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The ATP-sensitive potassium channel blocker glibenclamide prevents renal ischemia/reperfusion injury in rats

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The ATP-sensitive potassium channel blocker glibenclamide prevents renal ischemia/reperfusion injury in rats.

Background. Renal ischemia/reperfusion (I/R) is a complex neutrophil-mediated syndrome. Adenosine-triphosphate (ATP)-sensitive potassium (K_{ATP}) channels are involved in neutrophil migration in vivo. In the present study, we have investigated the effects of glibenclamide, a K_{ATP} channel blocker, in renal I/R injury in rats.

Methods. The left kidney of the rats was excised through a flank incision and ischemia was performed in the contralateral kidney by total interruption of renal artery flow for 45 minutes. Renal perfusion was reestablished, and the kidney and lungs were removed for analysis of vascular permeability, neutrophil accumulation, and content of cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-10] 4 and 24 hours later. Renal function was assessed by measuring creatinine, Na^+, and K^+ levels in the plasma and by determination of creatinine clearance. Drugs were administered subcutaneously after the onset of ischemia.

Results. Reperfusion of the ischemic kidney induced local (kidney) and remote (lung) inflammatory injury and marked renal dysfunction. Glibenclamide (20 mg/kg) significantly inhibited the reperfusion-associated increase in vascular permeability, neutrophil accumulation, increase in TNF-α levels and nuclear factor-κB (NF-κB) translocation. These inhibitory effects were noticed in the kidney and lungs. Moreover, glibenclamide markedly ameliorated the renal dysfunction at 4 and 24 hours.

Conclusion. Treatment with glibenclamide is associated with inhibition of neutrophil recruitment and amelioration of renal dysfunction following renal I/R. Glibenclamide may have a therapeutic role in the treatment of renal I/R injury, such as after renal transplantation.

Key words: cytokines, neutrophils, inflammation, kidney, ischemia and reperfusion, glibenclamide.

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Renal ischemia/reperfusion (I/R) is a complex syndrome involving several mechanisms, including renal vasoconstriction, tubular damage, and glomerular injury [1]. It is an important cause of renal dysfunction in renal transplantation and is associated with an increased rate of acute and chronic rejection [2]. Although the restoration of blood flow (i.e., reperfusion) is the treatment of choice following acute ischemia of a vascular territory, reperfusion of ischemic tissues can be accompanied by significant injury to local and remote organs, such as the lung, and systemic inflammatory events that may limit the beneficial effects of blood flow restoration [3]. The mechanisms proposed to explain the reperfusion-associated injury include anoxia, release of reactive oxygen species, such as superoxide radical and hydrogen peroxide, recruitment and activation of leukocytes and subsequent release of mediators of the inflammatory process [3–5]. Neutrophil and tumor necrosis factor-α (TNF-α) are among the cell types and inflammatory mediators, respectively, thought to be of major relevance in the pathophysiology of I/R injury [6–9]. It is believed that strategies limiting reperfusion-induced influx of neutrophils and production of TNF-α may be useful therapeutic adjuncts in the treatment of acute ischemia [9].

The response of neutrophils to inflammatory mediators is preceded or accompanied by changes in membrane potential [10–12], which increase the release of Ca^{+2} from intracellular stores and stimulate the uptake of extracellular Ca^{+2} [13]. These effects are thought to be primary steps in neutrophil migration and have been correlated with locomotion and chemotaxis [14, 15]. Glibenclamide has been shown to suppress neutrophil migration and chemotaxis and affects plasma exudation during acute inflammatory responses, effects likely related to its adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channel blocking activity [16]. In the present study we have investigated the effects of

glibenclamide in renal I/R injury in rats. For comparison, we have also evaluated the effects of the nonspecific channel blocker tetraethylammonium (TEA) and of the channel opener diazoxide.

METHODS

Animals

Male Wistar rats (200 to 220 g) obtained from the Bioscience Unit of our Institution were housed in standard conditions and had free access to commercial chow and water. All procedures described here had prior approval from the Institute Animal Ethics Committee.

I/R injury

Rats were anaesthetized with 185 mg/kg of ketamine plus 16 mg/kg of xylazine (intramuscularly) and a left nephrectomy was performed through a left flank incision. Renal ischemia required performing a right flank incision and dissecting the right renal pedicle so as to expose the renal vessels. A thick cotton thread was passed over the renal vascular pedicle to induce ischemia. Both ends of the thread were exteriorized through the back of the rat and tied tightly above a length of latex tubing on the external side of the skin [17]. The renal pedicle was released 45 minutes after occlusion by cutting the thread and pulling it out followed by 4 or 24 hours of reperfusion. Animals were allowed to recover from anesthesia without further interventions. Throughout the anesthesia, body temperature was kept at 36 to 38°C by placing the rats on a heating pad.

Glibenclamide (20 mg/kg) and a nonspecific potassium channel blocker, TEA (40 mg/kg), were administered through a single subcutaneous injection 40 minutes before the beginning reperfusion. In the groups submitted to 24 hours of reperfusion, glibenclamide was also administered 8 hours after reperfusion. The K_{ATP} channel opener, diazoxide (30 mg/kg), was given intraperitoneally 40 minutes before reperfusion period. Dimethyl sulfoxide (DMSO) and ethanol were employed as vehicle for administration of glibenclamide and diazoxide, respectively, as previously described [16], and TEA was dissolved in a saline solution. The dose of glibenclamide used here was based on previous work demonstrating that this dose inhibited neutrophil migration and fluid leakage in carrageenan-induced pleurisy in rats [16]. Doses of TEA and diazoxide were based on previous studies [16, 18]. Drugs or vehicle were injected in a final volume of 0.4 mL. Vehicle alone did not affect the parameters analyzed when compared to animals not injected with vehicle (data not shown). Sham-operated animals were submitted to a left nephrectomy and were used as control for the reperfusion-induced injury.

Evaluation of changes in vascular permeability

The extravasation of Evans blue dye into the kidney and left lung tissue was used as an index of increased vascular permeability [19]. Evans blue dye (20 mg/kg) was administered intravenously (1 mL/kg) via a femoral vein 5 minutes prior to reperfusion. After various times (45 minutes, 4 hours, or 24 hours) after reperfusion, the kidney and lungs were flushed with 20 mL of saline to wash the intravascular Evans blue dye and were cut and allowed to dry in a Petri dish for 24 hours at 37°C. The dry weight of the tissue was calculated and Evans blue dye was extracted using 3 mL of formamide (24 hours at room temperature). The amount of Evans blue dye in the tissue was obtained by comparing the extracted absorbance with that of a standard Evans blue dye curve read at 620 nm in an automatic plate reader. Results are presented as the amount of Evans blue dye per μg per 100 mg of tissue.

Myeloperoxidase (MPO) levels

The extent of neutrophil accumulation in the kidney and right lung tissue was measured by assaying MPO activity, as previously described [9]. A fragment of kidney and right lung of the animals that had undergone I/R (45 minutes, 4 hours, or 24 hours) injury was removed and snap frozen in liquid nitrogen. Upon thawing, the tissue (1 g of tissue per 19 mL of buffer) was homogenized in pH 4.7 buffer [0.1 mol/L NaCl, 0.02 mol/L $NaPO_4$, and 0.015 mol/L sodium ethylenediaminetetraacetic acid (EDTA)], centrifuged at 260 × *g* for 10 minutes and the pellet underwent hypotonic lysis (15 mL of 0.2% NaCl solution followed 39 seconds later by addition of an equal volume of a solution containing 1.6% NaCl and 5% glucose). After a further centrifugation, the pellet was then resuspended in 0.05 mol/L of $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide and rehomogenized. One milliliter aliquots of the suspension were transferred into 1.5 mL Eppendorf tubes followed by three freeze-thaw cycles using liquid nitrogen. These were then centrifuged for 15 minutes at 3000*g* and the pellet discarded. Samples of lung were diluted 1:40 prior to the assay and samples of kidney were not diluted. MPO activity was assayed by measuring the change in optical density at 450 nm using tetramethylbenzidine (1.6 mmol/L) and H_2O_2 (0.5 mmol/L). Results were expressed as MPO relative units/100 mg of tissue. A MPO relative unit represents the number of neutrophils obtained by comparing the optical density of tissue supernatant with that of rat peritoneal neutrophils processed in the same way. To this end, neutrophils were collected from the peritoneum of rats 8 to 12 hours after injection of 3 mL of 5% casein. A standard curve of neutrophil numbers versus optical density was obtained by processing casein-elicited neutrophils (>95% purity by

using this methodology), as above and assaying for MPO activity. Under the conditions described above, the assay failed to detect the peroxidase activity derived from monocytes/macrophages (data not shown).

Measurement of cytokine concentrations in kidney and lungs

TNF-α, interleukin (IL)-1β, and IL-10 concentrations were measured in kidney and lung of animals using enzyme-linked immunosorbent assay (ELISA) techniques previously described [20]. One hundred milligrams of kidney or lung of sham-operated and reperfused (4 or 24 hours) animals were homogenized in 1 mL of phosphate-buffered saline (PBS) (0.4 mmol/L NaCl and 10 mmol/L $NaPO_4$) containing antiproteases [0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.1 mmol/L benzethonium chloride, 10 mmol/L EDTA, and 20 kallikrein inhibitor unit (KI) aprotinin A] and 0.05% Tween 20. The samples were then centrifuged for 10 minutes at 3000*g* and the supernatant immediately used for ELISA assays at a 1:5 dilution in PBS. ELISA plates (Nunc MaxiSorb) were coated with sheep antirat TNF-α, IL-1β, or IL-10 polyclonal antibodies (1 to 2 μg/mL) overnight. The plates were washed three times and then blocked with 1% bovine serum albumin (BSA). After a further wash, plates were incubated with samples or recombinant rat cytokine and incubated overnight. The biotinylated polyclonal antibodies were used at a 1:1000 to 1:2000 dilution and the assays had a sensitivity of 16 pg/mL.

Measurement of mean arterial blood pressure

Mean arterial pressure (MAP) was measured by a polyethylene catheter placed in the right femoral artery. Blood pressure was measured during the 45 minutes of ischemia followed by 4 hours of reperfusion. The pressure signal was continuously monitored by a pressure transducer (TSD 104A) connected to an amplifier MP100 System (Biopac Systems, Inc., Goleta, CA, USA) and a computer-measuring program (Acqknowledge Software, Goleta, CA, USA).

Determination of blood glucose levels

Since the blockade of K_{ATP} channels with glibenclamide is clinically used as an oral hypoglycemic therapy, blood samples of animals were collected from vena cava at the end of experiments (4 hours or 24 hours of reperfusion) for assessment of glucose levels. For this, we used a commercial kit (Bioclin, Belo Horizonte, Brazil).

Measurement of renal function

Creatinine clearance was assessed as an index of glomerular filtration rate (GFR). Plasma samples were prepared from blood obtained by vena cava puncture. The concentrations of creatinine and urea were determined by colorimetric methods using commercially available kits (Bioclin) and were reported as mg/dL. Fractional excretions of Na^+ and K^+ were calculated as the ratio between urinary excretion and the amount filtered (GFR × plasma concentration).

Histologic analysis

Sections of kidney were obtained from representative animals in each of the treatment groups. The tissue was fixed in 10% formalin, embedded in paraffin and 4 μm thick section cuts. The sections were stained with hematoxylin and eosin and examined under a light microscope.

Preparation of nuclear extracts and band shift assay

Nuclear extracts were obtained from powdered kidney and processed according to a previously published method [21]. Tissue samples were obtained at 4 hours after reperfusion. Nuclear extracts were allowed to interact with the oligonucleotide containing nuclear factor-κB (NF-κB) consensus binding site 5′-AGT TGA GGG GAC TTT CCC AGC C-3′ labeled with [γ-^{22}P] ATP using T4 polynucleotide kinase. After DNA binding, complexes were resolved on nondenaturing 6% (wt/vol) polyacrylamide gels and exposed at −80°C.

Compounds

The following drugs were used: glibenclamide, TEA, diazoxide, Evans blue dye (Sigma Chemical Co.), xylazine (Bayer, São Paulo, Brazil), ketamine (Agribrands do Brasil Ltda, São Paulo, Brazil), creatinine, glucose and urea kits (Bioclin). ELISA kits for the detection of TNF-α, IL-1β, and IL-10 were a kind gift of Dr, S.P. Poole, National Institute for Biological Standards and Control, Potters Bar, United Kingdom. All solutions were prepared on the day of the experiment.

Statistical analysis

Results are shown as means ± SEM. Differences were compared by using analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc analysis. Results with a $P < 0.05$ were considered significant.

RESULTS

Model of I/R injury

Initial experiments were designed to optimize the kinetics of renal injury following ischemia of the renal vascular pedicle. An ischemic period of 45 minutes was used in all experiments and the time of reperfusion varied from 0 to 24 hours. As seen in Figure 1A, an increase of in vascular permeability in the kidney, as assessed by the extravasation of Evans blue dye, was first significant at 4 hours after renal reperfusion. In addition to the renal injury, there was a significant increase of vascular

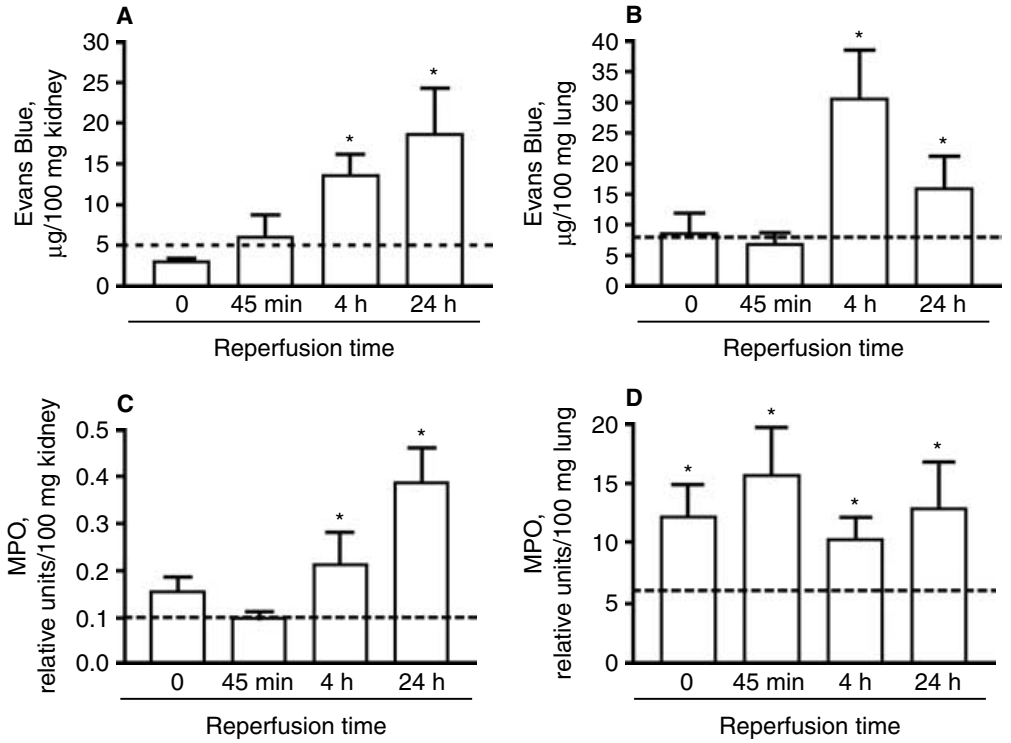

Fig. 1. Kinetics of the changes in vascular permeability (*A* and *B*) and in myeloperoxidase (MPO) contents (*C* and *D*) in the kidney and lungs following ischemia (I) (45 minutes) and reperfusion (R) (45 minutes to 24 hours) of renal vascular pedicle. Evaluation of changes in vascular permeability were assessed by Evans blue dye extravasation and neutrophil accumulation was assessed by measuring the tissue contents of MPO. A dotted line across the bars was inserted to represent levels of Evans blue dye or MPO in sham-operated animals. Results are shown as µg Evans blue dye per 100 mg of tissue or MPO, relative units per 100 mg of tissue and are the mean ± SEM of five to ten animals. $^*P < 0.05$ when compared to sham-operated animals.

permeability in the lung, an organ remote from the site of injury (Fig. 1B). In the lung, the first significant increase in vascular permeability was also detected 4 hours after renal reperfusion.

MPO activity was measured in kidney and lung tissues as a marker of neutrophil accumulation [22]. In the kidney, the increase in MPO activity was first significant at 4 hours after renal reperfusion and increased further at 24 hours (Fig. 1C). In the lungs, neutrophils appeared to be already trapped in the lungs in response to the ischemia (0 reperfusion time) and remained high in lung tissues thereafter (Fig. 1D).

MAP was registered during renal I/R. There was no change in MAP during the 45-minute ischemia. Unclamping of the renal pedicle produced a transient (5 minutes) elevation of MAP (from 86 ± 6 to 108 ± 9 mm Hg) (Fig. 2A).

Effects of the treatment with potassium channel blockers on I/R injury

Glibenclamide significantly inhibited the reperfusion-associated increase in vascular permeability in the kidney at 4 and 24 hours. The drug also inhibited the increase in vascular permeability in the lung after 4 hours of renal reperfusion. At 24 hours, the increase in vascular permeability in the lung of glibenclamide-treated animas had returned to baseline (Fig. 3A and B). Similarly, treatment with glibenclamide effectively prevented the accumulation of neutrophils in the kidney of reperfused animals at 4 and 24 hours (Fig. 3C). In contrast, the accumulation of neutrophils in the lungs was not altered by the administration of the drug (Fig. 3D), which is consistent with the observation that neutrophils accumulated in the lungs during ischemia (see Fig. 1) and the drug was administered after the onset of ischemia.

We also tested the effects of the pretreatment with the nonspecific potassium channel blocker, TEA, in animals submitted to 4 hours of reperfusion. In the latter experiments, the drug inhibited the increase in vascular permeability by 70% and 60%, in the kidney and lungs, respectively, but failed to affect neutrophil accumulation in either organ (Table 1).

Effects of the treatment with potassium channel blockers on the concentration of cytokines following I/R

The concentration of cytokines was measured in kidney and lung tissues at 4 hours or 24 hours of renal

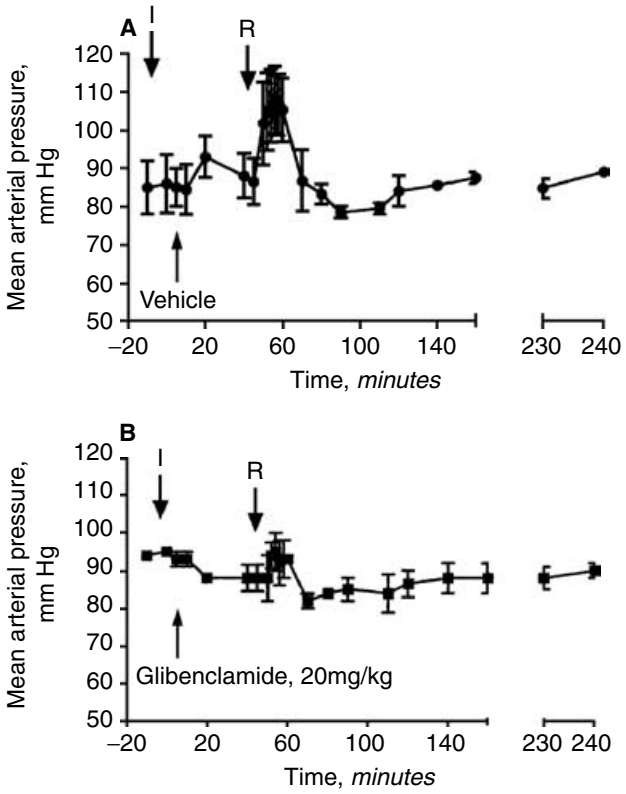

Fig. 2. Mean arterial pressure (MAP) in rats submitted to renal ischemia (I) and reperfusion (R) in control groups (*A*) and glibenclamide (20 mg/kg)-treated groups (*B*). The renal vascular pedicle was occluded during 45 minutes following 4 hours of reperfusion. Results are shown as mm Hg and are the mean ± SEM of four animals. Difference between the groups was not statistically significant ($P > 0.05$).

reperfusion. Renal I/R induced a marked elevation in the concentration of TNF-α in the kidney and lungs (Fig. 4A and C). Treatment with glibenclamide suppressed the reperfusion-induced TNF-α in the kidney (inhibition of approximately 90%) and abolished the production of the cytokine in the lungs at 4 hours of reperfusion (Fig. 4A and C). The drug also partially prevented the increase in the concentration of TNF-α in the lungs after 24 h of reperfusion (Fig. 4C).

There was also an increase in the concentration of IL-1β at 4 hours, but not at 24 hours, of reperfusion (Fig. 4B). Treatment with glibenclamide failed to affect the reperfusion-induced increased in the concentrations of IL-1β in the kidney (Fig. 4B). The concentration of this cytokine in the lung was similar in sham-operated and reperfused animals (data not shown). The concentration of the anti-inflammatory cytokine IL-10 was greatly elevated in the kidney at 4 hours, but not at 24 hours, after reperfusion of the ischemic kidney (Fig. 4D). This cytokine was not detectable in the lungs of sham-operated and reperfused animals (data not shown). Treatment with glibenclamide did not prevent the reperfusion-induced increase in IL-10 concentrations in the kidney (Fig. 4D).

For comparison, we evaluated the effects of TEA on the increases of TNF-α concentration induced by the ischemia followed by 4 hours of reperfusion. TEA had no significant effect on the reperfusion-induced increase in TNF-α concentrations in the kidney (I/R group 304 ± 66 pg/100 mg of tissue; treated with TEA 325 ± 45 pg/100 mg of tissue) ($N = 4$) or in the lungs (I/R group 53 ± 21 pg/100 mg of tissue; treated with TEA 12 ± 11 pg/100 mg of tissue) ($N = 4$).

In contrast to its effects on inflammatory parameters, glibenclamide had no significant effect on the transient changes in MAP induced by the reperfusion process (Fig. 2B).

Effects of glibenclamide on the changes of renal function secondary to I/R injury

As glibenclamide modified inflammatory parameters induced by the reperfusion process, next we evaluated whether changes in inflammation were reflected by changes in renal function. At 4 hours after reperfusion, there was a minor increase in plasma creatinine concentrations in vehicle- but not glibenclamide-treated animals (sham-operated 1.3 ± 0.1 mg/dL; I/R 4 hours, 1.8 ± 0.2 mg/dL; and I/R + glibenclamide 1.5 ± 0.2 mg/dL) ($N = 5$ in each group) ($P < 0.05$ for I/R versus sham). There were no changes in plasma Na^+ and K^+ concentrations (data not shown).

A 24 hours, reperfusion of the ischemic kidney resulted in a significant decrease in both urinary flow (Fig. 5A) and in GFR (Fig. 5B). These functional changes were prevented by treatment with glibenclamide (Fig. 5A and B). Moreover, there were 39- and sevenfold increases in fractional excretion of Na^+ and K^+, respectively (Fig. 5C and D). Treatment with glibenclamide did not change excretion of Na^+ but significantly inhibited the excretion of K^+ (Fig. 5C and D). There was also a significantly lesser increase in plasma concentration of creatinine (I/R 24 hours 2.4 ± 0.2 mg/dL and I/R + glibenclamide 1.8 ± 0.1 mg/dL) ($N = 5$ in each group) ($P < 0.05$).

Since glibenclamide is clinically used as an oral hypoglycemic therapy, we also measured blood glucose levels at the end of 4 or 24 hours of renal reperfusion. Glibenclamide did not induce hypoglycemia in animals submitted to 4 hours of reperfusion (sham-operated 254 ± 19 md/dL; I/R 279 ± 29 mg/dL; and I/R + glibenclamide 228 ± 51 mg/dL) ($N = 5$ in each group). In groups of animals submitted to 24 hours of renal reperfusion, the levels of blood glucose were reduced in presence of glibenclamide (I/R 228 ± 51 mg/dL and I/R + glibenclamide, 104 ± 23 mg/dL) ($N = 5$ in each group) ($P < 0.05$).

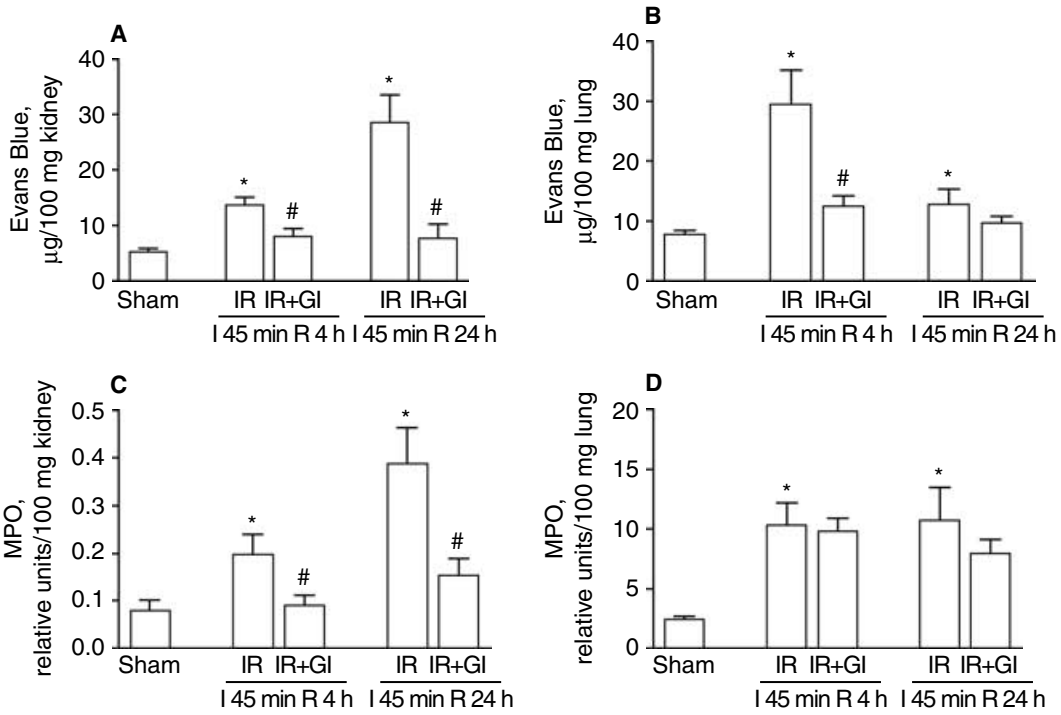

Fig. 3. Effects of the treatment with glibenclamide on vascular permeability (*A* and *B*) and on myeloperoxidase (MPO) contents (*C* and *D*) in the kidney and lung following ischemia(I) (45 minutes) and reperfusion (R) (4 or 24 hours) of renal vascular pedicle. Evaluation of changes in vascular permeability were assessed by Evans blue dye extravasation and neutrophil accumulation was assessed by measuring the tissue contents of MPO. Glibenclamide (20 mg/kg) was administered subcutaneously as described in the **Methods** section. Results are shown as µg Evans blue dye per 100 mg of tissue or MPO relative units per 100 mg of tissue and are the mean ± SEM of six to ten animals. $^{*}P < 0.05$ when compared to sham-operated animals; $\#P < 0.05$ when compared to I/R animals.

Table 1. Effects of the treatment with a nonselective potassium channel blocker, tetraethylammonium (TEA, 40mg/kg) on vascular permeability and on neutrophil accumulation in the kidney and lung following ischemia (45 min) and reperfusion (4 h) of renal vascular pedicle

	Evans blue (µg/100 mg tissue)		MPO Relative units/100 mg tissue	
	Kidney	Lung	Kidney	Lung
Sham-operated	5 ± 0.70	8 ± 0.90	0.03 ± 0.01	2 ± 0.30
I/R	14 ± 1.50*	29 ± 5.70*	0.2 ± 0.04*	10 ± 1.90*
I/R+TEA	4 ± 1.60#	12 ± 4.00*#	0.1 ± 0.04*	12 ± 3.00*

TEA (40 mg/kg) was administered subcutaneously 40 min prior to reperfusion as described in the Methods section. Values are mean ± SEM of 5–10 animals. $^{*}P < 0.05$ compared to sham-operated group, $^{\#}P < 0.05$ compared to ischemia and reperfusion (I/R) group.

Effects of the treatment with glibenclamide on the histologic analysis of renal tissue

Hematoxylin and eosin–stained histologic sections of the kidneys undergoing reperfusion injury showed that the changes were most prominent in the proximal tubules (Fig. 6B). There was shedding of the cells lining the tubules, sometimes complete, while other tubules showed single cell necrosis with nuclear pyknosis and cytoplasmic eosinophilia or vacuoles (Fig. 6B, insert). Overall the tissue was congested with foci of interstitial hemorrhage. The latter changes were more marked in the medulla. Infiltrating neutrophils could be detected scattered in the tissue. In contrast, sections of the glibenclamide-treated kidneys showed architectural and cytologic preservation of structure (Fig. 6C). Discreet vascular congestion was observed. Overall the morphologic features in treated animals were similar to those seen in control kidneys (Fig. 6A).

Effects of the treatment with glibenclamide on NF-κB translocation secondary to I/R injury

Translocation of the transcription factor NF-κB to the nucleus in the kidney during 4 hours of reperfusion was determined by electrophoretic mobility shift assays (EMSA) of kidney nuclear extracts. In the kidneys of sham-operated rats, there was little evidence of NF-κB translocation. I/R induced significant translocation of NF-κB, as assessed by gel shift assays, and the translocation was greatly prevented by glibenclamide treatment (Fig. 7).

The specifity of the DNA-protein complex formed was assayed by preincubating the nuclear extracts with 50-fold molar excess of unlabeled oligonucleotides before addition of the labeled oligonucleotide NF-κB. The DNA protein interaction was completely blocked by competing

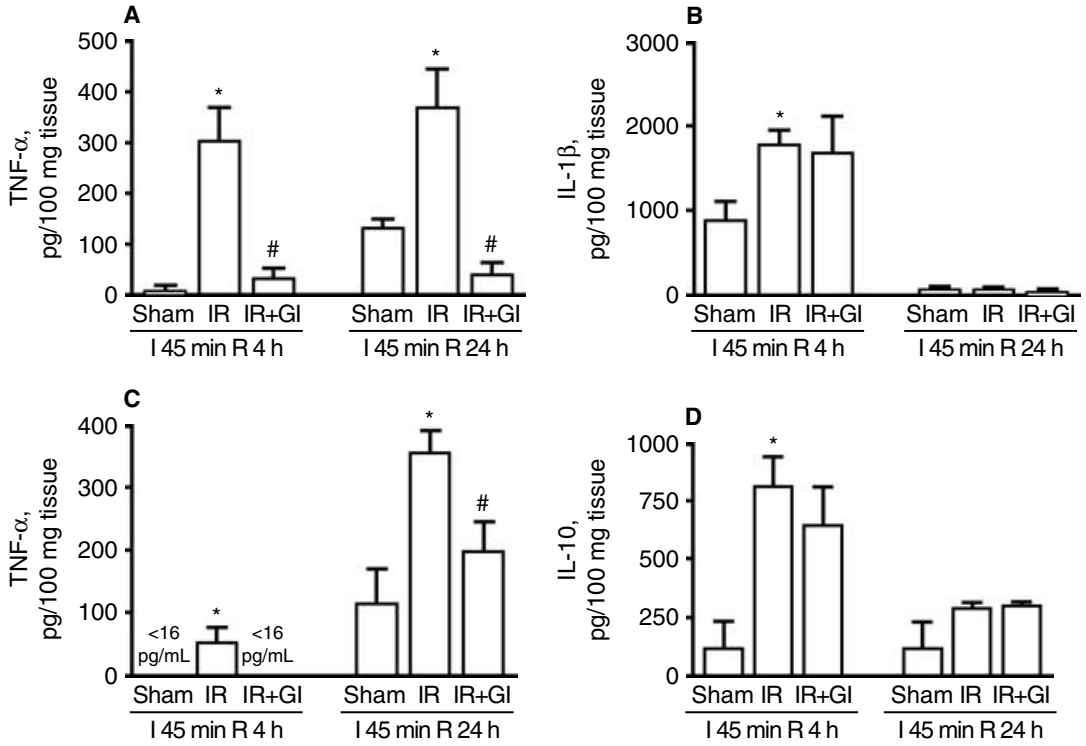

Fig. 4. Effects of the treatment with glibenclamide on the concentrations of tumor necrosis factor-α (TNF-α) (*A* and *C*), interleukin (IL)-1β (*B*), and IL-10 (*D*) in the kidney and lung following ischemia (I) (45 minutes) and reperfusion (R) (4 hours or 24 hours) of renal vascular pedicle. The concentrations of TNF-α, IL-1β, or IL-10 were assessed in the kidney and lung by using specific enzyme-linked immunosorbent assay (ELISA). Glibenclamide (GI) (20 mg/kg) was administered subcutaneously as described in the **Methods** section. Results are shown as pg of TNF-α, IL-1β, or IL-10 per 100 mg of tissue and are the mean ± SEM of six to 10 animals. Concentration of cytokines below the detection limit of the assay (16 pg/mL) was shown as < 16 pg/mL. $^*P < 0.05$ when compared to sham-operated animals; $\#P < 0.05$ when compared to I/R animals.

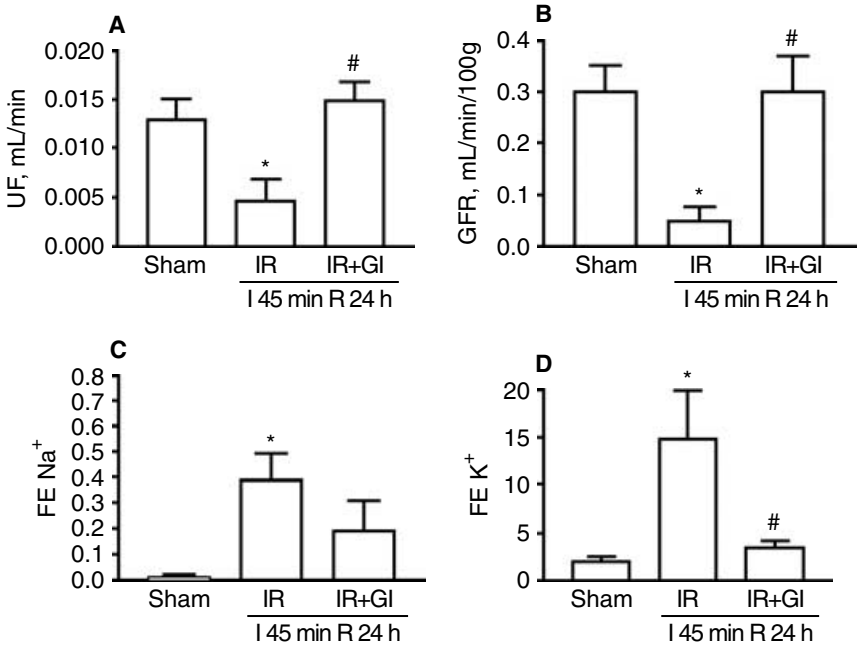

Fig. 5. Effect of glibenclamide (GI) on urinary volume (UF) (*A*), glomerular filtration rate (GFR) (*B*), fractional excretion of Na^+ ($FENa^+$) (*C*) and fractional excretion of K^+ ($FE\ K^+$) (*D*). Glibenclamide (20 mg/kg) was administered subcutaneously as described in the **Methods** section. Results are shown as mean ± SEM of seven animals. $^*P < 0.05$ when compared to sham-operated animals; $\#P < 0.05$ when compared to ischemia/reperfusion (I/R) animals.

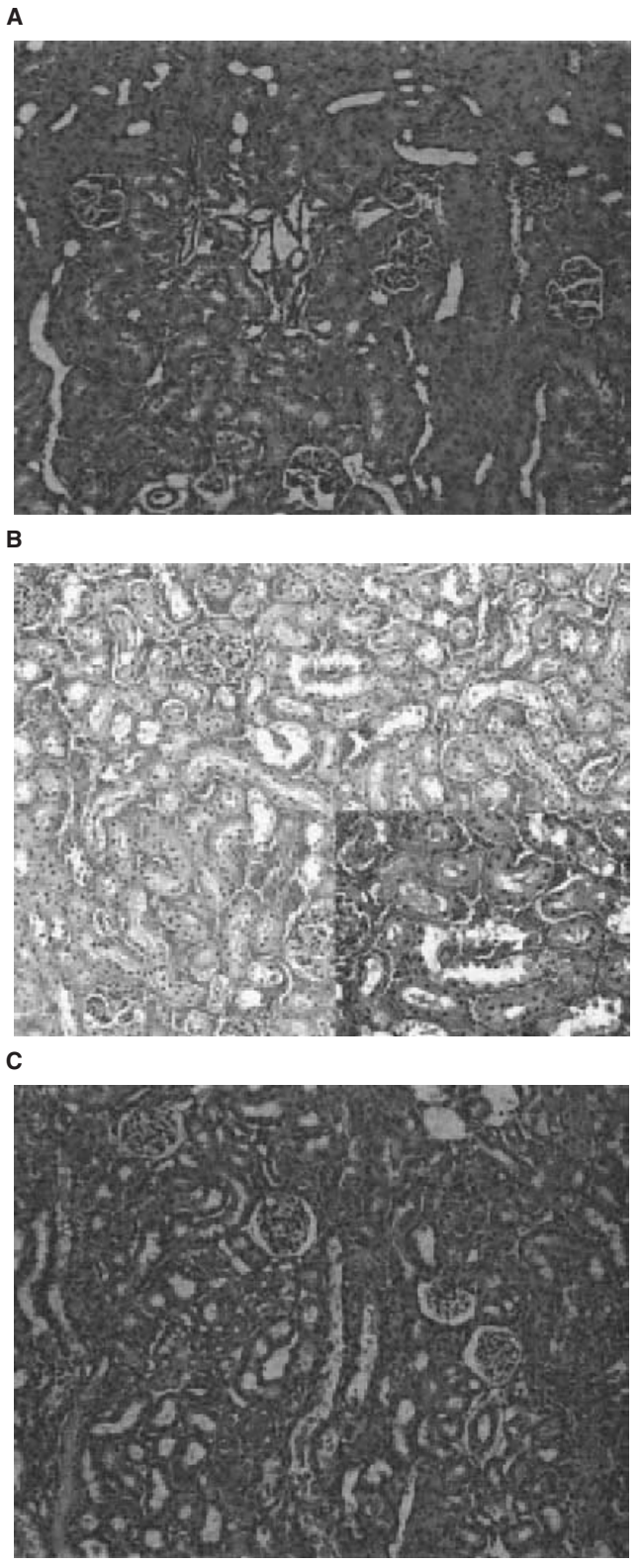

Fig. 6. Kidney damage following ischemia (I) (45 minutes) and reperfusion (R) (4 hours) of renal vascular pedicle. Rats were sham-operated (*A*), or submitted to ischemia and reperfusion (I/R) injury in the presence of vehicle (*B*) or glibenclamide (*C*). Glibenclamide (20 mg/kg) was administered subcutaneously 40 min prior to reperfusion as described in the **Methods** section. Kidneys were fixed and processed for histologic analysis after hemoxytolin and eoasin staining [×20 in (A, B, and C) and × 60 in the insert].

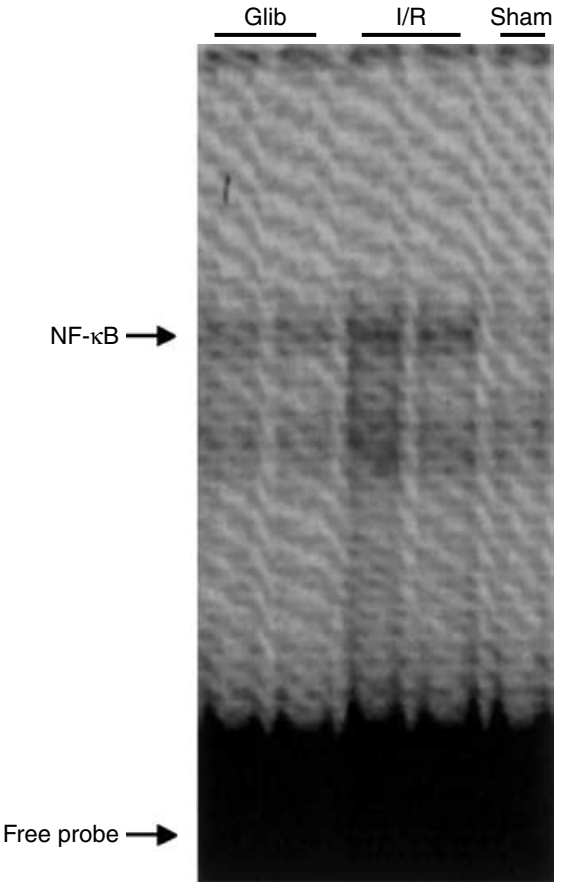

Fig. 7. Translocation of the transcription factor nuclear factor-κB (NF-κB) to the nucleus in the kidney during ischemia and reperfusion (I/R). Kidneys of sham-operated rats, or submitted to I/R injury in the presence of vehicle or glibenclamide (Glib) were analyzed. Glibenclamide (20 mg/kg) was administered subcutaneously 40 minutes prior to reperfusion as described in the **Methods** section. NF-κB was determined by electrophoretic mobility shift assays (EMSA) of kidney nuclear extracts.

with the related oligonucleotide (NF-κB) but was not affected by molar excess of unrelated oligonucleotide [activating protein-1 (AP-1)] confirming the specificity of the interaction (data not shown).

Effects of the treatment with diazoxide on the I/R injury

Next, we investigated the effects of the ATP-sensitive potassium channel opener diazoxide in the model of renal I/R injury. Treatment with diazoxide had no significant effect on the reperfusion-induced changes in vascular permeability in kidney (sham-operated 5 ± 0.6 Evans blue dye μg/100 mg of kidney; I/R 14 ± 1.5 μg; and I/R and diazoxide 12 ± 0.4 μg) ($N = 4$) or lungs (sham-operated 8 ± 0.9 μg Evans blue dye/100 mg of lung and I/R 29 ± 0.9 μg and 26 ± 8 μg) ($N = 4$). Similarly, diazoxide failed to affect the reperfusion-induced increase in the concentration of cytokines in kidney and lungs (data not shown). Nevertheless, the drug induced a further increase in the accumulation of neutrophils in the lungs (sham-operated

2 ± 0.3 neutrophils $\times 10^6$/100 mg of lung; I/R 10 ± 1.9 neutrophils; and I/R and diazoxide 19 ± 1 neutrophils) ($N = 4$) but not kidneys (sham-operated 0.03 ± 0.009 neutrophils $\times 10^6$/100 mg of kidney; I/R 0.2 ± 0.4 neutrophils; and I/R and diazoxide, 0.2 ± 0.1 neutrophils) ($N = 4$) of reperfused animals. As expected by its potent vasodilatory effects, administration of diazoxide induced a 50% fall in MAP shortly after its administration and persisted at low levels to the end of experiment (data not shown).

DISCUSSION

Reperfusion of an ischemic vascular bed is accompanied by local inflammatory injury that limits the potential benefits of blood flow restoration. For example, renal ischemia followed by reperfusion results in severe injury that may contribute to renal damage after organ transplantation [23]. Thus, strategies that limit reperfusion-associated renal damage may be useful during renal transplantation. In addition, to renal damage, it is clear that extensive I/R, such as that of the renal or intestinal vascular beds, may be accompanied by remote (lung) and systemic inflammation [9]. The latter may complicate the reperfusion syndrome and contribute to overall lethality. In this study, we evaluated whether the treatment with glibenclamide prevented renal I/R inflammatory injury and whether inhibition of inflammatory injury was accompanied by amelioration of renal dysfunction in rats.

Initial experiments were designed to investigate the kinetics of the reperfusion-induced increase in vascular permeability and neutrophil accumulation after ischemia of the renal vascular pedicle for 45 minutes. The time of ischemia employed here was based on previous studies demonstrating it was optimal to cause injury [24, 25]. Our results demonstrate that after 4 hours of renal reperfusion there was a marked neutrophil accumulation and increased vascular permeability in the kidney. In addition to causing local injury, 4 hours reperfusion of the kidney was accompanied by accumulation of neutrophils and increased vascular permeability in the lung, an organ remote from the site of ischemia. These effects were still present at 24 hours after reperfusion. In the lung, the neutrophil accumulation was also detected during the ischemic period, possibly reflecting the inflammation in response to the operation procedure necessary to remove the contralateral kidney. The presence of neutrophils and increased vascular permeability in local and remote organs has also been observed after different models of I/R [26–28].

We recently showed that the treatment of rats with glibenclamide dose-dependently decreased the exudation and neutrophil influx into the pleural cavity induced by carrageenan, N-formyl-methionyl-leucyl-phenylalanine and lipopolysaccharide [16]. Glibenclamide has been widely used as a K_{ATP} channel blocker [16, 29, 30], although it may exert effects unrelated to K_{ATP} channel blockage, such as thromboxane A_2 receptor antagonism [31] and Na^+/K^+-ATPase inhibition [32]. Although it cannot be ruled out that these other effects may be relevant for the ability of glibenclamide to prevent neutrophil migration, our previous study favored a more relevant effect of glibenclamide on potassium channels. Thus, both glibenclamide and a nonspecific K_{ATP} channel blocker prevented neutrophil influx, whereas minoxidil, a K_{ATP} channel opener, worsened the inflammatory response [16]. Treatment with glibenclamide, at a dose (20 mg/kg) similar to that previously shown to decrease the exudation and neutrophil influx into the pleural cavity [16], significantly inhibited reperfusion-induced neutrophil accumulation and increased vascular permeability in the kidney. Glibenclamide also inhibited the increase in vascular permeability in the lung, whereas the neutrophil accumulation in this remote organ was unaffected by glibenclamide. This is consistent with the results showing that increased neutrophil accumulation in the lung occurs during ischemia (see Fig. 1) and with the fact that the drug was administered after the onset of ischemia. Thus, it seems that both increase in vascular permeability and neutrophil infiltration consequent to 4 or 24 hours of reperfusion depends on the activation of K_{ATP} channels. TEA, a nonselective potassium channel blocker, was also effective to inhibit the increase in vascular permeability in both kidney and lungs. Surprisingly, this drug had no effect on neutrophil accumulation. One possibility for the absence of effect of TEA is the limited dose employed here since doses of TEA greater than 40 mg/kg were not tolerated by the animals and, thus, could not be used (data not shown). Another explanation would be that inhibition of several potassium channel subtypes may confound the results, as some subtypes may be pro- and other subtypes may be anti-inflammatory. Overall, the present results are consistent with the notion [16] that inhibition of K_{ATP} channels by glibenclamide is the mechanism by which the drug prevents renal I/R injury.

It has been shown that TNF-α is a proinflammatory cytokine capable of up-regulating its own expression and that of other genes important for the inflammatory response [33]. There was an increase in TNF-α concentrations in both kidney and lungs at 4 and 24 hours after reperfusion of the ischemic kidney. These results are in agreement with other studies showing the expression and role for TNF-α in reperfusion-induced tissue injury [9, 34–36]. Treatment with glibenclamide significantly inhibited the reperfusion-induced increase in TNF-α levels in the kidney and lungs. As TNF-α may have a role in the recruitment and activation of neutrophils in sites of I/R injury [34], the ability of glibenclamide to inhibit TNF-α production may account for its ability to prevent reperfusion-induced neutrophil accumulation and organ injury. On the other hand, we have previously shown that

the accumulation of neutrophils is a prerequisite for the production of TNF-α following intestinal I/R injury [9]. Whether the actions of glibenclamide are predominant on TNF-α production or neutrophil influx remains to be determined. Nevertheless, our studies showing that neutrophil migration may be directly affected by glibenclamide suggest that neutrophils are a major cellular target for the action of the drug.

In addition to TNF-α, previous studies have shown that the concentration of IL-1β is elevated and may have a pathophysiologic role following I/R injury [37]. In IL-1 receptor knockout or in wild-type mice treated with IL-1 receptor antagonist, the infiltration of polymorphonuclear leukocytes after renal I/R was attenuated compared to control mice [38]. In our model, renal I/R induced an increase in IL-1β levels in the kidney at 4 hours after reperfusion, whereas there was no increase of the cytokine in the lung. Treatment with glibenclamide had no significant effects on the reperfusion-induced increase of IL-1β in the kidney, suggesting that an effect on IL-1β production does not underlie the inhibitory effects of glibenclamide in our system.

Since IL-10 may be induced in reperfused tissue and may modulate reaction to injury [39], we measured the concentrations of this cytokine following renal I/R injury and observed a significant elevation of IL-10 concentrations in the kidney. Glibenclamide did not enhance or inhibit the reperfusion-induced elevation in IL-10 concentrations. Thus, it is unlikely that an effect on IL-10 expression explains the modulatory effects of glibenclamide in our system. Interestingly, whereas glibenclamide inhibited TNF-α production, this drug did not affect IL-10 production, suggesting that TNF-α is not an important inducer of IL-10 in the system. Nevertheless, there was a good correlation between the induction of IL-10 and IL-1β in the kidney after I/R. We have previously demonstrated that IL-1β is a major inducer of IL-10 during severe intestinal I/R injury [40]. Whether IL-1β participates in the cascade of events leading to IL-10 production during renal I/R injury is not yet clarified.

NF-κB is a nuclear transcription factor that may control the expression of proinflammatory mediators, including TNF-α [41]. In addition, proinflammatory mediators, such as TNF-α, function by activating NF-κB in various cell types. It is likely that these two systems amplify each other in complex inflammatory syndromes, such as after I/R. Here, we observed that the reperfusion-induced NF-κB expression in the kidney was inhibited by glibenclamide. The latter result is consistent with the ability of glibenclamide to prevent the production of TNF-α following renal I/R. Thus, the prevention of NF-κB translocation is part of the anti-inflammatory effects of glibenclamide in the system.

In addition to the inflammatory injury in the reperfused kidney, we also observed a marked renal dysfunction as assessed by a decrease in the GFR and urinary flow. Glibenclamide, in parallel to its anti-inflammatory effects, reversed the decrease in these renal parameters. Thus, our results are indicative that the anti-inflammatory effects of glibenclamide may improve the renal dysfunction consequent to renal I/R injury.

K_{ATP} channels are in various cell types other than leukocytes. In the kidney, they are present in cells of proximal tubule, thick ascending limb of Henle's loop, and cortical collecting duct [42]. It has been shown that a fall in intracellular ATP concentration induces the opening of K_{ATP} channels [43]. In the present study, we observed an increase in the fractional excretion of K^+ at 24 hours of reperfusion. Since glibenclamide inhibited the increase in the fractional excretion of K^+, it was reasonable to suggest that K_{ATP} channels in the kidney might be activated during renal ischemia which, in turn, would increase the renal excretion of K^+. However, as there was no change in the absolute renal K^+ excretion (data not shown), the observed changes in fractional K^+ excretion may reflect the changes in GFR rather than in K^+ secretion. Fractional excretion of Na^+ was also increased at 24 hours of reperfusion of ischemic kidney but was not affected by glibenclamide. Since the increase in the excretion of Na^+ was not accompanied by an increase in the GFR, it may be that renal ischemia followed by 24 hours reperfusion directly affected the tubular reabsorption of Na^+, probably without involvement of K_{ATP} channels.

Consistent with the expression of K_{ATP} on renal cells, a protective effect of glibenclamide has also been observed in experiments evaluating hypoxic renal injury ex vivo [29, 43]. However, at least one study suggests that glibenclamide enhanced renal I/R injury in isolated perfused rat kidney [30]. The reasons underlying the discrepancies of these studies are not clear but could be related to the different roles of K_{ATP} in various cell types in the kidney [42]. Moreover, none of the studies have used neutrophils or other leukocytes in their reperfusion system and neutrophils are thought to be major players of reperfusion injury [6–9]. Thus, it appears that in the in vivo system a role for glibenclamide on K_{ATP} of neutrophils may be more relevant for the ability of the drug to prevent inflammatory injury and ameliorate renal dysfunction.

Insulin has been shown to have potential anti-inflammatory activity by inhibiting the expression of the proinflammatory intercellular adhesion molecule-1 [44]. Glibenclamide is widely used as a hypoglycemiant because of its ability to increase pancreas insulin secretion by blockade of pancreatic K_{ATP} channels [45]. We measured blood glucose levels at 4 and 24 hours of renal reperfusion. Although levels of glucose were high in rats submitted to renal I/R, these values were similar between the groups. High levels of glucose may be due to the surgical stress (nephrectomy) carried out in all groups. Similar values of blood glucose have been

observed in rats submitted to nephrectomy [46]. After 24 hours of reperfusion, glibenclamide-treated animals significantly decreased blood glucose levels to those seen in nonmanipulated animals. It is unlikely that these glucose-normalizing effects of glibenclamide may have a role in our system but suggest the dose was sufficient for an effective blockade of K_{ATP} channels in the present study.

Although diazoxide induced a further increase in the accumulation of neutrophils in the lungs, this drug failed to affect the reperfusion-induced changes in vascular permeability and reperfusion-induced increase in the concentration of cytokines in kidney and lungs. One possibility is that the hypotensive effects of diazoxide may prevent the adequate development of an inflammatory response in the reperfused kidneys. Alternatively, a more likely explanation is that the absence of an effect of diazoxide is that the model of renal I/R used here was designed to produce a maximal inflammatory response. Future studies in milder models of renal reperfusion injury will help evaluating the latter possibility.

CONCLUSION

We demonstrated that glibenclamide is very effective at inhibiting the local injury following I/R of renal vascular pedicle in rats. Inhibition of injury was accompanied by amelioration of renal dysfunction. Altogether, our experiments are in agreement with other studies showing a role of K_{ATP} channels for neutrophil recruitment and suggest that glibenclamide could have a therapeutic role in the treatment of renal I/R injury, such as after renal transplantation.

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