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QUARTERLY

Nitric oxide — superoxide cooperation in the regulation of renal Na^+,K^+-ATPase✪

Jerzy Bełtowski✉, Andrzej Marciniak, Anna Jamroz-Wiśniewska and Ewelina Borkowska

Department of Pathophysiology, Medical University, Lublin, Poland

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The aim of this study was to investigate whether endogenous superoxide anion is involved in the regulation of renal Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase activities. The study was performed in male Wistar rats. Compounds modulating superoxide anion concentration were infused under general anaesthesia into the abdominal aorta proximally to the renal arteries. The activity of ATPases was assayed in isolated microsomal fraction. We found that infusion of a superoxide anion-generating mixture, xanthine oxidase (1 mU/min per kg) + hypoxanthine (0.2 μmol/min per kg), increased the medullary Na^+,K^+-ATPase activity by 49.5% but had no effect on cortical Na^+,K^+-ATPase and either cortical or medullary ouabain-sensitive H^+,K^+-ATPase. This effect was reproduced by elevating endogenous superoxide anion with a superoxide dismutase inhibitor, diethylthiocarbamate. In contrast, a superoxide dismutase mimetic, TEMPOL, decreased the medullary Na^+,K^+-ATPase activity. The inhibitory effect of TEMPOL was abolished by inhibitors of nitric oxide synthase (L-NAME), soluble guanylate cyclase (ODQ) and protein kinase G (KT5823). The stimulatory effect of diethylthiocarbamate was not observed in animals pretreated with a synthetic cGMP analogue, 8-bromo-cGMP. An inhibitor of NAD(P)H oxidase, apocynin (1 μmol/min per kg), decreased the Na^+,K^+-ATPase activity in the renal medulla and its effect was prevented by L-NAME, ODQ or KT5823. In contrast, a xanthine oxidase inhibitor, oxypurinol, administered at the same dose

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✉Corresponding author: Jerzy Bełtowski, Department of Pathophysiology, Medical University, K. Jaczewskiego 8, 20-090 Lublin, Poland; phone: (48 81) 742 5837; fax: (48 81) 742 5828; e-mail: patfiz@asklepios.am.lublin.pl

Abbreviations: 3-CP, 3-carbamoyl-proxyl; DETC, diethylthiocarbamate; 20-HETE, 20-hydroxy-eicosatetraenoic acid; HX, hypoxanthine; L-NAME, N^{ω}-nitro-L-arginine methyl ester; mTAL, medullary thick ascending limb; ODQ, 1-H-[1,2,4]-oxadiazolo-[4,3*a*]-quinoxaline-1-one; ROS, reactive oxygen species; SOD, superoxide dismutase; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl-piperidine 1-oxyl; XO, xanthine oxidase.

was without effect. These data suggest that NAD(P)H oxidase-derived superoxide anion increases Na^+,K^+-ATPase activity in the renal medulla by reducing the availability of NO. Excessive intrarenal generation of superoxide anion may upregulate medullary Na^+,K^+-ATPase leading to sodium retention and blood pressure elevation.

Nitric oxide (NO) plays an important role in the regulation of vascular tone and its deficiency may contribute to the pathogenesis of arterial hypertension. Reactive oxygen species (ROS), especially superoxide anion (O_2^-), scavenge NO by binding it to form peroxynitrite anion ($ONOO^-$). Oxidative stress causes NO depletion leading to vasoconstriction and blood pressure elevation (Schnackenberg, 2002a). Apart from modulating vascular tone, NO is involved in the regulation of sodium balance. Intrarenally produced NO increases natriuresis by inducing renal vasodilation, attenuating tubuloglomerular feedback, and inhibiting tubular Na^+ reabsorption (Ortiz & Garvin, 2002b). Experimentally induced intrarenal NO deficiency leads to Na^+ retention and blood pressure elevation (Cowley *et al.*, 2003). Recent studies indicate that renal tissue continuously produces superoxide anion (Zou *et al,* 2001), which regulates NO availability and limits its functional effects on the renal vasculature (Schnackenberg, 2002b), tubuloglomerular feedback (Ren *et al.*, 2002), and tubular transport (Ortiz & Garvin, 2002a; Garvin & Ortiz, 2003). Experimentally induced intrarenal oxidative stress causes arterial hypertension (Makino *et al.,* 2002), and excessive ROS production in the kidney is involved in some well-known models of animal hypertension such as spontaneously hypertensive rat (Welch *et al.,* 2000), Dahl salt-sensitive rat (Meng *et al.,* 2002), and hypertension associated with diabetic nephropathy (Schoonmaker *et al.,* 2000).

Na^+,K^+-ATPase, located in the basolateral membranes of renal tubular cells, drives active Na^+ reabsorption throughout the nephron and is involved in the regulation of extracellular fluid volume and blood pressure (Féraille & Doucet, 2001). *In vitro,* NO decreases the Na^+,K^+-ATPase activity in the proximal tubule (Aperia *et al.,* 1994; Zhang & Mayeoux, 2001), medullary thick ascending limb of Henle's loop (Kone & Higham, 1999), and in medullary tissue slices (Scavone *et al.,* 1995; Kang *et al.,* 2000). Recently, we have demonstrated that locally infused NO donors or the NO precursor L-arginine, decrease Na^+,K^+-ATPase activity in the renal medulla (Bełtowski *et al.*, 2003). In addition, an NO synthase inhibitor, L-NAME, increased Na^+,K^+-ATPase activity suggesting that renal sodium pump is tonically regulated by NO. Although the previously mentioned studies indicate that intrarenally produced superoxide anion regulates Na^+ transport, it is unclear whether it modulates the effect of NO on renal Na^+,K^+-ATPase.

In the present study we investigated the role of superoxide in the regulation of renal Na^+,K^+-ATPase by NO. In addition, we studied the effect of superoxide on a related enzyme, ouabain-sensitive H^+,K^+-ATPase, which is located in the apical membranes of tubular cells and is involved in K^+ reabsorption.

MATERIALS AND METHODS

Reagents. The specific inhibitors of protein kinases A and G, KT 5720 and KT 5823, respectively, were purchased from Kamiya Biomedical Co. (Thousand Oaks, CA, U.S.A.). The specific inhibitor of H^+,K^+-ATPases, 2-methyl 8-(phenylmethoxy)imidazol(1,2-α) pyridine-3-acetonitrile (Sch 28080) was kindly provided by the Schering-Plough Research Institute (Kenilworth, NJ, U.S.A.). Xanthine oxidase (lyophilized, from bovine milk) and other reagents were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Experimental protocol. All studies were performed on adult male Wistar rats weighing 250–300 g. The study protocol was ap-

proved by the Bioethics Committee of the Medical University of Lublin. The animals were anaesthetized with pentobarbital (50 mg/kg, i.p.) and a thin catheter was inserted through the femoral artery into the abdominal aorta proximally to the renal arteries for the infusion of the investigated substances. After the surgery, infusion with physiological saline (0.9% NaCl) was started at a rate of 66 μl/min. All investigated substances were infused as saline solution at a rate of 66 μl/min (4 ml/h). The total time of infusion was 60 min. Animals from the control group received 0.9% NaCl. The investigated drug was administered for 30 min, between the 1st and 30th or between the 31st and 60th minute of infusion. After the infusion, the abdominal cavity was opened and 5 ml of 0.9% NaCl was infused within 1–2 min through the catheter to remove erythrocytes from the kidneys. The kidneys were excised and the animals sacrificed by a lethal dose of pentobarbital.

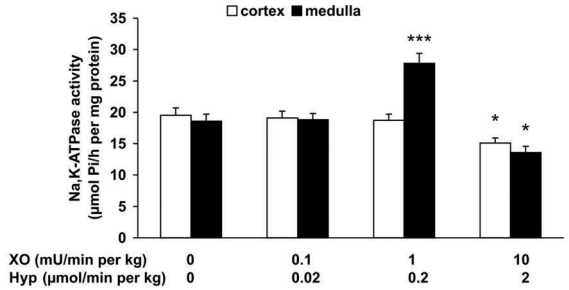
Enzyme assay. Microsomal fraction was isolated from the renal cortex and medulla as previously described (Bełtowski & Wójcicka, 2002). The ATPase activities were assayed by measuring the amount of inorganic phosphate (P_i) liberated from ATP. Na^+,K^+-ATPase activity was measured in a buffer containing 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 40 mM Tris/HCl (pH 7.4) and 3 mM Na_2ATP, and was calculated as the difference between the activities assayed in the absence and in the presence of 2 mM ouabain; both samples containing 0.2 mM Sch 28080 to block ouabain-sensitive H^+,K^+-ATPase. Ouabain-sensitive H^+,K^+-ATPase was measured in a medium containing 5 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 25 mM Tris/HCl (pH 7.4) and 5 mM Tris/ATP, and was calculated as the difference between the activities assayed in the absence and in the presence of 1 mM ouabain. Enzyme activities were expressed in μmole of P_i liberated from ATP by 1 mg of microsomal protein in 1 h (μmol/h per mg protein). P_i and protein concentrations were assayed by the method of Hurst (1964) and Lowry *et al.* (1951), respectively.

Statistics. Data are presented as mean ± S.E.M. from 8 animals in each group. Statistical significance was evaluated by unpaired Student's *t*-test or by ANOVA followed by Duncan's multiple range test for comparison of 2 or > 2 groups, respectively. $P < 0.05$ was considered significant.

RESULTS

Effect of exogenous superoxide on renal ATPases

To test whether superoxide has any effect on renal ATPases, we infused the O_2^--generating mixture of xanthine oxidase (XO) and hypoxanthine (Hyp) at three doses (Fig. 1). XO transfers electrons from Hyp to oxygen molecule forming O_2^- radical. The lowest tested dose (0.1 mU XO/min per kg + 0.02 μmol Hyp/min per kg) had no effect on the Na^+,K^+- ATPase and ouabain-sensitive H^+,K^+-ATPase activities in the renal cortex or medulla. The higher dose of 1 mU XO/min per kg + 0.2 Hyp μmol/min per kg increased the medullary Na^+,K^+-ATPase activity by 49.5% but had no effect on cortical Na^+,K^+-ATPase and cortical or medullary ouabain-sensitive H^+,K^+- ATPase. At the highest dose XO (10 mU/min per kg) and Hyp (2 μmol/min per kg) decreased the Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase activities in the renal cortex (by 22.6% and 28.9%, respectively) and in the renal medulla (by 26.9% and 24.4%, respectively). Thus, the XO/Hyp superoxide generating system had a dose-dependent effect on medullary Na^+,K^+-ATPase with stimulation observed at lower and inhibition at higher doses. No changes in renal ATPases were observed if either XO or Hyp were infused alone, or if XO was inactivated by boiling before the infusion (not shown).



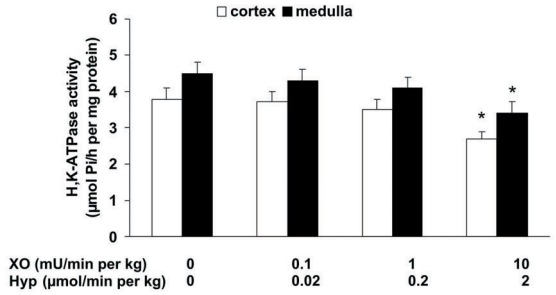
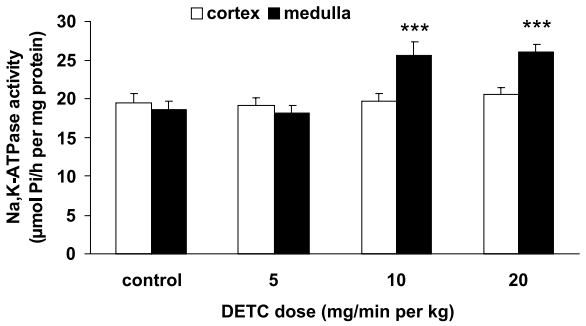


Figure 1. The effect of superoxide-generating mixture: xanthine oxidase (XO) + hypoxanthine (Hyp) on Na^+,K^+-ATPase (top panel) and ouabain-sensitive H^+,K^+-ATPase (bottom panel) activities in the renal cortex and medulla.

Control animals received 0.9% NaCl infusion for 60 min. Other groups received 30-min infusion of 0.9% NaCl and then were treated with different doses of XO and Hyp for another 30 min. ATPases were assayed in isolated microsomal fraction. Enzyme activities are expressed in μmole of inorganic phosphate liberated by the enzyme contained in 1 mg of microsomal protein in 1 h (μmole Pi/h per mg protein). N = 8 rats in each group. $^*P < 0.05$, $^{***}P < 0.001$ *vs* control group.



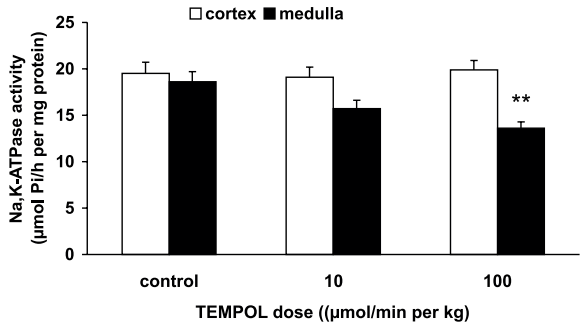


Figure 2. The effect of superoxide dismutase (SOD) inhibitor, diethylthiocarbamate (DETC, top panel) and SOD mimetic, TEMPOL (bottom panel) on Na^+,K^+-ATPase activity in the renal cortex and medulla.

$^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group.

Effect of endogenous superoxide on renal Na^+,K^+-ATPase

Superoxide anion is continuously produced in tissues but its steady-state level is very low because it is effectively scavenged by superoxide dismutase (SOD). To investigate whether endogenous O_2^- is involved in the regulation of renal ATPases, we measured their activities in animals treated with either an SOD inhibitor, diethyldithiocarbamate (DETC), or a SOD mimetic which catalytically scavenges O_2^-, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL). DETC infused at a dose of 5 mg/min per kg had no effect on medullary Na^+,K^+-ATPase, however, higher doses of this compound (10 and 20 mg/min per kg) increased its activity by 38.2% and 40.3%, respectively (Fig. 2). In contrast, DETC had no effect on cortical Na^+,K^+-ATPase (Fig. 2) as well as on cortical or medullary ouabain-sensitive H^+,K^+-ATPase (not shown). Thus, DETC reproduced only the stimulatory effect of XO/Hyp on medullary Na^+,K^+-ATPase. Infusion of TEMPOL at a dose of 100 μmol/min per kg decreased the medullary Na^+,K^+-ATPase activity by 26.9%. A lower dose of TEMPOL (10 μmol/min per kg) had no significant effect. In contrast to TEMPOL, a structurally related compound with no superoxide-scavenging properties, 3-carbamoyl-proxyl (3-CP), did not change the

medullary Na^+,K^+-ATPase activity (not shown). TEMPOL had no effect on cortical Na^+,K^+-ATPase and on cortical or medullary ouabain-sensitive H^+,K^+-ATPase. Taken together, these data suggest that endogenously produced superoxide increases the Na^+,K^+-ATPase activity specifically in the renal medulla.

Role of the NO-cGMP pathway in the regulation of Na^+,K^+-ATPase by O_2^-

In the vasculature, O_2^- scavenges NO thus decreasing its biological activity. TEMPOL exerts numerous effects by scavenging O_2^- and in this way protecting NO from inactivation. Thus, we hypothesized that TEMPOL decreased the medullary Na^+,K^+-ATPase activity by augmenting the inhibitory effect of NO. Consistently with our previous study (Bełtowski *et al.,* 2003), a nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 nmol/min per kg), increased the medullary Na^+,K^+-ATPase activity by 46.2% (Table 1). If DETC was infused after L-NAME, this SOD inhibitor had no additive stimulatory effect on medullary Na^+,K^+-ATPase. In addition, DETC had no effect on medullary Na^+,K^+-ATPase in animals pretreated with a membrane-permeable cGMP analogue, 8-bromo-cGMP. 8-Bromo-cGMP infused at a dose of 100 nmol/min per kg decreased the medullary Na^+,K^+-ATPase

Table 1. Interactions between compounds modulating endogenous superoxide and the NO-cGMP pathway in the regulation of Na^+,K^+-ATPase activity in the renal medulla

Treatment schedule		Na^+,K^+-ATPase activity (μmol P_i/h per mg protein)
0–30 min	31–60 min	
0.9% NaCl	0.9% NaCl	18.6 ± 1.1
L-NAME	0.9% NaCl	27.2 ± 1.4**
L-NAME	DETC	25.7 ± 1.8**
8-Br-cGMP	0.9% NaCl	13.2 ± 0.8**
8-Br-cGMP	DETC	13.4 ± 0.8**
0.9% NaCl	TEMPOL	13.6 ± 0.7**
L-NAME	TEMPOL	26.7 ± 1.2**
8-Br-cGMP	TEMPOL	13.0 ± 0.8**
ODQ	TEMPOL	18.4 ± 1.1
KT5823	TEMPOL	18.1 ± 1.0
KT5720	TEMPOL	13.4 ± 0.7**
0.9% NaCl	Apocynin	14.2 ± 0.7*
0.9% NaCl	Oxypurinol	18.1 ± 1.4
L-NAME	Apocynin	26.4 ± 1.2**
ODQ	Apocynin	18.8 ± 0.8
KT5823	Apocynin	18.4 ± 1.1
Apocynin	TEMPOL	13.7 ± 1**
Apocynin	DETC	13.5 ± 0.7**

The doses of drugs were as follows: TEMPOL, 100 μmol/min per kg; L-NAME, 8-Br-cGMP and ODQ, 100 nmol/min per kg; KT5823 and KT5720, 10 nmol/min per kg; apocynin and oxypurinol, 1 μmol/min per kg; DETC, 20 mg/min per kg; *P<0.05, **P<0.01 (compared to control by ANOVA and Duncan's test).

activity by 29.0%. If DETC was administered after 8-bromo-cGMP, the Na^+,K^+-ATPase activity was by 25.6% lower than in control animals and not significantly different to that in rats treated with 8-bromo-cGMP alone (Table 1). Thus, DETC had no effect on medullary Na^+,K^+-ATPase if the activity of the cGMP signalling pathway was clamped by an exogenous cGMP analogue. On the other hand, TEMPOL had no inhibitory effect on medullary Na^+,K^+-ATPase in animals pretreated with L-NAME or 8-bromo-cGMP (Table 1). In addition, the effect of TEMPOL on medullary Na^+,K^+-ATPase was abolished by pretreatment with a soluble guanylate cyclase inhibitor, 1-H-[1,2,4]-oxadiazolo-[4,3*a*]-quinoxalin-1-one (ODQ, 100 nmol/min per kg) or a protein kinase G inhibitor, KT5823 (10 nmol/min per kg), but not by a protein kinase A inhibitor, KT5720 (Table 1). ODQ, KT5823 and KT5720 administered alone had no effect on medullary Na^+,K^+- ATPase. These data indicate that TEMPOL decreases the medullary Na^+,K^+-ATPase activity by augmenting the biological action of NO.

Sources of O_2^- involved in the regulation of renal Na^+,K^+-ATPase

Superoxide may be generated in mammalian tissues by mitochondrial respiratory chain, NAD(P)H oxidase, xanthine oxidase, nitric oxide synthase, cyclooxygenase, lipooxygenase and cytochrome P450. In the present study, we tested the effect of specific inhibitors of xanthine oxidase (oxypurinol) and NAD(P)H oxidase (apocynin) on medullary Na^+,K^+-ATPase. Oxypurinol (1 μmol/min per kg) had no effect on Na^+,K^+-ATPase activity. In contrast, apocynin infused at the same dose decreased the medullary Na^+,K^+-ATPase activity by 23.7%. The effect of apocynin was abolished by L-NAME, ODQ or KT5823 (Table 1). Although apocynin or TEMPOL administered alone decreased the medullary Na^+,K^+-ATPase activity, their effect was not synergistic. In addition, the SOD inhibitor DETC did not increase Na^+,K^+-ATPase activity in apocynin-pretreated rats (Table 1). These data suggest that the Na^+,K^+-ATPase-regulating superoxide is generated mostly by NAD(P)H oxidase.

DISCUSSION

Recent studies indicate that O_2^- continuously produced within the kidney stimulates tubular sodium reabsorption. *In vivo*, infusion of DETC into the renal artery (Majid & Nishiyama, 2002) or into the medullary interstitium (Zou *et al.,* 2001) decreases sodium excretion and medullary blood flow without affecting the glomerular filtration rate, whereas TEMPOL has an opposite, natriuretic effect. Prolonged intrarenal DETC administration causes Na^+ retention and blood pressure elevation (Makino *et al.,* 2002). *In vitro* studies have demonstrated that superoxide anion stimulates chloride reabsorption in medullary thick ascending limb (mTAL) (Ortiz & Garvin, 2002a; 2002c). The results of the present study suggest that the antinatriuretic effect of endogenous superoxide is mediated, at least in part, by stimulation of the Na^+,K^+-ATPase activity specifically in the renal medulla.

Several lines of evidence suggest that superoxide increases the Na^+,K^+-ATPase activity by scavenging nitric oxide and attenuating its inhibitory effect on renal sodium pump. First, in our experimental model NO decreased the Na^+,K^+-ATPase activity in the renal medulla but not in the renal cortex, and had no effect on the ouabain-sensitive H^+,K^+-ATPase (Bełtowski *et al.,* 2003). This is consistent with the present observation that TEMPOL inhibits whereas DETC stimulates only medullary Na^+,K^+-ATPase. Second, the effect of TEMPOL was abolished by L-NAME, as well as by either soluble guanylate cyclase or protein kinase G blockade. Finally, the stimulatory effect of DETC was absent in animals with the NO system inhibited by L-NAME or

in rats treated with 8-bromo-cGMP. Taken together, these data indicate that manipulating the intrarenal superoxide level affects medullary Na^+,K^+- ATPase by modulating the NO-cGMP-PKG pathway. Although many biological effects of TEMPOL are associated with the preservation of endogenous NO, some NO-independent activities of this SOD mimetic have been described. In particular, superoxide stimulates whereas TEMPOL reduces the activity of renal sympathetic nerves (Shokoji *et al.,* 2004). Norepinephrine, released by renal sympathetic endings, stimulates tubular Na^+,K^+-ATPase (Aperia *et al.,* 1994), therefore, O_2^- could increase the Na^+,K^+-ATPase activity by activating sympathetic nerves. 20-Hydroxyeicosatetraenoic acid (20-HETE) inhibits Na^+ transport in different nephron segments including mTAL, partially by inhibiting tubular Na^+,K^+-ATPase (McGiff & Quilley, 1999). Recently, Hoagland *et al.* (2003) have demonstrated that TEMPOL may improve the natriuretic effect of 20-HETE by protecting it from ROS-mediated peroxidation. It remains to be established whether any of these or some other, yet unidentified NO-independent mechanisms are involved in the modulatory effect of O_2^- on medullary Na^+,K^+-ATPase.

NAD(P)H oxidase is a principal source of superoxide in the vasculature and is abundantly expressed also in the renal tubules (Geiszt *et al.*, 2000; Shiose *et al.,* 2001; Zou *et al.*, 2001, Chabrashvili *et al.,* 2002). Recently, Haque and Majid (2004) have demonstrated that mice lacking the catalytic subunit of NAD(P)H oxidase, $gp91^{phox}$, are characterized by a higher level of urinary NO metabolites and sodium excretion than wild-type animals. We demonstrated that an NAD(P)H oxidase inhibitor, apocynin, mimicked the inhibitory effect of TEMPOL on medullary Na^+,K^+-ATPase. When O_2^- generation is inhibited, neither TEMPOL nor DETC can modulate its level. In the present study TEMPOL had no additional inhibitory effect on Na^+,K^+-ATPase in apocynin-pretreated animals and apocynin abolished the stimulatory effect of DETC. These data strongly suggest that the superoxide involved in the regulation of renal Na^+,K^+-ATPase is generated mainly by NAD(P)H oxidase.

High dose of Hyp and XO decreased the Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase activities in the renal cortex and medulla. This is consistent with the findings of Racasan *et al.* (2003), who observed marked natriuresis despite reduced glomerular filtration rate in rats treated with comparable doses of Hyp and XO. This effect is most likely associated with inactivation of both renal ATPases by reactive oxygen species. Numerous studies have demonstrated a decrease in the Na^+,K^+-ATPase activity in various tissues including the kidney following oxidative stress (Guzman *et al.,* 1995; Rodrigo *et al.,* 2002; Zhang *et al.,* 2002; Varela *et al.,* 2004). However, the inhibitory effect of Hyp/XO on renal ATPases was not reproduced by increasing endogenous superoxide with an SOD inhibitor, suggesting that this effect is not physiologically important. Nevertheless, it may become significant in states associated with excessive ROS generation such as ischemia-reperfusion injury.

We are aware of several important limitations of the present study. General anesthesia could affect renal perfusion and decrease tissue pO_2, which is already low in the renal medulla under physiological conditions. Hypoxia stimulates NAD(P)H oxidase and O_2^- generation in the thick ascending limb (Li *et al.,* 2002). Thus, the experimental conditions applied in this study could overestimate the role of endogenous superoxide. In addition, NO reduces oxygen consumption by the renal tissue (Laycock *et al.,* 1998), and antioxidant treatment can increase intrarenal pO_2 by increasing NO availability (Zou & Cowley, 2003). Oxygen tension critically affects the regulation of renal Na^+,K^+-ATPase by cyclic nucleotide-dependent protein kinases (Kiroytscheva *et al.,* 1999). One could suggest that changes in O_2^- level modulate the effect of NO on re-

nal oxygenation rather than on tubular transport, and this indirectly affects other signalling pathways regulating Na^+,K^+-ATPase. However, scavenging superoxide is expected to improve renal oxygenation (Zou & Cowley, 2003), which directs the effect of cGMP or cAMP toward stimulation of renal Na^+,K^+-ATPase (Kiroytscheva *et al.,* 1999); an effect opposite to that observed in the present study.

In conclusion, the results of this study suggest that superoxide, continuously produced by NAD(P)H oxidase, increases the Na^+,K^+-ATPase activity in the renal medulla by reducing NO availability. This effect may be important in the regulation of tubular sodium reabsorption. Increased intrarenal O_2^- generation can upregulate medullary Na^+,K^+-ATPase leading to sodium retention and arterial hypertension. Physiological amounts of superoxide have no effect on renal ouabain-sensitive H^+,K^+-ATPase. In contrast, higher levels of ROS reduce both renal Na^+,K^+-ATPase and ouabain-sensitive H^+,K^+-ATPase activities.

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