IJMS
Vol 29, No 1, March 2004

Original Article

Effect of Endothelin-A Receptor Blockade on the Early Phase of Ischemia/Reperfusion-Induced Acute Renal Failure in Anesthetized Rats

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Abstract

Background: Previous studies have shown increases in endothelin (ET) concentration of plasma and renal tissues in acute renal failure (ARF). ET has a potent vasoconstrictor effect, through binding with its ET_A receptors, and may play some roles in renal hemodynamic dysfunction in ARF.

Objective: To examine the beneficial effect of a selective ET_A-receptor antagonist on renal dysfunction and tissue damage occurring during the early phase of ischemia/reperfusion-induced ARF.

Methods: Pentobarbital anesthetized rats were prepared for the measurement of blood pressure and renal function. A 0.5 hr clearance period was taken as control period, followed by 1 hr, and then a 4 hr experimental clearance period was taken. In ischemia/reperfusion (I/R) group, 30 min after the end of the control clearance period, renal ischemia was induced by bilateral renal artery clamping for 30 min. In drug-treated (I/R+D) group, a selective ET_A-receptor antagonist (UK-350,926) was infused iv at 50 µg/kg/min for 30 min before and 2 hr following occlusion of renal arteries. There was also a sham-operated group.

Results: In I/R group, renal ischemia lowered creatinine clearance (C_{Cr}) by 76% ($p<0.001$), but elevated urine flow rate (V^0) by 2.9-fold ($p<0.01$) and absolute Na^+ excretion ($U_{Na}V^0$) by 3.2-fold ($p<0.05$) during the 4 hr reperfusion period as compared to their own control values. In I/R+D group, V^0 and $U_{Na}V^0$ did not change significantly during the 4 hr experimental period, but the amount of decrease in C_{Cr} was equal to that of I/R group. Histological examination showed a mild degree of tissue damage in the cortex of I/R group but not in I/R+D and sham groups.

Conclusion: Administration of the ET_A-receptor antagonist does not prevent the fall of glomerular filtration, but it does ameliorate the damage of renal tissue and tubular reabsorption of Na^+ and water during 4 hrs of reperfusion following the ischemic challenge.

Iran J Med Sci 2004; 29(1):14-20.

Keywords • Acute renal failure• ischemia/reperfusion• ET_A-receptor antagonist

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Introduction

Endothelins (ETs) are 21-amino acid peptides initially isolated from endothelial cells and are the most potent vasoconstrictor known to date. There are three main closely related isoforms; ET_1, ET_2 and ET_3.[1] ET_1 is the major renal ET isoform and has been shown to cause vasoconstriction in both afferent and efferent arterioles, and even in arcuate and inter-lobular arteries.[2] Some stimuli such as hypoxia or ischemia induce ET_1 mRNA transcription, ET_1 synthesis and its secretion occur within minutes.[3] ETs bind to specific membrane receptors of which two types, ET_A and ET_B have been well characterized and both are present in the kidney. ET_A receptors are widely distributed in vascular smooth muscles and mediate most of the vasoconstrictor effects of ETs, while ET_B receptors are located mainly on the endothelial cells, where their activation leads to the production of nitric oxide (NO).[1,3]

Acute renal failure (ARF) is a common renal disease, which can be caused by an acute ischemic or toxic insult to the kidney, as well as acute obstruction of the urinary tract.[4] Ischemic damage to the kidney may occur during renal hypoperfusion. However, it is the period of ischemia followed by reperfusion which initiates renal damage and its subsequent failure.[4] There is considerable evidence showing that ET_1 concentration in plasma and renal tissue increases following ischemic or toxic challenges to the kidney, which parallels the reduction in renal blood flow and progression of renal dysfunction.[1,2,5] Mino *et al,* found that the treatment of ischemia/reperfusion (I/R)-induced ARF with a selective ET_A-receptor antagonist (BQ-123) prevented the decrease in creatinine clearance, increases in fractional Na^+ excretion, plasma creatinine and blood urea nitrogen (BUN), as well as necrotic tissue damage 24 hr after starting reperfusion.[6] In another study, administration of BQ-123 to rats with ischemic ARF resulted in a significantly better inulin clearance and net tubular reabsorption of Na^+ at 2 and 48 hr of reperfusion as well as a reduction in the severity of renal tissue damage 48 hr post-ischemia.[7] In a recent study, Johns and colleagues reported that pretreatment with a novel non-peptide selective ET_A-receptor antagonist (UK-350,926) attenuated the reduction in glomerular filtration rate (GFR) and associated anti-natriuresis in response to renal I/R, induced by 30 min of renal artery occlusion, over the 8 days period of observation.[8] It is evident from these studies that endogenous ET is involved in the renal dysfunction and histological lesions caused by ischemic challenges. The aim of the present study was to elucidate the pathophysiological role of endogenous ET and to investigate the impact of the selective ET_A-receptor antagonist, UK-350,926, on renal hemodynamic and excretory dysfunctions and tissue damage during the first few hours of reperfusion following an ischemic insult to the kidneys.

Method

Animal Preparation

The study protocols were done according to the international conventions on animal experimentation. Male Sprague Dawley rats weighing 290–340 g were anesthetized with sodium pentobarbital (60 mg/kg, ip; Sigma, UK). After tracheotomy, a heparinized cannula (20 U/ml in saline; Heparin sodium, Melsongen, Spain) was placed into the right femoral vein and an infusion of saline (150 mM NaCl, 3 ml/hr; Merck, NJ, USA) was begun and continued throughout the experiment. A bolus dose of sodium pentobarbital ((0.05 ml; 25 mg/ml) was administered when needed throughout the experiment. Another heparinized cannula was inserted into the carotid artery for the measurement of blood pressure (P23DC pressure transducer; Gould Statham Instruments, USA). The urinary bladder was cannulated for collection of urine. After a midline laparatomy, renal arteries and veins of both kidneys were carefully cleared and separated from each other. Body temperature was recorded by a thermistor probe (41TD, Yellow Spring Instrument Inc., USA) that was placed into the rectum and maintained at 37±1 ^{o}C with a heating pad. Arterial blood pressure was continuously recorded using a Grass 7 polygraph (Grass Instrument Co., USA). The animal was allowed to stabilize for 2 hrs before the start of the experimental protocol.

Experimental Protocol

A 0.5 hr control clearance period was taken for the measurement of the variables of basal levels of cardiovascular and renal functions, followed by 30 min period of iv infusion of UK-350,926 (Pfizer, USA) at 50 μg/kg/min in the group of ischemia/reperfusion plus drug (I/R+D; n=6) or saline in the ischemia/reperfusion (I/R; n=6) group. Then, renal ischemia was induced for 30 min by bilateral renal artery occlusion using non-traumatic clamps. After removal of the clamps, a 4 hr reperfusion (experimental clearance) period was taken. In I/R+D group, UK-350,926 was again infused (50 μg/kg/min) over the first 2 hrs of reperfusion period. In sham-operated (n=6) group, no

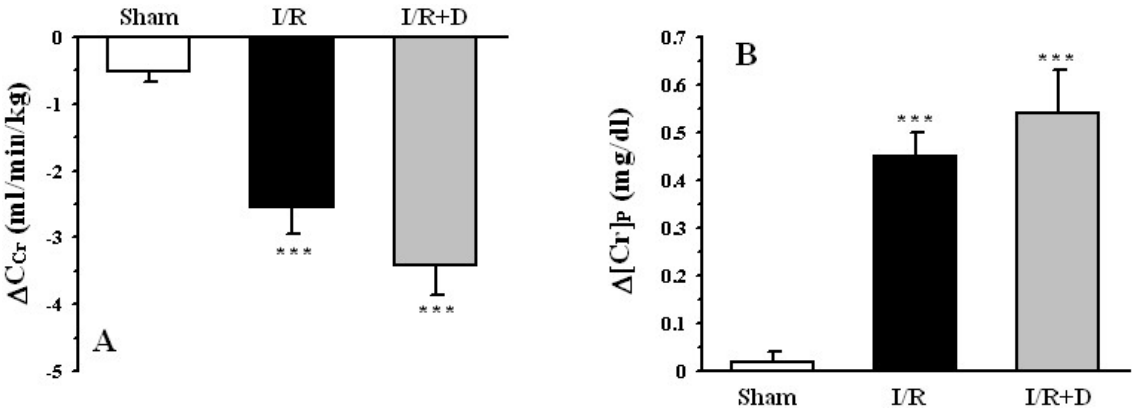

Fig 1: Effect of 30 min renal arterial clamping followed by 4 hrs reperfusion (I/R group), and infusion of ET_A-antagonist at 50 µg/kg/min (iv) for 30 min before occlusion of the renal arteries and 2 hrs after removal of the clamps (I/R+D group) on A) clearance of creatinine (C_{Cr}), and B) plasma concentration of creatinine ($[Cr]_P$). In sham group no drug was infused and the renal arteries were not occluded. Δ is difference between experimental and basal values in each group and data are expressed as mean±SEM. *p<0.05, **p<0.01, ***p<0.001 *vs.* sham group.

drug was infused and the renal arteries were not occluded.

Arterial blood samples (1 ml) were taken, at the end of control clearance period and at the beginning and end of experimental clearance period, to determine creatinine and Na^+ concentrations. The blood was collected into cooled syringes, quickly centrifuged, and the plasma was stored at -20 ^oC until assayed. The red blood cells were resuspended in an equal volume of saline and reinfused into the animal and, after 5 min equilibration, experimental protocol was continued. At the end of the experiment, the rat was killed by injecting an overdose of pentobarbital and both kidneys were preserved for future histological examination.

Drug preparation and dosage

The appropriate amount of UK-350,926 (generously provided by Pfizer Central Research, Sandwich; UK) was dissolved in 200 µl of 0.1 M NaOH, brought up to a final volume of 2 ml with normal saline and its pH adjusted to 7.0–7.5 with 0.01 M HCl. It was then, diluted with normal saline such that the UK-350,926 could be infused at 50 µg/kg/min. This dose was chosen based on the results of the previous study.[8]

Analyses

The urine produced during each clearance period was measured gravimetrically and urine flow rate (V^0, µl/min/kg) was calculated. Plasma and urine samples were assayed for creatinine and Na^+. Creatinine concentrations ([Cr]; mg/dl) were determined according to the Jaffe's method (Technicon RA-1000, USA), and Na^+ concentrations were measured by flame photometry (Hycel phf 104, France). Creatinine clearance (C_{Cr}; ml/min/kg) was taken as an estimate of GFR, and absolute sodium excretion rate ($U_{Na}V^0$; µmol/min/kg) and fractional sodium excretion (FE_{Na}; %) were calculated thereafter.

Histological examinations

The kidneys were decapsulated, sectioned transversely through the midline and placed in the buffered 10% formaldehyde (Merck, NJ, USA). The kidneys were processed by routine methods for paraffin embedding, stained with hematoxylin-eosin and examined under light microscopy.

Statistical analysis

Data are expressed as mean±SEM, and Student's paired t-test was used to compare basal and experimental values within groups. The difference (Δ) between experimental and basal values for each variable were calculated and given as mean±SEM. The difference values for each variable were compared between groups using one-way ANOVA followed by Duncan's post-hoc test. Significance was taken at $p<0.05$.

Results

In I/R group, C_{cr} was 3.31±0.24 ml/min/kg during control period. Thirty min of renal occlusion decreased it by 76% ($p<0.001$) during the 4 hr reperfusion period. C_{Cr} was also reduced, from its basal value, largely by 77% ($p<0.001$) in I/R+D group and slightly by 18% ($p<0.05$) in sham group during reperfusion period. Fig 1A compares the amounts of difference between experimental and basal values of creatinine clearances (ΔC_{Cr}) in the three experimental

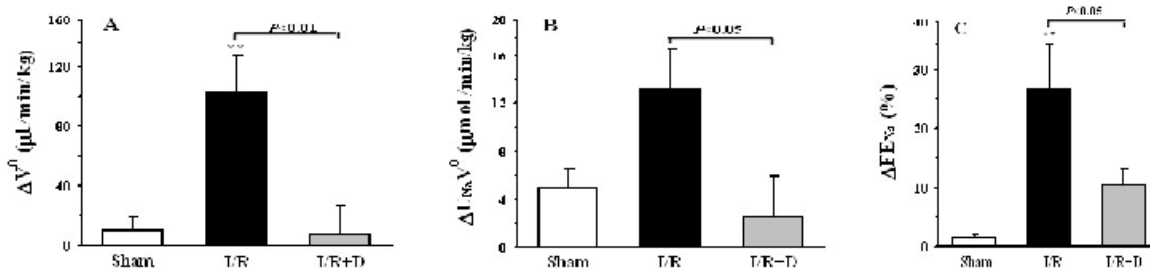

Fig 2: Effect of 30 min renal arterial clamping followed by 4 hrs reperfusion (I/R group) and infusion of the ET_A-antagonist at 50 µg/kg/min (iv) for 30 min before occlusion of renal arteries and 2 hrs after removal of the clamps (I/R+D group) on A) urine flow rate (V^0), B) absolute sodium excretion ($U_{Na}V^0$), and C) fractional sodium excretion (FE_{Na}). In sham group no drug was infused and the renal arteries were not occluded. Δ is difference between experimental and basal values in each group and data are expressed as mean±SEM. *p<0.05, **p<0.01, ***p<0.001 *vs.* sham group.

groups. The magnitudes of the ΔC_{Cr} in the two ischemic groups were not statistically different from each other but both were significantly (both p<0.001) greater than that of sham group.

In I/R and I/R+D groups, the decrement in C_{cr} raised $[Cr]_P$ to 1.23±0.07 and 1.11±0.12 mg/dl at the end of the experimental period from their basal values of 0.78±0.07 and 0.57±0.02 mg/dl, respectively, (both p<0.001). In sham group, basal value of $[Cr]_P$ was 0.67±0.08 mg/dl and remained stable during the experimental period (0.68±0.65 mg/dl). Fig 1B shows that the magnitudes of $\Delta[Cr]_P$ in I/R and I/R+D groups did not differ significantly from each other, but both of them were much higher than those of sham group (both p<0.001).

There were increments of about 2.9-fold in V^0 (p<0.01), 3.2-fold in $U_{Na}V^0$ (p<0.05) and 24-fold in FE_{Na} (p<0.05) during the 4 hr reperfusion period as compared to those of the control period (52.5±14.6 µl/min/kg, 5.92±2.03 µmol/min/kg and 1.15±0.34 %, respectively) in I/R group. In I/R+D group, while, FE_{Na} was elevated by 3.4-fold (p<0.05), but V^0 and $U_{Na}V^0$ were not significantly changed during the reperfusion period from those of the control period. In sham group, V^0 remained stable during the 4 hr experimental period, but there were small time-dependent increases in $U_{Na}V^0$ and FE_{Na} as compared to those of the basal values (both p<0.05). The magnitudes of ΔV^0 (Fig 2A) in sham and I/R+D groups were not significantly different from each other, whereas both of them were much smaller than that observed in I/R group (both p<0.01). Fig 2B shows that the $\Delta U_{Na}V^0$ in I/R group was significantly (p<0.05) greater than that of I/R+D group, while neither were statistically different from the $\Delta U_{Na}V^0$ values in sham group. There were no differences between the magnitudes of ΔFE_{Na} (Fig 2C) in sham and I/R+D groups, but both were smaller (p<0.01 and p<0.05, respectively) than the change recorded in I/R group.

In I/R and sham groups, the values of mean arterial pressure (MAP) during the experimental period (94.5±6.7 and 94.5±4.5 mmHg, respectively) were not significantly different from their basal values (109.2±7.2 and 98.3±4.5 mmHg, respectively). In I/R+D group, MAP during the experimental period (86.1±3.4 mmHg) was significantly (p<0.001) lower than in the control period (106.4±3.4 mmHg), which indicated that blockade of ET_A receptors had a hypotensive effect.

Histological examination of the kidneys showed that bilateral renal artery occlusion for 30 min followed by 4 hr reperfusion period resulted in a modest degree of cortical tissue damage in I/R group (Fig 3B). There were disappearance of brush borders in the proximal tubules, proteinaceous casts in the tubular lumen and vascular congestion. The glomeruli were morphologically normal, but the Bowman's capsules were enlarged. The kidneys of I/R+D group (Fig 3C) showed no sign of ischemia-induced histological injury and were morphologically similar to those of the sham group (Fig 3A).

DISCUSSION

Histological examination of the kidneys obtained from I/R group (Fig 3B) did not show morphological sign of necrosis. However, in all examined kidneys there was disruption of brush borders that was very evident in the proximal tubules. In addition, there were intratubular proteinaceous cast formation, vascular congestion and Bowman's capsular enlargement. These morphological alterations were limited to the cortex and there was no sign of injury at the medulla. This contrasts to the results of many other investigators,[6,7,9] who showed damage to the medulla, especially to outer layer. It should be noted that in these previous reports, kidneys, however,

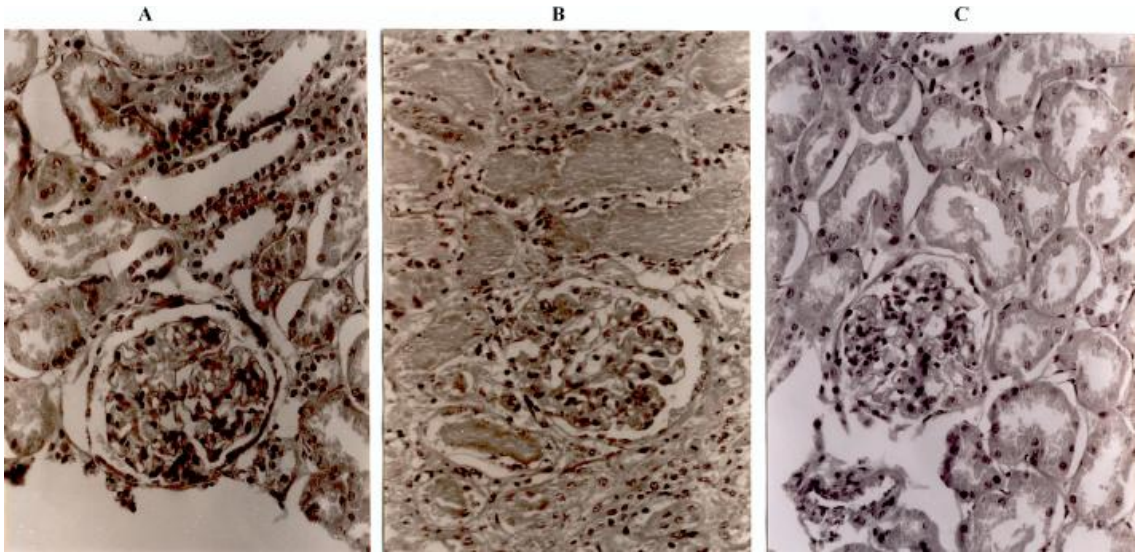

Fig 3: Phoromicrographs of the cortical zone of the rat kidneys which A) no drug was infused and the renal arteries were not occluded (sham group), B) renal arteries were clamped for 30 min followed by 4 hrs reperfusion (I/R group), and C) ET_A-antagonist at 50 µg/kg/min was infused (iv) for 30 min before occlusion of the renal arteries and 2 hrs after removal of the clamps (I/R+D group). The intratubular proteinaceous casts and Bowman's capsular enlargement are obvious in the I/R group, which are not seen in the sham and I/R+D groups. The kidney sections were stained with hematoxylin-eosin. Magnification x100.

were made ischemic for a longer duration—45 or 60 min—and were examined at least one day after the start of reperfusion. Thus, it can be argued that the longer period of impairment in medullarly perfusion, as well as prolongation of the time from the initial ischemic insult resulted in progressive and more profound tubular and vascular injury in this area. In addition, Wilhelm *et al,*[2] using an immunohistochemical analysis, revealed that renal ischemia increased expression of ET_1 uniformly throughout the endothelium of the cortical peritubular capillary network of the renal proximal and distal convoluted tubules. Based on this finding, they deduced that the ongoing vasoconstriction and resulting fall of blood flow in this vascular bed would cause hypoxia in the adjacent cells lining the tubules, leading to typical responses following renal ischemia.

There was no sign of ischemic injury in the kidneys of I/R+D group and they were morphologically similar to the kidneys of the sham group (Fig 3). Therefore, administration of the ET_A-receptor antagonist, for 30 min before and 2 hr after occlusion of renal arteries, was sufficient to protect the kidneys from ischemic tissue damage during the reperfusion period. Chan *et al.,*[7] showed that infusion of the ET into the renal artery of the isolated perfused rat kidney resulted in a dose-dependent reduction in renal perfusate flow, which was prevented by administration of a ET_A-receptor antagonist (BQ-123). A reduction in renal blood flow of up to 40%–50% has been frequently reported in ischemic reperfusion injury, which has been observed even during the maintenance phase of experimental models and human.[9] Therefore, it is likely that ET is involved in the ischemic injury by causing a persistent reduction in renal blood flow and oxygen supply. This is consistent with ET's potent and prolonged vasoconstrictor action that is due to the relatively irreversible and continued signal transduction activation up to 2 hr following endocytosis.[1] Thus, ET can be considered as an important mediator of persistent vasoconstriction in ischemia- or toxemia-induced renal failure.[1,9] Ischemic injury to the kidneys also stimulates the release of inflammatory mediators via an imbalance in ET and NO production[10] that in turn activates adhesion molecules on leukocytes and their binding to endothelial cells.[11,12] Vascular congestion was observed in the kidneys of I/R group, but not in those of I/R+D group, suggesting that the protective effect of ET_A blockade could have resulted from both antagonising vasoconstriction and amelioration of vascular congestion, which together would have contributed to an improved renal perfusion and oxygen supply during reperfusion period.

The 30 min occlusion of renal arteries caused 76% reduction in C_{cr}, as an estimate of GFR, during reperfusion period, which result in elevation of $[Cr]_p$ in I/R group. The severe reduction in GFR, which has also been observed in other types of experimentally-induced renal

injury,[4] was shown to be caused by a combination of intrarenal vasoconstriction,[13] tubular obstruction and back-leakage of glomerular filtrate, due to the loss of the gating function of tight junctions in the injured tubular epithelium.[14] The magnitudes of ΔC_{Cr} and $\Delta[Cr]_P$ in I/R+D group were equal to that of the I/R group (Fig 1), which indicated the blockade of ET_A receptors could not prevent the reduction in GFR during 4 hr of reperfusion period. As mentioned above, there was no evidence of any tubular or vascular damage or casts in I/R+D group (Fig 3C) suggesting that tubular obstruction and epithelial back-leak was unlikely to be involved, and intrarenal vasoconstriction was the main mechanism responsible for the reduction of GFR during the early phase of ischemic challenge to the kidney. Furthermore, the renal vasoconstrictor effect of ET, mediated through ET_A receptors, was blocked by UK-350,926 infusion in this group. Therefore, it is likely that there is production of vasoactive agents other than ET causing the intrarenal vasoconstriction during the early phase of the reperfusion induced by the ischemia. Indeed, there are a number of reports which suggest that treatment with a selective ET_A-receptor antagonist does not initially improve GFR in response to ischemia, but does so in the long-term,[8,15] while blockade of both ET_A and ET_B receptors together accelerates the short-term amelioration of GFR.[16,17]

Evaluation of the renal Na^+ excretory function showed that the ischemic insult to the kidneys resulted in an elevation of both $U_{Na}V^0$ and FE_{Na} during 4 hr reperfusion period in I/R group. The magnitude of $\Delta U_{Na}V^0$ in the I/R group was not statistically different from that of sham group (Fig 2B), which was mostly due to the lower filtered load of Na^+ because of the reduced GFR in I/R group. However, the magnitude of ΔFE_{Na} in I/R group was higher than that of sham group (Fig 2C) suggesting that renal tubular reabsorption of Na^+ was impaired during early phase of I/R-induced damages. It has been shown that sublethal injuries, such as loss of brush border and tight junction integrity of proximal tubular cells, markedly impairs the function of the renal tubular cells, even though the cells appear intact morphologically.[18]

The magnitudes of both $\Delta U_{Na}V^0$ and ΔFE_{Na} in I/R+D group were lower than those of I/R group (Fig 2), which suggests that treatment with the selective ET_A-antagonist did improve renal Na^+ reabsorption. The beneficial effect of the ET antagonist on tubular Na^+ reabsorption might be associated with its protective effect on ischemic tissue damage, by maintaining a normal number of correctly functioning tubular cells. It should be noted that there are many observations indicating a direct natriuretic effect of ET on tubular cells,[1] mostly mediated by ET_B receptors,[1] however, several groups have presented evidence for ET_A-mediated natriuresis.[15,19] Therefore, it is possible that part of the increase in Na^+ reabsorption following treatment by ET_A antagonist was due to the blockade of this ET_A-mediated natriuresis. Nevertheless, it is evident from our secretory functional and histological findings that the ET_A receptor contributes significantly to the acute epithelial injury of nephrons and its associated malfunction in tubular reabsorption of Na^+ caused by the ischemic insult.

V^0 was markedly increased during the reperfusion period as compared with the control period in I/R group but it was not changed significantly in sham and I/R+D groups. Therefore, the 30 min of renal ischemia resulted in a polyuria during the 4 hr reperfusion period, which could be associated with the concomitant natriuresis. By contrast, oliguria has been reported in over half of the clinical cases and some experimental animal models of ARF.[4] It has been observed severe ischemia induced by 1 hr renal arterial occlusion, decreased V^0 during the early phase of reperfusion.[16] Therefore, it seems that when the duration of ischemia is sufficiently long enough to cause widespread damage, the obstruction of the tubular lumen is so extensive that it reduces the flow of filtrate and results in oliguria. The magnitudes of ΔV^0 in sham and I/R+D groups were not different and were much lower than that of I/R group (Fig 2A), supporting the conclusion that the ET_A antagonist through an improvement in tubular Na^+ reabsorption was able to ameliorate the ischemia-induced diuresis.

In summary, the results obtained in this study indicate that renal ischemia for 30 min followed by 4 hr reperfusion caused a mild level of histological damage primarily limited to the cortex. These renal tissue injuries were accompanied by a severe reduction in GFR and elevation in Na^+ and water excretion. Administration of an ET_A-antagonist protected the kidneys from ischemic tissue damage and improved tubular Na^+ and water reabsorption, but did not ameliorate the reduction in GFR.

Acknowledgements

We wish to acknowledge the Research Council of Shiraz University of Medical Sciences, Shiraz, Iran, for the financial support of this study (grant Number: 79-971).

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