THE EFFECTS OF ENDOTHELIN RECEPTOR BLOCKADE ON THE COURSE OF EXPERIMENTAL POSTISCHAEMIC ACUTE RENAL FAILURE

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The main goal of this study was to examine the efficacy of endothelin (ET) receptor blockade in the course of experimental postischaemic acute renal failure (ARF). Experiments were performed on male adult Wistar rats. The right kidney was removed before renal ischaemia (by clamping the left renal artery for 45 minutes). The experimental groups received either bosentan (ETA/ETB-receptor antagonist; 10mg/kg/b.m.) or vehicle (saline) in the femoral vein 20 minutes before, during and 20 minutes after ischaemia. All parameters were measured 24 hours after reperfusion. The obtained results clearly demonstrate that bosentan yields beneficial effect on ARF. Bosentan improves both renal haemodynamic and functional parameters after ARF, while lesions of tubular epithelial cells, the principal targets of injury in ARF, are less serious in bosentan-treated rats than in the control ARF rats. This strongly suggests that endothelins have an important role in the development of ischaemia/reperfusion injury and contributes to the promotion of bosentan in future clinical practice.

Key words: acute renal failure, biochemistry, bosentan, endothelin receptor blockade, haemodynamic, tubular injury

INTRODUCTION

Acute renal failure (ARF) is defined as a sudden decline in glomerular filtration rate (GFR), usually associated with azotemia and a fall in urine output (oliguria or rarely anuria) resulting from ischemic or toxic injury of the kidney (Nissenson 1998). Acute renal failure is a devastating illness that is associated with a high risk of mortality. For example, simple acute renal failure in the presence of no other underlying illnesses has about 7 to 23% mortality, whereas the mortality of acute renal failure in an Intensive Care Unit setting is about 50 to 80% (Star 1998). There are four factors that have been considered to be most important in the initiation and maintenance of ARF. These are a decrease in glomerular capillary permeability, back-leak of glomerular filtrate, tubular obstruction and intrarenal vasoconstriction (Nissenson 1998). Each of these mechanisms

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contribute individually or in combination during the course of ARF. Although the term acute tubular necrosis accurately defines the population of cells most severely injured in this disease, accumulating evidence suggests that many renal tubular cells are sublethally injured and, while appearing intact morphologically, can still contribute to the tubular leakiness and obstruction associated with ARF (Liberthal, 1997).

The endothelins (ET_S) belong to a family of potent vasoconstrictor peptides that were first isolated from vascular endothelial cells in 1988 (Yanagisawa et al. 1988). The three isoforms of ET_S (ET_1, ET_2, and ET_3) are produced in renal tissue (Kohan, 1993). ET_S exert their effects via two types of receptors, classifed as ET_A and ET_B (Chandrashekar et al. 1994). ET_1 has been described as a very powerful vasoconstrictor agent isolated from vascular endothelium (Battistini et al., 1993) and it has unusually long-lasting vasoconstrictor activity (Kohan, 1993). Animal models of ARF, as well as studies during the maintenance phase of ARF in humans have demonstrated a marked reduction in total renal blood flow, averaging nearly 50% in most publications (Nissenson 1998). The study of Ruschitzka et al. (1999) demonstrates that in ARF an increase of circulating and vascular ET_1 and ET_A and ET_B receptor gene expression occurs, which in turn induces endothelial dysfunction and enhanced vasoconstriction in the renal as well as the systemic vasculature (Ruschitzka et al. 1999).

Numerous studies have shown that ET_S concentration is elevated in plasma of patients with ARF (Tomita et al. 1989) and in ischemic rats (López-Farré et al. 1991). The results obtained so far indicate that ischaemia-induced ARF is attenuated by the infusion of: anti-ET antibodies (López-Farré et al. 1991), ET-converting-enzyme inhibitor, phosphoramidon (Vemulapalli et al. 1993) and ET-receptor antagonists (Kusumoto et al. 1994), (Gellai et al. 1994α), (Bird et al. 1995).

The aim of our studies was to investigate the effects of the nonpeptide nonselective endothelin receptor antagonist *bosentan* on hemodynamic, biochemical and pathohistological parameters in rats with postischemic ARF.

MATERIALS AND METHODS

Materials

Male adult Wistar rats, weighting about 300 g, were bred in the Institute for Medical Research, Belgrade and fed on a standard chow for laboratory rats (Veterinarski zavod, Subotica, Yugoslavia). 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 the Canadian Council on Animal Care (CCAC). We used 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), a nonpeptide, potent and mixed ET_A and ET_B-receptor antagonist (gift of Dr Martine Clozel, Actelion Ltd, Allschwil, Switzerland) (Clozel et al. 1993).

Experimental procedure

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

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Experimental groups and model of acute renal failure

The animals were divided in to three groups: control sham operated rats (SHAM), control rats with acute renal failure (ARFcontrol) and rats with ARF and infusion of bosentan (ARFbosentan). In both ARF groups the right kidney was removed, and rats were subjected to renal ischemia by clamping the left renal artery for 45 minutes. The ARFbosentan group received bosentan (10 mg/kg b. m.; $n = 18$) in the femoral vein 20 minutes before, during and 20 minutes after the period of renal ischemia, while the ARFcontrol group ($n = 24$) received the vehicle (saline) via the same route. The SHAM group ($n = 17$) consisted of right nephrectomized rats and they received vehicle also. All rats were placed in individual metabolic cages immediately after infusion and surgical procedures.

Haemodynamic measurements 24 hours after reperfusion

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

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 total renal blood flow (RBF), using a Transonic T106 Small Animal Flowmeter (Transonic System Inc., Ithaca, NY, USA). Renal vascular resistance was calculated by dividing MAP by renal blood flow and expressed as Pa s kg/ μl b. m.

Biochemical measurements and morphology

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 on a IL 943-flame photometer (Instrumentation Laboratory, Milan, Italy). Plasma and urine protein concentrations were measured using the Randox commercial test (Crumlin, Antrim, UK). The standard formula was used to calculate creatinine clearance. Fractional excretion of electrolytes was calculated as a percent of creatinine clearance. Reabsorption rates of Na^+ and water at tubular sites were calculated from the formulae quoted in Kusaka et al. (1994). Renal failure index was also calculated.

Histological examination

The left kidney was examined morphologically, 24 hours after 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 cut and stained by periodic acid-Schiff reaction. Light microscopic (LM) evaluations were made and acute tubular lesions were graded on a scale from 0 to 4+ according to the degree of severity:

    0 = normal tubular cells,

    1+ = loss of luminal membrane or brush borders,

    2+ = swelling and vacuolization of cells,

    3+ = separation of cells from the basement membrane,

    4+ = same as 3+ with nude basement membrane.

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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.* (1978). The presence of tubular dilatation, cast formations, mononuclear infiltration and interstitial edema was scored with 1, and their absence with 0. The vascular changes (in arterial vessel walls) were graded according to the scoring system proposed by Mandal and coworkers (1977). The sum of these changes was the histopathological score for comparison between the groups.

PRA and ET measurement

In the remaining rats from each group ($n = 8$), plasma renin activity (PRA) and plasma ET were measured. Animals were guillotined and blood samples were collected into chilled, siliconised plastic centrifuge tubes containing Na_2EDTA (2 mg/ml) for PRA and both Na_2EDTA (1mg/ml) and aprotinin (1000 KIU/ml) for ET measurement. REN-CT2 angiotensin I radioimmunoassay (RIA) kit (CIS bio international, France) and RPA 535 Endothelin-1,2 Biotrack Assay System with Amprep C_2 RPN 1903 columns (Amersham, England), were used for their determination respectively.

Statistical analyses

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

RESULTS

Hemodynamic parameters

Mean arterial pressure and heart rate showed no statistically significant differences between the groups 24 hours after the period of ischemia (Figure 1.).

In the ARF*control* group renal blood flow (RBF) was significantly reduced and renal vascular resistance (RVR) was significantly increased compared to the SHAM group. In the ARF*bosentan* group bosentan infusion increased RBF and significantly reduces RVR in comparison to the ARF*control* (Figure 2.).

Biochemical parameters

Glomerular filtration rate (GFR; endogenous creatinine clearance) drastically dropped in the ARFcontrol vs. SHAM 24 hours after the period of ischemia. In the bosentan treated rats GFR was 76% higher compared to the ARFcontrol but the difference was not significant and the mean value was still significantly lower than in SHAM rats (Figure 3.).

Urine volume and plasma sodium, potassium and protein concentration showed no differences between the groups 24 hours after ARF (Table 1.). In the ARFcontrol, urine protein excretion was doubled in comparison to the SHAM animals but in ARFbosentan it had returned near to the level of sham operated rats (Figure 3.). Fractional excretion of sodium (FENa+) was increased 24 hours after induction of ischaemic ARF but was diminished in bosentan treated rats compared to the ARFcontrol (Figure 4.). ARF caused a marked decline in sodium and water reabsorption and increases renal failure index. In the ARFbosentan

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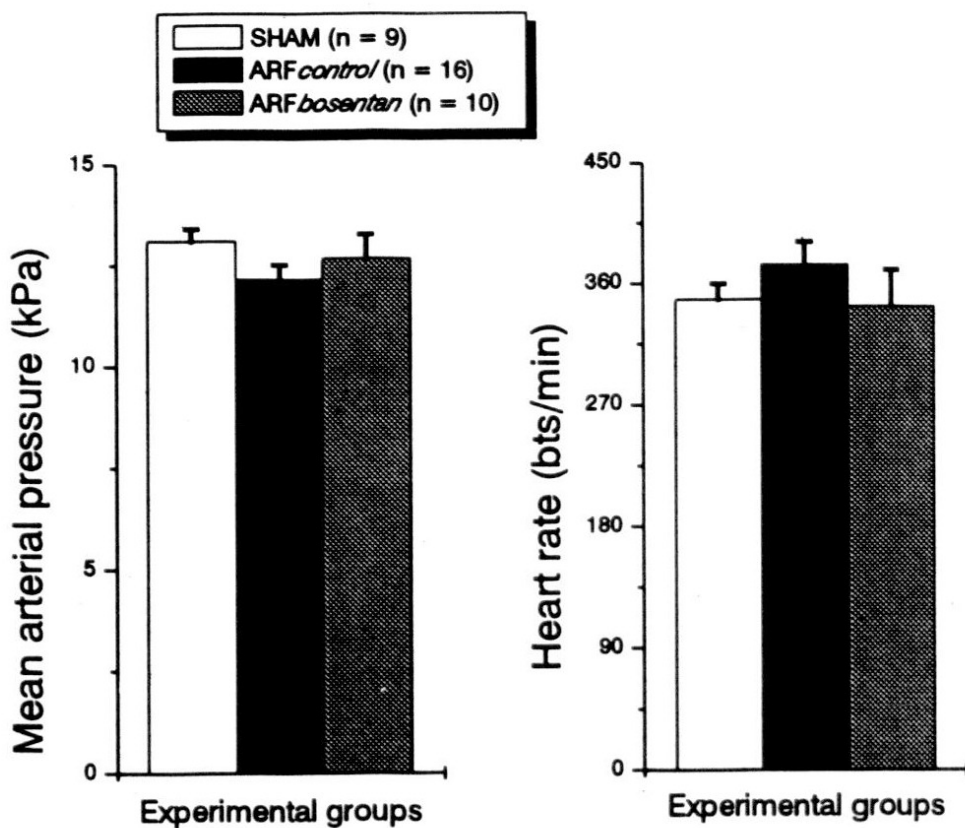of experimental postischaemic acute renal failure

Figure 1. Mean arterial pressure and heart rate 24 hours after ARF
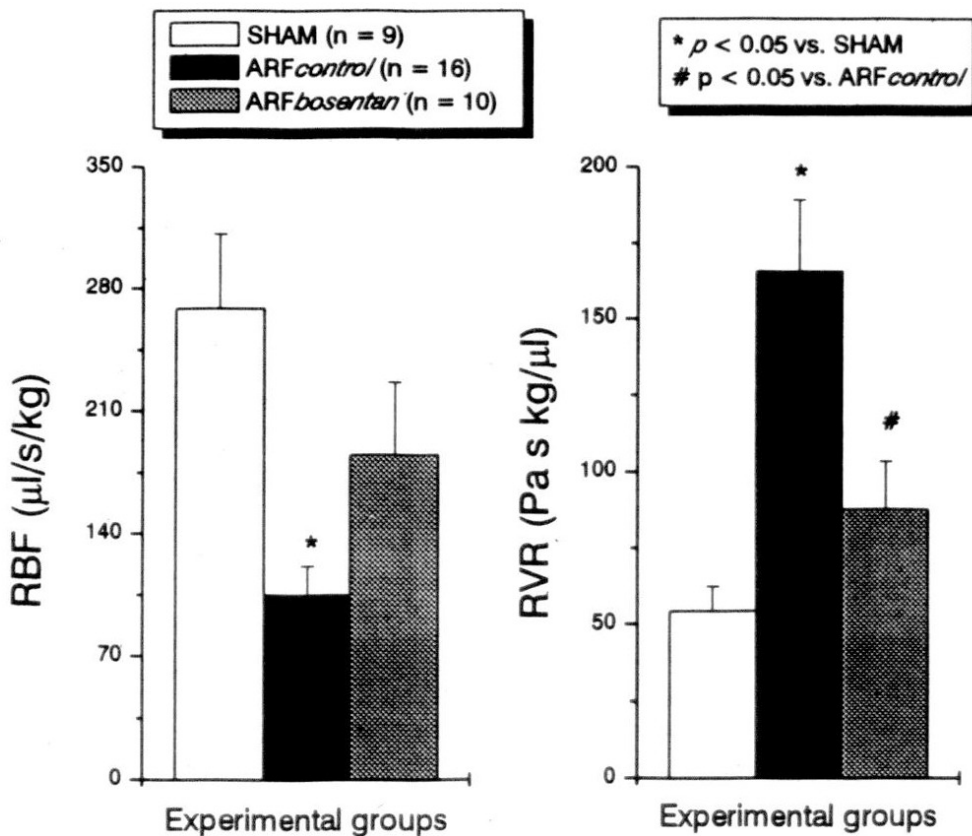
Figure 2. Renal blood flow (RBF) and renal vascular resistance (RVR) 24 hours after ARF
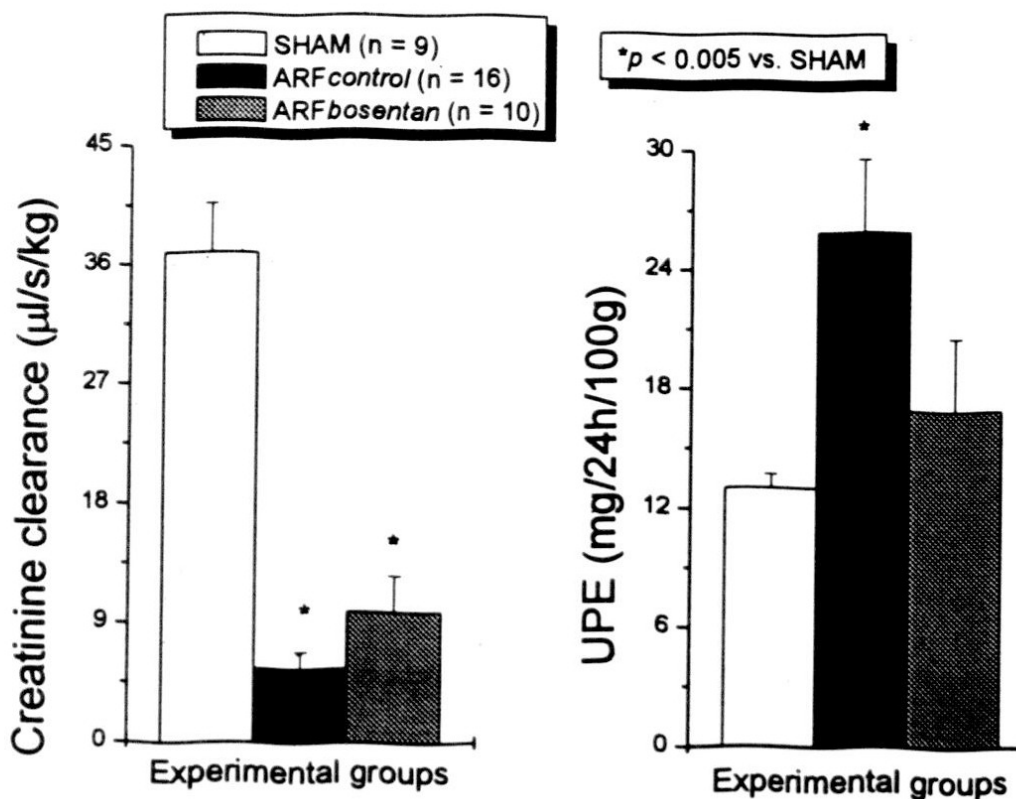
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Figure 3. Creatinine clearance and urinary protein excretion (UPE) 24 hours after ARF

Table 1. A) Urine volume, sodium and potassium in plasma (P_{Na}^+; P_K^+), B) protein in plasma (**PPr.**), sodium and water reabsorption in sham operated rats (**SHAM**), control animals with ARF (**ARF***control*) and bosentan treated animals with ARF (**ARF***bosentan*).

A

	Urine volume (ml/24/100g)	P_{Na}^+ (mmol/l)	P_K^+ (mmol/l)
SHAM (n = 9)	2.29 ± 0.37	140.7 ± 1.1	4.31 ± 0.15
ARFcontrol (n = 16)	3.27 ± 0.34	139.5 ± 1.1	4.96 ± 0.36
ARFbosentan (n = 10)	2.77 ± 0.34	138.1 ± 1.5	5.39 ± 0.33

B

	PPr. (g/l)	Sodium reabsorption (%)	Water reabsorption (%)
SHAM (n = 9)	49.90 ± 2.40	99.830 ± 0.081	99.14 ± 0.25
ARF*control* (n = 16)	47.51 ± 0.94	97.980 ± 0.660	90.82 ± 0.81*
ARF*bosentan* (n = 10)	48.90 ± 1.70	99.050 ± 0.620	93.40 ± 1.6*

Values represent means ± SEM. ; * $p < 0.05$ vs. SHAM; n - number of animals.

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group both sodium and water reabsorption were significantly higher and renal
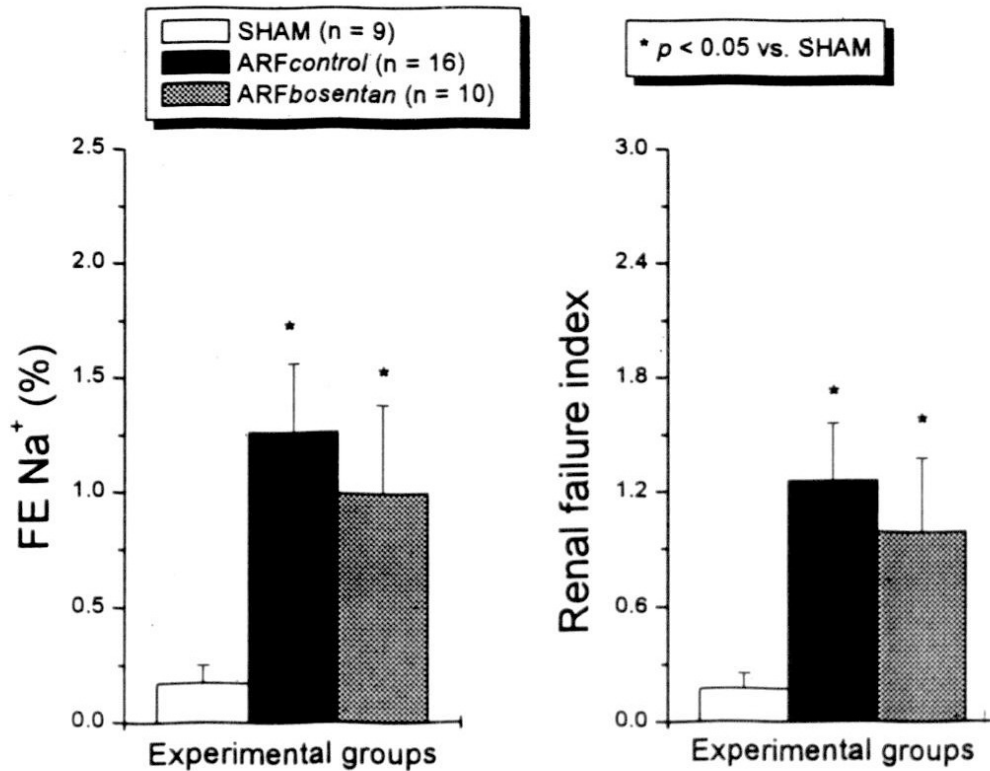failure index was markedly reduced compared to the ARFcontrol (Table 1.; Figure
4.).

Figure 4. Fractional sodium excretion (FeNa+) and renal failure index 24 hours after ARF

Histopathological analysis

Morphological examination of the renal tissue revealed significant
differences between experimental groups of animals. Glomeruli,
tubulointerstitium and blood vessels of the SHAM group were without any
changes on light microscopy examination. In a few kidney specimens only a small
number of PAS positive casts was observed in the tubular lumen (Figure 5.).
Kidneys in the ARFcontrol group showed dilatation of some segments of both
proximal and distal tubules with or without loss of brush border. Oedema of
proximal tubular epithelial cells was present in some other segments. The most
prominent lesions were widespread tubular necrosis such as focal infarctions in
the corticomedullary zone and a huge number of PAS positive casts in collecting
ducts. The intensity of interstitial oedema varied from specimens to specimens in
this group. Glomeruli and blood vessels were the same as in the sham-operated
group (Figure 6.).

In the bosentan-treated ARF group less intensive lesions were noticed in
comparison to the ARFcontrol group. Tubular dilatation was mild or even absent
in some kidney specimens of this group. In corticomedullary region tubular
necrosis was decreased, and focuses of infarction were smaller in size. Interstitial
oedema was rare. In addition, fewer casts was present in medulla. Glomeruli and
blood vessels were similar to those in the sham-operated rats (Figure 7.).

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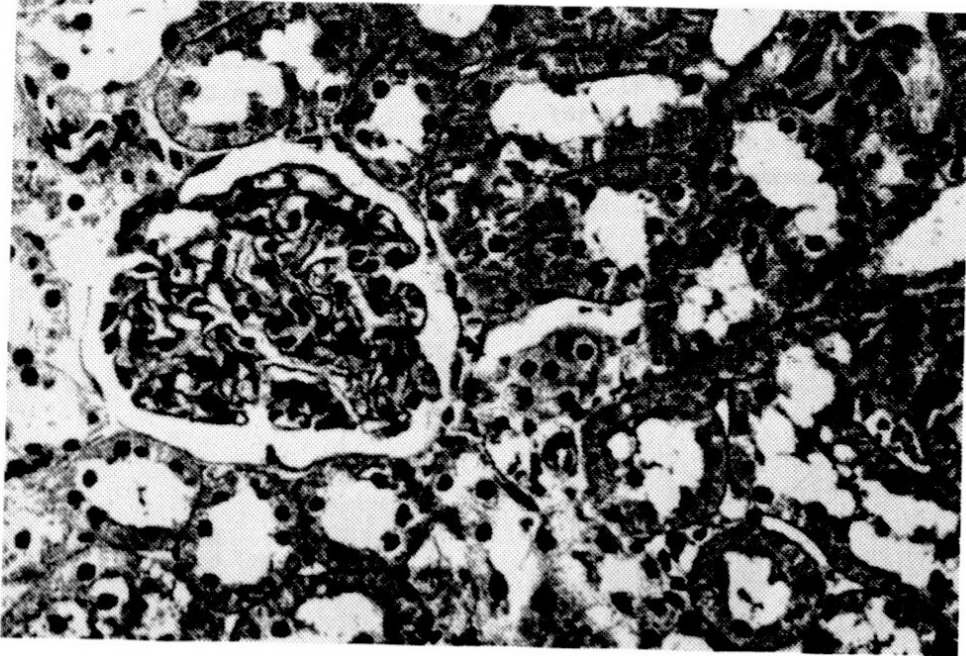of experimental postischaemic acute renal failure

Figure 5. Glomerulus, interstitium and tubules in sham operated rats with normal shape of kidney
structure (PAS X 320)

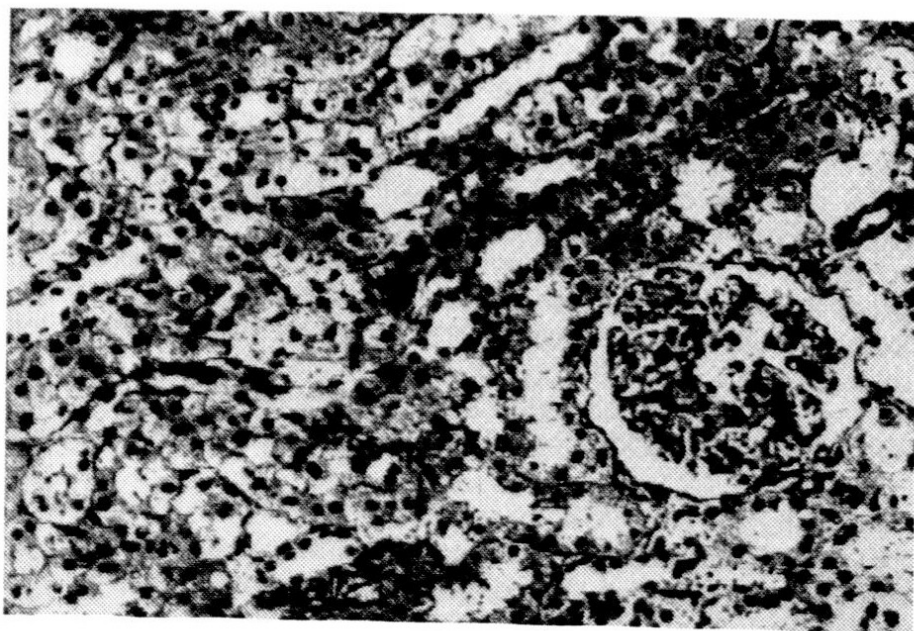Figure 6. Widespread corticomedullary necrosis, tubular dilatation and interstitial oedema in the
control group with ARF (PAS X 250)

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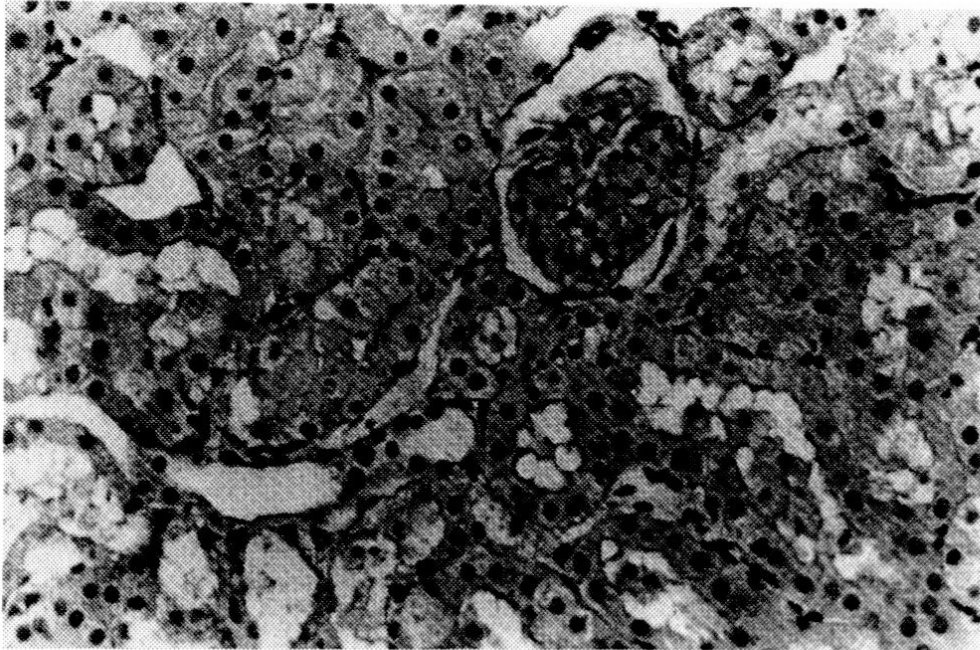of experimental postischaemic acute renal failure

Figure 7. Decreased corticomedullary tubular necrosis, less tubular dilatation, fewer casts and normal shaped glomerulus in the kidney of bosentan treated rats in comparison to the control group with ARF (PAS X 250)
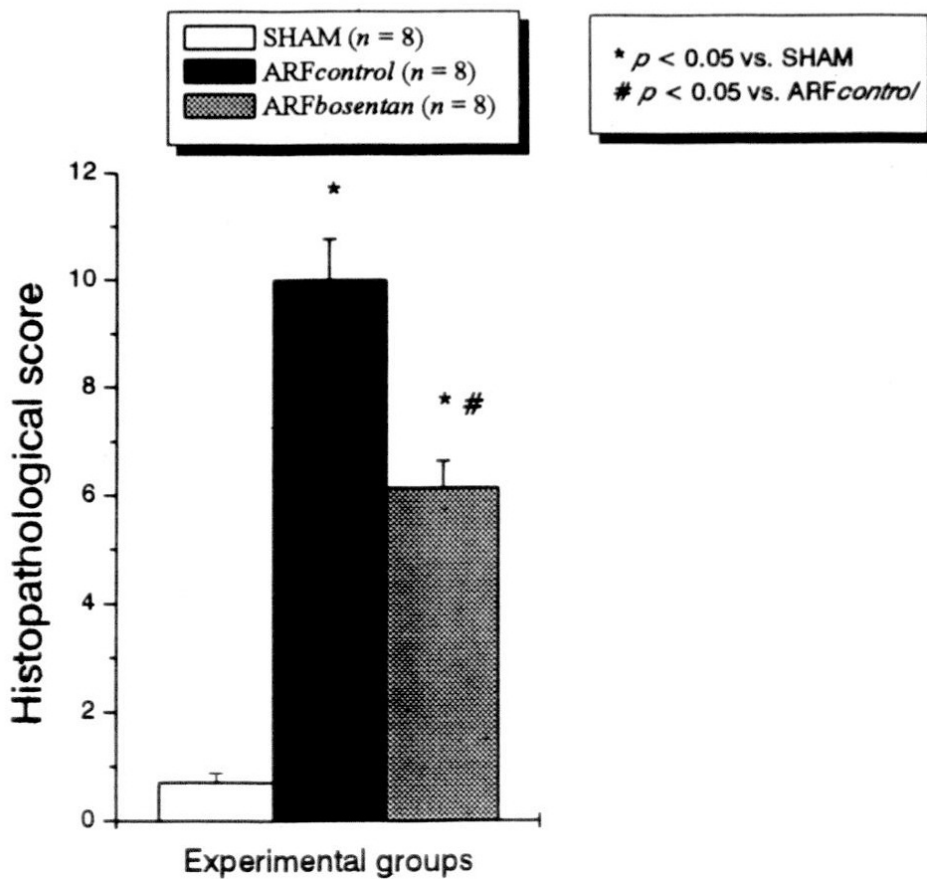

Figure 8. Histopathological score in the experimental groups 24 hours after ARF

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The histopathological score was drastically higher in all rats with ARF compared to sham-operated animals, but in the bosentan treated rats the sum of histopathological changes was much lower than in the ARFcontrol group (Figure 8.).

PRA and ET measurement

Circulating levels of angiotensin I (Ang I) and ET were found to rise 24 hours after induction of ARF. In the bosentan-treated animals with ARF, a further increase of plasma ET occurred, as a consequence of ET receptor blockade. However, the plasma level of angiotensin I in rats of this group was not different from the level in sham-operated rats (Figure 9.).
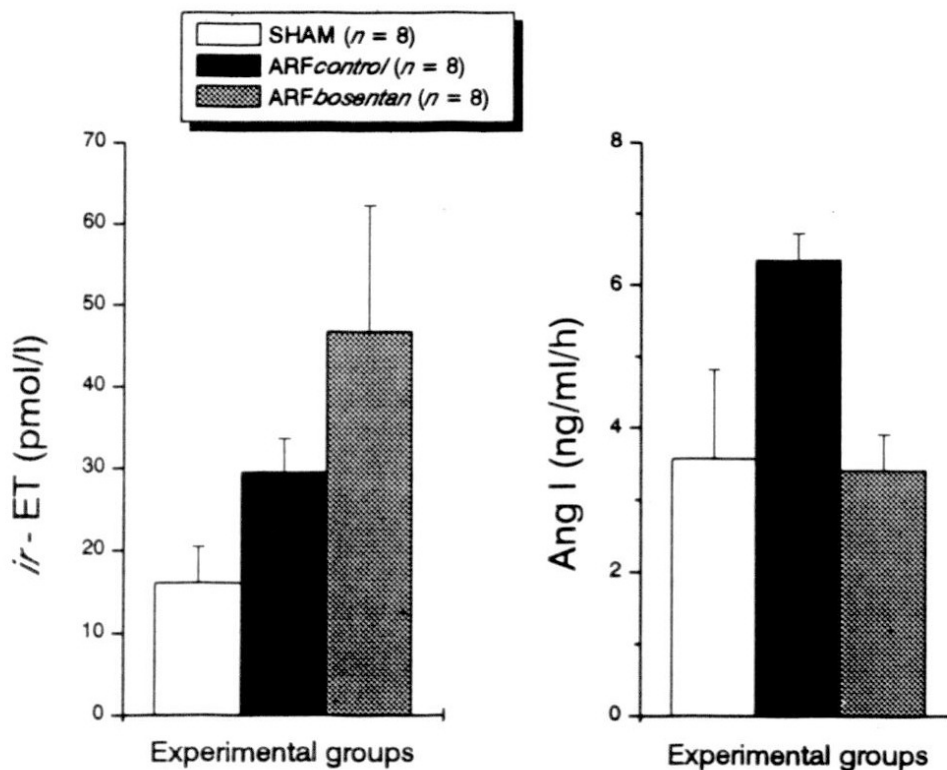

Figure 9. Plasma concentration of immunoreactive endothelin (ir ET) and angiotensin I (Ang I) 24 hours after ARF

DISCUSSION

The results of the present study demonstrate that bosentan, a mixed ET_A and ET_B-receptor antagonist, has beneficial effects on the early stage of postischaemic ARF. Bosentan improves both renal haemodynamic and functional parameters after ARF and its protective effects in ARF are also confirmed by the finding that destructive lesions of the kidney structure are less marked than the lesions in the control ARF group.

We chose to block both subtypes of endothelin receptors because both ET_A and ET_B receptors participate in the pathogenesis of ischaemia-induced ARF. Renal tissue expresses messenger RNA for both ET_A and ET_B receptors and hence both ET_A and ET_B receptors are present throughout the kidneys.

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Distribution of ET_A and ET_B receptors in the kidney is species dependent (Benigni and Remuzzi 1998). On the other hand, different patterns of ET receptor subtype distribution inside the same species depend on the method for detection and especially between *in vivo* and *in vitro* procedures (Hocher et al. 1995). Gellai et al. (1994a) found, using Scatchard analysis, that the distribution of ET_A and ET_B receptors in Sprague-Dawley rat kidney cortex, outer medulla and papilla was 50:50, 30:70 and 10:90, respectively. In comparison to the rat kidney, the glomeruli in the human kidney had a markedly lower density of ET receptors (Karet et al. 1993). However, by using Scatchard analysis, the ratio between ET_B and ET_A receptors in the kidney was shown to be similar in rats (Hocher et al. 1995) and humans (Karet et al. 1993).

The role of ET_A and ET_B receptors in the pathogenesis of ischemia-induced ARF was confirmed in experimental studies. Gellai et al. (1994b) studied the effects of an ET_A receptor antagonist (BQ123), given intravenously to Sprague-Dawley rats after they had been subjected to renal artery occlusion for 45 minutes. The authors found significantly increased survival rate (75%) among these rats, due to markedly improved tubular reabsorption of Na^+ and a moderate increase of GFR and K^+ excretion. These findings indicate that, in rats, the ET_A receptors mediate tubular epithelial function and play a significant role in the pathogenesis of ischemia-induced ARF. Bird et al. (1995) found that infusion of the ET-converting-enzyme inhibitor, phosphoramidon, protected the function and structure of rat kidney after 30 minutes of renal ischaemia, more than treatment with the ET_A receptor antagonist (BMS-182874). They concluded that both ET_A and non-ET_A receptors mediate ET-induced changes in ischaemic renal failure.

In the present study we investigated the effects of a nonselective ET_A/ET_B receptor antagonist, bosentan, on the course of postischaemic ARF. We found that systemic haemodynamic parameters changed neither after infusion of bosentan nor 24 hours later. However, RBF increased by 76% in the bosentan-treated animals, in comparison with control ARF rats, 24 hours after ischemic-reperfusion injury. This augmentation of RBF is a consequence of marked decrease of RVR in the bosentan-treated group. Following this improvement of renal haemodynamics, GFR became almost three times higher in the bosentan-treated group than in the ARF*control* group. However, the increase of creatinine secretion into the tubular lumen most likely contributes to the augmentation of GFR value in bosentan-treated rats. Such a conclusion stems from our finding that treatment with bosentan reduces tubular cell injury in acute postischaemic renal failure. We assume that tubular cell function is improved, as a result of less of tubular cell damage.

Urinary protein excretion doubled 24 hours after induction of ARF. Nevertheless, urinary protein excretion in ARF rats treated with bosentan almost returned to the level measured in sham-operated animals (Figure 3). This occurred, most probably, as a result of a marked decrease of glomerular capillary tonus in the bosentan-treated rats. Fractional sodium excretion (FE_{Na}^+) became ten times greater 24 hours after induction of ARF. However, the FE_{Na}^+ decreased by 31% in the bosentan-treated ARF group. Reabsorption rate of water and Na^+ was higher while renal failure index was markedly reduced (Table 1) in ARF rats treated with bosentan, compared to the ARF control. These effects of bosentan were most likely produced by stimulatory reabsorption of water and Na^+ at the tubular site. This conclusion stems from our finding that treatment with bosentan

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reduces tubular cell injury in ARF. The morphology of tubular epithelial cells in rats treated with bosentan shows clearly that only subtle changes can be found (Figure 7.). The recent results of Wilhelm *et al.* (1999) may contribute to our understanding of the latter findings. Namely, those authors found a marked increase of ET_1 in the peritubular capillary network of ischaemic kidneys and suggested that ET-induced vasoconstriction might have a pathophysiological role in ischaemic acute tubular necrosis. Another explanation for the increase of both water and sodium reabsorption, as well as for the decrease of urinary Na^+ excretion, which we observed in the bosentan-treated group with ARF, could come from a rise of renin secretion. However, our results showed that PRA was reduced in the bosentan-treated group, compared to the control ARF group (although this reduction did not reach a statistically significant level, see Figure 9.). Moreover, PRA was not different in the bosentan-treated ARF group in comparison to the sham-operated group. This reduction of PRA is presumably a consequence of the increase of RBF that, in turn, is caused by blockade of ET receptors. *In vitro* findings of Pacheco *et al.* (1996) suggest that ET_S inhibit renin secretion in rats via ET_A receptors. In our study however we used a nonselective antagonist of both ET_A and ET_B receptors and applied it in a dose that fully blocked both vasodepressive and vasoconstrictive effects of ET. We also found that in the group treated with bosentan the plasma ET level was raised 1.6-fold (in comparison with the control ARF group) which we see as a consequence and, hence, proof of successful ET receptor blockade.

To summarize, bosentan reduces glomerular and tubular cell injury in the early phase of acute postischaemic renal failure. This improvement of the course of ARF, after infusion of bosentan, implies that administration of this nonselective ET_A/ET_B receptor antagonist to patients with ARF would yield a favorable effect. However, Gellai *et al.* (1995) found only a moderate effect of another mixed ET receptor antagonist (SB 209670) upon the course of moderate and severe ARF in Sprague-Dawley rats, induced by occlusion of the renal artery for 30 and 45 minutes, respectively. The results of that study showed that even the highest dose of SB 209670 (corresponding to our dose of bosentan), had no effect in the rats when given before, during and after 45 minutes of renal ischaemia. We are inclined to interpret the discrepancy between those and our findings as a consequence of the different rat strains used and different choice of double ET receptor antagonist. Recently, Herrero *et al.* (1999) investigated the potential protective effect of bosentan against cold ischaemia-reperfusion injury in Lewis rats. They concluded that bosentan is effective in kidney protection from both renal function deterioration and tubular necrosis in cold induced ischaemia-reperfusion damage and that it might be useful in clinical renal transplantation. We believe that the results from that study, together with our results, contribute to the promotion of the dual, non-selective ET_A/ET_B receptor antagonist, bosentan, in future clinical practice.

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UTICAJI BLOKADE ENDOTELINSKIH RECEPTORA NA TOK POSTISHEMIČNE EKSPERIMENTALNE AKUTNE BUBREŽNE INSUFICIJENCIJE

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SADRŽAJ

Uticaji blokade receptora endotelinskih peptida na tok postishemične eksperimentalne akutne bubrežne insuficijencije (ABI), pretstavljali su glavni cilj naših istraživanja. Eksperimenti su izvođeni na odraslim pacovima Wistar soja. Urađena je nefrektomija desnog bubrega, a leva bubrežna arterija je klemovana u trajanju od 45 minuta. Eksperimentalne grupe su preko femoralne vene primale ili bosentan (antagonist ETA/ETB-receptora) ili fiziološki rastvor, kontinuirano, 20 minuta pre, tokom i 20 minuta posle perioda ishemije. Svi parametri su mereni 24 časa nakon perioda ishemije. Dobijeni rezultati jasno pokazuju da bozentan iskazuje povoljne uticaje na tok ABI. Bozentan poboljšava bubrežne hemodinamske i funkcionalne parametre, a lezije epitelnih ćelija tubula (glavnih mesta oštećenja u ABI), su manje izražene kod pacova tretiranih bosentan-om, nego kod kontrolnih životinja sa ABI. Sve to navodi na zaključak da endotelini imaju uticaja u razvoju ishemično/reperfuzionih oštećenja u ABI i da bosentan poseduje potencijal za primenu u kliničkoj praksi.