

Original Article

Administration of dexamethasone protects mice against ischemia/reperfusion induced renal injury by suppressing PI3K/AKT signaling

Jiong Zhang^{1*}, Ying Yao^{1*}, Fang Xiao², Xiaoqin Lan², Chong Yu¹, Ying Zhang¹, Cao Jiang¹, Juan Yang¹, Guangchang Pei¹, Yueqiang Li¹, Song Rong³, Shuang Hu⁴, Junhua Li¹, Gang Xu¹

¹Department of Nephrology, ²Department of Gastroenterology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, People's Republic of China; ³Department of Nephrology, Hannover Medical School, Hannover, Germany; ⁴The Center for Biomedical Research, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China. *Equal contributors.

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Abstract: Dexamethasone (DEX), a ligand for glucocorticoid receptor (GR), has long been used in the clinical practice due to its anti-inflammatory and immunosuppressive properties. Given that ischemia/reperfusion (IR)-induced renal injury is featured by the excessive immune response; the current study is therefore designed to address the impact of dexamethasone on IR-induced renal injury, a common disorder in the clinical settings. Precondition of mice with 4 mg/kg of dexamethasone significantly attenuated IR-induced injury as manifested by the improved renal function along with ameliorated pathological changes and suppressed inflammatory infiltration. Mechanistic studies revealed that dexamethasone promotes GR activation, and by which it attenuates the signals for PI3K/AKT activation. Attenuated PI3K/AKT signaling thus suppresses inflammatory response which then protects kidneys from IR-induced injury. All together, our data support that dexamethasone could be a good alternative therapy for prevention and treatment of IR-induced renal injury in the clinical practice.

Keywords: Inflammatory response, dexamethasone, ischemia/reperfusion injury, glucocorticoid receptor, PI3K/AKT signaling

Introduction

Renal ischemia reperfusion (IR) injury serves as a major risk factor for acute renal failure in diverse clinical settings such as aortic bypass surgery, cardiopulmonary surgery, and kidney transplantation [1-6]. The molecular mechanisms underlying renal IR injury are complex and are yet to fully addressed. However, there is compelling evidence that inflammatory mediators, adhesion molecules, and a variety of cytokines are implicated in disease pathoetiology [7-9]. Interestingly, the PI3K (phosphoinositide 3-kinase)/AKT pathway originally recognized to play a critical role in regulating cell growth and cell survival in different systems [10], has recently been rediscovered involved in the protection of myocardium and liver against IR injury by regulating inflammatory response [11, 12]. The PI3K kinase is com-

posed of a regulatory subunit p85 and a catalytic subunit p110 [10], and its activation relies on p85 activation. Once p85 got activated, it directs signals to phosphorylate AKT [10-13], which then provide signals to enhance inflammatory response [14]. Therefore, blockade of PI3K signaling has been noted to attenuate IR-induced renal injury [15].

For the past several decades, glucocorticoids (GCs) have been widely used in the clinical settings due to their anti-inflammatory and immunosuppressive properties [16]. Glucocorticoids are ligands for the glucocorticoid receptor (GR) that is widely distributed in different organs including liver and kidneys [17]. It has been demonstrated that glucocorticoids regulates the activity of transcription factors essential for inflammatory response [18], and therefore, dexamethasone, one of the GR ligands, has

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Table 1. Primers used for real-time PCR analysis

GENE	Species	Sense strand sequence	Anti-Sense strand sequence
IFN- γ	mice	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
TNF- α	mice	CTGAACCTCGGGGTGATCGG	GGCTTGCTACTCGAATTTGAGA
IL-6	mice	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
β -actin	mice	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT

been manifested to provide protection for liver and heart against IR-induced injury [19, 20]. However, the impact of dexamethasone on IR-induced renal injury is yet to be elucidated, we thus conducted studies in C57BL/6 mice to address this question.

Materials and methods

Animals

Male C57BL/6 mice (8wk-old, 25-30 g) were purchased from Hua Fukang Experimental Animal Center, Beijing, China. The mice were housed in a specific pathogen-free (SPF) facility and fed with laboratory chow and ad libitum water. After a minimum 7 days of acclimation, the mice were randomly allocated into three groups with each containing 6 mice: (1) I/R-saline group (IRI), in which the mice were subjected to renal ischemia for 1 h; (2) I/R-dexamethasone group (DEX), in which the mice were administered dexamethasone (4 mg/kg, i.p.) 1 h prior to I/R induction; and (3) sham-operated group (Sham), in which the mice were subjected to identical surgical procedure but without occlusion of both renal pedicles. The dosage for dexamethasone was determined according to the previous studies [21]. All studies were approved by the Institutional Animal Care and Use Committee at the Tongji Medical College, Huazhong University of Science and Technology.

IR induction in the kidneys

The mice were first anaesthetized by intraperitoneal injection of 1% sodium pentobarbital solution (6 ml/kg). An abdominal incision was made to exposure kidneys, and renal pedicles were bluntly dissected and a microvascular clamp was placed on the left renal pedicle for 60 min. During the experimental procedure, the mice were kept well-hydrated with warm saline and at a constant temperature of 32°C in an infant incubator. After removal of the clamp, the contralateral kidney was removed. The-

reafter, the incision was sutured, and the animals were allowed to recover with free access to food and water. The mice were sacrificed 24 h after reperfusion, and kidneys were harvested for further analysis.

Assessment of renal function

Blood samples were obtained from the inferior vena cava 24 h after reperfusion. Blood urea nitrogen (BUN) and serum creatinine (Cr) levels were assayed in the core laboratory of Tongji Hospital for assessment of renal function.

Histological analysis

Renal samples were fixed in formalin and then embedded in paraffin, and renal sections were next prepared and subjected to periodic acid-Schiff (PAS) staining as reported [22]. The histopathological changes in the cortex and medulla were evaluated by a pathologist in a blinded fashion using a five-point quantitative scale according to the degree of tubular necrosis, hemorrhage and cast formation as follows: 0, < 10%; 1, 10-25%; 2, 25-50%; 3, 50-75% and 4, 75-100% [23].

Immunohistochemical staining for myeloperoxidase

Renal myeloperoxidase (MPO) activity was assessed by immunohistochemistry as described previously [23]. Neutrophil infiltration was assessed quantitatively by counting the number of MPO positive cells per high-power field (\times 400) over 10 fields, and then presented as the average neutrophil counts.

Western blot analysis

Western blot analysis of protein levels for GR, p-GR, p85, p-p85, AKT, and p-AKT in renal tissues was carried out as reported [24]. Antibodies against p-GR (Abclonal, USA, 1:500), GR (Abclonal, USA, 1:1000), p85 (Abcam, USA, 1:500), p-p85 (Abcam, USA, 1:1000), p-AKT

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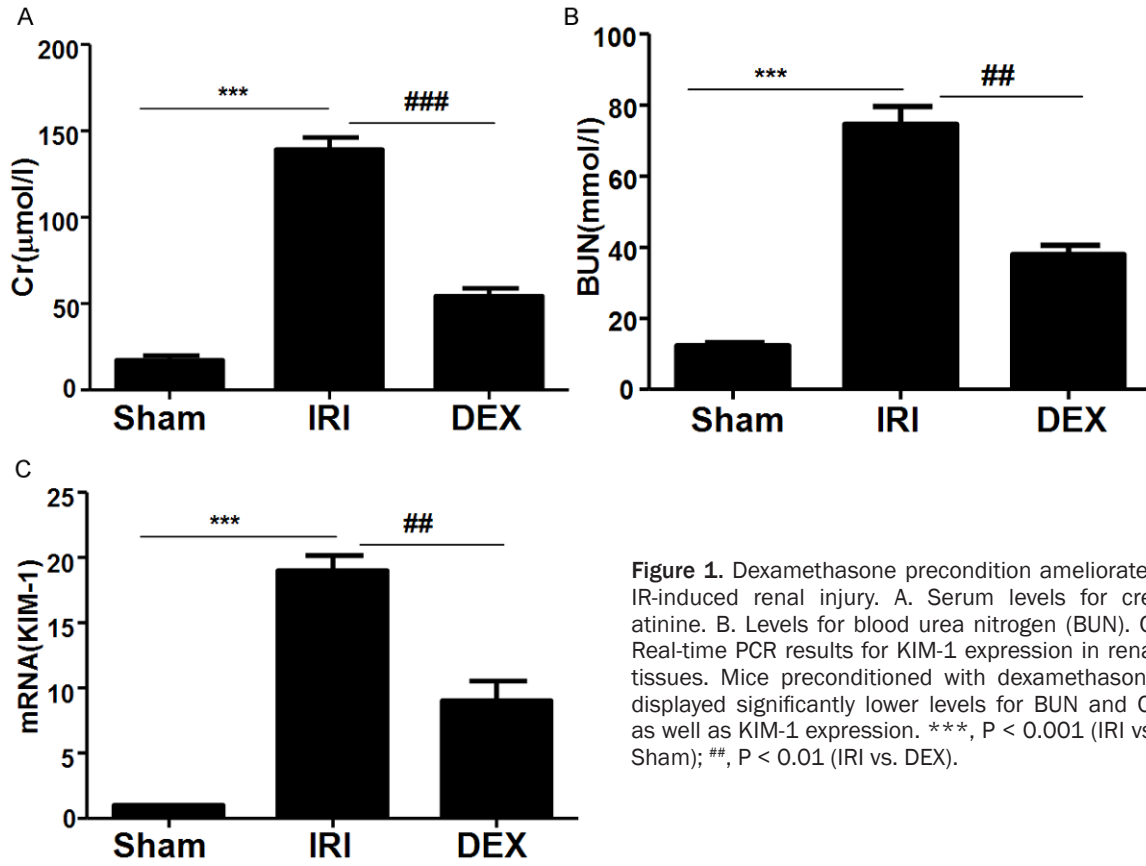


Figure 1. Dexamethasone precondition ameliorates IR-induced renal injury. A. Serum levels for creatinine. B. Levels for blood urea nitrogen (BUN). C. Real-time PCR results for KIM-1 expression in renal tissues. Mice preconditioned with dexamethasone displayed significantly lower levels for BUN and Cr as well as KIM-1 expression. ***, $P < 0.001$ (IRI vs. Sham); ##, $P < 0.01$ (IRI vs. DEX).

(CST, USA, 1:500), and AKT (CST, USA, 1:1000) were used to probe the membranes, followed by incubation with an HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). β -actin (Abmart, China, 1:300) was used for normalization. The reactive bands were visualized using the ECL-Plus reagent (Amersham, Piscataway, NJ) as instructed. The density of each reactive band was quantified using the Labworks image acquisition platform and its related analytic software (UVP, USA).

Real-time PCR analysis

Total RNA was isolated from renal tissues using Trizol according to the manufacturer's instructions (Takara, Japan). Four micrograms of total RNA were reverse transcribed into cDNA using the PrimeScript RT Master Mix (Takara, Japan) as instructed. Real-time PCR amplifications were carried out using the ABI 7500 system (Applied Biosystems, USA). PCR primers (Invitrogen, USA) for all analyzed genes are shown in **Table 1**. PCR was conducted at 95°C for 30 sec, followed by 40 cycles at 95°C for 5

sec, 60°C for 34 sec and 95°C for 15 sec. The amount of mRNA for each gene was normalized by β -actin, and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as reported [25].

Statistical analysis

All results are expressed as mean \pm SEM. Group comparisons were performed using the Student's *t*-test or analysis of variance and Survival analysis of the experiments. In all cases, P value < 0.05 was considered with statistical significance.

Results

Administration of dexamethasone protects mice against IR-induced renal injury

We first sought to establish the impact of dexamethasone on renal function following IR insult. For this purpose, we examined blood urea nitrogen (BUN) and serum creatinine (Cr) levels. As compared with those mice from Sham group, mice in Saline group (IRI) exhibited a 7-fold

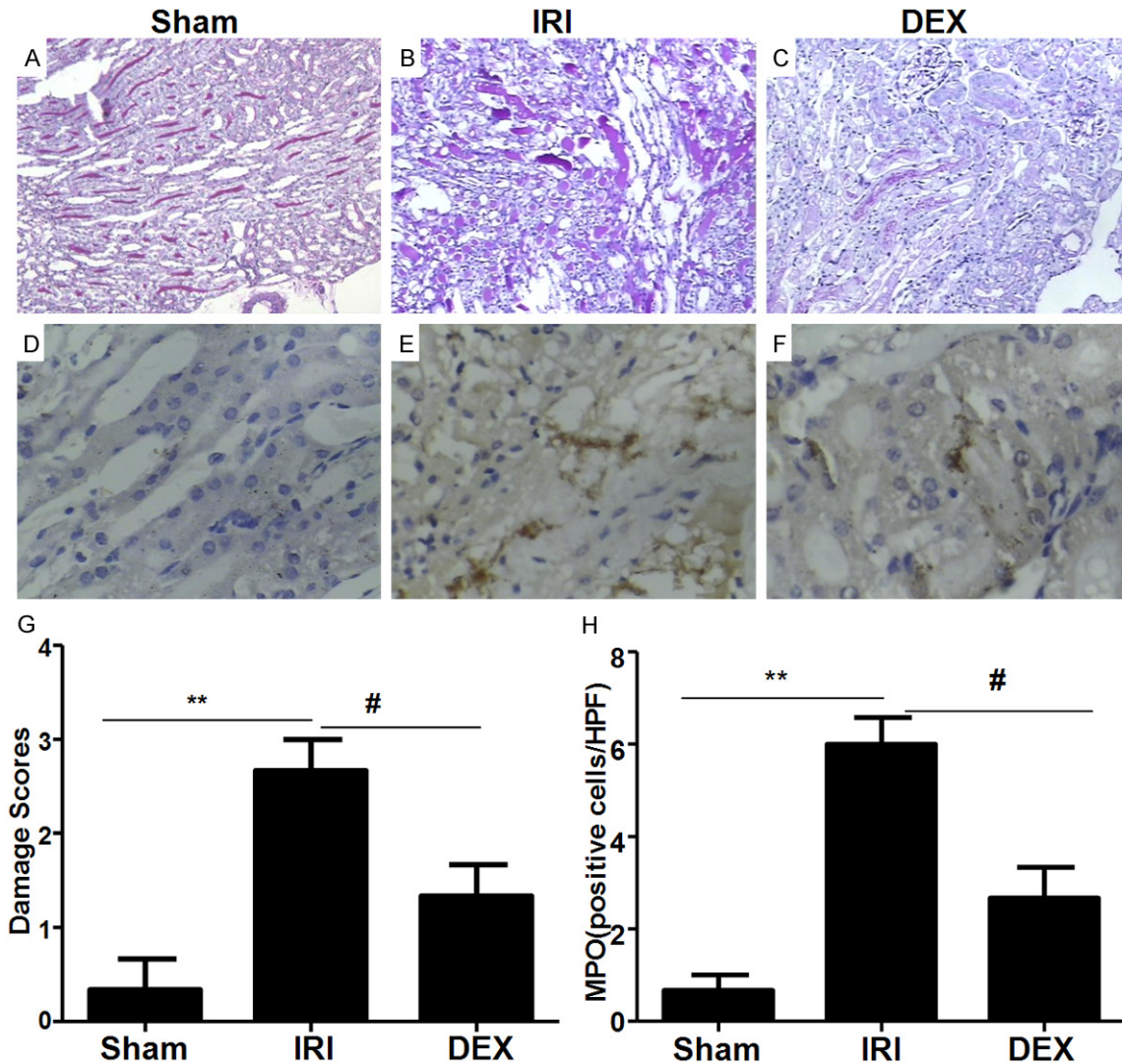


Figure 2. Histological analysis of renal sections. Representative PAS staining results for renal sections originated from: A. Sham mice; B. IRI mice; and C. DEX mice. Representative MPO staining results of renal sections originated from: D. Sham mice; E. IRI mice; and F. DEX mice. G. Semi-quantitative analysis of PAS staining for all mice included. H. Semi-quantitative assessment of MPO staining of all mice studied. Three mice were analyzed in each group, and the images are presented at $\times 200/\times 400$ magnifications. **, $P < 0.01$ (IRI vs. Sham); #, $P < 0.05$ (IRI vs. DEX).

higher levels of Cr (**Figure 1A**) and a 6-fold higher levels of BUN (**Figure 1B**), indicating that IR insult induced severe renal injury. Of Interestingly note, mice administered with dexamethasone showed a 1.3-fold lower levels of serum Cr (**Figure 1A**) and a 1-fold lower levels of BUN (**Figure 1B**) as compared with that of IRI mice. Given that the kidney injury molecule-1 (KIM-1) is a characteristic marker for renal injury, we next examined KIM-1 expression by real-time PCR. Similarly, a 18-fold higher KIM-1 expression was noted in IRI mice as compared with that of Sham mice (**Figure 1C**). However, dexamethasone administration attenuated KIM-

1 expression by approximately 1-fold (**Figure 1C**). Altogether, our data support that administration of dexamethasone provides protection for mice against IR-induced renal injury.

Dexamethasone attenuates IR-induced pathological changes in the kidney

To further confirm the above observations, we next conducted histological analysis of renal sections. Pathological changes relevant to IR injury in the renal sections were readily evident 24 h after reperfusion in mice from IRI group (**Figure 2B**) as compared with that of control

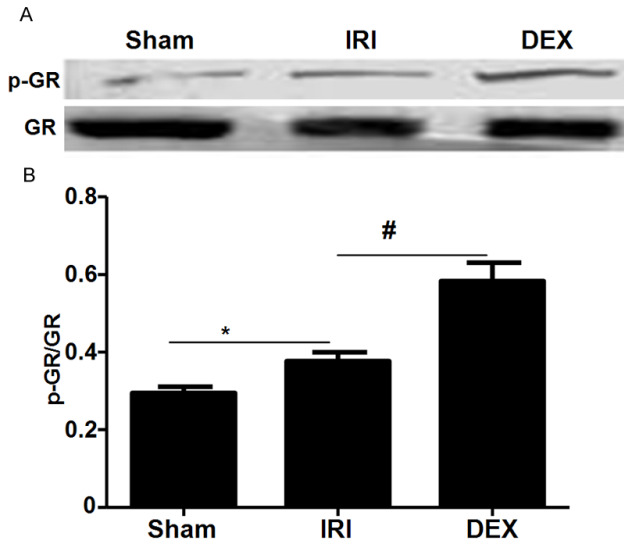


Figure 3. Precondition of dexamethasone promotes GR activation. Western blot analysis was employed to assess GR expression and activation by evaluating the levels for total GR and activated GR (p-GR). A. A representative result for Western blot analysis. B. Semi-quantitative analysis of 6 animals studied in each group. The relative amount of GR and p-GR in each group of mice was normalized by β -actin and presented as a ratio between p-GR and GR. *, $P < 0.05$ (IRI vs. Sham); #, $P < 0.01$ (DEX vs. IRI).

mice from Sham group (Figure 2A). Characteristically, the most pronounced injuries were observed in the cortex and outer stripe of the outer medulla manifested by the severe tubular necrosis, widespread degeneration of tubular architecture and detachment of epithelial cells from the basement membrane, intratubular cast formation, and luminal congestion with loss of brush border (Figure 2B). In contrast, renal sections originated from DEX mice demonstrated marked reduction for renal injury as manifested by more focal and less severe tubular necrosis (Figure 2C). In consistent with those observations, DEX mice displayed significantly lower pathological changes as compared with that of IRI mice (Figure 2G).

Next, we examined inflammatory infiltration, in which we assessed neutrophils by counting MPO positive cells through immunostaining. As expected, renal sections from Sham mice were negative for MPO staining (Figure 2D). However, MPO positive cells were readily detected in IRI (Figure 2E) and DEX mice (Figure 2F), but the number of MPO positive cells in renal sections of IRI mice were significantly higher than that of sections from DEX mice (Figure 2H).

Dexamethasone promotes IR-induced GR activation

As the first step to address the underlying mechanisms dexamethasone protection of mice against IR-induced injury, we examined glucocorticoid receptor (GR) activation, as dexamethasone is a ligand for GR. Unexpectedly, neither IR insult nor dexamethasone administration showed a perceptible impact on GR expression (Figure 3A, lower panel). However, the amount of activated GR (p-GR) noted in mice after IR insult was significantly higher as compared with that of control Sham mice (Figure 2A, upper panel). Nevertheless, administration of dexamethasone significantly promoted GR activation as manifested by the 50% higher levels of p-GR detected in DEX mice as compared with that of IRI mice (Figure 3B).

GR activation attenuates PI3K/AKT signaling

The above results prompted us to examine the impact of enhanced GR activation on PI3K/AKT signaling, a well recognized pathway with significant implications in IR-induced pathoetiology [11, 12, 15]. To this end, we first examined the activity for PI3K p85 regulatory subunit. We failed to detect a significant difference for total p85 between 3 groups of mice (Figure 4A). However, much higher levels of activated p85 (p-p85) were noted in IRI mice as compared with that of either DEX mice or control Sham mice (Figure 4B). Of note, DEX mice showed almost similar levels of p-p85 as that of control Sham mice. Since p85 activation would provide signals to phosphorylate AKT, we thus next examined AKT activity. Similar as p85, there was no difference for the total AKT (Figure 4C), but activated AKT (p-AKT) was significantly higher in IRI mice, and DEX mice displayed almost similar levels of p-AKT as that of Sham mice (Figure 4D). Collectively, our data suggest that dexamethasone enhanced GR activation, activated GR thus repressed PI3K p85 activity, which then attenuated AKT activation.

Attenuation of PI3K/AKT signaling suppresses inflammatory cytokine expression

The next important question is whether attenuated PI3K/AKT signaling would suppress the

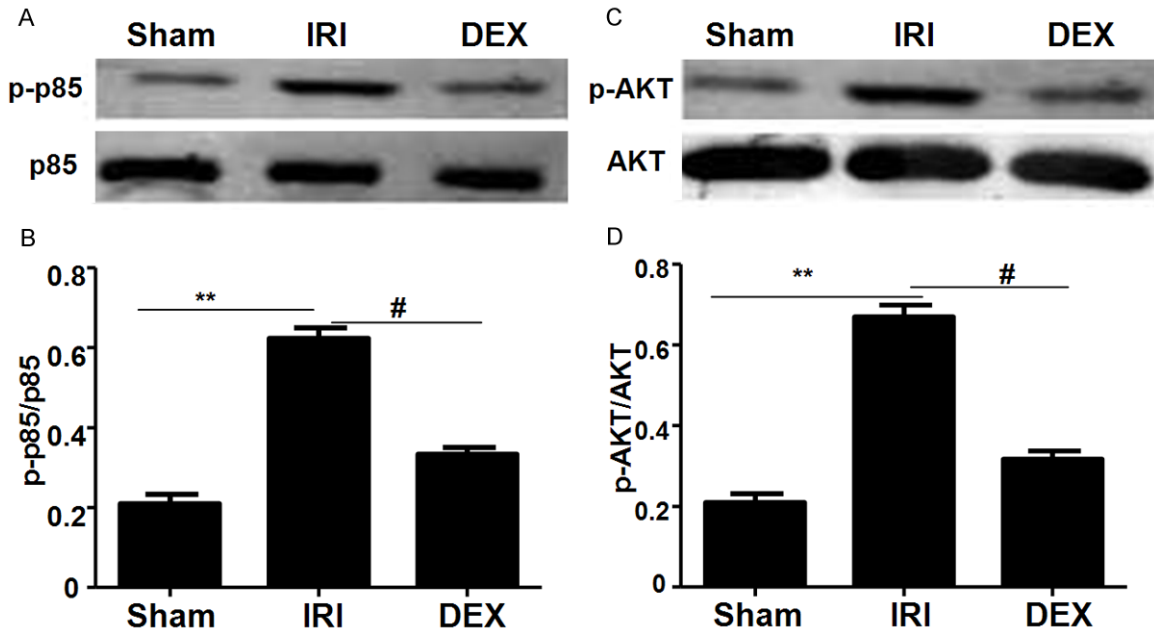


Figure 4. Administration of dexamethasone attenuates PI3K/AKT signaling. The p85 regulatory subunit was used for assessing the activity of PI3K, while the phosphorylated AKT (p-AKT) was employed for evaluating AKT activation. A. A representative Western blot result for p85 and p-p85. B. Results for quantitative analysis of p-p85 in all animals. C. Representative results for AKT Western blot analysis. D. Quantitative analysis of AKT activity in all mice studied. Similar as above, the relative activity for PI3K and AKT was assessed by a ratio between the phosphorylated form and total form. Six mice were included in each study group. **, $P < 0.001$ (IRI vs. Sham); #, $P < 0.01$ (IRI vs. DEX).

expression of inflammatory cytokines in the kidney. To address this issue, we selectively analyzed the expression of TNF- α , IL-6 and IFN- γ by real-time PCR in renal tissues after IR insult. It was noted that IR insult increased the expression of TNF- α by 9-fold (Figure 5A), IL-6 by 3.75-fold (Figure 5B), and IFN- γ by 5-fold as compared with that of control Sham mice. Remarkably, administration of dexamethasone inhibited IR-induced TNF- α expression by 49% (Figure 5A), IL-6 by 65% (Figure 5B), and IFN- γ by 57% (Figure 5C). Taken together, our data support that attenuation of PI3K/AKT signaling by administration of dexamethasone significantly suppressed IR-induced inflammatory cytokine expressions in the kidney.

Discussion

Although dexamethasone has long been used for anti-inflammatory therapy in the clinical settings, its exact impact on IR-induced renal injury, however, remains to be addressed. We thus induced IR injury in mice by blocking renal blood flow for a period of 60 min, and then examined IR injury 24 h after reperfusion. Blood tests revealed altered serum Cr and

BUN, while histological analyses demonstrated significant pathological changes manifested by the severe tubular necrosis, widespread degeneration of tubular architecture, detachment of epithelial cells from the basement membrane, intratubular cast formation, and luminal congestion with loss of brush border along with severe inflammatory infiltration. Remarkably, precondition of animals with 4 mg/kg of dexamethasone significantly attenuated those pathological changes associated with ameliorated renal injury. In line with our results, several previous studies suggest that treatment of mice with glucocorticoids can promote functional and morphologic tubular regeneration after I/R injury or cisplatin-induced acute kidney injury (ARF) [21, 26]. Taken these results into consideration, our data support that dexamethasone could be a novel therapeutic agent for prevention and treatment of IR-induced renal injury in the clinical practice.

To dissect the molecular mechanisms by which dexamethasone provides protection for mice against IR-induced renal injury, we first examined its impact on GR activation. Unexpectedly, either IR insult or dexamethasone administra-

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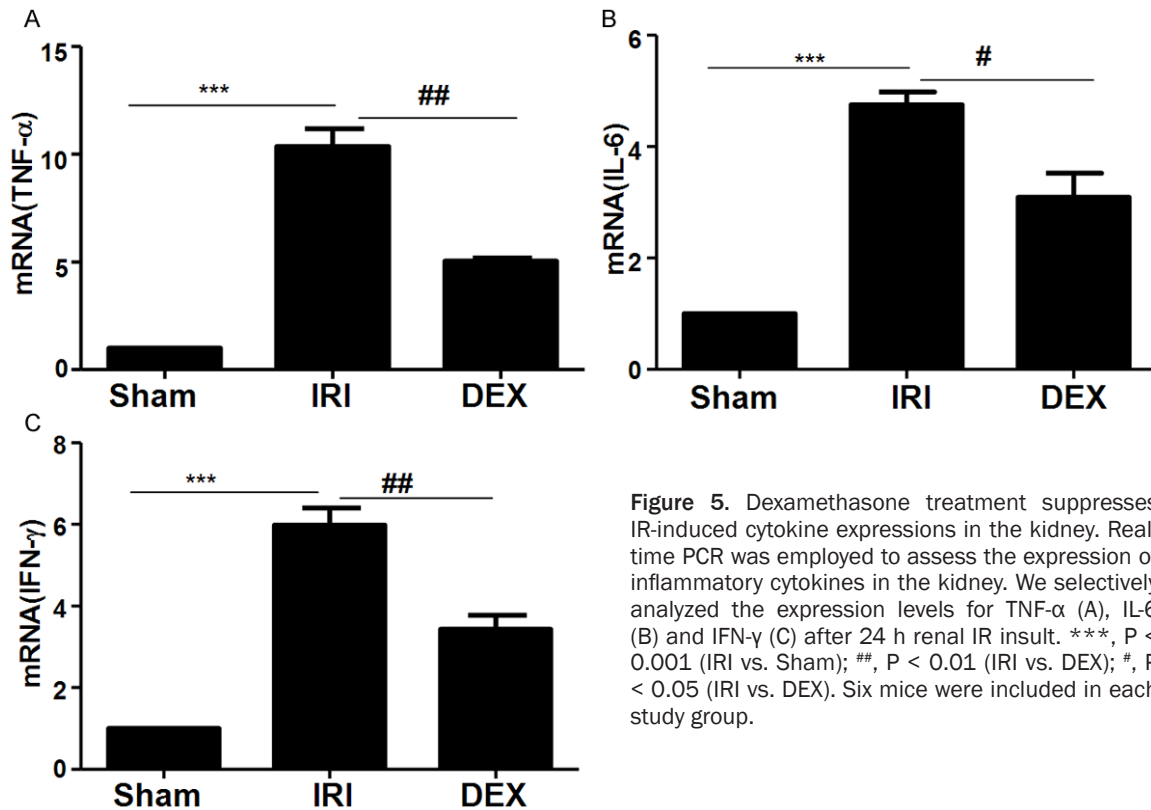


Figure 5. Dexamethasone treatment suppresses IR-induced cytokine expressions in the kidney. Real-time PCR was employed to assess the expression of inflammatory cytokines in the kidney. We selectively analyzed the expression levels for TNF- α (A), IL-6 (B) and IFN- γ (C) after 24 h renal IR insult. ***, $P < 0.001$ (IRI vs. Sham); ##, $P < 0.01$ (IRI vs. DEX); #, $P < 0.05$ (IRI vs. DEX). Six mice were included in each study group.

tion did not enhance GR expression. However, administration of dexamethasone increased p-GR by almost 1-fold. To our surprise, higher levels of p-GR were also observed in IRI mice as compared with that of control Sham mice, although its increase was significantly lower than that of dexamethasone preconditioned mice. This unexpected result is likely caused by the negative regulatory mechanisms, in which activation of negative feedback pathways is necessary to prevent overreactive and to stop prolonged immune response once a severe injury occurs in the body.

A number of studies revealed that GR ameliorates IR-induced liver/heart injury upon activation by cutting down inflammation [19, 20]. We thus next embarked on GR down stream pathways associated with regulation of immune response, in which the PI3K/AKT signaling could be a good candidate. Indeed, IR insult induced PI3K p85 regulatory subunit activation as manifested by the significantly higher levels of p-p85 in IRI mice as compared with that of control Sham mice. As expected, precondition of mice with dexamethasone significantly attenuated p85 activation. This result promoted us to examine AKT activity since phosphory-

lation of p85 would predispose AKT to activation. In consistent with the above results, dexamethasone significantly attenuated AKT activity as manifested by > 1-fold decrease of p-AKT. To further demonstrate that attenuated PI3K/AKT signaling inhibits inflammatory response, we next selectively examined TNF- α , IL-6 and IFN- γ expression in IR-insulted renal tissues. Indeed, mice preconditioned with dexamethasone displayed significantly lower levels of TNF- α , IL-6 and IFN- γ . Taken together, our data suggest that dexamethasone enhances GR activation, and by which it attenuates PI3K/AKT signaling, which then represses inflammatory response to protect kidneys from IR-induced injury.

Ischemic time and check point after reperfusion are critical factors relevant to the severity of IR injury in mice. The published data manifested variations in terms of experimental conditions such as temperature, duration of ischemia and so on. Particularly, the results could vary based on the strain of mice employed, for example C57BL/6 versus BALB/c. In our experimental condition, we employed C57BL/6 mice, and selected 60 min for ischemic insult and 24 h after reperfusion as the check point for exam-

ining IR injury. A similar degree of severity for renal injuries was noted in our model as compared with that of published data [27]. As aforementioned, evidence for renal injury was strongly supported by the pathological changes along with inflammatory infiltration, which was manifested by the presence of MPO positive cells and increased KIM-1 expression in the kidney. These data also support that I/R initiates a complex cascade of event, which eventually results in renal injury characterized by histological pathology and inflammatory infiltration [27].

The PI3K/AKT pathway has long been recognized to be important in regulating adaptive immune response. For example, different PI3K heterodimers critically control cell survival, proliferation, B- and T-cell receptor (BCR and TCR, respectively) signaling, and chemotaxis in B and T lymphocytes [28, 29]. More recently, it has increasingly been re-discovered that the PI3K/AKT pathway has broad and yet distinct roles also in innate immune cells, including neutrophils, mast cells, monocytes, macrophages and myeloid as well as plasmacytoid dendritic cells. For example, migration of innate immune cells into the site of injured tissues or organs involves dynamic reorganization of cytoskeleton and membrane structure, while PI3K signaling is essential for this process by providing cell polarity and pseudopodia extension [29]. There is also a report suggesting that inhibition of PI3K attenuates IR-induced renal injury [15]. We, therefore, in the current report did not conduct additional studies to demonstrate that suppressed PI3K/AKT signaling represses IR-induced immune response in the kidney. Given the capacity of dexamethasone precondition in prevention of IR-induced renal injury, it is worthy of note that GR activation induced by dexamethasone could involve additional pathways other than the PI3K/AKT signaling, such as the MAPK kinase cascade [23]. As a result, further studies with focus addressing the pathways involved in GR activation in the setting of IR insult would be necessary.

In summary, we demonstrated convincing evidence that precondition of animals with dexamethasone provides protection for mice against IR-induced renal injury as manifested by the improved renal function along with ameliorated pathological changes and suppressed inflammatory infiltration. Our mechanistic studies

demonstrated that dexamethasone promotes GR activation, and through which it attenuates PI3K/AKT signaling, which then suppresses inflammatory infiltration and protects kidneys from IR-induced injury. All together, our data support that dexamethasone could be a good alternative therapy for prevention and treatment of IR-induced renal injury in the clinical practice.

Acknowledgements

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

Address correspondence to: Dr. Gang Xu and Dr. Junhua Li, Department of Nephrology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China. Tel: 86-2783662848; Fax: 862783663187; E-mail: xugang@tjh.tjmu.edu.cn (Gang Xu); lijunhua333@163.com (Junhua Li)

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