Protective Effects of GYY4137 on Renal Ischaemia/Reperfusion Injury through Nrf2-Mediated Antioxidant Defence

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Keywords

GY4137 · Kidney · Reperfusion injury · Oxidative stress

Abstract

Introduction/Aims: Hydrogen sulfide (H₂S) is considered to be the third most important endogenous gasotransmitter in organisms. GYY4137 is a long-acting donor for H₂S, a gas transmitter that has been shown to prevent multi-organ damage in animal studies. We previously reported the effect of GYY4137 on cardiac ischaemia reperfusion injury (IRI) in diabetic mice. However, the role and mechanism of GYY4137 in renal IRI are poorly understood. The aims of this study were to determine whether GYY4137 can effectively alleviate the injury induced by renal ischaemia reperfusion and to explore its possible mechanism. **Methods:** Mice received right nephrectomy and clipping of the left renal pedicle for 45 min. GYY4137 was administered by intraperitoneal injection for 2 consecutive days before the operation. The model of hypoxia/reoxygenation injury was established in HK-2 cells, which were pre-treated with or without GYY4137. Renal histology, function, apoptosis, and oxidative stress were measured. Western blot was used to measure the target protein after renal IRI. **Results:** The results indicated that

GY4137 had a clear protective effect on renal IRI as reflected by the attenuation of renal dysfunction, renal tubule injury, and apoptosis. Moreover, GYY4137 remarkably reduced renal IRI-induced oxidative stress. GYY4137 significantly elevated the nuclear translocation of nuclear factor-erythroid-2-related factor 2 (Nrf2) and the expression of antioxidant enzymes regulated by Nrf2, including SOD, HO-1, and NQO-1. **Conclusions:** GYY4137 alleviates ischaemia reperfusion-induced renal injury through activating the antioxidant effect mediated by Nrf2 signalling.

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Introduction

Acute kidney injury (AKI) is a common complex clinical syndrome mainly caused by sepsis, rhabdomyolysis, renal ischaemia reperfusion injury (IRI), and other diseases, leading to kidney failure with a high rate of morbidity and mortality [1, 2]. Renal IRI is one of the main risk factors of clinical AKI. The pathological mechanisms of renal IRI are

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mainly related to oxidative stress, inflammation, and apoptosis [3, 4]. Therefore, effective measures to reduce renal IRI can decrease the occurrence of clinical AKI.

Many studies have shown that H₂S exerts protective effects to alleviate renal, myocardial, hepatic, and intestinal IRI in animal models [5–8]. However, the limitations of H₂S donor compounds have hindered their effective clinical application. Inorganic sulfide salts (Na₂S and NaHS) have effectively prevented renal IRI through up-regulation of heat shock proteins and suppression of inflammation, but their chemical composition is impure and their structure is unstable [9, 10]. Morpholin-4-ium-4-methoxyphenyl phosphinodithioate (GY4137) is a H₂S donor that releases H₂S at a slow steady rate at physiological pH and temperature [11]. GY4137 treatment also provides protective effects in myocardial ischaemic injury [11, 12]. However, whether this compound has a similar protective effect in renal IRI is unclear.

It has been shown that oxidative stress is one of the major causes of pathogenesis of renal IRI [13]. The scavenging of reactive free radicals depends on a series of antioxidant enzymes. The nuclear factor-erythroid-2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that exerts an important regulatory role in the expression of antioxidant and prevents cell injury induced by oxidative stress [14]. Studies have shown that Nrf2-deficient mice are significantly more susceptible to both ischaemic and nephrotoxic kidney injury [15]. During the early stage of renal IRI, Nrf2 hyperactivation can prevent the progression of renal tubule injury [16]. The above studies indicate that the activation of Nrf2 plays a pivotal role in renal IRI.

Based on the above background, we sought to investigate the effect and mechanism of GY4137 on renal IRI. The main purposes of this study were 2-fold: (1) to determine whether GY4137 alleviates renal injury after ischaemia reperfusion and (2) to determine whether renal protection is related to the antioxidant effect induced by activation of the Nrf2 signalling pathway.

Materials and Methods

Animals

Male C57BL/6 mice (25 ± 2 g, approximately 8 weeks old) were purchased from Shanghai SLRC Laboratory Animal Co., Ltd. (Shanghai, China). All mice were raised and given standard food and water ad libitum. The experimental protocol was approved by the Animal Care and Use Committee of Nanjing Medical University, and the animal procedures were carried out in accordance with the National Institutes of the Guide for the Care and Use of Laboratory Animals.

Experimental Protocol

The model of renal IRI-induced AKI was produced as previously described [17]. The mice were randomly separated into 4 groups: sham-operated (Sham); Sham plus GY4137 (GY); renal ischaemia reperfusion (IR); IR plus GY4137 (IR + GY). GY4137 was dissolved in saline. These mice were given 50 mg/kg GY4137 via intraperitoneal injection (Sigma-Aldrich, Germany) for 2 consecutive days before the operation [12]. Mice in the other groups received an equal amount of saline in the same way.

Measurement of Renal Function

At the end of the observation, blood and tissue samples were collected. Plasma creatinine (Cr) and blood urea nitrogen (BUN) levels were analysed using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China). The experiments were carried out in accordance with the instructions provided by suppliers.

Histological Evaluation and Renal Apoptosis

Renal tissue was assessed by haematoxylin and eosin staining (H&E) as previously mentioned [18]. Ten high-power fields (magnification, ×400) of cortical and extramedullary areas were randomly selected from each kidney. Histopathological damage was defined as cellular necrosis, loss of brush border, interstitial oedema, cellular vacuolation, dilatation of the lumen, and cast formation. These factors were scored as follows: no injury (0); mild: <25% (1); moderate: <50% (2); severe: <75% (3); and very severe: >75% (4) [19].

Apoptosis was measured by a terminal transferase dUTP nick-end labelling (TUNEL) kit (Roche Molecular Biochemicals Inc., Mannheim, Germany) according to the protocol provided by the manufacturer. For the evaluation of apoptosis, TUNEL-positive cells were counted using a microscope at ×400 magnification. Three fields were analysed randomly in each tissue section.

Measurement of Oxidative Stress

Malondialdehyde (MDA) and total superoxide dismutase (SOD) assay kits (Nanjing, China) were purchased from Jiancheng Bioengineering Institute. MDA levels in the kidney tissue or human renal tubular epithelial (HK-2) cells were measured using the thiobarbituric acid method. According to the instructions, the maximum absorbance of the reaction mixture was determined at 532 nm. Total SOD activity in kidney tissue or HK-2 cells was assessed using the hydroxylamine method, and the absorbance was measured at 550 nm. Tissue 15-F2t-isoprostane was assessed using a specific ELISA kit (Cayman Chemical) according to the instructions provided by the suppliers.

Cell Protocol

HK-2 cells were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). HK-2 cells were cultured in Dulbecco's modified Eagle medium (Thermo Fisher, Hudson, NH, USA) supplemented with 10% foetal bovine serum (GIBCO, Grand Island, NY, USA) in a 37°C humidified incubator with an atmosphere of 5% CO₂. The cells were sub-cultured or subjected to experimental procedures at 80–90% confluence. The hypoxia/reoxygenation (H/R) cell model was established by changing the medium to serum/glucose-free DMEM medium and then incubating in a Modular Incubator Chamber (Wuxi PuHe Biological Medicine Tech-

Fig. 1. GYY4137 alleviates renal injury. **A** H&E staining of kidney sections ($\times 400$). **B** Results of total histopathological scores reflecting tubular damage. **C, D** Circulating levels of BUN and Cr. $n = 5$ per group; $*p < 0.05$ compared with the IR group. H&E, haematoxylin and eosin; BUN, blood urea nitrogen; Cr, creatinine.

nology Co., Ltd., Wuxi, China). The chamber was flushed with 94% N_2 and 5% CO_2 . After 4 h of hypoxia, HK-2 cells were cultured in complete medium and then returned to a conventional incubator (5% CO_2 and 95% air) for 12 h of reoxygenation.

The HK-2 cell preparations were randomly divided into 6 groups: (1) Con group: cells were pre-treated with PBS for 48 h without performing hypoxia/reoxygenation; (2) GYY group: cells were pre-treated with 100 μM GYY4137 [12, 20] for 48 h without performing hypoxia/reoxygenation; (3) HR group: cells were pre-treated with PBS for 48 h before hypoxia/reoxygenation; (4) HR + GYY group: cells were pre-treated with 100 μM GYY4137 for 48 h before hypoxia/reoxygenation; (5) HR + GYY + siCON: cells were pre-treated with 100 μM GYY4137 and control siRNA for 48 h before hypoxia/reoxygenation; (6) HR + GYY + siNrf2: cells were pre-treated with 100 μM GYY4137 and Nrf2 siRNA (GenePharma Co., Shanghai, China) for 48 h before hypoxia/reoxygenation. Cells were transfected with 50 nM specific or control siRNA oligonucleotides using Lipofectamine^{TM} 2,000 transfection reagent (Invitrogen Ltd., Foster, CA, USA) according to the manufacturer's protocol.

Cell Viability Assay

Cell viability was measured by the Cell Counting Kit-8 (MCE, New Jersey, USA) according to the manufacturer's instructions. Culture medium samples were collected for measurement of LDH by a colorimetric method using a commercially available kit (Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

HK-2 cells were maintained in lysis buffer, supplemented with 1% DTT at 0°C for 20–60 min, and then centrifuged. Pro-

tein concentration was measured by using a bicinchoninic acid method (Sigma-Aldrich, Germany). Caspase-3 activity was measured by a caspase-3 colorimetric assay kit (Keygen Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions.

Western Blot Analysis

As described in [12], total cytosolic and nuclear components were extracted from renal tissues or cells with a Protein Extraction Kit (Keygen Biotech, China) containing a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich, Germany). Samples (50 μg per lane) were loaded and then separated by SDS-PAGE (10% gels) and transferred to PVDF membranes. The PVDF membranes were incubated with the following primary antibodies: Nrf2, HO-1, and NQO-1 (Abcam, Cambridge, UK; 1:1,000), Bax and Bcl-2 (Abmart, Shanghai, China), and cleaved caspase-3 (Cell Signalling Technology, Danvers, USA) at 4°C overnight. After extensive washing, primary antibody binding was tested with a secondary antibody (Cell Signalling Technology, Danvers, USA; 1:5,000). Protein bands were detected by using a standard ECL method, and images were measured by using a densitometer with analysis software (ImageJ 1.4).

Statistical Analysis

All values were expressed as the mean $\pm$ SD. Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test when the data involved 3 or more groups (GraphPad Prism, San Diego, CA). Student's t test was employed for pairwise comparisons. $p \leq 0.05$ was set as the significance level.

Fig. 2. GYY4137 decreases renal apoptosis after IRI. **A** Renal apoptosis detected by TUNEL staining ($\times 400$). **B** Western blotting of CC3, Bax, and Bcl-2. **C** Apoptosis quantification was performed by counting TUNEL-positive cells. **D, E** Bar graphs of CC3 and Bax/Bcl-2 ratio. $n = 5$ per group; $*p < 0.05$ compared with the IR group. IRI, ischaemia reperfusion injury; CC3, cleaved caspase-3; IR, renal ischaemia reperfusion.

Fig. 3. GYY4137 suppresses oxidative stress in the kidney after IRI. **A** Renal MDA levels assessed by a kit. **B** Renal 15-F2t-isoprostane levels assessed by a kit. $n = 5$ per group; $*p < 0.05$, compared with the IR group. IRI, ischaemia reperfusion injury; MDA, malondialdehyde; IR, renal ischaemia reperfusion.

Results

GYY4137 Attenuates Renal Injury after IR

Morphological damage to the kidney was evaluated by H&E staining. As shown in Figure 1A, the histological structures of the Sham and GYY groups were essentially normal. In contrast, the IR group had significantly damaged tissue structures, with necrotic tubules, loss of brush border, and cast formation. These pathological changes were markedly mitigated in the IR + GYY group compared with the IR group ($p < 0.05$; Fig. 1B).

The concentrations of plasma BUN and Cr are indicative of kidney function. As shown in Figure 1C and D,

plasma BUN and Cr levels in the IR group were markedly higher than those in the Sham group and GYY group ($p < 0.05$). In contrast, there was no significant difference between the Sham group and GYY group. Compared with the IR group, GYY4137 attenuated the increase of BUN and Cr levels after renal IRI ($p < 0.05$).

GYY4137 Decreases Renal Apoptosis after IRI

Quantification of TUNEL-positive renal cells (Fig. 2A, C) revealed more apoptotic cells in the IR group compared with the Sham and GYY groups ($p < 0.05$). TUNEL-positive renal cells were significantly reduced in the IR + GYY group compared with the IR group ($p < 0.05$).

Fig. 4. GYY4137 improves activation of the Nrf2 pathway in the kidney after IRI. **A** Representative Western blots. **B** Detection of total tissue SOD activity assessed by a kit. **C–F** Bar graph showing quantification of the immuno-reactive bands obtained as above. $n = 5$ per group; $*p < 0.05$ compared with the IR group. Nrf2, nuclear factor-erythroid-2-related factor 2; SOD, superoxide dismutase; R, renal ischaemia reperfusion.

Because activation of caspase-3 exerts an important role in the process of apoptosis, we assessed its expression in renal IRI. As shown in Figure 2B and D, the level of active caspase-3 in the IR + GYY group was significantly reduced compared with the IR group ($p < 0.05$). Bax expression in the IR group was significantly increased compared with the Sham group, while Bcl-2 expression was decreased. There were no significant differences between the Sham group and GYY group. Pre-treatment with GYY4137 reduced the Bax expression and increased the Bcl-2 expression in the kidney tissue when compared with the IR group ($p < 0.05$). The Bax/Bcl-2 ratio was extremely attenuated in the IR + GYY group ($p < 0.05$, Fig. 2E).

GYY4137 Suppresses Oxidative Stress in the Kidney after IRI

Compared with the Sham and GYY groups, the tissue MDA and 15-F2t-isoprostane levels in the IR group were significantly elevated ($p < 0.05$; Fig. 3A, B). Meanwhile, there was no significant difference between the Sham group and GYY group. However, the levels of these indi-

cators were much lower in the IR + GYY group than in the IR group ($p < 0.05$).

GYY4137 Regulates Expression of the Nrf2 Pathway

After renal IRI, nuclear translocation of Nrf2 was increased and the level of cytoplasmic Nrf2 decreased, as shown in Figure 4C and D. However, the nuclear translocation of Nrf2 in the IR + GYY group was significantly elevated compared with the IR group ($p < 0.05$). Furthermore, compared with the IR group, pre-treatment with GYY4137 markedly upregulated the levels of HO-1 and NQO-1, while SOD activity was increased in the kidney ($p < 0.05$; Fig. 4B, E, F).

GYY4137 Decreases Cellular Injury in vitro

As shown in Figure 5A and B, post-hypoxic cell death is reflected as a decrease in cell viability and an increase in LDH leakage. Compared with the HR group, GYY4137 significantly increased the cell viability and decreased LDH activity ($p < 0.05$).

As shown in Figure 5D and E, the levels of caspase-3 activity and the Bcl-2/Bax ratio were similar between the

Fig. 5. GYY4137 decreases cellular injury in vitro. **A** Cell viability assessed by the CCK-8 assay. **B** LDH leakage. **C** Representative Western blots. **D** Caspase-3 activity. **E** Bar graph showing quantification of the immunoreactive bands obtained as above. $n = 3$ or 5 per group; $*p < 0.05$ compared with the HR group. CCK-8, Cell Counting Kit-8.

Con group and GYY group. GYY4137 treatment significantly decreased caspase-3 activity and the Bax/Bcl-2 ratio when compared with the HR group ($p < 0.05$).

GYY4137 Suppresses Oxidative Stress Associated with the Nrf2 Pathway in vitro

As shown in Figure 6A and B, the levels of MDA and SOD activity were not different between the Con group and GYY group. Cells treated with GYY4137 displayed an apparent decrease in the level of MDA and an increase in SOD activity compared to the HR-induced cells ($p < 0.05$).

Figure 6E shows that HR-stimulated cells exhibit an upregulation in the nuclear translocation of Nrf2 relative to control cells. Pre-treatment with GYY4137 markedly upregulated the level of Nrf2 nuclear translocation in HR-stimulated HK-2 cells ($p < 0.05$). Meanwhile, the levels of HO-1 and NQO-1 expression as well as SOD activity in the HR + GYY group were significantly elevated when compared with the HR group ($p < 0.05$; Fig. 4B, F, G).

To investigate if Nrf2 is responsible for exerting the protective effects of GYY4137, we transfected Nrf2 siRNA into HK-2 cells and measured the efficiency of the silencing by Western blot. Treatment with Nrf2 siRNA

Fig. 6. GYY4137 suppresses oxidative stress associated with the Nrf2 pathway in vitro. **A** Cellular MDA levels assessed by a kit. **B** Cellular total SOD activity assessed by a kit. **C** Representative Western blots showing quantification of the immunoreactive bands obtained as above. $n = 3$ or 5 per group; $*p < 0.05$ compared with the HR group. MDA, malondialdehyde; Nrf2, nuclear factor-erythroid-2-related factor 2; SOD, superoxide dismutase.

Fig. 7. Nrf2 gene silencing abolishes the renoprotective effects of GYY4137. **A** Representative Western blots. **B** A bar chart indicates the relative protein expression of Nrf2. **C** Cell viability assessed by the CCK-8 assay. **D** Caspase-3 activity. Nrf2, nuclear factor-erythroid-2-related factor 2; CCK-8, Cell Counting Kit-8.

significantly decreased total Nrf2 expression (Fig. 7A, B). Nrf2 gene silencing abolished the effect of GYY4137 (Fig. 7C, D).

Discussion

Renal IRI is one of the major causes of AKI, leading to irreversible renal damage and high mortality. GYY4137, a water-soluble H₂S donor, has shown beneficial effects on myocardial IRI by limiting oxidative stress and apoptosis [6, 12, 21]. In this study, we showed that GYY4137 attenuated the deterioration of renal function and morphology in the renal IRI model. The renal protection likely relied on antioxidative capabilities because GYY4137 significantly increased the expression of antioxidant enzymes via activation of the Nrf2 pathway.

Our previous study found that GYY4137 protected against diabetic myocardial ischaemia/reperfusion injury via activation of the PHLPP-1/Akt/Nrf2 pathway. However, whether GYY4137 could alleviate renal IRI in mice had not been proven. In the *in vivo* or *in vitro* study, the results showed that GYY4137 significantly improved renal function, as reflected by the decrease in the levels of serum BUN and Cr as well as alleviated cellular injury and LDH leakage. Meanwhile, GYY4137 remarkably reversed the histopathological changes after renal IRI. Therefore, these results indicated that GYY4137 has a strong inhibitory effect on renal IRI *in vivo* and *in vitro*.

GYY4137 alleviates renal IRI, but its protective mechanism is unclear. Many researchers believe that apoptosis is an important cause of ischaemic renal dysfunction [22, 23]. During the process of apoptosis, the Bcl-2 protein family integrates life and death signals [24]. Our results indicate that GYY4137 pre-treatment can decrease expression of the pro-apoptotic protein Bax and increase expression of the anti-apoptotic protein Bcl-2. A previous study indicated that the ratio of Bax to Bcl-2 determines whether apoptosis is induced or inhibited [25]. Therefore, GYY4137 treatment can decrease the ratio of Bax to Bcl-2 to inhibit cellular apoptosis after renal IRI or HR-stimulated HK-2 injury. Because the intrinsic and extrinsic apoptotic pathways converge at the activation of caspase-3, we measured renal cleaved caspase-3 or cellular caspase-3 activity, thus reflecting the degree of apoptosis. Moreover, GYY4137 significantly attenuated the expression of cleaved caspase-3 after renal IRI and caspase-3 activity after cells induced by HR. Therefore, our results demonstrate that GYY4137 significantly reduces renal cell apoptosis.

Although the mechanism of renal IRI is multifactorial and complex, recent studies have shown that oxidative stress after ischaemia is an important part of the pathogenesis of renal IRI [26, 27]. It was shown that the antioxidant effect can exert a vital role in renoprotection [16, 28]. MDA and 15-F₂t-isoprostane are indicators of the degree of oxidative stress. Ischaemia/reperfusion injury leads to elevated levels of MDA and 15-F₂t-isoprostane, which are products of lipid peroxidation. Our results showed that pre-treatment with GYY4137 significantly alleviated the levels of MDA and 15-F₂t-isoprostane in the kidney. The scavenging of active free radicals is mainly achieved by a series of antioxidant enzymes. The transcription factor Nrf2 is activated by dissociation with the inhibitory factor Kelch-like ECH-associated protein 1 (Keap1) and transferred to the nucleus, where it binds to antioxidant reaction elements to regulate the expression of antioxidant enzymes, including SOD, HO-1, and NQO-1 [14]. In our study, GYY4137 treatment effectively promoted cytoplasmic Nrf2 into the nucleus, in which the nuclear translocation of Nrf2 was elevated. Meanwhile, pre-treatment with GYY4137 led to a significant increase in the expression of HO-1 and NQO-1 as well as SOD activity. To prove that Nrf2 plays an important role in renal protection mediated by GYY4137, we used the siRNA technology to silence the expression of Nrf2. Our results demonstrate that GYY4137-mediated renoprotection is abolished by Nrf2 siRNA. Therefore, our results indicate that GYY4137 decreases oxidative stress against renal IRI by increasing the nuclear localization of Nrf2 to improve the expression of antioxidant enzymes.

Conclusion

In summary, GYY4137-mitigated kidney injury protects against renal IRI via activation of the Nrf2 pathway, which mediates an antioxidant effect. Taken together, these data suggest that the H₂S donor GYY4137 may represent a potential new therapeutic approach for the treatment of renal IRI.

Statement of Ethics

The experimental protocol was approved by the Animal Care and Use Committee of Nanjing Medical University, and the animal procedures were carried out in accordance with the National Institutes of the Guide for the Care and Use of Laboratory Animals.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

S.G. conceived and designed the experiments. H.Z., Y.Q., and S.G. performed the experiments. S.G. and Y.W. analysed the data. S.G., Y.Q., Y.W., H.Z., and H.S. contributed to the discussion and wrote the paper.

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