Calpain inhibitor-1 reduces renal ischemia/reperfusion injury in the rat

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Background. Activation of the cysteine protease calpain has been implicated in renal ischemia/reperfusion (I/R) injury. The aim of this study was to investigate the effects of calpain inhibitor-1 (Cal I-1) in an in vivo model of renal I/R injury.

Methods. Male Wistar rats were administered Cal I-1 (10 mg/kg, IP) 30 minutes before undergoing bilateral renal ischemia (45 minutes) followed by reperfusion (6 hours). Plasma concentrations of urea, creatinine, Na+, γ-glutamyl transferase (γGT), aspartate aminotransferase (AST) and urinary Na+, glutathione S-transferase (GST), and N-acetyl-β-D-glucosaminidase (NAG) were measured for the assessment of renal dysfunction and I/R injury. Creatinine clearance (C_cr) and fractional excretion of Na+ (FE_{Na}) were used as indicators of glomerular and tubular function, respectively. Kidney myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels were measured for assessment of neutrophil infiltration and lipid peroxidation, respectively. Renal sections were used for histologic grading of renal injury and for immunohistochemical localization of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).

Results. Cal I-1 significantly reduced I/R-mediated increases in urea, creatinine, γGT, AST, NAG, and FE_{Na} and significantly improved C_cr. Cal I-1 also significantly reduced kidney MPO activity and MDA levels. Cal I-1 also reduced histologic evidence of I/R-mediated renal damage and caused a substantial reduction in the expression of iNOS and COX-2, both of which involve activation of nuclear factor-κB (NF-κB).

Conclusions. These results suggest that Cal I-1 reduces the renal dysfunction and injury associated with I/R of the kidney. We suggest that the mechanism could involve the inhibition of I/R-mediated activation of NF-κB.

Key words: kidney injury, inducible nitric oxide synthase, COX-2, nuclear factor-κB, acute renal failure.

Renal ischemia is one of the most common causes of acute renal failure (ARF), initiating a complex and interrelated sequence of events, resulting in injury to and the eventual death of renal cells [1, 2], with proximal tubule (PT) cells demonstrating particular susceptibility [2, 3]. Although reperfusion is essential for the survival of ischemic tissue, there is good evidence that reperfusion itself causes additional cellular injury (reperfusion injury) [3], which has been attributed to the generation of reactive oxygen species (ROS), adenosine 5'-triphosphate (ATP) depletion, neutrophil infiltration, phospholipase activation and membrane lipid alterations, cytoskeletal dysfunction, and intracellular Ca^{2+} accumulation [3-5]. Numerous studies have demonstrated a role for Ca^{2+} in the pathophysiology of renal injury associated with both hypoxia and ischemia/reperfusion (I/R) [5, 6]. Intracellular accumulation of Ca^{2+} under ischemic conditions results in the activation of Ca^{2+}-dependent enzymes such as calpain and nitric oxide synthase (NOS), which are implicated in the pathophysiology of ARF [6, 7].

Calpains are nonlysosomal neutral cysteine proteases that are activated by Ca^{2+} and that appear to be involved in Ca^{2+} signaling in mammalian cells [8]. To date, two major isoforms have been identified: calpain I (or μ-calpain) and calpain II (or m-calpain), which require low (μmol/L) and high (mmol/L) Ca^{2+} concentrations for activation, respectively [8]. Following activation, calpain selectively cleaves a specific subset of cellular proteins, including cytoskeletal and membrane proteins, several enzymes, and transcription factors [9], including nuclear factor-κB (NF-κB) [10], which is involved in the expression of including inducible NOS (iNOS) [11] and cyclooxygenase-2 (COX-2) [12].

Calpain inhibitor-1 (Cal I-1) is a cell-permeable peptide aldehyde that blocks the active site of calpain [13, 14]. Along with other inhibitors of calpain such as
E64 or PD150606 and the naturally occurring calpain inhibitor protein calpastatin, Cal I-1 has been used to investigate the role of the excessive activation of calpain in the pathophysiology of a variety of disorders, including cataract, restenosis, arthritis, stroke, and myocardial infarction [13, 14]. Several studies have demonstrated that calpain activation is implicated in the pathophysiology of I/R injury in several organs, including the brain [15], heart [16], and liver [17]. Interestingly, I/R has also been reported to attenuate calpastatin activity [18]. In the kidney, there is evidence from in vitro studies using rat and rabbit PT suspensions that calpain activation is involved in the cellular injury/death associated with hypoxia [19, 20] and exposure to toxic agents [21, 22], and that calpain inhibitors may be beneficial under these conditions [19, 20, 22]. Although there is some evidence that calpain is activated in the ischemic rat kidney in vivo [23], the beneficial actions of calpain inhibitors in animal models of renal I/R remain unclear [24].

Several studies have demonstrated that Cal I-1 can reduce the proteolytic cleavage of IkB-α/β from its complex with the NF-κB/Rel A unit and, hence, inhibit the activation of NF-κB and its subsequent translocation from the cytosol into the nucleus [25–27]. Thus, Cal I-1 can reduce the expression (for example, after exposure to endotoxin or inflammatory cytokines) of many NF-κB-dependent genes, including iNOS [26–28] and COX-2 [28]. However, to date, such an effect has not been demonstrated in renal tissues in vitro or in vivo.

Therefore, the aim of this study was to investigate the effects of Cal I-1 on renal dysfunction/injury caused by renal I/R in the anesthetized rat. In order to gain a better insight into the mechanism of action of Cal I-1, we also investigated the effects of Cal I-1 on the expression of iNOS and COX-2 in kidneys of rats subjected to renal I/R. Furthermore, the actions of Cal I-1 were compared with that of the serine protease inhibitor chymostatin.

METHODS

Animal preparation

In vivo studies were carried out using 56 male Wistar rats (Tuck, Rayleigh, Essex, UK) weighing 200 to 250 g, that received a standard diet and water ad libitum and were cared for in accordance with both the Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986, published by H.M.S.O. (London, UK) and the Institutional Animal Research Committee Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication number 85-23, revised 1996). All animals were anesthetized with sodium thiopentone (Intraval® Sodium, 120 mg/kg IP; Rhone Merieux Ltd., Essex, UK), and anesthesia was maintained by supplementary intravenous infusions of sodium thiopentone. Animals were prepared surgically for renal I/R as described previously [29]. Briefly, anesthetized rats were placed onto a thermostatically controlled heating mat (Harvard Apparatus Ltd., Kent, UK), and body temperature was maintained at 38 ± 1°C by means of a rectal probe attached to a homeothermic blanket. A tracheotomy was performed to maintain airway patency and to facilitate spontaneous respiration. The right carotid artery was cannulated (PP50, I.D. 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 (MAP) and derivation of the heart rate (HR) from the pulse waveform, which were displayed on a data-acquisition system (MacLab 8e, AD Instruments, Hastings, UK) installed on an Apple Macintosh computer. MAP and HR were monitored for the duration of each experiment. The jugular vein was cannulated (PP25, I.D. 0.40 mm; Portex) for the administration of drugs. A midline laparotomy was performed and the bladder was cannulated (PP90, I.D. 0.76 mm; Portex). Both kidneys were located, and the renal pedicles, containing the artery, vein, and nerve supplying each kidney, were carefully isolated.

Renal ischemia/reperfusion

As described previously [29], rats were allowed to stabilize for 30 minutes before they were subjected to bilateral renal occlusion for 45 minutes using artery clips to clamp the renal pedicles. Reperfusion commenced once the artery clips were removed (control animals). 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 (sham operated) where identical surgical procedures to control animals were used, except that they did not undergo bilateral renal clamping and were maintained under anesthesia for the duration of the experiment. At the end of all experiments, animals were killed by an overdose of sodium thiopentone.

Experimental protocol

Upon completion of surgical procedures, the animals were randomly allocated into seven groups.

(1) I/R control group. These animals underwent renal ischemia for 45 minutes followed by reperfusion for six hours (N = 12).

(2) I/R Cal I-1 group. These animals were administered Cal I-1 (10 mg/kg IP) 30 minutes prior to I/R as described previously (N = 12). Cal I-1 was dissolved in 50% (vol/vol) EtOH and 50% (vol/vol) saline. We have previously shown this dose and method of administration of Cal I-1 to (a) inhibit an increase in calpain activity [28], (b) reduce the expression of iNOS and COX-2 [28, 30], and (c) to attenuate the activation of NF-κB [30] associated with endotoxic and hemorrhagic shock in the rat [28, 30].
(3) **I/R chymostatin group.** These animals were administered chymostatin (10 mg/kg IP) 30 minutes prior to I/R (N = 6). Chymostatin was dissolved in 50% (vol/vol) EtOH and 50% (vol/vol) saline.

(4) **I/R vehicle group.** These animals were administered the vehicle for Cal I-1 and chymostatin [50% EtOH/50% saline (vol/vol) IP] 30 minutes prior to I/R (N = 6).

(5) **Sham group.** Sham-operated rats were subjected to identical surgical procedures described previously in this article, except for renal I/R, and they were maintained under anesthesia for the duration of the experiment (that is, 45 minutes + 6 hours, N = 12).

(6) **Sham Cal I-1 group.** These were identical to the sham-operated animals except for the pretreatment with Cal I-1 (10 mg/kg IP) 30 minutes prior to surgery (N = 4).

(7) **Sham vehicle group.** These were identical to sham-operated animals except they were pretreated with vehicle [50% EtOH/50% saline (vol/vol) IP] 30 minutes prior to the surgery (N = 4).

All animals in these seven groups received a continuous infusion of 0.9% (wt/vol) saline (4 mL/kg/h, IV) throughout the I/R period.

**Measurement of biochemical parameters**

At the end of the reperfusion period, blood (1 mL) samples were collected via the carotid artery. The samples were centrifuged (6000 r.p.m. for 3 minutes) to separate plasma. All plasma samples were analyzed for biochemical parameters within 24 hours after collection (Vetlab Services, Sussex, UK). Plasma concentrations of urea and creatinine were measured as indicators of impaired glomerular function [31]. Plasma concentrations of γ-glutamyl transferase (γGT) and aspartate aminotransferase (AST), enzymes that are both located in the PT [32], were used as indicators of renal reperfusion injury (Discussion section).

Urine samples were collected during the reperfusion period, and the volume of urine produced was recorded. Urine concentrations of creatinine and Na⁺ were measured (Vetlab Services) at the end of the reperfusion period and were used in conjunction with plasma concentrations to estimate creatinine clearance (C_Cr) and fractional excretion of Na⁺ (FE_Na) using standard formulae. These were used as respective indicators of glomerular and tubular function during the reperfusion period. Concentrations of urinary glutathione S-transferase (GST) and N-acetyl-β-D-glucosaminidase (NAG), specific indicators of tubular damage [33, 34], were also measured (Laboratory of Pharmacology, University of Lisbon, Lisbon, Portugal).

**Determination of myeloperoxidase activity**

Myeloperoxidase (MPO) activity in kidneys was used as an indicator of neutrophil infiltration using a previously described method [35]. Briefly, at the end of the experiments, kidney tissue was weighed and homogenized in a solution containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7.4) and centrifuged for 30 minutes at 20,000 × g at 4°C. An aliquot of supernatant was then removed and added to a reaction mixture containing 1.6 mmol/L tetramethylbenzidine and 0.1 mmol/L hydrogen peroxide (H₂O₂). 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 H₂O₂ at 37°C and was expressed in mIU/100 mg wet tissue.

**Determination of malondialdehyde levels**

Levels of malondialdehyde (MDA) in kidneys were determined as an indicator of lipid peroxidation following a protocol described previously [36]. Briefly, kidney tissue was weighed and homogenized in a 1.15% (wt/vol) KCl solution. A 100 μL aliquot of homogenate was then removed and added to a reaction mixture containing 200 μL 8.1% (wt/vol) lauryl sulfate, 1.5 mL 20% (vol/vol) acetic acid, 1.5 mL 0.8% (wt/vol) thiobarbituric acid, and 700 μL distilled water. Samples were then boiled for 1 hour at 95°C and centrifuged at 3000 × g for 10 minutes. The absorbance of the supernatant was measured spectrophotometrically at 650 nm. MDA levels were expressed as μmol/L MDA/100 mg wet tissue.

**Histologic evaluation**

At postmortem, a 5 mm section of kidney was removed and placed in formalin and processed through to wax. Five micrometer sections were cut and stained with hematoxylin and eosin. Histologic assessment of tubular necrosis was determined semiquantitatively using a method modified from McWhinnie et al [37]. Random cortical fields were observed using a ×20 objective. A graticule grid (25 squares) was used to determine the number of line intersects involving tubular profiles. One hundred intersections were examined for each kidney, and a score from 0 to 3 was given for each tubular profile involving an intersection: 0 = normal histology (Fig. 6A); 1 = tubular cell swelling, brush border loss, nuclear condensation, with up to one third of the tubular profile showing nuclear loss (Fig. 6B); 2 = same as for score 1, but greater than one third and less than two thirds of the tubular profile show nuclear loss (Fig. 6C); and 3 = greater than two thirds of the tubular profile showing nuclear loss (Fig. 6D).

The total score for each kidney was calculated by the addition of all 100 scores with a maximum score of 300.

**Immunohistochemical analysis of iNOS and COX-2 expression**

The expression of iNOS and COX-2 proteins was evaluated in kidneys from untreated (sham-operated), con-
trol, and animals administered Cal I-1 using immunohistochemical protocols described previously [38]. At the end of the experiment, kidneys were bisected, snap frozen in liquid nitrogen, and stored at −70°C. When required, samples were thawed, dehydrated using graded ethanol, and embedded in Paraplast, after which 8 μm sections were cut. After deparaffinization, endogenous peroxidase was quenched using 0.3% (vol/vol) H2O2 in 60% (vol/vol) methanol for 30 minutes. Sections were permeablized using 0.1% (vol/vol) Triton X-100 in phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.4) for 20 minutes. Nonspecific adsorption was minimized by incubating the section in 2% (vol/vol) normal goat serum (DBA, Milan, Italy) in PBS for 20 minutes. Sequential incubation for 15 minutes with avidin and biotin (DBA) was used to block endogenous binding sites. The sections were then incubated overnight with 1:1000 dilution of primary anti-iNOS or anti-COX-2 antibody (DBA) or with control solutions. Controls included buffer alone or nonspecific purified rabbit IgG (DBA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG (DBA) and avidin-biotin peroxidase complex (DBA).

To verify the binding specificity for iNOS or COX-2 (negative controls), some sections were also incubated with only the secondary antibody (no primary antibody). Positive controls for iNOS and COX-2 binding were performed on sections of kidney obtained from mice that were administered lipopolysaccharide (LPS; 50 mg/kg, IV).

Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). Cal I-1 was purchased from Calbiochem Novabiochem (Nottingham, UK). All solutions used for in vivo infusions were prepared using non-pyrogenic saline [0.9% (wt/vol) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK].

Statistical analysis

All values described in the text and figures are expressed as mean ± SEM for N observations. For in vivo studies, each data point represents biochemical measurements obtained from 6 to 12 separate animals. For histologic scoring, each data point represents analysis of kidneys taken from 6 to 12 individual animals. For immunohistochemical analysis, the figures shown are representative of at least three experiments performed on different experimental days. Statistical analysis was carried out using GraphPad Prism/InStat (GraphPad Software, Inc., San Diego, CA, USA). Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test. A P value of less than 0.05 was considered to be significant (NS, nonsignificant).

RESULTS

Effect of Cal I-1 and chymostatin on ischemia/reperfusion-mediated glomerular dysfunction

Animals that underwent renal I/R exhibited significant increases in the plasma concentrations of urea and creatinine compared with sham-operated animals (Fig. 1) and a significant reduction in C0 (Fig. 2A), suggesting a significant degree of glomerular dysfunction.

Pretreatment of rats with Cal I-1 prior to I/R produced relatively small, but significant, reductions in the plasma levels of urea and creatinine (Fig. 1) and increased C0 significantly (Fig. 2A). The serine protease inhibitor chymostatin, administered prior to I/R, did not have a significant effect on the increased plasma urea and creatinine concentrations or the reduced C0 associated with I/R (Figs. 1 and 2A). The administration of vehicle for
Cal I-1 and chymostatin [50% (vol/vol) EtOH] to rats prior to I/R did not result in any significant alterations on plasma levels of urea or creatinine or in $C_\text{Cr}$ compared with control animals (Figs. 1 and 2A). The administration of Cal I-1 or vehicle to sham-operated rats did not result in any alteration in plasma levels of urea or creatinine or in $C_\text{Cr}$ in comparison with sham-operated animals administered saline only (data not shown).

**Effects of Cal I-1 and chymostatin on ischemia/reperfusion-mediated tubular dysfunction/injury**

Fractional excretion of sodium ($\text{FE}_{\text{Na}}$), calculated using plasma and urinary concentrations of Na⁺ in association with urine production (urine flow, mL/min), was used as an indicator of tubular function. I/R produced a significant increase in $\text{FE}_{\text{Na}}$, suggesting tubular dysfunction (Fig. 2B); however, administration of Cal I-1 prior to I/R produced a significant reduction in $\text{FE}_{\text{Na}}$, suggesting improvement in tubular function (Fig. 2B). The administration of chymostatin or vehicle did not produce significant alterations in $\text{FE}_{\text{Na}}$ compared with control animals, and administration of Cal I-1 to sham-operated rats did not alter $\text{FE}_{\text{Na}}$ in comparison with values obtained from sham-operated animals administered saline only (Fig. 2B).

Renal I/R produced a significant increase in the urinary concentrations of both GST and NAG (Fig. 3), suggesting significant tubular injury. The administration of Cal I-1 significantly reduced both urinary GST and NAG concentrations, suggesting attenuation of tubular injury. The administration of chymostatin or vehicle did not produce significant alterations in urinary GST or NAG concentrations compared with control animals (Fig. 3). The administration of Cal I-1 or vehicle to the sham-operated animals did not alter GST or NAG con-
concentrations in comparison with values obtained from sham-operated animals administered saline only (data not shown).

**Effects of Cal I-1 and chymostatin on ischemia/reperfusion-mediated reperfusion injury**

Renal I/R produced significant increases in the plasma concentrations of γ-GT and AST in comparison with values obtained from sham-operated animals (Fig. 4). Plasma concentrations of γ-GT and AST, which were used as markers of reperfusion injury, were significantly reduced subsequent to pretreatment with Cal I-1 prior to I/R (Fig. 4). The administration of chymostatin or vehicle did not produce significant alterations in plasma levels of γ-GT or AST in comparison with control animals (Fig. 4).

The administration of Cal I-1 or vehicle to the sham-operated animals did not alter plasma concentrations of γ-GT or AST in comparison with values obtained from sham-operated animals administered saline only (data not shown).

**Effects of Cal I-1 and chymostatin on kidney MPO activity and MDA levels**

Rats subjected to renal I/R exhibited a substantial increase in kidney MPO activity and MDA levels, suggesting increased neutrophil infiltration and lipid peroxidation, respectively (Fig. 5). However, pretreatment of rats with Cal I-1 prior to I/R produced a significant reduction of MPO activity in comparison with the activity obtained from control rat kidneys (Fig. 5A). Similarly, pretreatment of rats with Cal I-1 produced a significant reduction of the MDA levels associated with I/R (Fig. 5B).

The administration of Cal I-1 or vehicle to the sham-operated animals did not alter plasma concentrations of γ-GT or AST in comparison with values obtained from sham-operated animals administered saline only (data not shown).
Fig. 6. Histologic evaluation of renal I/R. Histology of a normal proximal tubule (PT) (A) is compared with tubules revealing brush border loss, nuclear condensation, and cytoplasmic swelling and loss of nuclei in up to one third of the tubular profile (B). More severe cellular loss, with between one third and two thirds of the tubular profile denuded of nuclei (C), and greater than two thirds of nuclei lost (D) are also represented. HandE, ×700. (E) Effect of renal I/R on total severity score in the presence of calpain inhibitor-1 (Cal I-1, 10 mg/kg IP), chymostatin (Chymo, 10 mg/kg IP), or vehicle [50% (vol/vol) EtOH/saline]. *P < 0.05 vs. sham-operated group; +P < 0.05 vs. control group (45 minutes of ischemia and 6 hours of reperfusion).

5B). The administration of chymostatin or vehicle prior to I/R did not have a significant effect on MPO activity or MDA levels in comparison with values obtained from control animals (Fig. 5).

Effects of Cal I-1 and chymostatin on I/R-mediated renal histopathology

In comparison with the normal tubular histology observed in kidneys taken from sham-operated rats (Fig. 6A), animals that underwent renal I/R demonstrated the recognized features of severe acute tubular damage (Fig. 6D) [39]. These features included brush border loss, nuclear condensation, cytoplasmic swelling, and a consistent loss of significant numbers of nuclei from tubular profiles (Fig. 6D).

When compared with the total severity score measured from kidneys obtained from sham-operated animals, I/R produced a significant increase in total severity score, which was significantly reduced by administration of Cal I-1 prior to I/R (Fig. 6E). The administration of chymostatin or vehicle to rats prior to I/R did not have a significant effect on total severity score (Fig. 6E).

Immunohistochemical localization of iNOS and COX-2 formation

When compared with kidney sections obtained from sham-operated rats (Fig. 7A), immunohistochemical analysis of sections obtained from rats subjected to renal I/R revealed positive staining for iNOS (Fig. 7B). In contrast, substantially reduced staining was observed in the kidney sections obtained from rats administered Cal I-1 (Fig. 7C). The positive control for iNOS staining, performed on sections of kidney obtained from mice subjected to endotoxemia, demonstrated marked positive staining for iNOS protein (Fig. 7D). No positive staining for iNOS protein was observed in kidney sections from rats subjected to renal I/R, which were incubated with the secondary antibody only (no primary antibody–negative control; Fig. 7E).

In comparison with kidney sections obtained from sham-operated rats (Fig. 8A), sections prepared from rat kidneys subjected to I/R demonstrated marked staining for COX-2 (Fig. 8B). Kidneys obtained from rats administered Cal I-1 demonstrated markedly reduced staining for COX-2 upon comparison with kidneys obtained from control animals (Fig. 8C), suggesting a reduction in the expression of COX-2 subsequent to pretreatment with Cal I-1 prior to I/R. Staining for endogenous biotin was not detected in any sections. The positive control for COX-2 staining, performed on sections of kidney obtained from mice subjected to endotoxemia, demonstrated marked positive staining for COX-2 protein (Fig. 8D). In contrast, no positive staining for COX-2 protein could be observed in kidney sections from rats subjected to renal I/R that were incubated with the secondary antibody only (no primary antibody–negative control; Fig. 8E).

DISCUSSION

There is good evidence from both in vivo and in vitro studies that calpain activation plays an important role in the pathophysiology of renal injury mediated by hypoxia and I/R [23, 40]. We demonstrate here, to our knowledge for the first time, that the administration of Cal I-1 prior
to renal I/R significantly reduces the renal dysfunction and injury caused by I/R of the rat kidney. This conclusion is supported by four key findings: In a rat model of renal I/R, Cal I-1 significantly reduced the I/R-mediated increases in (1) the plasma levels of urea and creatinine, (2) the FE_{Na} and urinary concentrations of GST and NAG, (3) the plasma levels of γGT and AST, and (4) MPO activity and the levels of MDA in the kidney. Cal I-1 also significantly reduced the histologic evidence of I/R-mediated tubular injury and substantially reduced the immunohistochemical evidence of iNOS and COX-2 expression, suggesting that the protective mechanism provided by Cal I-1 may involve, to some degree, the inhibition of the activation of NF-κB.

Ischemia/reperfusion of the kidney causes both glomerular and tubular dysfunction [41]. In this study, the moderate but significant increases in the plasma concentrations of urea and creatinine subsequent to I/R suggest the impairment of glomerular function [31], and this was reflected by a significant reduction in C_{Cr} observed subsequent to I/R. However, I/R also resulted in significant increases in urinary NAG and GST concentrations, which can be regarded as markers for tubular injury and possibly tubular function [33, 34]. This was reflected by a significant increase in FE_{Na}. Taken together, these markers suggest a significant reduction in tubular function and increased tubular injury in this model of renal I/R.

Although there are recognized limitations in the use clearance techniques to measure C_{Cr} and FE_{Na} in renal models that involve extensive tubular injury, the compar-
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Fig. 8. Immunohistochemical localization of cyclooxygenase-2 (COX-2) in rat kidney following I/R. (A) Kidney section from Sham animal (OM ×80). (B) Control group (renal I/R only, OM ×32) with typical areas of COX-2 immunoreactivity indicated by arrows. COX-2 immunoreactivity of kidneys from rats treated with Cal I-1 (C) was markedly reduced in comparison with staining obtained from the control group (OM ×80). (D) Positive control for COX-2 immunoreactivity; Kidney sections were prepared from mice subjected to endotoxemia (LPS, 50 mg/kg IV). Typical areas of COX-2 immunoreactivity are indicated by arrows (OM ×80). (E) Negative control for COX-2 immunoreactivity; Kidney sections were prepared from rats subjected to I/R only and incubated with secondary antibody only (no primary antibody, OM ×125). Figures are representative of at least three experiments performed on different days.

Atively larger increase in the markers of tubular dysfunction/injury (FE\textsubscript{Na}, NAG, GST) suggests that there is a greater dysfunction and injury to the tubules rather than the glomeruli in this model of renal I/R. Marked tubular damage in models of I/R (such as the one used here) has been demonstrated previously [42] and was reflected, in this study, in the histopathological analysis of kidneys subsequent to I/R obtained from control animals.

Renal I/R also produced significant increases in plasma concentrations of \( \gamma \)GT and AST, both of which are regarded as nonspecific markers of extensive cellular disruption or necrosis [31] and were used in this study as markers of renal I/R. Both enzymes are present within the PT [32] and can be released into the urine subsequent to renal injury [43, 44]. Furthermore, a recent study has demonstrated that \( \gamma \)GT is significantly elevated subsequent to short-term ischemia of the rat kidney [45]. Therefore, it is certainly feasible that the increased plasma levels measured subsequent to renal I/R originated from the kidney after extensive damage to the tubular architecture. Renal I/R also caused a significant increase in both MPO activity, indicating neutrophil accumulation, and in MDA levels, indicating increased lipid peroxidation, and taken together, increased oxidative stress.

In this study, Cal I-1 produced a significant reduction of renal dysfunction and injury mediated by I/R of this kidney. Cal I-1 appears to provide a greater beneficial action against tubular, rather than glomerular, dysfunction. This is supported by our findings that compared
with rats subjected to I/R only, Cal I-1 produced relatively small, but significant, decreases in plasma urea and creatinine levels. This moderate effect on glomerular function was reflected by the small, but also significant, increase in Ccr. In contrast, Cal I-1 had a marked effect on markers of tubular dysfunction (FE_{Na}) and injury (urinary NAG and GST). This was further supported by the renal histopathology that revealed the recognized features of severe acute tubular damage in rat kidneys subjected to I/R only, which were markedly reduced by Cal I-1.

Calpain inhibitor-1 readily permeates cell membranes and subsequently inhibits calpain activation [13, 14]. Although not measured in this study, one potential mechanism of action by which Cal I-1 could produce its beneficial effects is via the inhibition of cysteine protease (calpain) activity. However, this is unlikely to provide a significant mechanism of protection in this model of renal I/R as (1) other cysteine protease inhibitors such as leupeptin and calpeptin do not protect against toxicant-mediated renal cell death and ischemic ARF, respectively [22, 24] and (2) serine protease inhibitors such as antipain have also been shown not to reduce ischemia-mediated ARF [22]. In this study, chymostatin, another potent inhibitor of serine proteases such as chymotrypsin and papain [46], did not affect I/R-mediated renal dysfunction and injury. This is in keeping previous reports from our laboratory that chymostatin, at the same dose as that used in this study, does not protect against the circulatory failure and multiple organ dysfunction mediated by endotoxic [28] or hemorrhagic shock [30]. Taken together, this supports the view that an inhibition of protease activity is unlikely to account for the beneficial action of Cal I-1 observed in this study.

Another putative mechanism for the beneficial actions observed with Cal I-1 in this study is related to its ability to attenuate the activation of NF-κB [25–28, 30]. NF-κB is a member of a family of dimers belonging to the Rel/NF-κB family of polypeptides, and the most frequently observed form of NF-κB is a dimer composed of two DNA-binding proteins, namely NF-κB (or p50) and RelA (or p65), although other dimeric combinations also exist [47]. In this study, Cal I-1 reduced the renal I/R-mediated expression of both iNOS and COX-2 protein, as demonstrated using immunohistochemistry, and it is our hypothesis that Cal I-1 mediates this effect via inhibition of the expression of NF-κB. This notion is supported by direct evidence obtained in this laboratory demonstrating that (1) Cal I-1 inhibits LPS-stimulated NF-κB DNA binding activity in RAW 264.7 macrophages [30] and that (2) Cal I-1 inhibits LPS-mediated degradation of IκBα, IκBβ, and IκBε in rat vascular smooth muscle cell cultures [30]. Furthermore, in our hands and in keeping with findings that we have previously reported [28, 30], chymostatin did not have any effect on the I/R-mediated expression of iNOS or COX-2 protein (data not shown).

Although this is the first report to our knowledge of such an effect of Cal I-1 in renal tissues, the mechanism(s) by which Cal I-1, but not chymostatin, potentially inhibits the activation of NF-κB and subsequently the attenuation of iNOS (and COX-2) expression certainly warrants further investigation.

In conclusion, this study demonstrates that Cal I-1 reduces the renal dysfunction and injury caused by renal I/R in the anesthetized rat. Cal I-1 also reduces the I/R-mediated expression of iNOS and COX-2 in the rat kidney. Thus, we speculate that the inhibition by Cal I-1 of the activation of NF-κB may contribute to the beneficial effects of Cal I-1 in I/R injury of the rat kidney.

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APPENDIX

Abbreviations used in this article are: ARF, acute renal failure; AST, aspartate aminotransferase; ATP, adenosine 5′-triphosphate; Cal I-1, calpain inhibitor-1; Ccr, creatinine clearance; COX-2, cyclooxygenase-2; FE_{Na}, fractional excretion of sodium; γGT, gamma-glutamyl transferase; GST, glutathione S-transferase; HR, heart rate; iNOS, inducible nitric oxide synthase; IR, ischemia/reperfusion; MAP, mean arterial pressure; MDA, malondialdehyde; MPO, myeloperoxidase; NAG, N-acetyl-β-d-glucosaminidase; NF-κB, nuclear factor-κB; NOS, nitric oxide synthase; PT, proximal tubule; ROS, reactive oxygen species.

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