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Renal protection by delayed ischaemic preconditioning is associated with inhibition of the inflammatory response and NF-κB activation

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Brief and sublethal ischaemia renders an organ tolerant to subsequent prolonged ischaemia, which is called ischaemic preconditioning (IPC). In regard to the beneficial effects and endogenous mechanisms of renal delayed IPC, few data are available. In this study, we aim at determining reno-protective effects of delayed IPC against ischaemia-reperfusion (I/R) injury, and illustrating whether these effects are associated with suppressing inflammation and nuclear factor-κB (NF-κB) activation. I/R injury was induced by clamping both renal pedicles for 40 min, followed by 24 h of reperfusion. The rats were subjected to ischaemia for 20 min (preconditioning) or sham surgery (non- preconditioning) at day 4 before I/R. Functional and morphological parameters were evaluated at 24 h after reperfusion. At the same time, macrophage (ED-1$^+$) infiltration, and the expression of intercellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor-α (TNF-α) were assessed by immunohistochemistry. Moreover, IκB-α degradation and NF-κB/DNA binding activity were analyzed. Compared with rats exposed to I/R injury, preconditioned rats had a significant decrease in levels of serum creatinine (Scr, 384.3 ± 21.8 μmol/L vs. 52.5 ± 21.7 μmol/L; $p < 0.001$), blood urea nitrogen (BUN, 40.4 ± 2.7 mmol/L vs. 15.9 ± 4.2 mmol/L; $p < 0.001$) and serum aspartate aminotransferase (AST, 486.7 ± 58.6 IU/L vs. 267.3 ± 43.9 IU/L; $p < 0.001$). Parallel to the above changes, preconditioned rats preserved structural integrity and decreased tubulointerstitial damage scores (3.4 ± 0.3 vs. 0.2 ± 0.05; $p < 0.001$) and ED-1$^+$ cell infiltration (25.3 ± 3.5 vs. 6.2 ± 1.2 cells/HPF, $p < 0.01$). Furthermore, our results showed that the expression of ICAM-1 and TNF-α, the degree of IκB-α degradation, and NF-κB/DNA binding activity were reduced by IPC. Taken together, our results demonstrated that delayed IPC offered both functional and histological protection, which may be related to suppression of inflammation in preconditioned kidneys. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS — kidney; ischaemic preconditioning; ischaemia-reperfusion; inflammation; nuclear factor-κB

INTRODUCTION

The kidney is vulnerable to the deleterious effects of renal ischaemia. Ischaemia-reperfusion (I/R) injury is the major cause of acute renal failure (ARF) in the native as well as in the transplanted kidney.[1–3] At present there is no specific treatment for this devastating clinical syndrome with high morbidity and mortality.

However, recent data from us and other investigators indicate that I/R injury may be avoidable by inducing ischaemic tolerance in various organs.[4–7] Brief and sublethal prior ischaemia renders an organ more tolerant to subsequent prolonged I/R injury, and this phenomenon is called 'ischaemic preconditioning' (IPC). There are two temporally and mechanistically distinct types of protection afforded by IPC,[4] acute and delayed IPC. The protective effects of acute preconditioning are protein synthesis-independent, mediated by post-translational protein modifications, and short-lived. Appearing about 24 h after the preconditioning ischaemia, the beneficial effects of delayed IPC require new protein synthesis and are

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sustained for days to weeks. Because of its promising and sustained effects against I/R injury, delayed IPC has been the topic of intense research interest in other organs such as cardiac muscle.[8,9] Conversely, there is a limited amount of information in the literature regarding renal delayed IPC,[6] and its endogenous protective mechanisms have not been adequately evaluated in the kidney.

Renal injury after ischaemia seems to be a consequence of tissue hypoxia from blood flow deficiency but also from the process of reperfusion, leading to an active inflammatory response. Inflammation is a significant component of renal I/R injury, playing a considerable role in its pathogenesis.[10,11] Nuclear factor-κB (NF-κB) is thought to play a central role in the regulation of inflammatory mediators.[12] In a previously reported study,[13] we demonstrated that NF-κB was activated following renal I/R injury, accompanied by macrophage infiltration and increased expression of several inflammatory molecules which are regulated by NF-κB, and that inhibition of NF-κB activation using NF-κB decoy strategy documented a strong renoprotective effect. Therefore, we hypothesize that renal protection by delayed IPC may be partially mediated by inhibiting inflammatory responses and NF-κB activation. A better understanding of the protective mechanisms underlying renal delayed IPC will allow promising therapies to be identified.

Thus, the major aims of this study were (1) to assess deleterious effects including renal dysfunction and morphological damage following I/R injury in rats, (2) to assess the degree of inflammatory responses, evaluated by macrophage infiltration and the expression of inflammatory markers, and the activity of NF-κB in this I/R model, (3) to determine whether delayed IPC can be induced in the kidney, and if so, (4) to demonstrate whether renoprotective effect of delayed IPC is associated with reducing inflammation and inhibiting NF-κB activity.

MATERIALS AND METHODS

Animals and surgical procedure

All experiments were performed with male Sprague-Dawley rats (250–280 g) from the Animal Centre, Shanghai Medical College, Fudan University (Shanghai, China). Rats were allowed free access to water and rodent food. All protocols were approved by the Institutional Animal Care Use Committee of Fudan University. Rats were randomly divided into three groups, including sham-operation (Sham, $n = 6$),

40 min of bilateral ischaemia (I/R, $n = 8$) and 20 min of bilateral ischaemia + 40 min of bilateral ischaemia (IPC, $n = 8$). The interval between the two surgeries was 4 days. Rats were anaesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and placed on a temperature-regulated table to maintain body temperature at 36–38°C. After a 10-min stabilization period, a midline laparotomy was performed. Bilateral ischaemia was induced by occluding both renal pedicles with non-traumatic vascular clamps for indicated times. The clamps were then removed, the kidney was observed for return of blood flow, and the incision was sutured. The sham-operated rats underwent the same surgical procedure, except that the clamp was not applied. The kidneys were harvested at 24 h after the second surgery. Blood was obtained via puncture of the inferior vena cava for measurement of serum creatinine (Scr) and blood urea nitrogen (BUN). Aspartate aminotransferase (AST), which is located in proximal tubules (PT) and released during reperfusion, was measured and used as an indicator of reperfusion injury.[14]

Histological examinations

For histological preparation, kidney slices from all experimental groups were fixed in 10% formalin solution overnight. After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin. Sections were examined by a renal pathologist, who was blind to the treatment groups. Abnormalities were graded using a semi-quantitative scale from 0 to 4,[15] according to tubular cell necrosis, tubular dilation, cellular or proteinaceous casts, interstitial oedema and interstitial leukocyte infiltration. Higher scores represented more severe damage: 0, normal kidney; 1, <25% involvement of the cortex or outer medulla; 2, 25–50% involvement of the cortex or outer medulla; 3, 50–75% involvement of the cortex or outer medulla; and 4, >75% involvement of the cortex or outer medulla.

Immunohistochemistry

The immunostaining was processed in 5 μm paraffinized sections. Briefly, after deparaffinization, endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min at room temperature. Sections were then subjected to microwave irradiation in citrate buffer to enhance antigen retrieval. Non-specific adsorption was minimized by incubating sections in

2% (v/v) normal goat serum in PBS for 20 min. Sections were then incubated with the primary antibodies against ED-1 (mouse anti-rat) (Chemicon International, Inc., Temecula, CA, USA), intercellular adhesion molecule-1 (ICAM-1) or tumor necrosis factor-α (TNF-α) (goat anti-rat) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Negative controls included buffer alone or equivalent concentrations of an irrelevant murine monoclonal antibody of normal IgG. Specific labelling was detected with appropriate biotin-conjugated secondary antibodies and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA). The color reaction was developed with 3,3-diaminobenzidine (Sigma) and sections were counterstained with hematoxylin. Evaluation of all sections was performed by an experienced renal pathologist, who was unaware of the origin of the sections. ED-1$^+$ cells were quantitatively measured by counting 20 randomly selected high-power fields (HPF; 400×) per section in the cortex and outer medulla areas.

Western blot analysis

The expression levels of IκB-α were examined by Western blotting. Kidney tissue was homogenized in lysis buffer that contained 50 mM Tris pH 7.4, 0.1% NP-40, 150 mM NaCl, 1 mM EDTA, 50 μg/ml PMSF, 1 μg/ml pepstatin, 2 μg/ml leupeptin and 2 μg/ml aprotinin. Samples were centrifuged at 14 000g for 15 min at 4°C, and the supernatant was collected and saved for assay. Protein samples (50 μg/Lane) were separated on 10% SDS–PAGE gels and then transferred to polyvinylidine difluoride membranes. Membranes were blocked with 5% non-fat milk and incubated with primary antibodies overnight at 4°C. Primary antibodies employed here were polyclonal rabbit antibodies against IκB-α (1:700 dilution) (Santa Cruz Biotechnology). After washing, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies. The same membranes were stripped and reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:5000) (Kangchen Biotechnology, China) to confirm equal loading. The proteins were detected using the enhanced chemiluminescence (ECL) system, which was captured on X-ray film. The expression levels were presented as ratios to the level of GAPDH expression.

Electrophoretic mobility shift assay (EMSA)

NF-κB/DNA binding activity was measured by an EMSA. Nuclear protein extracts were prepared from whole kidney tissue as described. Preparation for EMSA was performed with a commercial kit following the instructions of the manufacturer (Promega). Briefly, a double-stranded 22-mer oligonucleotide with the sequence 5 [prime]-AGT TGA GGG GAC TTT CCC AGG C-3 [prime] was end-labelled using [gamma-(^{32}) P]ATP and T4 polynucleotide kinase according to the manufacturer's protocol. This oligonucleotide has the consensus sequence for NF-κ B-binding, as indicated by underlines. Nuclear extracts (10 μg) were incubated in binding reaction medium with 0.5 ng of ^{32}P-end-labelled oligonucleotide for 30 min at room temperature. The DNA-protein complexes were analyzed on 5% polyacrylamide gels and autoradiographed. In competition assays, 50 ng of unlabelled NF-κB oligonucleotide was preincubated with the nuclear extracts before the addition of the labelled probe.

Statistical analysis

Results were presented as the mean ± SEM. Differences among three or more groups were evaluated using one-way ANOVA, followed by Dunnett test. The Kruskal-Wallis ANOVA on ranks was used for non-normally distributed data. $p < 0.05$ was considered statistically significant. Each animal group consisted of more than four rats.

RESULTS

Protective effects of IPC on renal function and morphology

During the 8-day follow-up (Figure 1), 20 min preconditioning ischaemia led to a slight but significant increase in levels of Scr and BUN at 1 day after ischaemia (Scr, 73.6 ± 16.7 μmol/L; BUN, 14.5 ± 4.0 mmol/L), which had declined to normal levels by day 4 post-ischaemia (Scr, 26.9 ± 2.5 μmol/L; BUN, 5.0 ± 1.3 mmol/L). The result indicates that the renal injury induced by 20 min ischaemia is mild, and can be soon reversed.

Animals that underwent renal I/R showed marked impairment of renal functional parameters with significant increase in both Scr and BUN levels, compared with basal levels from sham-operated rats (Figure 2A,B). In comparison with I/R, pretreatment with 20 min ischaemia produced a remarkable functional protection, as shown in the Scr (384.3 ± 21.8 μmol/L vs. 52.5 ± 21.7 μmol/L; $p < 0.001$) and BUN levels (40.4 ± 2.7 mmol/L vs.
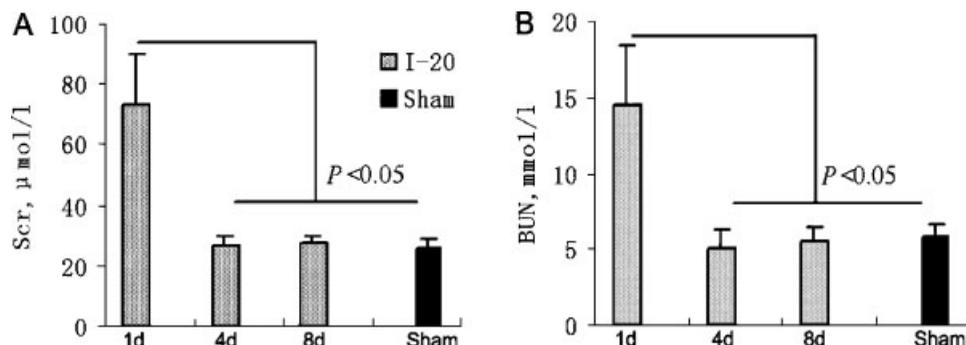
Figure 1.   Change in levels of Scr and BUN following 20 min preconditioning ischaemia (I-20). During the 8-day follow-up, there was a slight but significant increase in concentrations of Scr and BUN at day 1 post-ischaemia. Values are expressed as mean $\pm$ SEM ($n = 6$ per group)

15.9 ± 4.2 mmol/L; $p < 0.001$) at 24 h after reperfusion. Since AST is present within the PT and is regarded as a non-specific marker of extensive cellular disruption or necrosis, we used serum AST as a marker of reperfusion injury (Figure 2C). In comparison with values obtained from sham-operated rats, renal I/R produced an increase in serum AST levels, suggesting a significant reperfusion injury (283.9 ± 43.5 IU/L vs. 486.7 ± 58.6 IU/L; $p < 0.001$). In contrast, rats with ischaemic pretreatment had a marked reduction in serum AST, almost identical to that of sham-operated rats (267.3 ± 43.9 IU/L vs. 283.9 ± 43.5 IU/L; $p > 0.05$).

In rats subjected to I/R alone, parallel to the deterioration of functional parameters, histological examination revealed characteristic acute tubulointerstitial damage, including massive tubular epithelial cell necrosis, naked basement membranes, tubular dilation with proteinaceous or cellular casts, interstitial oedema and inflammatory cell infiltration. Morphological damage was most prominent in the outer medullary stripe, but also with patchy involvement of the cortical proximal segments. In addition, blood congestion in the outer zone of the inner stripe of the medulla was observed. In contrast, renal morphology of preconditioned rats was near normal, with mild degree of brush border loss and a few necrotic cells, which paralleled the improvement of renal function (Figure 3A). The Jablonski scale histology grading scores are shown in Figure 3B. When compared with rats that underwent I/R, preconditioned rats with 20 min renal ischaemia
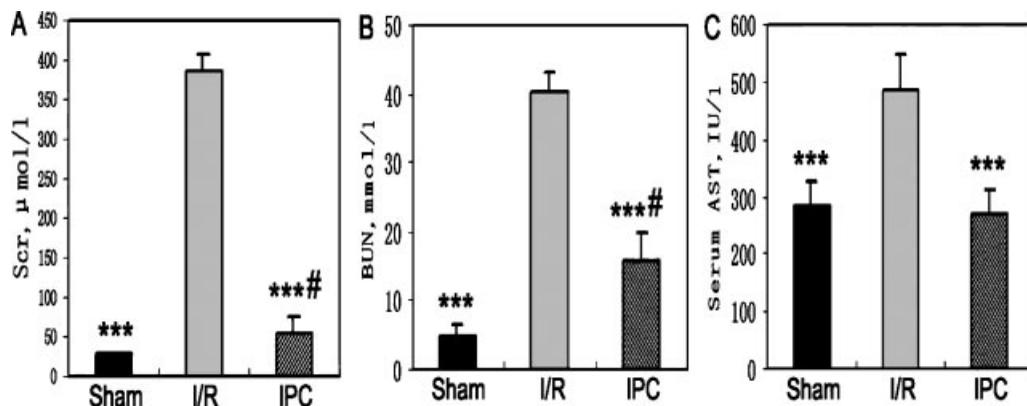

Figure 2.   Effect of IPC on renal function. Renal function was evaluated by levels of Scr (A), BUN (B) and serum AST (C). IPC significantly decreased the elevation of Scr, BUN and AST, caused by 40 min renal bilateral ischaemia and 24 h of reperfusion (I/R). Values are expressed as mean $\pm$ SEM ($n = 6$–8 per group). ***$p < 0.001$ versus I/R group, #$p < 0.05$ versus sham-operated group (Sham)
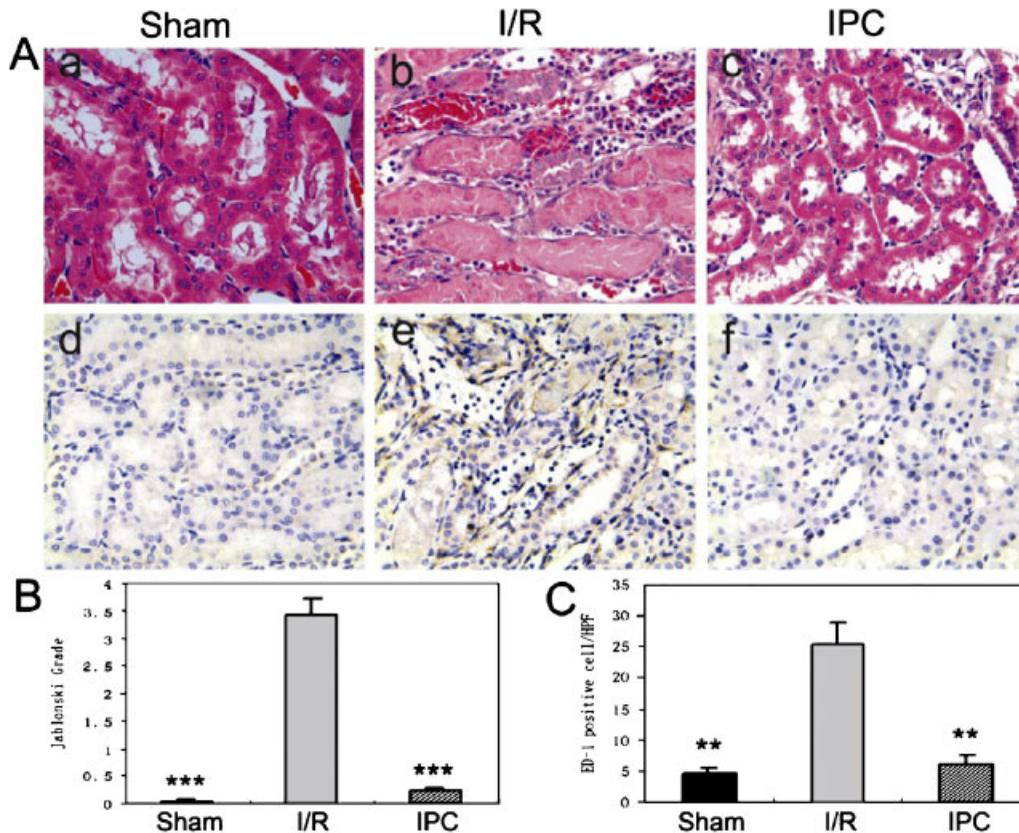
Figure 3. Effect of IPC on renal morphology and macrophage infiltration. The degree of acute tubulointerstitial damage was assessed by hematoxylin-eosin staining (a–c) and the Jablonski scale histology grading scores (B). Immunostaining for ED-1 (d–f) was performed, and infiltrating macrophage (ED-1$^+$) cells (C) were counted at 24 h following reperfusion. In contrast to I/R injury, ischaemic preconditiong (IPC) protected the integrity of renal internal architecture, and reduced macrophage infiltration. Magnification, 400×. Data represent mean ± SEM ($n = 6$–8 per group). $^{***}p < 0.001$, $^{**}p < 0.01$ versus I/R group

demonstrated significantly better scores in tubulointerstitial morphology (3.4 ± 0.3 vs. 0.2 ± 0.05; $p < 0.001$).

Effect of IPC on macrophage infiltration and the expression of ICAM-1 and TNF-α

ED-1$^+$ macrophages represent the predominant infiltrating cells during I/R injury,[16] and the efficacy of IPC in macrophage recruitment was studied by immunohistochemistry (Figure 3A). In sham-operated kidneys, a small number of ED-1$^+$ was present in peritubular and intraglomerular spaces (4.7 ± 0.9 cells/HPF). In rats subjected to I/R injury, ED-1$^+$ cells markedly accumulated into the tubulointerstitium, and increased to 25.3 ± 3.5 cells/HPF at 24 h post-ischaemia. Ischaemic pretreatments significantly ($p < 0.01$) decreased the number of macro-

phages to 6.2 ± 1.2 cells/HPF in preconditioned kidneys (Figure 3C).

At 24 h post-ischaemia, representative photomicrographs of immunostaining for ICAM-1 and TNF-α are illustrated in Figure 4. Sham-operated kidneys rarely stained for ICAM-1 and TNF-α. Kidneys with I/R injury showed a marked staining for ICAM-1 and TNF-α in the tubulointerstitium. The immunostaining was reduced significantly in kidneys with ischaemic pretreatment. Our results indicate delayed IPC downregulates the renal expression of inflammatory markers such as ICAM-1 and TNF-α.

Effect of IPC on NF-κB activation

In its inactive state, NF-κB is found in the cytoplasm bound to IκB-α, which prevents it from entering the nucleus.[17,18] Activation of NF-κB is preceded by the
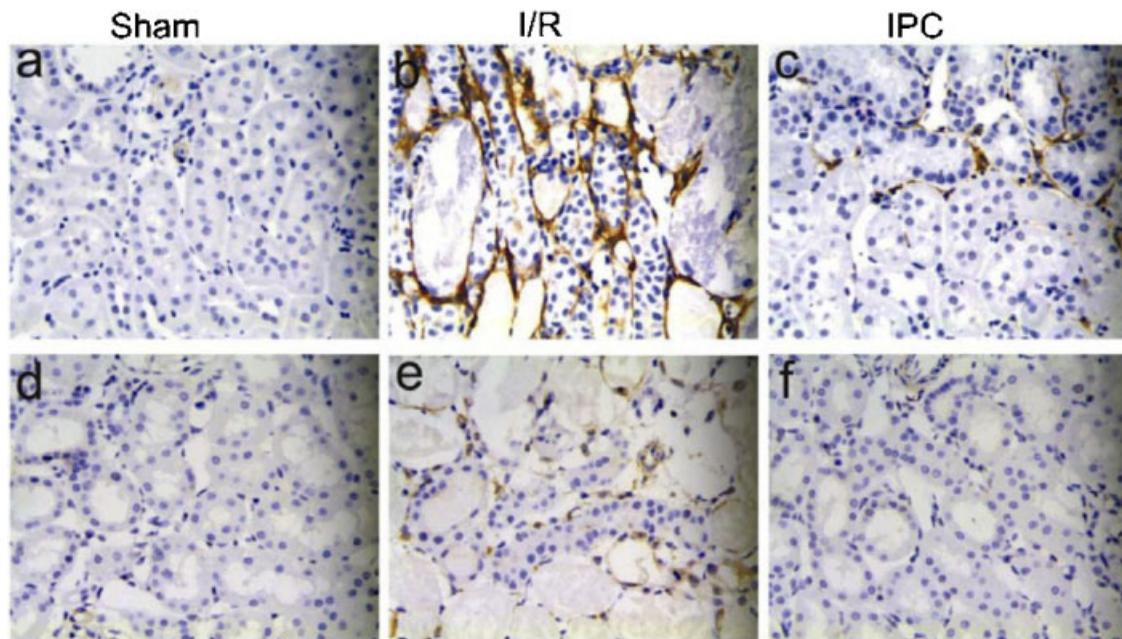
Figure 4. Immunostaining for ICAM-1 and TNF-α. The expression of ICAM-1 (a–c) and TNF-α (d–f) were faint in kidneys of sham-operated rats (Sham) and preconditioned rats (IPC). Their staining was much higher in kidneys subjected to I/R injury. Illustrations are representative of at least three experiments performed on different days. Magnification, 400×

phosphorylation, ubiquitination and proteolytic degradation of IκB-α. Therefore, we examined the effect of IPC on I/R-induced IκB-α degradation, by Western blot analysis using antibody against IκB-α (Figure 5A,B). I/R injury led to increased degradation of IκB-α. This process was almost reversed by IPC.

Release of NF-κB from IκB-α results in translocation of NF-κB to the nucleus, where it binds to specific sequences in the promoter regions of target genes. We next determined the effect of IPC on DNA binding ability of NF-KB (Figure 5C,D). I/R-induced NF-κB binding to target oligonucleotides was almost completely blocked by administration of IPC ($159.3 \pm 11.8\%$ vs. $110.2 \pm 8.8\%$; $p < 0.05$). Collectively, these results suggest that IPC suppresses I/R-induced NF-κB activation by reducing IκB-α degradation.

DISCUSSION

Preconditioning is a simple and harmless method used to render an organ tolerant to ischaemia.[19] Our findings in this study conclusively demonstrate that delayed IPC occurs in the kidney. Ischaemic pretreatment for 20 min significantly attenuated internal structural disruption, renal dysfunction

(increase in Scr and urea nitrogen), and reperfusion injury (increase in serum AST) caused by 40 min of bilateral renal ischaemia and 24 h of reperfusion. The above renoprotective effects were associated with reduced macrophage (ED-1^{+}) infiltration, downregulated expression of ICAM-1 and TNF-α and inhibition of NF-κB.

A previous study in mice has suggested that IPC is a dose-dependent phenomenon.[20] Therefore, in preliminary studies we tried to increase the protection of IPC by increasing the preconditioning time (10, 15 and 20 min, respectively, data not shown). It was found that 20 min was the optimal time of preconditioning ischaemia, which preserved both architectural integrity and renal function. During the 8-day follow up, we found there was a slight but significant increase in levels of Scr and BUN at 1 day after 20 min ischaemia. This finding indicates that 20 min ischaemia induces mild change in renal function and structure, but the change can be reversed completely due to endogenous regenerative systems. To allow the kidney to recover sufficiently from 20 min ischaemia, a 4-day interval between two ischaemic challenges was chosen.

Renal I/R injury caused severe architectural disruption, including massive tubular necrosis, luminal obstruction from debris, and loss of basement
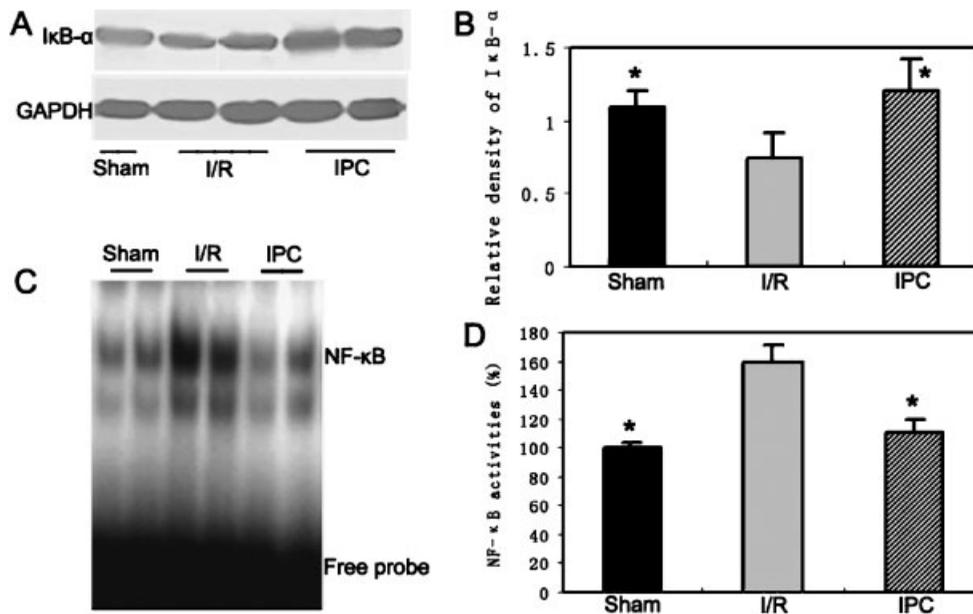
Figure 5.   Effect of IPC on NF-κB activation. Western blot analysis for IκB-α (A, B) and EMSA for NF-κB/DNA binding activity (C, D). While enhanced after I/R injury, ischaemic pretreatment (IPC) inhibited IκB-α degradation and NF-κB/DNA binding activity. Data represent mean $\pm$ SEM (n = 4–6 per group). $^*p < 0.05$ versus I/R group

membrane integrity, which led to subsequent renal dysfunction. Recent studies have brought evidence that macrophages as well as neutrophils are important leukocyte mediators of renal I/R injury.[16,21,22] Once present at the tubulointerstitium, macrophages interact with resident cells and extracellular matrix to generate a proinflammatory microenvironment that amplifies tissue injury and promotes scarring. In this study, macrophage infiltration in I/R group was accompanied by significant up-regulation of the adhesion molecule, ICAM-1. Furthermore, ICAM-1 became massively distributed along injured tubules and the interstitium, which corresponded with the localization of ED-1$^+$ cells in kidneys of the I/R group. ICAM-1 is critical for the recruitment of leukocytes to sites of inflammation. The role of ICAM-1 in renal injury was demonstrated by experiments in which the administration of ICAM-1 antibody was found to be protective and the kidneys of ICAM-1 knockout mice were protected against ischaemic injury.[23,24] Moreover, TNF-α is a key element in a network of proinflammatory cytokines and chemokines activated in the kidney following I/R.[25,26] There was an increase of TNF-α immunostaining in kidneys of the I/R group at 24 h after reperfusion. The result is in agreement with other studies showing the expression and role for

TNF-α in reperfusion-induced tissue injury.[27] Delayed IPC in the kidney not only decreased the infiltration of ED-1$^+$ cells, but also reduced the expression of ICAM-1 and TNF-α.

NF-κB is thought to play a central role in the regulation of inflammatory mediators, including ICAM-1 and TNF-α.[12] The reduction of TNF-α and ICAM-1 production in preconditioned kidneys may have been mediated by inhibiting NF-κB activation. NF-κB exists in an inactive form in the cytoplasm, binding to the inhibitory protein IκB. A variety of stimuli leads to proteolytic degradation of IκB, thus allowing NF-κB translocation into the nucleus where it binds to the κB binding site in the promoter regions of target genes, activating the transcription of these corresponding genes. Increasing data suggest a pivotal role of NF-κB in a variety of pathophysiological conditions, including renal I/R injury.[28–31] We also found degradation of IκB-α and subsequent activation of NF-κB were increased following I/R injury. In a previous study by us, NF-κB decoy treatment affected NF-κB activities in the kidney, prevented the infiltration of M/Mφ, decreased the expression of several inflammatory molecules including ICAM-1, and thus attenuated I/R injury in ischaemic ARF rats.[13] In this study, ischaemic pretreatment reduced

the degradation of IκB-α and the activation of NF-κB, which was consistent with the improvement of renal morphology and function in rats exposed to IPC. Concerning NF-κB's central role in regulating inflammatory response, therapies targeted at NF-κB may be more effective in mimicking renoprotective effects of delayed IPC than either anti- ICAM-1 or anti- TNF-α therapy alone.

In conclusion, the data from this study indicate delayed IPC can profoundly protect the kidney against both histological impairment and renal dysfunction induced by I/R. At the same time, delayed IPC showed a drastic inhibitory effect on the recruitment of macrophages, the expression of ICAM-1 and TNF-α, and the activation of NF-κB, thus abrogating the inflammatory response following I/R. Therefore, the renal protection of delayed IPC may be, at least in part, attributed to its anti-inflammatory effects. Elucidation of the molecular mechanisms that are involved in ischaemic tolerance and identification of drugs that mimic these protective effects have the potential to improve the prognosis of patients at risk of ischaemic injury. Additional studies to illustrate endogenous protective mechanisms, underlying delayed IPC in the kidney, are warranted.

ACKNOWLEDGEMENTS

REFERENCES

1. Molls RR, Savransky V, Liu M, *et al*. Keratinocyte-derived chemokine is an early biomarker of ischemic acute kidney injury. *Am J Physiol Renal Physiol* 2006; **290**: F1187–1193.
2. Sahna E, Parlakpinar H, Cihan OF, Turkoz Y, Acet A. Effects of aminoguanidine against renal ischaemia-reperfusion injury in rats. *Cell Biochem Funct* 2006; **24**: 137–141.
3. Molitoris BA. Transitioning to therapy in ischemic acute renal failure. *J Am Soc Nephrol* 2003; **14**: 265–267.
4. Okubo S, Xi L, Bernardo NL, Yoshida K, Kukreja RC. Myocardial preconditioning: basic concepts and potential mechanisms. *Mol Cell Biochem* 1999; **196**: 3–12.
5. Tomai F, Crea F, Chiariello L, Gioffrè PA. Ischemic preconditioning in humans: models, mediators, and clinical relevance. *Circulation* 1999; **100**: 559–563.
6. Park KM, Byun JY, Kramers C, Kim JI, Huang PL, Bonventre JV. Inducible nitric-oxide synthase is an important contributor to prolonged protective effects of ischemic preconditioning in the mouse kidney. *J Biol Chem* 2003; **278**: 27256–27266.
7. Muscari S, Bonafe' F, Gamberini C, Giordano E, Lenaz G, Caldarera CM. Ischemic preconditioning preserves proton leak-

8. age from mitochondrial membranes but not oxidative phosphorylation during heart reperfusion. *Cell Biochem Funct (in press)*.
8. Baxter GF, Goma FM, Yellon DM. Characterization of the infarct-limiting effect of delayed preconditioning: timecourse and dose-dependency studies in rabbit myocardium. *Basic Res Cardiol* 1997; **92**: 159–167.
9. Yamashita N, Hoshida S, Otsu K, Taniguchi N, Kuzuya T, Hori M. The involvement of cytokines in the second window of ischaemic preconditioning. *Br J Pharmacol* 2000; **131**: 415–422.
10. Bonventre JV, Zuk A. Ischemic acute renal failure: an inflammatory disease? *Kidney Int* 2004; **66**: 480–485.
11. Roelofs JJ, Rouschop KM, Leemans JC, *et al*. Tissue-type plasminogen activator modulates inflammatory responses and renal function in ischemia reperfusion injury. *J Am Soc Nephrol* 2006; **17**: 131–140.
12. Karin M, Delhase M, The I-κB kinase (IKK) and NF-κB: key elements of proinflammatory signalling. *Semin Immunol* 2000; **12**: 85–98.
13. Cao CC, Ding XQ, Ou ZL, *et al*. In vivo transfection of NF-κB decoy oligodeoxynucleotides attenuate renal ischemia/reperfusion injury in rats. *Kidney Int* 2004; **65**: 834–845.
14. Thiemermann C, Patel NS, Kvale EO, *et al*. High density lipoprotein (HDL) reduces renal ischemia/reperfusion. *J Am Soc Nephrol* 2003; **14**: 1833–1843.
15. Jablonski P, Howden BO, Rae DA, Birrell CS, Marshall VC, Tange J. An experimental model for assessment of renal recovery from warm ischemia. *Transplantation* 1983; **35**: 198–204.
16. Day YJ, Huang L, Ye H, Linden J, Okusa MD. Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages. *Am J Physiol Renal Physiol* 2005; **288**: F722–731.
17. Abraham E. NF-κB activation. *Crit Care Med* 2000; **28**: N100–N104.
18. Yamamoto Y, Gaynor RB. IκB kinases: key regulators of the NF-κB pathway. *Trends Biochem Sci* 2004; **29**: 72–79.
19. Torras J, Herrero-Fresneda I, Lloberas N, Riera M, Cruzado JM, Grinyó JM. Promising effects of ischemic preconditioning in renal transplantation. *Kidney Int* 2002; **61**: 2218–2227.
20. Park KM, Chen A, Bonventre JV. Prevention of kidney ischemia/reperfusion-induced functional injury and JNK, p38, and MAPK kinase activation by remote ischemic pretreatment. *J Biol Chem* 2001; **276**: 11870–11876.
21. Ysebaert DK, De Greef KE, Vercauteren SR, *et al*. Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury. *Nephrol Dial Transplant* 2000; **15**: 1562–1574.
22. Schrier RW, Wang W, Poole B, Mitra A. Acute renal failure: definitions, diagnosis, pathogenesis, and therapy. *J Clin Invest* 2004; **114**: 5–14.
23. Haller H, Dragun D, Miethke A, *et al*. Antisense oligonucleotides for ICAM-1 attenuate reperfusion injury and renal failure in the rat. *Kidney Int* 1996; **50**: 473–480.
24. Kelly KJ, Williams WW, Jr, Colvin RB, *et al*. Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *J Clin Invest* 1996; **97**: 1056–1063.
25. Donnahoo KK, Shames BD, Harken AH, Meldrum DR. Review article: the role of tumor necrosis factor in renal ischemia-reperfusion injury. *J Urol* 1999; **162**: 196–203.
26. Donnahoo KK, Meldrum DR, Shenkar R, Chung CS, Abraham E, Harken AH. Early renal ischemia, with or without reperfusion, activates NF kappaB and increases TNF-alpha bioactivity in the kidney. *J Urol* 2000; **163**: 1328–1332.

27. Belosjorow S, Bolle I, Duschin A, Heusch G, Schulz R. TNF-α antibodies are as effective as ischemic preconditioning in reducing infarct size in rabbits. *Am J Physiol Heart Circ Physiol* 2003; **284**: H927–H930.

28. Vos IH, Govers R, Grone HJ, *et al*. NF-kappaB decoy oligo-deoxynucleotides reduce monocyte infiltration in renal allografts. *FASEB J* 2000; **14**: 815–822.

29. Guijarro C, Egido J. Transcription factor-kappa B (NF-kappa B) and renal disease. *Kidney Int* 2001; **59**: 415–424.

30. Sung FL, Zhu TY, Au-Yeung KK, Siow YLOK. Enhanced MCP-1 expression during ischemia/reperfusion injury is mediated by oxidative stress and NF-kappaB. *Kidney Int* 2002; **62**: 1160–1170.

31. Chatterjee PK, Di Villa BRD, Sivarajah A, McDonald MC, Cuzzocrea S, Thiemermann C. Pyrrolidine dithiocarbamate reduces renal dysfunction and injury caused by ischemia/reperfusion of the rat kidney. *Eur J Pharmacol* 2003; **482**: 271–280.