**Original Article**

**GYY4137 attenuated renal ischemia-reperfusion injury by protecting against oxidative stress, apoptosis and inflammation**

Hongchao Zhao¹, Yang Du², Xiuheng Liu², Hengcheng Zhu²

¹Department of Urology, Hubei Provincial Hospital of Traditional Chinese Medicine, Wuhan, Hubei, China; ²Department of Urology, Renmin Hospital of Wuhan University, Wuhan, Hubei, China

Received October 10, 2016; Accepted December 8, 2016; Epub February 15, 2017; Published February 28, 2017

**Abstract:** Hydrogen sulfide (H₂S) has been known as a gasotransmitter in renal ischemia-reperfusion injury (RIRI) for years. GYY4137 as a novel, water-soluble, slow-releasing H₂S donor has attracted more and more concern recently. The aim of this study is to investigate the efficacy of GYY4137 in protecting against renal IRI. Male Sprague-Dawley rats were treated with GYY4137 10 ml at different concentration intraperitoneally (100, 200, 400 μmol/L). Then, rats were subjected to 45 min of left renal pedicle occlusion followed by reperfusion of 24 hours. We found that GYY4137 decreased Scr and BUN, alleviated histological injury and cellular apoptosis after renal I/R. GYY4137 also decreased MDA and increased SOD levels in serum, attenuated oxidative stress after renal I/R. Meanwhile, GYY4137 increased the expression of Bcl-2 and decreased the expression of Bax, NF-κB and caspase-3 activity to inhibit apoptosis. GYY4137 decreased the inflammatory factors significantly, included TNF-α, ICAM-1, IL-2 and IL-6. In conclusion, the results demonstrated that GYY4137 protects against renal ischemia-reperfusion injury by attenuating oxidative stress, apoptosis and inflammation. This might be a much promising therapy in ameliorating renal IRI in clinic.

**Keywords:** Hydrogen sulfide, ischemia reperfusion, renal injury, oxidative stress, apoptosis

**Introduction**

Renal ischemia-reperfusion injury (IRI) is an inevitable event during kidney transplantation and partial nephrectomy. The major aim of renal I/R is to reduce the bleeding and to optimize the operation time [1]. However, renal IRI is a common cause of acute kidney injury (AKI), which has complicated pathophysiological characteristics including renal tubular cell necrosis, cell apoptosis, extracellular matrix (ECM) degradation and interstitial inflammatory [2, 3]. It leads to an irreversible renal functional damage and bring patients tremendous pain. To solve this problem, many researches were carried on. Studies reported that H₂S has noteworthy effect on protecting against renal IRI by reducing oxidative stress [4].

Hydrogen sulfide (H₂S) has been recognized as a metabolic poison similar in potency to cyanide for a long time. No less than nitric oxide (NO) and carbon monoxide (CO), H₂S has been known as the third gaseous signaling molecule following NO and CO [5, 6]. Recently, H₂S has been confirmed as an effective molecule, which has the ability to protect against I/R of many organs, included center nervous, intestinal and heart [7-9]. To date, H₂S-releasing “drugs” have been already used in biological experiments. Nonetheless, it has been largely restricted to simple sulfide salts, most commonly sodium hydrosulfide (NaHS), which releases H₂S instantaneously in aqueous solution [4]. NaHS has been widely used in biological and pharmacological experiment as donor of H₂S. However, when dissolved in water, NaHS releases copious amounts of H₂S in a short time. The reaction occurs in seconds so that NaHS cannot be an effectively drug to mimic the time course of H₂S release in vivo [10].

A new drug-Morpholin-4-iium 4 methoxyphenyl (morpholino) phosphinodithioate (GYY4137) releases low but consistent concentrations of H₂S over several hours in aqueous solution at physi-
GYY4137 attenuated renal ischemia-reperfusion injury

ological pH and temperature, and better mimics the time course of H$_2$S release in vivo as a slow-release drug. GYY4137 is one of a series of compounds synthesized in this laboratory on the basis of the structure of Lawesson’s compound, which releases H$_2$S in organic solvents [11]. It has been already researched in myocardial ischemia reperfusion injury, acute lung injury and other diseases. And its protective ability has already been confirmed in these diseases [12, 13].

In this study, we firstly used GYY4137, as the slow-releasing H$_2$S donor, to detect the protective effects against renal IRI and to investigate the possible signaling mechanisms that involved. From the present results, we raise the possibility that the treatment of GYY4137 might be an effective therapeutic method to suppress and ameliorate the progress of renal IRI.

Materials and methods

Animal

Male Sprague-Dawley rats (n=48), weighing 250-280 g, aged 6-8 weeks, were obtained from the Renmin Hospital of Wuhan University Animal Center. All rats are housed in a standard environment, under a 12-h light/dark cycles, with access to water and a standard laboratory diet. All procedures and protocols used in the present study were approved by Experimental Animal Ethics Committee of Wuhan University, and the guidelines of the National Institutes of Health Guide for the Care and Use of laboratory animals were followed.

Surgical technique

Forty-eight rats were randomly divided into 6 groups (n=8, each): sham group, I/R group, I/R+GYY4137 groups (100, 200, 400 μmol/L, 10 ml) and sham+GYY4137 group (400 μmol/L, 10 ml). All animals were anesthetized by inhalation of 5% isoflurane, maintained under anesthesia with 2% isoflurane during surgery and 1% isoflurane during reperfusion. A right nephrectomy was initially performed via a midline abdominal incision to exclude the potential protective effects of contralateral kidney. The left renal pedicle was subsequently occluded via atraumatic clamping for 45 min followed by reperfusion. Animals in sham group underwent a surgical procedure identical to that of the ischemia and treatment groups but without cross-clamping of the renal pedicle. However, they received an equal volume of PBS. Rats were recovered for 24 h and then anesthetized to collect blood samples from heart. All of the left kidney tissues are divided into two parts. Half were immediately removed, frozen in liquid nitrogen, and stored at -80°C until processed. The other were removed and fixed in 10% formalin for histological examination. During the performance of these experiments, all animals were survived. At the end of the sampling, animals were sacrificed by bleeding during anesthesia.

Renal function detection

Blood samples were collected by cardiac puncture and placed at room temperature for 2 hours. Then we separated serum by centrifugation at 1,000 g for 15 min at 4°C and obtained the supernatants. Serum creatinine (Scr) and blood urea nitrogen (BUN) levels were determined at the Clinical Laboratory of Renmin Hospital of Wuhan University. Each measurement was performed in triplicate.

MDA & SOD

Malondialdehyde (MDA) level and superoxide dismutase (SOD) activity were detected in the supernatant of renal tissue. MDA level was measured by using a fluorometric method [14]. Briefly, 50 μL MDA buffer and 1 mL ddH$_2$O were added to 10 mL glass tubes. And 1 ml 29 mmol/L thiobarbituric acid was added and mixed for protein precipitating. All samples were incubated with boiling water for 1 hr. The mixture was quickly placed on ice for 10 min. Added 25 μL 5 mol/L HCl and 3.5 mL n-butanol then agitated for 10 min. The butanol phase was separated by centrifugation at 2000 g for 10 min. The absorbing values were measured by using the same steady-state fluorescence spectrophotometer (HORIBA Trading Co., Ltd., Shanghai, China) at 532 nm [15, 16]. SOD activity was determined in the supernatant of renal tissue. SOD estimation was detected by assessing the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with 2-(4-iiodophenyl)-3 (4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity is then measured by considering the degree of inhibition of this reaction [14, 17].
**Hematoxylin and eosin (H&E) staining**

Kidney damage was assessed using H&E staining. 24 h after surgery, left kidneys were removed and fixed in formalin (4% buffered neutral formalin). The histological evaluation was detected by the pathology department of Renmin hospital of Wuhan University, which was blinded to the treatments given, performed the morphological assessment. Transverse slices of the left kidneys were fixed by formalin (10% phosphate buffered) and then dehydrated. Paraffin-embedded kidney sections (4 mm) were stained by hematoxylin and eosin. Evaluation of the renal was based on the presence and extent of necrosis, cellular degeneration and vacuolization, tubular obstruction, and formation of luminal debris and casts.

**Tunel staining**

Terminal-deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining was performed to evaluate cellular apoptosis. The kidney tissues were fixed in 4% formaldehyde, embedded in paraffin, cut into 4-μm thickness sections and treated as indicated in manufacturer’s instructions of the in situ cell death detection kit (Roche, Mannheim, Germany). Thereafter, nuclei were co-stained with hematoxylin (Beyotime, Haimen, Jiangsu, China).

**Immunohistochemical staining**

Kidney tissue samples were stained by immunohistochemistry (IHC) with anti-NF-κB (p65) or anti-Bcl-2 monoclonal antibody (Roche) and tested for NF-κB (p65) or Bcl-2 protein expression with the OptiView® DAB IHC Detection kit and the OptiView® Amplification kit (Ventana Medical Systems, Inc., Tucson, AZ, USA). According to the manufacturer’s instruction manual, 4 μm thick FFPE sections were prepared for IHC staining, which was performed automatically using the Ventana BenchMark XT Stainer (Ventana Medical Systems Inc., Tucson, AZ, USA). The IHC stains were evaluated for expression of NF-κB (p65) and Bcl-2 by 3 pathologists (pathology department, Renmin hospital of Wuhan University).

**RNA isolated and real-time PCR**

Total RNA was isolated from left kidney tissue by using TRIzol® reagent (Invitrogen) according to the manufacturer’s protocol. The cDNA was synthesized from total RNA following the manufacturer’s protocols from Applied Biosystems® (Carsbad, CA).

Real-time PCR was performed by using SYBR® Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan) in a 25 μl reaction volume (2 μl cDNA template, 12.5 μl SYBR Premix Ex Taq, 1 μl forward primer, 1 μl reverse primer, and 0.5 μl ROX™ Reference Dye II) on a MJ Opticon Monitor Chromo4™ instrument (Bio-Rad Laboratories Inc., Hercules, CA). The following protocol was used for RNAs: 93°C for 4 min; 40 Cycles of 93°C for 30 s, 60°C for 30 s and 70°C for 30 s. The primers were designed and synthesized by GenePharma (Shanghai, China).

**Western blots analysis**

Kidney tissues were suspended in RIPA buffer (Roche Diagnostics Corp., Indianapolis, IN) and homogenized by a homogenizer (Beijing Kwinbon Biotechnology Co., Ltd., Beijing, China), then centrifuged at 10,000 g for 30 min to remove tissues debris. The protein content was determined with the bicinchoninic acid (BCA) protein assay. Protein samples were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MS) by Western blotting. The membranes were blocked in 5% skimmed milk and incubated for 1-2 h at room temperature. Then the membranes were incubated overnight with the appropriate primary antibodies respectively. In this study, antibodies directed against Bcl-2, NF-κB, caspase-3 (each at 1:500; Proteintech Group Inc., Chicago, IL) and GAPDH (1:1000; Cell Signaling Technology®, Danvers, MA) were used. After being washed with Tris-buffered saline containing 0.1% Tween 20 buffer (TBST) for 3 times, the membranes were incubated with secondary antibodies for 2 h at room temperature. The secondary antibodies were goat anti-rabbit horseradish peroxidase or goat anti-mouse horseradish peroxidase (Bio-Rad Laboratories Inc.) used at 1: 10,000-20,000, respectively. After incubated by the secondary antibodies, the membranes were washed with TBST for 3 times. The protein bands were detected by chemiluminescence systemand densitometric
GYY4137 attenuated renal ischemia-reperfusion injury

**Statistical analysis**

All data are expressed as mean ± SD and were analyzed by Student’s paired t test or one-way ANOVA for different groups (GraphPad Prism 5.0 software). A value of $P<0.05$ was considered statistically significant.

**Results**

GYY4137 reduced the damage of nephropathy function that caused by renal IRI

Serum creatinine (Scr) level and blood urea nitrogen (BUN) level has been measured to assess overall renal function. Compared with the sham group, I/R group demonstrated a significant rise in Scr (380 ± 6.40 vs. 80 ± 2.26...
μmol/L, P<0.05) and BUN (35 ± 0.72 vs. 8 ± 0.53 mmol/L, P<0.05) levels. When treated with different concentrations of GYY4137 in the surgery, the levels of Scr and BUN declined significantly. In the concentration of 200 μmol/L, the Scr (120 ± 4.89 vs. 380 ± 6.40 μmol/L, P<0.05) and BUN (15 ± 0.62 vs. 35 ± 0.72 mmol/L, P<0.05) declined significantly. However, when the concentration increased to 400 μmol/L, the decreased degree was the same as in the concentration of 200 μmol/L. (Figure 1).

GYY4137 reduced the damage of oxidative stress that caused by renal IRI

Superoxide dismutase (SOD) and malondialdehyde (MDA) were the indices of oxidative stress. Compared with the sham group, I/R group showed a significant reduction in the renal SOD (20 ± 0.73 vs. 32 ± 0.66 μ/g, P<0.05) activity. Treatment with GYY4137 in the surgery, especially in the concentration of 200 μmol/L, the levels of renal SOD activity (35 ± 0.75 vs. 20 ± 0.73 μ/g, P<0.05) significantly increased compared with the levels in the I/R group (Figure 2A). Compared with the sham group, I/R group showed a significant increase in the renal MDA concentration (3 ± 0.48 vs. 1.8 ± 0.09 μmol/100 mg, P<0.05). Treatment with 200 μmol/L GYY4137 during surgery, significantly reduced the levels of renal MDA (2 ± 0.16 vs. 3 ± 0.48 μmol/100 mg, P<0.05) compared with the I/R group (Figure 2B). But when the concentration increased to 400 μmol/L, the decreased degree has no difference from giving the concentration of 200 μmol/L.

GYY4137 slowed the progress of senescence, necrosis and apoptosis of kidney tissue after renal IRI

HE and TUNEL staining were performed to confirm the histopathological changes and apoptosis of the kidneys. Figure 3A shows the HE staining of kidneys tissue in each group. In the kidney sections of the sham group, there were no significant changes observed by light microscopy. Compared with sham group, the kidney sections of I/R group showed severe changes in the tubules, included the destruction of the tubular cells, flattening of the tubules, and necrosis of the straight proximal tubules. Significant tubular obstructions were present, especially in the more distal tubules. The GYY4137 treated tissues displayed lower amount of tubular cast formation. And no detectable cellular necrosis was seen.

The TUNEL staining showed that cellular apoptosis occurred in I/R group. And as it displayed in Figure 3B, the cellular apoptosis receded while treated with GYY4137. It indicated an effective therapeutic response that GYY4137 made in the progress of senescence, necrosis and apoptosis of kidney tissue after renal IRI.
GY4137 protected against the apoptosis via up-regulation of Bcl-2 and down-regulation of NF-κB (p65) and Bax

To find out how GYY4137 protected against renal IR injury, we used immunohistochemistry, western blot and real-time qPCR to detect the mechanism. The immunohistochemistry results showed a high expression of Bcl-2 in sham group. In I/R group, the Bcl-2 expression decreased significantly. When used GYY4137, the expression of Bcl-2 became high again. It means GYY4137 are able to reverse the decrease of Bcl-2 that caused by I/R (Figure 4A). Then we detected the expression of NF-κB (p65) and found that the expression of NF-κB (p65) were contrary to Bcl-2. The results showed a low expression of NF-κB (p65) in sham group, but a significant high expression in I/R group. Treated with GYY4137, the expression of NF-κB (p65) increase unconspicuous compared with the I/R group. It indicated that GYY4137 restrained the high expression of NF-κB (p65) that caused by I/R (Figure 4B).

Western blot results that the expression of Bcl-2 reduced in I/R group, but regained in GYY4137 group. Different concentrations of GYY4137 all showed a high expression of Bcl-2. The NF-κB (p65) expression showed a con-
GYY4137 attenuated renal ischemia-reperfusion injury

The expression of TNF-α, ICAM-1, IL-2 and IL-6 were detected by real-time PCR. All data are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA followed by post hoc Tukey test. *P<0.05 vs. sham group, #P<0.05 vs. I/R group.

GYY4137 reduced the inflammatory reaction that caused by renal IRI via down regulated TNF-A, ICAM-1, IL-2 and IL-6

We detected the expression of TNF-α, ICAM-1, IL-2 and IL-6 by real-time PCR. The results showed that the expression of these factors in mRNA level increased after renal IRI. However, in GYY4137 group, the expression of these inflammatory factors decreased significantly (Figure 5).

Discussion

In present study, we first built animal models. Before the surgery, we gave the rats intraperitoneal injection of GYY4137 or PBS. Then the rats were given 45 min ischemia to the functional kidneys which led to acute kidney injury. After 24 h, indices of renal function, oxidative stress, histological changes, apoptosis and inflammation were detected. Serum creatinine and BUN were used as markers of renal function in clinical practice as they are the simplest and most widely used indices [18]. SOD and MDA were used as indices of oxidative stress. Histological changes and apoptosis were demonstrated by HE staining and TUNEL staining. And inflammatory factor were detected by real-time PCR.

The results showed that GYY4137 can protect against the renal function damage by reversing the high levels of serum creatinine and BUN caused by renal I/R. Then the SOD and MDA detection indicated that GYY4137 can alleviate the damage of oxidative stress by increasing SOD and decreasing MDA. HE staining and TUNEL showed that GYY4137 can slow down the progress of senescence, necrosis and apoptosis of kidney tissue after renal I/R.

Further, we detected the mechanism by immunohistochemistry, western blot and RT-PCR. We found that I/R group showed down-regulation of Bcl-2 and up-regulation of NF-κB and Bax, while GYY4137 group present the reverse. NF-κB family, which is multifunctional nuclear factors which regulates several process, included inflammation, cells proliferation, apoptosis and death [19, 20]. The roles of NF-κB family and its signaling pathway in apoptosis has been the focus of intense investigation all over the world [21]. Researchers showed that NF-κB signaling pathway exerts anti-apoptotic function via many proteins, including Bcl-2, TRAF, JNK, FLIP, A20 and so on [22]. Researches claimed that the kinds and numbers of the subunit of NF-κB determined the apoptosis progression, especially p65 overexpression led to apoptosis [23]. Studies also reported that overexpression of Bcl-2 down-regulated the activity of NF-κB inhibiting apoptosis [24]. In present study, GYY4137 reduced the expression of NF-κB and reduced cell apoptosis.

Generally, the expression of Bcl-2 and Bax remain stable. When the expression of Bax increased, the number of Bax/Bax homodimer increase, so that cells’ sensibility to death signal aggrandized the cell apoptosis process started. In the other way, overexpression of Bcl-2 leads to a large number of dissociation of Bax/Bax homodimer and production of Bcl-2/Bax heterodimer, which is more stable and against the apoptosis [25]. Many studies suggest that the ratio of these two proteins is the key to determine the cell apoptosis and death.
GYY4137 attenuated renal ischemia-reperfusion injury

Research has reported that they protecting renal IRI via regulating apoptosis [27]. Recently, the relationship of caspase-3, Bcl-2 and Bax has been studied. Some studies claimed that Bcl-2 and Bax not only regulated the upstream region to control the activity of caspase-3, but also been a substrate to act on its downstream [28]. In present study, GYY4137 increased the expression of Bcl-2 and reduced the expression of Bax and caspase-3. So we make a point that GYY4137 has the ability to inhibit apoptosis.

Then we detected the inflammatory reaction by RT-PCR and got the results that GYY4137 reduced the expression of TNF-A, ICAM-1, IL-2 and IL-6 significantly. So we reach a decision that GYY4137 can release the inflammatory reaction and mitigate the renal damage.

In conclusion, we confirmed that GYY4137 significantly attenuated I/R injury in the kidney by improvement of functional indices, oxidative stress, histological changes and apoptosis. H2S had the effect of anti-inflammatory, anti-apoptosis, anti-oxidant and so on. GYY4137 as a promising H2S alternative medicine needs to be further investigated in cell experiment and clinic practice. Hope one day, it can be an effective therapy to control the bad consequence that caused by renal I/R and improve renal surgery.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hengcheng Zhu, Department of Urology, Renmin Hospital of Wuhan University, 99 Jiefang Road, Wuchang District, Wuhan 430060, Hubei, China. Tel: +86-13707133853; E-mail: zhuhcqi@126.com

References


