Simvastatin attenuates renal ischemia/reperfusion injury from oxidative stress via targeting Nrf2/HO-1 pathway

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Abstract. Ischemia-reperfusion (I/R) injury of the kidneys is commonly encountered in the clinic. The present study assessed the efficacy of simvastatin in preventing I/R-induced renal injury in a rat model and investigated the corresponding molecular mechanisms. Rats were divided into 3 groups, including a sham, I/R and I/R + simvastatin group. The results revealed that in the I/R group, the levels of blood urea nitrogen, serum creatinine and lactate dehydrogenase were significantly higher than those in the sham group, which was significantly inhibited by simvastatin pre-treatment. I/R significantly decreased superoxide dismutase activity compared with that in the sham group, which was largely rescued by simvastatin. Furthermore, I/R significantly increased the malondialdehyde content compared with that in the sham group, which was reduced by simvastatin. Hematoxylin-eosin staining revealed no obvious morphological abnormalities in the sham group, while I/R led to notable tubular cell swelling, vacuolization, cast formation and tubular necrosis, which was rescued by simvastatin. A terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay demonstrated that I/R significantly increased the number of apoptotic cells compared with that in the sham group, which was significantly inhibited by simvastatin. Western blot analysis demonstrated that simvastatin upregulated I/R-induced increases of nuclear factor erythroid-2-related factor 2 (Nrf2) and anti-oxidant enzyme heme oxygenase-1 (HO-1). Reverse-transcription quantitative PCR indicated that changes in the mRNA levels of Nrf2 and HO-1 were consistent with the western blot results. It was concluded that simvastatin treatment led to upregulation of HO-1 protein levels through activating the Nrf2 signaling pathway to ultimately protect the kidneys from I/R-associated oxidative damage.

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Introduction

Ischemia/reperfusion (I/R) injury of the kidneys represents a challenge in the clinic and is commonly encountered in renal transplantation, hemorrhagic shock, partial nephrectomy and accidental or iatrogenic trauma, brining about serious injury to multiple organs, including renal tubules, brain and heart (1). Therefore, it is urgent to find effective therapies and elucidate molecular mechanisms by which renal I/R injury may be attenuated. To date, a variety of signaling pathways that are correlated with renal I/R injury have been identified.

Oxidative stress has been validated to contribute to the pathogenesis of renal I/R injury (2). Renal I/R-induced oxidative stress generates high levels of reactive oxygen species (ROS). Consequently, overproduction of ROS results in lipid peroxidation, DNA mutation, apoptosis and necrosis, thus leading to cell death in various ways (3,4). Anti-oxidants, such as febuxostat, ligustrazine, sulforaphane, oxymatrine and gelsemine, have been demonstrated to protect murine kidneys against I/R injury (3-7). All of this evidence has indicated that renal I/R injury may be ameliorated via targeting oxidative stress.

The signaling pathway previously found to be associated with anti-oxidative stress is the nuclear factor erythroid-2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) pathway. Nrf2, a transcription factor, has the potential to bind with anti-oxidant response element (ARE), which is located at the promoter regions of a battery of anti-oxidant and detoxifying genes, including HO-1 (8). Furthermore, the anti-oxidant N-acetylcysteine was found to have a protective role in renal I/R injury via the Nrf2/HO-1 signaling pathway (9). The present study aimed to identify whether simvastatin is able to regulate oxidative stress and the Nrf2/HO-1 signaling pathway to prevent I/R-induced renal injury.

Statins, including simvastatin and pravastatin, limit catalysis in cholesterol biosynthesis via suppressing the activity of 3-hydroxyl-3-methyl coenzyme A (HMG-CoA) reductase (10). In addition, short-term pre-treatment with statins (HMG-CoA reductase inhibitors) was demonstrated to reduce post-ischemic acute renal failure in a uninephrectomized rat model, which may be achieved due to their anti-inflammatory

action (11). For instance, atorvastatin was found to attenuate renal I/R injury in rats via its anti-inflammatory action and reducing oxidative stress (12), and improve renal function via inhibiting active caspase-3 in rats (13). Simvastatin has the potential to lower cholesterol in patients with cardiovascular disease (14,15). In normocholesterolemic animals, simvastatin was reported to protect against stroke mediated by endothelial nitric oxide synthase (16), and to inhibit leukocyte-endothelial cell interactions and vascular inflammatory responses (17). In addition, simvastatin was demonstrated to attenuate I/R injury in rat hearts mediated by enhanced endothelial release of nitric oxide (18).

The present study aimed to investigate the effects of simvastatin on renal I/R injury and to provide a molecular foundation for the treatment of renal injury.

Materials and methods

Ethics statement and model construction. A total of 24 male adult Sprague-Dawley (SD) rats (8 weeks old; weight, 220-250 g) were obtained from Animal Core Facility of Nanjing Medical University (Nanjing, China), fed food and water freely under pathogen-free conditions and kept at 22±2˚C with 60±5% humidity under a 12-h light/dark cycle. Rats were randomly divided into 3 groups: Sham group, I/R group and I/R + simvastatin group, with 8 rats in each group. After acclimatization for approximately one week and fasting for 12 h, I/R injury was performed. In brief, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg, Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) and underwent right nephrectomy. Subsequent occlusion of the left renal hilus lasted for 45 min, followed by 24 h of reperfusion. Rats in the sham group were subjected to the same procedure but without any vessel occlusion. Rats in the I/R + simvastatin group received 2 ml simvastatin by intragastric administration (2 mg/kg; Hangzhou Moshadong Pharmaceutical Co. Ltd., Hangzhou, China) 60 min prior to occlusion, while rats in the other two groups received the same volume of 0.9% saline. The experimental protocol of the present study was approved by the Animal Ethics Review Committee of Nanjing Medical University (Nanjing, China). Procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Assessment of renal function. Renal function was assessed by determining the levels of blood urea nitrogen (BUN), serum creatinine (SCr) and lactate dehydrogenase (LDH). In brief, at the end of reperfusion, rats were sacrificed by intraperitoneal injection of pentobarbital sodium (150 mg/kg). Then blood samples were collected by cardiac puncture. Levels of SCr, BUN and LDH were evaluated by an automated analyzer (Siemens ADVIA 2400; Siemens AG, Munich, Germany). Left nephrectomy was performed and renal samples were fixed in 4% paraformaldehyde (PFA) or frozen in liquid nitrogen for further measurements.

Evaluation of oxidative stress in rat renal tissues. As sensitive indicators of oxidative stress, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were estimated to demonstrate the anti-oxidative effects of simvastatin in the tissues (19). SOD activity and MDA concentration were determined by commercialized assay kits (A001-1 and A003-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The optical density at 535 nm was read and results are presented in nm/mg.

Histological examination. Kidneys were fixed with 4% PFA, embedded in paraffin and sectioned into 4-μm slices, followed by staining with hematoxylin and eosin (HE). In brief, slices were de-waxed in xylene, re-hydrated by a graded series of alcohols and rinsed with distilled water with subsequent staining with hematoxylin for 3-5 min. Prior to change of solution from 70% alcohol to with 1% HCl, slices were washed with distilled water for 15-30 min. Thereafter, slices were stained with eosin for 1-4 min. Following dehydration and differentiation in alcohol to remove the blue cytoplasm, making nucleus more clear, slices were observed under a microscope (Olympus, Tokyo, Japan). The degree of tubular damage was estimated utilizing a semi-quantitative scale according to the following criteria: 0, normal kidney tissue; 1, minimal damage (<5% area, outer medulla or the cortex); 2, mild damage (5-25% area, outer medulla or cortex); 3, moderate damage (25-75% area outer medulla or cortex); and 4, severe damage (>75% area, outer medulla or cortex).

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay. At 24 h post-ischemia, apoptotic cells in kidney tissues were evaluated by a TUNEL assay kit (11684817910; Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions. In brief, 4-mm slices were treated with 20 mg/ml proteinase K, incubated in a nucleotide mixture of fluorescein-12-dUTP and TdT. Cells were regarded as TUNEL-positive if their nuclei were stained by 3,3-diaminobenzidine. In each section, ten areas were randomly selected. TUNEL-positive cells were counted in a double-blinded manner.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Kidneys were homogenized in TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total RNA was immediately treated with DNaseI (Invitrogen; Thermo Fisher Scientific, Inc.) and reversely transcribed into complementary DNA (cDNA) by PrimeScript™ 1st Strand cDNA Synthesis Kit according to the manufacturer's instructions (6110A; Takara Bio Inc., Otsu, Japan). Reactions were performed using the SYBR® Premix Ex Taq™ II (Perfect Real Time) Kit (DRR041A; Takara Bio, Inc., Otsu, Japan) in an ABI Prism 7300 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). After an initial denaturation at 95˚C for 5 min, the PCR reaction conditions were as follows: 35 cycles of denaturation at 95˚C for 30 sec, anneal at 55˚C for 30 sec, and extension at 72˚C for 30 sec. PCR amplification of cDNA were performed in triplicate. GAPDH was used as an endogenous control. The mean value of each sample was expressed as the cycle threshold (ΔCt). Gene expression was determined as the difference in ΔCt between target gene and GAPDH (20). The primer sequences were as follows: HO-1

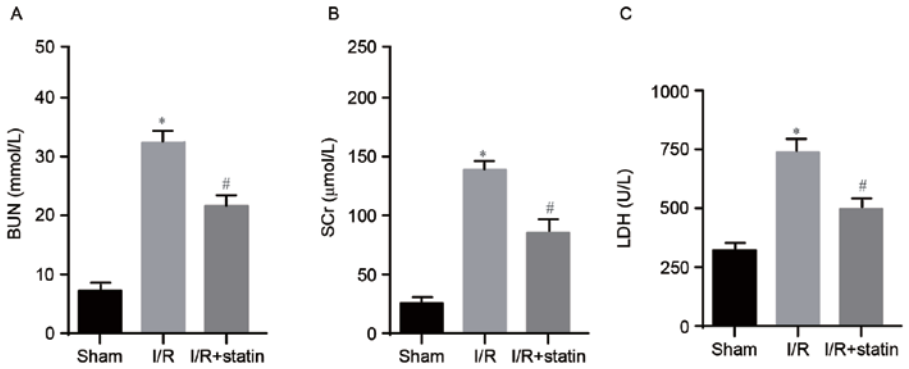

Figure 1. Simvastatin pre-treatment ameliorates I/R induced renal injury. In the I/R group, levels of (A) BUN, (B) SCr and (C) LDH were significantly elevated compared with those in the sham group, which were all markedly downregulated by simvastatin pre-treatment. *P<0.05, I/R group vs. sham group; #P<0.05, I/R + statin group vs. I/R group. I/R, ischemia-reperfusion; BUN, blood urea nitrogen; SCr, serum creatinine; LDH, lactate dehydrogenase.

forward, 5'-AAGATTGCCCAGAAAGCCCTGGAC-3' and reverse, 5'-AACTGTCGCCACCAGAAAGCTGAG-3'; Nrf2 forward, 5'-AGTCGCTTGCCCTGGATATTC-3' and reverse, 5'-GCCGGAGTCAGAGTCATTGAA-3'; GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAA GATGGTGATGGGATTTC-3'.

Western blot analysis. Rats were sacrificed with pentobarbital sodium (150 mg/kg), and the kidneys were removed, washed with ice-cold PBS and homogenized in radioimmunoprecipitation assay lysis buffer (Roche Diagnostics). Protein concentrations were assayed with a NanoDrop instrument (Thermo Fisher Scientific, Inc., USA) and 40 μg of protein was separated by 10% SDS-PAGE, and electro-transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked in 5% bovine serum albumin (BS043E; Biosharp, Hefei, China) for 1 h at room temperature and then incubated with primary antibody Nrf2 (1:1,000; sc-81342), HO-1 (1:1,000; sc-136256), β-actin (1:1,000; sc-58673; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. After rinsing with Tris-buffered saline containing Tween 20 (TBST) for 3 times, membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (A0216; 1:1,000; Beyotime Institute of Biotechnology; Haimen, China) for 1 h at room temperature followed by rinsing with TBST. Targeted proteins were visualized with super signal west pico chemiluminescent substrate (Thermo Fisher Scientific, Inc.). The membranes were visualized by exposure to X-ray film in the dark. β-actin was used as an endogenous control. Western blot analyses were performed 3 times in triplicate.

Statistical analysis. Data were evenly distributed and expressed as the mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance followed by a post-hoc analysis using Dunnett's test. P<0.05 was considered to indicate a significant difference.

Results

Simvastatin pre-treatment ameliorates I/R-induced renal dysfunction. Renal function was assessed via the levels of BUN, SCr and LDH, which were determined by an automated analyzer. In comparison with the sham group, I/R significantly induced upregulation of BUN (Fig. 1A), SCr (Fig. 1B) and LDH (Fig. 1C), which were all notably repressed by simvastatin pre-treatment. This inferred that simvastatin had a protective role in I/R-induced renal dysfunction.

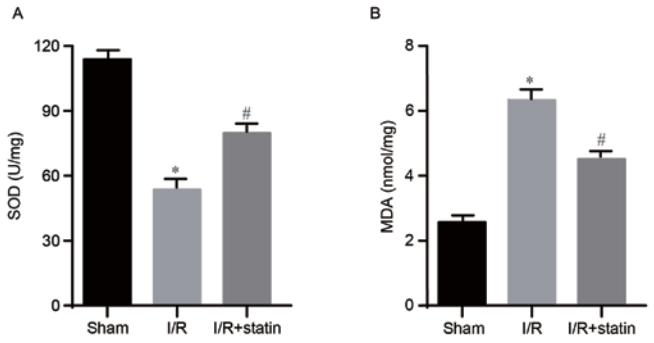

Figure 2. Simvastatin pre-treatment reduces I/R-induced oxidative stress. (A) I/R significantly decreased SOD activity compared with that in the sham group, which was largely rescued by simvastatin. (B) I/R significantly increased the MDA content compared with that in the sham group, which was reduced by simvastatin pre-treatment. *P<0.05, I/R group vs. sham group; #P<0.05, I/R + statin group vs. I/R group. SOD, superoxide dismutase; MDA, malondialdehyde; I/R, ischemia-reperfusion.

Simvastatin pre-treatment reduces I/R-induced oxidative stress. The present study then assessed whether the protective role of simvastatin relies on inhibition of oxidative stress. As SOD activity and MDA concentration are markers for oxidative stress, they were explored using commercial assay kits. Compared with the sham group, I/R led to significantly lower SOD activity, which was predominantly rescued by simvastatin pre-treatment (Fig. 2A). Furthermore, compared with the sham group, a significantly higher MDA concentration was determined in the I/R group, which was notably reduced by simvastatin (Fig. 2B). These data suggested that simvastatin indeed affected oxidative stress in the kidney.

Simvastatin pre-treatment alleviates I/R-induced renal histological injury. The present study then assessed whether simvastatin influences the pathology of renal tubules. Morphological changes in tissues were assessed by HE staining. In the I/R group, renal tubules displayed severe pathological changes, including loss of brush borders, congestion, tubular

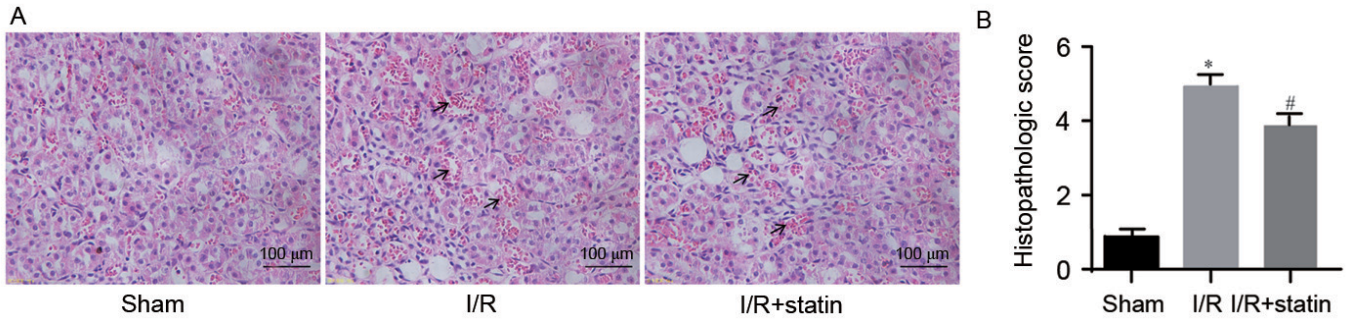

Figure 3. Simvastatin pre-treatment alleviates I/R-induced renal injury. (A) Histological analysis with hematoxylin and eosin staining (magnification, x400; scale bar, 100 μm) revealed no obvious morphological abnormalities in the sham group. I/R generated serious tubular cell swelling, vacuolization, cast formation and tubular necrosis, which were rescued by simvastatin. (B) Corresponding histopathological score were calculated and provided. *P<0.05, I/R group vs. sham group; #P<0.05, I/R + statin group vs. I/R group. I/R, ischemia-reperfusion; statin, simvastatin.

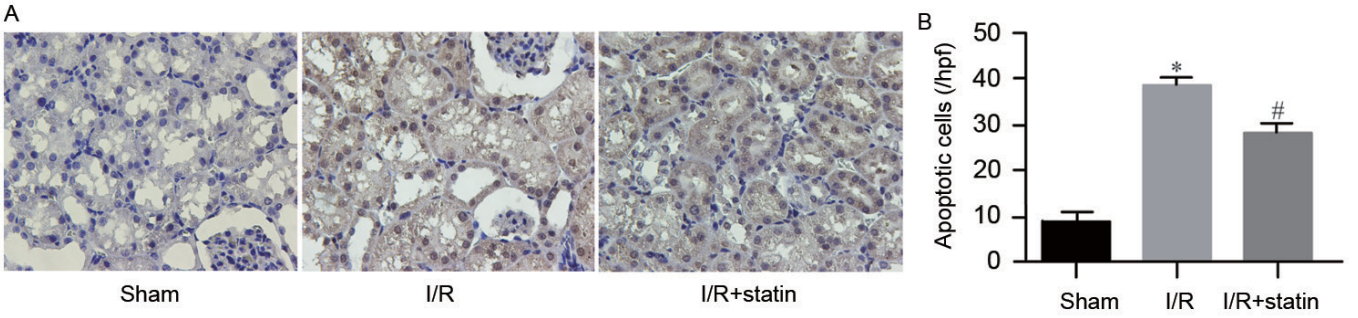

Figure 4. Simvastatin pre-treatment suppresses I/R-induced apoptosis in tubular epithelial cells. (A) Representative photomicrographs of TUNEL-stained renal tissue samples (magnification, x400). (B) Quantification of TUNEL-stained cells. I/R significantly increased the number of apoptotic cells compared with that in the sham group, which was significantly reduced by simvastatin. *P<0.05, I/R group vs. sham group; #P<0.05, I/R + statin group vs. I/R group. I/R, ischemia-reperfusion; hpf, high-power field; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; statin, simvastatin.

cell swelling and tubular dilation. Of note, a marked amelioration was observed in the I/R + simvastatin group (Fig. 3A). The degree of tubular damage in the 3 groups was also estimated (Fig. 3B). The data revealed that simvastatin protected against renal I/R injury by preserving renal tubule pathology.

Simvastatin pre-treatment suppresses I/R-induced tubular epithelial cell apoptosis. Furthermore, the present study explored whether simvastatin affected tubular epithelial cell apoptosis. The TUNEL assay revealed that the amount of apoptotic cells in the sham group was low, while I/R resulted in a significantly elevated number of apoptotic cells, which was significantly reduced by pre-treatment with simvastatin (Fig. 4A). Quantified rates of apoptotic cells are exhibited in Fig. 4B. These results demonstrated that simvastatin prevented tubular epithelial cells from I/R injury-induced apoptosis.

Simvastatin pre-treatment further increases Nrf2/HO-1 levels after renal I/R. To elucidate the molecular mechanisms of action of simvastatin in renal I/R injury, the present study assessed the expression levels of Nrf2/HO-1 in ischemic renal tissues by RT-qPCR and western blot analyses. Western blot analysis demonstrated that, compared with that in the sham group, Nrf2 was upregulated in the I/R group, while it was further increased in the simvastatin group; however, no significant effect was noted among the 3 groups with regard to HO-1 protein expression, while the same increasing trend was observed (Fig. 5A and B). RT-qPCR demonstrated that I/R induced an elevation of the mRNA levels of Nrf2 and HO-1, which were further increased by simvastatin treatment (Fig. 5C). These results indicated that the Nrf2/HO-1 signaling pathway is involved in the protective effects of simvastatin in renal I/R injury.

Discussion

The present study found that simvastatin ameliorated I/R-induced renal dysfunction and oxidative stress via activating the Nrf2/HO-1 signaling pathway, thus providing a possible molecular foundation for the effective treatment of renal injury.

Multiple antioxidants, including febuxostat, ligustrazine, sulforaphane, oxymatrine and gelsemine, have been reported to protect murine kidneys against I/R injury by attenuation of oxidative stress (3-7).

Similarly, statins have also been reported to attenuate oxidative stress generated by I/R injury. For instance, atorvastatin was found to attenuate rat renal I/R injury by reducing oxidative stress (12) and inhibiting active caspase-3 in rats (13). Simvastatin preserves microvascular barrier function by inhibiting the ischemia-induced release of vasoactive angiopoietin-2 and endothelin-1 as well as the tubule

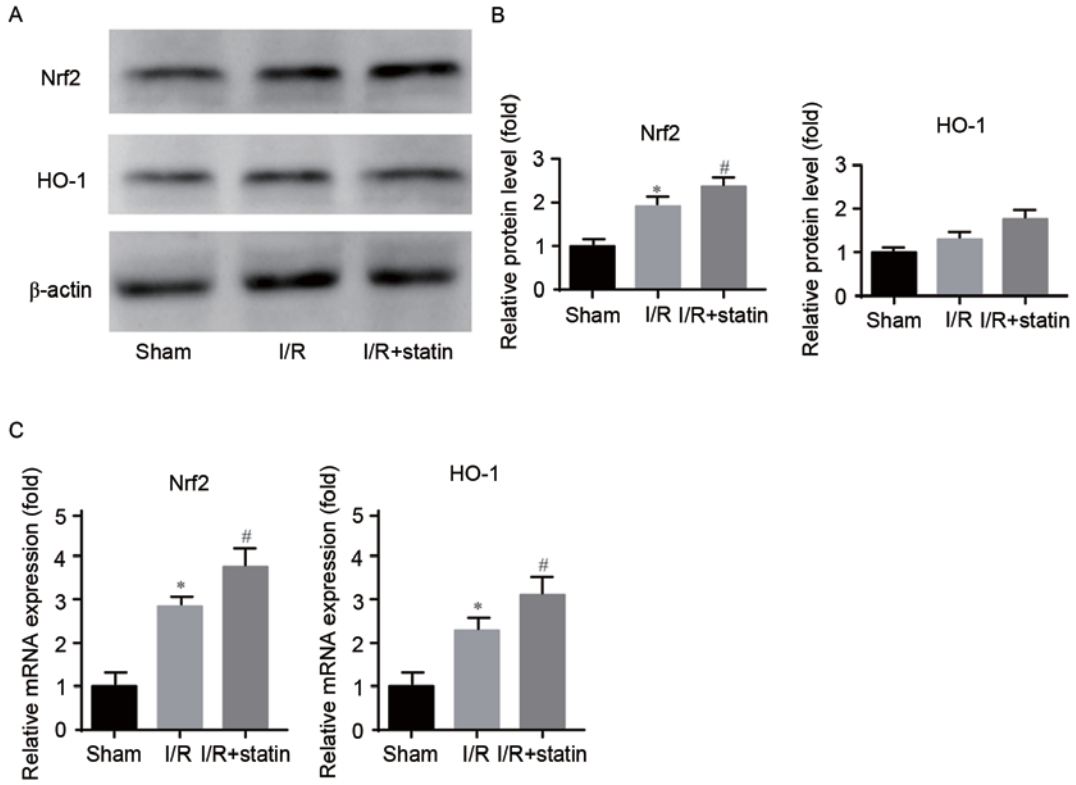

Figure 5. Simvastatin pre-treatment enhances renal I/R-induced upregulation of Nrf2 and HO-1 expression. (A) Representative western blot images for Nrf2 and HO-1 in the three groups. (B) Densitometrically quantified expression Nrf2 and HO-1. Pre-treatment with simvastatin led to a further upregulation of Nrf2 after I/R. While a similar trend was noted for HO-1, differences between groups were not statistically significant. (C) mRNA levels of Nrf2 and HO-1 determined by reverse-transcription quantitative polymerase chain reaction analysis. I/R-induced upregulation of Nrf2 and HO-1 was further enhanced by pre-treatment with simvastatin. *P<0.05, I/R group vs. sham group; #P<0.05, I/R+ statin group vs. I/R group. I/R, ischemia-reperfusion; statin, simvastatin; Nrf2, nuclear factor erythroid-2-related factor 2; HO-1 heme oxygenase 1.

interstitial injury markers kidney injury molecule-1 and SCr following I/R (21). The acute protective effects of simvastatin pre-treatment in rat renal I/R injury were also proved by Todorovic *et al* (22).

The present study aimed to investigate a possible molecular foundation of simvastatin pre-treatment for the attenuation of renal injury. BUN, SCr and LDH are pivotal indexes for kidney function. In the sham group, the concentrations of BUN, SCr and LDH were relatively low, while I/R induced a significant elevation of these parameters, which was notably reduced by simvastatin pre-treatment, suggesting a protective role of simvastatin in renal I/R injury. However, whether any other indicators were affected by simvastatin remains elusive.

Inordinate or aberrant ROS is implicated in the pathogenesis of tissue injury (23,24). As an organ with the ability to generate ROS, the kidney has long been studied and is also vulnerable to damage caused by ROS (25,26).

Furthermore, reperfusion has the ability to produce excess ROS, while downregulation of SOD leads to oxidative stress in the kidney (16,27). As the most crucial endogenous antioxidant enzyme, SOD scavenges oxygen free radicals and prevents mitochondria from damage through cytotoxic agents. The improved activities of these endogenous antioxidant enzymes provide protection against oxidative stress. As acknowledged, SOD activity and MDA content reflect the anti-oxidative ability of tissues (28). As one of the products of lipid peroxidation, MDA is generated by the reaction of ROS with polyunsaturated fatty acids. Therefore, the present study examined SOD activity and the MDA concentration in kidney tissues of the three different groups. The results demonstrated that SOD activity and MDA concentration were normal in the sham group, and that simvastatin pre-treatment predominantly rescued I/R-induced reduction of SOD activity and upregulation of MDA. Based on these results, it is not difficult to infer that simvastatin exerts its protective effect on renal I/R injury via suppressing the oxidative stress response.

ROS was reported to induce apoptosis during reperfusion (29). Apoptosis, which has been frequently observed in animal models of I/R-induced kidney injury and in human acute tubular necrosis, is crucial in the initiation of I/R-induced tissue injury (30). The present study evaluated changes in apoptotic rates in kidneys from different treatment groups. The results demonstrated a small amount of apoptotic tubular epithelial cells in the sham group, and the number of tubular epithelial cells with TUNEL-positive nuclei in the simvastatin-pre-treated group was significantly lower than that in the I/R group, which suggested that simvastatin ameliorated apoptosis. Furthermore, decreased production of MDA may have also contributed to the protection against tubular epithelial cell apoptosis. The corresponding signaling pathways were then further explored.

Signaling pathways associated with renal I/R injury have remained to be fully elucidated. Under physiological conditions, the Nrf2 signaling pathway is sequestered by virtue of binding with Kelch-like ECH-associated protein 1 (Keap 1) (31).

However, the pathological conditions of oxidative stress may lead to the dissociation of the Nrf2-Keap 1 complex and the nuclear translocation of Nrf2, where it may bind with ARE and HO-1 to ultimately offset cellular oxidative stress (8,32). The Nrf2/HO-1 signaling pathway was reported to be tightly associated with ROS scavenging during the process of oxidative stress and the attenuation thereof. Of note, the Nrf2/HO-1 pathway has been reported to represent a therapeutic target in renal I/R injury (9).

However, the effects of simvastatin on the expression of Nrf2 and HO-1 in ischemic kidneys have remained to be elucidated. In the present study, western blot and RT-qPCR analyses were utilized to investigate their levels. As for the protein levels, simvastatin pre-treatment predominantly further elevated I/R-induced elevation of Nrf2, but no significant difference was noted among the 3 groups with regard to HO-1 protein expression. At the mRNA level, I/R-induced upregulation of Nrf2 and HO-1 were further enhanced by simvastatin, which strongly suggested that simvastatin pre-treatment provided renoprotection by activating Nrf2 and its target gene HO-1.

Simvastatin has also been reported to activate Nrf2-associated pathways in other cells types. For instance, simvastatin was discovered to lower ROS levels by activating Nrf2 via the phosphoinositide-3 kinase (PI3K)/Akt pathway in ST-2 cells (33), to induce HO-1 via Nrf2 activation through the extracellular signal-regulated kinase and PI3K/Akt pathway in the HCT116 and HT-29 colon cancer cell lines (34), and to activate Keap1/Nrf2 signaling in rat liver primary hepatocytes (35).

Taken together, it is possible to draw the conclusion that simvastatin upregulates HO-1 mRNA levels through activating the Nrf2 signaling pathway to ultimately protect the kidney from the I/R injury-associated oxidative stress response, renal tubule pathology and tubular epithelial cell apoptosis.

However, three questions remain unanswered: i) While the Nrf2 pathway was activated in the I/R + simvastatin group in comparison with the sham or I/R group, resulting in upregulation of HO-1, SOD activity was reduced in the I/R and IR + simvastatin groups compared with that in the sham group. One possible explanation may be that the SOD activity or its expression is impaired by ROS. ii) The effect on other molecules downstream of the Nrf2 pathway, such as glutamate-cysteine ligase catalytic subunit and quinine oxidoreductase 1 in the experimental groups remains to be clarified. iii) The exact involvement of the Nrf2/HO-1 signaling pathway in the protection of the kidney from I/R injury after treatment with simvastatin. These questions will be addressed in future studies.

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