Nephrol Dial Transplant (2007) 22: 2476–2484
doi:10.1093/ndt/gfm213
Advance Access publication 2 June 2007

Original Article

NDT
Nephrology Dialysis Transplantation

Bosentan and losartan ameliorate acute renal failure associated with mild but not strong NO blockade

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Abstract

Background. Acute renal failure (ARF) is a devastating illness, especially when it occurs in various conditions with impaired nitric oxide (NO) synthesis, such as arterial hypertension, heart failure and some renal diseases. We have directed our investigations to effects of both angiotensin II (AII) and endothelin (ET) receptor blockade associated with mild or strong NO deficiency on haemodynamic, biochemical and morphological parameters in experimental post-ischaemic ARF.

Methods. In this study, we used bosentan (dual, ET_A/ET_B-receptor antagonist), losartan (non-peptide, competitive antagonist of type I AII receptor), and NG-nitro-L-arginine methyl ester (L-NAME), inhibitor of NO synthesis. Experiments were performed in anaesthetized, adult male Wistar rats. The right kidney was removed and the renal ischaemia was performed by clamping the left renal artery for 45 min. Experimental groups received receptor antagonists (bosentan or losartan) or vehicle (saline) in the femoral vein 20 min before, during and 20 min after the period of ischaemia. L-NAME was given as i.v. bolus before each antagonist infusion. All parameters were measured 24 h after reperfusion.

Results. Our results showed that strong NO blockade overcame effects of both ET and AII receptor blockade in experimental post-ischaemic ARF. In addition, the AII receptor blockade had a harmful effect on this condition, probably due to disturbed autoregulatory renal function. On the other hand, ET and AII receptor blockade in mild NO blockade associated with reperfusion injury, improves the most haemodynamic, biochemical and morphological parameters.

Conclusions. We concluded that experimental post-ischaemic ARF is neither AII nor ET mediated in case of strong NO blockade, but, in more realistic conditions of mild NO deficiency, these peptides represent significant players whose receptor blockade expressed relevant therapeutic potential.

Keywords: acute renal ischaemia; angiotensin; endothelin; L-NAME; rats

Introduction

Acute renal failure (ARF) occurs frequently in hospitalized patients, and it is frequently associated with significant morbidity and mortality. Many therapeutic strategies have been undertaken to prevent acute renal injury, and, when ARF occurs, to improve renal function and reduce mortality [1]. ARF in the critical care setting is defined as the abrupt decline in glomerular filtration rate (GFR), resulting from ischaemic or toxic injury of the kidney. Renal failure is often only one of several organ-system failures that are present in intensive care patient population.

Recent evidence suggests that there are several factors involved in the initiation and maintenance of ARF. These factors include a decrease of glomerular capillary permeability, back-leak of glomerular filtrate, tubular obstruction, intrarenal vasoconstriction, etc. Both sublethal and lethal cell injuries have been found in ARF, the latter leads either to necrosis or to apoptosis. The finding that balance between endothelin (ET) and endothelium-derived nitric oxide (NO) is shifted in ARF, in favour of ET, which then leads to intrarenal vasoconstriction, is receiving considerable attention as a major contribution to the understanding of the pathogenesis of ARF [2]. It yields therapeutic manoeuvres targeted at restoring this balance, and relieving intrarenal vasoconstriction. Recent observations indicate that angiotensin II (AII), ET and NO may play an important role in the progression of HgCl2-induced ARF through the acceleration of

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proximal tubule epithelial cell injury and the deterioration of glomerular haemodynamics [3]. AII and ET-1, major endogenous vasoconstrictors in ARF, can modulate the effects of each other, and therefore contribute to general balance of vasoconstrictor-vasodilatator modulation in ARF. On the other hand, recent findings are consistent with a rationale for administration of both ET and AII blockers in treating NO-deficient conditions, such as arterial hypertension, heart failure, and chronic renal diseases [4]. Regarding that, the aim of our study was to evaluate the interaction of these vasoconstrictors on haemodynamic, biochemical and histological parameters in experimental post-ischaemic ARF associated with mild or strong NO deficiency.

Materials and methods

Materials

Male adult Wistar rats, weighing about 300 g, were bred in the Institute for Medical Research, Belgrade and fed with a standard chow for laboratory rats (Veterinarski zavod, Subotica, Serbia). All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals. The investigation also conformed to the principles and guidelines of Conseil de l'Europe (published in the Official Daily N. L358/1-358/6, 18 December 1986), the US National Institutes of Health (Guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23), and the Canadian Council on Animal Care (CCAC).

We used the following:

(i) Bosentan (Ro 47-0203/001; 4-*tert*-butyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-metoxy-phenoxy)-2,2'-bispyrimidine-4-yl]-benzenesulphonamide sodium salt), non-peptide, potent and mixed ET_A and ET_B-receptor antagonist (gift of Dr Martine Clozel, Actelion Ltd, Allschwil, Switzerland).
(ii) Losartan (DUP 153), AII type 1 receptor blocker was obtained from Du Pont, (Wilmington, DE, USA).
(iii) L-NAME (N^G-L-Arginine Methyl Ester, Sigma); a NO synthesis inhibitor.

Experimental protocol

All our experiments were performed in anaesthetized (35 mg/kg b.m. sodium pentobarbital; intra peritoneal—i.p.) rats.

Experimental groups and model of ARF

The animals were divided into the following experimental groups: L-NAME + ARF, L-NAME + ARF + bosentan and L-NAME + ARF + losartan. There were two control groups, control sham-operated rats (SHAM) and control rats with ARF (ARF control). In all ARF groups, the right kidney was removed, and the rats were subjected to renal ischaemia by clamping the left renal artery for 45 min. The sham group consisted of right nephrectomized rats and received the vehicle via the same route as all other experimental groups. All rats were placed in individual metabolic cages immediately after infusion and surgery procedures.

Investigation was designed in two separately performed set-ups, with high (10 mg/kg b.m.) and with low (3 mg/kg b.m.) dosage of the L-NAME infusion, combined with adequate receptor antagonist treatment or vehicle. The experimental groups received receptor antagonists bosentan (10 mg/kg b.m.) and losartan (10 mg/kg b.m.), or vehicle (saline) in the femoral vein 20 min before, during and 20 min after the period of ischaemia. This dose of bosentan was used according to the studies of Closel *et al*. [5], where the initial depressor and sustained pressor response to ET-1 was abolished and the maximal effects of bosentan were usually reached at 10 mg/kg i.v. bolus. The applied dose of losartan represents the most commonly used dose for similar cardiovascular experiments. Thus, Ortiz *et al*. [6] published that losartan in a dose of 10 mg/kg i.v. bolus blocked almost completely ($95.6 \pm 2.3\%$) the pressor effects of a bolus dose of 250 ng/kg of AII for at least 60 min.

L-NAME was given as i.v. bolus before antagonists or vehicle infusion. Inhibition of NO synthesis by L-NAME blocks all three NO synthase isoforms, resulting in hypertension in rats [7]. The dose of 10 mg/kg i.v. bolus was applied according to results published by Ortiz *et al*. [6], which revealed that this dose produces the maximum blood pressure and renal blood flow effects. On the other hand, the dose of 3 mg/kg i.v. bolus, as an example of mild NO deficiency, was chosen concerning works of Wang [8] and Filep [9], where this dose had significant effects on blood presssure and renal function in animal models.

Haemodynamic measurements 24 h after reperfusion

Haemodynamic parameters were measured after the 24 h urine collection period. All animals were anaesthetized (35 mg/kg sodium pentobarbital; i.p.). Mean arterial pressure (MAP) and heart rate (HR) were determined directly through a femoral artery catheter (PE-50, Clay-Adams, Parsippany, NY, USA), using a low-volume displacement transducer (P23 Db, Statham, Oxnard, CA, USA) and recorded on a direct writing recorder.

Cardiac output (CO) was determined by modified Coleman's application [10] of the dye dilution technique as previously described [11]. Total vascular resistance (TVR) was calculated by dividing MAP by CO and expressed as mmHg min kg/ml.

For the blood flow measurement, the left renal artery was gently separated. An ultrasonic flow probe (1RB, internal diameter = 1 mm) was placed around the artery for the measurement of renal blood flow (RBF), using a Transonic T106 Small Animal Flowmeter (Transonic System Inc., Ithaca, NY, USA). Renal vascular resistance (RVR) was calculated by dividing MAP by renal blood flow and expressed as mmHg min kg/ml.

Biochemical measurements 24 h after reperfusion

Urinary and plasma concentrations of creatinine were determined using a Beckman 42 spectrophotometer. Concentrations of sodium (Na^+) and potassium (K^+) in the plasma and urine were measured using IL 943-flame photometer (Instrumentation Laboratory, Milan, Italy). Plasma and urine protein concentrations were measured using Randox commercial test (Crumlin, Antrim, UK). Standard formula was used to calculate creatinine clearance.

Fractional excretion of electrolytes was calculated as a percentage of creatinine clearance. Reabsorption rates of Na^+ and water at tubular sites were calculated from the formulae quoted in Kusaka *et al.* [12]. Renal failure index was also calculated.

Histological examination

The left kidney was examined morphologically, 24 h after the period of reperfusion. The renal tissue was fixed in 10% buffered formalin solution. Later, the kidney was dehydrated in alcohol, blocked in paraffin wax, and 5 μm thick sections were sliced and stained by haematoxylin eosine (H&E) and by periodic acid-Schiff (PAS) reaction. By light microscopy the following parameters were semi-quantitatively evaluated on a scale from 0 to 4 according to the degree of lesions: intensity and spread of tubular necrosis, number of intraluminal cast formations, swelling and vacuolization of cells, loss of luminal membrane or brush borders, tubular dilatation, interstitial oedema and separation of cells from tubular basal membrane. The severity of congestion i.e. the accumulation of red blood cells in glomeruli, peritubular capillaries and intrarenal veins, was graded on a scale from 1 to 3, as described by Mandal *et al.* [13,14]. The sum of these changes represented the histopathological score for comparison between groups.

Statistical analysis

The results are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was applied. When the ANOVA results were significant, Bonferroni's *t*-test was used to determine the level of significance and a *P*-value <0.05 was considered to be significant (Primer of Biostatistics, by Stanton A. Glanz).

Results

Haemodynamic parameters

ARF groups treated with 10 mg/kg b.m. L-NAME. The MAP was significantly increased in all L-NAME-treated groups in comparison with both control groups. HR was significantly lower in L-NAME + ARF and L-NAME + ARF + bosentan groups, however, in the L-NAME + ARF + losartan group HR was similar to the SHAM (Table 1).

The CO was not different between experimental groups, but it was markedly reduced in comparison with the controls. Although TVR was increased in all L-NAME treated groups, it was significantly higher only in the L-NAME + ARF + bosentan group compared with both control groups (0.54 ± 0.06 *vs* 0.34 ± 0.04/0.28 ± 0.02 mmHg min kg/ml; Table 1).

After ARF induction, RBF was dramatically reduced in all ARF groups independently of treatment procedure. The L-NAME administration increased RVR in all experimental groups in comparison with SHAM (Figure 1).

ARF groups treated with 3 mg/kg b.m. L-NAME. The MAP was significantly increased in the L-NAME+ ARF group in comparison with control groups (125.00 ± 6.41 *vs* 91.11 ± 2.72/95.18 ± 2.85 mmHg; Table 2). However, bosentan and losartan decreased it approximately to control values. HR was significantly lower in all L-NAME-treated groups as compared with the controls (Table 2).

The CO was not different between the groups. TVR was significantly increased in the L-NAME + ARF group *vs* ARF control group (0.47 ± 0.04 *vs* 0.28 ± 0.02 mmHg min kg/ml; Table 2).

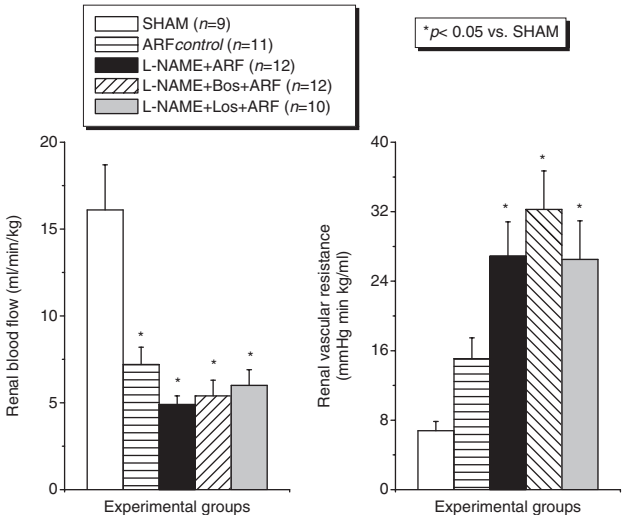

Fig. 1. Renal blood flow and renal vascular resistance in experimental groups after 10 mg/kg b.m. L-NAME infusion.

Table 1. Haemodynamic parameters and histopathological score after 10 mg/kg b.m. L-NAME infusion

Groups	MAP (mmHg)	HR (beats)	CO (ml/min/kg)	TVR (mmHg min kg/ml)	Histopathological score
SHAM ($n=9$)	91.11 ± 2.72	374.44 ± 17.00	307.00 ± 32.00	0.34 ± 0.04	0.71 ± 0.18
ARF control ($n=11$)	95.18 ± 2.82	335.00 ± 13.19	331.00 ± 24.40	0.28 ± 0.02	9.75 ± 0.70*
L-NAME + ARF ($n=2$)	126.15 ± 3.53**	290.00 ± 15.02*	267.50 ± 28.70	0.47 ± 0.06	10.67 ± 0.56*
L-NAME + Bos + ARF ($n=2$)	129.15 ± 2.63*,**	302.75 ± 17.83*	244.00 ± 22.40	0.54 ± 0.06*,**	8.22 ± 1.00*
L-NAME + Los + ARF ($n=0$)	117.38 ± 4.65*,**	372.50 ± 10.65	284.50 ± 19.90	0.45 ± 0.04	12.00 ± 0.53*,†

*$P < 0.05$ compared with sham; **$P < 0.05$ compared with ARF control.
†$P < 0.05$ compared to L-NAME + Bos + ARF
MAP-mean arterial pressure; HR-heart rate; CO-cardiac output; TVR-total vascular resistance

Table 2. Haemodynamic parameters and histopathological score after 3 mg/kg b.m. L-NAME infusion

Groups	MAP (mmHg)	HR (beats)	CO (ml/min/kg)	TVR (mmHg min kg/ml)	Histopathological score
SHAM ($n=9$)	91.11 ± 2.72	374.44 ± 17.00	307.00 ± 32.00	0.34 ± 0.04	0.71 ± 0.18
ARF control ($n=11$)	95.18 ± 2.85	335.00 ± 13.19	331.00 ± 24.40	0.28 ± 0.02	$9.75 \pm 0.70^{*}$
L-NAME + ARF ($n=8$)	$125 \pm 6.41^{*,**}$	$297.50 \pm 7^{*}$	273.80 ± 12.20	$0.47 \pm 0.04^{**}$	$10.50 \pm 0.34^{*}$
L-NAME + Bos + ARF ($n=8$)	$102.50 \pm 6.90^{***}$	$305 \pm 1.89^{*}$	281.50 ± 24.30	0.38 ± 0.04	$5.71 \pm 0.29^{*,**,***}$
L-NAME + Los + ARF ($n=9$)	$89.44 \pm 5.46^{***}$	$297.78 \pm 6.41^{*}$	231.70 ± 28.10	0.42 ± 0.04	$6.50 \pm 0.43^{*,**,***}$

$^{*}P < 0.05$ compared with sham; $^{**}P < 0.05$ compared with ARF control; and $^{***}P < 0.05$ compared with L-NAME + ARF.
MAP-mean arterial pressure; HR-heart rate; CO-cardiac output; TVR-total vascularr esistance.

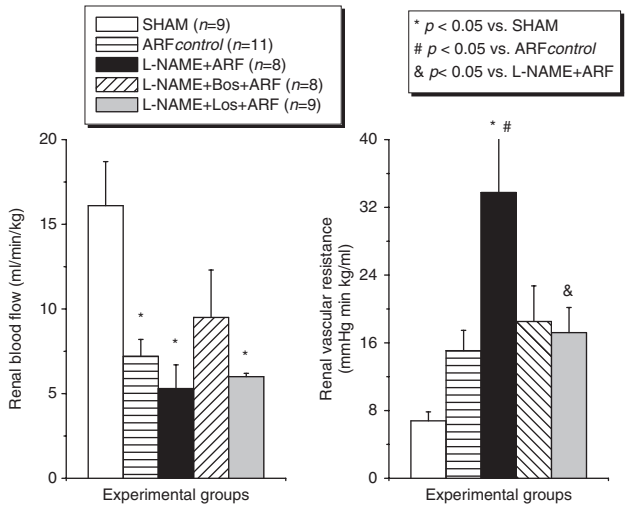

Fig. 2. Renal blood flow and renal vascular resistance in experimental groups after 3 mg/kg b.m. L-NAME infusion.

Renal ischaemia markedly diminished RBF in all ARF groups as compared with SHAM. L-NAME or receptor antagonists had no influence on the RBF value (Figure 2). RVR was significantly higher in the L-NAME+ARF group compared to ARF control (33.74 ± 6.71 *vs* 15.07 ± 2.40 mmHg min kg/ml). However, bosentan and losartan reduced it almost to the value of ARF control group (Table 2).

Biochemical parameters

ARF groups treated with 10 mg/kg b.m. L-NAME. GFR, presented as an endogenous creatinine clearance, fell in all ARF groups 24 h after the period of ischaemia. In the L-NAME + ARF group, decrease in GFR was smaller as compared with other groups with ARF, but not statistically significant (Table 3).

ARF induced higher fractional excretion of sodium ($FE_{Na}{}^{+}$), however, $FE_{Na}{}^{+}$ was strongly increased in the L-NAME + ARF groups treated with antagonists (Table 3).

The value of the urine protein excretion (UPE) was doubled in the ARF control with respect to sham animals (278.31 ± 46.67 *vs* 130.51 ± 6.91 mg/24 h/kg) and it was similar to the values of all experimental groups (Table 3).

Plasma potassium concentration ($P_K{}^{+}$) was significantly increased in the group of L-NAME + ARF + losartan as compared with SHAM and ARF control (7.17 ± 0.75 *vs* $4.31 \pm 0.15/4.93 \pm 0.15$ mmol/l). Other groups showed no relevant differences (Table 3).

Renal failure index (RFI) notably rose after ARF and was extremely high in both groups with receptor antagonist infusion (Table 3).

ARF groups treated with 3 mg/kg b.m. L-NAME. GFR in the L-NAME + ARF group was similar to the ARF control group. Although both applied receptor antagonists improved GFR, only bosentan revealed significant improvement as compared with ARF control (1.74 ± 0.39 *vs* 0.54 ± 0.17 ml/min/kg; Table 4).

Bosentan and losartan treatment reduced $FE_{Na}{}^{+}$ as compared with L-NAME + ARF group, and significantly reduced it as compared with ARF control group (Table 4).

UPE had similar values in all L-NAME treated groups but it was markedly lower compared with the ARF control. $P_K{}^{+}$ showed no relevant differences between the groups. RFI was reduced moderately in bosentan- and losartan-treated groups, as compared with L-NAME + ARF group (Table 4).

Histological studies

Morphological examination of the renal tissue revealed significant differences between experimental groups of animals.

Glomeruli, tubulointerstitium and blood vessels of the sham-operated animals were without any changes on the light microscopy examination. In a few kidney specimens, only a small number of PAS-positive casts were observed in the tubular lumen (Figure 3A).

Kidneys in the ARF control group showed dilatation of some segments of proximal and distal tubules with or without loss of brush border of proximal tubular epithelium. Swelling of some proximal tubular epithelial cells was present. The most prominent lesions were widespread tubular necrosis in the corticomedullary zone and a huge number of PAS-positive casts in the lumina of distal tubuli and collecting ducts. The intensity of interstitial oedema varied among

Table 3. Biochemical parameters and renal failure index after 10 mg/kg b.m. L-NAME infusion

Groups	Creatinine clearance (ml/min/kg)	Fractional excretion of sodium (%)	Urine protein excretion (mg/24 h/kg)	Plasma potassium concentration (mmol/l)	Renal failure Index
SHAM ($n=9$)	2.22 ± 0.23	1.18 ± 0.56	130.51 ± 6.91	4.31 ± 0.15	0.23 ± 0.10
ARF control ($n=11$)	$0.23 \pm 0.03^{*}$	11.04 ± 2.43	278.31 ± 46.67	4.93 ± 0.29	2.17 ± 0.48
L-NAME + ARF ($n=12$)	$0.63 \pm 0.20^{*}$	9.73 ± 3.61	172.86 ± 14.69	5.47 ± 0.54	1.88 ± 0.66
L-NAME + Bos + ARF ($n=12$)	$0.17 \pm 0.03^{*}$	$49.44 \pm 10.28^{*}$	313.74 ± 44.94	6.03 ± 0.39	$9.19 \pm 2.14^{*}$
L-NAME + Los + ARF ($n=10$)	$0.14 \pm 0.026^{*}$	$74.31 \pm 21.60^{*,**,***}$	333.62 ± 108.03	$7.17 \pm 0.75^{*,**}$	$14.95 \pm 4.14^{*,**,***}$

$^{*}P < 0.05$ compared with sham; $^{**}P < 0.05$ compared with ARF control; $^{***}P < 0.05$ compared with L-NAME + ARF.

Table 4. Biochemical parameters and renal failure index after 3 mg/kg b.m. L-NAME infusion

Groups	Creatinine clearance (ml/min/kg)	Fractional excretion of sodium (%)	Urine protein excretion (mg/24 h/kg)	Plasma potassium concentration (mmol/l)	Renal failure index
SHAM ($n=9$)	2.22 ± 0.23	1.18 ± 0.56	130.51 ± 6.91	4.31 ± 0.15	0.23 ± 0.10
ARF control ($n=11$)	$0.23 \pm 0.03^{*}$	$11.04 \pm 2.43^{*}$	$278.31 \pm 46.67^{*}$	4.93 ± 0.29	2.17 ± 0.48
L-NAME + ARF ($n=8$)	$0.54 \pm 0.17^{*}$	4.65 ± 3.01	$95.70 \pm 13.70^{**}$	4.09 ± 0.14	6.2 ± 3.90
L-NAME + Bos + ARF ($n=8$)	$1.74 \pm 0.39^{**}$	$1.64 \pm 0.55^{**}$	$93.20 \pm 14.60^{**}$	4.54 ± 0.35	2.16 ± 0.70
L-NAME + Los + ARF ($n=9$)	$1.06 \pm 0.56^{*}$	$2.06 \pm 0.90^{**}$	$78.80 \pm 18.90^{**}$	4.37 ± 0.29	2.76 ± 1.22

$^{*}P < 0.05$ compared to sham; $^{**}P < 0.05$ compared with ARF control.

specimens in this group. Glomeruli and blood vessels were the same as in the sham-operated group (Figure 3B).

ARF groups treated with 10 mg/kg b.m. L-NAME. In the L-NAME + ARF group, the picture was similar to the ARF control, with more widespread and intensive tubular necrosis (Figure 3C).

In some specimens of the L-NAME + ARF + bosentan group, tubular necrosis in corticomedullary junction was less intense and less widespread in comparison with the control animals with ARF (Figure 3D).

Unexpectedly, kidney specimens of L-NAME + ARF + losartan group showed intensive and more widespread tubular necrosis in the corticomedullary region. Tubular dilatation and a huge number of PAS-positive casts in collecting ducts were more prominent as compared with the ARF control group (Figure 3E).

ARF groups treated with 3 mg/kg b.m. L-NAME. Confluent tubular necrosis in the corticomedullary zone, a huge number of PAS-positive casts in lumina of tubuli and collecting ducts and tubular dilatation with pronounced loss of brush border of proximal tubular epithelium, were all present in the L-NAME + ARF group. Focal tubular necrosis and PAS-positive luminal casts in the cortex were also noticed in this group (Figure 4A).

Interestingly, in the L-NAME + ARF + bosentan group, tubular necrosis was less widespread and predominantly focal, proximal tubular dilatation was slightly prominent, and casts formation was very rarely present. The loss of brush border of proximal tubular epithelium was less evident (Figure 4B).

The morphology of the kidneys of the L-NAME + ARF + losartan group was similar to the kidneys of the L-NAME + ARF + bosentan group, with more prominent tubular dilatation in the corticomedullary zone (Figure 4C).

Discussion

Recent observations indicate that NO has a crucial role in the pathogenesis of ischaemic ARF, and that spontaneous NO donors may be clinically effective in ischaemic ARF [15,16]. However, ET-1 plays a significant role in the pathogenesis of ischaemic ARF [17], in renal failure that occurs in patients with acute liver failure [18], as well as in haemodynamic events in radiocontrast-induced nephropathy [19]. ET-1 receptor antagonists provide a potential therapeutic tool due to the fact that bosentan could attenuate cyclosporine A renal toxicity in a double-blind, placebo-controlled crossover study [20]. Also, it has been demonstrated that AT1 receptor blockade accelerates recovery of renal function in the post-ischaemic kidney [21].

In the present study, bolus administration of 10 mg/kg b.m. L-NAME increased both MAP and TVR, similarly to long-term L-NAME application in the studies reported by Jerkić *et al.* [16]. ET and AII receptor blockades had no influence on those parameters in the mentioned conditions. Although MAP

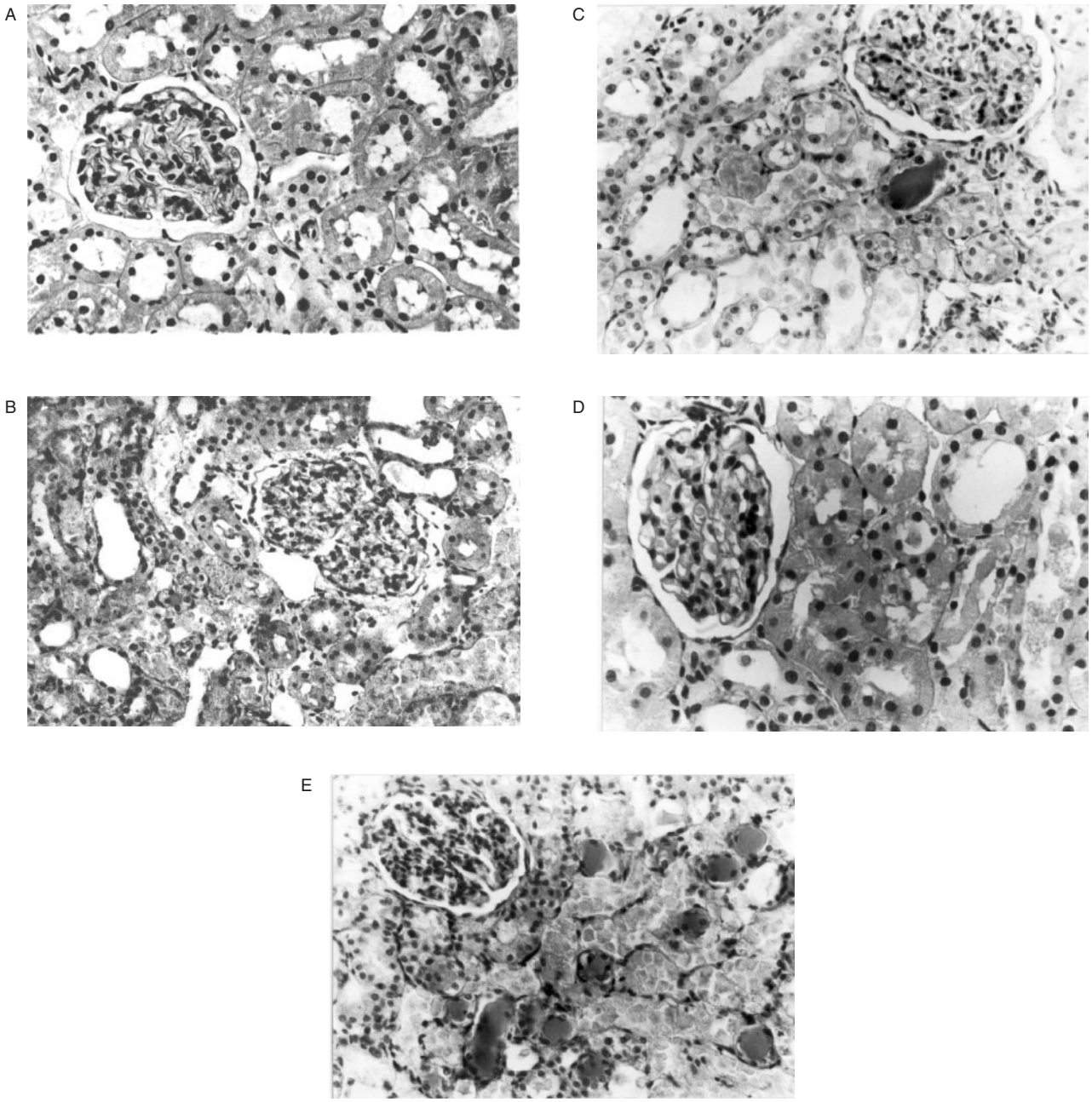

Fig. 3. Histology of the kidney after 10 mg/kg b.m. L-NAME infusion; (**A**) a normal shape of the glomerulus and tubulointerstitium in the sham-operated animals (**B**) glomerulus, tubular dilatation and necrosis, PAS-positive casts in the ARF *control* group; (**C**) intensive tubular necrosis, tubular dilatation and interstitial oedema in the L-NAME + ARF group; (**D**) moderately intensive tubular necrosis in bosentan-treated rats; (**E**) intensive and widespread tubular necrosis, tubular dilatation and a huge number of PAS-positive casts in losartan-treated rats (PASX320).

had a tendency to decrease in the L-NAME + ARF + losartan group in comparison with other L-NAME groups, this result was not statistically significant. This is in accordance with results of Turskstra *et al.* [22] who showed that short-term NO synthase (NOS) blockade caused a dose-dependent pressor and renal vasoconstrictor response and that AT1-receptor blockade restored systemic pressor and renal vasoconstrictive effects of mild NOS inhibition, but failed to exert vasorelaxation during strong NOS blockade. Also, the mentioned results supported our findings that after 3 mg/kg b.m. L-NAME infusion MAP significantly fell in losartan-treated animals with ARF. In the L-NAME + ARF + bosentan group, after strong NO blockade we obtained MAP values similar to the ARF group treated with L-NAME alone. Filep [9] has demonstrated that the pressor effect of L-NAME (given in a dose of 3 mg/kg b.m.) was markedly reduced by

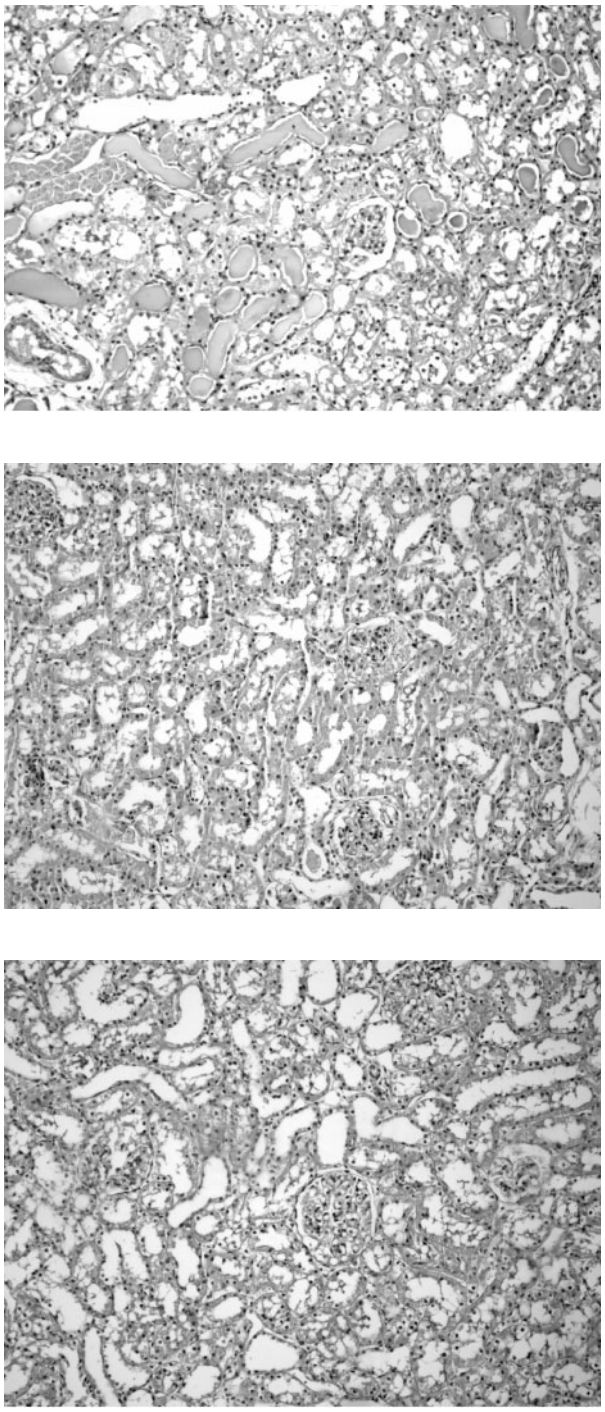

Fig. 4. Histology of the kidney after 3 mg/kg b.m. L-NAME infusion; (A) focal necrotic arrays, a huge number of PAS-positive casts in collecting ducts, tubular dilatation with pronounced loss of the brush border of proximal tubular epithelium in the cortex of the L-NAME + ARF group; (B) slightly prominent proximal tubular dilatation, rarely casts formation and less pronounced loss of brush border of the proximal tubular epithelium in L-NAME + ARF + bosentan group; (C) more pronounced tubular dilatation in the corticomedullary zone of the L-NAME + ARF + losartan group.

bosentan in normal Wistar rats, which is in correspondence with the reduction of MAP in our results obtained by administration of 3 mg/kg b.m. L-NAME together with bosentan in ischaemic rats. On the other hand, results of Qiu *et al.* [23] have shown that bosentan blunted (but did not normalize) the rise in MAP after acute L-NAME application in the dosage of 10 mg/kg b.m. in chronically catheterized Spargue-Dawley rats. The absence of this effect in our experiment could be explained by different rat strain and by production of other vasoconstrictors in post-ischaemic condition.

Present study revealed that renal haemodynamic alterations induced by ARF accompanied with NO synthesis inhibition by L-NAME, are more intensive than those induced by ARF alone. The worsening of ischaemia-induced renal vasoconstriction caused by L-NAME suggests that endogenous NO is functionally present and may be an important mediator in the regulation of renal haemodynamics in the post-ischaemic ARF. Conger *et al.* [24] demonstrated in 1980 that endothelium-dependent relaxation was clearly impaired after ischaemia, but their later publication [25] disclosed that attenuated response to endothelium-dependent vasodilators could not be interpreted due to decrease of NOS activity. Namely, the authors suggest that the increase of NOS activity is a response to ischaemia-induced renal vasoconstrictor activity. In our experiment, both ET and AII receptor antagonists slightly improve RBF after strong NOS blockade compared with the group treated only with L-NAME, but strongly increased RBF after moderate NO deficiency. Qiu and Baylis [26] suggest that endogenous ET and AII partially mediate the glomerular haemodynamic response to acute NOS inhibition. The authors also indicate that actions of ET and AII are mainly additive, because vasoconstrictive responses to acute NOS inhibition are mostly prevented when both vasoconstrictor systems are blocked. In addition, results of Fortepiani *et al.* [27] have demonstrated that treatment with bosentan did not attenuate the arterial hypertension of the L-NAME-treated rats, but normalized their GFR and RBF. Our results demonstrated that it was not the case in post-ischaemic conditions associated with strong NO inhibition, but in the condition of mild NO deficiency, bosentan clearly improved RBF and GFR. Impairment of GFR and RBF induced by strong NOS blockade in our bosentan-treated rats could be partially explained by complete blockade of ET-B receptors in addition to NO inhibition, because enhanced auto-regulatory renal blood flow efficiency due to NO inhibition includes preferential activation of the ET-B receptors [28].

Twenty-four hours after ARF induction, GFR was drastically reduced followed by enhanced $FE_{Na}{}^{+}$. These biochemical parameters were not influenced by inhibition of NOS induced by L-NAME (10 mg/kg b.m.) alone or in association with receptor antagonists. Results of Treeck *et al.* [29] have shown that after pre-treatment with L-NAME (19 mg/kg b.m.), losartan increased total GFR, in the absence of ARF, which is

somehow in agreement with our results, obtained by mild NO blockade associated with ARF. Otherwise, our results with strong NO deficiency suggest, similar to the observation of Jover *et al*. [30], that AII, through its effects on AT1 receptors, may play an important role in the maintenance of L-NAME hypertension, but regulation of both GFR and filtration fraction encompasses non-AII-mediated mechanisms. In addition, tubular cell injury which is associated with ARF, additionally diminished regular vascular response to vasoactive substances.

Increase of $FE_{Na}{}^{+}$ in both groups treated with receptor antagonists could be a consequence of receptor blockade and tubular cell injury. It is well known that AII has a dominant role in sodium retention due to stimulation of aldosterone secretion from the adrenal cortex and due to action on intraluminal AT1 receptors of proximal and distal tubules [31]. Blockade of AT1 and ET receptors, as well as sublethal and necrotic injuries of tubular cells (caused by ischaemia), could be the reason for the noticeable increase of RFI and increase of $P_K{}^{+}$. Results of Jerkić *et al*. [17] have shown that losartan (10 mg/kg b.m.) significantly reduced UPE after reperfusion injury in Wistar rats. Results of the present study demonstrate that mild NO inhibition leads to diminished UPE independently of receptor blockades.

Slight improvement of morphological renal changes in bosentan-treated rats did not support a crucial role of ET in induction of kidney lesions caused by strong NO inhibition in post-ischaemic ARF. Our previous results have shown that ET-1 dual receptor blockade improved histopathological changes induced by ischaemia in the kidney of normotensive Wistar rats [17]. Other authors reported also that ET receptor antagonists improve both renal function and renal histological features in the setting of renal ischaemia-reperfusion injury [32,33]. In the present study, our results show that ET peptides probably do have an important role in inducing renal damages caused by mild NO deficiency, because dual ET blockade improves the most morphological parameters in the ischaemic kidney.

Prominent tubular necrosis in the corticomedullary region, tubular dilatation and a huge number of PAS-positive casts in collecting ducts in rats treated with losartan, indicate harmful effects of AII receptor blockade in ARF associated with strong NO blockade. However, our previous results [17] have shown that losartan had no beneficial effects on renal function and morphology in normotensive rats with ischaemic ARF. In addition, there is a well-known precaution for use of AII blockade in patients with ARF in clinical units, because this blockade reduced blood pressure and GFR. In the present study, blood pressure in the losartan-treated group was high, but GFR was still significantly reduced. Taken together, these results indicate that the most important consequence of strong NO deficiency in the post-ischaemic kidney is renal vasoconstriction, which could be suppressed neither by dual ET receptor blockade nor by AT1 receptor blockade. In our opinion, strong NO deficiency is a main reason for non-improved kidney function and morphology in the present experimental conditions. Additionally, NO participates in several vital processes in the kidney which encompass regulation of glomerular and medullary haemodynamics, tubuloglomerular feedback response, renin release, extracellular fluid volume [34] and regulation of Na^{+}-K^{+}-ATPase, Na^{+}/H^{+} exchangers and paracellular permeability of proximal tubular cells [35]. Kontogiannis and Burns [21] have demonstrated that renal ischaemia–reperfusion injury caused an early increase of intrarenal AII levels and that blockade of AT1 receptors with losartan accelerated recovery of renal function after bilateral renal pedicle occlusion for 60 min. These results are in concordance with results of the present study, which show that AII partly contributes to functional disturbance and morphological lesions after moderate NO deficiency in ischaemic kidney.

In summary, our results have shown that strong NO blockade overcomes the effects of both ET and AII receptor blockade in experimental post-ischaemic ARF. Thus, we could conclude that renal vasoconstriction, decline of kidney function and morphological lesions, due to strong NOS blockade, were not mediated either by ET-1 or by AII in post-ischaemic ARF. Also, as a consequence of disturbed tubuloglomerular feedback response, additional AII receptor blockade may be harmful in the condition of strong NO deficiency and ARF. Non-selective ET receptor blockade excludes ET-B receptors from enhanced auto-regulatory renal blood flow efficiency due to NO inhibition and therefore contributes to further impairment of renal function and morphology. Since ET and AII receptor blockade in more realistic mild NO blockade associated with reperfusion injury improved the most haemodynamic, biochemical and morphological parameters, this could have relevant therapeutic potential.

Acknowledgements. The authors are grateful for excellent technical assistance to Zaga Jovanović, Belgrade, Serbia. This work was supported by a grant (project No. 145054) from the Ministry of Science and Enviromental Protection of Serbia.

Conflict of interest statement. None declared.

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Received for publication: 17.2.06
Accepted in revised form: 19.3.07