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INFLUENCE OF RENAL DENERVATION ON RENAL EFFECTS OF ACUTE NITRIC OXIDE AND ET_A/ET_B RECEPTOR INHIBITION IN CONSCIOUS NORMOTENSIVE RATS

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The role of renal nerves in the effects of concomitant NO synthase and non-selective ET_A/ET_B receptor inhibition on renal function was investigated in conscious normotensive Wistar rats. NO synthase inhibition alone (10 mg/kg b. w. i.v. L-NAME) in sham-operated rats with intact renal nerves induced an increase in systolic, diastolic and mean arterial pressure, urine flow rate, sodium, chloride and calcium excretion ($p<0.05$). The effect of L-NAME was markedly reduced by bosentan (10 mg/kg b.w. i.v.) and the values of urine flow rate, sodium, chloride and calcium excretions returned to control level ($p<0.05$). L-NAME administration one week after a bilateral renal denervation increased blood pressure to a similar extent as in sham-operated rats but decreased urine flow rate ($p<0.05$) and did not change electrolyte excretion. ET_A/ET_B receptor inhibition with bosentan during NO synthase inhibition in the renal denervated rats did not produce changes in urine flow rate or electrolyte excretion. NO synthase inhibition as well as concurrent NO synthase and ET_A/ET_B receptor inhibition did not change clearance of inulin or paraaminohippuric acid in sham-operated or renal denervated rats. These results indicate that renal sympathetic nerves play an important modulatory role in NO and endothelin induced effects on renal excretory function.

Key words: *bilateral renal denervation, L-NAME, bosentan, Wistar rats, renal excretory function*

INTRODUCTION

It is well known that renal sympathetic nerves participate in the regulation of renal haemodynamics and transport processes in the nephron (1). Furthermore, both nitric oxide (NO) and endothelins (ET) have been implicated in the regulation of renal function. NO inhibits sodium reabsorption in the proximal tubule (2) as

well as sodium reabsorption and vasopressin-stimulated osmotic water permeability in the cortical collecting duct (3). ET inhibit sodium reabsorption in the proximal tubule, the loop of Henle and the inner medullary collecting duct (4) as well as vasopressin-induced water reabsorption (5). Thus, renal nerves, NO and ET are important regulators of renal blood flow, sodium and water excretion.

Previous observations have indicated the existence of complex interaction between renal nerves, NO and ET. It has been shown that the intrarenal infusion of an NO donor reduces (6), whereas NO synthase (NOS) inhibition potentiates the action of renal nerve stimulation (7). It has been also demonstrated that ET-1 functions as an inhibitory modulator of the renal noradrenergic neurotransmission (8). Some studies indicate that ET are partially involved in the haemodynamic changes during NOS inhibition (9, 10), but its role in the renal effects is controversial (11).

There is little information about a possible interaction between the renal nerves, NO and ET in the regulation of renal excretory function and blood pressure. The available data provide evidence that NO and ET regulate the renal sympathetic nerve activity. However, the contribution of renal nerves to the effects of NO and ET within the kidney is not well studied. Moreover, the participation of renal nerves in the regulation of the changes of renal excretory function due to concurrent NO synthase and ET_A/ET_B receptor inhibition is largely unknown.

This study was designed to investigate the interactions between renal nerves, NO and ET in the regulation of renal function. To accomplish this, we studied the short-term changes of the renal function and arterial blood pressure as a result of NOS inhibition in the absence or presence of non-selective ET_A /ET_B receptor blockade in conscious rats subjected to bilateral renal denervation. Since the kidney contains ET_A and ET_B receptors, and ET have some opposite effects, possibly mediated by different receptors, the complete blockade of the actions of endogenous ET requires antagonism of both ET_A and ET_B receptors (12). Therefore, the purpose of this study was to investigate the role of renal nerves in the effects of concomitant NOS and non-selective ET_A/ET_B receptor inhibition on renal function in conscious normotensive rats.

MATERIAL AND METHODS

The experiments were carried out on conscious, male Wistar rats weighing 290-310 g. The rats were kept in individual cages for a minimum of one week before the experiments and were housed under standard conditions: temperature 22 °C, light/dark cycle 12/12 hours, free access to tap water and food. The study was performed in accordance with institutional care and use of laboratory animal quidelines, Medical University, Sofia.

For surgical preparation the rats were anaesthetized with pentobarbital sodium (Nembutal) 35 mg/kg b.w. given intraperitoneally. Bilateral renal denervation was performed in about half of the animals prior to vascular and bladder catheterization. After a retroperitoneal flank incision the kidneys were denervated by stripping all visible nerves from the renal arteries and by coating the

renal arteries and veins with a 10% solution of phenol in absolute alcohol. Sham operation was performed exposing the kidneys through retroperitoneal flank incision. One week was allowed for the rats to recover. The effectiveness of the denervation was assessed by noradrenaline concentration in renal cortical tissue homogenate on the day of sacrifice by high-performance liquid chromatography using a modified procedure of Lozanov et al. (13).

During general anaesthesia (35 mg/kg b.w. Nembutal) through a small suprapubic incision a modified polyethylene catheter was placed into the urinary bladder eliminating its dead space. The catheter was tunnelled and exteriorised on the flank. Urine was collected in a small plastic tube secured in a plastic loop sutured to the skin during the surgery. A catheter was also placed into the femoral vein for N^{Ω}-nitro-L-arginine methyl ester (L-NAME) or L-NAME and bosentan administration and inulin and paraaminohippuric acid (PAH) infusion. For blood pressure measurement a catheter was placed into the femoral artery. The catheters were tunnelled to the back of the neck and exteriorized. Thereafter, the rats were returned into their individual metabolic cages. To avoid clotting the femoral artery and vein catheters were flushed with 20 IU/ml heparin in 0.9 % sterile saline. 24 hours were allowed for the rats to recover from the surgical intervention and the experiments were performed on conscious, freely moving animals.

Control experiments. Blood pressure was measured and urine was collected over a time period of 100 min for two clearance periods in the sham-operated rats with intact renal nerves (n=14) and in the renal denervated rats one week after bilateral renal denervation (n=12).

NO synthase inhibition. Renal excretory function, blood pressure and heart rate were investigated in the sham-operated rats (n=15) as well as in the renal denervated rats (n=12). The animals were studied during a 40-min control clearance period, a 20-min equilibration, and a 40-min experimental clearance period. Intravenous bolus injection of 10 mg/kg b.w. L-NAME (Sigma), (11) was performed after the end of the control period. Twenty minutes later, urine collection and blood pressure registration were started again.

NO synthase and non-selective ET_A/ET_B receptor inhibition. Renal excretory function, blood pressure and heart rate were investigated in the sham-operated rats (n=13) and in the renal denervated rats (n=10). Intravenous bolus injection of 10 mg/kg b.w. L-NAME was performed after the end of the 40-min control clearance period. Ten min later non-selective ET_A/ET_B receptor inhibitor, bosentan (10 mg/kg b.w.), (14) was applied by intravenous bolus injection. Ten minutes after bosentan administration urine collection and blood pressure registration were completed in the course of 40-min experimental clearance period.

Vehicle administration. To eliminate any possible effect of the vehicle (0.9 % saline) after the end of the control period 200 µl sterile saline was applied as a bolus injection and after 20-min equilibration blood pressure was measured and urine was collected during a 40-min clearance period in 8 sham-operated and in 8 renal denervated rats.

A priming dose of 50 mg/kg b.w. inulin (Sigma) and 12 mg/kg b.w. PAH, (Merck) was applied as intravenous bolus injection to rapidly attain high plasma levels of these substances. Immediately after that, a continuous intravenous infusion of inulin (1.8 mg/kg.min) and PAH (0.70 mg/kg.min) was started at a pump rate of 25 µl/min. Plasma and urine concentrations of inulin were determined by the anthrone method (15). Glomerular filtration rate (GFR) was estimated by the clearance of inulin. Urinary and plasma concentrations of PAH were determined by the modified method of Smith et al. (16). Effective renal plasma flow (ERPF) was estimated from the clearance of PAH and renal blood flow (RBF) was calculated as ERPF/1- Haematocrit.

Urine volume was assessed gravimetrically to establish urine flow rate (V). Haematocrit was obtained from all blood samples. Plasma and urine concentrations of sodium and potassium were determined by flame photometry (Corning 410 C), while of chloride by using analyzer (Corning 925). Plasma and urine concentrations of calcium were determined spectrophotometrically with commercially available kits (SGM Italia). Osmolality was determined by using a vapour pressure

osmometer (Vescor 5500A). On the basis of these data excretion rates of sodium (U_{Na} V), potassium (U_K V), chloride (U_{Cl} V), calcium (U_{Ca} V) and their fractional excretions were calculated.

Throughout the study, arterial blood pressure was measured directly in the femoral artery by Gould/Statham P23ID pressure transducer connected to computerized data acquisition system Biopac MP100WS. After analogue-to-digital conversion, values of systolic (SAP), diastolic (DAP) and mean arterial pressure (MAP) in every heart cycle were determined by the AcqKnowledge 2.0 software. Renal vascular resistance (RVR) was calculated as MAP/RBF.

Statistical analysis was performed using the Student's t-test, Wilcoxon's test for unpaired data, or one-way analysis of variance when appropriate. All results are presented as mean±SEM. Differences at a level of $p<0.05$ were considered statistically significant.

RESULTS

The application of 10 mg/kg b. w. L-NAME as a single i.v. injection produced a rapid and pronounced increase in SAP, MAP and DAP that was sustained over the 60-min period of observation. The increase in blood pressure was nearly maximal 5 min after L-NAME administration. During concomitant L-NAME and bosentan administration, blood pressure increased to a similar extent as with L-NAME alone and was constant until the end of the experiment. In the sham-operated and in the renal denervated rats renal excretory function and blood pressure were stable throughout the time control experiments. The investigated variables did not change after the injection of the vehicle.

L-NAME induced an increase in SAP, MAP and DAP by 14%, 24% and 36% in the sham-operated rats ($p<0.01$) and in the renal denervated rats by 13.6%, 25.3% and 34% ($p<0.01$). RVR was enhanced by L-NAME in both sham-operated and renal denervated rats ($p<0.05$), (*Table 1*). The pressor effect of L-NAME on SAP MAP and DAP as well as the effect on RVR in both sham-operated and renal denervated rats were not affected by bosentan (*Table 1*).

In the sham-operated rats, L-NAME increased urine flow rate from 4.99±0.53 to 9.71±1.18 µl/min.100 g b.w. ($p<0.05$) and decreased urine osmolality from 835.7±52.7 to 606.0±44.8 mosm/kg H_2O ($p<0.05$). Sodium excretion increased from 210.1±20.8 to 714.4±109.1 nmol/min.100 g b.w. ($p<0.05$). Chloride excretion increased from 203.6±24.6 to 837.4±129.3 nmol/min.100 g b.w. and calcium excretion increased from 5.4±0.71 to 8.25±0.73 nmol/min.100 g b.w., ($p<0.05$), (Fig. 1A).

The effect of L-NAME on urine flow rate as well as sodium, chloride and calcium excretions in the sham-operated rats with intact renal nerves was markedly reduced by bosentan and the values returned to control level (*Fig. 1A*).

Chronic renal denervation did not change SAP, MAP, DAP, RVR, C_{in}, C_{PAH}, sodium, potassium or chloride excretions. Urine flow rate increased from 4.99±0.53 to 6.48±0.49 µl/min.100 g b.w. ($p<0.05$), while urine osmolality decreased from 835.7±52.7 to 626.4±40.0 mosm/kg H_2O ($p<0.05$). As a result of the denervation renal norepinephrine content decreased from 9.1±0.62 to 0.51±0.02 nmol/g renal tissue ($p<0.001$).

Table 1. Changes in blood pressure, clearance of inulin, clearance of paraaminohippuric acid (PAH) and renal vascular resistance due to L-NAME induced nitric oxide synthase inhibition as well as concurrent nitric oxide synthase and ET_A/ET_B receptor inhibition in sham-operated Wistar rats with intact renal nerves and in Wistar rats one week after bilateral renal denervation

Sham-operated rats with intact renal nerves	Control	L-NAME	L-NAME+Bo
SAP	136.8±1.4	156.4±3.0 *	157.7±5.0 *
MAP	104.2±0.87	130.3±3.0 *	127.2±4.3 *
DAP	83.2±1.1	113.2±3.1 *	109.5±4.1 *
C_{in}	0.84±0.13	0.79±0.07	0.82±0.06
C_{PAH}	2.26±0.24	2.32±0.13	2.36±0.24
RVR	26.7±0.9	32.4±1.4 *	32.9±1.6 *
Renal denervated rats	Control	L-NAME	L-NAME+Bo
SAP	135.6±4.1	153.9±4.0 *	157.9±9.8 *
MAP	101.7±2.2	125.6±3.9 *	125.0±7.6 *
DAP	79.4±1.6	106.4±3.8 *	106.8±6.6 *
C_{in}	0.75±0.03	0.72±0.08	0.71±0.04
C_{PAH}	2.25±0.17	2.07±0.24	2.31±0.40
RVR	24.7±1.1	33.1±1.4 *	29.8±2.0 *

L-NAME - administration of 10 mg/kg b.w. i.v N^{Ω} -nitro-L-arginine methyl ester; L-NAME+Bo - concomitant administration of L-NAME and ET_A/ET_B receptor blocker Bosentan 10 mg/kg b.w. i.v.. SAP - systolic arterial pressure, mmHg; MAP - mean arterial pressure, mmHg; DAP - diastolic arterial pressure, mmHg; C_{in} - clearance of inulin ml/min.100 g b.w.; C_{PAH} clearance of paraaminohippuric acid ml/min.100 g b.w., RVR - renal vascular resistance, mmHg/ml.min.100 g b.w. Statistically significant differences as compared to control (*)

In the renal denervated rats, L-NAME administration decreased urine flow rate from 6.48±0.49 to 4.47±0.77 µl/min.100 g b.w. ($p<0.05$). Sodium excretion was 238.0±18.9 nmol/min.100 g b.w. before and 262.2±63.6 nmol/min.100 g b.w. after L-NAME administration, chloride excretion was 219.0±58.5 and 300.8±80.4 nmol/min.100 g b.w. respectively. Calcium excretion was 7.1±0.9 nmol/min.100 g b.w. before and 6.6±0.66 nmol/min.100 g b.w. after L-NAME (Fig. 1B). Potassium excretion was 598.9±47.2 nmol/min.100 g b.w. before L-NAME and 663.3±80.5 nmol/min.100 g b.w.after L-NAME administration (n.s.).

In the renal denervated rats, bosentan administration during NOS inhibition did not produce changes in urine flow rate, sodium, chloride, calcium or potassium excretions. Urine flow rate was 5.68±0.73 µl/min.100 g b.w., sodium, chloride and calcium excretions were 313.8±38.4 nmol/min.100 g b.w., 323.5±67.3 nmol/min.100 g b.w. and 6.56±0.82 nmol/min.100 g b.w. respectively (*Fig. 1B*). Potassium excretion was 557.6±74.4 nmol/min.100 g b.w..

NO synthase inhibition with L-NAME and concurrent NO synthase inhibition and ET_A/ET_B receptor blockade did not alter C_{PAH} and C_{in} in both sham-operated and renal denervated rats. Haematocrit did not change in any of the groups investigated.

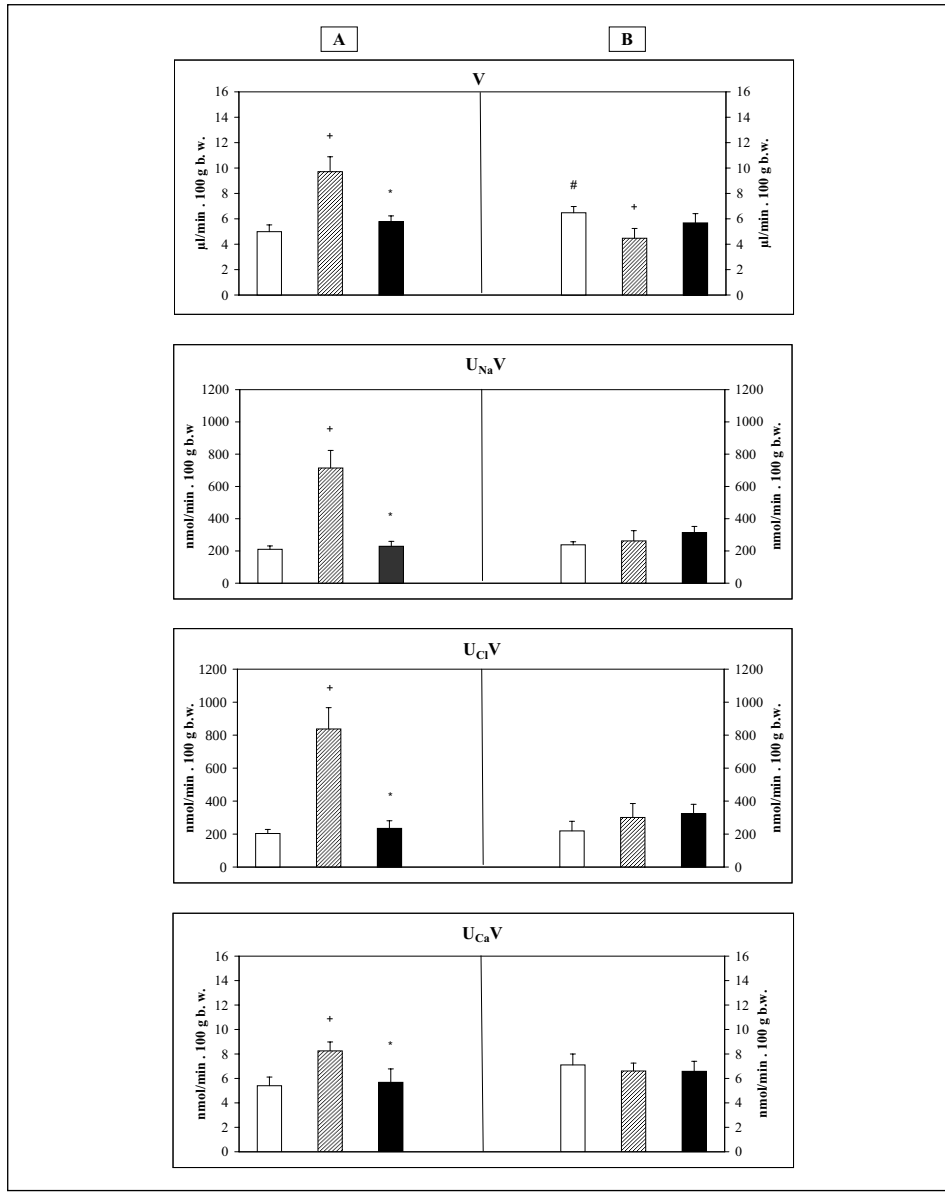

Fig. 1. Changes in urine flow rate, sodium excretion ($U_{Na}V$), chloride excretion ($U_{Cl}V$) and calcium excretion ($U_{Ca}V$) due to L-NAME induced nitric oxide synthase inhibition as well as concurrent nitric oxide synthase and ET_A/ET_B receptor inhibition in sham-operated Wistar rats with intact renal nerves (A) and in Wistar rats one week after bilateral renal denervation (B)
Open bars - control; Hatched bars - L-NAME administration 10 mg/kg b.w. i.v.; Solid bars - concurrent L-NAME (10 mg/kg b.w. i.v) and Bosentan (10 mg/kg b.w. i.v) administration
Statistically significant differences as compared to control ($^+$) as compared to L-NAME (*) and as compared to intact renal nerves (#).

DISCUSSION

The acute NOS inhibition led to a similar prompt and sustained increase in arterial blood pressure in both renal denervated and sham-operated rats. The pressor effect of L-NAME was not affected by ET_A/ET_B receptor inhibition in both sham-operated and renal denervated rats. Therefore, endogenous ET as well as renal nerves did not play an important role in the maintenance of the elevated blood pressure level observed in normotensive rats when NO formation was acutely inhibited.

Urine flow rate as well as excretion rates of sodium, chloride and calcium increased after NOS inhibition in the sham-operated rats despite the fact that GFR and thus, the filtered load of these electrolytes did not increase. Consistent with the findings of the present study are reports that L-NAME produced an increase in arterial pressure and excretion of sodium and water in normotensive rats (17, 18). Both the natriuretic and pressor responses were similar in magnitude to the responses obtained in the present study. The effect of NOS inhibition on water and electrolyte excretions could be due to elimination of a direct modulatory action of NO on tubular sodium transport, pressure natriuresis and changes of RBF or GFR. It was shown that renal blood flow autoregulation was maintained during an acute inhibition of NO sythesis (19). In the proximal tubule the action of NO was reported to be inhibitory (2, 20) or stimulatory (21), whereas the action in the thick ascending limb of Henle's loop is inhibitory (3, 22). Since in our experiments C_{in} and C_{PAH} did not change, the increase in sodium, chloride and calcium excretions after NOS inhibition is likely to be a result of some direct modulation in the tubular transport as well as of pressure diuresis and saluresis.

In marked contrast to the absence of alterations in arterial pressure, ET_A/ET_B receptor inhibition in the sham-operated rats with intact renal nerves caused reversing of the renal effects which was due to NOS inhibition. This interesting result indicates a dissociation of the ability of ET to prevent the elevation in blood pressure and to normalize the renal excretory response to acute NOS inhibition. It was reported that ET_A /ET_B receptor blockade per se did not modify RBF autoregulation (11). We have recently demonstrated that non-selective ET_A/ET_B receptor blockade with intravenous bolus injection of 10 mg/kg b.w. bosentan decrease urine flow rate and sodium excretion without any changes in C_{in} or RBF calculated from C_{PAH} (23). This indicates that endogenous ET may affect considerably renal function by influencing renal tubular transport processes. During NOS inhibition there is an imbalance between ET-1- and NO-mediated effects on renal excretory function. Therefore, the changes in renal function observed after NOS inhibition are due, at least in part, to the fact that NO buffers the influence of endogenous ET within the kidney. The present study documented for the first time that endothelin receptor antagonist attenuate the marked increase in urinary chloride and calcium excretion as a result of NOS inhibition.

The present study additionally demonstrated that in contrast to the sham-operated rats in the renal denervated rats urine flow rate decreased after NOS inhibition. NO might exert different actions - one by inhibiting basal levels of fluid reabsorption, the other by facilitating neurally stimulated fluid reabsorption (20). Moreover, it was demonstrated that there was a tonic inhibitory action of NO on water excretion, which was renal nerve independent, whereas its impact on sodium handling appeared to be dependent upon a background level of renal nerve activity (24). Therefore, the natriuretic and chloruretic responses following NOS inhibition are more closely related to renal nerves. The effects of NOS inhibition on urinary excretion of sodium and chloride were more pronounced in the rats with innervated kidneys. The available data show an interaction between renal nerves and NO in the control of renal tubular transport. The presence of NO was essential for renal nerves to increase fluid transport through the epithelial cells (20). Micropuncture studies have shown that NOS inhibition causes a reduction in proximal fluid reabsorption in control rats, but this effect is abolished by renal denervation (21, 25). These observations indicate that the action of NO required the participation of renal sympathetic nerves. Such modulation may not be equally important in the renal cortex or medulla. There is evidence that blood circulation in the medulla is less sensitive to the renal nerve activity than that in the cortex (26) and that adequate perfusion of the medulla is largely dependent on NO generated by nNOS, whereas the activity of all three isoforms is needed to maintain cortical blood flow (27). This may contribute to the more pronounced effect of L-NAME on pressure-diuresis and pressure-saluresis in innervated than in denervated kidneys. In our experiments, renal denervation attenuated the effects of NOS inhibition. These observations are compatible with the hypothesis that the effects of NOS inhibition on renal excretory function are partly mediated through renal sympathetic nerves.

The next question addressed here was whether ET_A/ET_B receptor blockade would modify the effects of the previous inhibition of NOS in chronically denervated rats. In contrast to the sham-operated rats with intact renal nerves, ET_A/ET_B receptor blockade during NOS inhibition in the renal denervated rats did not alter renal excretory function. It has recently been demonstrated that endogenous ET stimulate eNOS gene expression in the inner medullary collecting duct cells where it acts to promote sodium excretion through a mechanism that may involve activation of eNOS and increase in NO production (28). ET-1 produced by inner medullary collecting duct cells acts mainly on ET_B receptors of the vasa recta endothelial cells to activate eNOS and to generate NO, which acts on both inner medullary collecting duct cells and on vasa recta (4). Interestingly, no responses to bosentan were observed in the renal denervated rats when NO synthesis was inhibited. The results of this study demonstrated that non-selective ET_A/ET_B receptor blockade markedly diminish renal excretory response to NOS inhibition in the presence of renal nerves. Therefore, renal

sympathetic nerve activity is necessary for the influence of the endogenous ET on L-NAME induced changes in renal excretory function. An interaction between endogenous endothelins and renal sympathetic nerves was also shown in a prior study, whereby renal nerves modulate the effects of systemically applied ET_A receptor antagonist (28). We also observed that chronic renal denervation in normotensive rats was followed by a decrease in renal papillary ET-1 concentration (29). Such influence of renal denervation on renal papillary ET-1 content may account for the observed in the present study differences between renal excretory function of rats with intact and rats with denervated kidneys. Therefore, the results now obtained are consistent with the role of renal nerves in modulating renal excretory function during concomitant NOS and ET_A/ET_B receptor inhibition.

In conclusion, our data suggest that in the presence of renal nerves the effects of NO synthase inhibition on renal excretory function is endothelin dependent. Therefore, the changed renal excretory function during acute NO synthase inhibition may be due to unopposed endothelin-induced effect. The diuretic, natriuretic, chloruretic and calciuretic effects of NO synthase inhibition were eliminated by renal denervation. ET_A/ET_B receptor blockade during NO synthase inhibition in renal denervated rats did not alter renal excretory function. This observation indicates that NO, ET and renal nerves interact to maintain renal excretory function. These results indicate that renal sympathetic nerves play an important modulatory role in the NO and endothelin induced effects on renal excretory function.

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