

RESEARCH PAPER

AMPK activator AICAR ameliorates ischaemia reperfusion injury in the rat kidney

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BACKGROUND AND PURPOSE

Renal ischaemia/reperfusion (RI/R) injury is a major cause of acute kidney injury (AKI) and an important determinant of long-term kidney dysfunction. AMP-kinase and histone deacetylase sirtuin 1 (SIRT1) regulate cellular metabolism and are activated during hypoxia. We investigated whether AMP-kinase activator AICAR (5-amino-4-imidazolecarboxamide riboside-1-β-D-ribofuranoside) ameliorates RI/R injury and whether SIRT1 is involved in the pathogenesis.

EXPERIMENTAL APPROACH

Eight-week-old Sprague Dawley rats were divided into five groups: (i) sham-operated group; (ii) I/R group (40 min bilateral ischaemia followed by 24 h of reperfusion); (iii) I/R group + AICAR 50 mg·kg⁻¹ i.v. given 60 min before operation; (iv). I/R group + AICAR 160 mg·kg⁻¹ i.v.; (v) I/R group + AICAR 500 mg·kg⁻¹ i.v. Serum creatinine and urea levels were measured. Acute tubular necrosis (ATN), monocyte/macrophage infiltration and nitrotyrosine expression were scored. Kidney AMP-activated protein kinase (AMPK) and SIRT1 expressions were measured.

KEY RESULTS

Highest dose of AICAR decreased serum creatinine and urea levels, attenuated I/R injury-induced nitrosative stress and monocyte/macrophage infiltration, and ameliorated the development of ATN. Kidney I/R injury was associated with decreased AMPK phosphorylation and a fivefold increase in kidney SIRT1 expression. AICAR increased pAMPK/AMPK ratio and prevented the I/R-induced increase in renal SIRT1 expression.

CONCLUSIONS AND IMPLICATIONS

AICAR protects against the development of ATN after kidney I/R injury. Activators of kidney AMP kinase may thus represent a novel therapeutic approach to patients susceptible to AKI and to those undergoing kidney transplantation. The present study also suggests a role for SIRT1 in the pathogenesis of RI/R injury.

Abbreviations

AFOS, alkaline phosphatase; AICAR, 5-amino-4-imidazolecarboxamide riboside-1-β-D-ribofuranoside; AKI, acute kidney injury; ALAT, alanine aminotransferase; AMPK, AMP-activated protein kinase; ASAT, aspartate aminotransferase; ATN, acute tubular necrosis; eNOS, endothelial nitric oxide synthase; FOXO, Forkhead box protein; LKB1, liver kinase B1; MnSOD, manganese superoxide dismutase; NAD, nicotinamide adenine dinucleotide; Nox1, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1; PGC-1α, peroxisome proliferator-activated receptor γ-coactivator 1-α; RI/R renal ischaemia/reperfusion; SIRT1, sirtuin 1 (silent information regulator 1); ZMP, 5-aminoimidazole-4-carboxamide-1-β-D-furanosyl 5'-monophosphate

Introduction

Acute kidney injury (AKI) occurs in 5% of all patients admitted to hospital and is associated with a 2- to 15-fold increase in mortality (Kunzendorf *et al.*, 2010). AKI is the generic term for sudden decrease in renal function resulting in decreased urine output and retention of nitrogenous (creatinine and urea) and non-nitrogenous waste products in the blood (Lameire *et al.*, 2005). Although the pathophysiology of AKI is complex and the aetiologies diverse, there is now accumulating evidence to indicate that hypotension, hypoperfusion, hypoxia, oxidative stress and renal vasoconstriction contribute to the pathogenesis (Kunzendorf *et al.*, 2010). These detrimental changes in the renal haemodynamics and cellular metabolism have been shown to result in tubular and endothelial cell injury, mitochondrial damage and further renal vasoconstriction due to activation of the sympathetic nervous system and renin-angiotensin-aldosterone axis (Noiri *et al.*, 2001; Devarajan, 2006; Hasegawa *et al.*, 2010). Currently, there is no specific drug therapy for AKI, and recommendations for the prevention and non-dialysis treatment of AKI include mainly i.v. isotonic hydration, avoidance of nephrotoxins, maintenance of adequate systemic blood pressure, cardiac output and renal blood flow, as well as restoration and/or increase in urine flow (Lameire *et al.*, 2005).

AMP-activated protein kinase (AMPK) is a ubiquitously expressed heterotrimeric kinase that acts as a highly conserved ultrasensitive energy sensor and participates in the regulation of energy-generating and energy-consuming pathways (Hallows *et al.*, 2010). It is activated by an upstream kinase liver kinase B1 (LKB1) and when the intracellular AMP/ATP ratio is increased. It has been shown that AMPK plays an important role in the regulation of ion transport, podocyte function and diabetic renal hypertrophy in the kidneys (Hallows *et al.*, 2010). AMPK is also a key regulator of lipid and glucose metabolism. Previous studies have provided evidence that AMPK is rapidly activated by acute renal ischaemia; however, the functional significance of AMPK activation remains undefined (Hallows *et al.*, 2010). Mount *et al.* (2005) showed recently that although renal ischaemia activates AMPK within 1 min, the downstream target for AMPK, namely endothelial nitric oxide synthase (eNOS), was not phosphorylated. It is also unclear whether long-term pharmacological activation of AMPK provides protection against renal ischaemia/reperfusion (RI/R) injury.

Sirtuin 1 (SIRT1), a member of highly conserved nicotinamide adenine dinucleotide (NAD)-dependent class III histone deacetylases, is another ultrasensitive energy sensor. SIRT1 increases cellular stress resistance and genomic stability, and regulates cellular senescence via deacetylation of its target proteins such as p53, Forkhead box protein (FOXO) transcription factors and PPAR γ -coactivator 1- α (PGC-1 α) (Hao and Haase, 2010). Interestingly, SIRT1 activation has been shown to protect the kidney medulla from oxidative injury (He *et al.*, 2010), and a close interplay between AMPK and SIRT1 in the regulation of cellular metabolism and inflammation has recently become apparent (Ruderman *et al.*, 2010).

AICAR (5-amino-4-imidazolecarboxamide riboside-1- β -D-ribofuranoside) is an adenosine analogue, which

binds directly to AMPK leading to allosteric modification and activation of AMPK. AICAR is taken up by adenosine transporters and subsequently phosphorylated to ZMP (5-aminoimidazole-4-carboxamide-1- β -D-furanosyl 5'-monophosphate) in the cell (Wong *et al.*, 2009). ZMP in turn mimics AMP in AMPK signalling (Merrill *et al.*, 1997). The aim of present study was to investigate whether AICAR ameliorates renal I/R injury. We were able to demonstrate that AICAR protected against the development of acute tubular necrosis (ATN) after kidney I/R injury. The beneficial effects of AICAR were mainly due to AMPK activation. However, AICAR also attenuated I/R injury-induced nitrosative stress, renal monocyte/macrophage infiltration and I/R injury-induced SIRT1 overexpression. Activators of kidney AMP kinase may thus represent a novel therapeutic approach to the treatment of AKI.

Methods

Experimental animals, RI/R, AICAR administration and sample preparation

Sixty-one 6-week-old male Sprague Dawley rats were purchased from Charles River Laboratories (Research Models and Services, Sulzfeld, Germany). The protocols were approved by the Animal Experimentation Committee of the University of Helsinki, Finland, and the Provincial State Office of Southern Finland (approval number STH059A), whose standards correspond to those of the American Physiological Society. Rats were kept under 12-h light/12-h dark cycle and they had free access to food and water. After a 1 week adaptation period, the rats were divided into five groups: (i) sham-operated controls receiving vehicle (0.9% NaCl 5 mL·kg⁻¹ i.v.; $n = 17$), (ii) I/R group receiving vehicle ($n = 19$), (iii) I/R group treated with AICAR (Bepharma Ltd, Shanghai, China) 50 mg·kg⁻¹ i.v. ($n = 8$), (iv) I/R group treated with AICAR 160 mg·kg⁻¹ i.v. ($n = 9$), (v) I/R group treated with AICAR 500 mg·kg⁻¹ i.v. ($n = 8$). In a separate study, the effects of the highest dose of AICAR (500 mg·kg⁻¹ i.v.) were examined in sham-operated rats ($n = 6$ in sham-operated rats receiving vehicle and $n = 6$ in sham-operated rats treated with AICAR). AICAR was dissolved in saline (0.9% NaCl) and given i.v. to the tail vein 60 min before the operation; the others received the same volume of vehicle.

An established model of renal I/R injury was used (Kennedy and Erlich, 2008). Rats were anaesthetized with isoflurane (anaesthesia induction in a chamber with 4–5% isoflurane at the flow rate 1.5 L·min⁻¹), intubated and 1.5% isoflurane at rate 1.5 L·min⁻¹ was used to maintain anaesthesia. Abdominal incisions were made and the renal pedicles were bluntly dissected. Bilateral renal ischaemia was induced by clamping renal pedicles for 40 min with microvascular clamps. Control animals were subjected to sham operation without renal pedicle clamping. The rats were hydrated with warm saline during the operation and the body temperature was maintained constantly at 37°C by using a heating pad until awake. The wounds were sutured after removal of the clips, and the animals were allowed to recover. Buprenorphine (0.1 mg·kg⁻¹ s.c.) was used as post-operative analgesia; 24 h after the operation, the rats were

again anaesthetized with isoflurane, and blood samples were collected from inferior vena cava with a 5 mL syringe and a 22G needle for biochemical measurements. The kidneys were excised, washed with ice-cold saline, blotted dry and weighed. Left kidney was used for Western Blot, qRT-PCR and histological examinations. Tissue samples for histology were fixed in 10% formalin and processed to paraffin with routine methodology. Samples for immunohistochemistry were snap-frozen in -38°C isopentane. For protein and gene expression studies, renal samples were snap-frozen in liquid nitrogen. Samples were stored at -80°C until assayed.

Kidney histology

For histological examination, 4 μm thick paraffin sections were cut and stained with haematoxylin eosin ($n = 8\text{--}19$ per group). Renal samples were visually examined by a pathologist (PF) with a Leica DMR microscope (Leica Microsystems AG, Heerbrugg, Switzerland) and morphological changes from the whole cross-sectional area of cortex and medulla were assessed according to the ATN scoring system adopted from Dragun *et al.* (2001; magnification $\times 200$, ≥ 20 fields per kidney section quantified using the ATN-scoring system). Evaluation of histopathological changes included the loss of tubular brush border, tubular dilatation, cast formation and cell lysis. Tissue damage was quantified in a blinded manner and scored according to the percentage of damaged tubules in the sample: 0, no damage; 1, less than 25% damage; 2, 25–50% damage; 3, 50–75% damage; and 4, more than 75% damage.

Immunohistochemistry

For immunohistochemistry, frozen kidneys were processed and semi-quantitative scoring of inflammatory cells and kidney nitrotyrosine expression was performed ($n = 8\text{--}19$) as described in detail elsewhere (Helkamaa *et al.*, 2003). The relative amount of antibody-positive signal in cortical and medullary areas per sample was determined with computerized densitometry (Leica IM500 and Leica QWIN software, Leica Microsystems AG, Heerbrugg, Switzerland). Primary monoclonal antibody against rat monocyte/macrophage ED-1 (Serotec Ltd, Oslo, Norway) and polyclonal antibody against nitrotyrosine (Upstate Biotechnology, Lake Placid, NY, USA), as well as peroxidase-conjugated rabbit anti-mouse and biotinylated anti-rabbit (Vector Laboratories Inc., Burlingame CA, USA) secondary antibodies (DAKO A/S, Glostrup, Denmark) were used.

Western blotting

Proteins from kidney samples ($n = 5\text{--}8$ per group) were isolated with lysis buffer (NaCl 136 mM, Na_2HPO_4 8 mM, KCl 163.27 mM, KH_2PO_4 1.46 mM, Tween 20 0.001%, and complete protease inhibitors; Roche Diagnostics, Neuilly-Sur-Seine, France). Tissue samples were homogenized using a Bertin Precellys 24 homogenizer (Bertin Technologies, Aix en Provence, France), ceramic beads (Precellys CK14, Bertin Technologies) and a protocol consisting of 5000 rpm for 50 s repeated twice. Nuclear proteins for assessment of SIRT1 were extracted with Microcon's YM-10 filters and Centrifugal Filter Units (Millipore, Bedford, MA, USA) according to the instructions of the manufacturer. This protocol was first described by

Schreiber *et al.* (1989). Samples were electrophoretically separated by 8% SDS-PAGE (20 μg total protein of the whole cell lysate per lane). Each lane corresponded to one rat and all five groups were run on one gel. Proteins were transferred to a PVDF membrane (Immobilon-P®, Millipore, Bedford, MA, USA) and blocked in 5% non-fat milk-TBS + 0.01% Tween-20® buffer. The membranes were probed with the following primary antibodies; AMPK (AMPK α , 1/1000, Cell Signaling, Beverly, MA, USA), pAMPK (pAMPK α , 1/1000, Cell Signaling) and SIRT1 (Anti-Sir2, 1/1000, Upstate, Millipore, Temecula, CA, USA). Tubulin was used as the loading control (Anti α tubulin, 1/3000; Abcam, Cambridge, MA, USA). Horseradish peroxidase-conjugated anti-rabbit secondary antibody (Chemicon, Temecula, CA, USA) was subjected to enhanced chemiluminescence solution (ECLplus, Amersham Biosciences, Buckinghamshire, UK). The relative protein expressions in separate samples from the membranes were quantified with a Fluorescent Image Analyzer (FUJIFILM Corp, Tokyo, Japan).

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed using the LightCycler instrument (Roche Diagnostics, Neuilly sur Seine, France) for detection of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 (Nox1), mitochondrial superoxide dismutase (MnSOD), catalase and ribosomal 18S mRNA, as described elsewhere (Louhelainen *et al.*, 2009). Briefly, total RNA from the rat kidneys ($n = 7\text{--}9$ per group) was collected with TRIzol (Gibco, Invitrogen, Carlsbad, CA, USA), treated with deoxyribonuclease 1 (Sigma Chemicals, St. Louis, MO, USA) and reverse transcribed to cDNA by reverse-transcription enzyme (Im-Prom-II reverse transcription system, Promega, Madison, WI, USA). One microlitre of cDNA was subjected to quantitative real-time PCR for the detection of Nox1, as well as MnSOD, catalase and ribosomal 18S mRNA. The following primers were used: Nox1-2 sequence (5'-3') down TTCTGCCGGGAGCGATAA, up GGAGTTGCAGGAGTCCTCATTTT; MnSOD down CCTC GGTGACGTTTCAGATTGT, up TTAACGCGCAGATCATGCA; catalase down GGGCTGGGCTCAATGC, up TCAGCGA CCGAGGGAT; and 18 s down TTTTCGTCCTACTACCTCCCCG, up ACATCCAAGGAAGGCAGCAG. The samples were amplified using FastStart DNA Master SYBR Green 1 (Roche Diagnostics, Neuilly sur Seine, France) according to the protocol of the manufacturer. The quantities of the PCR products were quantified with an external standard curve amplified from purified PCR product.

Analysis of protein carbonyls

For detection of protein carbonyls in the kidney ($n = 7\text{--}9$ samples per group), we used commercially available OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Biochemical determinations

Creatinine, urea and electrolytes from serum samples, as well as serum lipids and liver enzymes were measured by routine laboratory techniques (ADVIA 1650 Chemistry System, Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA).

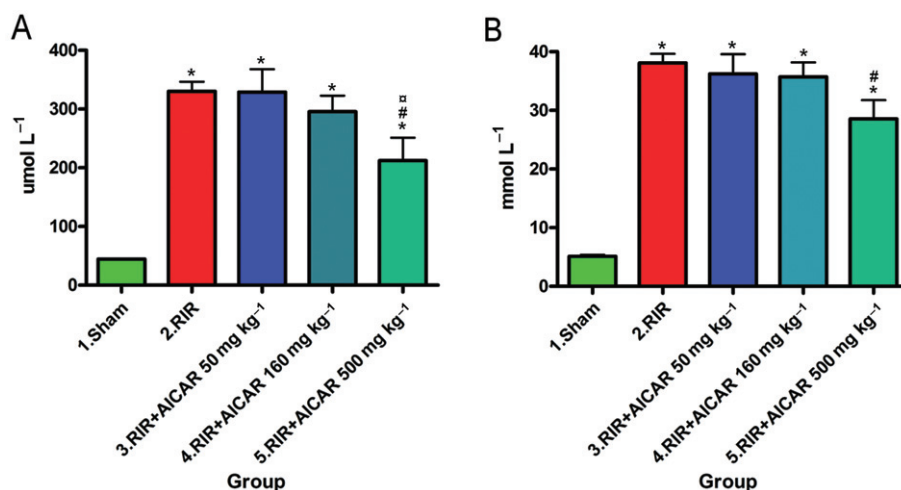


Figure 1

Effects of AICAR on serum creatinine (A) and serum urea concentrations (B) in rats with RI/R injury. Sham denotes sham-operated rats; RIR, rats with renal I/R injury; RIR + AICAR50, rats with I/R injury treated with AICAR at the dose 50 mg·kg⁻¹ i.v.; RIR + AICAR160, rats with I/R injury treated with AICAR at the dose 160 mg·kg⁻¹ i.v.; RIR + AICAR500, rats with I/R injury treated with AICAR at the dose 500 mg·kg⁻¹ i.v. AICAR, 5-amino-4-imidazolecarboxamide riboside-1-β-D-ribofuranoside. Means ± SEM are given, $n = 8$ –19 in each group. * $P < 0.05$ versus sham; # $P < 0.05$ versus RIR; $P < 0.05$ versus RIR+AICAR50.

Statistical analysis

Data are presented as the mean + SEM. Statistically significant differences in mean values were tested by ANOVA and Bonferroni's *post hoc* test, or by Student's *t*-test, when appropriate. The differences were considered significant when $P < 0.05$.

Results

Effects of AICAR on serum creatinine and urea levels in I/R injury

Acute kidney I/R injury was associated with a 7.4-fold increase in serum creatinine concentration and a 7.5-fold increase in serum urea level as compared to sham-operated controls (Figure 1A and B). In rats with I/R injury, the highest dose of AICAR decreased serum creatinine and urea concentrations by 35% and 25%, respectively, whereas the lower AICAR doses did not significantly influence serum creatinine and urea levels (Figure 1). In sham-operated rats, AICAR treatment did not influence serum urea level; however, AICAR slightly but significantly increased serum creatinine concentration by 10% (Supporting Information Table S1).

Effects of AICAR on kidney morphology in I/R injury

Histopathological analysis of the kidneys harvested 24 h after I/R showed marked injury of the renal parenchyma comprising vast necrosis of the tubuloepithelial cells, tubular dilatation and cast formation (Figure 2). The highest dose of AICAR significantly ameliorated I/R injury-induced ATN.

Effects of AICAR on monocyte/macrophage recruitment and kidney nitrotyrosine expression in I/R injury

Acute kidney I/R injury was associated with a 23-fold increase in the number of ED-1-positive inflammatory cells in the

kidney (Figure 3), and a 12.5-fold increase in renal nitrotyrosine expression (Figure 4) as compared to sham-operated controls. The difference in the number of renal ED-1-positive cells between sham-operated group and the groups with two highest AICAR doses did not reach statistical significance (Figure 3). AICAR at a dose of 160 mg·kg⁻¹ also significantly ameliorated renal nitrosative stress (Figure 4).

Effects of AICAR on kidney AMPK expression in I/R injury

To investigate the cellular mechanisms mediating the effects of AICAR in I/R injury, we measured the total expression and phosphorylation level of AMPK in the kidneys by Western blot. Kidneys harvested 24 h after I/R injury showed a 40% decrease in the ratio of phosphorylated/total AMPK expression as compared to sham-operated rats, whereas the total expression of AMPK in the kidney remained unaltered (Figure 5). AICAR at the highest treatment dose increased significantly the phosphorylation level of AMPK in the kidneys (Figure 5).

Effects of AICAR on kidney SIRT1 expression in I/R injury

Acute kidney I/R injury was associated with a fourfold increase in kidney SIRT1 expression measured from the nuclear protein fraction as compared to sham-operated controls (Figure 6). AICAR treatment prevented the I/R injury-induced increase in kidney SIRT1 expression in a dose-dependent manner (Figure 6).

Effects of AICAR on oxidative stress markers in I/R injury

To further investigate the role of oxidative stress in the pathophysiology of renal I/R injury, we measured the renal mRNA expression of pro-oxidative Nox1, and the mRNA expressions

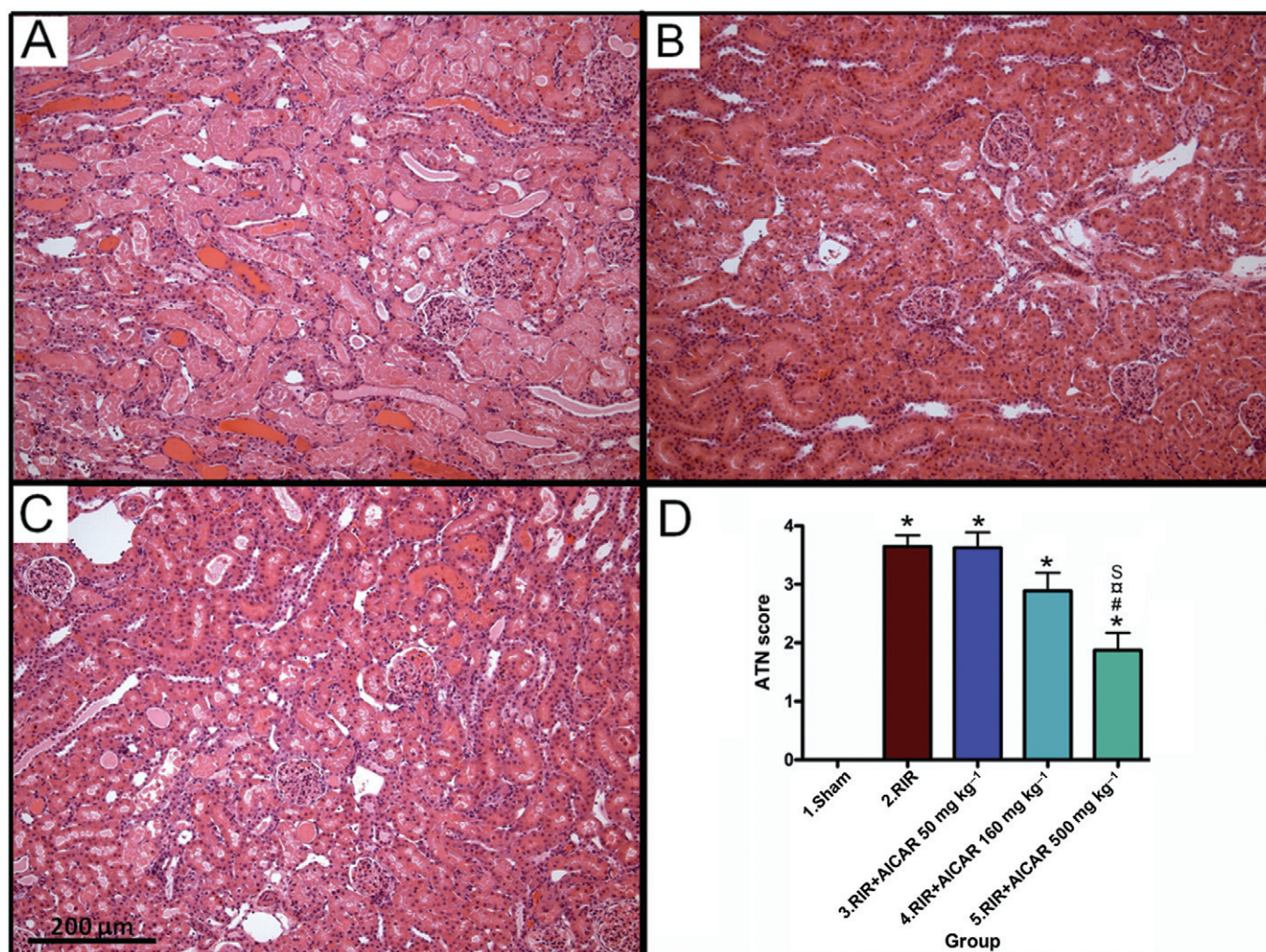


Figure 2

Effects of AICAR on ATN in rats with RI/R injury. Representative photomicrographs from untreated rats with I/R injury (A), sham-operated rats (B) and rats with I/R injury treated with 500 mg·kg⁻¹ AICAR (C) are given. Magnification $\times 100$, scale bar 200 μ m. Quantification of ATN with a scoring system is presented in (D). Means $\pm$ SEM are given, $n = 8$ –19 in each group. * $P < 0.05$ versus sham; # $P < 0.05$ versus RIR; * $P < 0.05$ versus RIR + AICAR50; S $P < 0.05$ versus RIR + AICAR160. For abbreviations, see Figure legend 1.

of the anti-oxidative catalase and MnSOD. I/R injury was associated with approximately 90% decrease in renal catalase mRNA expression (Figure 7A), and a 2.3-fold increase in renal Nox1 mRNA expression (Figure 7B), whereas no change was found in MnSOD mRNA expression in the kidneys (Figure 7C). The highest dose of AICAR decreased renal Nox1 mRNA expression, whereas the renal mRNA expressions of catalase or MnSOD were not influenced by AICAR treatment (Figure 7A–C).

I/R injury in the kidneys was associated with a non-significant 50% increase in the level of renal protein carbonyls (Figure 7D). AICAR treatment did not influence the concentration of protein carbonyls in the kidneys (Figure 7D).

Effects of AICAR on serum biochemistry in I/R injury

Acute kidney I/R injury was associated with 98%, 26% and 509% increases in the serum concentrations of alanine ami-

notransferase (ALAT), alkaline phosphatase (AFOS) and aspartate aminotransferase (ASAT), respectively, as compared to sham-operated controls, indicating AKI-induced hepatic injury (Table 1). AKI also markedly increased the serum levels of serum γ -glutamyl transferase and serum glutamate dehydrogenase (Table 1). The highest dose of AICAR decreased serum ASAT level but did not restore the other markers of I/R injury-induced hepatic damage/dysfunction (Table 1).

Serum potassium and phosphate concentrations were increased and the level of serum chloride decreased in rats with I/R injury (Table 1). These changes in serum electrolytes were attenuated by highest AICAR dose.

Neither I/R injury nor drug treatment influenced serum sodium, calcium, albumin, protein, cholesterol or triglyceride concentration.

In sham-operated rats, AICAR treatment increased the serum concentration of ALAT by 1.3-fold and that of ASAT by 1.4-fold (Supporting Information Table S1).

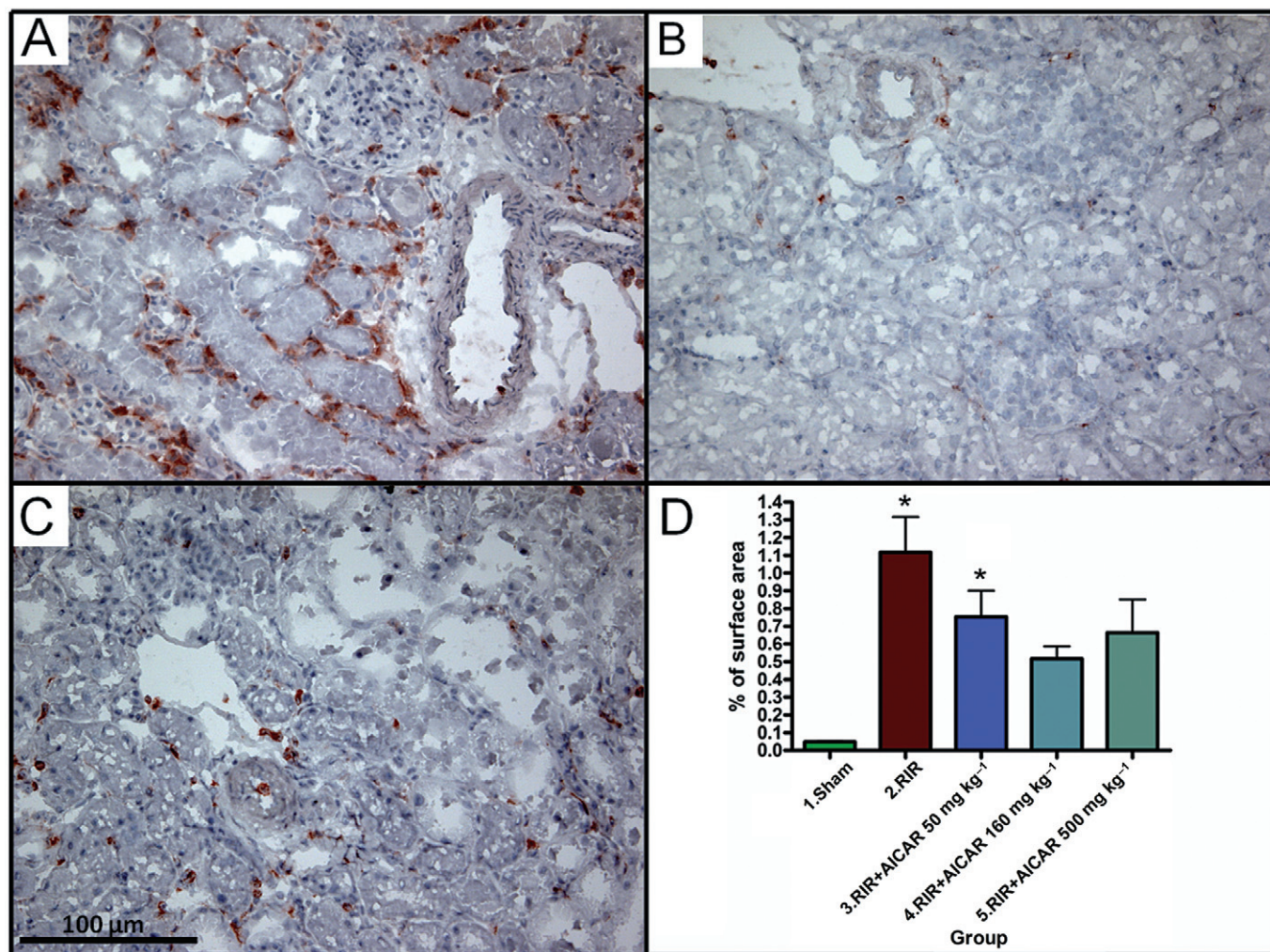


Figure 3

Effects of AICAR on monocyte/macrophage infiltration (ED1-immunopositive cells) in rats with RI/R injury. Representative photomicrographs from untreated rats with I/R injury (A), sham-operated rats (B) and rats with I/R injury treated with 500 mg·kg⁻¹ AICAR (C) are given. Magnification × 200, scale bar 100 μm. Quantification of ED1-positive cells in the kidney is presented in (D). Means ± SEM are given, $n = 8-19$ in each group. * $P < 0.05$ versus sham; # $P < 0.05$ versus RIR. For abbreviations, see Figure 1 legend.

Discussion

The acute response initiated by ischaemia-reperfusion injury in the kidneys is characterized by increased production of reactive oxygen species (ROS) and induction of pro-inflammatory cytokines, leading to inflammatory response and ATN (Bonventre and Yang, 2011). The important finding of the present study was that AICAR ameliorated the development of ATN in a rat I/R injury. AICAR attenuated I/R injury-induced nitrosative stress and monocyte/macrophage infiltration in the kidneys. We here also reported that kidney I/R injury is associated with decreased AMPK phosphorylation and a fivefold increase in the expression SIRT1 in the kidneys. AICAR increased AMPK phosphorylation and prevented the I/R-induced increase in renal SIRT1 expression.

Previous studies have provided evidence that AICAR protects against I/R injury in several tissues, such as heart and liver (Bullough *et al.*, 1994; Alkhulaifi and Pugsley, 1995; Gali-

nanes *et al.*, 1995; Mathew *et al.*, 1995; Peralta *et al.*, 2001). Lin *et al.* (2004) were the first to demonstrate that the combination therapy with AICAR and N-acetyl cysteine attenuates renal I/R injury and improves the outcome of the transplanted kidney after prolonged cold preservation. Consistent with the present study, Lee *et al.* (2009) have reported recently that preconditioning of the kidneys by AICAR, given as a single low-dose treatment 10 min before ischaemia, ameliorates renal I/R injury. Furthermore, in the same study, the authors demonstrated that acute AICAR treatment phosphorylated AMPK in the sham-operated rats. In contrast to the study by Lee *et al.* (2009), here we investigated the effects of AICAR in rats with kidney I/R injury by using three different AICAR doses. Furthermore, we examined the effects of I/R injury as well as the long-term effects of AICAR treatment on two major metabolic sensors, namely AMPK and SIRT1, from kidney samples harvested 24 h after the reperfusion. We assessed the renal effects of AICAR by combining functional

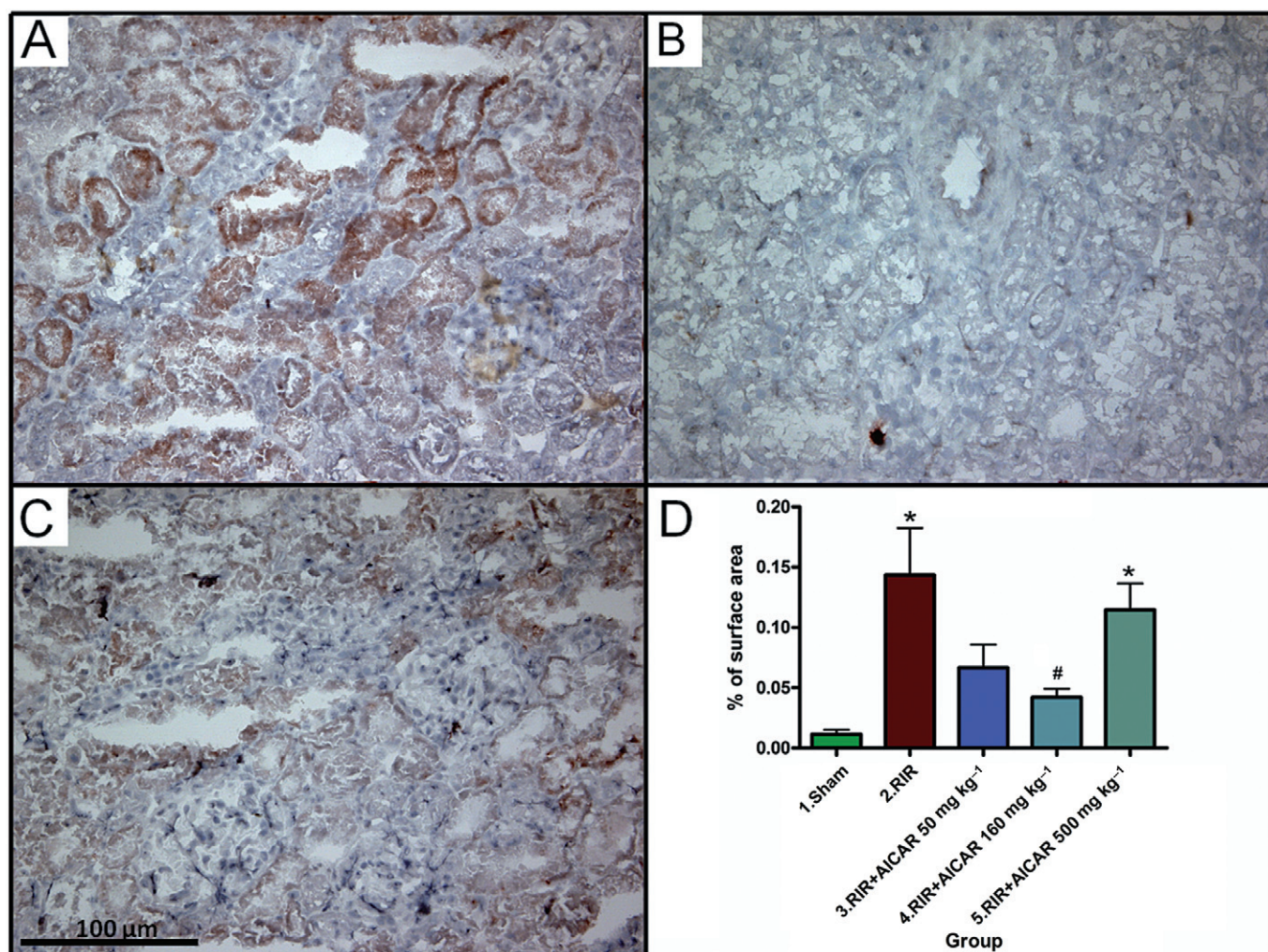


Figure 4

Effects of AICAR on nitrosative stress measured as kidney nitrotyrosine expression in rats with RI/R injury. Representative photomicrographs from untreated rats with I/R injury (A), sham-operated rats (B) and rats with I/R injury treated with 500 mg·kg⁻¹ AICAR (C) are given. Magnification × 200, scale bar 100 μm. Quantification of nitrotyrosine expression in the kidney is presented in (D). Means ± SEM are given, $n = 8-19$ in each group. * $P < 0.05$ versus sham; # $P < 0.05$ versus RIR. For abbreviations, see Figure 1 legend.

data with the widely used histological scoring for ATN. Furthermore, renal nitrotyrosine expression and the number of infiltrated monocytes/macrophages were used in the quantification of local nitrosative stress and leucocyte recruitment, respectively. To further investigate the role of oxidative stress in the pathophysiology of AKI, we measured the renal mRNA expression of pro-oxidative Nox1 and of anti-oxidative catalase and MnSOD. We also quantified protein oxidation from the kidney samples by using a commercially available protein carbonyl ELISA kit. It was found that renal I/R injury is associated with significant leucocyte infiltration, renal nitrosative stress, up-regulation of Nox1 and down-regulation of catalase mRNA expression, as well as a non-significant increase in the level of protein carbonyls in the kidneys. These findings strongly suggest the involvement of oxidative stress in the pathogenesis of renal I/R injury. Interestingly, the renoprotective effects of AICAR were associated with a decrease in nitrosative stress and amelioration of monocyte/macrophage

infiltration in the kidneys. AICAR also ameliorated I/R-induced increase in renal Nox1 expression. Our study is thus in very good accordance with the previous study by Kim *et al.* (2008) reporting suppression of ROS formation by AICAR and studies by Lin *et al.* (2004) and Gaskin *et al.* (2009) demonstrating the anti-inflammatory actions of AICAR. It should also be noted that the anti-oxidative and anti-inflammatory effects of AICAR could also explain, at least in part, improvement of urea and creatinine clearance, as AICAR has been shown to increase the bioavailability of NO (Morrow *et al.*, 2003; Gaskin *et al.*, 2007), and could thereby improve post-ischaemic blood flow in the kidneys (Versteilen *et al.*, 2006).

AMPK is a heterotrimeric serine/threonine kinase that plays a central role in the regulation of cellular energy metabolism. AMPK consists of a catalytic α subunit ($\alpha 1$, $\alpha 2$) and regulatory, non-catalytic β ($\beta 1$, $\beta 2$) and γ ($\gamma 1$, $\gamma 2$, $\gamma 3$) subunits. In the kidney, the $\alpha 1$ subunit is highly expressed, whereas the expression of the $\alpha 2$ subunit is barely detectable

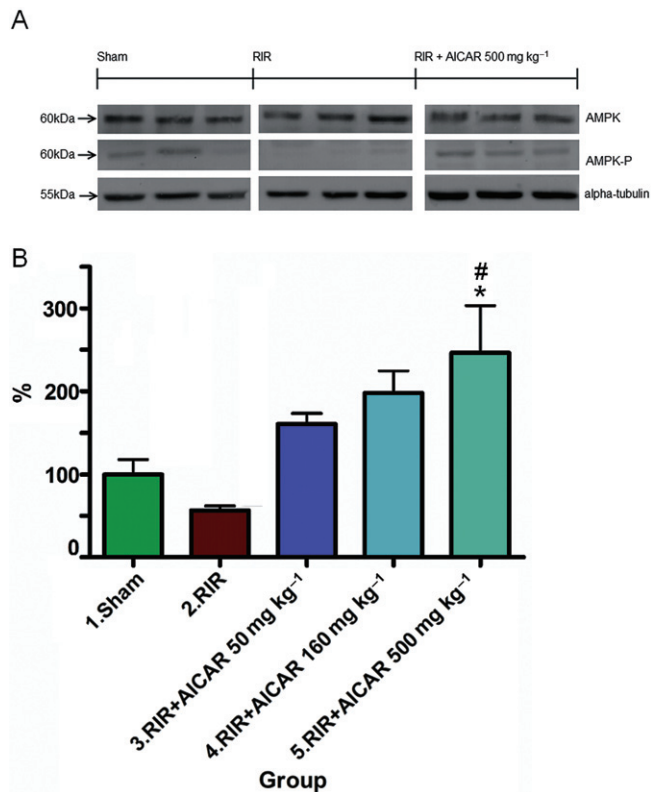


Figure 5

Effects of AICAR on kidney AMPK expression in rats with RI/R injury. The expressions of total AMPK, phosphorylated AMPK and house-keeping protein α -tubulin, measured by Western blot (20 μ g total protein of the whole cell lysate per lane), are given in (A). The phosphorylation level of AMPK measured as p-AMPK/AMPK ratio is given in (B). Means $\pm$ SEM from three different runs are given, $n = 4-5$ in each group. * $P < 0.05$ versus sham; # $P < 0.05$ versus RIR. For abbreviations, see Figure 1 legend.

(Kim and Tian, 2010). Mount *et al.* (2005) demonstrated recently that acute renal ischaemia leading to energy depletion and falling levels of ATP with a simultaneous rise in AMP activates AMPK within 1 min and that the activation of AMPK remains for a least 30 min. At present, data on AMPK activity at later phases after reperfusion are sparse. In the present study, we demonstrated that kidneys harvested 24 h after I/R injury showed a 40% decrease in AMPK activity when measured as the ratio of phosphorylated/total AMPK expression. These findings suggest an acute compensatory/regulatory role for AMPK in kidney I/R injury. In contrast, AICAR treatment increased the phosphorylation level of AMPK in the kidney. Although AICAR has been shown to exert several mechanisms of action, we believe that renoprotection found in the present study was largely due to AMPK activation. Previous studies have provided evidence that AICAR suppresses the production of ROS (Kim *et al.*, 2008), inhibits pro-inflammatory NF- κ B signalling (Katerelos *et al.*, 2010), and inhibits inflammation in MRL/lpr mouse mesangial cells (Peairs *et al.*, 2009) through AMPK activation. Further studies are warranted to examine the upstream AMPK kinases as well as the substrates that are phosphorylated by AMPK in the kidney.

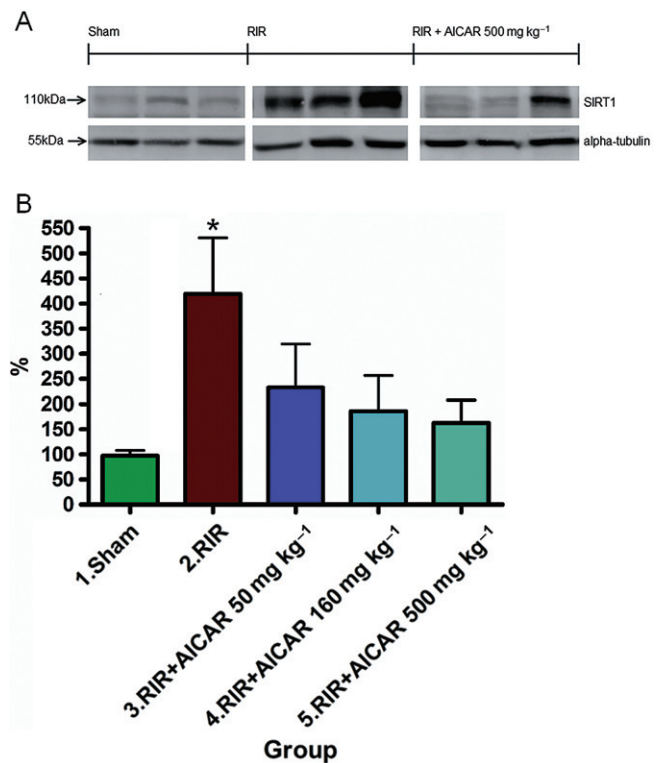


Figure 6

Effects of AICAR on kidney SIRT1 expression in rats with RI/R injury. The expression of SIRT1 and housekeeping protein α -tubulin, measured by Western blot (20 μ g nuclear protein fraction per lane), are given in (A). Kidney SIRT1 expression measured as SIRT1/ α -tubulin ratio is given in (B). Means $\pm$ SEM from three different runs are given, $n = 4-5$ per group in each gel. * $P < 0.05$ versus sham. For abbreviations, see Figure 1 legend.

SIRT1 is a member of the sirtuin family that are highly conserved NAD-dependent enzymes regulating life span in lower organisms and act as metabolic sensors of the cell (Finkel *et al.*, 2009). SIRT1 increases cellular stress resistance, genomic stability and regulates cellular senescence and energy metabolism via deacetylation of the target proteins such as P53, FOXO transcription factors and PGC-1. In the kidney, SIRT1 has been shown to be cytoprotective and to participate in the regulation of blood pressure and sodium homeostasis (Hao and Haase, 2010). SIRT1 activation protects the kidney medulla from oxidative injury (He *et al.*, 2010). Furthermore, kidney-specific overexpression of SIRT1 (Hasegawa *et al.*, 2010) and SIRT1 activation by resveratrol (Kim *et al.*, 2008) have been shown to ameliorate cisplatin-induced AKI. Interestingly, SIRT1 has also been shown to protect the heart from I/R injury (Hsu *et al.*, 2010; Nadtochiy *et al.*, 2011a,b). In the present study, renal SIRT1 expression was increased by fourfold in the kidney when measured 24 h after I/R injury. Our findings thus suggest sustained SIRT1 activation after kidney I/R injury whereas AMPK activation seems to occur only during the acute kidney ischaemia. We also reported here that AICAR treatment prevented the I/R injury-induced increase in kidney SIRT1 expression. It could therefore be postulated that amelioration of the renal nitrosative

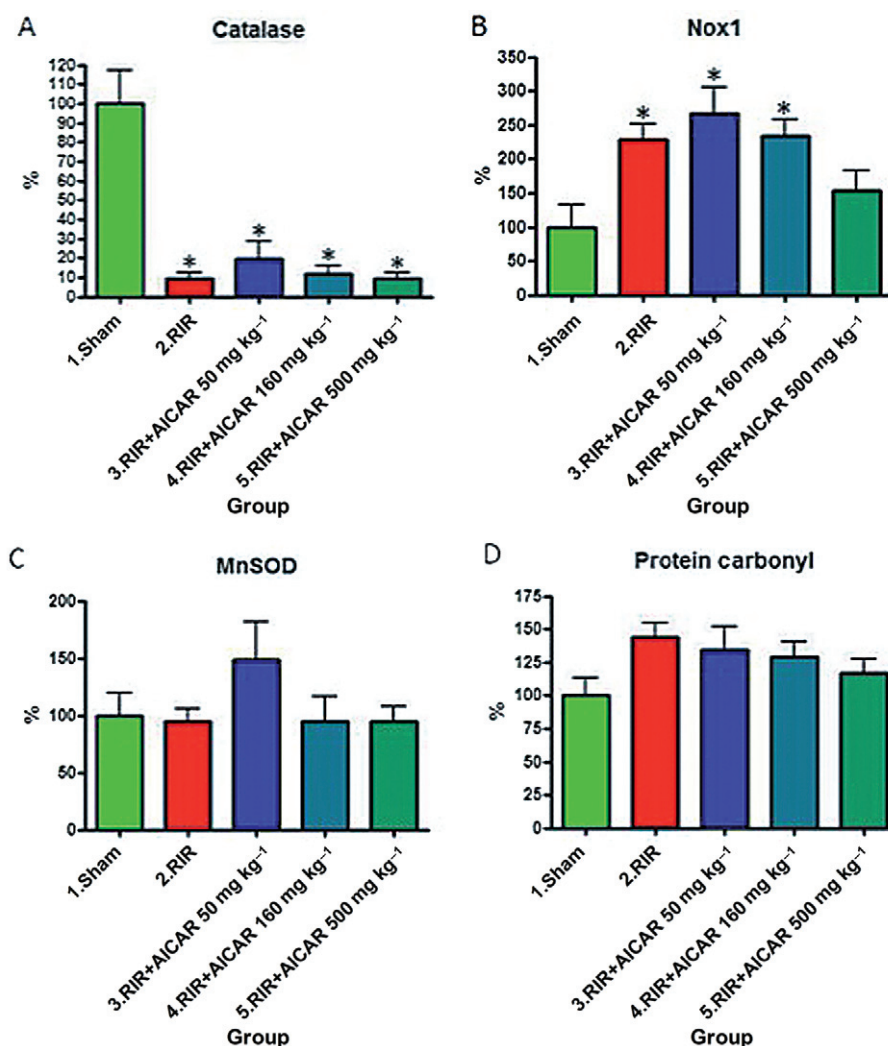


Figure 7

Effects of AICAR on oxidative stress markers in rats with RI/R injury. Renal mRNA expression of catalase (A), Nox1 (B) and mitochondrial superoxide dismutase (MnSOD; C) are given. The levels of protein carbonyls in the kidneys are shown in (D). Means $\pm$ SEM are given, $n = 6-9$ in each group. * $P < 0.05$ versus sham. For abbreviations, see Figure 1 legend.

stress and monocyte/macrophage infiltration by AICAR leading to improved excretion of nitrogenous waste and tissue morphology diminish the need for SIRT1 overexpression after I/R injury.

The morbidity and mortality from AKI is high in part due to extra-renal complications such as hepatic dysfunction. Development of liver injury may also lead to other extra-renal complications such as respiratory failure, intestinal barrier destruction and systemic inflammatory response syndrome (Elapavaluru and Kellum, 2007). Park *et al.* showed recently rapid peri-portal hepatocyte necrosis, vacuolization, neutrophil infiltration and up-regulation of pro-inflammatory genes (TNF- α , IL-17A, IL-6) in the liver after AKI, suggesting an intestinal source of hepatic injury (Park *et al.*, 2011). Interestingly, the highest dose of AICAR decreased the serum ASAT level in rats with renal I/R injury. In contrast, AICAR treatment produced a slight (13–36%) but significant increase in liver enzymes in sham-operated rats.

Further studies are thus warranted to investigate the molecular mechanisms mediating the effects of AICAR on liver function.

In conclusion, using a rat model of kidney I/R injury, we here reported that AICAR protects against the development of ATN via mechanisms linked to decreased nitrosative stress and monocyte/macrophage infiltration and activation of AMPK in the kidneys. The present study thus suggests a therapeutic role for AMPK activators in patients susceptible to AKI and to those undergoing kidney transplantation.

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Table 1

Effects of AICAR treatment on serum biochemistry in rats with kidney I/R injury

Variable	Sham (n = 17)	RIR (n = 19)	RIR + AICAR50 (n = 8)	RIR + AICAR160 (n = 9)	RIR + AICAR500 (n = 8)	ANOVA P-value
s-ALAT (U·L ⁻¹)	39.3 ± 2.8	77.7 ± 4.2*	67 ± 7.1*	59 ± 4.0*	62 ± 6.2*	0.2413
s-AFOS (U·L ⁻¹)	257.9 ± 15.3	324.6 ± 17.7	293 ± 17.1	292 ± 23.1	321 ± 37.8	0.2840
s-ASAT (U·L ⁻¹)	120.4 ± 5.9	612.5 ± 42.1*	648 ± 96.3*	505 ± 52.1*	374 ± 49.2*#	<0.0001
s-GGT (U·L ⁻¹)	0.0 ± 0	27.3 ± 2.6	26.1 ± 6.0	19.2 ± 2.8	13.2 ± 2.5	<0.0001
s-GLDH (U·L ⁻¹)	10.0 ± 0.8	198.8 ± 19.8*	195 ± 28.0*	201 ± 24.4*	129 ± 31.7*	<0.0001
s-Protein(g·L ⁻¹)	52.1 ± 0.8	52.5 ± 0.8	53.6 ± 1.3	52.6 ± 1.8	53.3 ± 1.2	0.4843
s-Albumin (g·L ⁻¹)	27.9 ± 0.4	27.6 ± 0.5	28.4 ± 0.7	27.7 ± 0.8	28.0 ± 0.5	0.4838
s-K (mmol·L ⁻¹)	4.7 ± 0.1	6.9 ± 0.3*	6.4 ± 0.3*	6.1 ± 0.2*	5.6 ± 0.3#	<0.0001
s-Na (mmol·L ⁻¹)	143.3 ± 1.7	142.6 ± 2.2	149 ± 1.0	145 ± 1.9	150 ± 0.9	0.0267
s-Cl (mmol·L ⁻¹)	99.6 ± 0.9	91.5 ± 1.2*	98 ± 2.7#	95 ± 1.9	100 ± 2.0#	0.1513
s-Calsium (mmol·L ⁻¹)	2.4 ± 0.0	2.4 ± 0.0	2.55 ± 0.1	2.47 ± 0.1	2.50 ± 0.0	0.0002
s-Pi (mmol·L ⁻¹)	2.9 ± 0.1	4.7 ± 0.3*	4.3 ± 0.3*	4.2 ± 0.3*	3.7 ± 0.3	<0.0001
s-Chol (mmol·L ⁻¹)	1.6 ± 0.1	1.6 ± 0.1	1.50 ± 0.1	1.45 ± 0.1	1.67 ± 0.1	0.8392
s-Trigly (mmol·L ⁻¹)	0.8 ± 0.0	0.9 ± 0.1	0.89 ± 0.1	0.76 ± 0.1	0.71 ± 0.1	0.0406

Sham denotes sham-operated rats; RIR, rats with renal I/R injury; RIR+AICAR50, rats with I/R injury treated with AICAR at the dose 50 mg·kg⁻¹ i.v.; RIR+AICAR160, rats with I/R injury treated with AICAR at the dose 160 mg·kg⁻¹ i.v.; RIR+AICAR500, rats with I/R injury treated with AICAR at the dose 500 mg·kg⁻¹ i.v. Means ± SEM are given, n = 8–19 in each group.

*P < 0.05 versus sham; #P < 0.05 versus RIR.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Effects of AICAR treatment on serum biochemistry in sham-operated rats. Sham denotes sham-operated rats; Sham+AICAR500, sham operated-rats treated with AICAR at the dose 500 mg·kg⁻¹ i.v. Means ± SEM are given, *n* = 6 in both groups

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