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Original Article

HMGB1-TLR4 signaling participates in renal ischemia reperfusion injury and could be attenuated by dexamethasone-mediated inhibition of the ERK/NF-κB pathway

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Abstract: Studies have shown that the HMGB1-TLR4 (High-mobility group protein B1, toll-like receptor 4) pathway participates in renal ischemic reperfusion injury (IRI) and that dexamethasone (DEX) could protect the kidney against IRI. This study aims to examine the protective effects of DEX on renal IRI and further explore the possible mechanism of action. During mouse renal IRI, HMGB1-TLR4 signals changed markedly including HMGB1 translocation and TLR4 up-regulation, resulting in histological damage and an increase in MPO expression. Treatment with DEX markedly decreased the damage to renal function (serum Cr and BUN; kidney KIM-1 expression) and the histological pathology of the kidney after renal IRI. The activation of GR by DEX did not suppress p38 and JNK activity but inhibited ERK phosphorylation. Treatment with DEX also attenuated IκB-α phosphorylation and further reduced NF-κB expression in the nucleus by decreasing acetylation of the p65 subunit. Furthermore, the HMGB1-TLR4 inflammatory pathway was inhibited via the attenuated translocation of HMGB1 from the nucleus to the cytoplasm and the down-regulation of TLR4 expression through DEX treatment. The inhibition of HMGB1 translocation may interact with acetyltransferase and attenuate HMGB1 acetylation. As a result, the levels of cytokines (TNF-α, IL-6, and IL-1β) were down-regulated and inflammatory cell infiltration after renal IRI was attenuated by treatment with DEX. This study demonstrated that the HMGB1-TLR4 pathway may play a critical role in renal IRI. DEX may attenuate renal IRI by suppressing ERK and NF-κB activation, followed by attenuating the HMGB1-TLR4 pathway through inhibiting acetyltransferases.

Keywords: Dexamethasone, ischemia-reperfusion injury, HMGB1-TLR4, ERK, NF-κB, acetylation

Introduction

Acute kidney injury (AKI) is a common clinical complication with high morbidity and mortality rates [1]. The pathogenesis of ischemia reperfusion injury (IRI) induced AKI is common but complex, and studies show that apoptosis [2], endothelial dysfunction and inflammation [3, 4], and other factors are involved in renal dysfunction [3]. In particular, the inflammatory processes induced by renal IRI have been widely investigated, but the exact mechanism is still unclear.

In a previous study, we showed that the inflammatory factor HMGB1, which was first described as a nuclear protein, acts as an essential early mediator in ischemia-induced AKI; we showed that targeting HMGB1 could inhibit renal tubular apoptosis and inflammation [5]. However, since the exact mechanism of AKI remains unknown, further inflammatory factors and their interactions still need to be investigated.

Nuclear factor-kappa B (NF-κB) is a transcription factor that regulates inflammatory responses. This protein remains quiescent in the cyto-

plasm when in a complex with IκB-α. In response to a pro-inflammatory stimulus, IκBα is phosphorylated, leading to NF-κB translocation from the cytoplasm to the nucleus where NF-κB activates the transcription of a cascade of pro-inflammatory chemokines and cytokines that induce the inflammatory response [6, 7]. Multiple reports have examined renal IR injury-induced NF-κB activation [8-10]. Mitogen-activated protein kinase (MAPK) signaling pathways are a series of parallel cascades of serine/threonine kinases (including ERK, p38, and JNK) that are activated by diverse extracellular, physical and chemical stresses and that regulate cell proliferation, differentiation and survival. In addition, MAPK activation may be linked to NF-κB translocation to the nucleus [11]; therefore, a close relationship may exist between the NF-κB and MAPK pathways in the inflammatory response [9, 10].

Dexamethasone (DEX), a glucocorticoid (GC), has been widely used clinically due to its anti-inflammatory and immunosuppressive effects [12]. GCs are GR ligands that are expressed in a number of tissues, including the lung, liver and kidney, and others [13]. GCs have been reported to regulate the MAPK signaling pathway [14], and GR ligands, including DEX, have been shown to reduce myocardial/intestinal IRI *in vivo*. The protective effects of GR agonists are involved in GR activation [15, 16]. Thus, the aim of the present study was to determine whether DEX has a protective effect on renal IRI and whether the protection involves the suppression of NF-κB activation mediated by the MAPK signaling pathway and involves the regulation of the HMGB1-TLR4 signal.

Material and methods

Animals and group

Male C57BL/6 mice (Hua Fukang Experimental Animal Center, Beijing, China) were provided with a standard laboratory diet and water ad libitum and were cared for using a protocol approved by the Institutional Animal Care and Use Committee of the Tongji Medical College, Huazhong University of Science and Technology. At the start of the experiments, the mice were 8-10 weeks of age, weighing 25-30 g. After a minimum 7-day acclimation period, the mice were randomly allocated into the following three groups: (1) ischemia/reperfusion (IR)-saline group-the mice were subjected to renal ischemia for 1 h (n=10); (2) IR-DEX group-the mice were treated with DEX (4 mg/kg, i.p.) 1 h prior to I/R (n=10); and (3) sham-operated group-the mice were subjected to the identical surgical procedure without occlusion of both renal pedicles (n=10). The dose of DEX was determined according to a previously reported method [17].

Briefly, the mice were anesthetized with an intraperitoneal injection of 1% sodium pentobarbital solution (6 ml/kg). Following abdominal incisions, the renal pedicles were bluntly dissected, and a microvascular clamp was placed on the left renal pedicle for 60 min. During the procedure, the mice were kept well-hydrated with warm saline and at a constant temperature of 32°C in an infant incubator. After the removal of the clamp, the contralateral kidney was removed. Next, the incisions were sutured, and the animals were allowed to recover with free access to food and water. The mice were sacrificed at 24 h after reperfusion, and blood samples and the left kidneys were harvested for further analysis.

Assessment of renal function

The blood samples were obtained from the inferior vena cava 24 h after reperfusion. The blood urea nitrogen (BUN) and serum creatinine (Cr) levels were measured in the core laboratory of Tongji Hospital (Wuhan, China) to assess renal function.

Renal morphological changes

The renal tissue samples harvested from mice 24 h post-reperfusion were fixed in formalin and then embedded in paraffin. Four-micrometer sections were stained with periodic acid-Schiff (PAS) stain. The histopathological changes in the cortex and medulla were evaluated by a pathologist in a blinded fashion using a semi-quantitative scale designed to evaluate the degree of tubular necrosis, hemorrhage and cast formation on a five-point scale based on the area of involvement as follows: 0, < 10%; 1, 10-25%; 2, 25-50%; 3, 50-75%; and 4, 75-100% [18].

Immunohistochemical staining

The renal tissues were removed 24 hours after ischemia reperfusion, fixed with formalin, and embedded in paraffin. Serial sections were cut at a thickness of 5 μm. The tissues were sub-

jected to immunohistochemical staining for HMGB1 (Abcam, 1:200) and TLR-4 (Abcam, 1:50) using the SP method. The dyeing process was conducted in strict accordance with the kit protocol. The brown coloring observed under the microscope represented HMGB1 and TLR-4 positive staining, which showed the expression of HMGB1 and TLR-4.

Immunohistochemical staining for myeloperoxidase

Renal myeloperoxidase (MPO) activity, which signifies neutrophil infiltration, in the kidney was evaluated by immunohistochemistry as previously described [18]. Neutrophil infiltration was assessed quantitatively by counting the number of neutrophils per high-power field (×400) over 10 fields, then averaging the neutrophil counts.

Western blotting

The expression of ERK, p-ERK, p38, p-p38, JNK, p-JNK, β-actin, p65, p-p65, Acep65, p-IκB-α, IκB-α, KIM-1, HMGB1, TLR4 and histones in renal tissues were determined by western blotting for total tissue protein samples or cytoplasmic and nuclear protein fractions. To obtain whole-cell lysates, the renal tissue was homogenized in RIPA buffer (Wuhan Goodbio Technology Co. Ltd, Wuhan, China) with phosphatase inhibitor (Wuhan Goodbio Technology) and protease inhibitors (1:50, Roche, Basel, Switzerland). The homogenate was centrifuged at 12,000 rpm for 30 min at 4°C. The cytoplasmic and nuclear proteins were isolated using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, China). The total cytoplasmic and nuclear protein concentrations were determined using the BCA Protein Assay Kit (Beyotime), and the samples were stored at -80°C for western blot analysis. Polyclonal rabbit antibodies against ERK (Cell Signal Technology, Danvers, MA, USA, 1:2000), p-ERK (CST, 1:2000), p38 (CST, 1:2000), p-p38 (CST, 1:500), JNK (CST, 1:1000), p-JNK (CST, 1:400), p65 (CST, 1:500), p-p65 (CST, 1:500), Acep65 (Acetyl NF-κB p65 (Acetyl-Lys310), CST, 1:1000), IκB-α (Abclonal, 1:1000), p-IκB-α (Abclonal, 1:500), HMGB1 (Abcam, 1:1000), TLR-4 (Abcam, 1:500), KIM-1 (Abcam, 1:500), β-actin (Abmart, Shanghai, China, 1:1000), and histones (Abclonal, 1:2000) were used as primary antibodies. Anti-rabbit/mouse antibody conjugated to HRP (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as the secondary antibody. β-Actin or histones was used as an intrinsic quality control. The bands were incubated in ECL-Plus reagent (Amersham, Piscataway, NJ, USA) and were detected on a BioMax MR Film (Kodak, Rochester, NY, USA). The density of the bands was quantified using a Labworks image acquisition and analysis software (UVP, Upland, CA, USA).

Immunoprecipitation

The renal tissue was homogenized in RIPA buffer (Wuhan Goodbio Technology) with phosphatase inhibitor (Wuhan Goodbio Technology) and protease inhibitors (1:50, Roche, Basel, Switzerland). The homogenate was centrifuged at 12,000 rpm for 30 min at 4°C. The protein concentration was determined using the BCA Protein Assay Kit (Beyotime), and the sample was subsequently were mixed with anti-HMGB1 primary antibody (ABclonal Biotech Co., Ltd, Cambridge, MA, USA) coupled to agarose beads (Cell Signaling Technology, Inc. Danvers, MA, USA). The immunoprecipitates were separated via SDS-PAGE, transferred to PVDF membranes (Roche, Ltd, Basel, Switzerland) and blotted with antibodies against acetyl-lysine (Cell Signaling Technology, Inc. Danvers, MA, USA). The bands were incubated in ECL-Plus reagent (Wuhan Goodbio Technology Co. Ltd, Wuhan, China), and chemiluminescence was detected using BioMax MR Film (Kodak, Rochester, NY). The X-ray films were scanned using a Bioimaging System (UVP, California, USA).

Quantitative Real-Time PCR

The total RNA of the kidney tissue was isolated with TRIzol according to the manufacturer's instructions (Takara, Otsu, Japan). The extracted RNA was resuspended with 20 μl of diethyl pyrocarbonate-treated water, and the concentration was measured using a spectrophotometer (Eppendorf, Hamburg, Germany). Four micrograms of total RNA were reverse transcribed into cDNA using PrimeScript RT Master Mix (Takara). The reaction mix (20 μl) was incubated at 37°C for 15 minutes for reverse transcription and finally at 85°C for 5 seconds for reverse transcriptase inactivation. Real-Time PCR assays were performed using a Real-Time PCR system (ABI 7500, Applied Biosystems,

Table 1. Primers used for Real-Time PCR analysis

Gene	Species	Sense strand sequence	Anti-Sense strand sequence
KIM-1	Mouse	ACATATCGTGGAATCACAACGAC	ACTGCTCTTCTGATAGGTGACA
TNF-α	Mouse	CTGAACTTCGGGGTGATCGG	GGCTTGTCACTCGAATTTTGAGA
IL-1β	Mouse	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL-6	Mouse	ACATATCGTGGAATCACAACGAC	ACTGCTCTTCTGATAGGTGACA
B-actin	Mouse	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT

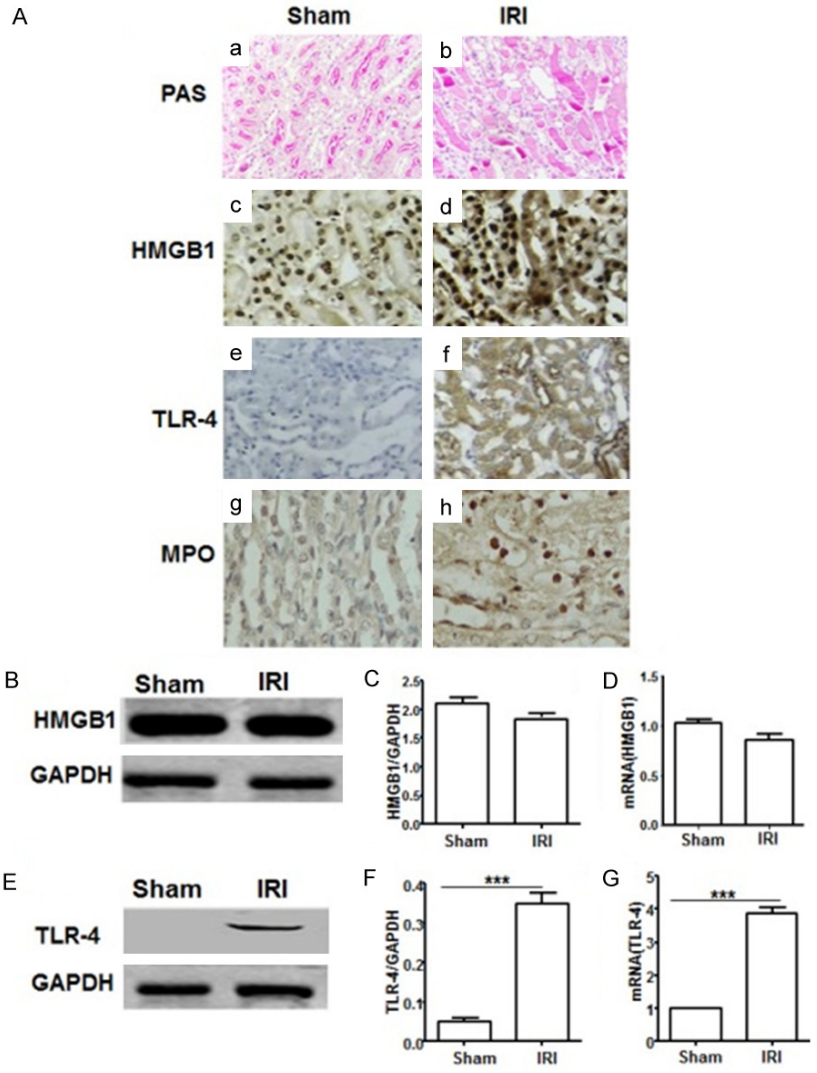

Figure 1. Altered HMGB1-TLR4 pathway expression during mouse kidney IRI. (A) PAS staining was performed to detect renal histological injury after IRI (a, b); immunohistochemistry was performed to detect HMGB1 (c, d), TLR4 (e, f) and MPO (g, h) in sham mice and IRI mice (original magnification, ×400). (B) Total kidney tissue protein of sham mice and IRI mice was tested for HMGB1 by western blot. (C) The quantitative analysis of B. (D) Total kidney tissue mRNA of sham mice and IRI mice was tested for HMGB1 by Real-Time PCR. (E) Total kidney tissue protein of sham mice and IRI mice was tested for TLR4 by western blot. (F) The quantitative analysis of (E) (***$P < 0.01$). (G) Total kidney tissue mRNA of sham mice and IRI mice was tested for TLR4 by Real-Time PCR (***$P < 0.001$).

Carlsbad, CA, USA). The primers (Invitrogen, Carlsbad, CA, USA) used for the assays are shown in **Table 1**. The cDNA was detected in 96-well plates in duplicate using SYBR Green I (Takara). The Real-Time PCR conditions were 95°C for 30 seconds followed by 40 cycles at 95°C for 5 seconds, and 60°C for 30 seconds. The mRNA quantity for each gene was normalized to β-actin, and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as previously reported [19].

ELISA analysis

The levels of inflammatory mediators (TNF-α, IL-1β and IL-6) in the serum were quantified using specific ELISA kits for rats according to the manufacturer's instructions (Biosource International Inc, Camarillo, CA, USA).

Statistical analysis

All results are expressed as the means ± SEM of at least three independent experiments. Statistical analysis of data was performed by Student's *t*-test or one-way ANOVA using Graph Pad Prism 5 software for two groups or multiple group comparison. The threshold for statistical significance was set at $P < 0.05$.

Results

Altered expression of HMGB1-TLR4 pathway during mouse kidney IRI

Twenty-four hours after ischemia reperfusion injury, the kidneys were collected for PAS,

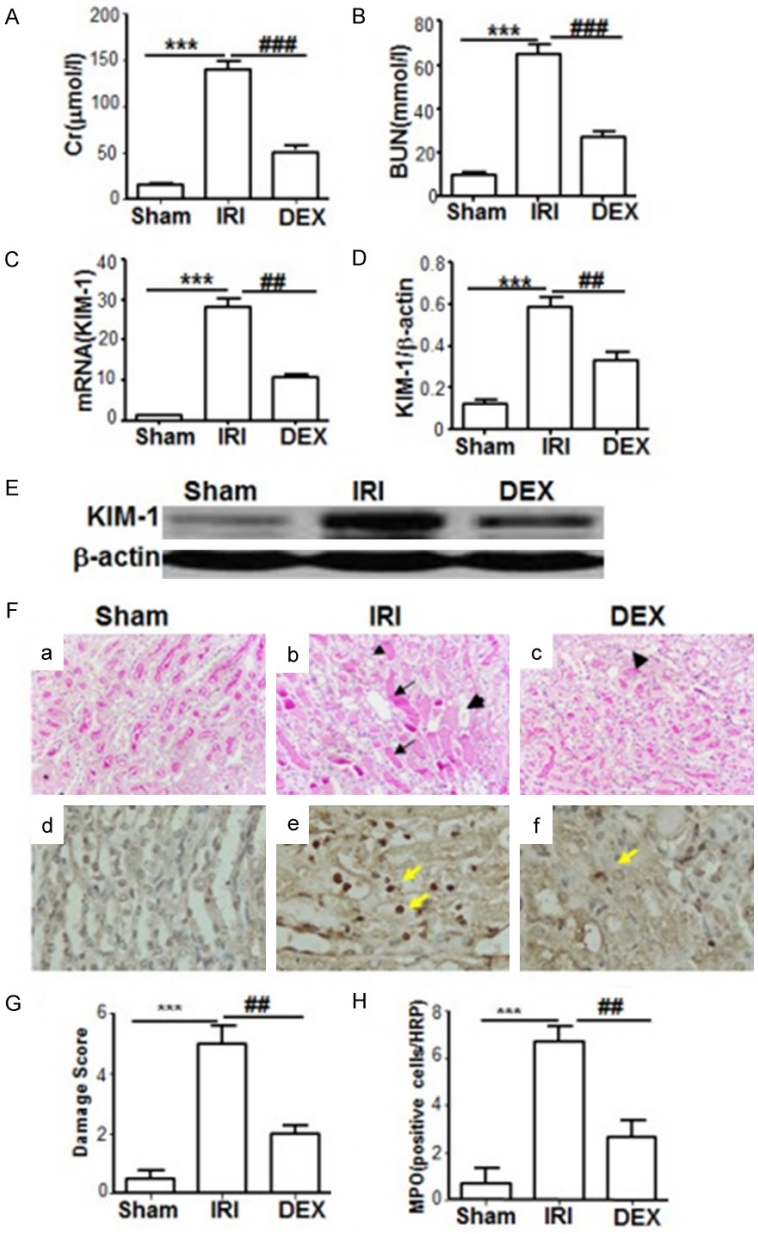

Figure 2. DEX pre-treatment ameliorates renal function damage and histological injury after kidney IRI. The samples were harvested from control mice (Sham), operated mice (IRI) and dexamethasone-treated mice (DEX). (A and B) Serum creatinine (Cr) and urea nitrogen (BUN) were measured in the three groups (***P < 0.001, ###P < 0.001). (C) KIM-1 levels in whole kidney tissues were tested by Real-Time PCR (***P < 0.001, ##P < 0.01). (D and E) KIM-1 levels of whole kidney tissues were tested by western blot; (D) is the statistical analysis of (E) (***P < 0.001, ##P < 0.01). (F) Histological changes were assessed using PAS staining (a-c); inflammatory cell infiltration was determined using immunohistochemistry for MPO (d-f) (magnification, ×400). Thin black arrows show cast formation, thick black arrows show injured tubular cells and yellow arrows show MPO-positive cells. (G) Semi-quantitative assay of histological injury shown in (F) (***P < 0.001, ##P < 0.01). (H) Semi-quantitative assay of MPO in (F) (***P < 0.001, ##P < 0.01).

immunohistochemistry staining, PCR and western blot for HMGB1, TLR4, and other proteins.

Compared with the sham group, renal I/R induced much more severe pathological injury, particularly in terms of tubular necrosis and cast formation at the site of corticomedullary junction (**Figure 1A**). The inflammatory factor HMGB1 was predominantly expressed in the nucleus of renal parenchyma cells and especially renal tubular epithelial cells in the sham group, but after 24 hours of IRI, HMGB1 was expressed in both the nucleus and cytoplasm. Interestingly, the western blot and PCR analyses demonstrated no significant differences between the two groups in terms of total HMGB1 expression in the kidneys (**Figure 1B-D**). The expression of TLR-4 was markedly up-regulated after 24 hours of IRI compared to the negative expression in the sham group; the western blot and PCR analyses confirmed that these differences were significant (P < 0.001) (**Figure 1E-G**). The expression of MPO, a marker of neutrophil infiltration, increased significantly in the IRI group; by contrast, MPO expression was negative in the sham group (**Figure 1A**).

DEX pre-treatment ameliorates IRI-induced renal dysfunction and histological injury of the kidney

The protective role of DEX on IR-induced renal injury is unclear; therefore, we established a renal IRI model and pre-treated mice with dexamethasone to investigate its potential biological effects *in vivo*. Serum Cr, BUN and the expression of kidney injury molecule (KIM-1) were examined. The animals that underwent renal I/R exhibited elevated levels of Cr (147±13 μmol/l)

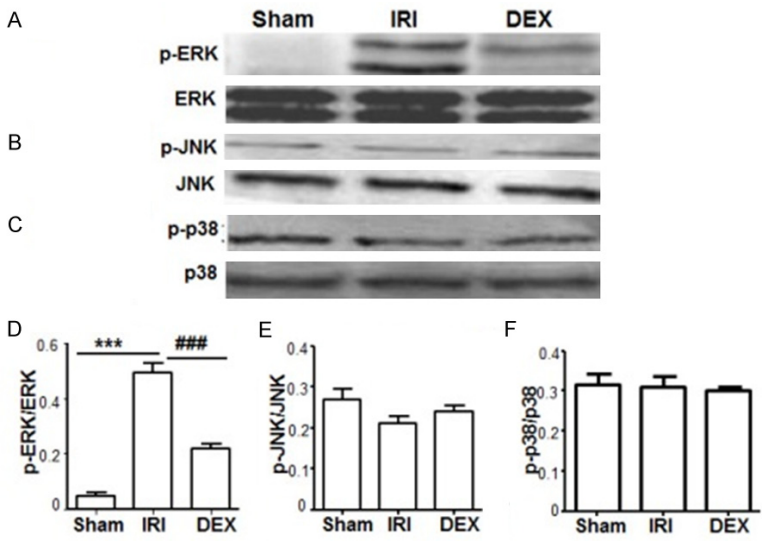

Figure 3. The expression and activation of ERK, p38 and JNK in IRI kidneys were regulated by dexamethasone treatment. The samples were harvested from control mice (Sham), operated mice (IRI) and dexamethasone-treated mice (DEX). (A) The expression of ERK and p-ERK in the three groups were measured by western blot for total protein. (B) The expression of JNK and p-JNK in the three groups were measured by western blot for total protein. (C) The expression of p38 and p-p38 in the three groups were measured by western blot for total protein. (D-F) Quantitative assays of (A-C), respectively (***$P < 0.001$, ###$P < 0.001$).

and BUN (70±4 mmol/l) and increased KIM-1 mRNA (30-fold) and protein expression levels compared to the sham-operated animals. This result reflects a significant decrease of renal dysfunction ($P < 0.001$). Compared with I/R animals, the pre-treatment with DEX significantly decreased the Cr serum levels (60±7.6 μmol/l), BUN (37±3 mmol/l) and the KIM-1 mRNA (15-fold) and protein expression levels ($P < 0.01$) (**Figure 2A-E**).

The characteristic histopathological features of IRI were evident at 24 h of reperfusion in the kidneys obtained from the I/R-treated mice compared with the sham-operated mice. Specifically, the most severe and pronounced injuries were observed in the cortex and the outer stripe of the outer medulla with a typical tubular necrosis pattern, which included widespread degeneration of the tubular architecture, detachment of epithelial cells from the basement membrane, tubular cell necrosis, cast formation and luminal congestion with the loss of the brush border (**Figure 2Fb**). In contrast, renal sections obtained from mice pretreated with DEX showed a remarkable reduction of the histological features of renal injury, which consisted of more focal and mild characteristics of tubular necrosis (**Figure 2Fc**). A semi-quantitative assessment of the histological lesions showed a significantly higher score in the I/R-treated mice compared with the DEX-treated mice at 24 h of reperfusion ($P < 0.01$, **Figure 2G**). The presence of inflammatory cell infiltration was documented using the MPO assay (**Figure 2F**). Significant variability was observed in the number of MPO-positive cells in the renal tissue among the three groups. The kidneys from the animals that were subjected to renal I/R appeared to have a greater number of MPO-positive cells compared with the sham group ($P < 0.01$). In contrast, a significant reduction in cell infiltration was observed among the DEX-treated mice compared with the IRI group. A semi-quantitative assessment of the MPO-positive cells showed a significantly higher number of these cells in the IRI mice compared with the DEX-treated mice at 24 h of reperfusion ($P < 0.01$, **Figure 2H**).

The expression and activation of ERK, p38 and JNK in kidneys subjected to IRI after DEX treatment

Inflammation pathways play a crucial role in IRI-damaged kidneys. Thus, suffered from IRI, the expression of MAPK (ERK, p38 and JNK) pathway proteins were detected using western blotting to further determine the mechanism. As shown in **Figure 3**, no difference in the expression of total ERK, p38 and JNK were observed between the sham, IRI and DEX groups. The kidneys from the IRI group showed significantly increased phosphorylation of ERK (p-ERK), the activated ERK form, compared with the sham group ($P < 0.001$); however, the p-ERK level was markedly decreased in the kidneys of animals pre-treated with dexamethasone. A quantitative assessment showed a significant increase in p-ERK expression in the I/R group, which was significantly decreased in the DEX group (**Figure**

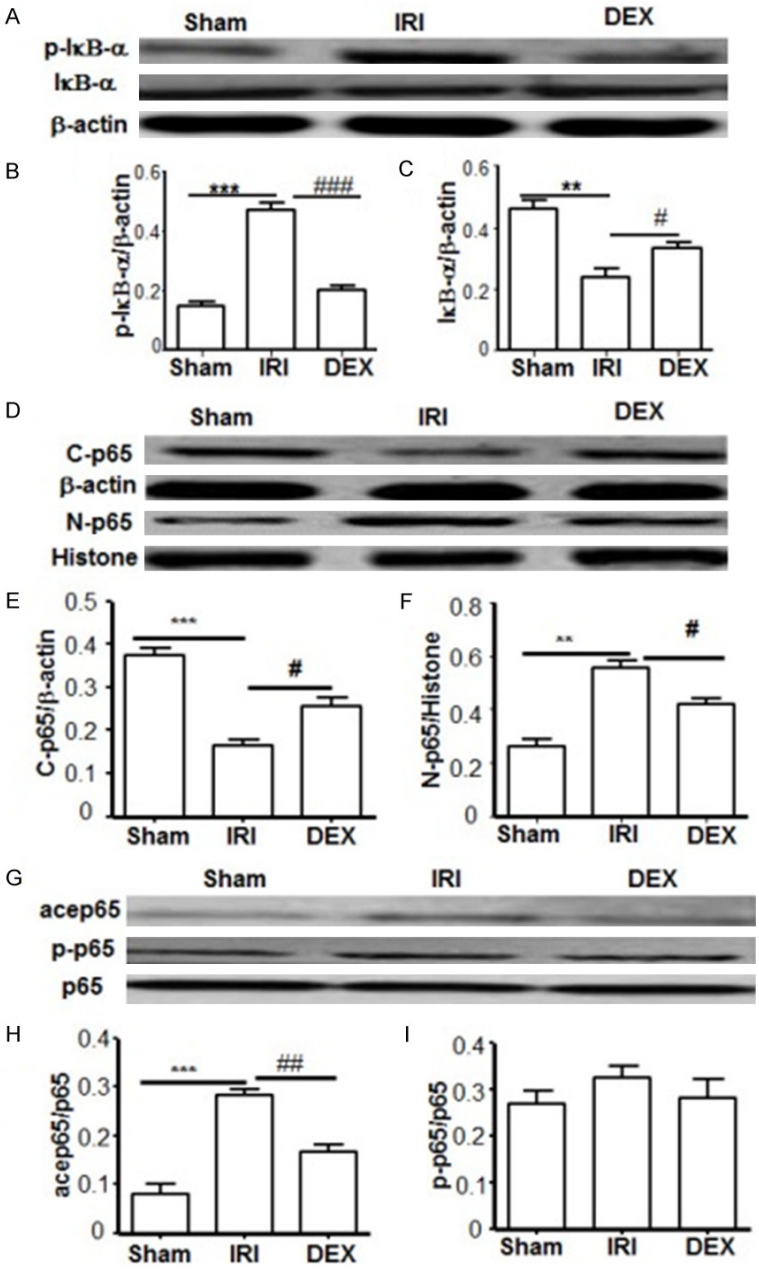

Figure 4. Effect of DEX on NF-κB activation in renal ischemia reperfusion injury. The samples were harvested from control mice (Sham), operated mice (IRI) and dexamethasone-treated mice (DEX). (A) The expression of IκB-α and p-IκB-α in the three groups were measured by western blot for total protein. (B and C) The quantification of (A) (****P* < 0.001, ***P* < 0.01, ###*P* < 0.001, #*P* < 0.05). (D) The expression of p65 was tested in the nuclear and cytoplasmic fractions of kidney tissue proteins. (E and F) The quantification of (D) (****P* < 0.001, ***P* < 0.01, #*P* < 0.05). (G) The expression of p65, p-p65 and p65 acetylation in the three groups were measured by western blot for total protein. (H and I) The quantification of (G) (****P* < 0.001, ##*P* < 0.01).

3A, **3D**, *P* < 0.001). Interestingly, no differences were observed in the phosphorylation of p38 and JNK between the sham, IRI and DEX groups; these results were confirmed by the quantitative assessment (**Figure 3B**, **3C**, **3E**, **3F**). Taken together, these findings demonstrated that in ERK activation may play a critical role in IRI-induced kidney damage.

Effect of DEX on NF-κB activation in renal ischemia reperfusion injury

MAPKs/NF-κB acts as an inflammatory regulator, and NF-κB is reportedly activated by the phosphorylation and acetylation of its p65 subunit [20, 21]. To ascertain the mechanism of DEX-mediated reno-protection, we performed western blots to assess whether DEX treatment inhibits the activation of NF-κB. The inhibition of NF-κB activation by IκB-α was first detected (**Figure 4A-C**, western blot and semi-quantitative assessment). Compared to the sham group, the expression of IκB-α and phosphorylated IκB-α (p-IκB-α) in the IRI group were significantly decreased and increased, respectively. Moreover, DEX treatment markedly reversed these alterations, indicating that IκB-α may be activated during renal IRI and the activation could be inhibited by DEX treatment.

Western blotting was also conducted using the nuclear and cytoplasmic protein fractions to confirm the results indicating the activation and translocation of p65. The kidneys from the IRI group demonstrated a notable increase of p65 expression in the nucleus (N-p65) and a significant reduction of p65 expression in the cytoplasm (C-p65) compared with the sham group (*P* < 0.01). However, these alterations were significantly suppressed in the kidneys of animals pre-treated with dexamethasone (*P* < 0.05) (**Figure 4D-F**). These data indicate that during

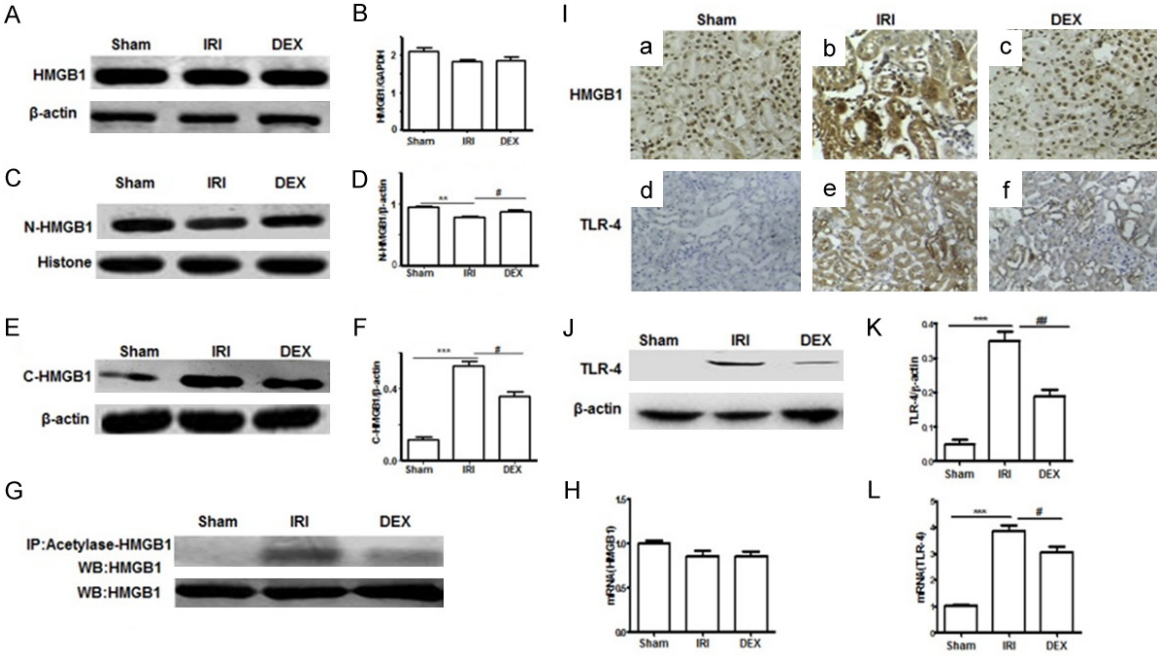

Figure 5. HMGB1 acetylation, migration variation and the expression of TLR4 in the kidneys of IRI mice after DEX treatment. The samples were harvested from control mice (Sham), operated mice (IRI) and dexamethasone-treated mice (DEX). (A and B) The total protein levels of HMGB1 of the three groups. (B) is the quantitative analysis of (A). (C and D) HMGB1 expression in the nuclear fraction of kidney proteins in the three groups was measured by western blot. (D) is the quantitative analysis of (C) (***P* < 0.01, #*P* < 0.05). (E and F) HMGB1 expression in the cytoplasmic fraction of kidney proteins in the three groups was measured by western blot. (F) is the quantitative analysis of (E) (****P* < 0.001, #*P* < 0.05). (G) Immunoprecipitation was performed to test the interaction of HMGB1 and acetylase in the three groups. (H) The mRNA levels of HMGB1 were tested by Real-Time PCR. (I) Immunohistochemistry staining of HMGB1 and TLR4 were performed in all groups to show their localization and expression (original magnification, ×400). (J and K) The expression of TLR4 in total kidney protein was tested by western blot; (K) is the quantitative analysis of (J) (****P* < 0.001, ##*P* < 0.01). (L) The mRNA expression of TLR4 in the kidney was tested by Real-Time PCR (****P* < 0.001, #*P* < 0.05).

renal IRI, p65 may translocate from the cytoplasm to the nucleus to play a pro-inflammatory role. Furthermore, activated p65 was detected in the sham, IRI, and DEX groups (**Figure 4G-I**), though there were no differences in the expression of phosphorylated p65 (p-p65) and total p65 between the three groups. However, the acetylation of p65 (ace-p65) was significantly up-regulated in the IRI group compared with the sham group. DEX treatment reversed this regulation (*P* < 0.01), which demonstrated that during IRI, the active form of p65 may be acetylated p65 but not phosphorylated p65.

HMGB1 acetylation, variations in cell migration, and TLR4 expression in the IRI-damaged kidney after DEX treatment

To explore the molecular mechanisms of DEX-induced reno-protection, we assessed the expression of TLR4 and its ligand, nuclear protein high mobility group box-1 (HMGB1), in the kidneys. HMGB1 and TLR4 signaling play a pivotal role in the coordination of inflammatory responses in renal IRI [22, 23]. As shown in **Figure 5A**, **5B** and **5H**, there were no differences in the total HMGB1 protein and mRNA in the kidney tissues between the sham, IRI and DEX groups after 24 h of renal IRI. In fact, the location of HMGB1 in the kidney was altered after renal IRI. Western blot and IP analyses demonstrated that the expression of HMGB1 in the cytoplasm and the physical interaction between HMGB1 and acetylase were dramatically elevated, and the level of nuclear HMGB1 was significantly down-regulated in the IRI group compared to the sham group. However, pretreatment with DEX significantly attenuated this alteration compared to the IRI group (**Figure 5C-G**). Furthermore, immunohistochemical sta-

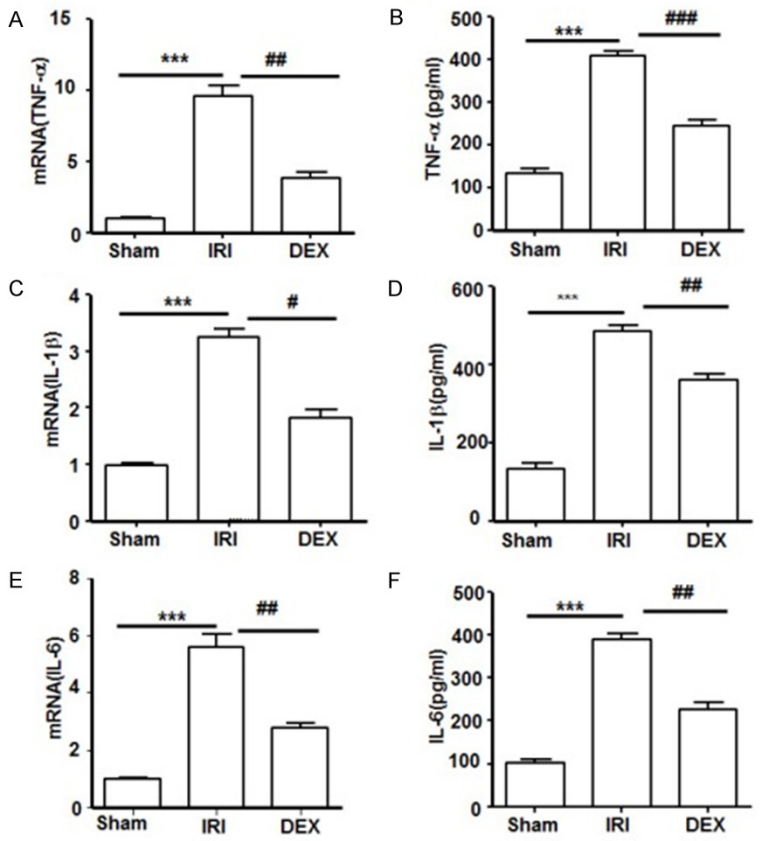

Figure 6. DEX suppresses inflammation by inhibiting the release of inflammatory factors. The samples were harvested from control mice (Sham), operated mice (IRI) and dexamethasone-treated mice (DEX). (A and B) Real-Time PCR and ELISA assays were used to test the levels of TNF-α in the kidney and serum, respectively (***$P < 0.001$, ###$P < 0.001$, ##$P < 0.01$). (C and D) Real-Time PCR and ELISA assays were used to test the levels of IL-1β in the kidney and serum, respectively (***$P < 0.001$, ##$P < 0.01$, #$P < 0.05$). (E and F) Real-Time PCR and ELISA assays were used to test the levels of IL-6 in the kidney and serum, respectively (***$P < 0.001$, ##$P < 0.01$).

ining confirmed the western blot results, which showed that in the sham group, little HMGB1 was expressed in the cytoplasm and that most HMGB1 was localized to the nucleus. By contrast, IRI induced high expression of HMGB1 in the cytoplasm and low HMGB1 expression in the nucleus, but DEX reversed these alterations (**Figure 5I**).

Western blotting, PCR, and immunohistochemical staining were used to detect the HMGB1 receptor TLR4. The mRNA and protein levels of TLR4, though very low in the sham group, were markedly increased in the IRI group (**Figure 5I-L**). Notably, pre-treatment with DEX resulted in a dramatic decrease in TLR4 expression compared to the IRI group (**Figure 5I**, **5J**). A quantitative assessment showed a significant reduction in TLR-4 expression in the sham, IRI and Dex groups (**Figure 5I-L**; $P < 0.05$). Together, these findings suggest that the pro-inflammatory signaling mediated by HMGB1 and TLR4 in renal IRI may be partially dependent on a GR-mediated mechanism.

DEX suppresses inflammation by inhibiting the release of inflammatory factors

Inflammation is an important cause of renal IRI. Inflammatory cytokines were detected to further understand the mechanism of renal IRI. As shown in **Figure 6A**, **6C** and **6E**, the mRNA levels of TNF-α, IL-1β and IL-6 in the IRI group were approximately 9.3-fold, 6.4-fold, and 3-fold the levels of the sham group after 24 h reperfusion, respectively. Interestingly, dexamethasone pre-treatment remarkably attenuated the increased level of TNF-α, IL-1β and IL-6 compared with the IRI group (42% of I/R group, $P < 0.01$, 53% of I/R group, $P < 0.05$, and 47% of I/R group, $P < 0.01$, respectively). Furthermore, the serum levels of TNF-α, IL-1β and IL-6 were also reduced by DEX treatment (**Figure 6B**, **6D**, **6F**).

Discussion

In this study, serious renal ischemia for 1 h followed by reperfusion after 24 h resulted in significant renal dysfunction, which was detected by a marked increase in creatinine, urea nitrogen and KIM-1. The differences in the levels of these biochemical parameters and the mRNA expression levels may have resulted from differences in the experimental animals (C57BL/6 vs. BALB/c) or differences in the experimental conditions (i.e., temperature or duration of ischemia). However, similarly severe renal damage has been previously reported [5]. Evidence of renal injury was also supported by histological

scoring. Based on morphological criteria and the MPO method, the type of kidney damage was characterized as a combination of pathological changes and inflammatory cell infiltration. These findings are consistent with previous studies reporting that renal IRI initiates a complex cascade of events that eventually result in injury, renal pathological changes and inflammatory cell infiltration [5, 24].

Renal injury after ischemia/reperfusion results from a complex sequence of events. As reported, acute ischemia induces adenosine triphosphate deficits and initiates renal cell damage. After reperfusion, the primary injury is exacerbated by the cascade amplification of inflammatory responses, such as the activation of inflammatory cells, cytokine secretion, and additional inflammation that results in apoptosis or necrosis of the renal parenchyma cells [25]. In our previous study, the inflammatory factor HMGB1 was shown to act as the link between the initiation of cell damage and the inflammatory cascade during renal IRI, though the exact mechanism remained uncertain [5]. In the present study, we investigated alterations in the expression of HMGB1 and its receptor TLR4, during IRI. We also assessed leukocyte activation (MPO up-regulation), which demonstrated that the HMGB1-TLR4 pathway plays a critical role in renal IRI. However, further aspects of this mechanism still must be investigated.

Clinical applications have shown that dexamethasone (DEX), an anti-inflammatory agent, is widely used in patients with inflammatory diseases. DEX has also been reported to protect the kidney from IRI, but the exact mechanism remained unstudied [26]. In the present study, we explored the effects of DEX in a model of severe kidney injury induced by an ischemic period of 60 min followed by a reperfusion period of 24 h. Our results showed that DEX pretreatment before the onset of ischemia led to a significant reduction in kidney damage, especially tubular injury, and a remarkable reduction of renal functional impairment as assessed by biochemical parameters and KIM-1. The detection of MPO expression by immunohistochemistry, which indicates inflammatory cell infiltration, also demonstrated that DEX could markedly inhibit the accumulation of leukocytes during renal IRI. All these data provide evidence that DEX, a glucocorticoid receptor (GR) agonist, can activate GR and promote the strong inhibition of inflammation. Furthermore, in our previous study, we showed that GR activation is further increased, which may be modulated by DEX in renal tissue in IRI. Activated GR may inhibit kidney inflammation resulting from IRI [17]. Previous studies have also shown that GR activation decreases myocardial/intestinal IRI by reducing inflammation [27, 28].

NF-κB activation regulates inflammation in renal IRI [29], and this protein is activated by the phosphorylation or acetylation of its p65 subunit [20, 30]. To further ascertain the DEX anti-inflammatory mechanisms in renal IRI, we also explored the effect of IRI on the NF-κB signal pathway in renal tissue with and without DEX treatment. This study first demonstrated that DEX suppresses NF-κB activation by inhibiting the acetylation of p65 at Lys310, but not p65 phosphorylation, in renal IRI. To ascertain the mechanism underlying DEX inhibition of p65 acetylation of Lys310 in renal IRI, we analyzed the MAPK signaling pathway because MAPK has a close relationship with NF-κB activation, and NF-κB activation may be activated by MAPK activation in IRI [31]. The MAPK signaling pathway is involved in the regulation of diverse biological phenomena, including inflammation, apoptosis, necrosis and others [32, 33] and is also activated by GR activation [34]. These study results demonstrate that renal IRI leads to ERK pathway activation but not the activation of the p38 and JNK pathways, which is consistent with previous studies, suggesting that the ERK signaling pathway may be linked to the pathophysiology of various renal disorders, including I/R [26]. Recently, the ERK signaling pathway has been shown to play an important role in DEX-mediated protective actions on liver/heart injury [26, 27]. However, this influence may be tissue-specific, and limited information supports a similar role for ERK in the ischemic kidney. The protective effect of DEX against IRI seems to be associated with anti-inflammatory effects induced by the suppression of NF-κB activation. Moreover, the MAPK/NF-κB signaling pathway likely plays a pivotal role in the pathophysiology of renal IR injury and may play a key role in DEX-mediated protective effects.

ERK/NF-κB may be deeply involved in IR-induced renal injury, but the initiation of the inflammatory cascade during ischemia-induced

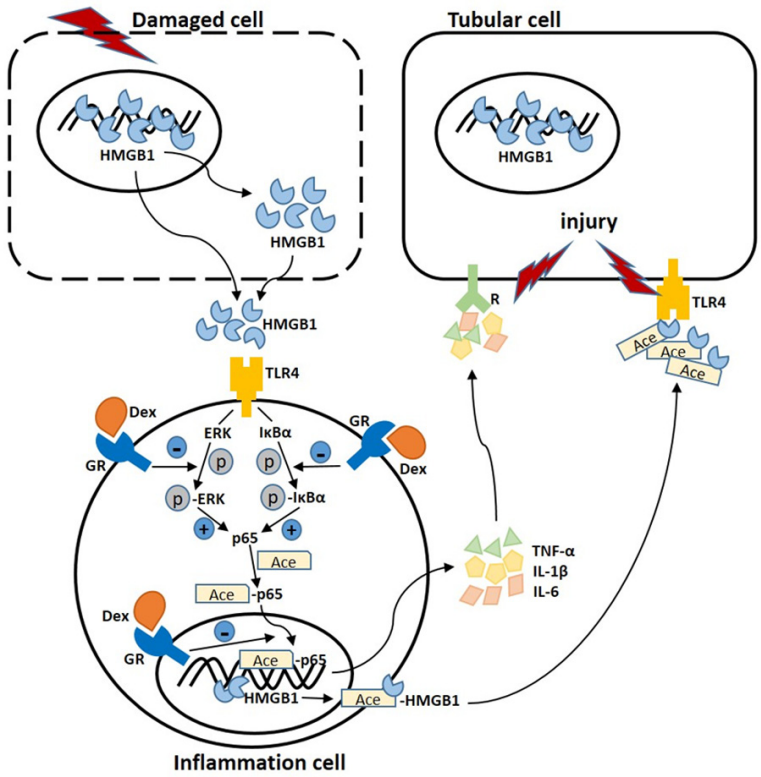

Figure 7. Schematic diagram illustrating inhibition of HMGB1-TLR4 signaling pathway by dexamethasone in renal ischemia reperfusion injury. HMGB1 was shown to passively release from damaged or necrotic cells and bind to TLR4 on inflammation cells during renal ischemia reperfusion injury. Activation of TLR4 by HMGB1 causes EKR and IκBα phosphorylation and interaction with NF-κB p65 subunit, promoting p65 acetylation and translocation to the nucleus. Activated p65 in nucleus binds with DNA and leads to HMGB1 acetylation and active release out of inflammation cells together with other inflammatory factors, for instance, TNF-α, IL-1β and IL-6. Released inflammatory factors bind to their receptors on renal tubular cells. This ligand-receptor interaction forms an inflammatory cascade vicious circle and lead to further damage. It is possible that administration with dexamethasone activates glucocorticoid receptor, which then inhibits ERK and IκBα phosphorylation and decreases p65 acetylation, thereby inhibits HMGB1-TLR4 pathway during kidney ischemia-reperfusion injury. Dex, dexamethasone; GR, glucocorticoid receptor; R, receptor; Circled plus denotes possible role in activating the kinase cascade; Circled minus denotes suppression of the possible kinase cascade; Circled P denotes phosphorylation; Squared Ace denotes acetylation.

renal damage must be further investigated. HMGB1 was originally described as a nuclear protein that acts as a transcription factor by binding to DNA and modifying gene expression; however, HMGB1 was also recently described as a cytokine that acts as a central mediator of inflammation [35]. In our previous study, we showed that the inflammatory factor HMGB1 acts as an essential early mediator in ischemia-induced AKI and that targeting HMGB1 could inhibit renal tubular apoptosis and inflammation [5]. In the present investigation, we investigated whether inhibiting the ERK/NF-κB pathway with DEX could also attenuate the HMGB1-TLR4 pathway, demonstrating that although HMGB1 acts as an inflammatory cytokine, the total amount of HMGB1 in the kidney was not altered during renal IRI. Further investigations showed that nuclear HMGB1 may translocate to the cytoplasm and may even be exported from the cells, where HMGB1 could act as a pro-inflammatory cytokine. This translocation of HMGB1 may be executed by intracellular acetylation, which may also result in the inverse translocation of the acetylated form of subunit p65 of NF-κB from the cytoplasm to the nucleus. It could be deduced that acetyltransferase may play an important role in the translocation of NF-κB and HMGB1 between the cytoplasm and nucleus, but further investigations must be done in the near future.

Our study also showed that renal IRI results in the remarkable up-regulation of the mRNA and protein levels of IL-6, IL-1β and TNF-α in the tubules and serum. This up-regulation was especially notable in the proximal tubular cells and serum at 24 h after reperfusion, indicating that the up-regulation of inflammatory factors contributes to inflammation in severe renal IRI. Previous studies conducted in similar animal models of renal IRI have also shown that severe ischemia leads to the increased expression of inflammatory cytokines [5]. The effects of DEX on IRI-induced inflammation were investigated by quantifying the mRNA levels of inflammation factors and MPO infiltration. The inhibitory effects of DEX on IRI-induced inflammation was determined by the expression of inflammation factors and inflammatory cells, which confirmed previous

data suggesting that the inhibition of inflammation is one of the most promising protective mechanisms of GC [36]. In mice subjected to IRI or to cisplatin-induced acute kidney injury (ARF), GC treatment promotes functional and morphological tubular regeneration [37].

Nevertheless, whether the DEX-mediated protective effect observed in renal IRI is only associated with the activation of the ERK signaling pathway and the subsequent down-regulation of NF-κB mediated inflammation, especially via the inhibition of the HMGB1-TLR4 pathway, remains unknown. Recent studies suggest that DEX-mediated protection against renal IRI may also involve MAPK-mediated apoptosis [38]. According to our results, apoptosis could not be excluded as a factor in the DEX-mediated protective effect against renal IRI through the ERK or other signal pathways. Alternative protective mechanisms that may be activated downstream of the GC/GR system include the regulation of apoptosis and inflammation. To study this hypothesis, we will conduct an experiment to assess the effect of DEX on apoptosis in renal IRI.

Our study demonstrated that DEX pretreatment before the onset of ischemia confers marked protection against ischemia-induced renal injury. Based on these findings, DEX treatment may be representative of a novel therapeutic approach due to its capacity to preserve renal function and directly protect renal tissue. DEX may exert these effects by inhibiting MAPK-ERK activation, NF-κB subunit p65 acetylation, and the subsequent translocation of HMGB1, thereby further attenuating inflammation during renal IRI (**Figure 7**). However, the mechanism by which DEX protects renal cells against IRI is not fully understood. Improved understanding of the DEX-mediated signaling cascade is needed to further elucidate the mechanism of action and the benefits of DEX therapy for potential incorporation into clinical practice in the future.

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Disclosure of conflict of interest

None.

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