

Inhibition of inducible nitric oxide synthase reduces renal ischemia/reperfusion injury

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Inhibition of inducible nitric oxide synthase reduces renal ischemia/reperfusion injury.

Background. Nitric oxide (NO), produced via inducible nitric oxide synthase (iNOS), is implicated in the pathophysiology of renal ischemia/reperfusion (I/R) injury. The aim of this study was to investigate the effects of the iNOS inhibitors L-N⁶-(1-*iminoethyl*)lysine (L-NIL) and aminoethyl-isothiourea (AE-ITU) on (a) renal dysfunction and injury mediated by bilateral I/R of rat kidneys *in vivo* and (b) cytokine-stimulated NO production by primary cultures of rat proximal tubule (PT) cells.

Methods. Male Wistar rats subjected to bilateral renal ischemia (45 min) followed by reperfusion (6 h). Rats were administered either L-NIL (3 mg/kg IV bolus 15 min prior to I/R followed by 1 mg/kg/h throughout I/R) or AE-ITU (1 mg/kg IV bolus 15 min prior to I/R followed by 1 mg/kg/h throughout I/R). Serum and urinary biochemical indicators of renal dysfunction and injury were measured; serum creatinine (S_{Cr}, glomerular dysfunction), fractional excretion of Na⁺ (FE_{Na}, tubular dysfunction), serum aspartate aminotransferase (sAST, I/R injury) and urinary *N*-acetyl-β-D-glucosaminidase (uNAG, tubular injury). Additionally, renal sections were used for histological grading of renal injury and for immunological evidence of nitrotyrosine formation. Nitrate/nitrite levels in plasma were measured using the Griess assay and used as an indicator of NO production. Primary cultures of rat PT cells were incubated with interferon-γ (IFN-γ, 100 IU/mL) and lipopolysaccharide (LPS, 10 μg/mL) for 24 h, either in the absence or presence of increasing concentrations of L-NIL or AE-ITU (0.001 to 1 mmol/L) after which nitrite/nitrate levels were measured using the Griess assay.

Results. L-NIL and AE-ITU significantly reduced the I/R-mediated increases in S_{Cr}, FE_{Na}, sAST and uNAG, indicating attenuation of I/R-mediated renal dysfunction and injury. Specifically, L-NIL and AE-ITU reduced the I/R-mediated glomerular and tubular dysfunction and biochemical and histological evidence of tubular injury. Both L-NIL and AE-ITU attenuated the plasma levels of nitrate (indicating reduced NO production) and the immunohistochemical evidence of the formation of nitrotyrosine. *In vitro*, L-NIL and AE-ITU both

significantly reduced cytokine-stimulated NO production by primary cultures of rat PT cells in a dose-dependent manner.

Conclusions. These results suggest that L-NIL and AE-ITU reduce the renal dysfunction and injury associated with I/R of the kidney, via inhibition of iNOS activity and subsequent reduction of NO (and peroxynitrite) generation. We propose that selective and specific inhibitors of iNOS activity may be useful against the NO-mediated renal dysfunction and injury associated with I/R of the kidney.

Despite significant advances in critical care medicine, acute renal failure (ARF) remains a major clinical problem, and mortality associated with ARF has not decreased substantially over the last 50 years [1, 2]. The mortality rate of ARF remains between 50 to 70% among patients in intensive care who require dialysis, and ranges between 25 and 100% in post-operative patients suffering ARF [3–5]. Additionally, ARF is frequently implicated in the pathophysiology of other life-threatening complications including sepsis and multiple-organ failure (MOF) [6, 7]. 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 [1, 8]. The prognosis is complicated by the fact that reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage (reperfusion-injury) [9], contributing to the renal dysfunction and injury associated with ischemia/reperfusion (I/R) of the kidney [1, 8, 9]. Furthermore, it appears that the proximal tubule (PT) is particularly susceptible to injury caused by renal I/R [10, 11]. Subsequent to renal transplantation, renal I/R is also a major cause of early allograft rejection and adversely affects the long-term survival of the allograft [12]. Thus, motivated by the fact that previous interventions against ARF have proved to be largely ineffective and that dialysis still remains the only effective therapy [2], development of novel therapeutic interventions with which to reduce renal dysfunction and injury mediated by I/R of

the kidney, and associated ARF, have been topics of intense research interest.

Nitric oxide (NO), generated by any one of the three isoforms of nitric oxide synthase (NOS) plays an important role in renal function, both under normal and pathophysiological conditions [13, 14]. All three isoforms of NOS have been located in the kidney; the endothelial and neuronal (constitutive) isoforms have been identified in the renal vasculature and macula densa, respectively [15] and inducible NOS (iNOS) can be induced in the kidney by cytokines, lipopolysaccharide and during I/R [15–17] leading to renal cell injury [18]. Several *in vivo* and *in vitro* investigations have demonstrated how inhibition of the expression or activity of iNOS, or absence of iNOS itself, can ameliorate or prevent renal I/R injury [19–23], suggesting that NO generated by iNOS contributes to renal I/R injury.

Furthermore, it is now clear that the production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radicals contribute to renal I/R injury (and associated ARF) [9, 11]. Traditionally, ROS have been considered to exert their effects through a direct toxic action on target cells. For example, ROS cause DNA damage during renal I/R and oxidative stress [24–26] leading to the activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP), depletion of NAD⁺ and adenosine 5'-triphosphate (ATP) and ultimately cell death [25, 26]. Thus, various antioxidant strategies such as the use of TEMPOL or desferrioxamine have been shown to provide beneficial actions against renal dysfunction and injury mediated by I/R of the kidney [27]. Furthermore, NO reacts with superoxide anion to form peroxynitrite [28], which causes injury via direct oxidant injury and protein tyrosine nitration [28, 29]. Specifically in the kidney, peroxynitrite generation has been implicated in the pathophysiology both renal I/R and hypoxia-reoxygenation injury, respectively [18, 30, 31].

Here we compare the effects L-N⁶-(1-iminoethyl)lysine (L-NIL) and aminoethyl-isothiourea (AE-ITU), both relatively selective inhibitors of iNOS activity [32–34] on (a) renal dysfunction and injury in an *in vivo* model of renal I/R injury and (b) cytokine-stimulated NO production by primary cultures of rat PT cells.

METHODS

Renal ischemia/reperfusion

In vivo studies were carried out using 47 male Wistar rats (Tuck, Rayleigh, Essex, UK) weighing 215 to 330 g. Rats were subjected to renal bilateral renal ischemia for 45 minutes followed by reperfusion for six hours as described previously [22, 26, 27]. Upon completion of surgical procedures, the animals were randomly allocated into six groups:

I/R Control group. Control animals underwent renal

ischemia for 45 minutes followed by reperfusion for six hours ($N = 10$).

I/R L-NIL group. Animals were administered L-NIL (3 mg/kg IV) 15 minutes prior to I/R followed by infusion (1 mg/kg/h, IV) throughout the reperfusion period ($N = 9$).

I/R AE-ITU group. Animals were administered AE-ITU (1 mg/kg IV) 15 minutes prior to I/R followed by infusion (1 mg/kg/h, IV) throughout the reperfusion period ($N = 8$).

Sham Control group. Sham-operated rats were subjected to identical surgical procedures described above except for renal I/R and maintained under anesthesia for the duration of the experiment (45 min + 6 h, $N = 12$).

Sham L-NIL group. These animals were identical to Sham-operated animals except for treatment with L-NIL as described above ($N = 4$).

Sham AE-ITU group. They were identical to Sham-operated animals except for treatment with AE-ITU as described above ($N = 4$).

Rats in the Sham and I/R Control groups received an IV bolus of saline and infusion throughout I/R at the same rate as that administered to the L-NIL and AE-ITU group.

Measurement of biochemical parameters

At the end of the reperfusion period, blood (1 mL) samples and urine samples were collected and used for the measurement of biochemical renal parameters as described previously [22, 26, 27]. Briefly, serum samples were used for the measurement of serum urea (S_{Ur}) and creatinine (S_{Cr}) levels, which were used as indicators of impaired glomerular function [22, 26, 27], and aspartate aminotransferase (AST), which was used as an indicator of renal I/R injury [22, 27]. Urine samples were collected during the reperfusion period and the volume of urine produced recorded. Urine concentrations of creatinine were measured and used in conjunction with S_{Cr} and urine flow to estimate the glomerular filtration rate in the form of creatinine clearance (C_{Cr}), which was used as an indicator of glomerular function [22, 26, 27]. In a similar fashion, urinary and serum concentrations of Na⁺ were measured and used in conjunction with urine flow to calculate fractional excretion of Na⁺ (FE_{Na}), which was used as an indicator of tubular dysfunction [22, 26, 27]. Additionally, concentrations of urinary *N*-acetyl- β -D-glucosaminidase (NAG), an indicator of tubular damage, and possibly tubular function, were measured (Dr. Joaquim Chaves, Clinica Medica e Diagnostico, Lisbon, Portugal; personal communication) [22, 27].

Measurement of plasma nitrite/nitrate concentrations

Nitrite and nitrate are the primary oxidation products of NO subsequent to reaction with oxygen and, therefore, the nitrite/nitrate concentration in plasma was used

as an indicator of NO synthesis. Nitrite/nitrate levels in rat plasma were measured as previously described [35] after enzymatic conversion of nitrate to nitrite using nitrate reductase. Subsequently, total nitrite in the plasma was assayed by adding 100 μ L Griess reagent (0.05% (wt/vol) naphthaethylenediamine dihydrochloride and 0.5% (wt/vol) sulphanilamide in 2.5% (vol/vol) 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} of a standard solution of sodium nitrate (also stoichiometrically converted to nitrite) prepared in saline.

Histological evaluation

Renal sections were prepared as described previously and used for the assessment of renal I/R injury [23, 27]. Briefly, 100 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; 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 shows 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 with a maximum score of 300.

Immunohistochemical localization of nitrotyrosine

Tyrosine nitration, which was used as an index of the nitrosylation of protein by peroxynitrite and/or ROS, was determined using immunohistochemistry as previously described [27]. Briefly, sections were incubated overnight with a 1:1000 dilution of primary anti-nitrotyrosine monoclonal antibody (DBA, Milan, Italy). Separate sections were also incubated with control solutions consisting of PBS alone or a 1:500 dilution of non-specific purified rabbit IgG (DBA). Specific labeling was detected using a biotin-conjugated goat anti-rabbit IgG (DBA) and avidin-biotin peroxidase (DBA). Samples were then viewed under a light microscope.

Isolation and culture of rat proximal tubular cells

Proximal tubule cells were isolated from kidneys obtained from 12 male Wistar rats (260 to 350 g) using collagenase digestion, differential sieving and Percoll density centrifugation as described previously [25, 27]. Rat PT cells were cultured in minimum essential medium (MEM) containing 10% (vol/vol) FCS in a humidified 5% CO_2 /95% air atmosphere at 37°C and medium was changed every 48 hours until the cells reached confluence.

Experimental design

Confluent primary cultures of rat PT cells were incubated with 100 IU/mL interferon- γ (IFN- γ) and 10 μ g/mL

bacterial lipopolysaccharide (LPS) in combination in the absence or presence of increasing concentrations of L-NIL (0.001 to 1 mmol/L) or AE-ITU (0.001 to 1 mmol/L) for 24 hours. Upon completion of incubations, NO levels in incubation medium was measured as nitrite/nitrate after conversion of nitrate to nitrite using nitrate reductase as described above and previously [35].

Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). LPS was obtained from *E. coli* serotype 0.127:B8 (Sigma).

All solutions used for in vivo infusions were prepared using non-pyrogenic saline [0.9% (wt/vol) NaCl; Baxter Healthcare Ltd., Norfolk, UK].

Statistical analysis

All values described in the text and figures are expressed as mean \pm standard error (SE) of the mean for the number (N) of observations. For in vivo studies and histological scoring, each data point represents biochemical measurements obtained from N separate animals. For immunohistochemical analysis, the figures shown are representative of at least three experiments performed on different experimental days. For in vitro studies involving PT cell cultures, measurements were taken from cultures obtained from 12 separate isolations from 12 separate animals. For the determination of NO production (Griess assays performed on from rat plasma and incubation medium from PT cells), experiments were performed in triplicate. Statistical analysis was carried out using GraphPad Prism/Instat 1.1 (GraphPad Software, San Diego, CA, USA). Data were analyzed using one-way ANOVA followed by Bonferroni's post hoc test and a P value of less than 0.05 was considered to be significant (NS = not significant).

RESULTS

Effect of L-NIL and AE-ITU on ischemia/reperfusion-mediated glomerular dysfunction

Animals that underwent renal I/R exhibited significant increases in the serum concentrations of creatinine compared to Sham-operated animals (Fig. 1), suggesting a significant degree of glomerular dysfunction. This was reflected by a significant increase in serum levels of urea [from 6 ± 0.4 μ mol/L (Sham-operated group) to 24 ± 1 μ mol/L (I/R only group), $P < 0.05$], also indicating glomerular dysfunction mediated by renal I/R. Renal I/R also produced a significant reduction in C_{cr} [from 1.1 ± 0.2 mL/min (Sham-operated group) to 0.013 ± 0.005 mL/min (I/R only group), $P < 0.05$], which was used as an indicator of glomerular filtration rate and thus, glomerular function.

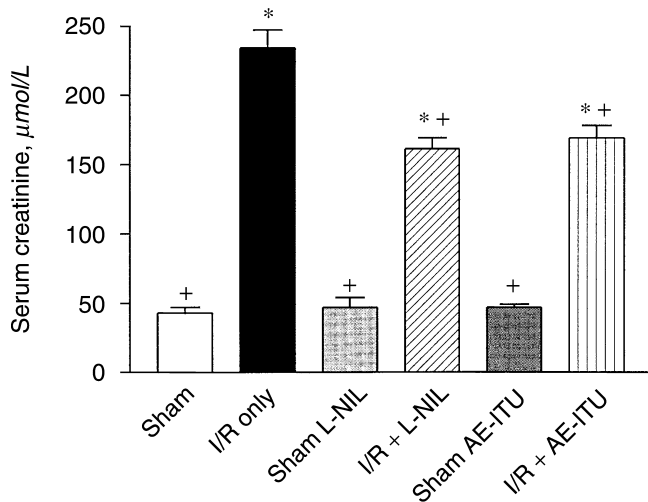


Fig. 1. Alterations in serum creatinine concentrations during renal ischemia/reperfusion (I/R) in the presence of L-NIL (3 mg/kg IV bolus followed by 1 mg/kg/h IV infusion), or AE-ITU (1 mg/kg IV bolus followed by 1 mg/kg/h IV infusion). * $P < 0.05$ vs. sham-operated group, + $P < 0.05$ vs. I/R only group.

Treatment of rats with L-NIL prior to and throughout I/R produced a relatively small, but significant, reduction in the serum levels of creatinine (Fig. 1). Administration of AE-ITU prior to and during I/R had a similar, significant effect on I/R-mediated increases in serum creatinine levels (Fig. 1).

Administration of L-NIL or AE-ITU prior to and throughout I/R produced a similar significant attenuation of I/R-mediated increase in serum urea [from 24 ± 1 µmol/L (I/R only group) to 19 ± 2 µmol/L (I/R + L-NIL group), $P < 0.05$ vs. I/R only group, and 21 ± 1 µmol/L (I/R + AE-ITU group), $P < 0.05$ vs. I/R only group]. Administration of L-NIL or AE-ITU prior to and throughout I/R also produced a significant increase in the reduced C_{Cr} associated with renal I/R [from 0.013 ± 0.005 mL/min (I/R only group) to 0.73 ± 0.13 mL/min (I/R + L-NIL group), $P < 0.05$ vs. I/R only group, and 1.10 ± 0.12 mL/min (I/R + AE-ITU group), $P < 0.05$ vs. I/R only group].

Administration L-NIL or AE-ITU to Sham-operated rats did not result in any alteration in serum levels of creatinine on comparison to Sham-operated rats that were administered saline only (Fig. 1). Similarly, administration L-NIL or AE-ITU to Sham-operated rats did not result in any alteration in serum urea levels or in C_{Cr} on comparison to Sham-operated rats, which were administered saline only (data not shown).

Effect of L-NIL and AE-ITU on ischemia/reperfusion-mediated tubular dysfunction/injury

Fractional excretion of sodium (FE_{Na}), calculated using plasma and urinary concentrations of Na^+ in associa-

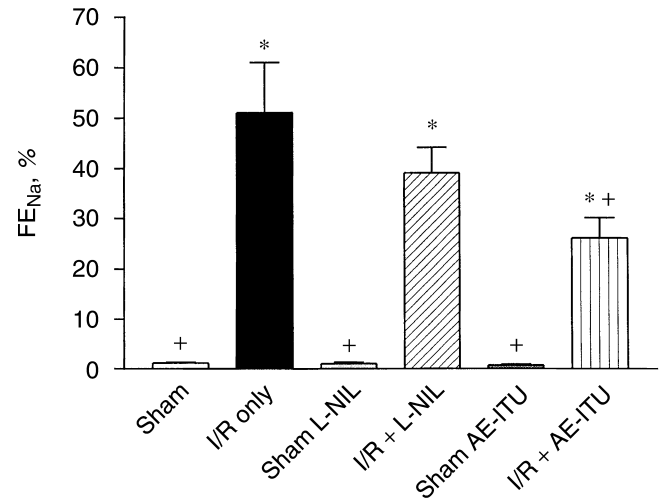


Fig. 2. Effect of renal I/R on fractional excretion of Na^+ (FE_{Na}) in the presence of L-NIL (3 mg/kg IV bolus followed by 1 mg/kg/h IV infusion), or AE-ITU (1 mg/kg IV bolus followed by 1 mg/kg/h IV infusion). * $P < 0.05$ vs. sham-operated group, + $P < 0.05$ vs. I/R only group.

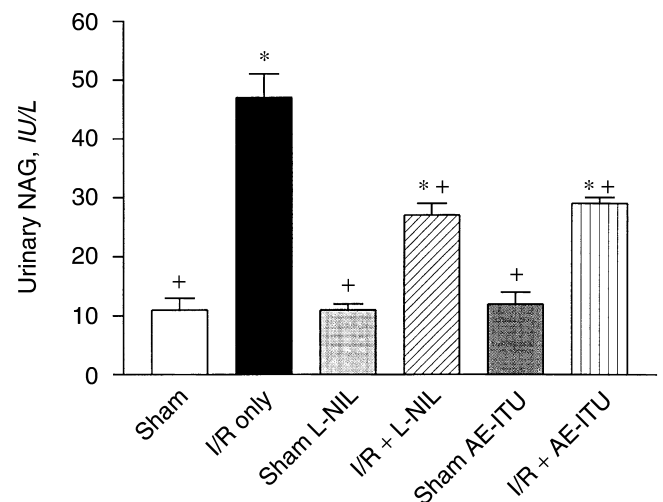


Fig. 3. Alterations in urinary *N*-acetyl- β -*D*-glucosaminidase levels during renal I/R, in the presence of L-NIL (3 mg/kg IV bolus followed by 1 mg/kg/h IV infusion), or AE-ITU (1 mg/kg IV bolus followed by 1 mg/kg/h IV infusion). * $P < 0.05$ vs. sham-operated group, + $P < 0.05$ vs. I/R only group.

tion with urine production (urine flow, mL/min), was used as an indicator of tubular function. I/R produced a significant increase in FE_{Na} suggesting tubular dysfunction (Fig. 2). Administration of L-NIL or AE-ITU prior to and during I/R produced a reduction in the I/R-mediated increase in FE_{Na} , suggesting improvement in tubular function (Fig. 2). Administration of L-NIL or AE-ITU to Sham-operated rats did not alter FE_{Na} on comparison to values obtained from Sham-operated rats that were administered saline only (Fig. 2).

Renal I/R produced a significant increase in the urinary concentrations of NAG (Fig. 3), suggesting signifi-

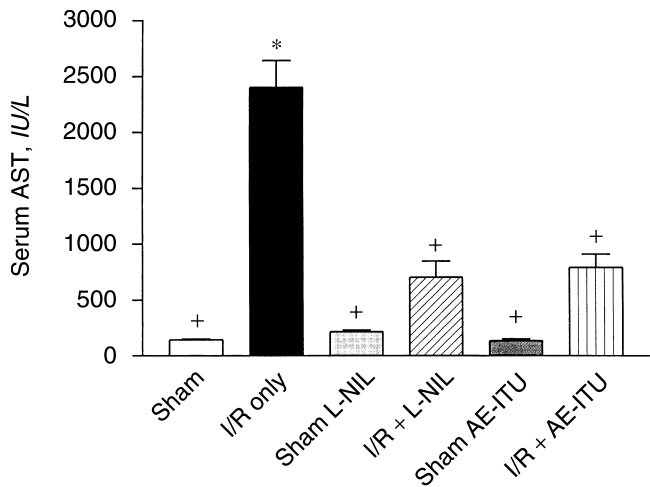


Fig. 4. Effect of renal I/R on serum aspartate aminotransferase concentrations during renal I/R in the presence of L-NIL (3 mg/kg IV bolus followed by 1 mg/kg/h IV infusion), or AE-ITU (1 mg/kg IV bolus followed by 1 mg/kg/h IV infusion). * $P < 0.05$ vs. sham-operated group, + $P < 0.05$ vs. I/R only group.

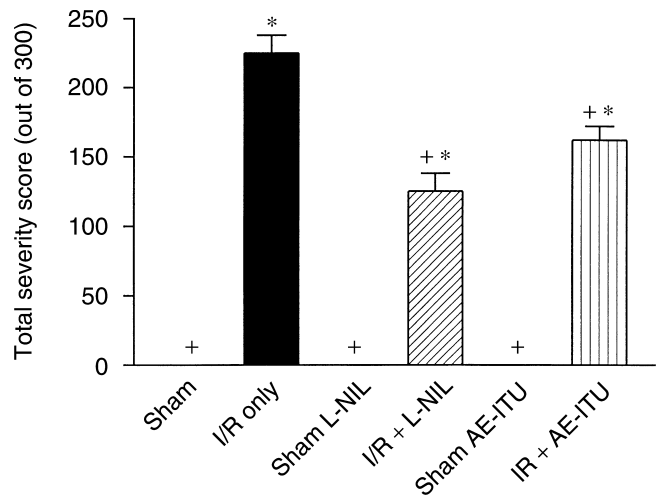


Fig. 5. Effect of renal I/R on the total severity score in the presence of L-NIL (3 mg/kg IV bolus followed by 1 mg/kg/h IV infusion), or AE-ITU (1 mg/kg IV bolus followed by 1 mg/kg/h IV infusion). * $P < 0.05$ vs. sham-operated group, + $P < 0.05$ vs. I/R only group.

cant tubular injury. Administration of L-NIL or AE-ITU prior to and during I/R significantly reduced I/R-mediated increase in urinary NAG, suggesting attenuation of tubular injury (Fig. 3). Administration of L-NIL or AE-ITU to Sham-operated rats did not alter NAG concentrations on comparison to values obtained from Sham-operated rats, which were administered saline only (Fig. 3).

Effect of L-NIL and AE-ITU on ischemia/reperfusion injury

Renal I/R produced a significant increase in the serum concentrations of AST on comparison with values obtained from Sham-operated animals (Fig. 4). Serum concentrations of AST, which was used as marker of I/R-injury, was significantly reduced subsequent to administration of L-NIL or AE-ITU prior to and during I/R (Fig. 4). Administration of L-NIL or AE-ITU to Sham-operated rats did not alter serum concentrations of AST in comparison to values obtained from Sham-operated rats that were administered saline only (Fig. 4).

Effects of L-NIL and AE-ITU on ischemia/reperfusion-mediated renal histopathology

When compared to the total severity score measured from kidneys obtained from Sham-operated animals, renal I/R produced a significant increase in total severity score, indicating significant tubular injury (Fig. 5). Total severity scores were significantly reduced by administration of L-NIL or AE-ITU prior to and during I/R (Fig. 5). Administration of L-NIL or AE-ITU to Sham-operated rats did not alter the total severity score in comparison to values obtained from Sham-operated rats that were administered saline only (Fig. 5).

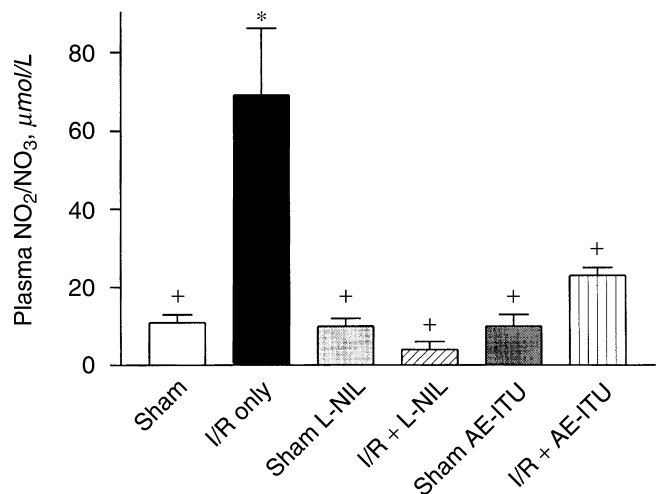


Fig. 6. Effect of L-NIL and AE-ITU on NO₂/NO₃ production mediated by renal I/R; L-NIL was administered at 3 mg/kg IV bolus followed by 1 mg/kg/h IV infusion. AE-ITU was administered at 1 mg/kg IV bolus followed by 1 mg/kg/h IV infusion. * $P < 0.05$ vs. sham-operated group, + $P < 0.05$ vs. I/R only group.

Effect of L-NIL and AE-ITU on ischemia/reperfusion-mediated increase in plasma nitrite/nitrate levels

Renal I/R resulted in a significant increase in the plasma levels of nitrite/nitrate on comparison with values obtained from the plasma of Sham-operated animals (Fig. 6). Increased plasma nitrite/nitrate levels mediated by renal I/R were significantly reduced after administration of L-NIL or AE-ITU to rats prior to and during renal I/R (Fig. 6). Administration of L-NIL or AE-ITU to Sham-operated rats did not alter plasma nitrite/nitrate levels

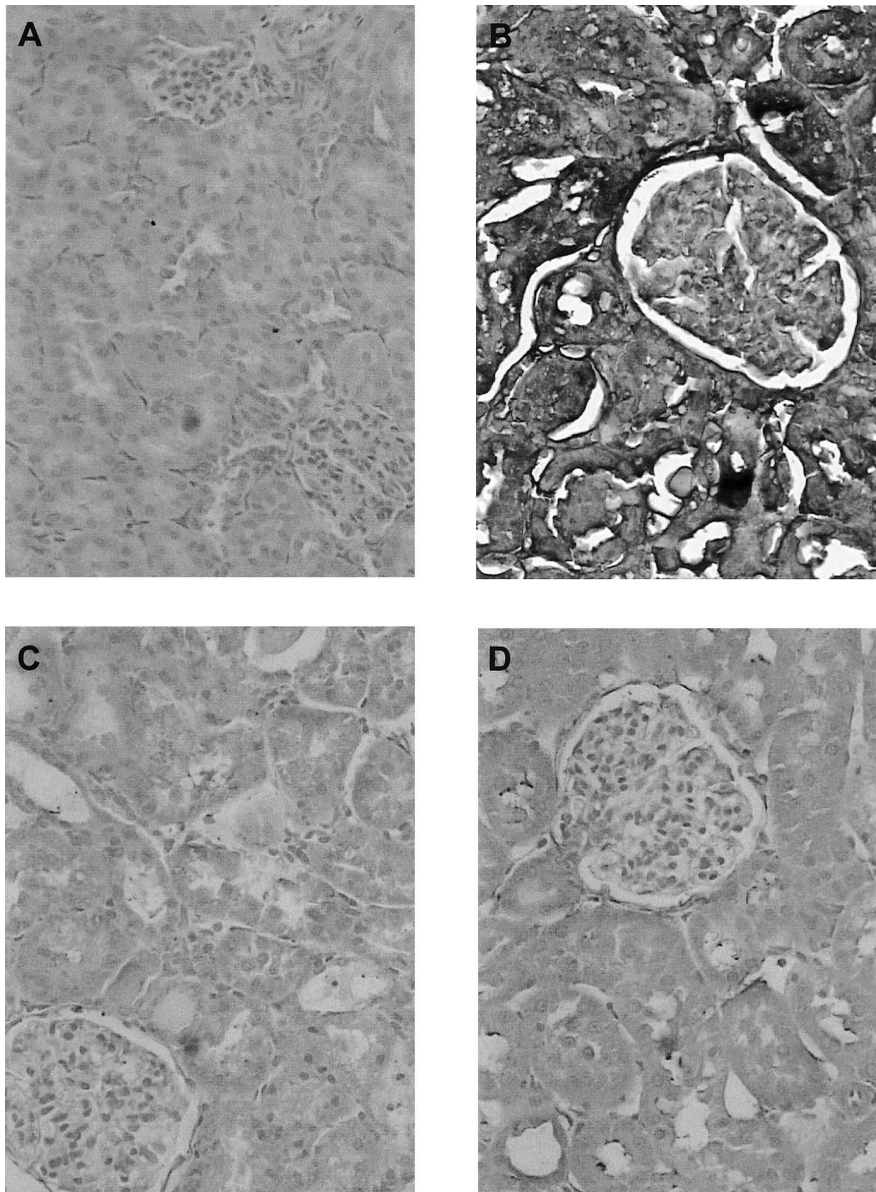


Fig. 7. Immunohistochemical evidence of peroxynitrite formation in rat kidney following I/R. (A) Kidney section from a Sham animal (original magnification, OM, $\times 32$) and (B) from a rat subjected to I/R only (OM $\times 80$). In comparison, immunoreactivity of kidney sections from rats subjected to I/R but administered (C) L-NIL (3 mg/kg IV bolus followed by 1 mg/kg/h IV infusion) or (D) AE-ITU (1 mg/kg IV bolus followed by 1 mg/kg/h IV infusion), was markedly reduced (OM $\times 80$).

in comparison to values obtained from Sham-operated rats that were administered saline only (Fig. 6).

Effect of L-NIL and AE-ITU on nitrotyrosine formation during renal ischemia/reperfusion

In comparison to renal sections obtained from Sham-operated rats which were administered saline only (Fig. 7A), immunohistochemical analysis of renal sections obtained from rats subjected to renal I/R revealed positive staining for nitrotyrosine (Fig. 7B). In contrast, substantially reduced staining was observed in the kidney sections obtained from rats, which were administered L-NIL (Fig. 7C) or AE-ITU (Fig. 7D). No evidence of staining for nitrotyrosine was observed in kidney sections

obtained from sham-operated rats, which were administered L-NIL or AE-ITU (data not shown).

Effect of L-NIL and AE-ITU on cytokine-stimulated nitric oxide production by primary cultures of rat proximal tubular cells

Isolation of primary cultures of rat PT cells resulted in confluent monolayers exhibiting typical 'cobblestone' morphology associated with epithelial cells that did not exhibit any evidence of fibroblast contamination. As discussed previously [22, 26], suspensions and cultures of rat PT cells obtained using the isolation method described here have been previously characterized using phase-contrast and electron-scanning microscopy, enzyme histo-

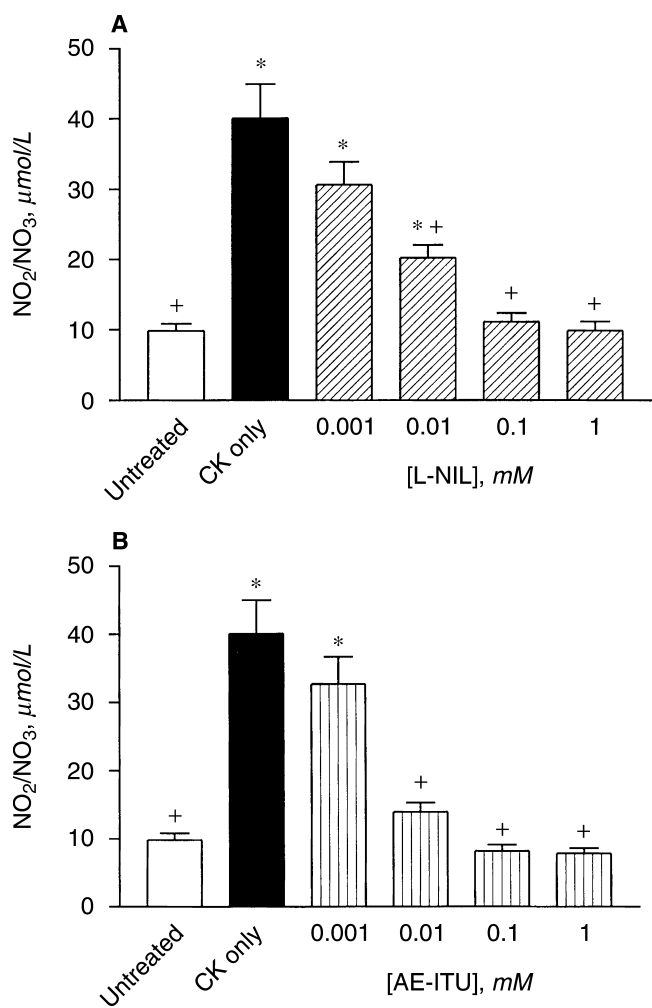


Fig. 8. Effect of L-NIL and AE-ITU on cytokine-stimulated NO₂/NO₃ production by primary cultures of rat proximal tubule (PT) cells. Rat PT cells were incubated with incubation medium only (Untreated) or with a combination of IFN- γ (100 IU/mL) and LPS (10 μ g/mL) for 24 hours. PT cells were incubated with IFN- γ and LPS in the presence of increasing concentrations of (A) L-NIL (0.001 to 1 mmol/L) or (B) AE-ITU (0.001 to 1 mmol/L). * P < 0.05 vs. untreated group, + P < 0.05 vs. CK only group.

chemistry and the demonstration of PT specific transport mechanisms (such as, D-glucose and anionic/cationic transport). These studies have demonstrated that the cells isolated using this technique are of PT origin and maintain PT-specific properties throughout culture and during experiments performed using primary cultures.

Incubation of rat PT cell cultures with IFN- γ and LPS in combination for 24 hours produced a significant increase in NO production as determined by measurement of nitrite/nitrate levels in incubation medium using the Griess assay (Fig. 8). Incubation of PT cells with IFN- γ and LPS in the presence of increasing concentrations of L-NIL (0.001 to 1 mmol/L) resulted in a significant, concentration-dependent inhibition of NO production (Fig. 8A). A similar, significant, concentration-dependent in-

hibition of NO production was observed when PT cells were incubated with IFN- γ and LPS in the presence of increasing concentrations of AE-ITU (0.001 to 1 mmol/L; Fig. 8B).

DISCUSSION

There is good evidence from both in vivo and in vitro studies that formation of NO plays an important role in the pathophysiology of renal injury mediated by hypoxia and I/R [18–22, 30, 31]. We demonstrate here, to our knowledge for the first time, that administration of L-NIL and AE-ITU, two relatively selective inhibitors of iNOS activity, significantly reduces the renal dysfunction and injury caused by I/R of the rat kidney. This conclusion is supported by the following specific, key findings: In a rat model of renal I/R, L-NIL and AE-ITU reduced the renal I/R-mediated increases in (a) serum levels of urea and creatinine (and improved creatinine clearance), (b) fractional excretion of Na⁺ (FE_{Na}) and urinary concentrations of NAG, (c) serum levels of AST and (d) plasma levels of NO. L-NIL and AE-ITU also significantly reduced the histological evidence of I/R-mediated tubular injury and substantially reduced the immunohistochemical evidence of nitrotyrosine expression. Finally, both L-NIL and AE-ITU significantly inhibited the cytokine-stimulated NO production by primary cultures of rat PT cells.

Ischemia/reperfusion of the kidney causes both glomerular and tubular dysfunction [36]. In this study, the large increase in the serum concentrations of urea and creatinine subsequent to I/R suggests significant impairment of glomerular function, which was reflected by a significant reduction in creatinine clearance. Renal I/R also resulted in a large increase in FE_{Na}, suggesting significant impairment of tubular function. Renal I/R also caused an increase in urinary NAG concentrations, which can be regarded as a marker, possibly for tubular function, but definitely for tubular injury [37]. Evidence of tubular injury also was supported by the histological scoring of renal injury as there was marked tubular injury. Renal I/R also produced significant increases in serum levels of AST, which can be regarded as a non-specific marker of extensive cellular disruption or necrosis [38] and, as previously discussed [23, 27] was used in this study as a marker of renal I/R.

In this study, both L-NIL and AE-ITU produced significant reductions of renal dysfunction and injury mediated by I/R of this kidney. The degree of protection of the kidney against I/R-mediated renal dysfunction and injury afforded by L-NIL and AE-ITU were similar. However, it is interesting to consider the mechanism(s) by which L-NIL and AE-ITU produce their beneficial effects against renal dysfunction and injury mediated by I/R. There is now good evidence that NO derived from NOS plays an important role in renal function, both under nor-

mal and pathophysiological conditions [11, 12]. High levels of NO produced by iNOS have been implicated in the renal dysfunction/injury associated with either renal I/R [23] or during endotoxic and hemorrhagic shock [39, 40]. Several *in vivo* and *in vitro* investigations have demonstrated that inhibition of iNOS expression (such as using inhibitors of nuclear factor- κ B or tyrosine kinase activation), iNOS activity (such as using inhibitors of iNOS activity), absence of iNOS itself (using iNOS^{-/-} mice), or scavenging of NO (for example, using hemoglobin) can ameliorate or prevent NO-mediated renal injury [17–23], suggesting that NO, generated by iNOS, contributes to renal I/R injury. Furthermore, the generation and action of ROS plays an important role in pathophysiology of renal I/R [17, 18]. For instance, the combination of superoxide and NO leads to the formation of peroxynitrite, which also contributes to renal I/R injury [31]. Taken together, along with formation of pro-inflammatory cytokines [such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6 or IL-8], the expression on endothelium and neutrophils of adhesion molecules [such as vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1)], and the overproduction of other vasoactive mediators [for example, eicosanoids via cyclo-oxygenase-2 (COX-2)], formation of both ROS and RNS contribute significantly to ischemic acute tubular necrosis (ATN) leading to ARF [9, 11, 18, 30].

Pathophysiological conditions such as renal I/R and endotoxemia cause the expression of iNOS mRNA and protein within the kidney [18, 30, 41, 42], increases eNOS activity [43] but does not affect expression of nNOS [44]. Although endogenous NO plays an important role in the control of regional blood flow and intrarenal hemodynamics (such as in supporting medullary reperfusion) [45], the contribution of NO to changes in renal function following injury is still relatively poorly understood. Early studies suggested that inhibition of all isoforms of NOS by non-selective NOS inhibitors by, for example, N^G-monomethyl-L-arginine (L-NMMA), causes a concomitant inhibition of eNOS activity, causing excessive vasoconstriction and hence, exacerbates organ ischemia, microvascular thrombosis and mortality [46]. This is supported by the finding that a high dose of N^G-nitro-L-arginine methyl ester (L-NAME), a relatively selective inhibitor of eNOS activity, reduces renal function in rats with or without endotoxemia [47] and augments the degree of kidney injury (glomerular thrombosis) and reduces perfusion in a rodent model of endotoxic shock [47, 48]. Thus, the beneficial hemodynamic effects of non-selective NOS inhibitors may well be due to inhibition of iNOS activity, while the reported adverse effects may be due to inhibition of eNOS activity [49]. In contrast, inhibition of NOS activity by L-NMMA in models of endotoxic shock attenuates renal dysfunction without influencing blood pressure or total renal blood flow, and can increase urine output [35].

However, it was not clear whether these effects were due to reduction of NO production from eNOS or iNOS [35].

This study compared the effects of L-NIL and AE-ITU, which are both relatively selective inhibitors of iNOS activity [32–34, 50, 51]. We demonstrate here that the administration of L-NIL provides a significant reduction of the renal dysfunction and injury associated with I/R of the rat kidney. In a recent study, L-NIL (3 mg/kg) reduced renal I/R injury and reduced peroxynitrite levels in a rat model of bilateral renal occlusion (40 min I, 6 h R) [31]. However, this effect was not observed at a higher dose of L-NIL (10 mg/kg), suggesting a loss of isoform selectivity that was reflected in the finding that nitrite/nitrate levels in the plasma of sham-operated rats were significantly reduced [31].

The beneficial role of AE-ITU in renal I/R has not been previously studied. Our current study demonstrates that AE-ITU (at a dose comparable to that of L-NIL used in this study) provides significant beneficial actions against the renal dysfunction and injury associated with I/R of the rat kidney. AE-ITU is a relatively selective inhibitor of iNOS activity that has been used to investigate the role of iNOS in LPS-mediated shock [52]. AE-ITU belongs to the group of non-amino acid isothioureas, which are potent and selective inhibitors of human nitric oxide synthases [33]. Some members of this class of compound, such as ethyl-thiourea and isopropyl-thiourea, are potent inhibitors of NOS, but without isoform selectivity [51], whereas others such as AE-ITU and S-methylisothiourea (SMT) are relatively selective inhibitors of iNOS activity compared to N^G-substituted L-arginine analogs [51]. It should, however, be noted that AE-ITU has several non-specific effects which are unrelated to the ability of this compound to inhibit iNOS activity, such as via ROS-scavenging activity or via reduction of the expression of iNOS in Gram-positive shock [53, 54].

In this study, renal I/R caused an increase in the immunological evidence of the generation of peroxynitrite that was identified as increased nitration of proteins. Generation of peroxynitrite causes renal injury via direct oxidant injury and protein tyrosine nitration [28, 29]. Respective *in vivo* and *in vitro* studies have demonstrated that formation of peroxynitrite contributes to the pathophysiology of both renal I/R and hypoxia-reoxygenation injury, respectively [30, 31]. *In vivo*, peroxynitrite formation has been demonstrated subsequent to renal I/R [31] and *in vitro*, hypoxia-reoxygenation injury of isolated PTs leads to the generation of peroxynitrite [30]. Furthermore, peroxynitrite impairs the adhesion of the tubular membrane to the basement membrane leading to the tubular obstruction often observed in ARF [55]. Both L-NIL and AE-ITU reduced evidence of peroxynitrite formation that is most likely due to their inhibitory activity of iNOS leading to reduced NO levels. It is highly likely that the residual renal dysfunction and injury caused by I/R of the

kidney was caused by ROS such as hydroxyl radical and superoxide anions, the presence of the latter increased by the reduced NO levels (and thus decreased conversion to peroxynitrite). Interestingly, when AE-ITU was administered at a lower dose than that of L-NIL, it produced a greater reduction of I/R-mediated increase in FE_{Na} , suggesting a more potent action on tubular dysfunction than L-NIL (Fig. 2). As the PT is particularly susceptible to injury from ROS [8–11], this may be evidence of ROS-scavenging activity of AE-ITU separate to its activity as an inhibitor of iNOS activity [54]. However, this will require further investigation.

In conclusion, we demonstrate that AE-ITU and L-NIL, two selective inhibitors of iNOS activity, (a) reduce the degree of renal dysfunction and injury caused by I/R of the rat kidney in vivo, (b) reduce both I/R and cytokine-stimulated NO production in vivo and in primary cultures of PT cells, and (c) reduce the evidence of I/R-mediated peroxynitrite production in vivo. We, therefore, propose that the beneficial effects of L-NIL and AE-ITU observed in our study are due to inhibition of iNOS activity and subsequent NO production. We propose that selective inhibition of iNOS activity may be of therapeutic benefit in conditions associated with renal I/R and ischemic ARF.

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APPENDIX

Abbreviations are: AE-ITU, aminoethyl-isothiourea; ARF, acute renal failure; ATN, acute tubular necrosis; C_{Cr} , creatinine clearance; CK, cytokine; COX-2, cyclo-oxygenase-2; eNOS, endothelial nitric oxide synthase; FE_{Na} , fractional excretion of Na^+ ; I/R, ischemia/reperfusion; iNOS, inducible nitric oxide synthase; ICAM-1, intercellular cell adhesion molecule-1; IL, interleukin; IFN- γ , interferon- γ ; L-NAME, N^G -nitro-L-arginine methyl ester; L-NMMA, N^G -monomethyl-L-arginine, L-NIL, L- N^6 -(1-iminoethyl)lysine; LPS, lipopolysaccharide; MEM, minimum essential medium; MOF, multiple organ failure; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PT, proximal tubule; RNS, reactive nitrogen species; ROS, reactive oxygen species; sAST, serum aspartate aminotransferase; S_{Cr} , serum creatinine; SMT, S-methylisothiourea; TNF- α , tumor necrosis factor- α ; uNAG; urinary N-acetyl- β -D-glucosaminidase; VCAM-1, vascular cell adhesion molecule-1.

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