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Differential effects of caspase inhibitors on the renal dysfunction and injury caused by ischemia–reperfusion of the rat kidney

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Abstract

Caspase activation has been implicated in the development of ischemia–reperfusion injury. Here, we investigate the effects of different caspase inhibitors on the renal dysfunction and injury caused by ischemia–reperfusion of the rat kidney. Bilateral clamping of renal pedicles (45 min) followed by reperfusion (6 h) caused significant renal dysfunction and marked renal injury. Caspase-1 inhibitor II (*N*-acetyl-L-tyrosyl-L-valyl-*N*-[(1S)-1-(carboxymethyl)-3-chloro-2-oxo-propyl]-L-alaninamide, Ac-YVAD-CMK, 3 mg/kg, administered i.p.) significantly reduced biochemical and histological evidence of renal dysfunction and injury. However, although caspase-3 inhibitor I (*N*-acetyl-L-aspartyl-L-glutamyl-*N*-(2-carboxyl-1-formylethyl]-L-valinamide, Ac-DEVD-CHO, 3 mg/kg, administered i.p.) produced a significant improvement of renal (glomerular) dysfunction (reduction of serum creatinine levels), it was not able to reduce tubular dysfunction and injury. Furthermore, the pan-caspase inhibitor caspase inhibitor III (*N*-*tert*-butoxycarbonyl-aspartyl(OMe)-fluoromethylketone, Boc-D-FMK, 3 mg/kg, administered i.p.) did not reduce renal dysfunction and injury. Both caspase-1 and -3 inhibitors markedly reduced the evidence of oxidative and nitrosative stress in rat kidneys subjected to ischemia–reperfusion. Overall, these results demonstrate that inhibition of caspase-1 reduces renal ischemia–reperfusion injury to a greater extent than caspase-3 inhibition, supporting the notion that the mode of acute cell death in our model of renal ischemia–reperfusion is primarily via necrosis. Furthermore, our finding that a pan-caspase inhibitor did not reduce the renal dysfunction and injury suggests that activation of some caspases during ischemia–reperfusion could provide protection against acute ischemic renal injury. Overall, these results demonstrate that inhibition of caspase-1 activity reduces renal ischemia–reperfusion injury and that this therapeutic strategy may be of benefit against ischemic acute renal failure.

Keywords: Renal/kidney; Ischemia; Reperfusion injury; Caspase; Caspase inhibitor

1. Introduction

Despite significant advances in critical care medicine, acute renal failure remains a major clinical problem, causing considerable morbidity and mortality that has not decreased significantly over the last 50 years (Chatterjee and Thiemermann, 2003a). Previous interventions against acute renal failure have proved to be largely negative, and dialysis remains the only effective therapy (Chatterjee and Thiemermann, 2003a). Thus, the development of novel therapeutic interventions against acute renal failure has remained a topic of intense research interest (Chatterjee and Thiemermann, 2003a; Venkataraman and Kellum, 2003).

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It is now well established that (i) renal ischemia initiates a complex and interrelated sequence of events resulting in the injury and death of renal cells and (ii) reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage (reperfusion injury; Molitoris, 1992; Paller, 1994a; Weight et al., 1996). Together, ischemia–reperfusion of the kidney contributes to the renal dysfunction and injury associated with ischemic acute renal failure (Thadhani et al., 1996; Weight et al., 1996) involving both apoptotic and necrotic renal cell death pathways (Padanilam, 2003).

Two groups of cysteine proteases, caspases and calpains, are involved in the development of acute renal injury caused by, e.g., ischemia–reperfusion (Padanilam, 2003). Calpains, which are calcium-dependent cysteine proteases, are activated by increases in intracellular calcium during renal ischemia–reperfusion (Liu et al., 2001), and calpain inhibitors such as 3-(4-Iodophenyl)-2-mercapto-(*Z*)-2-propenoic acid (PD150606) and calpain inhibitor-1 can provide protection (Chatterjee et al., 2001; Liu et al., 2001). Caspases consist of a family of proteases, which have roles in the development of apoptosis and inflammation of the kidney (Daemen et al., 1999; Saikumar and Venkatachalam, 2003) and have been classified on the basis of their substrate specificities and functional subfamilies. Specifically, caspases can be grouped according to those which promote apoptotic cell death (group III enzymes: caspase-6, -8, -9, and -10 are activators or initiators, whereas group II enzymes: caspase-2, -3, and -7 are effectors) and those which activate proinflammatory cytokines (group I enzymes: caspase-1, -4, -5, and -13) (Nicholson, 1999).

There is now evidence that chemokine induction following renal ischemia–reperfusion is dependent on caspase activation (Daemen et al., 2001a) and that caspases may interact with calpains during renal ischemia–reperfusion injury, specifically, that levels of the endogenous calpain inhibitor calpastatin are reduced by ischemia–reperfusion and that this can be prevented by pretreatment with caspase inhibitors, suggesting that caspases may promote calpain activity via degradation of its intrinsic inhibitor (Shi et al., 2000).

Recent evidence suggests that caspase-1 is implicated in the development of renal ischemia–reperfusion injury (Kaushal et al., 1998; Kaushal, 2003). Caspase-1 expression is up-regulated during ischemia–reperfusion of the kidney (Kaushal et al., 1998; Kaushal, 2003). Furthermore, caspase-1 knockout mice are protected against renal ischemia–reperfusion injury with reductions in both tubular necrosis and infiltration of polymorphonuclear neutrophils (Melnikov et al., 2001). Activation of caspase-3 leads to apoptosis, and its activation has been implicated in several disease states including heart failure and myocardial ischemia–reperfusion injury and stroke (Yaoita et al., 1998; Narula et al., 1999; Chapman et al., 2002; Joly et al., 2004). Specifically within the kidney, renal ischemia–reperfusion leads to an increased expression of caspase-3 over prolonged ischemic periods promoting apoptosis (Kaushal et al., 1998; Chien et al., 2000).

The beneficial effects of caspase inhibition during ischemia–reperfusion injury of the heart and brain have recently been reported (Endres et al., 1998; Yaoita et al., 1998; Okamura et al., 2000; Chapman et al., 2002; Kilic et al., 2004). There is also some evidence that caspase inhibition can provide protection against endotoxin-induced myocardial dysfunction in rats (Fauvel et al., 2001; Nevière et al., 2001). Caspase inhibition can reduce renal inflammation associated with glomerulonephritis (Daemen et al., 1999; Yang et al., 2003), and another study has reported that a pan-caspase inhibitor (caspase inhibitor I, *N*-benzyloxycarbonyl-L-valyl-L-alanyl-L-aspartyl(OMe)-fluoromethylketone, Z-VAD-FMK), which inhibits caspases-1, -3, -4, and -7, protected ischemic mouse kidneys against apoptosis and subsequent inflammation (Daemen et al., 1999). However, although that study by Daemen et al. demonstrates a role for caspases in the development of renal ischemia–reperfusion injury, the role of individual caspases such as caspase-1 and -3 cannot be determined from such studies.

The effects of caspase inhibitors against the renal dysfunction and acute injury caused by ischemia–reperfusion injury of the kidney have therefore not been investigated in detail. The present study was therefore designed to evaluate the effectiveness of three different caspase inhibitors (a caspase-1 inhibitor, a caspase-3 inhibitor, and a "pan-caspase" inhibitor; Thornberry and Lazebnik, 1998) in an established in vivo rat model of renal ischemia–reperfusion injury (Chatterjee and Thiemermann, 2003b). Specifically, the effects of the caspase inhibitors, caspase-1 inhibitor II (interleukin-1β converting enzyme inhibitor II, *N*-acetyl-L-tyrosyl-L-valyl-*N*-[(1S)-1-(carboxymethyl)-3-chloro-2-oxo-propyl]-L-alaninamide Ac-YVAD-CMK), caspase-3 inhibitor I (CPP32/apopain inhibitor, *N*-acetyl-L-aspartyl-L-glutamyl-*N*-(2-carboxyl-1-formylethyl]-L-valinamide, Ac-DEVD-CHO), and the pan-caspase inhibitor caspase inhibitor III (*N*-*tert*-butoxycarbonyl-aspartyl(OMe)-fluoromethylketone, Boc-D-FMK), were investigated. Once the ability of these caspase inhibitors to reduce the renal dysfunction and injury was established, we investigated the potential mechanisms involved in any beneficial effect. Specifically, the ability of caspase inhibitors to reduce the oxidative and nitrosative stress associated with renal ischemia–reperfusion was elucidated using a combination of biochemical and immunohistological assays.

2. Materials and methods

2.1. Animals and experimental design

Male Wistar rats (12 weeks of age, Tuck, Rayleigh, Essex, UK) were housed in a light-controlled room with a

12-h light–dark cycle and were allowed ad libitum access to food and water. Animal care and experimental protocols were performed in accordance with the Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office, London, UK and were approved by the local University Ethical Committee. Rats were anesthetized with sodium thiopentone (Intraval® Sodium, 120 mg/kg i.p.; Rhone Merieux, Essex, UK), and anesthesia was maintained by supplementary injections (approximately 10 mg/kg i.v.) of sodium thiopentone. Rats were randomly allocated into the following 10 groups:

(i) I/R+saline group. Rats were administered i.p., 2 ml/kg 0.9% w/v saline 30 min prior to renal ischemia for 45 min followed by reperfusion for 6 h (n=12).
(ii) I/R+vehicle group. Rats were administered i.p., 2 ml/kg 10% v/v dimethylsulfoxide (DMSO), the vehicle for caspase inhibitors, 30 min prior to ischemia–reperfusion (n=12).
(iii) I/R+Casp-1 inhib group. Rats were administered i.p., 2 ml/kg caspase-1 inhibitor II, 3 mg/kg, prepared in 10% v/v dimethylsulfoxide (n=6).
(iv) I/R+Casp-3 inhib group. Rats were administered i.p., 2 ml/kg caspase-3 inhibitor I, 3 mg/kg, prepared in 10% v/v dimethylsulfoxide (n=6).
(v) I/R+Pan-casp inhib group. Rats were administered i.p., 2 ml/kg caspase inhibitor III, 3 mg/kg, prepared in 10% v/v dimethylsulfoxide (n=6).
(vi) Sham+Saline group. Rats subjected to identical surgical procedures, except for renal ischemia–reperfusion, were maintained under anesthesia for the duration of the experiment (30 min+45 min+6 h). At the beginning of the experiment, rats were administered 2 ml/kg 0.9% w/v saline i.p. (n=12).
(vii) Sham+DMSO group. Identical to Sham+Saline rats except for the administration of 10% v/v dimethylsulfoxide prepared in 0.9% w/v saline (2 ml/kg i.p.) 30 min prior to the commencement of the experimental period (n=12).
(viii) Sham+Casp-1 inhib group. Identical to Sham+Saline rats except for the administration of 2 ml/kg caspase-1 inhibitor II, 3 mg/kg, prepared in 10% v/v dimethylsulfoxide, 30 min prior to the commencement of the experimental period (n=4).
(ix) Sham+Casp-3 inhib group. Identical to Sham+Saline rats except for the administration of 2 ml/kg caspase-3 inhibitor I, 3 mg/kg, prepared in 10% v/v dimethylsulfoxide, 30 min prior to the commencement of the experimental period (n=4).
(x) Sham+Pan-casp inhib group. Identical to Sham+Saline rats except for the administration of 2 ml/kg caspase inhibitor III, 3 mg/kg, prepared in 10% v/v dimethylsulfoxide, 30 min prior to the commencement of the experimental period (n=4).

All rats received an i.v. infusion of 0.9% w/v saline throughout the experimental period at a rate of 2 ml/kg/h. The time course and doses of caspase inhibitors used were based on those previously shown by others to effectively attenuate the apoptosis in rat and mouse models of ischemia–reperfusion injury (Yaoita et al., 1998; Daemen et al., 1999; Okamura et al., 2000) and myocardial dysfunction in rats caused by endotoxin (Fauvel et al., 2001; Nevière et al., 2001).

2.2. Surgical procedures

Surgical preparation of rats and the protocol used to produce renal ischemia–reperfusion were identical to those described previously (Chatterjee et al., 2001; Chatterjee and Thiemermann, 2003b). Briefly, anesthetized rats were placed onto a thermostatically controlled heating mat (Harvard Apparatus, Kent, UK). A tracheotomy was performed to maintain airway patency and to facilitate spontaneous respiration. The right carotid artery was cannulated (PP50, internal diameter 0.58 mm, Portex, Kent, UK) and connected to a pressure transducer (Senso-Nor 840, Horten, Norway) for the measurement of mean arterial blood pressure and heart rate, which were displayed on a data acquisition system (MacLab 8e, AD Instruments, Hastings, UK) installed on a Dell Dimension 4100 Personal computer (Dell Computer, Glasgow, UK). The jugular vein was cannulated (PP25, internal diameter 0.40 mm, Portex) for the administration of anesthesia or 0.9% w/v saline as required. A midline laparotomy was performed, and the bladder was cannulated (PP90, internal diameter 0.76 mm, Portex). Both kidneys were located, and the renal pedicles containing the artery, vein, and nerve supplying each kidney were carefully isolated. For rats subjected to ischemia–reperfusion, bilateral renal occlusion for 45 min was performed using 3.5 cm Dieffenbach 'bulldog' arterial clips (Holborn Surgical and Medical Instruments, Margate, Kent, UK), which were used to clamp the renal pedicles. Reperfusion commenced once the artery clips were removed. Occlusion was verified visually by change in the color of the kidneys to a paler shade and reperfusion by a blush. Other rats were subjected to sham operation, which underwent identical surgical procedures to rats subjected to renal ischemia–reperfusion but did not undergo bilateral renal clamping and were maintained under anesthesia for the duration of the experiment (45 min+6 h). Throughout surgery and the experimental period, rat body temperature was maintained at 37±1 °C by means of a rectal probe attached to a homoeothermic blanket. At the end of all experiments, rats were euthanised using an overdose of sodium thiopentone.

2.3. Measurement of biochemical parameters

At the end of the experimental period, blood samples were collected via the carotid artery into tubes containing

serum gel. The samples were centrifuged (6000 rpm for 3 min) to separate serum from which biochemical parameters were measured (Vetlab Services, Sussex, UK). Serum creatinine levels were used as an indicator of renal (glomerular) function (Chatterjee et al., 2001; Chatterjee and Thiemermann, 2003b). Aspartate aminotransferase and γ-glutamyltransferase, enzymes both located in the proximal tubule, were used as indicators of reperfusion injury (Chatterjee et al., 2001; Chatterjee and Thiemermann, 2003b). Urine samples were collected throughout the reperfusion period and the volume of urine produced recorded. Urine concentrations of Na^+ were measured (Vetlab Services) and used in conjunction with serum Na^+ levels to estimate fractional excretion of Na^+ (as an indicator of tubular dysfunction), using standard formulae, which were used as indicators of renal function (Chatterjee et al., 2001; Chatterjee and Thiemermann, 2003b). Urinary *N*-acetyl-β-D-glucosaminidase activity, a specific indicator of tubular injury and possibly also tubular dysfunction (Chatterjee et al., 2001; Chatterjee and Thiemermann 2003b), was also measured (Clinica Medica é Diagnóstico Dr Joaquim Chaves, Lisbon, Portugal).

2.4. Histological evaluation

Renal sections were prepared as described previously (Chatterjee et al., 2001) and used for histological assessment of ischemia–reperfusion injury. Briefly, 100 intersections were examined, and a score from 0 to 3 was given for each tubular profile involving an intersection: 0: normal histology; 1: tubular cell swelling, brush border loss, nuclear condensation, with up to 1/3 of tubular profile showing nuclear loss; 2: as for score 1 but greater than 1/3 and less than 2/3 of tubular profile showing nuclear loss; and 3: greater than 2/3 of tubular profile shows nuclear loss. The total score for each kidney was calculated by addition of all 100 scores (maximum score 300).

2.5. Immunohistochemical localization of intercellular adhesion molecule-1 and nitrotyrosine

Evidence of intercellular adhesion molecule (ICAM)-1 expression and nitrotyrosine formation was determined using immunohistochemical protocols, as described recently (Chatterjee et al., 2004a,b). Briefly, kidney sections were incubated overnight at 4 °C with primary anti-ICAM-1 or antinitrotyrosine antibodies (1:500 v/v in phosphate buffered saline, [PBS, 0.01 M, pH 7.4] DBA, Milan, Italy). Separate sections were also incubated, with control solutions consisting of PBS alone or a 1:500 dilution of nonspecific purified rabbit immunoglobulin G (DBA). Specific labelling was detected using a biotin-conjugated goat antirabbit immunoglobulin G (DBA) and avidin–biotin peroxidase (DBA). Samples were then viewed under a light microscope.

2.6. Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity in kidneys was used as an indicator of polymorphonuclear neutrophil infiltration, using a method previously described (Chatterjee et al., 2001). Briefly, at the end of the experiments, kidney tissue was weighed and homogenised in a solution containing 0.5% (w/v) hexadecyltrimethylammonium bromide dissolved in 10 mM PBS and centrifuged for 30 min at 20,000 ×*g* at 4 °C. An aliquot of supernatant was then removed and added to a reaction mixture containing 1.6 mM tetramethylbenzidine and 0.1 mM hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme required to degrade 1 μmol of hydrogen peroxide at 37 °C and was expressed in U/g wet tissue.

2.7. Determination of malondialdehyde levels

Levels of malondialdehyde (MDA) in kidneys were determined as an indicator of lipid peroxidation following a previously described protocol (Chatterjee et al., 2001). Briefly, kidney tissue was weighed and homogenised in a 1.15% w/v potassium chloride solution. A 100-μl aliquot of homogenate was then removed and added to a reaction mixture containing 200 μl 8.1% w/v lauryl sulfate, 1.5 ml 20% v/v acetic acid, 1.5 ml 0.8% w/v thiobarbituric acid, and 700 μl distilled water. Samples were then boiled for 1 h at 95 °C and centrifuged at 3000 ×*g* for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 650 nm. MDA levels were expressed as μM/100 mg wet tissue.

2.8. Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich (Poole, Dorset, UK). The caspase inhibitors, caspase-1 inhibitor II (interleukin-1β converting enzyme inhibitor II, Ac-YVAD-CMK), caspase-3 inhibitor I (CPP32/apopain inhibitor, Ac-DEVD-CHO), and the pan-caspase inhibitor caspase inhibitor III (Boc-D-FMK), were purchased from Calbiochem Biochemicals and Immunochemicals, (Merck Biosciences, Nottingham, UK). All stock solutions were prepared using nonpyrogenic saline (0.9% w/v NaCl; Baxter Healthcare, Thetford, Norfolk, UK).

2.9. Statistical analysis

All values described in the text and figures are expressed as mean±standard deviation (S.D.) for *n* observations. Each data point represents biochemical measurements obtained from up to 12 separate rats. For histological and immunohistochemical analysis, the figures shown are representative of at least 3 experiments performed on different experimental days. Statistical analysis was carried out using

GraphPad Prism 3.02/Instat 1.1 (GraphPad Software, San Diego, CA, USA). Data were analyzed using one-way analysis of variance followed by Dunnett's post hoc test, and a P value of less than 0.05 was considered to be significant.

3. Results

The mean±S.D. for the weights of the rats used in this study was 264±35 g (n=78). On comparison with sham rats, renal ischemia–reperfusion produced significant increases in serum, urinary, and histological markers of renal dysfunction and injury, as described in detail below (Figs. 1–3). When compared to rats used as shams, renal ischemia–reperfusion (in the presence or absence of caspase inhibitors) did not have a significant effect on urine flow (0.010±0.009 ml/min, n=78).

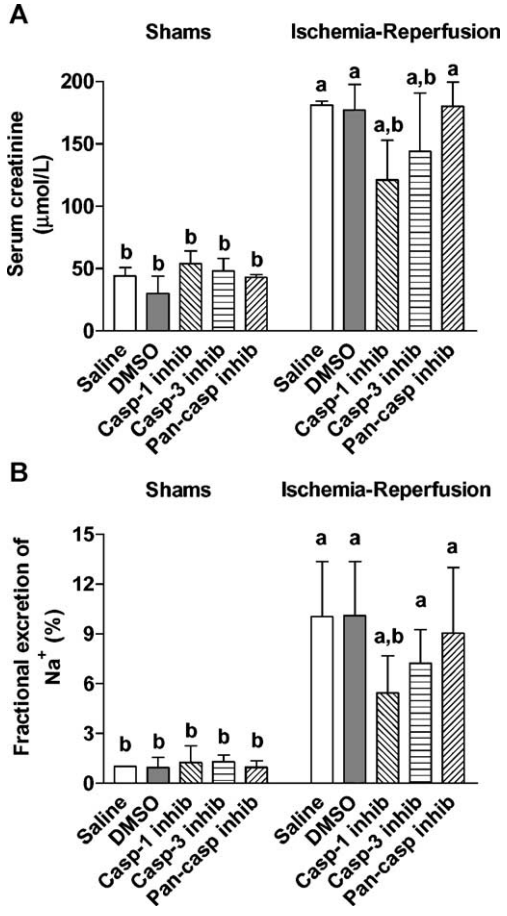

Fig. 1. Effect of caspase inhibitors on renal dysfunction. Serum creatinine levels (A) and fractional excretion of Na^+ (B) were measured subsequent to sham-operation (Shams) or renal ischemia–reperfusion (Ischemia–Reperfusion) in the absence or presence of caspase-1 inhibitor II (Casp-1 inhib), caspase-3 inhibitor I (Casp-3 inhib), or a pan-caspase inhibitor (Pan-casp inhib), 3 mg/kg, administered 30 min prior to renal ischemia–reperfusion. P<0.05 vs. DMSO (Sham) group (a), P<0.05 vs. DMSO (Ischemia–Reperfusion) group (b), n=4–12 rats.

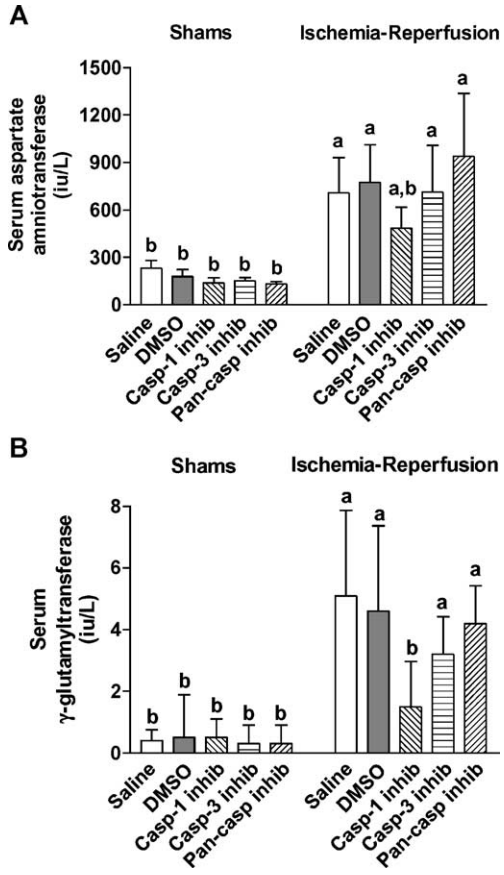

Fig. 2. Effect of caspase inhibitors on reperfusion injury. Serum aspartate aminotransferase (A) and γ-glutamyltransferase (B) levels were measured subsequent to sham-operation (Shams) or renal ischemia–reperfusion (Ischemia–Reperfusion) in the absence or presence of caspase-1 inhibitor II (Casp-1 inhib), caspase-3 inhibitor I (Casp-3 inhib), or a pan-caspase inhibitor (Pan-casp inhib), 3 mg/kg, administered 30 min prior to renal ischemia–reperfusion. P<0.05 vs. DMSO (Sham) group (a), P<0.05 vs. DMSO (Ischemia–Reperfusion) group (b), n=4–12 rats.

3.1. Effect of caspase inhibitors on renal dysfunction caused by ischemia–reperfusion of the rat kidney

Rats subjected to renal ischemia–reperfusion demonstrated significantly increased serum levels of creatinine compared to sham-operated rats (Fig. 1A). This was reflected by a significant increase in fractional excretion of Na^+ (Fig. 1B). Compared to rats subjected to ischemia–reperfusion only, administration of caspase-1 inhibitor II (3 mg/kg, i.p. 30 min prior to ischemia–reperfusion) produced a significant reduction in serum creatinine levels (Fig. 1A) and in fractional excretion of Na^+ (Fig. 1B). Administration of caspase-3 inhibitor I (3 mg/kg, i.p. 30 min prior to ischemia–reperfusion) also produced a significant reduction in serum creatinine levels compared to rats subjected to renal ischemia–reperfusion only (Fig. 1A). However, although caspase-3 inhibitor I also reduced fractional excretion of Na^+ compared to rats subjected to renal ischemia–reperfusion, this effect did not reach significance (Fig. 1B). Administration of the pan-caspase inhibitor

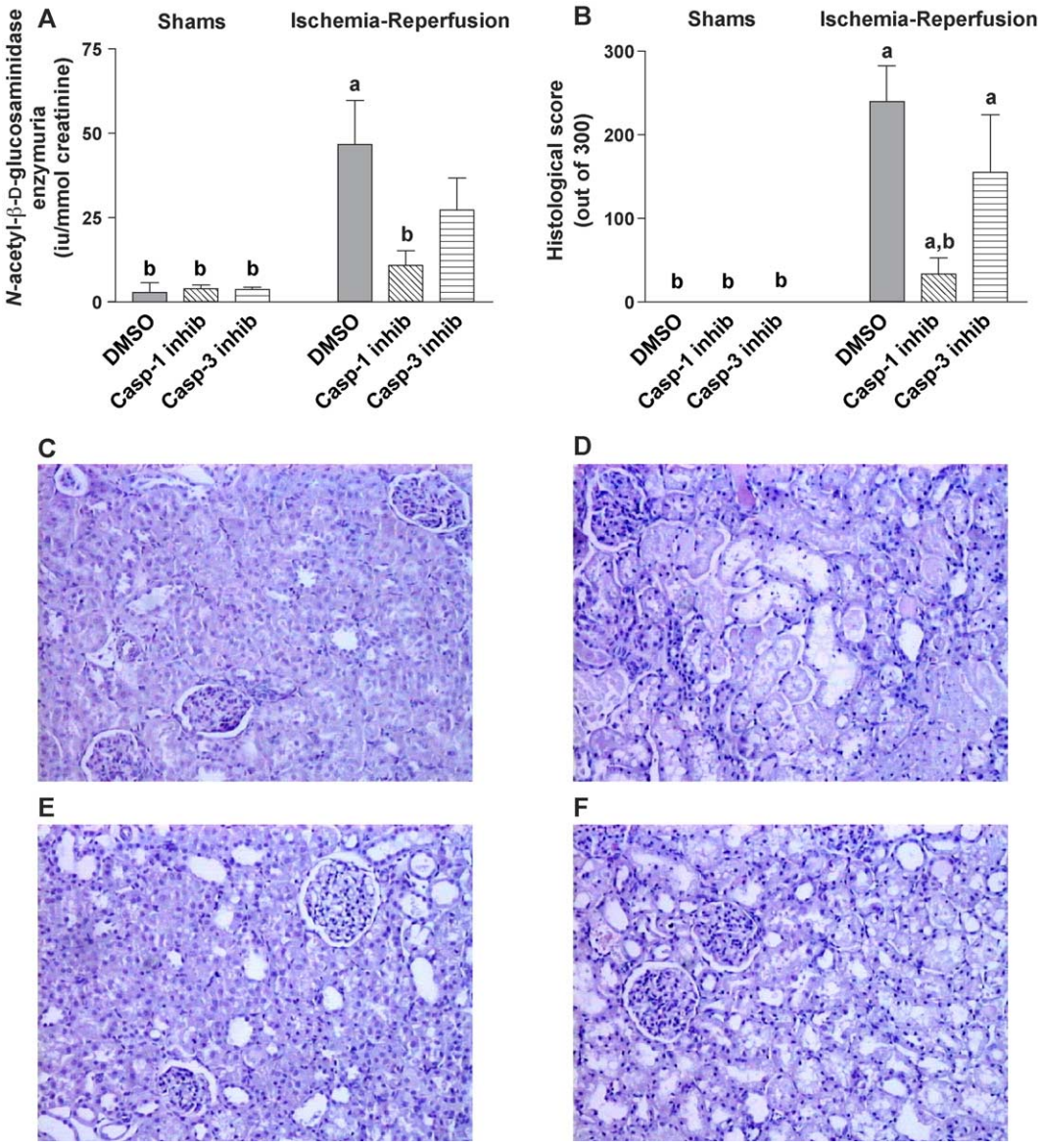

Fig. 3. Effect of caspase inhibitors on renal (tubular) injury. Urinary *N*-acetyl-β-D-glucosaminidase activity (A) and histological score (B) were measured subsequent to sham-operation (Shams) or renal ischemia–reperfusion (Ischemia–Reperfusion) in the absence or presence of caspase-1 inhibitor II (Casp-1 inhib) or caspase-3 inhibitor I (Casp-3 inhib), 3 mg/kg, administered 30 min prior to renal ischemia–reperfusion. $P<0.05$ vs. DMSO (Sham) group (a), $P<0.05$ vs. DMSO (Ischemia–Reperfusion) group (b), $n=4$–12 rats. A kidney section taken from a sham-operated rat (C) is compared with that taken from a rat subjected to 45-min ischemia followed by 6-h reperfusion (D). Kidney sections taken from rats subjected to renal ischemia–reperfusion after administration of caspase-1 inhibitor II (E) or caspase-3 inhibitor I (F), 3 mg/kg, 30 min prior to ischemia–reperfusion are also represented. Hemotoxylin and eosin, original magnification ×125, figures are representative of at least 3 experiments performed on different days.

caspase inhibitor III to rats (3 mg/kg, i.p. 30 min prior to ischemia–reperfusion) did not have any effect on either the increased serum creatinine levels or fractional excretion of Na^+ associated with renal ischemia–reperfusion (Fig. 1A,B).

Administration of caspase inhibitors to sham-operated rats did not have any effect on baseline serum creatinine levels (Fig. 1A) or cause any alteration in fractional excretion of Na^+ (Fig. 1B). Administration of the vehicle for the caspase inhibitors (10% v/v dimethylsulfoxide) to rats subjected to renal ischemia–reperfusion did not have any effect on the renal dysfunction obtained on comparison with similar rats administered saline only (Fig. 1A,B).

3.2. Effect of caspase inhibitors on reperfusion injury caused by renal ischemia–reperfusion

Rats subjected to renal ischemia–reperfusion demonstrated significantly increased serum levels of aspartate aminotransferase and γ-glutamyltransferase compared with sham-operated rats (Fig. 2A,B). Compared to rats subjected to renal ischemia–reperfusion only, administration of caspase-1 inhibitor II produced a significant reduction in both serum aspartate aminotransferase and γ-glutamyltransferase levels (Fig. 2A,B). Administration of caspase-3 inhibitor I did not produce any reduction of

serum aspartate aminotransferase and γ-glutamyltransferase levels compared with rats subjected to ischemia–reperfusion only (Fig. 2A,B). Administration of the pan-caspase inhibitor caspase inhibitor III did not have a significant effect on either increased serum aspartate aminotransferase or γ-glutamyltransferase levels associated with renal ischemia–reperfusion (Fig. 2A,B).

Administration of caspase inhibitors to sham-operated rats did not have any effect on baseline serum aspartate aminotransferase or γ-glutamyltransferase levels (Fig. 2A,B). Furthermore, administration of the vehicle for the caspase inhibitors (10% v/v dimethylsulfoxide) to rats subjected to renal ischemia–reperfusion did not have any effect on the increased serum levels of aspartate aminotransferase or γ-glutamyltransferase obtained on comparison with rats administered saline only (Fig. 2A,B).

3.3. Effect of caspase inhibitors on renal injury caused by renal ischemia–reperfusion

Rats subjected to renal ischemia–reperfusion demonstrated significantly increased levels of urinary *N*-acetyl-β-D-glucosaminidase activity on comparison with sham-operated rats (Fig. 3A). Administration of caspase-1 inhibitor II produced a significant reduction in the urinary *N*-acetyl-β-D-glucosaminidase activity associated with renal ischemia–reperfusion (Fig. 3A). Administration of caspase-3 inhibitor I also produced a reduction in urinary *N*-acetyl-β-D-glucosaminidase activity compared with rats subjected to ischemia–reperfusion only, however, this effect was not significant (Fig. 3A). Administration of caspase inhibitors to sham-operated rats did not have any effect on baseline urinary *N*-acetyl-β-D-glucosaminidase activity (Fig. 3A).

This profile of tubular injury was reflected in the histological scoring of renal injury (Fig. 3B). On comparison with the histological score measured from kidneys obtained from sham-operated animals, renal ischemia–reperfusion produced a significant increase in histological score (Fig. 3B). Administration of caspase-1 inhibitor II significantly reduced the histological score when compared to that obtained from rats subjected to renal ischemia–reperfusion only (Fig. 3B). However, although administration of caspase-3 inhibitor I also produced a reduction in renal injury, this was not a significant effect (Fig. 3B).

On comparison with the renal histology observed in kidneys taken from sham-operated rats which were administered DMSO only (Fig. 3C), rats which were subjected to renal ischemia–reperfusion demonstrated the characteristic features of renal injury including degeneration of tubular structure, tubular dilatation, swelling and necrosis, and luminal congestion (Fig. 3D). In contrast, renal sections obtained from rats administered caspase-1 inhibitor II prior to renal ischemia–reperfusion demonstrated marked reduc-

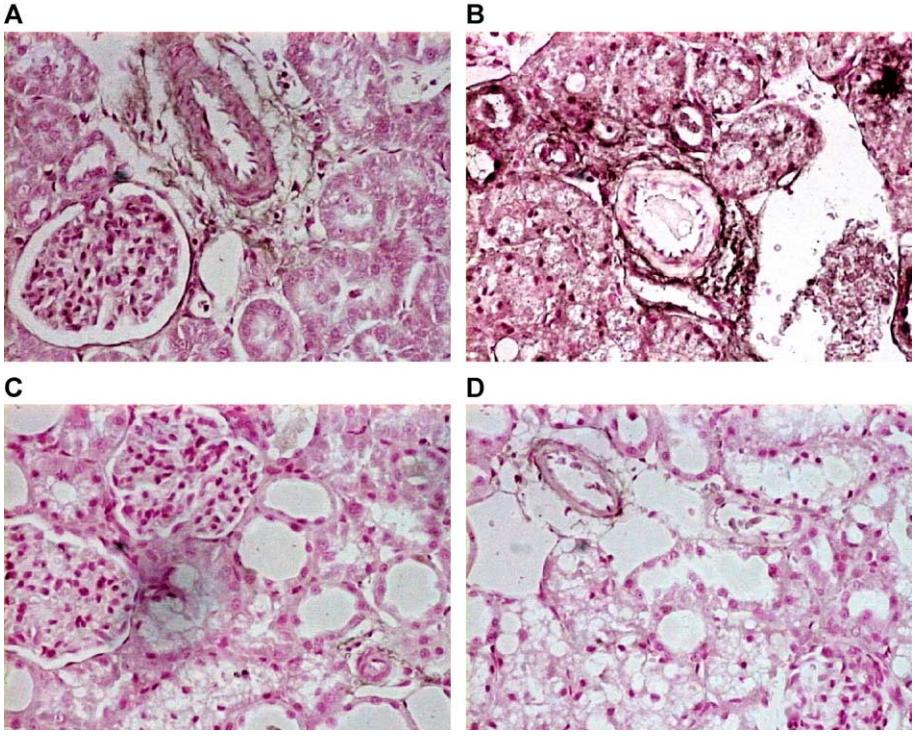

Fig. 4. Effect of caspase inhibitors on ICAM-1 expression. Kidney sections were incubated at 4 °C overnight with 1:500 dilution of a primary antibody directed against ICAM-1. Kidney sections taken from a sham-operated rat (A) administered the vehicle for caspase inhibitors (10% v/v DMSO) and rat subjected to 45-min ischemia followed by 6-h reperfusion and administered DMSO (B). Kidney sections taken from rats subjected to renal ischemia–reperfusion after administration of caspase-1 inhibitor II (C) or caspase-3 inhibitor I (D), 3 mg/kg, 30 min prior to ischemia–reperfusion are also represented. Original magnification ×250, figures are representative of at least 3 experiments performed on different days.

tion of renal injury (Fig. 3E). Kidney sections obtained from rats administered caspase-3 inhibitor I prior to ischemia–reperfusion also demonstrated reduction of renal injury, although some evidence of renal injury was still present (Fig. 3F).

3.4. Effect of caspase inhibitors on ICAM-1 expression during renal ischemia–reperfusion

Kidneys obtained from rats subjected to ischemia–reperfusion demonstrated marked staining for the adhesion molecule ICAM-1 when compared with kidneys obtained from sham-operated rats (Fig. 4A,B), suggesting adhesion molecule expression during reperfusion. Kidneys obtained from rats administered caspase-1 inhibitor II demonstrated markedly reduced staining for ICAM-1 (Fig. 4C) when compared with kidneys obtained from rats subjected to renal ischemia–reperfusion only, suggesting a reduction in the expression of this adhesion molecule during reperfusion. A similar degree of reduction of ICAM-1 staining was observed in the kidneys of rats administered caspase-3 inhibitor I prior to renal ischemia–reperfusion (Fig. 4D). Due to the inability of caspase inhibitor III to reduce the renal dysfunction caused by ischemia–reperfusion of the rat kidney, ICAM-1 expression was not determined in the kidneys of rats subjected to ischemia–reperfusion and which were administered caspase inhibitor III.

3.5. Effect of caspase inhibitors on nitrotyrosine formation during renal ischemia–reperfusion

When compared to kidney sections obtained from sham-operated rats (Fig. 5A), immunohistochemical analysis of sections obtained from rats subjected to renal ischemia–reperfusion revealed positive staining for nitrotyrosine (Fig. 5B). In contrast, reduced staining was observed in kidney sections obtained from rats subjected to renal ischemia–reperfusion but which were administered caspase-1 inhibitor II (Fig. 5C) or caspase-3 inhibitor I (Fig. 5D) prior to ischemia–reperfusion. Nitrotyrosine formation was not determined in the kidneys of rats subjected to ischemia–reperfusion and which were administered caspase inhibitor III.

3.6. Effect of caspase inhibitors on kidney myeloperoxidase activity and malondialdehyde levels during renal ischemia–reperfusion

Compared to sham-operated animals, rats subjected to renal ischemia–reperfusion exhibited a substantial increase in kidney MPO activity (Fig. 6), suggesting increased polymorphonuclear neutrophil infiltration into renal tissues. However, administration of caspase-1 inhibitor II or caspase-3 inhibitor I prior to ischemia–reperfusion produced a significant reduction of MPO activity on

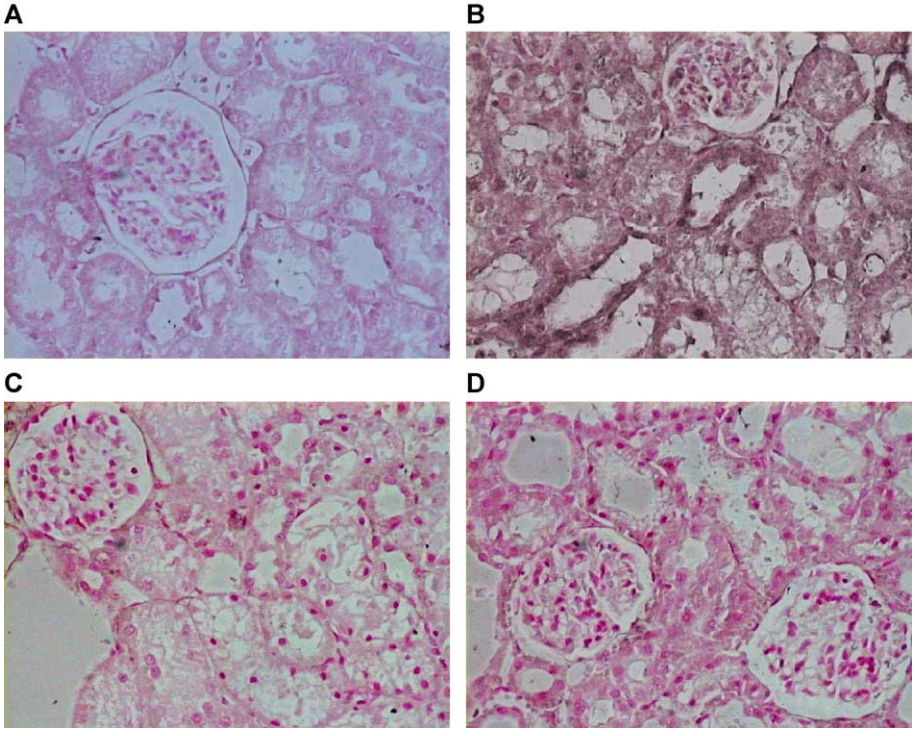

Fig. 5. Effect of caspase inhibitors on nitrotyrosine formation. Kidney sections were incubated at 4 °C overnight with 1:500 dilution of a primary antibody directed against nitrotyrosine. Kidney sections taken from a sham-operated rat administered DMSO (A) and a rat subjected to 45-min ischemia followed by 6-h reperfusion and administered DMSO (B). Kidney sections taken from rats subjected to renal ischemia–reperfusion after administration of caspase-1 inhibitor II (C) or caspase-3 inhibitor I (D) 3 mg/kg 30 min prior to ischemia–reperfusion are also represented. Original magnification ×250, figures are representative of at least 3 experiments performed on different days.

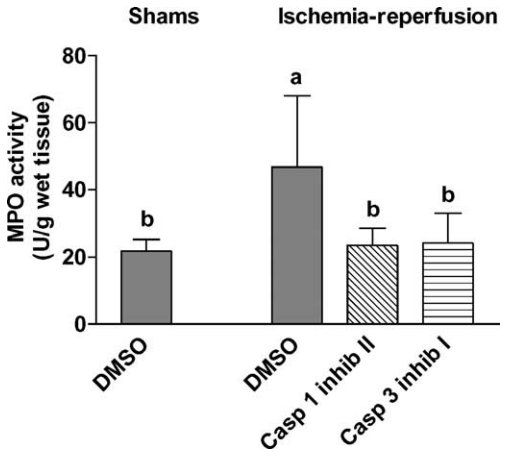

Fig. 6. Effect of caspase inhibitors on kidney myeloperoxidase (MPO) activity. MPO activity was measured subsequent to sham-operation (Shams) or renal ischemia–reperfusion (Ischemia–Reperfusion) in the absence or presence of caspase-1 inhibitor II (Casp-1 inhib) or caspase-3 inhibitor I (Casp-3 inhib), 3 mg/kg, administered 30 min prior to renal ischemia–reperfusion. $P<0.05$ vs. DMSO (Sham) group (a), $P<0.05$ vs. DMSO (Ischemia–Reperfusion) group (b), $n=4$ rats.

comparison with that obtained from control (ischemia–reperfusion only) rat kidneys (Fig. 6). Rats subjected to renal ischemia–reperfusion also exhibited a substantial increase in kidney MDA levels on comparison with sham-operated rats (Fig. 7), suggesting increased lipid peroxidation subsequent to oxidative stress. However, administration of both caspase-1 inhibitor II or caspase-3 inhibitor I prior to ischemia–reperfusion produced significant reductions in MDA levels on comparison with MDA levels obtained from the kidneys of rats subjected to ischemia–reperfusion only (Fig. 7). MPO activity and MDA levels were not determined in the kidneys of rats subjected to ischemia–reperfusion and which were administered caspase inhibitor III.

4. Discussion

In the present study, we show that ischemia–reperfusion of the rat kidney results in a significant renal dysfunction and injury, and together with evidence of increased oxidative and nitrosative stress, confirmed a recognizable pattern of renal dysfunction and injury caused by ischemia–reperfusion of the kidney (Paller, 1994a; Thadhani et al., 1996; Weight et al., 1996; Kribben et al., 1999; Sheridan and Bonventre, 2001). We demonstrate here that administration of caspase inhibitors prior to ischemia–reperfusion of the kidney can provide varying degrees of protection against renal dysfunction and injury and reduce the degree of renal oxidative and nitrosative stress. Specifically, administration of a caspase-1 inhibitor (caspase-1 inhibitor II) provided the largest degree of protection against renal dysfunction and injury. Administration of a caspase-3 inhibitor (caspase-3 inhibitor I) provided markedly less protection, whereas administration of a pan-caspase inhibitor (caspase inhibitor III) had no effect on the renal dysfunction and injury caused by ischemia–reperfusion of the rat kidney. However, both caspase-1 inhibitor II and caspase-3 inhibitor I reduced oxidative and nitrosative stress of the kidney to a similar degree.

Specifically, we report here that caspase-1 inhibitor II, a cell-permeable and irreversible inhibitor of caspase-1 and -4 (Brenner et al., 1998; Thornberry and Lazebnik, 1998), was able to reduce significantly the renal dysfunction and injury caused by ischemia–reperfusion of the rat kidney. Caspase-1 is a proinflammatory protease, which mediates apoptosis and activates cytokines such as interleukins-1 and -18 within the kidney (Daemen et al., 2001b; Melnikov et al., 2001). Caspase-1 is implicated in the development of renal ischemia–reperfusion injury (Kaushal et al., 1998; Kaushal, 2003), and its expression is up-regulated during ischemia–reperfusion of the kidney (Kaushal et al., 1998; Kaushal, 2003). Furthermore, caspase-1 knockout mice are protected against renal ischemia–reperfusion injury with reductions in both tubular necrosis and infiltration of polymorphonuclear neutrophils (Melnikov et al., 2001). This is supported by the findings of our study in which caspase I inhibitor II was able to reduce both renal ICAM-1 expression and MPO activity, suggesting a reduction of polymorphonuclear neutrophil infiltration (and thus oxidative stress).

We also report here that caspase-3 inhibitor I, a very potent, specific, and reversible inhibitor of caspase-3 and also caspase-6, -7, -8, and -10 (Garcia-Calvo et al., 1998; Thornberry and Lazebnik, 1998), was not able to provide effective protection against renal ischemia–reperfusion injury. Activation of caspase-3 leads to apoptosis and has been implicated in several disease states including heart failure and myocardial ischemia–reperfusion injury and

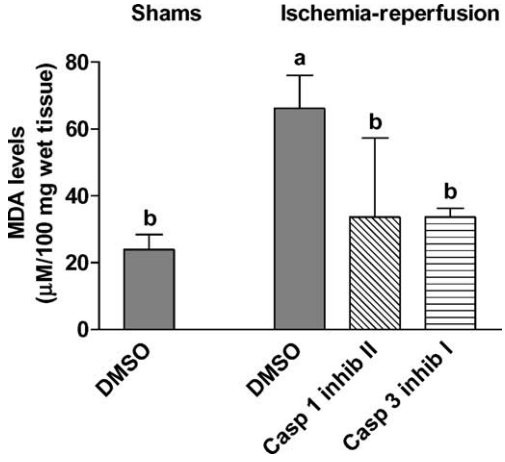

Fig. 7. Effect of caspase inhibitors on kidney malondialdehyde (MDA) levels. MDA levels were measured subsequent to sham-operation (Shams) or renal ischemia–reperfusion (Ischemia–Reperfusion) in the absence or presence of caspase-1 inhibitor II (Casp-1 inhib) or caspase-3 inhibitor I (Casp-3 inhib), 3 mg/kg, administered 30 min prior to renal ischemia–reperfusion. $P<0.05$ vs. DMSO (Sham) group (a), $P<0.05$ vs. DMSO (Ischemia–Reperfusion) group (b), $n=4$ rats.

stroke (Yaoita et al., 1998; Narula et al., 1999; Chapman et al., 2002; Joly et al., 2004). Specifically within the kidney, renal ischemia–reperfusion leads to an increased expression of caspase-3 with prolonged ischemia promoting apoptosis (Kaushal et al., 1998; Chien et al., 2000). The inability of the caspase-3 inhibitor to reduce renal injury in our model of ischemia–reperfusion injury is not altogether surprising as the mode of cell death associated with severe models of ischemic acute renal failure such as the one used in this study is predominantly via necrosis, whereas caspase-3 activation mediates apoptosis. It is therefore possible that caspase-3 inhibition would have provided more benefit after longer periods of reperfusion, during which apoptosis overtakes necrosis as the primary form of cell death (Lieberthal and Levine, 1996; Ueda and Shah, 2000; Padanilam 2003), similar to the course of neonatal ischemia in the rat brain (Joly et al., 2004).

In this study, we also observed that the pan-caspase inhibitor caspase inhibitor III a cell-permeable, irreversible, broad-spectrum caspase inhibitor, which inhibits the activity of all caspases (D'Mello et al., 1998; Thornberry and Lazebnik, 1998), did not produce any beneficial effects against the renal dysfunction and injury caused by ischemia–reperfusion. These findings were similar to those of Joly et al. (2004) who investigated the effects of caspase inhibitor III on ischemic injury in the neonatal rat brain. Specifically, it was shown that although this pan-caspase inhibitor was able to reduce caspase-3 activity by almost 80%, there was no significant reduction of infarct volume (Joly et al., 2004). As the pan-caspase inhibitor used in our study also inhibits caspase-1 activity, it seems surprising that this agent was not able to reduce the renal dysfunction and injury caused by ischemia–reperfusion; however, it is possible that this pan-caspase inhibitor also inhibited the activity of caspases which may have protective functions during ischemia–reperfusion. This is supported by evidence that caspases can regulate cell cycle progression (Los et al., 2001), and as tubular regeneration is a fundamental part of renal recovery after ischemia–reperfusion injury (Gobe et al., 1999; Padanilam, 2003), it is possible that inhibition of all caspases during ischemia–reperfusion is detrimental. Finally, as this pan-caspase inhibitor also inhibits caspase-3 activity, it may be possible that caspase inhibitor III may be able to provide protection in the later stages of reperfusion injury by reducing the degree of apoptosis.

Overall, our study investigates the beneficial action of caspase-1 inhibition in a short-term model of ischemic acute renal failure; however, investigation of the effects of caspase inhibitors on the course of renal ischemia–reperfusion injury over the course of days is certainly warranted, especially to investigate if the antiapoptotic effects of caspase-3 inhibition may also provide benefits against renal ischemia–reperfusion injury. It appears that a pan-caspase inhibitor, which inhibits all caspases, did not provide any protection against renal ischemia–reperfusion injury in the short term, possibly due to inhibition of caspases which may provide protection against ischemia–reperfusion injury. This aspect of renal caspase function certainly warrants further investigation.

In conclusion, the results presented here indicate that inhibition of caspase-1 activity may be useful in enhancing the tolerance of the kidney against renal dysfunction and injury in situations where renal tissues may be subjected to periods of ischemia followed by reperfusion, e.g., during aortovascular surgery or renal transplantation.

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