

**URINARY MAGNESIUM AND CALCIUM EXCRETION AFTER NEURONAL NITRIC OXIDE SYNTHASE INHIBITION IN NORMOTENSIVE AND SPONTANEOUSLY HYPERTENSIVE RATS**

Ivan Chakalov, Georgi Lyutov

Department of Physiology, Medical Faculty, Medical University – Sofia, Bulgaria  
[chakalov\\_dc@yahoo.com](mailto:chakalov_dc@yahoo.com)

**Summary**

It has been supposed that altered expression of nitric oxide synthase isoenzymes is a factor involved of the pathogenesis of hypertension.

The present study was aimed to establish the participation of nitric oxide generate by neuronal nitric oxide synthase (nNOS) in the regulation of water, magnesium and calcium excretion in normotensive Wistar rats and spontaneously hypertensive rats (SHR).

Experiments were carried out on conscious, male, normotensive Wistar rats (n=10) and spontaneously hypertensive rats (SHR, n=10) at the same age of 12-14 weeks. In the SHR group were included animals with systolic blood pressure over 170 mmHg. Selective nNOS inhibition was performed by 2 mg/kg 7-nitroindazole (7-NI), applied through previously implanted in femoral vein catheter (Portex OD: 0.96, ID: 058). Urine was collected through modified polyethylene catheter inserted in the bladder in two (40 min) clearance periods: control and 20 min after 7-NI application. All surgical preparations were performed 24 hours before experiments under general anesthesia (Nembutal – 35 mg/kg, i.p.). Urine flow rate was determined gravimetrically, magnesium and calcium concentration was measured by flame atomic absorption spectrophotometry (Perkin-Elmer, Analyst 300). The urine flow rate, calcium and magnesium excretion did not differ between Wistar rats and SHR. The nNOS inhibition did not change urine flow rate calcium and magnesium excretion in normotensive rats. In SHR nNOS inhibition decreased urine flow rate in SHR from  $4.78 \pm 0.69$  to  $2.70 \pm 0.45 \mu\text{l} \cdot \text{min}^{-1} \cdot 100 \text{ g b.w.}$ , ( $p < 0.05$ ) and decreased magnesium excretion from  $19.02 \pm 2.59$  to  $11.41 \pm 2.13 \text{ nmol} \cdot \text{min}^{-1} \cdot 100 \text{ g b.w.}$ , ( $p < 0.05$ ). Calcium excretion in SHR did not change after nNOS inhibition ( $4.03 \pm 0.64$  and  $4.01 \pm 0.49 \text{ nmol/min/100 g b.w.}$ ). In normotensive rats nitric oxide generated by nNOS is not involved in the regulation of water, calcium and magnesium excretion. However in SHR nitric oxide generated by neuronal nitric oxide synthase participate in the maintenance of condition necessary for reabsorbtion of magnesium.

**Key words:** Wistar rats, SHR, renal excretory function, 7-nitroindazole

### **Introduction**

Many physiological functions rely on the precise maintenance of body calcium ( $\text{Ca}^{2+}$ ) and magnesium ( $\text{Mg}^{2+}$ ) balance, which is tightly regulated by the concerted actions of intestinal absorption, renal reabsorption, and exchange with bone.<sup>[1]</sup> The homeostatic mechanism involves several hormones, which comprise among parathyroid hormone, vitamin D and others <sup>[2]</sup>. Extracellular calcium concentrations in humans are thousands times higher than within cells. Maintenance of such gradient requires specific regulation including intracellular stores, Ca binding proteins and transmembrane protein systems<sup>[3]</sup>. The kidney plays an important role in the homeostasis of divalent ions. Most calcium and magnesium reabsorption occurs in the proximal tubules and the thick ascending limb of Henle's loop via a passive paracellular pathway. At the level of the distal convoluted tubule and the connecting tubule, calcium is reabsorbed via an active transcellular route. Reabsorption of divalents in these latter segments is regulated in a  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -specific manner and determines the final excretion in the urine <sup>[1]</sup>.

Magnesium is a vasodilator which may play a role in the regulation of blood pressure. Both magnesium and calcium depletion can occur in selected tissues in spontaneously hypertensive rats (SHR). Magnesium depletion in the vascular smooth muscle of this hypertension model is contributory factor in the hypertension <sup>[4]</sup>.

The SHR model replicates the clinical progression of hypertension in humans. Decreased nitric oxide (NO) bioavailability is factor related with development of hypertension in SHR. The NO play an important role of regulation of blood pressure, and renal excretory function <sup>[5]</sup>.

The conditionally essential amino acid L-arginine is the substrate for nitric oxide (NO) synthesis, a key second messenger involved in many physiological functions <sup>[6]</sup>, which production is mediated by group of enzymes called nitric oxide synthases (NOS). NO is synthesized by three different NO synthase (NOS) isoforms, including neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS) <sup>[7]</sup>.

There is a body of evidence indicating that NO can act as a modulator of neurotransmission within the central nervous system, at sympathetic ganglia, and at peripheral neuroeffector junctions <sup>[8]</sup>. NO facilitates the proximal tubule transport which is stimulated by the renal nerves. Therefore, the presence of NO is of very high importance for the renal nerves to be able to stimulate the transport in the proximal epithelial cells <sup>[9]</sup>. NOS localization in the intracellular membrane is important in increasing NO production to aid water homeostasis <sup>[10]</sup>. Increased nitric oxide synthase (NOS) activity contributes to preglomerular vasodilation and subsequent glomerular hyperfiltration <sup>[11]</sup>.

The increased urine flow rate observed after nonselective NOS inhibition with L-NAME is not exclusively the result of a pressure diuresis but are somewhat dependent on the renal sympathetic nerve activity <sup>[12]</sup>.

Decreased renal neuronal nitric oxide synthase (nNOS) is present in various chronic kidney diseases <sup>[13]</sup>. However, there is no enough information about the effect of nNOS in renal excretory function and development of hypertension.

In our recent study we used selective inhibition of neuronal nitric oxide synthase with 7-nitroindazole (7-NI) to investigate if NO produced by nNOS there is any effect on parameters of renal function: urine flow rate, magnesium and calcium excretion in normotensive rats and spontaneously hypertensive rats.

### Materials and methods

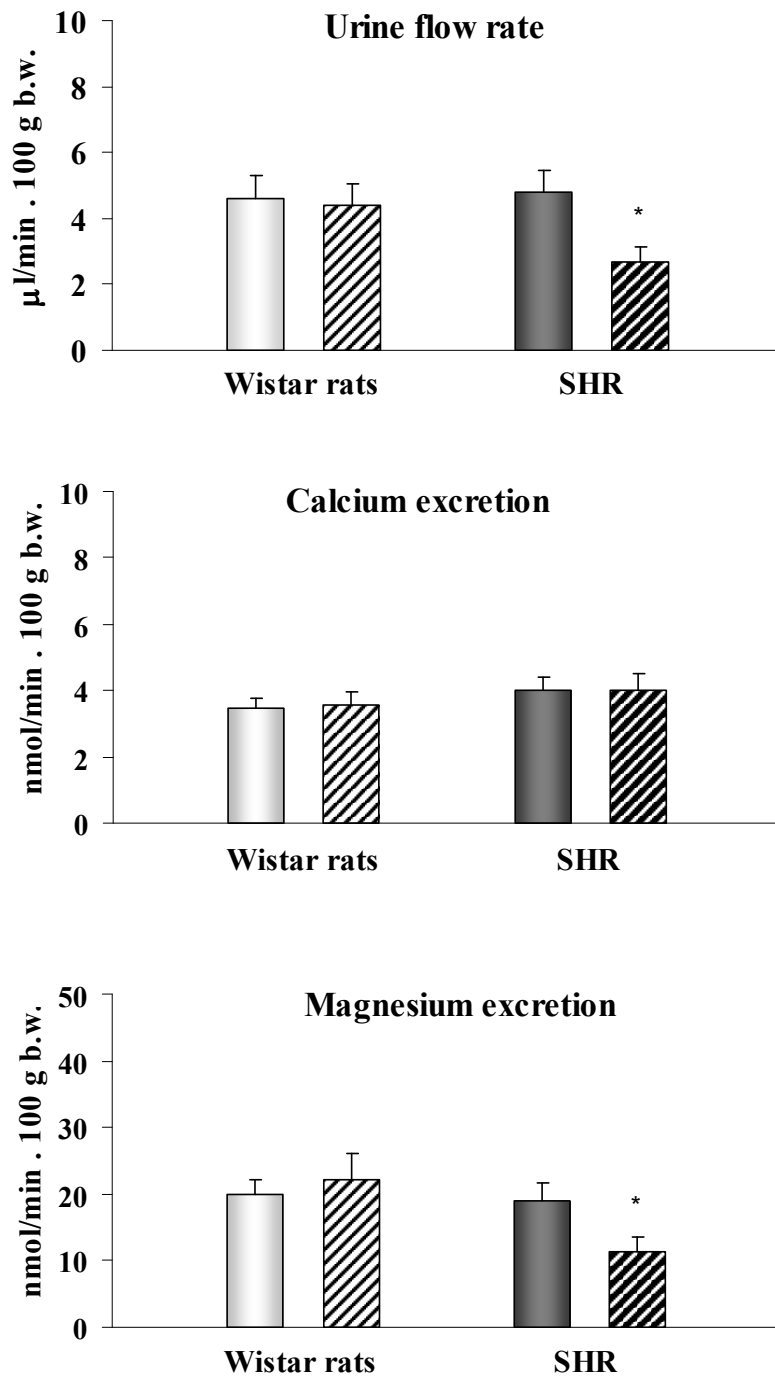
Experiments were carried out on conscious, male, normotensive Wistar rats (n=10) and spontaneously hypertensive rats (SHR, n=10) at the same age of 12-14 weeks. The study was performed in accordance with institutional care and use of laboratory animal guidelines, Medical University, Sofia. The animals were housed under standard condition: 12/12hours light / dark cycle; 22 °C room temperature; free access to tap water and standard rat chow. In the SHR group were included animals with systolic blood pressure over 170 mmHg, previously measured by tile cuff method (Ugo-Basile 58500). One day before the experiments all animals were subject to surgical preparations under general anesthesia Pentobarbital Sodium (Nembutal, Sigma), 35 mg/kg b.w., applied intraperitoneally. Catheter (Portex OD: 0.96, ID: 058) was inserted in to femoral vein for drug application and modified polyethylene catheter was placed in to the bladder for urine collection. The venous catheter was filed with 20 IU/ml heparin in sterile saline to avoid clotting. The experiments were performed on conscious animals 24 hours after surgical intervention. Urine was collected during two 40 minute long periods: control and 20 min after selective neuronal nitric oxide synthase (nNOS) inhibition. Inhibition of nNOS was performed by intravenous infusion of 7-nitroindazole (2 mg .kg<sup>-1</sup>. min<sup>-1</sup>), dissolved in 0.9 % warm NaCl .

Urine flow rate was determined gravimetrically, magnesium and calcium concentration in plasma and urine were measured by flame atomic absorption spectrophotometry (Perkin-Elmer, Analyst 300). On the basis of this data the excretion of magnesium and calcium were calculated.

All results were present as mean ± SEM. Student's t-test was used for comparison between two means. Differences at a probability level of p<0.05 were considered significant.

### Results

The systolic arterial blood pressure in SHR was higher compared to normotensive Wistar rats: 182.3±2.4 and 132±2.7 mmHg, (p<0.01). Urine flow rate, magnesium and calcium excretion did not differ between Wistar and SHR. The selective nNOS inhibition did not alter urine flow rate in Wistar rats: 4.58±0.72 and 4.38±0.69 µl.min<sup>-1</sup>.100 g b.w. However in SHR nNOS inhibition decreased urine flow rate from 4.78±0.69 to 2.70±0.45 µl/min/100 g b.w., (p<0.05). The nNOS inhibition did not change calcium 3.46±0.28 vs. 3.97±0.70 nmol/min/100 g b.w and magnesium (19.97±2.26 vs. 22.13±3.90 nmol/min/100 g b.w) excretion in normotensive rats. In SHR application of 7-NI provoke a decrease in magnesium excretion from 19.02±2.59 to 11.41±2.13 nmol/min/100 g b.w., (p<0.05). The calcium excretion in SHR did not change during 7-NI infusion (4.03±0.64 and 4.01±0.49 nmol/min/100 g b.w.).



**Figure 1.** Urine flow rate, magnesium and calcium excretion in normotensive Wistar rats and in spontaneously hypertensive rats SHR in control period (no hatched bars) and during selective nNOS synthase inhibition by 7-nitroindazole, applied intravenous in dose 2 mg.kg<sup>-1</sup>. min<sup>-1</sup> hatched bars)

\* p<0.05 statistically significant difference as a result of 7-NI application

### Discussion

In normotensive Wistar rats application of nNOS inhibitor 7-nitroindazole did not change the urine flow rate, magnesium and calcium excretion, whereas in spontaneously hypertensive rats, nNOS inhibition produced significant decrease in urine flow rate and magnesium excretion without changing calcium excretion.

We observed that in normotensive rats nitric oxide generated by nNOS is not involved in the regulation of water, calcium and magnesium excretion. However, in SHR nitric oxide generated by neuronal nitric oxide synthase participate in the maintenance of condition necessary for reabsorption of magnesium.

The neuronal isoform of the enzyme nitric oxide synthase (nNOS) has been localized in the kidney to the thick ascending limb the loop of Henle and the macula densa [14].

Immunocytochemical studies revealed decreased staining of nNOS in the macula densa, collecting ducts and in the glomerulus of SHR compared to Wistar rats [15].

The neuronal isoform of NOS is expressed in macula densa cells where it functions to blunt the tubuloglomerular feedback (TGF) response. Studies in intact SHR kidneys suggest a diminished role for NO in tubular and vascular regulation. Thus SHR have impaired pressure natriuresis that is corrected by infusion of L-arginine and enhanced tubuloglomerular feedback responses that have been ascribed to diminished blunting by macula densa-derived NO generated from nNOS. The tubuloglomerular feedback mechanism play main role in the regulation of water and electrolyte excretion by regulation of tonus of afferent arteriola [16]. NO produced in macula densa cells by nNOS contribute to vasoconstriction caused by TGF mechanism. In SHR is determined decreased sensibility of the TGF mechanism [17]. Any defect in NO generation in the juxta glomerular apparatus could contribute to heightened tubuloglomerular feedback response, enhanced renal vascular resistance, and hypertension [17]. It is determined that the effect of NO generate by nNOS on TGF is decreased in SHR although that nNOS expression is increased [17]. Urine flow rate increase after in vivo stimulation of NO production in kidney, and urine flow rate decrease after inhibition the generation of NO. This diuretic effect is not proportional with changes in glomerular filtration and renal blood flow. In nNOS deficiency mouse in the proximal tubule is determined high levels of reabsorption of water, nNOS stimulated effects on the proximal tubule. On the other side in nNOS deficiency mouse are determined low levels of reabsorption of water in the whole kidney compare to normal mouse. Consequently nNOS stimulated transport generally in the proximal tubule and thus effects of NO on the proximal tubule are depended of concentration and interact with other mechanisms. Calcium filtration depends on transport systems in the nephron. About 60% of filtrated calcium turn back by reabsorption in the proximal tubule, and 10% in distal convoluted tubules. In the descending limb of Henle calcium reabsorption is 20%, about 8% in distal tubules and 1-2% in the cortical tubules. About 1% of filtrated calcium and 10% of all calcium go out of the body trough the urine. A greater fraction of the ingested amount magnesium is reabsorbed in magnesium depletion; a lesser fraction is reabsorbed when body magnesium stores are excessive. However, the mechanisms involved in this regulation are not fully understood. The kidney must excrete an amount equivalent to the daily intake of magnesium by absorption from the intestine, or about 120 mg/day. Normally 5–20% of the filtered load of magnesium is excreted, but the amount excreted can approach the filtered load with excessive magnesium intake, and it can even exceed the filtered load in

the extreme. Alternatively, filtered magnesium can be reabsorbed almost completely in states of magnesium depletion. Presumably, these changes in renal handling occur because of hormonal influences, but these mechanisms are at present unknown. The proximal tubule passively reabsorbs a far smaller fraction of magnesium (only 20–30%) than of sodium, potassium, or calcium. Although the magnesium concentration in the tubular fluid of the proximal tubule and the descending limb of the loop of Henle rises considerably as fluid is reabsorbed, its reabsorption is limited due to the low magnesium permeability of the junctional complexes in these segments of the nephron. However, about two-thirds of the filtered magnesium is reabsorbed passively in the thick ascending limb of the loop of Henle. The elevated luminal concentration of magnesium and the lumen-positive voltage in these segments provide a large driving force for this passive reabsorption. A small amount of the filtered magnesium is also reabsorbed in the distal convoluted tubule, the connecting tubule and collecting duct by mechanisms that have not been identified but appear to be passive. Therefore there is a relationship between magnesium excretion and water excretion, magnesium reabsorption is passive and magnesium excretion depends on urine flow rate. We suggest that in the model of hypertension where there is defect in NO synthase system neuronal isoform of the enzyme play an important role of regulation of water and magnesium homeostasis, because there is a diminished role for NO from nNOS in blunting of TGF in SHR which cannot be ascribed to limited NOS expression. The changes in magnesium excretion are particularly dependent on water reabsorption, and indirectly controlled of TGF. Besides glomerulotubular balance, i.e., the normal flow dependence of tubular reabsorption in every nephron segment, the juxtaglomerular apparatus significantly contributes to the fine coordination between glomerular filtration and tubular reabsorption through the mechanism of tubuloglomerular feedback (TGF). The neuronal type isoform of NOS is expressed densely in macula densa cells where it functions to blunt the tubuloglomerular feedback (TGF) response [18]. In accordance with the fact that spontaneously hypertensive rats has an enhanced TGF compare to Wistar rats we supposed that in SHR the blunting effect of NO produced by nNOS has a more important role in the regulation of TGF. The enhanced of TGF lead to increase of water reabsorption and decrease in urine flow rate and as a results decrease in magnesium excretion in SHR. Calcium concentration it is not water depended and did not change any of the two groups.

We can concluded that NO generate by nNOS is involved in regulation of urine flow rate and regulation of magnesium balance in models of spontaneous hypertension.

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