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Inhibitors of calpain activation (PD150606 and E-64) and renal ischemia-reperfusion injury

Prabal K. Chatterjee [a,*], Zoran Todorovic [b], Ahila Sivarajah [a], Helder Mota-Filipe [c], Paul A.J. Brown [d], Keith N. Stewart [e], Emanuela Mazzon [f], Salvatore Cuzzocrea [f], Christoph Thiemermann [a]

[a] *Centre for Experimental Medicine, Nephrology and Critical Care, William Harvey Research Institute, Queen Mary-University of London, London, UK*
[b] *Department of Clinical Pharmacology, Pharmacology & Toxicology, School of Medicine, University of Belgrade, Serbia, Yugoslavia*
[c] *Laboratory of Pharmacology, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal*
[d] *Department of Pathology, University of Aberdeen, Aberdeen, UK*
[e] *Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, UK*
[f] *Department of Clinical and Experimental Medicine & Pharmacology, Institute of Pharmacology, School of Medicine, University of Messina, Messina, Italy*

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Abstract

Calpain activation has been implicated in the development of ischemia-reperfusion (I-R) injury. Here we investigate the effects of two inhibitors of calpain activity, PD150606 and E-64, on the renal dysfunction and injury caused by I-R of rat kidneys in vivo. Male Wistar rats were administered PD150606 or E-64 (3 mg/kg i.p.) or vehicle (10%, v/v, DMSO) 30 min prior to I-R. Rats were subjected to bilateral renal ischemia (45 min) followed by reperfusion (6 h). Serum and urinary biochemical indicators of renal dysfunction and injury were measured; serum creatinine (for glomerular dysfunction), fractional excretion of Na^+ (FE_{Na}, for tubular dysfunction) and urinary *N*-acetyl-β-D-glucosaminidase (NAG, for tubular injury). Additionally, kidney tissues were used for histological analysis of renal injury, immunohistochemical analysis of intercellular adhesion molecule-1 (ICAM-1) expression and nitrotyrosine formation. Renal myeloperoxidase (MPO) activity (for polymorphonuclear leukocyte infiltration) and malondialdehyde (MDA) levels (for tissue lipid peroxidation) were determined. Both PD150606 and E-64 significantly reduced the increases in serum creatinine, FE_{Na} and NAG caused by renal I-R, indicating attenuation of renal dysfunction and injury and reduced histological evidence of renal damage caused by I-R. Both PD150606 and E-64 markedly reduced the evidence of oxidative stress (ICAM-1 expression, MPO activity, MDA levels) and nitrosative stress (nitrotyrosine formation) in rat kidneys subjected to I-R. These findings provide the first evidence that calpain inhibitors can reduce the renal dysfunction and injury caused by I-R of the kidney and may be useful in enhancing the tolerance of the kidney against renal injury associated with aortovascular surgery or renal transplantation.

Keywords: Renal/kidney; Ischemia; Reperfusion-injury; Calpain inhibitor; PD150606; E-64

1. Introduction

Despite significant advances in critical care medicine, ARF remains a major clinical problem, causing considerable morbidity and mortality that has not decreased significantly over the last 50 years [1]. As previous pharmacological interventions against ARF have proven to be largely negative in the clinical setting, the development of novel therapeutic interventions against ARF has remained a topic of intense research interest [1]. Renal ischemia is a major cause of ARF, initiating a complex and interrelated sequence of events resulting in injury to, and the eventual death of renal cells via both apoptotic and necrotic renal cell death pathways [2,3]. Furthermore, although essential for the survival of ischemic renal tissue,

Abbreviations: ARF, acute renal failure; FE_{Na}, fractional excretion of Na^+; ICAM-1, intercellular adhesion molecule-1; I-R, ischemia-reperfusion; MDA, malondialdehyde; MPO, myeloperoxidase; NAG, *N*-acetyl-β-D-glucosaminidase; NF-κB, nuclear factor-kappaB; PMN, polymorphonuclear leukocyte

* Corresponding author. Tel.: +44 1273 642078; fax: +44 1273 642674.
E-mail address: p.k.chatterjee@brighton.ac.uk (P.K. Chatterjee).

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renal reperfusion causes additional damage (reperfusion injury) and together, I-R of the kidney leads to ischemic ARF [4].

Two groups of cysteine proteases, caspases and calpains, are involved in the development of the acute renal injury caused by I-R of the kidney [3]. Calpains, which are calcium-dependent non-lysosomal cysteine proteases, participate in several normal physiological cellular processes including signal transduction involving calcium signalling, remodelling of cytoskeletal-membrane attachment and apoptosis [5–7]. Two major isoforms of calpain were originally identified: calpain 1 (or μ-calpain, CAPN1) and calpain 2 (m-calpain, CAPN2), which require low (μM) and high (mM) calcium concentrations for activation, respectively [5]. More recently, 14 mammalian calpain genes and proteins have been characterised, many of which are implicated in pathological conditions [5–8]. For example, calpains 1 and 2 have been associated with stroke, traumatic brain injury, Alzheimer's disease and the development of cataracts, mutations of calpain 3 are implicated in limb-girdle muscular dystrophy and cataracts, the calpain 9 gene is down-regulated in gastric cancer and calpains 8 and 10 are implicated in the pathophysiology of type 2 diabetes mellitus [5]. Thus, excessive calpain activation, subsequent to intracellular calcium accumulation, has been identified in a variety of disorders. Several studies have demonstrated that calpain activation is involved in the development of I-R injury in several organs including the brain [9,10], heart [11–13] and liver [14,15]. I-R also inhibits the activity of the endogenous calpain inhibitor, calpastatin [16], thereby contributing to excessive calpain activation. Excessive activation of calpain has been implicated in the pathophysiology of ischemic ARF [17–19] and in vitro studies have demonstrated that calpain activation is involved in the cellular injury and death caused by renal hypoxia and nephrotoxic agents [20–23]. There is also evidence that calpain is activated in vivo in the ischemic rat kidney [24].

Many calpain inhibitors have been developed over the previous decade [25–27] and subsequently, the beneficial effects of calpain inhibition during I-R of many organs have been reported. Specifically, agents such as PD150606, E-64, calpain inhibitor-1, NS-7, A-705239 and calpastatin have been used to investigate the role of excessive calpain activation in the brain and heart [11,23,28–33]. Furthermore, the ability of calpain inhibitor-1 to reduce the organ injury caused by hemorrhagic shock (which involves I-R of several major organs including the kidney) has also been reported [34]. With respect to the kidney, PD150606, SJA7019, SJA7029, *Z*-Leu-Phe-COOH, *Z*-Leu-Abu-CONH-CH_2-CH(OH)-Ph and *Z*-Leu-Phe-CONH-Et have been shown to protect rabbit renal proximal tubular cells against antimycin A-induced cell death in vitro [23,35,36]. The α-mercaptoacrylic acid derivative PD150606 [3-(4-iodophenyl)-2-mercapto-(*Z*)-2-propenoic acid] is a non-peptide, cell-permeable, uncompetitive and selective calpain inhibitor which binds to the Ca^{2+}-binding domain of calpains 1 and 2 with high affinity only when the substrate is bound to protease [26,29,37]. Although the effectiveness of PD150606 in in vivo models of ischemic disease has yet to be fully elucidated, one investigation has recently shown that intracerebroventricular administration of 100 μM PD150606 into rat brains subjected to ischemic insult was able to effectively inhibit hippocampal calpain activity [38]. In contrast, several in vitro investigations have revealed the beneficial effects of inhibition of calpain activation by PD150606. For example, PD150606 was able to provide inhibit calpain activation in two intact cell systems and thereby provide neuroprotection [29,38]. At 100 μM, PD150606 was able to effectively reduce calpain activation and reduce cell death in rat and rabbit renal proximal tubules subjected to hypoxia or nephrotoxins [21,39,40]. E-64 [*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane] is both structurally and pharmacologically different to PD150606 [26]. E-64 is an irreversible, general inhibitor of cysteine proteases which, at 40 μM, was able to effectively reduce fodrin breakdown in slices of rat cerebral cortex subsequent to calpain activation caused by hypoxia/hypoglycaemia and thereby provide neuroprotection [28]. E-64 was also able to effectively reduce calpain activation and reduce cell death in rabbit renal proximal tubules suspensions exposed to diverse nephrotoxins such as antimycin A and bromohydroquinone [40] and reduce proteinuria in an in vivo experimental model of glomerulonephritis [41]. Although not as cell permeable as PD150606, there is some evidence that the uptake and subsequent effectiveness of E-64 may be related to a generalised increase in membrane permeability [42] which can prevail during renal I-R [4,43]. Furthermore, E-64d, an esterified and more cell-permeable analogue of E-64, has been shown to reduce calpain activation in rat myocardial tissues after global ischemia [12].

Few studies have investigated the effects of calpain inhibitors on the renal dysfunction and injury caused by I-R of the kidney in vivo. We have previously demonstrated that calpain inhibitor-1 can reduce renal dysfunction and injury caused by I-R of the rat kidney via attenuation of the expression of pro-inflammatory genes primarily via inhibition of the transcription factor NF-κB [34,44]. The present study was designed to evaluate the effectiveness of more potent and specific calpain inhibitors, specifically, the structurally and chemically distinct inhibitors PD150606 and E-64, in an established in vivo rat model of renal I-R injury [45]. Subsequently the ability of these calpain inhibitors to reduce the oxidative and nitrosative stress associated with renal I-R was elucidated using a combination of established biochemical and immunohistological assays.

Table 1
Experimental groups studied

Experimental group	Number of rats (*N*)	Protocol
Renal I-R saline	12	Rats subjected to renal ischemia for 45 min followed by reperfusion for 6 h
Renal I-R DMSO	12	Rats administered the vehicle for PD150606 and E-64 (10%, v/v, DMSO) i.p. 30 min pre I-R
Renal I-R PD150606	8	Rats administered PD150606 (3 mg/kg) i.p. 30 min pre I-R
Renal I-R E-64	6	Rats administered E-64 (3 mg/kg) i.p. 30 min pre I-R
Sham saline	12	Rats subjected to identical surgical procedures described above except for renal I-R
Sham DMSO	12	Identical to Sham Saline group except for the administration of the vehicle (10%, v/v, DMSO) i.p. 30 min earlier
Sham PD150606	4	Identical to Sham Saline group except for the administration of PD150606 (3 mg/kg) i.p. 30 min earlier
Sham E-64	4	Identical to Sham Saline group except for E-64 administration (3 mg/kg) i.p. 30 min earlier

2. Materials and methods

2.1. Experimental protocol

In vivo studies were carried out using 70 male Wistar rats (Tuck) weighing 200–250 g and receiving a standard diet and water ad libitum. Animals were cared for 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. Rats were anesthetised using sodium thiopentone (Intraval® Sodium, 120 mg/kg i.p.; Rhone Merieux Ltd.) and anesthesia was maintained using supplementary i.v. injections of sodium thiopentone. Animals were prepared surgically as described previously [44,45] and were randomly allocated into eight groups (Table 1). All animals received a continuous infusion of 0.9% (w/v) saline (4 ml $(kg\ h)^{-1}$, i.v.).

The doses of PD150606 and E-64 used in this study (3 mg/kg) and regimen of administration were based on previous investigations in which we have shown that similar doses of another calpain inhibitor (calpain inhibitor-1) were able to reduce significantly the renal dysfunction and injury associated with I-R and hemorrhagic shock [34,44]. Furthermore, a dose of 3 mg/kg was chosen in order to provide plasma levels in rats of a similar magnitude to that shown to effectively reduce calpain activation in in vitro studies. Specifically, 100 μM PD150606 and E-64 were able to effectively reduce calpain activation and reduce cell death in rat and rabbit renal proximal tubules subjected to hypoxia or nephrotoxins [21,39,40]. Based on the molecular weights of PD150606 and E-64 (306.1 and 357.4, respectively) and a 25 ml plasma volume of a 250 g rat, we calculated that administration of 3 mg/kg would produce plasma levels of approximate 100 μM. At higher dosing concentrations, e.g. 10 mg/kg, both calpain inhibitors suffered from insolubility.

2.2. Measurement of biochemical parameters

At the end of the reperfusion period, blood (1 ml) samples were collected via the carotid artery into tubes containing serum gel, centrifuged (6000 rpm for 3 min) to separate serum and analysed within 24 h (Vetlab Services). Serum creatinine levels were measured as an indicator of glomerular function [44,45]. Urine concentrations of Na^+ were measured (Vetlab Services) and used in conjunction with serum Na^+ concentrations to estimate FE_{Na} which was used as an indicator of tubular function. Urinary concentrations of NAG, an indicator of tubular injury [44,45], were measured (Clínica Médica é Diagnóstico Dr. Joaquim Chaves, Lisbon, Portugal) and adjusted for urinary creatinine levels.

2.3. Histological evaluation

Renal sections were prepared as described previously [46] and used for histological assessment of I-R 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).

2.4. Immunohistochemical analysis

Evidence of ICAM-1 expression and nitrotyrosine formation were determined using immunohistochemical protocols as described recently [46,47]. Briefly, kidney sections were incubated overnight at 4 °C with primary anti-ICAM-1 or anti-nitrotyrosine antibodies (1:500, v/v, in PBS [0.01 M, pH 7.4], DBA). Sections were also incubated with control solutions containing either PBS alone or a 1:500 dilution of non-specific purified rabbit immunoglobulin G (DBA). Specific labelling was detected using a biotin-conjugated goat anti-rabbit immunoglobulin G (DBA) and avidin–biotin peroxidase (DBA) and viewed under a light microscope. Immunohistochemistry photographs were assessed using densitometry as previously described [48] using Optilab Graftek software on a Macintosh personal computer.

2.5. Determination of myeloperoxidase activity

MPO activity in kidneys was used as an indicator of PMN infiltration, using a protocol previously described [44]. Briefly, kidney tissue was weighed and homogenised in a solution containing 0.5% (w/v) hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.4) 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 and MPO activity was defined as the quantity of enzyme required to degrade 1 μmol of hydrogen peroxide at 37 °C.

2.6. Determination of malondialdehye levels

Levels of MDA in kidneys were determined as an indicator of lipid peroxidation as described previously [44]. 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 sulphate, 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.

2.7. Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd. PD150606 and E-64 were purchased from Calbiochem Novabiochem. All solutions used for in vivo infusions were prepared using non-pyrogenic saline (0.9%, w/v, NaCl; Baxter Healthcare Ltd.).

2.8. Statistical analysis

All values described in the text and figures are expressed as SEM for *N* observations. For in vivo studies, each data point represents biochemical measurements or histological scores obtained from 4–12 separate animals. For histological and 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 1.1 (GraphPad Software) using one-way analysis of variance (ANOVA) followed by Dunnett's post-significance testing. A *P*-value of less than 0.05 was considered to indicate significance (NS: non-significant).

3. Results

3.1. Effect of PD150606 and E-64 on renal dysfunction caused by I-R

Compared to Sham-operated animals, rats which underwent renal I-R exhibited a significant increase in both serum creatinine concentrations and FE_{Na} (Fig. 1A and B) suggesting a significant degree of glomerular and tubular dysfunction, respectively. Pre-treatment of rats with PD150606 or E-64 prior to I-R produced significant reductions in both serum levels of creatinine and FE_{Na} (Fig. 1A and B). Administration of vehicle for PD150606 and E-64 (10%, v/v, DMSO) to rats prior to I-R did not result in any significant alterations of serum creatinine levels or FE_{Na} compared to animals administered saline only (Fig. 1A and B). Administration of PD150606, E-64 or vehicle to Sham-operated rats did not result in any alteration in serum creatinine levels or in FE_{Na} on comparison with Sham-operated animals administered saline only (Fig. 1A and B).

3.2. Effect of PD150606 and E-64 on renal injury caused by I-R

Renal I-R produced a significant increase in NAG enzymuria suggesting significant tubular injury which

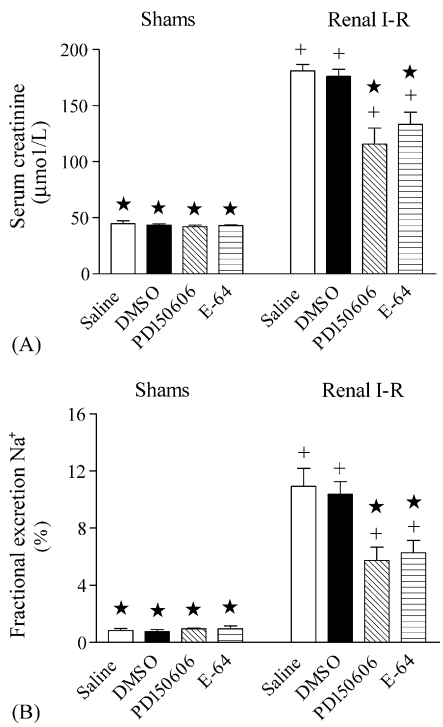

Fig. 1. Effect of calpain inhibitors on renal dysfunction. Serum creatinine levels (A) and fractional excretion of Na^+ (B) were measured subsequent to Sham-operation (Shams) or renal I-R (Renal I-R) in the absence or presence of PD150606 or E-64, 3 mg/kg, administered 30 min prior to renal I-R. (+) $P < 0.05$ vs. DMSO (Sham) group, (★) $P < 0.05$ vs. DMSO (Renal I-R) group, $N = 4$–12 rats.

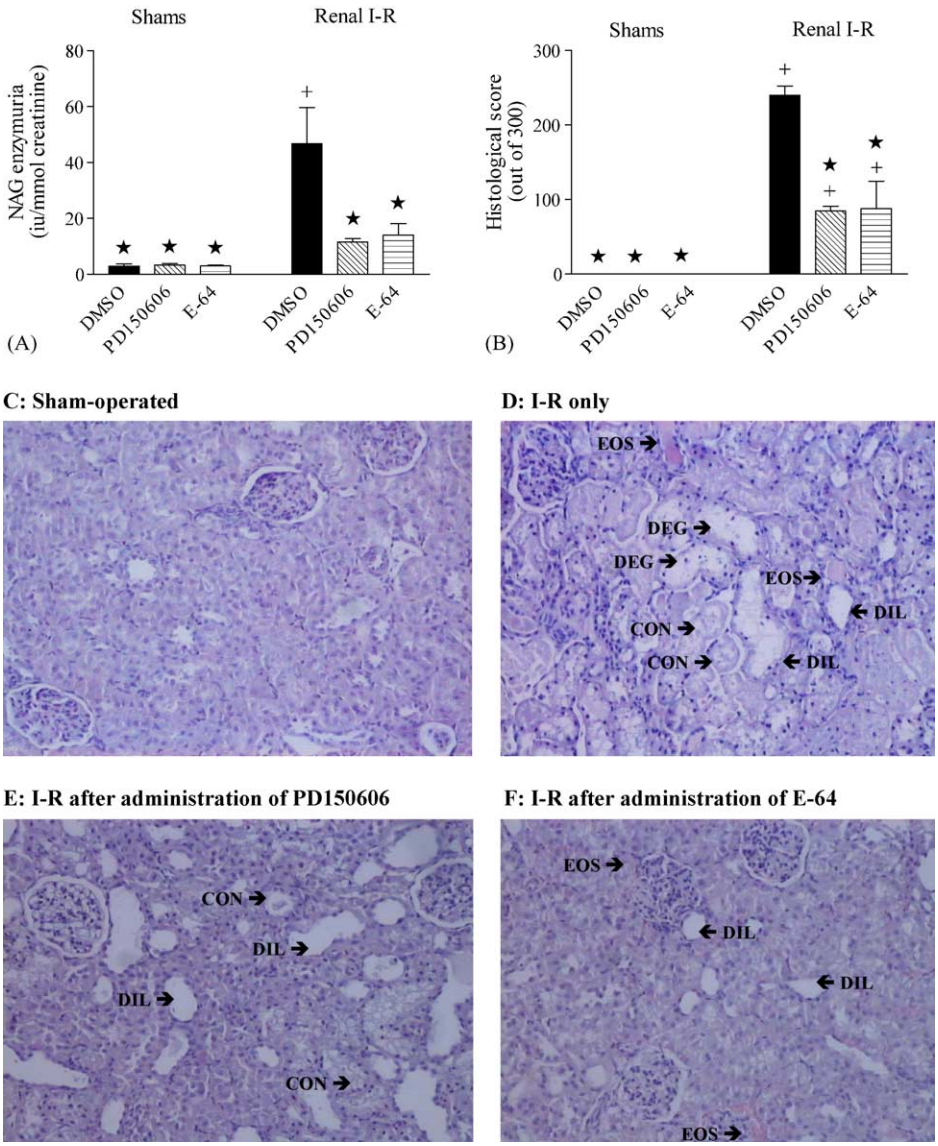

Fig. 2. Effect of calpain inhibitors on renal (tubular) injury. Urinary *N*-acetyl-β-D-glucosaminidase activity (A) and histological score (B) were measured subsequent to Sham-operation (Shams) or renal I-R (Renal I-R) in the absence or presence of PD150606 or E-64, 3 mg/kg, administered 30 min prior to renal I-R. (+) $P < 0.05$ vs. DMSO (Sham) group, (★) $P < 0.05$ vs. DMSO (Renal I-R) group, $N = 4$–12 rats. A kidney section obtained from a Sham-operated rat (C) is compared to that taken from a rat subjected to 45 min ischemia followed by 6 h reperfusion (D). Renal sections obtained from rats subjected to renal I-R after administration of PD150606 (E) or E-64 (F) 3 mg/kg 30 min prior to I-R, are also represented. Rats that underwent I-R demonstrated the recognised histological features of renal injury (D–F). Specifically this included tubular degeneration (DEG), e.g. loss of nuclei, congestion (CON), dilatation (DIL) and eosinophilia (EOS). Hemotoxylin and eosin, original magnification ×125, figures are representative of at least three experiments performed on different days.

was significantly reduced by administration of PD150606 or E-64 prior to I-R (Fig. 2 A). Administration of PD150606 or E-64 to Sham-operated animals did not alter NAG enzymuria when compared to that measured in 'Sham DMSO' rats (Fig. 2A). When compared to the histological score measured from kidneys obtained from Sham-operated animals, I-R produced a significant increase in histological score suggesting marked renal injury caused by I-R (Fig. 2B). Histological evidence of renal injury was significantly reduced by administration of PD150606 or E-64 prior to I-R (Fig. 2B). Administration of PD150606 or E-64 to Sham-operated rats did not have a significant effect on histological score when compared to the histological scores measured in 'Sham DMSO' rats (Fig. 2B). Compared to the normal histological features of kidney sections obtained from Sham-operated rats (Fig. 2C), rats which were subjected to renal I-R demonstrated the characteristic histological features of renal injury such as tubular degeneration and dilatation, luminal congestion and eosinophilia (as indicated in Fig. 2D). In contrast, renal sections obtained from rats administered PD150606 or E-64 prior to renal I-R demonstrated marked reduction of renal injury (Fig. 2E and F). Specifically, although some tubular dilation and luminal congestion

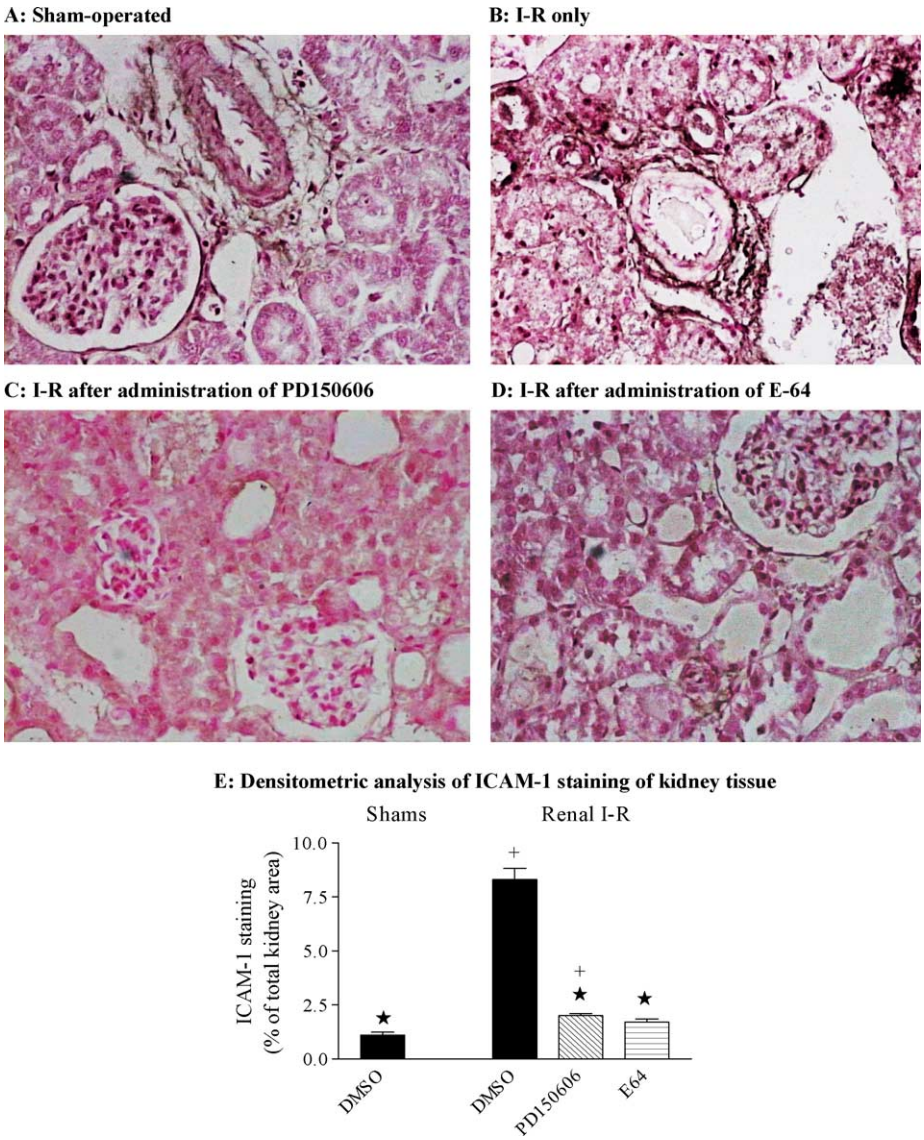

Fig. 3. Effect of calpain inhibitors on ICAM-1 expression. Kidneys sections were incubated at 4 °C overnight with 1:500 dilution of a primary antibody directed against ICAM-1. Kidney sections taken from (A) a Sham-operated rat, (B) a rat subjected to 45 min ischemia followed by 6 h, a rat subjected to renal I-R after administration of PD150606 (C) or E-64 (D) 3 mg/kg 30 min prior to I-R. Original magnification ×250, figures are representative of at least three experiments performed on different days. (E) Densitometric analysis of ICAM-1 staining of kidney tissue sections obtained from rat subsequent to Sham-operation (Shams) or renal I-R (Renal I-R) in the absence or presence of PD150606 or E-64, 3 mg/kg, administered 30 min prior to renal I-R. (+) $P < 0.05$ vs. DMSO (Sham) group, (★) $P < 0.05$ vs. DMSO (Renal I-R) group, $N = 6$ rats.

were still apparent in kidney sections from rats pre-treated with PD150606 prior to renal I-R (Fig. 2E), tubular degeneration was significantly reduced (Fig. 2B). Similarly, although some tubular dilation and eosinophilia were observed in kidney sections from rats pre-treated with E-64 prior to renal I-R (Fig. 2F), tubular degeneration was also significantly reduced (Fig. 2B).

3.3. Effect of PD150606 and E-64 on ICAM-1 expression during renal I-R

On immunohistochemical analysis, kidney sections obtained from Sham-operated rats demonstrated a small degree of staining for constitutive expression of the adhesion molecule ICAM-1 in the vascular endothelium of normal tissues (Fig. 3A). In contrast, kidney sections prepared from rats subjected to I-R demonstrated marked staining for ICAM-1 (Fig. 3B), suggesting increased adhesion molecule expression during reperfusion. Kidneys obtained from rats administered PD150606 demonstrated markedly reduced staining for ICAM-1 (Fig. 3C) when compared with kidneys obtained from rats subjected to renal I-R 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 E-64 prior to renal I-R (Fig. 3D). These findings were confirmed by densitometic analysis (Fig. 3E).

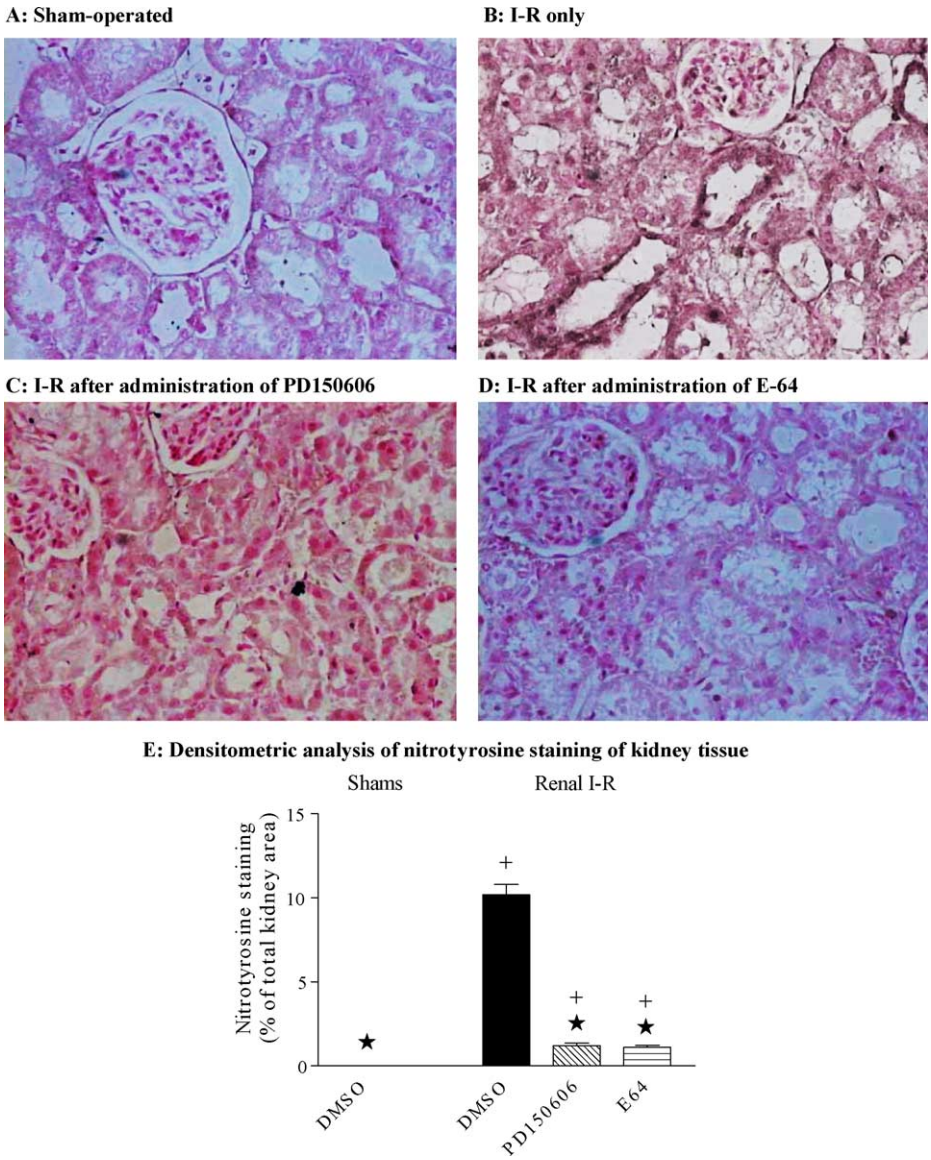

Fig. 4. Effect of calpain inhibitors on nitrotyrosine formation. Kidneys sections were incubated at 4 °C overnight with 1:500 dilution of a primary antibody directed against nitrotyrosine. Kidney sections taken from (A) a Sham-operated rat, (B) a rat subjected to 45 min ischemia followed by 6 h reperfusion and rats subjected to renal I-R after administration of PD150606 (C) or E-64 (D) 3 mg/kg 30 min prior to I-R. Original magnification ×250, figures are representative of at least three experiments performed on different days. (E) Densitometric analysis of nitrotyrosine staining of kidney tissue sections obtained from rat subsequent to Sham-operation (Shams) or renal I-R (Renal I-R) in the absence or presence of PD150606 or E-64, 3 mg/kg, administered 30 min prior to renal I-R. (+) $P < 0.05$ vs. DMSO (Sham) group, (★) $P < 0.05$ vs. DMSO (Renal I-R) group, $N = 6$ rats.

3.4. Effect of PD150606 and E-64 on nitrotyrosine formation during renal I-R

Compared to immunohistochemical analysis of kidney sections obtained from Sham-operated rats (Fig. 4A), rats subjected to renal I-R revealed positive staining for nitrotyrosine (Fig. 4B). In contrast, reduced nitrotyrosine staining was observed in kidney sections obtained from rats subjected to renal I-R but which were administered PD150606 (Fig. 4C) or E-64 (Fig. 4D) prior to ischemia. These findings were confirmed by densitometic analysis (Fig. 4E).

3.5. Effects of PD150606 and E-64 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 PMN infiltration into renal tissues and lipid peroxidation, respectively (Fig. 5A and B). However, pre-treatment of rats with PD150606 or E-64 prior to I-R produced a significant reduction of MPO activity on comparison with the activity obtained from 'Renal I-R DMSO' rat kidneys (Fig. 5A). Similarly, pre-treatment of rats with PD150606 or E-64 produced a significant reduction of the

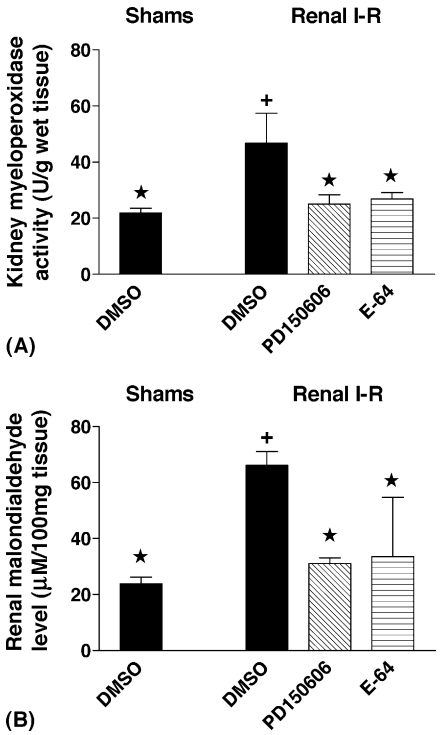

Fig. 5. Effect of calpain inhibitors on kidney myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels. MPO activity (A) and MDA levels (B) were measured subsequent to Sham-operation (Shams) or renal I-R (Renal I-R) in the absence or presence of PD150606 or E-64, 3 mg/kg, administered 30 min prior to renal I-R. (+) $P < 0.05$ vs. DMSO (Sham) group, (★) $P < 0.05$ vs. DMSO (Renal I-R) group, $N = 4$ rats.

MDA levels associated with I-R (Fig. 5B). Reduction of both parameters by PD150606 and E-64 produced levels which were not significantly different to those measured from the renal tissues of 'Sham DMSO' rats (Fig. 5A and B).

4. Discussion

There is now 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 [19–21,23,24,39]. We demonstrate here, for the first time, that administration of the calpain inhibitors PD150606 and E-64 prior to renal I-R reduces significantly the renal dysfunction and injury caused by I-R of the rat kidney. This conclusion is supported by the following key findings: in a rat model of renal I-R, both PD150606 and E-64 significantly reduced the I-R-mediated increases in: (i) serum levels of creatinine, (ii) fractional excretion of Na^+ (FE_{Na}), (iii) NAG enzymuria and (iv) histological evidence of I-R-mediated renal injury. Both PD150606 and E-64 reduced oxidative and nitrosative stress of the kidney by a similar degree. Specifically, PD150606 and E-64 reduced the expression of ICAM-1 and MPO activity, indicating attenuation of PMN infiltration into kidney tissues and reduced MDA levels, indicating decreased lipid peroxidation. Together these findings suggest that PD150606 reduced oxidative stress of the kidney caused by I-R. Finally, the reduction of nitrotyrosine formation observed in kidney tissues subjected to I-R but pre-treated with PD150606 or E-64 indicate decreased formation of peroxynitrite, and therefore, nitrosative stress.

In this study, both PD150606 and E-64 produced significant reductions in renal dysfunction and injury mediated by I-R of this kidney. Although both produced a similar degree of renoprotection, both also provided a greater beneficial action against tubular, rather than glomerular, dysfunction. This is supported by our findings that, compared to rats subjected to I-R only (Sham Saline group), PD150606 and E-64 produced a relatively small, but significant, decrease in serum creatinine levels. In contrast, both PD150606 and E-64 had a marked effect on markers of tubular dysfunction (FE_{Na}) and injury (NAG) and this profile of injury was further supported by histological analysis. In view of the fact that the proximal tubule of the kidney is most susceptible to I-R injury and that calpain activation is implicated [23,43,49], it is not surprising that both PD150606 and E-64 were able to demonstrate a marked beneficial effect of tubular dysfunction and injury. Overall, our study investigates the beneficial action of calpain inhibition in a short-term model of ischemic acute renal failure; however, investigation of the effects of calpain inhibitors on the course of renal I-R injury over the course of days is certainly warranted, especially to investigate if the reduction in oxidative and nitrosative stress mediated by PD150606 and E-64 reported here can provide longer-term benefits against renal I-R injury.

PD150606 and E-64 are chemically distinct but potent inhibitors of calpain activation. PD150606 is a reversible non-peptide inhibitor which is a derivative of α-mercaptoacrylic acid which inhibits both μ- and m-calpain, whereas E-64 is an irreversible peptide inhibitor [25,26]. Although not measured directly in this study, both agents are likely to have produced a significant inhibition of cysteine protease (calpain) activity and there is good evidence from several in vitro studies that such inhibition can reduce renal cell death [35,36,39]. The contribution of the inhibition of calpain activity during renal I-R in vivo warrants further investigation in view of reports that other cysteine protease inhibitors such as leupeptin and calpeptin *do not* protect against toxicant-mediated renal cell death and ischemic ARF, respectively [40,50]. Furthermore, inhibitors of serine proteases such as antipain do not reduce ischemia-mediated ARF [40] and we have previously shown that chymostatin, another potent inhibitor of serine protease, does not reduce renal dysfunction and injury caused by I-R [44].

The beneficial effects of calpain inhibition during I-R of several organs have been reported. Specifically, agents such as E-64 and PD150606 have been used to investigate the role of excessive calpain activation in the brain and

heart. However, there are few studies in which the effects of calpain inhibitors on the renal dysfunction and injury caused by I-R of the kidney have been investigated. We have previously demonstrated that calpain inhibitor-1 can reduce the renal dysfunction and injury associated with I-R and hemorrhagic shock of the rat kidney via attenuation of the expression of pro-inflammatory genes primarily via inhibition of the activation of the transcription factor NF-κB [34,44]. Although not investigated in this study, another putative mechanism for the beneficial actions observed using PD150606 and E-64 in this study could involve inhibition of NF-κB activation. 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 [51]. There is good evidence that inhibitors of the proteosome pathway can block NF-κB activation [52,53] and we, and others, have demonstrated that calpain inhibitor-1 can block NF-κB activation and subsequently reduce the expression of pro-inflammatory genes in vitro and in models of endotoxic and hemorrhagic shock [34,44,54,55].

The potential of PD150606 and E-64 to modulate the activation of NF-κB certainly warrants further and more detailed investigation. Although there is some evidence that E-64 (and calpain inhibitor II) do not block NF-κB activation in human cells [56], we have demonstrated here that both PD150606 and E-64 were able to attenuate PMN infiltration into renal tissues (reduced MPO activity) subsequent to reduced ICAM-1 expression during renal I-R. Renal expression of ICAM-1 is dependent on activation of NF-κB during I-R [57,58] and we have previously demonstrated that inhibition of the activation of NF-κB during renal I-R will reduce ICAM-1 expression [46,59]. As there is some evidence that calpain inhibition may actually *induce* PMN adhesion, polarisation and rapid chemokinesis in the absence of exogenous activators [60], the results of our study suggest that the reduction of PMN recruitment by PD150606 and E-64 is secondary to the reduced activation of NF-κB during renal I-R activation followed by attenuated ICAM-1 expression. Although this will require further investigation for the calpain inhibitors used in this study, there is already some evidence that other calpain inhibitors (e.g. calpain inhibitor-1) are also able to reduce NF-κB activation during I-R and shock, leading to reduced gene expression [34,44]—an action which appears to be separate to their inhibition of calpain activation.

In this study, we evaluated the effectiveness of more potent and specific calpain inhibitors, specifically, the structurally and chemically different calpain inhibitors PD150606 and E-64, in an established in vivo rat model of renal I-R injury. We demonstrate here, for the first time, that PD150606 and E-64 reduce the renal dysfunction and injury caused by renal I-R in the anesthetised rat. In conclusion, our results indicate that PD150606, E-64 and similar agents may be useful in enhancing the tolerance of the kidney against renal dysfunction and injury in situations where renal tissues are subjected to I-R, e.g. during aortovascular surgery, renal transplantation or possibly hemorrhagic shock.

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