

Metformin attenuated the inflammation after renal ischemia/reperfusion and suppressed apoptosis of renal tubular epithelial cell in rats¹

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ABSTRACT

PURPOSE: To investigate the effect of metformin on renal tubular epithelial cell apoptosis and inflammation after kidney ischemia/reperfusion in rats.

METHODS: Eighteen SD rats were randomly divided into three groups: Sham (S), Ischemia/reperfusion (I/R), and Metformin (E). Before establishing the I/R model, group E was administered metformin for three days, while groups S and I/R were administered equal volumes of saline. After three days, a right nephrectomy was performed on all groups, after which the left kidneys of groups E and I/R rats were subjected to 45 min renal ischemia. Renal function, histology, and cell apoptosis were assessed. AMPK, pAMPK, COX-2, and Caspase 3 were also detected.

RESULTS: Compared to I/R group, Caspase 3 and COX-2 levels were decreased in group E. COX-2, Caspase3 and pAMPK levels were higher in groups E and I/R than in group S. The pAMPK level of group E was higher than that of I/R group, while COX-2 and caspase 3 were lower in group E than they were in the other groups. There was no significant difference between E and I/R groups in AMPK levels.

CONCLUSION: Metformin preconditioning attenuated the inflammation caused by ischemia/reperfusion and inhibited the apoptosis of renal tubular epithelial cells.

Key words: Metformin. Ischemia. Reperfusion. Inflammation. Apoptosis. Kidney. Rats.

Introduction

Kidney ischemia-reperfusion (I/R) injury is a common clinical, pathological process. Acute renal failure is a clinical disease, which occurs in 5% of the total incidences of hospitalized patients and reaches 25% in severe illness¹. Ischemia is the main cause of acute renal failure, and it causes injury to the kidney. Although the recovery of blood flow is essential for the survival of ischemic kidney tissue, this may paradoxically cause additional damage to the kidney. Renal tubular epithelial cell apoptosis is the main pathophysiological change that occurs in I/R, and it determines the extent of damage to the kidney function.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK), contributes to meeting the energy requirements of ischemic tissue by inhibiting synthesis metabolism, promoting catabolism, and increasing the synthesis of adenosine triphosphate (ATP) while reducing its consumption. It has been confirmed that metformin relieves inflammatory responses following cardiac ischemia and protects cardiac function by activating AMPK². It has also been proven that AMPK agonist alleviate the ischemia/reperfusion injury of renal epithelial cells in vitro and in animals such as Sprague-Dawley (SD) rats and canines^{3,4}. Furthermore, AMPK agonists have anti-inflammatory effects, which inhibit the expression of inflammation factors such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, and cyclooxygenase (COX2)⁵⁻⁷. Studies have proven that COX2 is associated with aggravation of inflammatory responses in numerous diseases^{8,9} including kidney I/R injury¹⁰. Moreover, it has been verified that the expression level of COX2 increased after I/R in the kidney of rats¹¹ while inhibition of COX2 was beneficial and improved renal function¹⁰.

Metformin may aggravate renal insufficiency, which in its severe form is a contraindication for the administration of metformin. However, we hypothesized that low doses of metformin may attenuate renal I/R injury by increasing the energy supply to the ischemic tissue and reducing the expression of inflammatory cytokines. Furthermore, the simultaneous occurrence of these metformin-induced actions may be beneficial and further protect the renal function.

Methods

The experimental animal protocol used in our study was approved by the Animal Ethics Review Committee of the Wuhan University, and the procedures were carried out accordance with the recommendations in the Guide for the Care and Use of Laboratory

Animals of the National Institutes of Health (NIH, Bethesda, MD, USA). Rats were kept in an air-filtered, homoiothermal (20–22°C), and light-controlled (8 a.m.–8 p.m.) room, and allowed free access to a standard diet.

Eighteen healthy adult male SD rats, weighing 220–250 g were provided by the Experimental Animal Center of the Medical College of Wuhan University (Wuhan, China). The rats were anesthetized by intraperitoneal (ip) injection of pentobarbital (50 mg/kg), administered 500 U of heparin ip, and then placed on an electric heating pad to maintain their body temperature at 37°C. The rats were divided into three groups using a random number table ($n = 6$). The groups included the sham operated (S), I/R, and metformin (E) groups. All the groups underwent a right nephrectomy while the I/R group alone was further subjected to 45 min of renal ischemia of left the kidney after which the renal arteriovenous perfusion was restored. For 3 days prior to establishing the I/R model, group E was administered metformin (0.125 mg·kg⁻¹·d⁻¹, ip) while the S and I/R groups were administered the same volume of normal saline.

Reagents and drugs

The cell apoptosis detection kit was purchased from Roche Co., Ltd (Basel, Switzerland), while the polyclonal rabbit anti-rat antibodies against caspase 3, AMPK, pAMPK, and COX2 were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-rat β -actin monoclonal antibody and horseradish peroxidase (HRP)-tagged goat anti-rabbit IgG antibody were both purchased from the Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. The SC-2048 enhanced chemiluminescence (ECL) kit, and metformin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma-Aldrich Corp. (St. Louis, USA), respectively.

Detection of blood urea nitrogen (BUN) and creatinine (Cr)

After a 24-h reperfusion, all the rats were euthanized and 5-mL blood samples were collected from the inferior vena cava, and centrifuged for the determination of concentration of blood urea nitrogen (BUN) and creatinine (Cr). These parameters were measured in the blood using standard techniques with an Olympus AU 2700 Analyzer (Olympus Optical Co., Ltd., Tokyo, Japan).

Hematoxylin and eosin (H&E) staining and immunohistochemical analysis

After the blood samples had been collected, the left kidneys of all rats were harvested, and tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin according to standard procedures. Then the tissue was sectioned onto slides, gradually deparaffinized, rehydrated, and subsequently observed using hematoxylin and eosin (H&E) staining. The morphological assessment was performed by an experienced renal pathologist who was blinded to the experimental grouping, using standard methodology. The histological grading standard used was the Jablonski grade¹², which was classified into levels 0–4. A StreptAvidin-Biotin Complex (SABC) immunohistochemical staining kit was used to detect the expression of COX2 and Caspase-3, and all steps were performed strictly adhering to the manufacturer’s instructions. Furthermore, we determined the expression rate and spatial distribution of the COX-2 and Caspase-3 positive cells.

Determination of cell apoptosis

To observe cell apoptosis induced by ischemia, an in-situ apoptosis detection kit (Promega, USA) was used and a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed following the manufacturer’s instructions. The normal and apoptotic cell nuclei were blue-green and different shades of brown, respectively. Five high-power fields of vision in the distribution areas of the apoptotic cells in each slide were chosen, and the average number of apoptotic cells per 100 cells was calculated. The apoptotic index (AI) was expressed as a percentage (%).

Western blot analysis

The kidney tissue protein samples were extracted and quantitated using the bicinchoninic acid (BCA) assay,

and stored at -70°C until analyzed. Briefly, protein samples were prepared and separated (50 µg/lane) using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline plus Tween (TBST) buffer and then incubated with polyclonal primary antibodies against caspase-3, COX-2, AMPK, and pAMPK (1:100 each) and monoclonal rabbit anti-rat β-actin (1:2000) overnight at 4°C. After extensively washing the membranes with TBST buffer, they were incubated with their respective secondary antibodies for 1 h. The proteins were detected using an ECL system kit (Pierce Biotechnology, Beijing, China) and captured on light-sensitive X-ray film (Kodak, Shanghai, China). The optical densities were analyzed using the ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

All data was statistically analyzed using the statistical package for the social sciences (SPSS) version 18.0 (SPSS Inc., Chicago, IL, USA). The means of the different groups were compared using the Student’s *t*-test and differences were considered statistically significant *p*<0.05.

Results

Effect of metformin on renal function

Rats in the I/R and E groups showed significant increases in serum BUN and Cr levels compared with the S group rats (*p*<0.05). Furthermore, the I/R-induced increase improved following treatment with metformin; however, there was no significant difference between the I/R and E groups (Table 1).

TABLE 1 - Metformin preconditioning reduced Jablonski grade and apoptosis index.

Group	BUN	Cr	Jablonski grade	Apoptosis index %
S	5.01+0.16	30.33+0.67	0.33+0.21	1.67+0.33
I/R	40.77+1.42*	326.5+10.22*	3.67+0.21*	34.50+1.12*
E	38.53+1.42*#	297.3+11.44*#	2.50+0.34*##	27.50+1.12*##

**p*<0.05 vs S group, #*p*>0.05 vs I/R group, ##*p*<0.05 vs I/R group. BUN, blood urea nitrogen; Cr, creatinine; S, sham-operated; I/R, ischemia/perfusion; E, metformin.

Effect of metformin on renal histopathological changes

The H&E stained kidney tissue sections showed that there were no obvious glomerular and renal tubular necrotizing pathological changes in the S group. However, kidney I/R resulted in significant renal injury, as evidenced by acute tubular necrosis, renal tubular expansion, renal tubular epithelial cell exfoliation, and development of proteinaceous casts. There were less renal tubular necrotizing changes in the group E than there were in the I/R group. The Jablonski grade analysis of severe acute tubular necrosis results revealed significantly lower scores for the E group

than for the I/R group. Metformin preconditioning significantly reduced the Jablonski grade and AI in group E compared with that of the I/R group ($p < 0.05$). Compared to I/R group, Metformin preconditioning improved the renal dysfunction induced by I/R injury, although there were no significant differences between groups E and I/R ($p > 0.05$). Compared with group S, the I/R and E groups exhibited higher serum Cr and BUN levels as well as Jablonski grade and AI indices ($p < 0.05$, Table 1 and Figure 1). Furthermore, compared to the S group, the results of the TUNEL staining also revealed a significant increase in the AI of the E and I/R groups ($p < 0.05$), although that of group E was lower than that of the I/R group was ($p < 0.05$, Table 1, Figure 2).

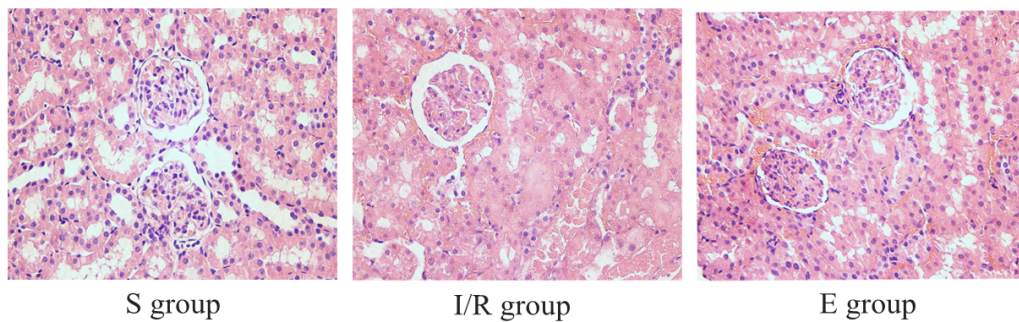


FIGURE 1 - Metformin preconditioning improved morphological feature after renal ischemia/reperfusion (IR) injury. Compared to group S, I/R and E groups exhibited more tubular necrosis, renal tubular expansion, renal tubular epithelial cellular exfoliation, and development of proteinaceous casts. Renal tubular necrotizing pathological changes in group E were fewer than in I/R group. Jablonski grade of severe acute tubular necrosis was significantly lower in group E than in I/R group ($p < 0.05$, magnification $\times 200$). S, sham operated; E, metformin.

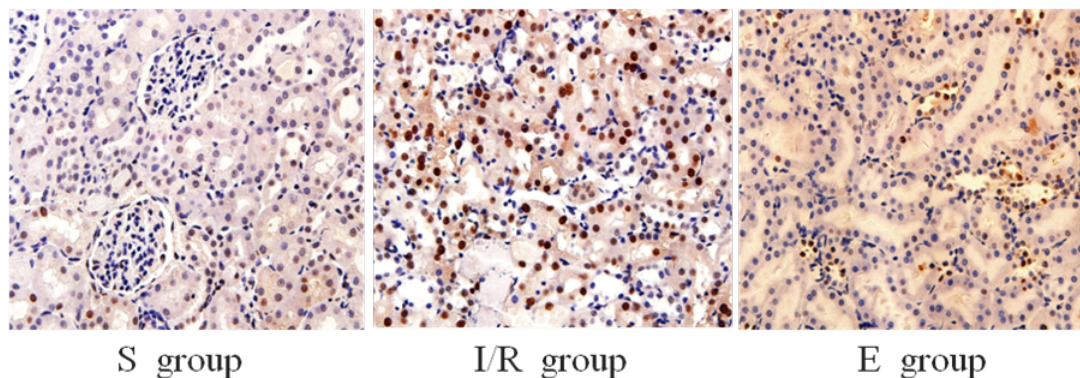


FIGURE 2 - Metformin preconditioning attenuated apoptosis of tubular epithelial cells. TUNEL staining shows apoptosis index increased significantly in groups E and I/R compared with group S ($p < 0.05$). Apoptosis index of group E was lower than that of I/R group ($p < 0.05$, magnification $\times 200$). TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; S, sham operated; I/R, ischemia reperfusion; E, metformin.

Effect of metformin on expression of pAMPK, COX-2, and Caspase-3

The results of the immunohistochemistry analysis showed that the expression levels of COX-2 and Caspase-3 in the E and I/R groups were significantly higher than those in the S group were. However, the E group levels were much lower than those of the I/R group were (Figure 3). Furthermore, the western

blot analysis revealed that the expressions of COX-2, Caspase-3, and pAMPK in groups E and I/R were significantly higher than those in the S group were ($p < 0.05$), while the expression of pAMPK was much more in group E than it was in the I/R group ($p < 0.05$). In addition, the expression levels of COX-2 and Caspase 3 were lower in group E than they were in the I/R group ($p < 0.05$). However, there was no significant difference in the expression of AMPK between groups E and I/R ($p > 0.05$, Figure 4).

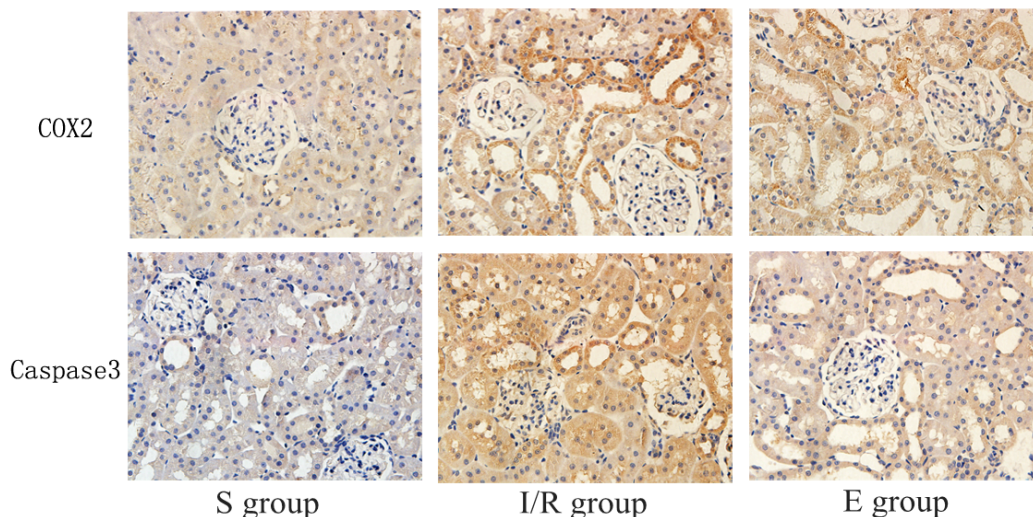


FIGURE 3 - Effect of metformin preconditioning on cyclooxygenase (COX)-2 and Caspase 3 expressions after renal ischemia-reperfusion (I/R). Immunohistochemistry shows expression levels of COX2 and Caspase3 in groups R and I/R were obviously higher than they were in group S. However, expression levels of COX2 and Caspase 3 in group E were much lower than they were in I/R group (magnification $\times 200$). S, sham operated; I/R, ischemia reperfusion; E, metformin.

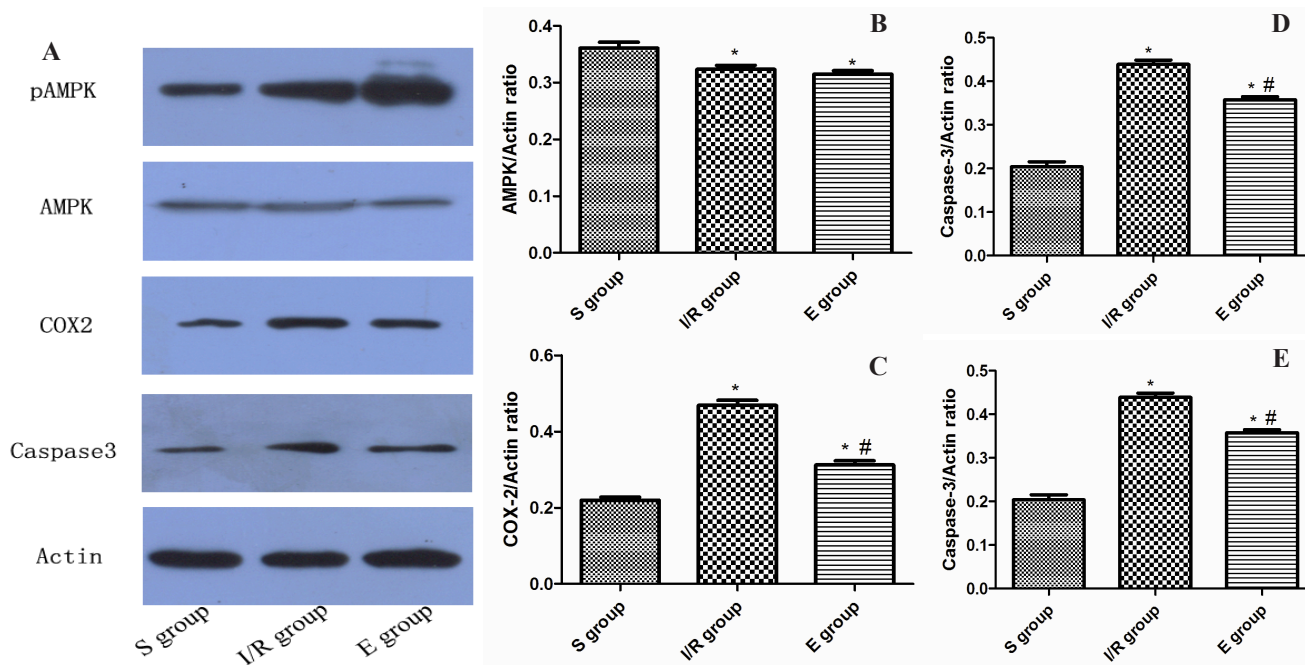


FIGURE 4 - Effect of metformin preconditioning on expression of adenosine monophosphate (AMP)-activated protein kinase (AMPK), phosphorylated (p)AMPK, cyclooxygenase (COX)-2 and Caspase 3. Expressions of COX-2, Caspase 3, and pAMPK in groups E and I/R were significantly higher than they were in group S ($p < 0.05$). Expression of pAMPK was significantly higher in group E than it was in I/R group ($p < 0.05$), while levels of COX-2 and Caspase 3 were lower in group E than they were in I/R group ($p < 0.05$). However, no significant differences were found in expression of AMPK between groups E and I/R ($p > 0.05$). Bars represent means \pm S.E.M. ($n = 6$), * $p < 0.05$ vs S group, # $p < 0.05$ vs I/R group. S, sham operated; I/R, ischemia reperfusion; E, metformin.

Discussion

I/R injury is known to be induced by the ischemia that occurs after blood flow recovery and restoration to tissues and organs, which further aggravates the injury. It is generally accepted that I/R injury is associated with a series of inflammatory reaction processes, which include endothelial cell activation, expression of adhesion molecules, adhesion, aggregation and activation of leukocytes and platelets, release of oxygen free radicals, and cellular calcium overload as well as apoptosis that is mediated by these processes. Acute kidney injury caused by I/R is a clinical syndrome that leads to rapid kidney dysfunction and high mortality rates^{13,14}. However, the mechanisms underlying the development of I/R injury have not been fully elucidated.

The main characteristics of ischemic diseases include inflammatory responses, metabolic disorders, and cell necrosis^{15,16}. AMPK is an evolutionarily conserved serine/threonine kinase and one of the key energy regulatory factors, which could inhibit constructive metabolism and promote the oxidation of fatty acid, glucose uptake, and glycolysis following its activation¹⁷. All these processes facilitate the increase in energy supply to the ischemic tissue.

There are several known physiological, pharmacological, and natural activators of AMPK, and a few of these are currently used clinically^{18,19} such as metformin. Accumulating evidence has shown that metformin inhibits the proliferation of prostate, ovarian, colon, and pancreatic cancer cells²⁰⁻²². Furthermore, studies with metformin have shown that inflammatory mediators such as TNF- α , IL-6, interferon (INF)- γ , and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) were decreased following supplementation with metformin²³⁻²⁵. Nevertheless, the effect of metformin on the kidney ischemia/reperfusion has not been studied yet. Therefore, in the present study, we investigated the effects of metformin on renal tubular epithelial cell apoptosis and inflammation in a rat model of kidney I/R injury.

Metformin is an oral hypoglycemic agent that is commonly used in the treatment of type 2 diabetes mellitus. Furthermore, metformin has been shown to inhibit the activation of complex 1 in the Krebs cycle and upregulate the ratio of AMP/ATP, which activates AMPK²⁶. Therefore, we further sought to determine if the effects of metformin in I/R injury are associated with the activation of AMPK signaling. Studies have shown that metformin protected the activity of Na-K-ATPase after ischemia and maintained the polarity of the renal tubular epithelial cells²⁷ and, thereby, protected kidney tissue after IR injury perhaps by activating the AMPK cascade. In this study, we found that pretreatment with metformin increased the level of pAMPK.

Inflammatory responses are also important factors that contribute to worsening of renal function after ischemia-reperfusion. Studies show that AICAR (an AMPK agonist) inhibited inducible nitric oxide synthase (iNOS) and COX-2 produced by macrophages. COX-2 is the key enzyme in prostanoid synthesis, and an increase in its expression enhances the synthesis of proinflammatory factors such as prostaglandin (PG) E₂ and PGI₂, which might further aggravate the inflammatory response. In our study, we found that treatment with metformin reduced the levels of the inflammatory mediator, COX-2 and the proapoptotic factor, caspase-3 in rats following I/R injury. Ariane Bischoff *et al.*²⁸ found that SC58125, a selective inhibitor of COX-2 alleviated renal tubular epithelial cell apoptosis after kidney I/R. We speculated that metformin reduced the activation of macrophages following ischemia and inhibited the expression of COX-2 and Caspase-3 and, thereby, attenuated the inflammatory responses and apoptosis of the renal tubular epithelial cell. Furthermore, these effects may have been mediated by the activation of AMPK, and were beneficial in protecting the kidney tissue against I/R injury.

Conclusions

Metformin played a role in alleviating the inflammatory responses and apoptosis of renal tubular epithelial cell, which was beneficial in protecting the kidney from I/R injury. However, metformin did not significantly improve the kidney function in the acute phase of I/R injury. The results of this rodent study might be useful as a foundation for further research to elucidate the specific underlying protective mechanisms of metformin in renal I/R injury.

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