was determined in rats of different groups 24 h after ischemia/reperfusion. E, Western blotting was used to detect the protein levels of IL-6 and TGF-β in rats of different groups 24 h after ischemia/reperfusion. *P < 0.05 compared with the WT + sham group; #P < 0.05 compared with the WT + I/R group; &P < 0.05 compared with the SR-A^{-/-} + I/R group.

group 24 h after I/R (all $P > 0.05$). The mRNA and protein levels of IL-6 and TGF-β were significantly increased 24 h after I/R in the WT + I/R group compared with the WT + sham group (all $P < 0.05$). The mRNA and protein levels of IL-6 and TGF-β were significantly decreased 24 h after I/R in the WT + I/R + NaHS, SR-A^{-/-} + I/R and SR-A^{-/-} + I/R + NaHS groups compared with the WT + I/R group (all $P < 0.05$). The mRNA and protein levels of IL-6 and TGF-β in the SR-A^{-/-} + I/R group were higher than those in the SR-A^{-/-} + sham group ($P < 0.05$). No marked difference in the mRNA and protein levels of IL-6 and TGF-β were observed between the SR-A^{-/-} + I/R + NaHS group and the SR-A^{-/-} + I/R group ($P > 0.05$) (Fig. 3).

Comparison of the apoptosis indexes of renal tissues among the six groups

There was no significant difference of the apoptotic index between the SR-A^{-/-} + sham group and the WT + sham group 24 h after I/R ($P > 0.05$). The apoptotic index was remarkably increased in the WT + I/R group compared with the WT + sham group 24 h after I/R ($P < 0.05$). The apoptotic indexes were significantly decreased 24 h after I/R in the WT

Fig. 4. Detection of apoptosis levels in rats of different groups 24 h after ischemia/reperfusion using TUNEL (n = 8). Notes: **P* < 0.05 compared with the WT + sham group; #*P* < 0.05 compared with the WT + I/R group; &*P* < 0.05 compared with the SR-A^{-/-} + I/R group.

+ I/R + NaHS and SR-A^{-/-} + sham groups compared with the WT + I/R group (all *P* < 0.05). The apoptotic indexes of the WT + I/R + NaHS and SR-A^{-/-} + I/R groups were remarkably elevated compared with that of the WT + sham group (both *P* < 0.01); no marked difference in the apoptotic index was observed between the SR-A^{-/-} + I/R + NaHS group and the SR-A^{-/-} + I/R group (*P* > 0.05) (Fig. 4).

Comparison of SR-A expression in the renal tissues of rats among the six groups

The SR-A mRNA and protein levels were significantly decreased in the WT + I/R group compared with the WT + sham group 24 h after I/R (both *P* < 0.01). The SR-A mRNA and protein levels were significantly decreased 24 h after I/R in the SR-A^{-/-} + sham, SR-A^{-/-} + I/R and SR-A^{-/-} + I/R + NaHS groups compared with the WT + I/R group 24 h after I/R, while the levels in the SR-A^{-/-} + I/R + NaHS were obviously elevated (all *P* < 0.05) (Fig. 5).

Comparison of stress-autophagy levels of rats among the six groups

Our data showed that there were no significant differences in the mRNA and protein levels of the autophagy genes LC3 II/I, p-eIF2α and P62 between the WT + sham group and the SR-A^{-/-} + sham group 24 h after I/R (all *P* > 0.05). The protein levels of LC3I/II and p-eIF2α in the WT + I/R group, WT + I/R + NaHS group, SR-A^{-/-} + I/R group, and SR-A^{-/-} + I/R + NaHS group were significantly elevated compared with those in the WT + sham group 24 h after I/R, while the expression of P62 was apparently reduced (all *P* < 0.01). The mRNA and protein levels of LC3 II/I and p-eIF2α were significantly lower than those in the WT + IR group 24 h after I/R. The levels in the WT + I/R + NaHS and SR-A^{-/-} + I/R + NaHS groups were significantly reduced, but the trend for P62 was the opposite (all *P* < 0.05). The autophagy in the SR-A^{-/-} + I/R group was higher than that in the SR-A^{-/-} + sham group, and no marked differences in the protein levels of LC3 II/I, p-eIF2α and P62 were observed between the SR-A^{-/-} + I/R + NaHS group and the SR-A^{-/-} + I/R group (all *P* > 0.05) (Fig. 6A). Electron microscopy was used to observe autophagosome ultrastructure, and a large quantity of double-membraned autophagosomes was observed near the cell nuclei in the WT + I/R group (white arrow). A small amount of autophagosomes was observed near the cell nuclei in the WT + I/R + NaHS, SR-A^{-/-} + I/R and SR-A^{-/-} + I/R + NaHS groups (white arrows). However, no autophagosomes

Fig. 5. The expression of SR-A in rats of different groups 24 h after ischemia/reperfusion (n = 5). Notes: A, Western blotting was used to detect the expression of SR-A protein in rats of different groups 24 h after ischemia/reperfusion. B, qRT-PCR was used to detect the mRNA expression of SR-A in the renal tissues of rats in different groups 24 h after ischemia/reperfusion. **P* < 0.05 compared with the WT + sham group; #*P* < 0.05 compared with the WT + I/R group.

were found in the WT + sham or SR-A^{-/-} + sham groups (Fig. 6B). Additionally, qRT-PCR was used to detect ER stress pathway-related genes (Fig. 6C). The levels of the ER stress pathway genes GRP78, GPR94, ATF4, IRE1, XBP1, ATF6, and eIF2α were significantly increased in the WT + I/R group, WT + I/R + NaHS group, SR-A^{-/-} + I/R group and SR-A^{-/-} + I/R + NaHS group while decreased after the administration of NaHS compared with the WT + sham group (*P* < 0.05). I/R also induced the activation of genes involved in the ER stress pathway in the rats with SR-A knockdown. The expression of the above genes was not statistically significantly different between the SR-A^{-/-} + I/R + NaHS group and the SR-A^{-/-} + I/R group (all *P* > 0.05), which demonstrates that exogenous H₂S regulates SR-A ER stress through the PERK, ATF6 and IRE1 pathways.

Discussion

This study aimed to investigate the role of the exogenous H₂S-mediated SR-A signaling pathway in improving renal I/R outcomes by regulating ER stress/autophagy. Our data suggest a possible mechanism in which the inhibition of the SR-A signaling pathway will decrease urinary protein, BUN, SCR and MDA, increase SOD activity, and inhibit inflammation, apoptosis and ER stress/autophagy, which implies that exogenous H₂S may attenuate renal

We found that urinary protein, BUN and SCR were increased in renal IRI, while SR-A gene knockout, with increased H₂S, showed reduced levels of urinary protein. BUN and SCR increased SOD activity significantly, thus providing protective effects for renal IRI. Elevated levels of proteins in the urine have been widely regarded as a highly important determinant of renal complications in a large variety of populations and settings [28]. BUN and SCR are both considered specific markers to measure kidney function, and BUN concentration has been correlated with adverse outcomes. BUN has also been included in myocardial infarction risk prediction models, and the measurement of SCR has been used to diagnose kidney disease [29, 30]. SOD has been considered to play a protective role in renal IRI and is widely expressed in the kidneys and the vasculature. It might be considered as the most relevant molecule against ischemia-induced changes in the kidney, including the maintenance of renal blood flow [31]. Other than increased urinary protein, BUN and SCR may influence multi-organ damage, and SOD can decrease oxidative stress and injury and improve the

Fig. 6. Detection of endoplasmic reticulum stress/autophagy in rats of different groups 24 h after ischemia/reperfusion (n = 5). Notes: A, Western blotting was used to detect LC3 II/I and P62 protein levels in rats of different groups 24 h after ischemia/reperfusion. B, Electron microscopy was used to observe the structure of autophagosomes in the renal tissues of rats from different groups, and the white arrows represent the autophagosomes with bilayer membrane structures. C, qRT-PCR was used to detect the mRNA expression of GRP78, GPR94, ATF4, IRE1, XBP1, ATF6, and eIF2α in the renal tissues of rats in different groups 24 h after ischemia/reperfusion. **P* < 0.05 compared with the WT + sham group; #*P* < 0.05 compared with the WT + I/R group; &*P* < 0.05 compared with the SR-A^{-/-} + I/R group.

recovery of renal blood flow following renal I/R. SR-α is a critical molecule to increase oxidative stress, inflammation, and fibrosis in the pathogenesis of chronic kidney disease, and its deficiency can protect mice from progressive nephropathy, indicating that SR-α gene knockout may positively regulate urinary protein, BUN and SCR and positively modulate SOD [28, 31-33]. It is involved in the regulation of renal function and increases urinary sodium excretion through tubular and vascular actions in the kidney, and it can also alleviate renal IRI via antioxidant, anti-apoptotic, and anti-inflammatory effects, which the exact opposite of the roles of urinary protein. BUN and SCR are associated with the role of SOD in renal injury, implying that H₂S may inhibit urinary protein, BUN and SCR to increase SOD [25]. Mahavithakul et al. have suggested that the level of SCR in animals with SR deficiency was lower than that of control animals, indicating better-maintained kidney function [32]. H₂S therapy contributed to a remarkable reduction in kidney injury, with reduced SCR levels, and it protects glomerular function, thus providing a promising new approach to ameliorate renal IRI [34]. Because urinary protein levels, BUN and SCR are renal injury markers that are all increased in renal injury, it is not difficult to deduce that they are positively correlated with each other [28]. Once SCR is decreased, urinary protein and BUN will also show a downward trend [35]. The studies mentioned above are all in accordance with our result that increased H₂S or SR deficiency can lead to the reduction of urinary protein, BUN and SCR. Thus, renal IRI may be improved.

Our study also found that the mRNA and protein levels of IL-6 and TGF-β were higher in renal IRI, while SR-α gene knockout and increased H₂S could lower them, thereby attenuating renal IRI. As a pleiotropic cytokine, IL-6 produced by tubular epithelial cells primarily participates in regulating immune responses and exacerbating inflammatory

responses, and its levels in renal I/R were increased, which is regarded as a predictor of sustained and acute renal failure [36]. TGF- β is part of a large family of cytokines that regulate cellular responses in development and tissue repair, and TGF- β activation can lead to fibrogenic foci and trigger fibrogenesis in chronic renal injury [37]. The levels of IL-6 and TGF- β were higher in patients with nephropathy, promoting inflammation or fibrosis, while SR-A deficiency helped inhibit the TGF- β pathway and reduce inflammation to attenuate the injury [33]. IL-6 is closely associated with inflammation, suggesting that SR-A deficiency may negatively affect the expression of IL-6 and TGF- β [38]. H₂S has been reported to be involved in the negative regulation of inflammatory responses related to a variety of cytokines, thus suggesting that H₂S has the potential to decrease IL-6 and TGF- β [39]. The findings of Yang et al. suggested that SR-A gene knockout protects mice from progressive nephropathy by suppressing the TGF- β pathway [33]. When H₂S was decreased, IL-6 levels were increased, suggesting their negative correlation [39]. We also found that GRP78, eIF2 α , P62, GRP94, ATF4, IRE1, XBP1 and ATF6 were all expressed more highly in renal IRI, while SR-A gene knockout increased H₂S-inhibited autophagy by lowering their expression. Previous studies have demonstrated that the ER is involved in the cell death pathway, conversion of LC3 I/II and increased expression of P62 [40]. GRP78, CHOP, XBP1s, etc. are the ER stress markers that are often analyzed to investigate the mechanism of the ER stress-mediated apoptotic pathway [41]. The up-regulation of GRP78 is involved in autophagy activation, and the phosphorylation of eIF2 α is essential for autophagy. LC3 has been used as a specific marker to monitor autophagy, and the amount of LC3-II (conjugated form of LC3) is closely correlated with the number of autophagosomes [42-44]. H₂S can suppress ER stress, and ER stress is able to induce autophagy. Therefore, it is reasonable to conclude that H₂S can also suppress autophagy. As GRP78, eIF2 α and LC3-II are positively associated with autophagy, it is logical to deduce that H₂S can help decrease the levels of GRP78, eIF2 α and LC3-II [45, 46].

In summary, our findings may offer a novel therapy for the treatment of renal IRI. However, the concrete mechanism of how SR-A mediates H₂S remains unknown, and further studies are needed to develop this study.

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Disclosure Statement

There are no conflicts of interests concerning the publication of this paper.

References

1. Zhang Z, Zahedi K, Barone S, Tehrani K, Rabb H, Matlin K, Casero RA, Soleimani M: Overexpression of SSAT in kidney cells recapitulates various phenotypic aspects of kidney ischemia-reperfusion injury. *J Am Soc Nephrol* 2004;15:1844-1852.
2. Wu H, Chen G, Wyburn KR, Yin J, Bertolino P, Eris JM, Alexander SI, Sharland AF, Chadban SJ: TLR4 activation mediates kidney ischemia/reperfusion injury. *J Clin Invest* 2007;117:2847-2859.
3. Mehrabi A, Mood Zh A, Sadeghi M, Schmied BM, Muller SA, Welsch T, Kuttymuratov G, Wente MN, Weitz J, Zeier M, Morath C, Riediger C, Schemmer P, Encke J, Buchler MW, Schmidt J: Thymoglobulin and ischemia reperfusion injury in kidney and liver transplantation. *Nephrol Dial Transplant* 2007;22 Suppl 8:viii54-viii60.
4. Beiras-Fernandez A, Thein E, Chappel D, Gallego R, Fernandez-Roel D, Kemming G, Hammer C: Polyclonal anti-thymocyte globulins influence apoptosis in reperfused tissues after ischaemia in a non-human primate model. *Transpl Int* 2004;17:453-457.

- 5 Aydin Z, van Zonneveld AJ, de Fijter JW, Rabelink TJ: New horizons in prevention and treatment of ischaemic injury to kidney transplants. *Nephrol Dial Transplant* 2007;22:342-346.
- 6 Wu H, Huang T, Ying L, Han C, Li D, Xu Y, Zhang M, Mou S, Dong Z: MiR-155 is Involved in Renal Ischemia-Reperfusion Injury via Direct Targeting of FoxO3a and Regulating Renal Tubular Cell Pyroptosis. *Cell Physiol Biochem* 2016;40:1692-1705.
- 7 Li Y, Zhong D, Lei L, Jia Y, Zhou H, Yang B: Propofol Prevents Renal Ischemia-Reperfusion Injury via Inhibiting the Oxidative Stress Pathways. *Cell Physiol Biochem* 2015;37:14-26.
- 8 Preising C, Schneider R, Bucher M, Gekle M, Sauvant C: Regulation of Expression of Renal Organic Anion Transporters OAT1 and OAT3 in a Model of Ischemia/Reperfusion Injury. *Cell Physiol Biochem* 2015;37:11-23.
- 9 Decuypere JP, Ceulemans LJ, Agostinis P, Monbaliu D, Naesens M, Pirenne J, Jochmans I: Autophagy in the Kidney: Implications for Ischemia-Reperfusion Injury and Therapy. *Am J Kidney Dis* 2011;57:705-715.
- 10 Liu XH, Zhang ZY, Sun S, Wu XD: Ischemic postconditioning protects myocardium from ischemia-reperfusion injury through attenuating endoplasmic reticulum stress. *Shock* 2008;30:432-425.
- 11 Guan X, Qian Y, Shen Y, Zhang L, Du Y, Dai H, Qian J, Yan Y: Autophagy protects renal tubular cells against ischemia / reperfusion injury in a time-dependent manner. *Cell Physiol Biochem* 2011;27:285-298.
- 12 Zhang HY, Wang ZG, Lu XH, Kong XX, Wu FZ, Lin L, Tan X, Ye LB, Xiao J: Endoplasmic reticulum stress: relevance and therapeutics in central nervous system diseases. *Mol Neurobiol* 2015;51:1305-1352.
- 13 Petrovski G, Das S, Juhasz B, Kertesz A, Tosaki A, Das DK: Cardioprotection by endoplasmic reticulum stress-induced autophagy. *Antioxid Redox Signal* 2011;14:2191-2200.
- 14 Bobrovnikova-Marjon E, Pytel D, Riese MJ, Vaites LP, Singh N, Koretzky GA, Gajjar ES, Diehl JA: PERK utilizes intrinsic lipid kinase activity to generate phosphatidic acid, mediate Akt activation, and promote adipocyte differentiation. *Mol Cell Biol* 2012;32:2268-2278.
- 15 Birkenfeld AL, Lee HY, Majumdar S, Jurczak MJ, Campese AP, Jochimsen FR, Frederick DW, Guigni B, Kahn M, Zhang D, Weismann D, Arafat AM, Pfeiffer AF, Lieske-Hadomi S, Ron D, Samuel VT, Shulman GI: Influence of the hepatic eukaryotic initiation factor 2alpha (eIF2alpha) endoplasmic reticulum (ER) stress response pathway on insulin-mediated ER stress, hepatic lipid metabolism and peripheral glucose metabolism. *J Biol Chem* 2011;286:36163-36170.
- 16 Ni M, Zhang Y, Lee AS: Beyond the endoplasmic reticulum: atypical GRP78 in cell viability, signalling and therapeutic targeting. *Biochem J* 2012;444:1-12.
- 17 Mahfoudh-Boussaid A, Zaoui M, Hadj-Ayed K, Miled AH, Ghoul-Mazgar S, Saidane-Mosbahi D, Rosello-Catafau J, Ben Abdallah H: Attenuation of endoplasmic reticulum stress and mitochondrial injury in kidney with ischemia-reperfusion conditioning application and trimetazidine treatment. *J Biomed Sci* 2012;19:71.
- 18 Yorimitsu T, Nair U, Niggli V, Klionsky DJ: Endoplasmic reticulum stress triggers autophagy. *J Biol Chem* 2006;281:30299-30303.
- 19 Kolattukudy PE, Liu J: Regulation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant protein-1/CCR2 pathway. *Circ Res* 2012;110:174-189.
- 20 Platt N, Gordon S: Is the class A macrophage scavenger receptor (SR-A) multifunctional? - The mouse's tale. *J Clin Invest* 2001;108:649-654.
- 21 Wang S, Timm D, Seimon TA, Sadler A, Kolodgie FD, Virmani R, Tabas I: Signal transducer and activator of transcription-1 is critical for apoptosis in macrophages subjected to endoplasmic reticulum stress in vitro and advanced atherosclerotic lesions in vivo. *Circulation* 2008;117:940-951.
- 22 Kelley JL, Ozment TR, Li C, Schweitzer JB, Williams DL: Scavenger receptor-A (CD204): a two-edged sword in health and disease. *Crit Rev Immunol* 2014;34:241-261.
- 23 Jonansen D, Ytrehus K, Baxter GF: Exogenous hydrogen sulfide (H₂S) protects against regional myocardial ischemia-reperfusion injury--Evidence for a role of K⁺ ATP channels. *Basic Res Cardiol* 2006;101:53-60.
- 24 Ji Y, Pang QF, Xu G, Wang L, Wang JK, Zeng YM: Exogenous hydrogen sulfide postconditioning protects isolated rat hearts against ischemia-reperfusion injury. *Eur J Pharmacol* 2008;587:1-7.
- 25 Wu D, Wang J, Li H, Xue M, Ji A, Li Y: Role of Hydrogen Sulfide in Ischemia-Reperfusion Injury. *Oxid Med Cell Longev* 2015;2015:186908.
- 26 Gerasimova E, Lebedeva J, Yakovlev A, Zefirov A, Giniatullin R, Sitdikova G: Mechanisms of hydrogen sulfide (H₂S) action on synaptic transmission at the mouse neuromuscular junction. *Neuroscience* 2015;303:577-585.

- 27 Bian Y, Deng C, Li W, Lei Z, Li Y, Li X: A Comparative Study on the Biological Characteristics of Human Adipose-Derived Stem Cells from Lipectomy and Liposuction. *PLoS One* 2016;11:e0162343.
- 28 Lambers Heerspink HJ, Gansevoort RT, Brenner BM, Cooper ME, Parving HH, Shahinfar S, de Zeeuw D: Comparison of different measures of urinary protein excretion for prediction of renal events. *J Am Soc Nephrol* 2010;21:1355-1360.
- 29 Kirtane AJ, Leder DM, Waikar SS, Chertow GM, Ray KK, Pinto DS, Karmaliotis D, Burger AJ, Murphy SA, Cannon CP, Braunwald E, Gibson CM, Group TS: Serum blood urea nitrogen as an independent marker of subsequent mortality among patients with acute coronary syndromes and normal to mildly reduced glomerular filtration rates. *J Am Coll Cardiol* 2005;45:1781-1786.
- 30 Chang YK, Lee SJ, Chung BH, Kim YO, Shin YS: Renal histology in patients with elevated serum creatinine and concurrent normal urinalysis. *Clin Nephrol* 2012;77:283-289.
- 31 Schneider MP, Sullivan JC, Wach PF, Boesen EI, Yamamoto T, Fukai T, Harrison DG, Pollock DM, Block PC: Protective role of extracellular superoxide dismutase in renal ischemia/reperfusion injury. *Am J Physiol* 2010;298:R1374-R1381.
- 32 Leelahavanichkul A, Bocharov AV, Kurlander R, Baranova IN, Vishnyakova TG, Souza AL, Liu X, Dong X, Vaisman B, Amar M, Sviridov D, Chen Z, Remaley AT, Csako G, Patterson AP, Yuen PS, et al: Class B scavenger receptor types I and II and CD36 targeting improves survival and acute outcomes in mice. *J Immunol* 2012;188:2749-2758.
- 33 Wang W, He B, Shi W, Liang X, Ma J, Shan Z, Hu Z, Danesh FR: Deletion of scavenger receptor A protects mice from progressive nephropathy independent of lipid control during diet-induced hyperlipidemia. *Kidney Int* 2012;81:1002-1014.
- 34 Hunter JP, Hosgood SA, Patel M, Rose R, Read K, Nicholson ML: Effects of hydrogen sulphide in an experimental model of renal ischaemia-reperfusion injury. *Int J Surg* 2012;99:1665-1671.
- 35 Maimoona A, Naeem I, Saddiqe Z, Jameel K: A review of biological, pharmacological and clinical aspects of French maritime pine bark extract. *J Ethnopharmacol* 2013;133:261-277.
- 36 Patel NS, Chatterjee PK, Di Paola R, Mazzon E, Britti D, De Luca A, Cuzzocrea S, Thiemermann C: Endogenous interleukin-6 enhances the renal dysfunction, and inflammation caused by ischemia/reperfusion. *J Pharmacol Exp Ther* 2005;312:170-177.
- 37 Bottinger EP: TGF-beta in renal injury and disease. *Semin Nephrol* 2007;27:309-320.
- 38 Kalliakmani P, Nakopoulou L, Tsakalieris C, Papasotiriou M, Goumenos DS: Urinary interleukin-6 (IL-6) and transforming growth factor-beta levels in corticosteroid-treated patients with IgA nephropathy. *Clin Nephrol* 2006;66:144-149.
- 39 Li T, Zhao B, Wang C, Wang J, Li W, Jin H, Tang C, Du J: Regulatory effects of hydrogen sulfide on IL-6, IL-8 and IL-10 levels in the plasma and pulmonary tissue of rats with acute lung injury. *Exp Biol Med* (Maywood) 2008;233:1081-1087.
- 40 Suparji NS, Chan G, Saito H, Akiyama NM, In LL, Awang K, Hasima Nagoor N: Geranylated 4-Phenylcoumarins Exhibit Anticancer Effects Against Human Prostate Cancer Cells through Caspase-Independent Mechanism. *PLoS One* 2016;11:e0151472.
- 41 Yang Y, Sun M, Han X, Ma H, Ma W, Wang Z, Pei X, Wang Y: Endoplasmic reticulum stress-mediated apoptotic pathway involved in corpus luteum regression in rats. *Reprod Sci* 2015;22:572-584.
- 42 Li XY, Zhang L, Song DD, Zhou J, Han R, Qin ZH, Sheng R: Endoplasmic reticulum chaperone GRP78 is involved in autophagy activation induced by ischemic preconditioning in neural cells. *Mol Brain* 2015;8:20.
- 43 Weng X, Wang D, Weiss S, Zhong H, Osman I, Gardner LB: Phosphorylation of eIF2alpha triggered by mTORC1 inhibition and PP6C activation is required for autophagy and is aberrant in PP6C-mutated glioma. *Sci Signal* 2015;8:ra27.
- 44 Kuma A, Matsui M, Mizushima N: LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: caution in the interpretation of LC3 localization. *Autophagy* 2007;3:323-328.
- 45 Li MH, Tang JP, Zhang P, Li X, Wang CY, Wei HJ, Yang XF, Zou W, Tang XQ: Disturbance of endogenous hydrogen sulfide generation and endoplasmic reticulum stress in hippocampus are involved in homocysteine-induced defect in learning and memory of rats. *Behav Brain Res* 2014;262:35-41.
- 46 Yorimitsu T, Klionsky DJ: Endoplasmic reticulum stress: a new pathway to induce autophagy. *Autophagy* 2007;3:160-162.