British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01231.x
www.brjpharmacol.org

RESEARCH PAPER

Nicorandil ameliorates ischaemia-reperfusion injury in the rat kidney

Shogo Shimizu[1], Motoaki Saito[1], Yukako Kinoshita[1], Fumiya Ohmasa[1], Fotios Dimitriadis[1], Kohei Shomori[2], Atsushi Hayashi[3] and Keisuke Satoh[1]

[1]*Division of Molecular Pharmacology, Tottori University School of Medicine, Yonago, Japan,* [2]*Division of Organ Pathology, Tottori University School of Medicine, Yonago, Japan, and* [3]*Department of Pediatrics, Yonago Medical Center, Yonago, Japan*

Correspondence
Dr Motoaki Saito, Division of Molecular Pharmacology, Tottori University School of Medicine, 86 Nishimachi, Yonago 683-8503, Japan. E-mail: saitomo@med.tottori-u.ac.jp

Keywords
nicorandil; ischaemia-reperfusion injury; ATP-sensitive potassium channel; NO donor

Received
26 May 2010
Revised
21 September 2010
Accepted
28 October 2010

BACKGROUND AND PURPOSE

Nicorandil, an ATP-sensitive potassium (K_{ATP}) channel opener and nitric oxide donor, is used in the treatment of angina and acute heart failure. Here we investigated the effects of two K_{ATP} channel openers, nicorandil and cromakalim on ischaemia reperfusion (I-R) injury in the kidney.

EXPERIMENTAL APPROACH

Right nephrectomy was performed in 8-week-old male Sprague-Dawley rats and they were then divided into six groups: control group; I-R, including 30 min of left renal ischaemia followed by 24 h of reperfusion; I-R groups plus nicorandil 3 or 10 mg·kg^{-1} i.p.; and I-R groups plus cromakalim 100 or 300 μg·kg^{-1} i.p. After reperfusion, renal function was estimated by serum creatinine (SCr), urinary albumin : creatinine ratio (ACR) and urinary β2-microglobulin (β2-MG). Levels of K_{ATP} channel subtypes were investigated by Western blot. Kidney sections were stained for 4-hydroxy-2-nonenal and 8-hydroxy-2′-deoxyguanosine.

KEY RESULTS

Renal I-R induced significant increases in SCr, ACR and β2-MG levels compared with the control animals. Treatment with K_{ATP} channel openers reduced urinary β2-MG levels, raised by I-R. Both $K_{IR}6.1$ and $K_{IR}6.2$ channels were expressed. Expression of $K_{IR}6.2$ channels in the I-R group was lower than in the control group, which was restored to normal by treatment with K_{ATP} channel openers. Histologically, severe acute tubular damage was observed in the I-R kidney and this damage was ameliorated by K_{ATP} channel openers, dose-dependently.

CONCLUSIONS AND IMPLICATIONS

ATP-sensitive potassium channel openers protected against proximal tubule damage after I-R injury. Nicorandil could represent a powerful additional component in the treatment of patients undergoing partial nephrectomy or renal transplantation.

Abbreviations

4-HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; CKD, chronic kidney disease; ELISA, enzyme-linked immunosorbent assay; IPreC, ischaemic preconditioning; I-R, ischaemia-reperfusion; K_{ATP} channel, ATP-sensitive potassium channel; OSOM, outer stripe of outer medulla; PAS, periodic acid-Schiff; ROS, reactive oxygen species; β2-MG, β2-microglobulin

Introduction

There is accumulating evidence that the numbers of patients with chronic kidney disease (CKD) are increasing all over the world. Data from the USA suggest that for every patient with end-stage renal disease, there are more than 200 with overt CKD (stage 3 or 4) and almost 5000 with covert disease (stage 1 or 2). The kidneys are particularly susceptible to ischaemic injury because of clinical conditions such as renal transplantation, treatment of suprarenal aneurysms, renal

artery reconstructions, contrast agent-induced nephropathy, cardiac arrest and shock. Recent studies demonstrate that nephron-sparing surgery is the gold standard in surgical procedures for small renal malignancies. During this operation, it is necessary to occlude the renal artery in order to remove the renal mass. The restoration of the blood flow after the removal of the renal mass resembles the ischaemia-reperfusion (I-R) phenomenon in the kidney (Van Poppel, 2010). Especially in CKD patients, it is important to decrease any additional damage to the kidney caused by I-R injury in order to avoid likely consequences such as haemodialysis, peritoneal dialysis or kidney replacement therapies. Renal I-R is a complex syndrome involving several mechanisms, including renal vasoconstriction, tubular damage and glomerular injury (Bird *et al.*, 1988). Although reperfusion is essential for the survival of ischaemic tissue, there is good evidence that reperfusion itself causes additional cellular injury (reperfusion injury) (Paller *et al.*, 1984), which has been attributed to the generation of reactive oxygen species (ROS), depletion of ATP, neutrophil infiltration, phospholipase activation and membrane lipid alterations, cytoskeletal dysfunction and intracellular Ca^{2+} accumulation (Paller *et al.*, 1984; Bonventre, 1993).

Ischaemic preconditioning (IPreC) is an evolutionarily conserved endogenous mechanism whereby short periods of non-lethal exposure to hypoxia alleviate damage caused by subsequent I-R (Lee and Emala, 2001). Many studies in cardiac protection with IPreC suggest that the mechanism of cardiac IPreC involves the activation and opening of ATP-sensitive potassium (K_{ATP}) channels (Auchampach and Gross, 1993; Van Winkle *et al.*, 1994). K_{ATP} channel agonists may thus have a direct effect on the underlying pathophysiological mechanism of the tissue injury after an I-R insult. For example, K_{ATP} channel agonists have been suggested to exert cytoprotective effects in the myocardium (Zager *et al.*, 2006). In addition, a recent study has shown that the K_{ATP} channel opener diazoxide prevents renal I-R injury (Sun *et al.*, 2008). On the other hand, there are some reports indicating that the physiological effect of renal IPreC *in vivo* is independent of the action of K_{ATP} channels (Lee and Emala, 2001; Joo *et al.*, 2006). Moreover, in another study, opening of K_{ATP} channels has been reported to exacerbate both ischaemic/hypoxic tubular necrosis and post-ischaemic acute renal failure (Pompermayer *et al.*, 2005). Thus, the role of K_{ATP} channel openers in the pathogenesis of renal I-R injury remains a matter of debate.

Nicorandil (2-nicotiamidoethyl-nitrate ester), a K_{ATP} channel opener and an nitric oxide (N0) donor, is used in the treatment of angina and acute heart failure (Taira, 1989). There is increasing evidence demonstrating that nicorandil protects myocardial tissue against I-R injury. For example, nicorandil has been proven to improve recovery of the post-ischaemic contractile dysfunction of the heart and to reduce the infarct size in several animal models and in humans (Mizunuma *et al.*, 1995; Patel *et al.*, 1999). Recently, the impact of nicorandil in angina study has shown that nicorandil reduced the incidence of major cardiovascular events in patients with angina pectoris (The IONA Study Group, 2002). Cromakalim is a potassium-channel opening vasodilator. The active isomer is levcromakalim. Cromakalim acts on K_{ATP} channels causing membrane hyperpolarization of smooth muscle cells, making them more difficult to excite and to contract. Intrarenal infusion of cromakalim significantly increases renal blood flow, glomerular filtration rate, urine flow and renin release. It is suggested that cromakalim does not inhibit sodium transport at the medullary portion of the ascending limb of the loop of Henle and that it may increase the delivery of sodium to the loop of Henle (Tamaki *et al.*, 1991).

A number of laboratories have demonstrated that, in animal models, NO provides potent cytoprotection following focal I-R injury of the heart, liver, brain and kidney (Nandagopal *et al.*, 2001). In view of these reports, it is possible that nicorandil could also ameliorate I-R injury in the kidney, because of its properties as a K_{ATP} channel opener and as an NO donor. Currently, there is no information available about the protective effects of the K_{ATP} channel openers on I-R injury in the kidney. In the present study, we hypothesized that nicorandil might prevent the renal injury caused by I-R and we investigated the effects and the possible mechanisms of action of the K_{ATP} channel openers, nicorandil and cromakalim, on the I-R injury in the rat kidney.

Methods

Animal preparation

All animal care and experimental procedures were performed in accordance with the guidelines set by the Tottori University Committee for Animal Experimentation. Eight-week-old male Sprague-Dawley rats weighing 260–300 g (SLC, Shizuoka, Japan) were divided into six groups: sham operated-control group (n = 5); I-R group, including 30 min of left renal ischaemia followed by 24 h of reperfusion (n = 9); I-R groups plus nicorandil 3 or 10 mg·kg^{-1} i.p. [groups Nic 3 (n = 6) and Nic 10 (n = 6) respectively]; and I-R groups plus cromakalim 100 or 300 μg·kg^{-1} i.p. [groups Cro 100 (n = 4) and Cro 300 (n = 5) respectively]. Nicorandil was dissolved in saline (0.9% NaCl, pH 7.4), and cromakalim stock solutions were made in 99.9% ethanol and diluted in saline (approximately 30 volumes) before use (total volume: approximately 0.3 mL).

After anaesthesia with sodium pentobarbital (50 mg·kg^{-1}, i.p.), the abdomen was opened in the midline, and then right nephrectomy was performed and then I-R was induced in the left kidney using a small clip (Sugita standard aneurysm clip, holding force 145 g; Mizuho Ikakogyo, Tokyo, Japan), which was placed on the renal vascular pedicle for 30 min to induce ischaemia. Sham operations were performed in a similar manner, except that the renal vessels were not clamped. Each drug was administered 10 min prior to the induction of ischaemia in the left kidney. After 24 h of reperfusion, rats were anaesthetized (sodium pentobarbital; 50 mg·kg^{-1}, i.p.) and blood samples (10 mL) were taken from the vena cava. The left kidney was removed and tissue samples of the cortex were immediately frozen at –80°C until used, or fixed in 10% phosphate-buffered formalin fixation solution. Urinary samples were collected by housing the rats in metabolic cages. In order to confirm I-R properly, the blood flow of the left kidney was measured with a Laser Doppler Flow meter (BRL-100, Bioresearch Co., Nagoya, Japan) during

the ischaemia period and the 30 min of reperfusion phase, according to the method described previously (Shimizu *et al.*, 2009).

As our preliminary experiments indicated, there were no significant differences in serum creatinine (SCr), urinary albumin and β2-microglobulin (β2-MG) values between sham-operated control rats treated with saline, sham-operated rats with nicorandil (10 mg·kg^{-1}) and sham-operated rats with cromakalim (300 μg·kg^{-1}); the saline-treated rats were used as controls. Moreover, these drug doses did not affect the blood pressure of the rats. In addition, preliminary work from this and other laboratories indicated that the drug should be given before ischaemia so that the K_{ATP} channels would be open when first exposed to molecular oxygen during the reperfusion phase (Saito and Sakai, 1998; Ismail *et al.*, 2007).

Measurement of serum creatinine, urinary excretion and albumin excretion

The serum and urinary creatinine levels in experimental rats were measured with the Jaffe method (Creatinine-test Wako; Wako Pure Chemical Industries, Osaka, Japan), according to the kit manufacturer's instructions. The urinary levels of albumin were determined with the enzyme-linked immunosorbent assay (ELISA) method (Nephrat II, Exocell Inc., Philadelphia, PA, USA) according to the manufacturer's instructions (Okada *et al.*, 2008). β2-MG, a specific indicator of tubular damage, was measured in urine with ELISA, using kits obtained from Panafarm laboratories (Rat Beta-2-microglobulin, Tokyo, Japan).

Histological examination of the rat kidney

After fixation, the kidney samples were embedded in paraffin and tissue sections (5 μm) were cut from the paraffin blocks. All the kidney specimens were stained using haematoxylin and eosin, periodic acid-Schiff (PAS) and Masson trichrome. Each section was examined under a light microscope at a magnification of 40–400. Histological evaluations were performed by experienced renal pathologist (A.H.), without knowledge of the treatment groups. For the histopathological analysis of renal damage induced by I-R, proximal tubules from three areas of the kidney [outer cortex, inner cortex and outer stripe of outer medulla (OSOM)] were evaluated, and a semi-quantitative analysis of histological damage was conducted on these areas, according to the methods of Miyaji *et al.* (2001) and Zhou *et al.* (2003). Approximately one hundred tubules per section were selected randomly at ×400 magnification, and scored according to the following criteria: 0, normal; 1, areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis and desquamation involving <25% of the tubular profile; 2, similar changes involving >25% but <50% of the tubular profile; 3, similar changes involving >50% but <75% of the tubular profile; 4, similar changes involving >75% of the tubular profile according to Miyaji *et al.* (2001). The Jablonski grading scale (0–4) was used for the assessment of I-R-induced necrosis of the overall proximal tubules, as in Jablonski *et al.* (1983) (Table 1). In both analyses, about one hundred tubules were scored in each section and the total scores divided by the number of tubules scored, thus providing a mean score for each section.

Table 1

Jablonski grading scale

Score 0 = Normal
Score 1 = Necrosis of individual cells
Score 2 = Necrosis of all cells in adjacent PCT, with survival of surrounding tubules
Score 3 = Necrosis confined to distal third of PCT with band(s) of necrosis extending across inner cortex
Score 4 = Necrosis of all three segments of PCT

Modified from Jablonski *et al.* (1983).
PCT, proximal convoluted tubule.

Immunohistochemistry

After deparaffinization with xylene and rehydration in graded alcohols, sections were subjected to antigen retrieval using a microwave in a 10 mM citrate buffer (pH 6.0) for 20 min. All incubations were performed in a humidified chamber. For saturation of non-specific binding sites, the sections were preincubated with 1.5% normal horse serum (Vectastain, Vector Laboratories, CA, USA) at room temperature for 30 min. Then the sections were incubated overnight at 4°C using a mouse monoclonal antibody against 4-hydroxy-2-nonenal (4-HNE) (1:4, Japan Institute for the Control of Aging, Shizuoka, Japan) and a mouse monoclonal antibody against 8-hydroxy-2′-deoxyguanosine (8-OHdG) (1:10, Japan Institute for the Control of Aging, Shizuoka, Japan). After washing with phosphate-buffered saline for 10 min, biotinylated horse anti-mouse IgG (1:200) was applied onto the tissue sections, and incubated for 30 min at room temperature. Immunoreaction was performed with an avidin–biotin complex alkaline phosphatase kit (Vectastain, Vector Laboratories, Burlingame, CA, USA). The sections were counterstained with haematoxylin or methyl green. Negative control sections, which were incubated in the absence of the primary antibody, were also processed and evaluated for specificity or background staining levels.

Western blot

ATP-sensitive potassium channels play an important role in many cellular functions by coupling cell metabolism to electrical activity. K_{IR}6.1 and K_{IR}6.2 are members of the family of inwardly rectifying potassium channels (nomenclature follows Alexander *et al.*, 2009). β-tubulin was used as a loading control for normalization. Rat kidney was lysed by sonication in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA plus protease inhibitor cocktail (Roche). Protein was quantified using colour-producing solution (Wako). Samples were separated on 12% SDS–PAGE and transferred on a nylon membrane (Immobilon P, Millipore, Bangalore, India) using a semi-dry transfer blotter (BioRad, Hercules, CA, USA). Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline–0.1% Tween 20 (TBST). The blots were then incubated with one of the following: primary rabbit anti-K_{IR}6.1 (H80) antibody, primary goat anti-K_{IR}6.2 (G16), primary rabbit anti-β tubulin (H235)

(Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200, 1:100 or 1:500 dilution overnight at 4°C in TBST. Membranes were subsequently washed with large volumes of TBST (3 × 5 min) and the immobilized antibody was conjugated with a diluted horseradish peroxidase-labelled secondary antibody donkey anti-rabbit (1:5000) (Amersham Life Science, Buckinghamshire, UK) for $K_{IR}6.1$ or horseradish peroxidase conjugated horse anti-goat IgG (1:5000) (PI-9500, Vector Laboratories, Burlingame, CA, USA) for $K_{IR}6.2$, for 1 h at room temperature in TBST. After thorough washing with TBST, membranes were incubated with enhanced chemiluminescence detection reagents and images were captured in a LAS-4000 plus imager (GE Healthcare) (Takamura *et al.*, 2008). Densitometric analysis was performed using Scion Image Software (version Beta 4.0.2) (Scion Corp., Frederick, MD, USA).

Statistical analysis

All data were expressed as mean ± SEM. The statistical significance of differences between group mean values was established using the Student's *t*-test. To establish the statistical significance of differences between more than two group mean values, we used ANOVA followed by Fisher's test. Statistical analysis of histological results was performed using Kruskal–Wallis tests and Mann–Whitney *U*-tests with Bonferroni correction. $P < 0.05$ was regarded as the level of significance.

Materials

Nicorandil was kindly supplied by Chugai Pharmaceutical Co. Ltd (Tokyo, Japan). Cromakalim was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals are available commercially.

Results

Effects of nicorandil and cromakalim on ischaemia reperfusion-mediated glomerular and proximal tubular injury

Figure 1 shows the data of SCr and urinary albumin : creatinine ratio (ACR) as biomarkers of glomerular damage and urinary levels of β2-MG as a biomarker of proximal tubular damage. Renal I-R induced significant increases in the SCr levels and ACR in comparison with values obtained from control rats (Figure 1). However, there were no significant differences between the groups treated with either low or high dose of nicorandil (3 or 10 mg·kg^{-1}) or cromakalim (100 or 300 μg·kg^{-1}) and Cont group or I-R group. The ACR values in the I-R group treated with the higher dose of nicorandil (10 mg·kg^{-1}) returned to the control value. In contrast to these parameters, I-R in the kidney significantly increased the urinary levels of β2-MG compared with control animals. Treatments with both low and high doses of nicorandil or cromakalim decreased the urinary β2-MG levels, raised by the I-R procedure.

Histological analysis of renal tissue in the rat

Figures 2–4 show PAS stained specimens of all groups of the outer cortex, inner cortex and OSOM. In comparison with

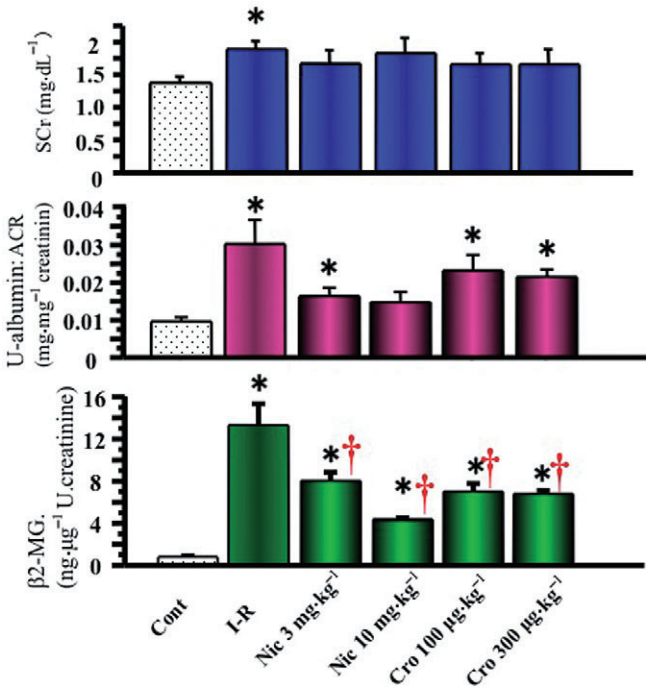

Figure 1

Effects of nicorandil and cromakalim on ischaemia reperfusion (I-R)-mediated glomerular and proximal tubular injury. Renal damage induced by I-R was assessed by measuring serum creatinine (SCr), urinary albumin : creatinine ratio (ACR) and urinary β2-microglobulin (β2-MG). β2-MG values were normalized against urinary creatinine content. Data are shown as mean ± SEM of six to eight separate determinations in each group. *Significantly different from control (Cont) group ($P < 0.05$). †Significantly different from I-R group ($P < 0.05$).

the control rat tubules, kidneys from the I-R group demonstrated severe acute tubular damage. These features included brush border loss, nuclear condensation, cytoplasmic swelling and a consistent loss of nuclei from tubular control profiles. However, significant damage in glomerular or distal tubules was not observed in the kidneys after I-R. The changes in the proximal tubules were drastically attenuated in rats subjected to I-R injury after treatment with nicorandil or cromakalim especially with the higher doses of both drugs (Groups Nic 10 mg·kg^{-1} and Cro 300 μg·kg^{-1}). We also evaluated the histological damage in the proximal tubules and these histological scores are shown in Table 2. The scores of the tubules in the outer cortex, the inner cortex and the OSOM as well as the Jablonski score in the I-R rat kidney were significantly higher than those in the control rat kidney. Treatment with the lower dose of nicorandil (3 mg·kg^{-1}) did not improve the outer cortex and the Jablonski scores but the higher dose of nicorandil (10 mg·kg^{-1}) improved all scores and returned them to control levels, except for the OSOM score. On the other hand, treatment with either dose of cromakalim (100 or 300 μg·kg^{-1}) did not improve any scores, compared with those in the I-R group.

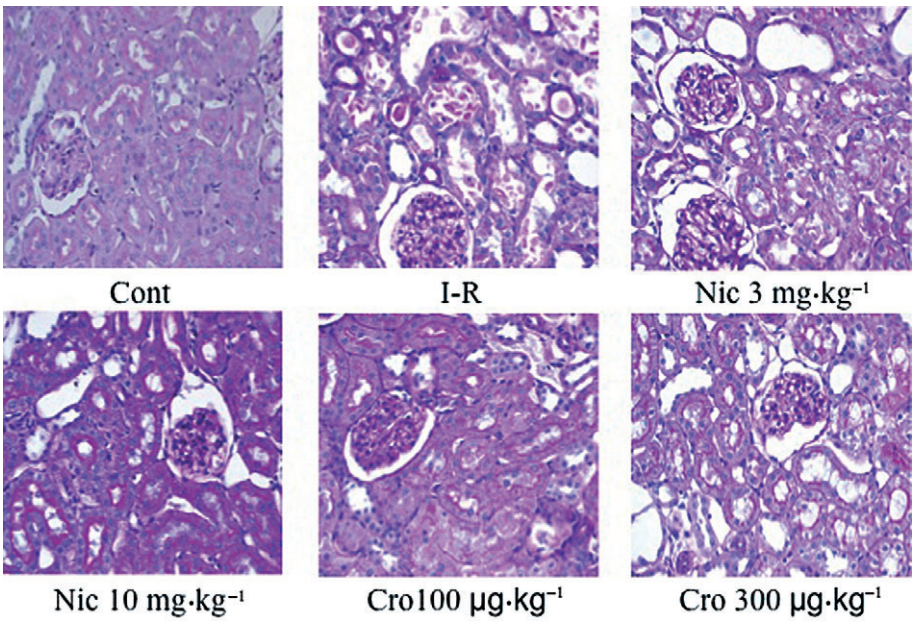

Figure 2

Periodic acid-Schiff (PAS) staining of outer cortex in rat kidney. Sections of kidneys from control (Cont), ischaemia reperfusion (I-R) untreated (I-R), I-R with nicorandil (Nic) 3 or 10 mg·kg^{-1} and I-R with cromakalim (Cro) 100 or 300 μg·kg^{-1} groups. Original magnification: ×400.

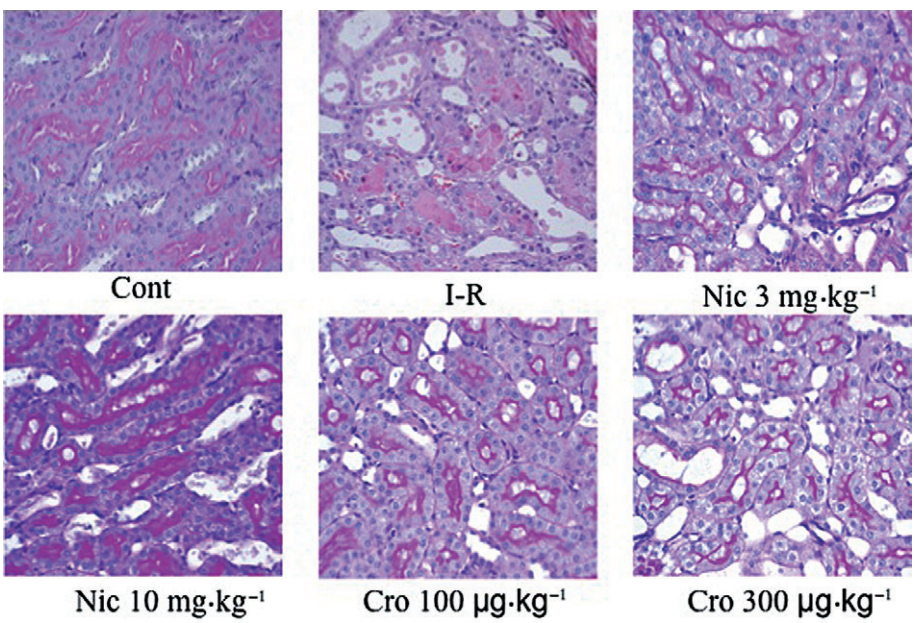

Figure 3

Periodic acid-Schiff (PAS) staining of inner cortex in rat kidney. Sections of kidneys from control (Cont), ischaemia reperfusion (I-R) untreated (I-R), I-R with nicorandil (Nic) 3 or 10 mg·kg^{-1} and I-R with cromakalim (Cro) 100 or 300 μg·kg^{-1} groups. Original magnification: ×400.

Immunohistochemical localization of products of lipid peroxidation and oxidative DNA damage

Lipid peroxidation was assessed by immunohistochemical staining for 4-HNE, using anti-4-HNE antibody (Figure 5). In the Cont group, 4-HNE immunoreactivity was rarely observed. In the I-R group, immunoreactivity for 4-HNE was detected at the proximal and distal tubule, whereas significant immunoreactivity was not observed in glomerulus. The strongest immunoreactivity for 4-HNE was observed in the lumen of the proximal tubules, which appeared as red dots.

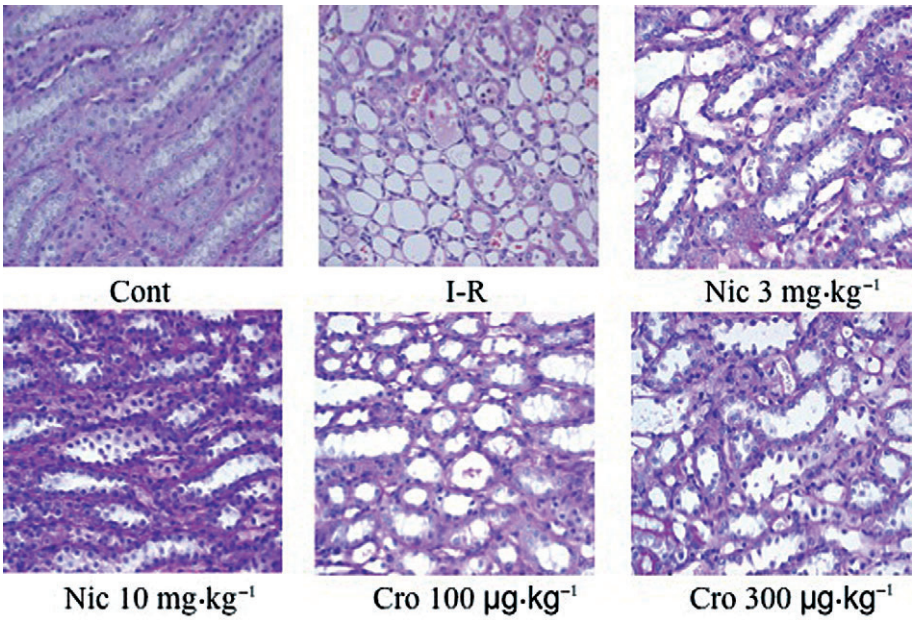

Figure 4

Periodic acid-Schiff (PAS) staining of the outer stripe of the outer renal medulla in rat kidney. Sections of kidneys from control (Cont), ischaemia reperfusion (I-R) untreated (I-R), I-R with nicorandil (Nic) 3 or 10 mg·kg^{-1} and I-R with cromakalim (Cro) 100 or 300 μg·kg^{-1} groups. Original magnification: ×400.

Table 2

Score of damage to proximal tubules in rat kidney following ischaemia reperfusion (I-R) and pretreatment with nicorandil (Nic) or cromakalim (Cro)

	Miyaji score Outer cortex	Inner cortex	OSOM	Jablonski score
Cont	0.002 ± 0.002	0.000 ± 0.000	0.014 ± 0.006	0.000 ± 0.000
I-R	0.883 ± 0.141*	1.615 ± 0.131*	1.671 ± 0.243*	3.250 ± 0.236*
Nic 3 mg·kg^{-1}	0.467 ± 0.231	1.241 ± 0.418	0.890 ± 0.368	2.667 ± 0.422
Nic 10 mg·kg^{-1}	0.080 ± 0.023†	0.205 ± 0.107†	0.351 ± 0.266	0.500 ± 0.224†‡
Cro 100 μg·kg^{-1}	0.248 ± 0.068	0.458 ± 0.117	0.428 ± 0.112	1.750 ± 0.250
Cro 300 μg·kg^{-1}	0.090 ± 0.066	0.173 ± 0.142	0.088 ± 0.072	0.750 ± 0.671

Nicorandil was given at 3 or 10 mg·kg^{-1} and cromakalim at 100 or 300 μg·kg^{-1}; 10 min before the standard I-R procedure (30 min ischaemia + 24 h reperfusion). Data are shown as mean ± SEM of four to eight separate determinations in each group.
*Significantly different from control group ($P < 0.05$).
†Significantly different from I-R group ($P < 0.05$).
‡Significantly different from Nic 3 group ($P < 0.05$).
OSOM, outer stripe of outer medulla.

This immunoreactivity for 4-HNE in the proximal tubules was weaker, after treatment with nicorandil or cromakalim, in a dose-dependent manner.

We also identified oxidative DNA damage immunohistochemically using anti-8-OHdG antibody (Figure 6). In the Cont group, 8-OHdG immunoreactivity was rarely observed. In the I-R group, immunoreactivity for 8-OHdG was detected at the proximal and distal tubule, whereas significant immunoreactivity was not observed in glomerulus. The strongest immunoreactivity for 8-OHdG was detected in the epithelium at the proximal tubule and is represented by red dots. This immunoreactivity for 8-OHdG, in the proximal tubules, was reduced by treatment with nicorandil or cromakalim, in a dose-dependent manner.

Western blot analysis of the expression of mitochondrial ATP-sensitive potassium channel subunits, $K_{IR}6.1$ and $K_{IR}6.2$

Figure 7 shows the expression of the $K_{IR}6.1$ and $K_{IR}6.2$ channel subunits in the cortex of the kidney, using

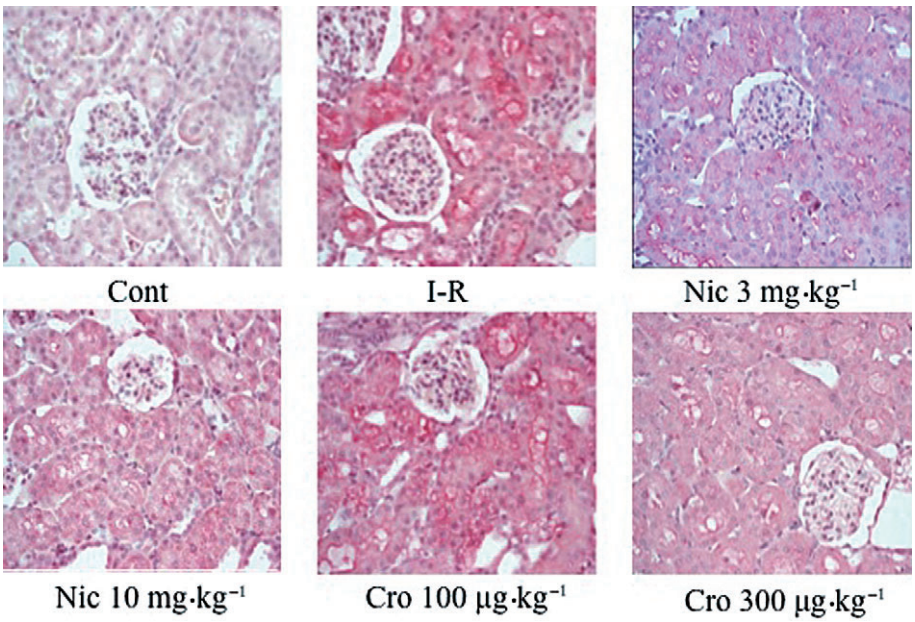

Figure 5

Immunohistochemical location of 4-hydroxy-2-nonenal (4-HNE) in rat kidney. Lipid peroxidation was assessed with anti-4-HNE antibody 4-HNE-positive cells shown as red stain. In the Cont group, 4-HNE immunoreactivity was rarely observed. In the ischaemia reperfusion (I-R) group, immunoreactivity for 4-HNE was detected at the proximal and distal tubule, whereas, significant immunoreactivity was not observed in glomeruli. The strongest immunoreactivity for 4-HNE was observed in the lumen of proximal tubules. This immunoreactivity for 4-HNE in the proximal tubules was weaker after treatment with nicorandil or cromakalim, in a dose-dependent manner.

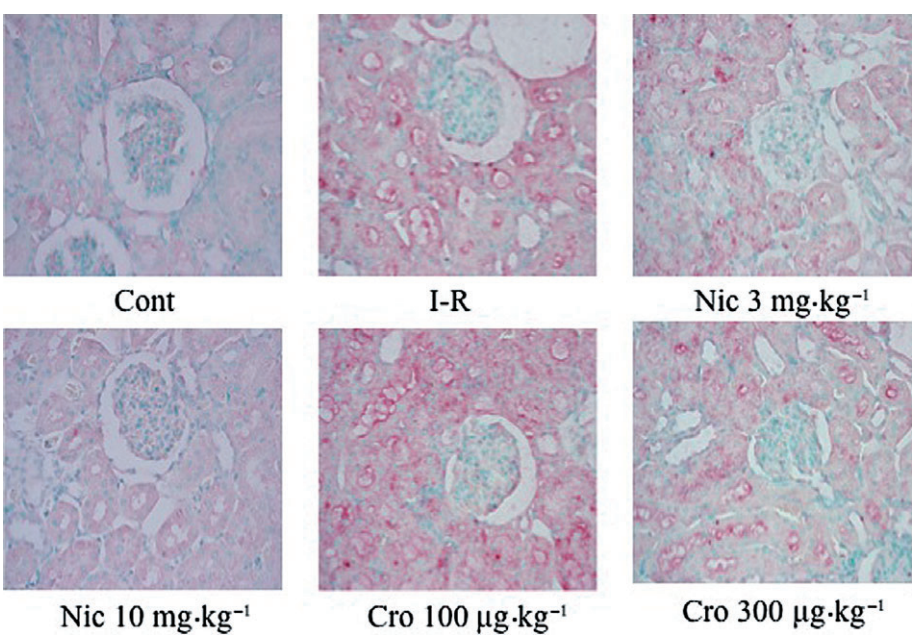

Figure 6

Immunohistochemical location of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in rat kidney. 8-OHdG-positive cells show red stain. In the Cont group, 8-OHdG immunoreactivity was rarely observed. In the ischaemia reperfusion (I-R) group, immunoreactivity for 8-OHdG was detected at the proximal and distal tubule, whereas, significant immunoreactivity was not observed in glomeruli. The strongest immunoreactivity for 8-OHdG was detected in the epithelium at the proximal tubule, seen as red dots. This immunoreactivity for 8-OHdG in the proximal tubles was reduced after treatment with nicorandil or cromakalim in a dose-dependent manner.

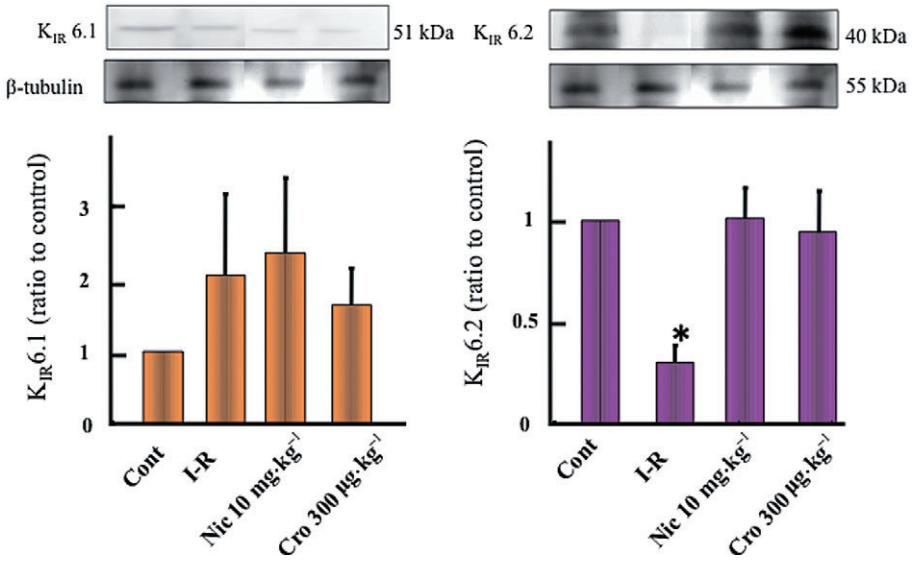

Figure 7

Western blot analysis of ATP-sensitive K channel subunit $K_{IR}6.1$ or $K_{IR}6.2$, and β-tubulin protein expression. Expression of $K_{IR}6.1$ and $K_{IR}6.2$ channels were detected in kidneys from all groups of rats (each group, $n = 4$). In contrast to $K_{IR}6.1$, the expression of $K_{IR}6.2$ channels in the ischaemia reperfusion (I-R) group was lower than that in the control group. This down-regulation of $K_{IR}6.2$ channels was totally prevented by treatment with nicorandil or cromakalim, at their higher doses. *Significantly different from the other groups ($P < 0.05$).

immunoblot analysis. In control rats, both $K_{IR}6.1$ and $K_{IR}6.2$ channels were detected in the kidney. There were no significant differences of expression of $K_{IR}6.1$ between any groups. In contrast, the expression of $K_{IR}6.2$ in the I-R group was significantly lower than in the control group. This down-regulation of expression of $K_{IR}6.2$ was totally prevented by treatment with the higher dose of nicorandil (10 mg·kg^{-1}) or the higher dose of cromakalim (300 μg·kg^{-1}).

Discussion

In this study, we clearly demonstrated that proximal tubule cells were injured by induction of I-R, and that pretreatment with either nicorandil or cromakalim prevented this injury in a dose-dependent fashion. Furthermore, our results suggest that nicorandil ameliorated I-R-induced renal damage by reducing oxidative damage via activation of K_{ATP} channels and possibly NO production.

There is increasing evidence from both *in vivo* and *in vitro* studies that K_{ATP} channel activation plays an important role in the pathophysiology of myocardial injury mediated by hypoxia and I-R (O'Rourke, 2004). The role of K_{ATP} channels in the cardiovascular system has been well studied and one of their most relevant features is that the opening of these channels can protect cardiac myocytes against ischaemic injury. More specifically, sarco-K_{ATP} channels are reported to regulate the cell energy metabolism in two ways. First, opening of these K_{ATP} channels accelerates phase 3 repolarization and slows down depolarization, which will shorten action potential duration and prevent the reversal of Na^+/Ca^{2+} exchanger resulting in inhibition of Ca^{2+} entry in the cell. Second, opening of these channels induces hyperpolarization of the membrane potential (Zhou *et al.*, 2005). On the other hand, in an energy crisis such as I-R injury, mitochondrial production of ATP is impaired by changes of mitochondrial membrane potential, imbalanced trans-membrane ions transport or overproduction of ROS. The opening of mitochondrial K_{ATP} channels prevents the mitochondria from these disturbances by controlling the mitochondrial Ca^{2+} concentration and matrix swelling.

Renal ischaemia, whether caused by shock or during partial nephrectomy and transplantation, is a major cause of acute renal failure. Although reperfusion is essential for the survival of the ischaemic kidney, reperfusion itself causes additional injury (Martins *et al.*, 2006). The aetiology of renal injury due to I-R is complex with many mediators, including oxidant production, cytokines and ROS. The ischaemic phase of the renal injury is characterized by a rapid depletion of ATP (Lieberthal and Nigam, 1998; Chandra *et al.*, 2000). Recent studies demonstrated that tubular cell apoptosis is a primary and major contributor to the pathophysiology of renal I-R and determined the outcome of the renal damage (Bonegio and Lieberthal, 2002; Padanilam, 2003). Pompermayer *et al.* (2005) found that treatment with glibenclamide, a K_{ATP} channel closer, 5 min after the induction of ischaemia, inhibited neutrophil recruitment and improved renal dysfunction following renal I-R. The authors also suggested that glibenclamide may have a therapeutic role in the treatment of renal I-R injury. In contrast to the previous report, Sun *et al.* (2008) demonstrated that treatment with diazoxide 10 min before induction of ischaemia significantly ameliorated I-R injury in the kidney, in which mitochondrial K_{ATP} channels would be open when first exposed to molecular oxygen on reperfusion. The findings of Sun *et al.* agree with our present results showing that K_{ATP} channel openers prevented renal I-R injury. In this earlier report (Sun *et al.*, 2008), diazoxide was administered 40 min before reperfusion, which means that diazox-

ide was provided after renal artery occlusion (5 min after the induction of ischaemia), and also that diazoxide did not penetrate the mitochondria, as Pompermayer *et al.* (2005) showed in their study. In our present study, the K_{ATP} channel openers, nicorandil and cromakalim, were given before ischaemia, in order to evaluate their potential beneficial effects in clinical use during partial nephrectomy for small renal cell carcinomas. Thus, the different doses of K_{ATP} channel openers or closers and the different timing of administration of these drugs may explain the differences between our data and those of Pompermayer *et al.* (2005). Furthermore, Zager *et al.* (2006) reported that levosimendan, a K_{ATP} channel opener, conferred almost complete protection against lipopolysaccharide (LPS)-induced acute renal failure, without any apparent reduction in the LPS-induced inflammatory response, and that neither levosimendan nor diazoxide altered tubule injury mediated by ATP depletion. Conversely, glibenclamide induced a marked and direct cytotoxic effect. They concluded that K_{ATP} channel agonists have a margin of safety if employed in situations (sepsis syndrome, heart failure) in which severe renal vasoconstriction might lead to ischaemic acute renal failure (Zager *et al.*, 2006).

Nicorandil is reported to be a selective mitochondrial K_{ATP} channel opener, and cromakalim is reported to be a non-selective K_{ATP} channel opener (Date *et al.*, 2005). Nicorandil consists of an *N*-(2-hydroxyethyl) nicotinamide group and an organic nitrate moiety and therefore is thought to have dual actions, a nitrate-like action and an action on K_{ATP} channels (Markham *et al.*, 2000). Like other nitrates, nicorandil activates cytoplasmic guanylate cyclase leading to an increase in cellular levels of cGMP with a reduction in cytosolic calcium and thus relaxation of the vascular smooth muscle (Flaherty, 1989). As a K_{ATP} channel opener, nicorandil increases the efflux of potassium ions from the cell, leading to a more negative resting membrane potential (hyperpolarization), and also shortens the action potential duration. Through its dual action, nicorandil reduces both preload (nitrate-like effect) and afterload (K_{ATP} channel opener effect), and improves coronary blood flow (Gomma *et al.*, 2001). Clinical studies have shown that nicorandil improves functional and clinical outcomes in patients with acute myocardial infarction (The IONA Study Group, 2002; Sugimoto *et al.*, 2003).

Segawa *et al.* (2001) reported that the inhibitory effects of nicorandil on rat mesangial cell proliferation are mediated via the protein kinase G pathway. Sudo *et al.* (2009) reported that nicorandil suppressed the development of mesangioproliferative glomerulonephritis in anti-Thy1 nephritis rats. Nicorandil significantly prevented overexpression of type-1 collagen, fibronectin, transforming growth factor-β and platelet-derived growth mRNAs in the cortex. These data indicate that nicorandil prevents mesangial proliferation and extracellular matrix production. This is quite interesting, because nicorandil has beneficial effects not only on tubules (from our present data) but also on glomerulus (from Sudo *et al.*). However, no study has investigated whether nicorandil could be used to treat renal I-R injury. K_{ATP} channels are present in various cell types in the kidney, including the proximal tubule, the thick ascending limb of the loop of Henle and the cortical collecting duct (Quast, 1996).

As it is important to investigate which K_{ATP} channel subtype is concerned in the rat renal I-R, we analysed rat kidneys by Western blot and found both $K_{IR}6.1$ and $K_{IR}6.2$ subtypes. However, the expression of $K_{IR}6.2$ channels in the I-R group was significantly lower than in the control group. This down-regulation of the $K_{IR}6.2$ expression was reversed after treatment with nicorandil or cromakalim. Nicorandil activates SUR2x-containing K_{ATP} channels by interacting with the 17th transmembrane α-helix of SUR2x (Reimann *et al.*, 2001). Moreover, nicorandil activates SUR2x/$K_{IR}6.2$ channels more potently and efficiently (Yamada *et al.*, 2004). Sun *et al.* (2008) reported that diazoxide activated the expression of $K_{IR}6.2$ in the rat kidney. These data would be compatible with our present results. The $K_{IR}6.2$ channel subunit has been shown to provide protection during hypoxia-induced generalized seizure, using mutant mice lacking this subunit of K_{ATP} channels (Yamada *et al.*, 2001). Héron-Milhavet *et al.* (2004) also reported a protective effect of K_{ATP} channels, as transgenic overexpression of $K_{IR}6.2$ in the forebrain significantly protected mice from hypoxic–ischaemic injury and neuronal damage seen in stroke. However, there is no information available about the relationship between the $K_{IR}6.2$ subunit and renal I-R injury. Our work may indicate the possibility that the $K_{IR}6.2$ subunit protects against renal I-R injury in rats, similar to the protective effect which is found in brain and in the heart. Our data show that I-R induces down-regulation of the expression of $K_{IR}6.2$ channels in the kidney and that treatment with nicorandil reversed this down-regulation. Such down-regulation may occur in other organs as well, under hypoxic conditions, if ischaemia and hypoxia are a genrla stimulus of down-regulation of the expression of the $K_{IR}6.2$ subunit. However, further studies are needed to clarify the mechanism responsible for this phenomenon.

I-R of the kidney causes both glomerular and tubular dysfunction (Xie and Guo, 2006). In this study, we demonstrated that I-R induced damages mainly in the renal tubules and not in the glomerulus (Figures 2–4). Nicorandil produced a reduction of the renal dysfunction and the injury mediated by I-R of the kidney. Nicorandil appears to provide a greater beneficial action against tubular dysfunction, rather than glomerular dysfunction. This is supported by our findings that compared with the rats subjected to I-R, nicorandil did not produce significant decreases in SCr and ACR levels. In contrast, nicorandil had a marked effect on a marker of tubular dysfunction injury, the urinary β2-MG levels. This finding was supported by the renal histopathology that revealed severe acute tubular damage in rat kidneys subjected to I-R, whereas the animals treated with nicorandil showed a marked reduction of the tubular damage. The strongest immunoreactivity for 4-HNE as well as for 8-OHdG was detected in proximal and distal tubules, but not in glomerulus. These data support our biochemical studies. Furthermore, our data confirm that mitochondrial K_{ATP} channel activation effectively blocks oxidative damage, as estimated by immunohistochemistry for 4-HNE and 8-OHdG.

In conclusion, this study provides strong evidence that nicorandil has a protective effect against proximal tubule damage after I-R injury in the rat kidney. Under these circumstances, nicorandil or other mitochondrial K_{ATP} channel openers may have a major therapeutic role by preventing oxidative injury, via expression of $K_{IR}6.2$ channels. Our results strongly suggest potential clinical benefits of nicorandil in

patients undergoing partial nephrectomy or renal transplantation, based on its dual action as a K_{ATP} channel opener and as an NO donor.

Acknowledgements

We would like to thank Dr Katsumi Higaki, Mr Itaru Satoh and Ms Natsuko Ikubo for their help and technical support and we are grateful to Dr Darryl T. Martin for his assistance with this manuscript.

Conflicts of interest

None.

References

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC). 4th edn. Br J Pharmacol 158 (Suppl. 1): S1–S254.

Auchampach JA, Gross GJ (1993). Adenosine A_1 receptors, K_{ATP} channels, and ischemic preconditioning in dogs. Am J Physiol 264: 1321–1336.

Bird JE, Milhoan K, Wilson CB, Young SG, Mundy CA, Parthasarathy S *et al.* (1988). Ischemic acute renal failure and antioxidant therapy in the rat. The relation between glomerular and tubular dysfunction. J Clin Invest 81: 1630–1638.

Bonegio R, Lieberthal W (2002). Role of apoptosis in the pathogenesis of acute renal failure. Curr Opin Nephrol Hypertens 11: 301–308.

Bonventre JV (1993). Mechanisms of ischemic acute renal failure. Kidney Int 43: 1160–1178.

Chandra J, Samali A, Orrenius S (2000). Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 29: 323–333.

Date T, Taniguchi I, Inada K, Matsuo S, Miyanaga S, Yamane T *et al.* (2005). Nicorandil inhibits serum starvation-induced apoptosis in vascular endothelial cells. J Caridiovas Pharmacol 46: 721–726.

Flaherty JT (1989). Nitrate tolerance. A review of the evidence. Drugs 37: 523–550.

Gomma AH, Purcell HJ, Fox KM (2001). Potassium channel openers in myocardial ischaemia: therapeutic potential of nicorandil. Drugs 61: 1705–1710.

Héron-Milhavet L, Xue-Jun Y, Vannucci SJ, Wood TL, Willing LB, Stannard B *et al.* (2004). Protection against hypoxia-ischemic injury in transgenic mice overexpressing Kir6.2 channel pore in forebrain. Mol Cell Neurosci 25: 585–593.

Ismail HA, Khalifa MM, Hassan MK, Ashour OM (2007). Investigation of the mechanisms underlying the gastroprotective effect of nicorandil. Pharmacology 79: 76–85.

Jablonski P, Howden BO, Rae DA, Birrell CS, Marshall VC, Tange J (1983). An experimental model for assessment of renal recovery from warm ischemia. Transplantation 35: 198–204.

Joo JD, Kim M, D'Agati VD, Lee HT (2006). Ischemic preconditioning provides both acute and delayed protection against renal ischemia and reperfusion injury in mice. J Am Soc Nephrol 17: 3115–3123.

Lee HT, Emala CW (2001). Protein kinase C and G(i/o) proteins are involved in adenosine- and ischemic preconditioning-mediated renal protection. J Am Soc Nephrol 12: 233–240.

Lieberthal W, Nigam SK (1998). Acute renal failure. I. Relative importance of proximal vs. distal tubular injury. Am J Physiol 275: 623–631.

Markham A, Plosker GL, Goa KL (2000). Nicorandil. An updated review of its use in ischaemic heart disease with emphasis on its cardioprotective effects. Drugs 60: 955–974.

Martins PN, Chandraker A, Tullius SG (2006). Modifying graft immunogenicity and immune response prior to transplantation: potential clinical applications of donor and graft treatment. Transpl Int 19: 351–359.

Miyaji T, Kato A, Yasuda H, Fujigaki Y, Hishida A (2001). Role of the increase in p21 in cisplatin-induced acute renal failure in rats. J Am Soc Nephrol 12: 900–908.

Mizunuma T, Nithipaticom K, Gross GJ (1995). Effects of nicorandil and glyceryl trinitrate on infarct size, adenosine release, and neutrophil infiltration in the dog. Cardiovasc Res 29: 482–489.

Nandagopal K, Dawson TM, Dawson VL (2001). Critical role for nitric oxide signaling in cardiac and neuronal ischemic preconditioning and tolerance. J Pharmacol Exp Ther 297: 474–478.

O'Rourke B (2004). Evidence for mitochondrial K^+ channels and their role in cardioprotection. Circ Res 94: 420–432.

Okada S, Saito M, Kazuyama E, Hanada T, Kawaba Y, Hayashi A *et al.* (2008). Effects of N-hexacosanol on nitric oxide synthase system in diabetic rat nephropathy. Mol Cell Biochem 315: 169–177.

Padanilam BJ (2003). Cell death induced by acute renal injury: a perspective on the contributions of apoptosis and necrosis. Am J Physiol Renal Physiol 284: 608–627.

Paller MS, Hoidal JR, Ferris TF (1984). Oxygen free radicals in ischemic acute renal failure in the rat. J Clin Invest 74: 1156–1164.

Patel DJ, Purcell HJ, Fox KM (1999). Cardioprotection by opening of the K_{ATP} channel in unstable angina: is this a clinical manifestation of myocardial preconditioning? Results of a randomized study with nicorandil. CESAR 2 investigation. Clinical European studies in angina and revascularization. Eur Heart J 20: 51–57.

Pompermayer K, Souza DG, Lara GG, Silveira KD, Cassali GD, Andrade AA *et al.* (2005). The ATP-sensitive potassium channel blocker glibenclamide prevents renal ischemia/reperfusion injury in rats. Kidney Int 67: 1785–1796.

Quast U (1996). ATP-sensitive K^+ channels in the kidney. Naunyn Schmiedebergs Arch Pharmacol 354: 213–225.

Reimann F, Ashcroft FM, Gribble FM (2001). Structural basis for the interference between nicorandil and sulfonylurea action. Diabetes 50: 2253–2259.

Saito K, Sakai K (1998). Possible involvement of K_{ATP} channel activation of depressor response to vasoactive neuropeptides in rats. Jpn J Pharmacol 76: 227–231.

Segawa K, Minami K, Shiga Y, Shiraishi M, Sata T, Nakashima Y *et al.* (2001). Inhibitory effects of nicorandil on rat mesangial cell proliferation via the protein kinase G pathway. Nephron 87: 263–268.

Shimizu S, Saito M, Kinoshita Y, Shomori K, Satoh I, Satoh K (2009). Ischemic preconditioning and postconditioning are effective strategies to reduce testicular torsion-detorsion injury. J Urol 182: 1637–1643.

Sudo H, Hirata M, Kanada H, Yorozu K, Tashiro Y, Serizawa K *et al.* (2009). Nicorandil improves glomerular injury in rats with mesangioproliferative glomerulonephritis via inhibition of proproliferative and profibrotic growth factors. J Pharmacol Sci 111: 53–59.

Sugimoto K, Ito H, Iwakura K, Ikushima M, Kato A, Kimura R *et al.* (2003). Intravenous nicorandil in conjunction with coronary reperfusion therapy is associated with better clinical and functional outcomes in patients with acute myocardial infarction. Circ J 67: 295–300.

Sun Z, Zhang X, Ito K, Li Y, Montgomery RA, Tachibana S *et al.* (2008). Amelioration of oxidative mitochondrial DNA damage and deletion after renal ischemic injury by the K_{ATP} channel opener diazoxide. Am J Physiol Renal Physiol 294: 491–498.

Taira N (1989). Nicorandil as a hybrid between nitrates and potassium channel activators. Am J Cardiol 63: 18–24.

Takamura A, Higaki K, Kajimaki K, Otsuka S, Ninomiya H, Matsuda J *et al.* (2008). Enhanced autophagy and mitochondrial aberrations in murine G(M1)-gangliosidosis. Biochem Biophys Res Commun 367: 616–622.

Tamaki T, Hasui K, Shoji T, Fukui K, Iwao H, Abe Y (1991). Effects of cromakalim on renal hemodynamics and function in dogs: comparison with nicardipine. J Cardiovasc Pharmacol 17: 305–309.

The IONA Study Group (2002). Effect of nicorandil on coronary events in patients with stable angina: the impact of nicorandil in angina (IONA) randomized trial. Lancet 359: 1269–1275.

Van Poppel H (2010). Efficacy and safety of nephron-sparing surgery. Int J Urol 17: 314–322.

Van Winkle DM, Chien GL, Wolff RA, Soifer BE, Kuzume K, Davis RF (1994). Cardioprotection provided by adenosine receptor activation is abolished by blockade of the K_{ATP} channel. Am J Physiol 266: 829–839.

Xie J, Guo Q (2006). Apoptosis antagonizing transcription factor protects renal tubule cells against oxidative damage and apoptosis induced by ischemia-reperfusion. J Am Soc Nephrol 17: 3336–3346.

Yamada K, Ji JJ, Yuan H, Miki T, Sato S, Horimoto N *et al.* (2001). Protective role of ATP-sensitive potassium channels in hypoxia-induced generalized seizure. Science 292: 1543–1546.

Yamada M, Ishii M, Hibino H, Kurachi Y (2004). Mutation in nucleotide-binding domains of sulfonylurea receptor 2 evokes Na-ATP-dependent activation of ATP-sensitive K^+ channels: implication for dimerization of nucleotide-binding domains to induce channel opening. Mol Pharmacol 66: 807–816.

Zager RA, Johnson AC, Lund S, Hanson SY, Abrass CK (2006). Levosimendan protects against experimental endotoxemic acute renal failure. Am J Physiol Renal Physiol 290: 1453–1462.

Zhou H, Kato A, Yasuda H, Odamaki M, Itoh H, Hishida A (2003). The induction of heat shock protein-72 attenuates cisplatin-induced acute renal failure in rats. Pflugers Arch 446: 116–124.

Zhou M-L, Huang Y, Liu D-P, Liang C-C (2005). K_{ATP} channel: with cell metabolism and role in the cardiovascular system. In J Biochem Cell Boil 37: 751–764.