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Pyrrolidine dithiocarbamate reduces renal dysfunction and injury caused by ischemia/reperfusion of the rat kidney

Prabal K. Chatterjee[a,1], Roberta D'Emmanuele di Villa Bianca[b], Ahila Sivarajah[a], Michelle C. McDonald[a], Salvatore Cuzzocrea[c], Christoph Thiemermann[a,*]

[a] *Department of Experimental Medicine, Nephrology and Critical Care, William Harvey Research Institute, Queen Mary, University of London, Charterhouse Square, London EC1M 6BQ, UK*
[b] *Department of Experimental Pharmacology, University of Naples, Naples, Italy*
[c] *Department of Clinical and Experimental Medicine and Pharmacology, Institute of Pharmacology, School of Medicine, University of Messina, Messina, Italy*

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Abstract

Dithiocarbamates can modulate the expression of genes associated with inflammation or development of ischemia/reperfusion injury. Here, we investigate the effects of pyrrolidine dithiocarbamate, an inhibitor of nuclear factor (NF)-κB activation, 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. Pyrrolidine dithiocarbamate (100 mg/kg, administered i.v.) significantly reduced biochemical and histological evidence of renal dysfunction and injury caused by ischemia/reperfusion of the rat kidney. Furthermore, pyrrolidine dithiocarbamate markedly reduced the expression of inducible nitric oxide synthase (iNOS) protein and significantly reduced serum levels of nitric oxide. Finally, pyrrolidine dithiocarbamate inhibited the activation of NF-κB by preventing its translocation from the cytoplasm into the nuclei of renal cells. These results demonstrate that pyrrolidine dithiocarbamate reduces renal ischemia/reperfusion injury and that dithiocarbamates may provide beneficial actions against ischemic acute renal failure.

Keywords: Renal/kidney; Reperfusion-injury; Dithiocarbamates; Pyrrolidine dithiocarbamate; Nuclear factor-κB; Inducible nitric oxide synthase; Nitric oxide

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 (Lameire and Vanholder, 2001). Previous interventions against acute renal failure have proved to be largely negative and dialysis still remains the only effective therapy (Star, 1998). Thus, the development of novel therapeutic interventions against acute renal failure has remained a topic of intense research interest (Star, 1998; Lameire and Vanholder, 2001). Renal ischemia initiates a complex and interrelated sequence of events, resulting in the injury and death of renal cells (Lieberthal and Levine, 1996; Thadhani et al., 1996). Reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage (reperfusion injury) (Paller, 1994a; Weight et al., 1996). Together, ischemia/reperfusion of the kidney contribute to the renal dysfunction and injury associated with ischemic acute renal failure (Thadhani et al., 1996; Weight et al., 1996).

The transcription factor nuclear factor (NF)-κB, a heterotrimeric complex composed of p50, p65 (Rel A) and inhibitor (I)κB-α (IκB-α) (Boone et al., 2002; Sun and Andersson, 2002), plays a pivotal role in the expression of numerous genes and mediators involved in normal and pathophysiological processes including inducible nitric oxide synthase (iNOS), cyclooxygenase-2, adhesion molecules and numerous cytokines and chemokines (Xie et al., 1994; Baeuerle and Baichwal, 1997; Barnes and Karin, 1997; Sun and Andersson, 2002). The expression of several of these genes and mediators has been implicated in the pathophysiology of renal disease and development of acute renal failure, indicating an important role for NF-κB activation (Guijarro and Egido, 2001; Wardle, 2001).

* Corresponding author. Tel.: +44-20-7882-6025; fax: +44-20-7251-1685.
E-mail address: c.thiemermann@qmul.ac.uk (C. Thiemermann).
[1] Current address: Department of Pharmacology, School of Pharmacy and Biomolecular Sciences, University of Brighton, Cockcroft Building, Moulsecoomb, Brighton BN2 4GJ, UK.

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Dithiocarbamates represent a class of antioxidants, which are also potent inhibitors of NF-κB (Schreck et al., 1992). The metal-chelating properties of the diethyl derivative of dithiocarbamate (diethyldithiocarbamate) have been exploited for decades for the treatment of metal poisoning in humans (Sunderman, 1992) and diethyldithiocarbamate has been used to retard the onset of acquired immune deficiency syndrome in human immunodeficiency virus-infected individuals via inhibition of NF-κB activation (Reisinger et al., 1990; Schreck et al., 1991). Of the known dithiocarbamates, the most effective NF-κB inhibitor is the pyrrolidine derivative of dithiocarbamate (pyrrolidine dithiocarbamate), which has been attributed to its ability to traverse cell membranes and its prolonged stability in solution at physiological pH (Topping and Jones, 1988).

The potential for the modulation of NF-κB activation by dithiocarbamates suggests that these agents may offer therapeutic benefit against renal disease states involving ischemia/reperfusion injury or inflammation. Pyrrolidine dithiocarbamate has been shown to reduce chronic inflammation in a rat model of zymosan-induced multiple organ failure (Cuzzocrea et al., 2003). In models of chronic renal failure, inhibition of NF-κB activation by pyrrolidine dithiocarbamate provides beneficial actions against established renal disease including adriamycin-induced nephropathy and Heymann nephritis (Mudge et al., 2001; Rangan et al., 2001). Pyrrolidine dithiocarbamate also inhibits NF-κB activation leading to amelioration of renal inflammation caused by angiotensin II (Muller et al., 2000) and reduces cortical tubulointerstitial injury in proteinuric rats (Rangan et al., 1999a). Recently, pyrrolidine dithiocarbamate has been shown to reduce the development of renal dysfunction in an in vivo model of zymosan-induced multiple organ failure in mice (Cuzzocrea et al., 2003). In vitro, inhibition of NF-κB by pyrrolidine dithiocarbamate attenuates cytokine-stimulated iNOS expression in mesangial cells (Eberhardt et al., 1994; Satriano and Schlondorff, 1994) and cytokine expression in rat proximal tubular cells incubated with bacterial lipopolysaccharide (Rangan et al., 1999b). Recently, inhibition of NF-κB activation by pyrrolidine dithiocarbamate has been shown to reduce nephropathy (including tubular injury and interstitial fibrosis) caused by chronic administration of tacrolimus (FK506) to rats (Tamada et al., 2003). Although the beneficial effects of pyrrolidine dithiocarbamate on chronic and acute inflammation (Cuzzocrea et al., 2002) and ischemia/reperfusion injury of the heart, lung and muscle have recently been reported (Chandrasekar et al., 1998; Ross et al., 2000; Sasaki et al., 2000; Lille et al., 2001; Long et al., 2003), the effects of pyrrolidine dithiocarbamate against the renal dysfunction and injury caused by ischemia/reperfusion injury of the kidney have not been investigated. The present study was therefore designed to evaluate the effectiveness of pyrrolidine dithiocarbamate in an established in vivo rat model of renal ischemia/reperfusion injury and elucidate the potential mechanisms involved in any beneficial effect.

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 anesthetised with sodium thiopentone (Intraval® Sodium, 120 mg/kg i.p.; Rhone Merieux, Essex, UK) and anesthesia was maintained by supplementary injections (~ 10 mg/kg i.v.) of sodium thiopentone. Rats were randomly allocated into the following four groups: (i) I/R + Saline group. Rats were subjected to renal ischemia for 45 min followed by reperfusion for 6 h ($n = 12$), (ii) I/R + PDTC group. Rats were administered pyrrolidine dithiocarbamate (100 mg/kg, i.v. bolus) 30 min prior to commencement of ischemia/reperfusion ($n = 12$), (iii) Sham + Saline group. Rats were subjected to identical surgical procedures except for renal ischemia/reperfusion, and were maintained under anesthesia for the duration of the experiment (30 min + 45 min + 6 h, $n = 12$). Rats were administered an i.v. bolus of saline (2 ml/kg, vehicle for pyrrolidine dithiocarbamate), (iv) Sham + PDTC group. Identical to Sham-operated rats except for the administration of pyrrolidine dithiocarbamate (100 mg/kg i.v. bolus) 30 min prior to commencing prior to the commencement of the experimental period ($n = 12$). All rats received an i.v. infusion of saline throughout the experimental period at a rate of 2 ml/kg/h. The time course and doses of pyrrolidine dithiocarbamate used were based on those previously shown by us, and others, to effectively attenuate the expression of inflammatory genes in rat models of ischemia/reperfusion, renal disease, acute and chronic inflammation and endotoxic shock (Liu et al., 1999a,b; Rangan et al., 1999a; Sasaki et al., 2000; Cuzzocrea et al., 2002, 2003).

2.2. Surgical procedures

Surgical preparation of rats and the protocol used to produce renal ischemia/reperfusion were identical to that described previously (Chatterjee et al., 2000b, Chaterjee and Thiemermann, 2003). Anesthetised 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 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 reperfusion period and before euthanasia, blood (1 ml) samples were collected via the carotid artery into tubes containing serum gel. The samples were centrifuged (6000 rpm for 3 min) to separate serum. All serum samples were analyzed for biochemical parameters within 24 h after collection (Vetlab Services, Sussex, UK). Serum creatinine concentrations were measured and used as indicators of renal function (Chatterjee et al., 2000b; Chaterjee and Thiemermann, 2003). Urine samples were collected during the reperfusion period and the volume produced recorded. Urine concentrations of creatinine and Na^+ were measured (Vetlab Services) at the end of the reperfusion period and used in conjunction with serum creatinine and Na^+ concentrations to estimate creatinine clearance and fractional excretion of Na^+ using standard formulae, which were used as respective indicators of glomerular and tubular function (Chatterjee et al., 2000b; Chaterjee and Thiemermann, 2003).

2.4. Measurement of serum nitrite/nitrate concentrations

Serum concentrations of nitrite and nitrate were used as an indicator of nitric oxide synthesis and were measured as previously described (Millar and Thiemermann, 1997) after enzymatic conversion of nitrate using nitrate reductase. Subsequently, total nitrite was assayed by adding 100 μl Griess reagent [0.05% (w/v) naphthalethylenediamine dihydrochloride and 0.5% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid] to each sample. Optical density at 550 nm (OD_{550}) was measured (Molecular Devices microplate reader, Richmond, CA, USA) and total nitrite/nitrate concentration for each sample was calculated by comparison of the OD_{550} to a standard curve prepared using a standard solution of sodium nitrate (also stoichiometrically converted to nitrite) prepared in saline.

2.5. Histological examination of renal sections

Rat kidneys were fixed for 1 week at room temperature in 10% (w/v) formaldehyde in phosphate-buffered saline (PBS, 0.01 M, pH 7.4). Kidneys were then dehydrated using graded ethanol, embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA) and 8-μm sections prepared. Sections were deparaffinized with xylene, stained with hematoxylin and eosin and viewed under a light microscope (Dialux 22 Leitz) (Cuzzocrea et al., 2002, 2003).

2.6. Immunohistochemical localization of iNOS and p65

After fixation and preparation of 8-μm deparaffinised renal sections as described above, peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Sections were then permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). Evidence of iNOS expression was determined using immunohistochemistry as previously described (Cuzzocrea et al., 2002, 2003). Localization of p65 (Rel A), which was used as an indicator of NF-κB activation in vivo, was determined as previously described (Cuzzocrea et al., 2002). The presence of p65 in the cytoplasm of renal cells was used as an indication that the NF-κB heterotrimeric complex was still in its "dormant" or inactive form with p50 and IκB as the 'IκB–NF-κB complex'. In contrast, localization of p65 in the nucleus indicated that the NF-κB (p50 and p65) had translocated into the nucleus and was therefore able to activate transcription of NF-κB-dependent genes. Sections were incubated overnight with primary anti-iNOS or anti-p65 antibody (1:500 dilution) and specific labeling detected using a biotin-conjugated goat anti-rabbit immunoglobulin G and avidin–biotin peroxidase complex (DBA).

2.7. Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company (Poole, Dorset, UK). Pyrrolidine dithiocarbamate was purchased from Alexis Biochemicals, Nottingham, UK. All stock solutions were prepared using non-pyrogenic saline [0.9% (w/v) NaCl; Baxter Healthcare, Thetford, Norfolk, UK].

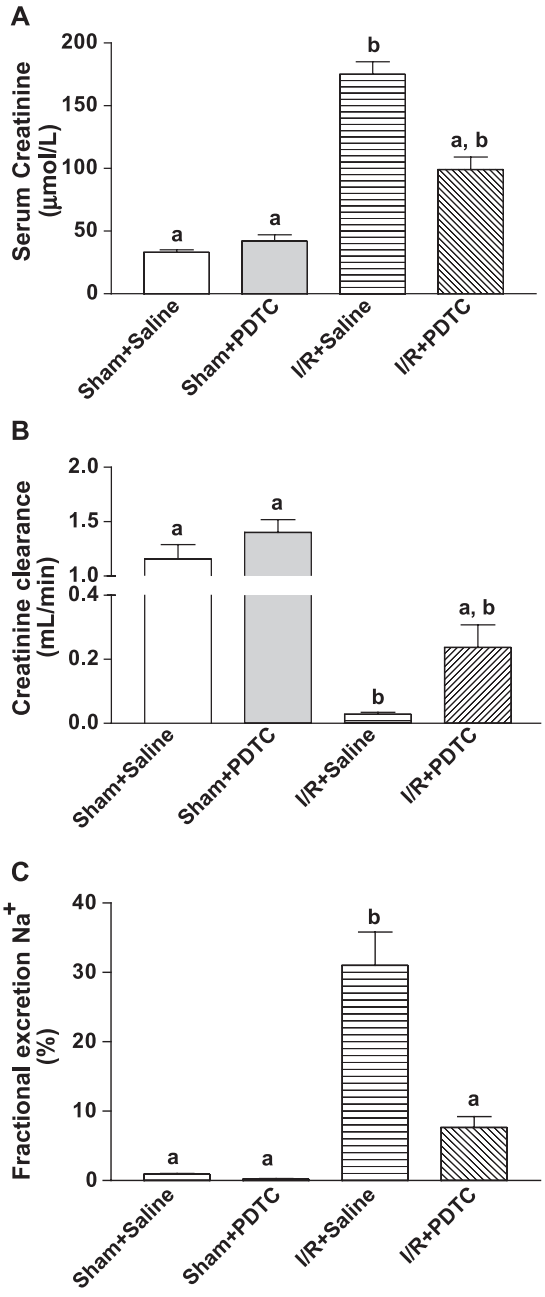

Fig. 1. Effect of pyrrolidine dithiocarbamate on renal dysfunction. Serum creatinine levels (A), creatinine clearance (B) and fractional excretion of Na^+(C) were measured subsequent to Sham-operation (Sham + Saline) or renal ischemia/reperfusion in the absence (I/R + Saline) or presence (I/R + PDTC) of pyrrolidine dithiocarbamate (100 mg/kg), administered 30 min prior to renal ischemia/reperfusion. $^{a}P < 0.05$ vs. I/R + Saline group, $^{b}P < 0.05$ vs. Sham + Saline group, $N = 12$ rats.

2.8. Statistical analysis

All values described in the text and figures are expressed as mean ± standard error of the mean (S.E.M.) 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 three 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.

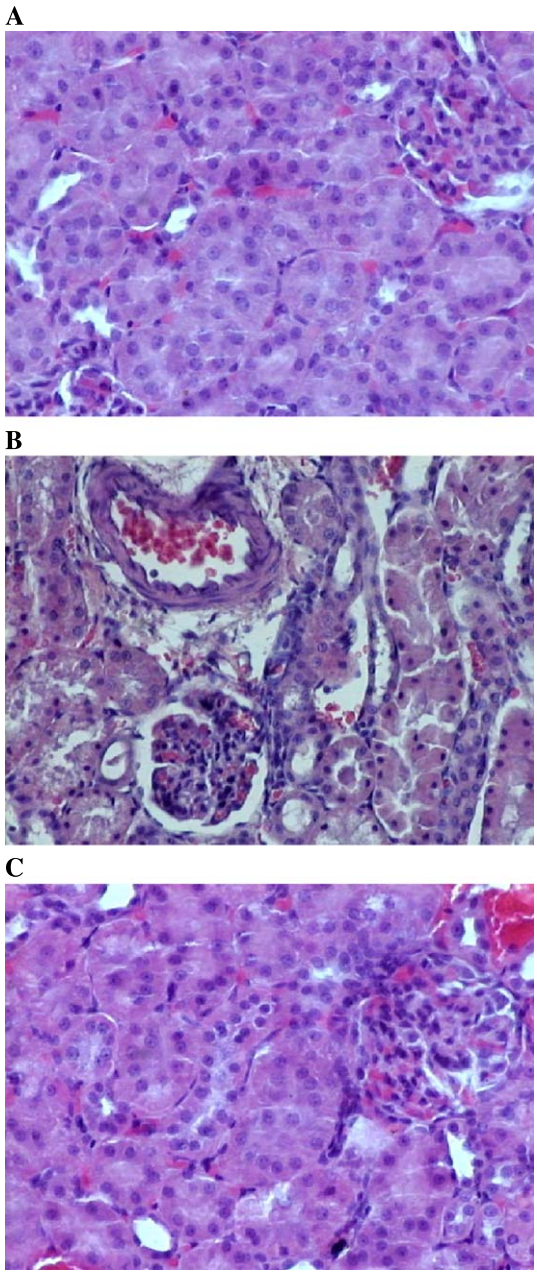

Fig. 2. Histological analysis of renal ischemia/reperfusion injury—effects of pyrrolidine dithiocarbamate. A kidney section taken from a Sham-operated rat (A) is compared to that taken from a rat subjected to renal ischemia/reperfusion (B). A kidney section taken from a rat subjected to renal ischemia/reperfusion (45-min ischemia/6-h reperfusion) after administration of 100 mg/kg pyrrolidine dithiocarbamate 30 min prior to ischemia/reperfusion (C) is also represented. Hemotoxylin and eosin, original magnification × 250, figures are representative of at least three experiments performed on different days.

3. Results

The mean ± S.E.M. for the weights of the rats used in this study was 267 ± 3 g, $n = 48$). On comparison with Sham rats, renal ischemia/reperfusion produced significant increases in serum and histological markers of renal dysfunction and injury as described in detail below (Figs. 1 and 2). When compared to rats used as Shams, renal ischemia/reperfusion (in the presence or absence of pyrrolidine dithiocarbamate) did not have a significant effect on urine flow (0.017 ± 0.001 ml/min, $n = 48$).

3.1. Effect of pyrrolidine dithiocarbamate on the 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 decrease in creatinine clearance (Fig. 1B). Renal ischemia/reperfusion also produced a significant increase in fractional excretion of Na^+ (Fig. 1C). Compared to rats subjected to ischemia/reperfusion-only, administration of pyrrolidine dithiocarbamate produced a significant reduction in serum creatinine levels (Fig. 1A) and in fractional excretion of Na^+ (Fig. 1C). Administration of pyrrolidine dithiocarbamate to rats subjected to ischemia/reperfusion also produced a significant improvement of creatinine clearance (Fig. 1B). Administration of pyrrolidine dithiocarbamate to Sham-operated rats did not have any effect on baseline serum creatinine (Fig. 1A) or cause any alteration in creatinine clearance or fractional excretion of Na^+ (Fig. 1B,C).

3.2. Effects of pyrrolidine dithiocarbamate on the renal histopathological changes caused by ischemia/reperfusion of the rat kidney

On comparison with the renal histology observed in kidneys taken from Sham-operated rats (Fig. 2A), rats that were subjected to renal ischemia/reperfusion demonstrated the recognized features of renal injury (Fig. 2B). Specifically, degeneration of tubular structure, tubular dilatation, swelling

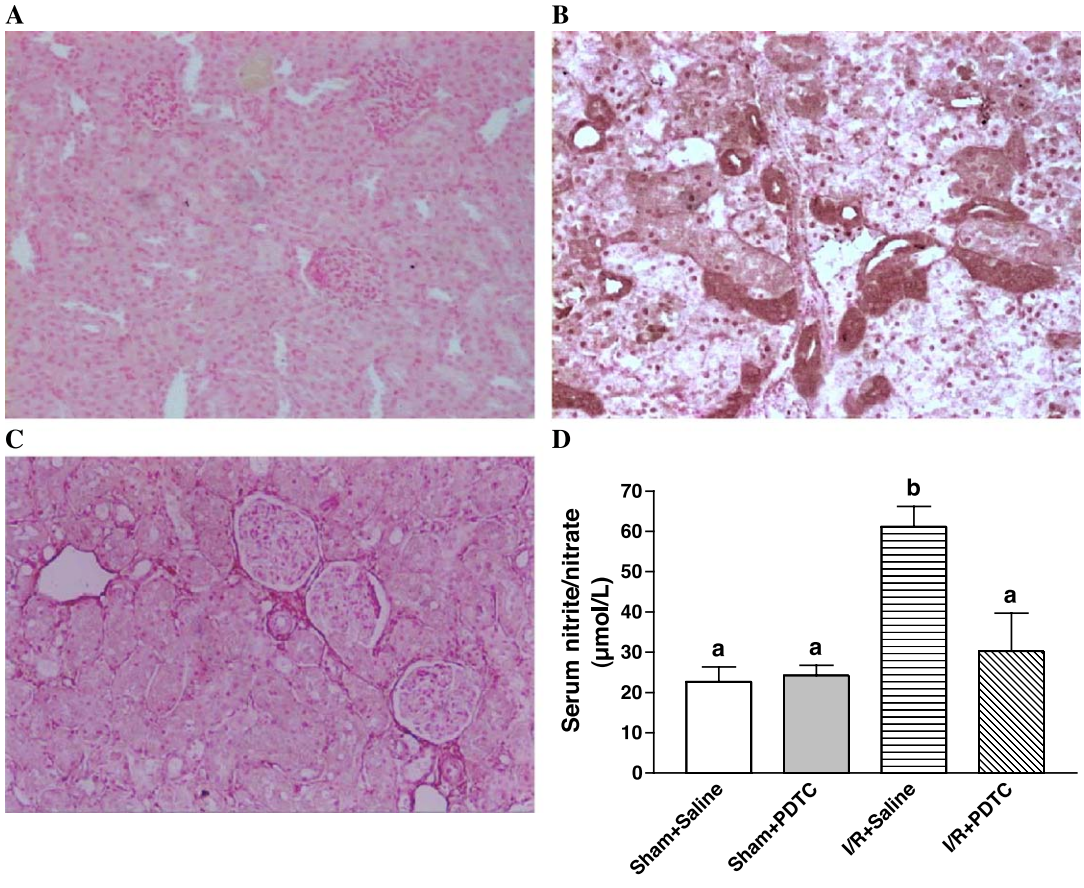

Fig. 3. Effect of pyrrolidine dithiocarbamate on iNOS expression and NO production during renal ischemia/reperfusion. iNOS expression in rat kidney sections incubated overnight with 1:500 dilution of primary antibody directed against iNOS. (A) Sham-operated rat kidney section (B) I/R + Saline and (C) I/R + PDTC (100 mg/kg). Original magnification × 125, figures are representative of at least three experiments performed on different days. (D) Effect of pyrrolidine dithiocarbamate on serum NO_2/NO_3 levels subsequent to sham-operation in the presence or absence of pyrrolidine dithiocarbamate (100 mg/kg), or renal ischemia/reperfusion in the absence (I/R + Saline) or presence of pyrrolidine dithiocarbamate (I/R + PDTC, 100 mg/kg). $^aP < 0.05$ vs. I/R + Saline group, $^bP < 0.05$ vs. Sham + Saline group, $N = 12$ rats.

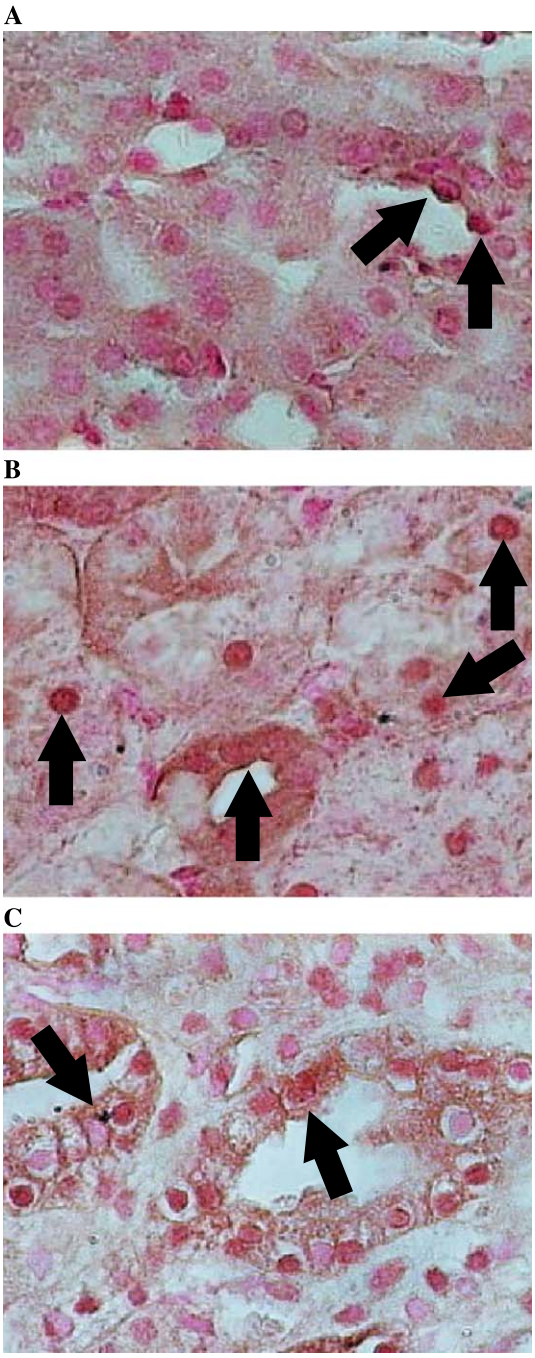

Fig. 4. Effect of pyrrolidine dithiocarbamate on NF-κB translocation. Representative immunohistochemistry sections of kidney obtained from (A) rats subjected to sham treatment (saline), (B) rats subjected to renal ischemia/reperfusion only and (C) rats subjected to ischemia/reperfusion and treated with the pyrrolidine dithiocarbamate (100 mg/kg). In the renal section from sham-treated rat (A), positive (brown) staining for NF-κB was found in the cytoplasm (indicated by arrows). After renal ischemia/reperfusion (B), positive (brown) staining for NF-κB was located in the nucleus of renal cells (indicated by arrows). In the renal section obtained from pyrrolidine dithiocarbamate-treated rats (C), positive (brown) staining was localized to the cytoplasm indicating an effect of pyrrolidine dithiocarbamate on NF-κB translocation during ischemia/reperfusion. Original magnification: ×1200. Figure is representative of at least three experiments performed on different experimental days.

and necrosis, luminal congestion and the presence of eosinophilia were observed. In contrast, renal sections obtained from rats administered pyrrolidine dithiocarbamate prior to renal ischemia/reperfusion demonstrated marked reduction of the histological features of renal injury (Fig. 2C).

3.3. Effects of pyrrolidine dithiocarbamate on expression of iNOS and subsequent production of nitric oxide during ischemia/reperfusion of the kidney of the rat

When compared to kidney sections obtained from Sham-operated rats (Fig. 3A), immunohistochemical analysis of sections obtained from rats subjected to renal ischemia/reperfusion revealed positive staining for iNOS protein (Fig. 3B). In contrast, reduced staining was observed in kidney sections obtained from rats administered pyrrolidine dithiocarbamate and subjected to renal ischemia/reperfusion (Fig. 3C). Renal ischemia/reperfusion resulted in a significant increase in the serum levels of nitrite/nitrate (Fig. 3D), which were significantly reduced by administration of pyrrolidine dithiocarbamate prior to renal ischemia/reperfusion (Fig. 3D).

3.4. Effect of pyrrolidine dithiocarbamate on the nuclear translocation of p65 in renal cells during ischemia/reperfusion of the kidney of the rat

Immunohistochemical (brown) staining for p65 was located in the cytoplasm in kidney sections obtained from Sham-operated rats (Fig. 4A) indicating that NF-κB was present in its inactive or dormant form as a heterotrimeric complex with p50 and IκB. Immunohistochemical analysis of kidney sections obtained after renal ischemia/reperfusion demonstrated positive staining for p65 which was localized in the nucleus of renal cells (Fig. 4B), suggesting translocation of p65. In kidney sections prepared from pyrrolidine dithiocarbamate-treated rats, positive staining for p65 was mostly located in the cytoplasm of tubular cells indicating an inhibitory effect of pyrrolidine dithiocarbamate on p65 translocation (and thus inhibition of NK-κB activation) (Fig. 4C).

4. Discussion

In the present study, we have shown that renal ischemia/reperfusion of the rat kidney results in a significant reduction in renal function as indicated by increased serum creatinine levels and a marked decrease in creatinine clearance. Renal dysfunction also correlated with increased fractional excretion of Na^+ indicating tubular dysfunction. Renal ischemia/reperfusion injury was also documented using histological analysis. All these data, together with increased expression of iNOS confirmed a well-known pattern of renal dysfunction and injury caused by ischemia/reperfusion of the kidney (Thadhani et al., 1996; Weight et al., 1996; Kribben et al., 1999; Sheridan and Bonventre, 2001) and are in keeping

with the notion that renal ischemia/reperfusion causes both glomerular and tubular dysfunction (Paller, 1994b). Specifically, marked tubular damage occurs during renal ischemia/reperfusion (Venkatachalam et al., 1978), which was confirmed in the histological analysis of kidneys obtained from rats subjected to ischemia/reperfusion. We demonstrate here for the first time, that administration of pyrrolidine dithiocarbamate prior to ischemia/reperfusion produces a significant reduction of the renal dysfunction and injury caused by ischemia/reperfusion of the rat kidney. In this study, administration of pyrrolidine dithiocarbamate provided a greater beneficial effect against tubular dysfunction than glomerular dysfunction caused by renal ischemia/reperfusion as indicated by the actions of pyrrolidine dithiocarbamate on creatinine clearance and fractional excretion of Na^+. In this model of renal ischemia/reperfusion injury in the rat, induction of iNOS during reperfusion followed by nitric oxide production causes greater tubular dysfunction than glomerular dysfunction (Chatterjee et al., 2002a,b). Although nitric oxide causes both glomerular and tubular damage, there is good evidence from in vitro and in vivo studies that nitric oxide has a stronger pathological effect on PT cells where they can target mitochondrial respiration amongst other mechanisms of injury (Lieberthal, 1998). This is supported by the findings in this study, specifically, that pyrrolidine dithiocarbamate inhibited the activation of NF-κB and iNOS expression in the proximal tubules and attenuated nitric oxide production that consequently reduced tubular dysfunction to a greater degree than glomerular dysfunction.

It is interesting to consider the mechanisms by which pyrrolidine dithiocarbamate (and other dithiocarbamates) provide these observed beneficial effects. The results reported here, specifically that pyrrolidine dithiocarbamate reduced p65 translocation, iNOS expression and subsequent nitric oxide production during renal ischemia/reperfusion, suggest that protective effects of pyrrolidine dithiocarbamate are mediated via the inhibition of the activation of NF-κB. Activation of NF-κB during renal ischemia/reperfusion leads to the expression of numerous genes implicated in the development of renal injury and in the pathophysiology of acute renal failure including iNOS (Lieberthal, 1998; Kribben et al., 1999). Furthermore, the activation of NF-κB is a common end-point of several signal transduction pathways, including the activation of phosphatidylcholine-specific phospholipase C, protein kinase C, protein tyrosine kinases and mitogen-activated protein kinases amongst other signaling factors (Kolesnick and Goled, 1994; Novogrodsky et al., 1994; Schultze et al., 1994; Sun and Andersson, 2002). The role of NF-κB activation in the development of renal disease has recently been reviewed (Guijarro and Egido, 2001; Wardle, 2001), supporting our previous studies which have suggested that inhibition of the activation of NF-κB may be beneficial against renal dysfunction associated with ischemia/reperfusion and shock (Chatterjee et al., 2001; McDonald et al., 2001) and those of others which report that inhibition of NF-κB activation can alleviate renal inflammation (Rangan et al, 1999a; Muller et al., 2000; Mudge et al., 2001; Rangan et al., 2001). Pyrrolidine dithiocarbamate, in common with other dithiocarbamates, inhibits NF-κB activation during ischemia/reperfusion, inflammation and shock (Liu et al., 1999a,b; Sasaki et al., 2000; Cuzzocrea et al., 2002, 2003) via prevention of the phosphorylation of IκB kinases, thereby preventing the degradation of IκB (Liu et al., 1999a,b). This is supported by numerous reports from in vitro studies utilizing cultured renal cells describing the inhibitory effects of pyrrolidine dithiocarbamate on the activation of NF-κB (Eberhardt et al., 1994; Satriano and Schlondorff, 1994; Kone et al., 1995; Walker et al., 1995; Zoja et al., 1998; Rangan et al., 1999b). We demonstrate here that pyrrolidine dithiocarbamate attenuates the activation of NF-κB in vivo and subsequently, the expression of downstream target genes (e.g. iNOS) and therefore propose that the inhibition of NF-κB activation by pyrrolidine dithiocarbamate contributes to the renoprotective effects observed in this study. However, it should be noted that even though there is good evidence to support a link between the activation of NF-κB in the ischemic kidney (Donnahoo et al., 2000) and its role in the expression of iNOS expression and development of subsequent ischemia/reperfusion injury (Tsoulfas and Geller, 2001), there is also convincing evidence that iNOS expression can be independent of NF-κB activation. Pyrrolidine dithiocarbamate has been shown to differentially modulate interleukin-1β and cyclic AMP-induced nitric oxide synthase expression in rat renal mesangial cells suggesting that cyclic AMP triggers a separate signaling pathway not involving NF-κB (Eberhardt et al., 1994). A recent study has demonstrated that cytokine-stimulated iNOS expression in human kidney epithelial cells involves the activation of tyrosine kinases including Janus kinase-2, protein kinase C and p38 mitogen activated protein kinase (Poljakovic et al., 2003). Specifically, activation of several subgroups of the mitogen-activated protein kinase family has been show to mediate cytokine-stimulated iNOS expression independently of NF-κB including Janus kinase-2 (Doi et al., 2002; Nakasima et al., 1999), p42/p44 mitogen-activated protein kinase (Doi et al., 2000) and recently, extracellular signal-regulated kinase (Inn-Oc et al., 2003; Marcus et al., 2003). In the presence of pro-inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α, inhibition of these pathways can reduce the expression of iNOS without affecting the activation of NF-κB suggesting that NF-κB and iNOS can be regulated independently (Doi et al., 2000, 2002). We have recently demonstrated that the tyrosine kinase inhibitor tyrphostin AG126 can significantly reduce renal dysfunction and injury caused by renal ischemia/reperfusion in rats (Chatterjee et al., 2003).

In addition to the effects of pyrrolidine dithiocarbamate on NF-κB inhibition, there may be other mechanisms contributing to the anti-inflammatory property of pyrrolidine dithiocarbamate. Further biological effects of pyrrolidine dithiocarbamate that have been considered include (i) the interference with reactive oxygen metabolism (Satriano and

Schlondorff, 1994), (ii) the chelation of divalent metal ions (Sundermann, 1991) and (iii) its influence on intracellular thiol levels (Mihm et al., 1991). The recognized strong antioxidant effect of pyrrolidine dithiocarbamate may have also contributed to the reduction in renal dysfunction and injury caused by ischemia/reperfusion in this study. Especially, inhibition of reactive oxygen intermediates (Schmidt et al., 1995) and superoxide anions generated by xanthine oxidase (Liu et al., 1997) are possibly involved in the protective effect of pyrrolidine dithiocarbamate. In general, antioxidants are known to exhibit beneficial effects in renal ischemia/reperfusion injury (Chatterjee et al., 2000a, Patel et al., 2002). Recent evidence also suggests that the activation of NF-κB may also be under the control of oxidant/antioxidant balance and it is feasible that the ability of pyrrolidine dithiocarbamate to inhibit NF-κB activation is connected to its antioxidant activity (Flohe et al., 1997; Roberts and Cowsert, 1998; Bowie and O'Neill, 2000). This hypothesis is based primarily on the observation that low doses of peroxides, including H_2O_2 and *tert*-butyl-hydroperoxide can induce NF-κB activation whereas some antioxidants prevent it (Chen et al., 1996; Flohe et al., 1997). However, although pyrrolidine dithiocarbamate is an antioxidant, recent evidence from a study using renal proximal tubular cells also suggests that this property may not be responsible for its ability to inhibit NF-κB (Woods et al., 1999). Overall, the results obtained in this study demonstrate that pyrrolidine dithiocarbamate inhibits the degradation of IκB-α degradation, thus inhibiting NF-κB activation.

Metal ion chelation by pyrrolidine dithiocarbamate is unlikely to have provided major beneficial actions in this model of renal ischemia/reperfusion as it have already been demonstrated that it is not possible to overcome pyrrolidine dithiocarbamate-mediated blockade of NF-κB activation using Co^{2+}, Mn^{2+} or Fe^{2+} (Ziegler-Heitbrock et al., 1993). Likewise, the influence of pyrrolidine dithiocarbamate on intracellular thiol levels is unlikely to have contributed significantly to the beneficial effects obtained using pyrrolidine dithiocarbamate, since other substances which interfere with thiol binding, such as *N*-acetyl-L-cysteine, inhibit NF-κB at higher, subtoxic concentrations (Meyer et al., 1993). Paradoxically, the pro-oxidant and metal-chelating properties of pyrrolidine dithiocarbamate could also be involved in its ability to inhibit NF-κB (Pinkus et al., 1996). In this regard, pyrrolidine dithiocarbamate appears to act catalytically at micromolar concentrations to cause the oxidation of several hundred molar equivalents of intracellular glutathione (Pinkus et al., 1996) and may explain the steep concentration gradient of pyrrolidine dithiocarbamate-mediated inhibition of NF-κB activation reported in other studies (Liu et al., 1999a,b).

Overall, our study investigates the beneficial action of pyrrolidine dithiocarbamate in a short-term model of ischemic acute renal failure; however, investigation of the effects of pyrrolidine dithiocarbamate on renal ischemia/reperfusion injury over the course of days is certainly warranted. Such studies would provide useful information to ensure that there are no negative consequences to the inhibition of NF-κB since the expression of numerous genes are modulated—some of which may be protective genes activated in response to renal ischemia/reperfusion. Furthermore, the effect of administration of pyrrolidine dithiocarbamate after ischemia and during reperfusion also warrants further investigation, as a protective effect under these conditions would greatly broaden its potential clinical application. In conclusion, our results indicate that pyrrolidine dithiocarbamate provides beneficial actions against renal dysfunction and injury mediated by ischemia/reperfusion of the kidney. The mechanism of action of pyrrolidine dithiocarbamate involves reduction of the activation of NF-κB (thereby reducing expression of NF-κB-dependent genes such as iNOS and subsequent production of nitric oxide). We propose that pyrrolidine dithiocarbamate, or compounds utilizing similar mechanisms of action, may be useful in enhancing the tolerance of the kidney against renal dysfunction and injury in situations where renal tissues are subject to ischemia/reperfusion, e.g. during aortovascular surgery or renal transplantation and that in general, dithiocarbamates may provide beneficial actions against ischemic acute renal failure.

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