Inhibition of angiotensin type 1 receptor impairs renal ability of K conservation in response to K restriction

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IT IS WELL ESTABLISHED THAT an increase in K intake stimulates superoxide generation and PTK activation is not known. Two lines of evidence from our previous experiments have strongly suggested that ANG II may be involved in mediating the effect of K restriction on renal K excretion: 1) ANG II inhibited ROMK channel activity in the CCD from K-restricted rats and 2) the effect of ANG II was abolished by blocking NADPH oxidase (NOX) and attenuated by inhibiting PTK (35). Because the effect of ANG II on ROMK channels is blocked by losartan, the type 1 angiotensin receptor (AT1R) may be involved in mediating the effect of K restriction on ROMK channels and renal K secretion. Thus the aim of the present study is to test the hypothesis that AT1R may be involved in suppressing renal K secretion in response to K depletion by stimulating superoxide anion generation, which further activates c-Src expression and MAPK phosphorylation.

METHODS

Metabolic cage. Sprague-Dawley rats (5–6 wk, either sex) were purchased from Taconic Farms (Germantown, NY). Rats were divided into the following four groups: 1) control group in which rats were received a normal K diet (NK) plus vehicle (0.5 ml water containing 0.1% ethanol) for 7 days; 2) NK + losartan group in which rats were on a NK diet plus losartan (2 mg/100 g body wt) resolved in 0.5 ml water containing 0.1% ethanol; 3) K-deficient diet (KD) group in which rats were fed a KD diet for 7 days plus vehicle; and 4) KD + losartan group in which rats were fed with a KD diet and treated with losartan (2 mg/100 g body wt). Animals were adapted in a metabolic cage for 3 days before experiments, and both vehicle and losartan were administered one time a day using gavage. Data regarding the 24-h food intake, body weight, and urine output were recorded. Urinary and plasma K concentrations were measured by a flame photometer, and daily K excretion was calculated as 24-h meq/l. At the end of 7-day metabolic cage studies, animals were anesthetized through intraperitoneal injection of Nembutal (6 mg/100 g) to draw the blood through cardiac puncture. After animals were killed, both kidneys were removed immediately for further experiments, including measurement of superoxide or Western blot. The animal use is approved by the Institutional Animal Care and Use Committee of New York Medical College.

Measurement of superoxide anion. Renal cortex and outer medulla (OM) were isolated and cut into a small piece with a sharp blade. Renal samples (100 mg wet tissue) were then suspended in air-

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The role of AT₁R in mediating the effect of K restriction on superoxide anion generation was studied in rats on a normal K diet (NK), a K-deficient diet (KD), and losartan-treated rats. The kidney and superoxide anions and related products were responsible for mediating the effect of K restriction on the phosphorylation of p38 and MAPK. We confirmed our previous finding that K restriction significantly increased the phosphorylation of p38 and ERK by 80% and 100% (n = 3 animals), respectively. Inhibition of AT₁R abolished the effect of K restriction on both p38 and ERK, and the effect of K restriction on superoxide production was reduced by 10%. The results suggest that AT₁R is involved in the regulation of superoxide anion generation.

To determine the role of AT₁R in mediating the effect of K restriction on superoxide anion generation, we used lucigenin methods to measure the superoxide anion level in renal cortex and OM from rats on a NK diet and a KD diet for 7 days with or without losartan treatment. We confirmed the previous finding that K restriction significantly increased superoxide generation by 120 ± 10% (n = 4) in renal cortex and OM compared with that in a NK diet (Fig. 1). Treatment of rats with losartan had no significant effect on superoxide generation in rats on a NK diet. However, inhibiting AT₁R almost completely abolished the effect of K restriction on superoxide anion production (Fig. 1). This suggests that stimulation of AT₁R is at least partially responsible for the effect of K restriction on renal superoxide anion generation.

Our previous study has demonstrated that K restriction stimulates the phosphorylation of p38 and ERK in the kidney and that superoxide anions and related products are responsible for mediating the effect of K restriction on the phosphorylation of MAPK and c-Src (1, 2). If AT₁R is involved in mediating the effect of K restriction on superoxide generation, it is conceivable that inhibiting AT₁R should attenuate the effect of K restriction on MAPK phosphorylation and c-Src expression. Thus we examined whether losartan treatment diminished the effect of K restriction on MAPK and c-Src. We confirmed our previous finding that K restriction significantly increased the phosphorylation of p38 and ERK by 80 ± 10 and 100 ± 10% (n = 3 animals), respectively (Fig. 2). However, inhibiting AT₁R has largely abolished the effect of K restriction on both p38 and ERK. Data summarized in Fig. 2, bottom, show that, after losartan treatment, K restriction increased modestly p38 and ERK phosphorylation by 12 ± 4 and 35 ± 8% (n = 3), respectively. Also, inhibiting AT₁R abolished the effect of K restriction on c-Src expression. Figure 3 is a Western blot demonstrating that K restriction increased c-Src expression in renal cortex and OM by 120 ± 15% (n = 3 animals) compared with those on a NK diet. However, in losartan-treated rats, c-Src expression was not significantly different from the control value. Thus results suggest that AT₁R is involved in...
mediating the effect of K restriction on MAPK phosphorylation and c-Src expression.

We have previously demonstrated that K restriction significantly decreased the channel activity of ROMK-like SK in the CCD and that suppressing superoxide generation with tempol abolished the inhibitory effect of K restriction on SK channels (3). If inhibiting AT1R diminished the effect of K restriction on superoxide generation, it is expected that losartan treatment should increase SK channel activity in the CCD of rats on a KD diet. Thus we used the patch-clamp technique to examine the SK channel activity in the CCD from rats on a NK or KD diet. We confirmed the previous finding that K restriction decreased the $N_P_0$ from $1.25 \pm 0.3$ to $0.55 \pm 0.16$ ($n = 17$ patches). However, the $N_P_0$ of ROMK-like SK channels in the CCD from rats treated with losartan was $1.14 \pm 0.3$ ($n = 35$) which was significantly higher than those in the CCD of nontreated rats on a KD diet (Fig. 4). Because we have previously demonstrated that the K depletion-induced decrease SK channel activity was at least partially mediated by stimulating tyrosine phosphorylation of ROMK channel (2), we speculate that inhibiting AT1R should also attenuate the effect of K

Fig. 2. Western blot showing the effect of losartan treatment on p38 (left) and extracellular signal-regulated kinase (ERK, right) phosphorylation in renal cortex and OM (mixture) from rats on a NK or KD diet. *Significant difference from the other three groups.

Fig. 3. Western blot showing the expression of c-Src in renal cortex and OM from rats on NK, NK + losartan, KD, and KD + losartan. *Significant difference from the other three groups.

Fig. 4. Effect of losartan treatment on ROMK-like small-conductance K (SK) channel activity defined by $N_P_0$, the product of channel no. ($N$) and open probability ($P_o$), in the cortical collecting duct (CCD) from rats on a NK and KD diet. *Significant difference from the other three groups.
Thus inhibiting AT1R impairs the renal ability for K conservation. To detect the tyrosine-phosphorylated ROMK proteins, we immunoprecipitated renal tissue with either 4G10, an antibody that reacts with tyrosine-phosphorylated proteins, or ROMK antibody and followed by immunoblotting with either ROMK (Fig. 4, top) or 4G10 antibodies (Fig. 4, middle). Figure 5 is a typical recording from four such experiments showing that K restriction significantly increased ROMK tyrosine phosphorylation by 95 ± 15%. Moreover, from inspection of Fig. 5, it is apparent that that effect was completely abolished by inhibiting AT1R.

Because ROMK-like SK channels are mainly responsible for K secretion in rats under a NK diet (11, 38), increase in SK channel activity in the CCD during K restriction is expected to increase urinary K loss. This speculation was tested by the metabolic cage: SD rats from the same age were divided into the following four groups: 1) control group (NK diet for 7 days), 2) NK + losartan group (NK diet plus losartan for 7 days), 3) KD group (KD diet for 7 days), and 4) KD + losartan group (KD diet and treated with losartan). Figure 6 summarizes the results of 24-h urinary K excretion in four groups after 7 days in their corresponding K diet. K restriction significantly decreased 24-h urinary K excretion from 2.37 ± 0.15 to 0.35 ± 0.03 meq/l·100 g body wt⁻¹ (n = 4). Losartan treatment has no significant effect on urinary K excretion (2.06 ± 0.35 meq/l) in rats on a NK diet but significantly increased urinary K excretion to 0.50 ± 0.03 meq/l in rats on a KD diet. Consequently, losartan-treated rats on a KD diet developed a severe hypokalemia (2.5 ± 0.2 mM) that is significantly lower than 2.9 ± 0.3 mM in nontreated rats on a KD diet (n = 4). Thus inhibiting AT1R impairs the renal ability for K conservation in response to K restriction.

**DISCUSSION**

Dietary K intake plays a key role in the regulation of renal K secretion. HK intake stimulates whereas K restriction decreases renal K secretion (19, 20, 33). The K restriction-induced decrease in renal K secretion is achieved by both inhibition of apical ROMK-like SK and BK channels in PC and by stimulation of K absorption through H-K-ATPase in intercalated cells (36, 37). Recently, a study by Lee et al. (15) demonstrated that increasing K loading with food intake in the stomach stimulates renal K secretion even if plasma K concentration remains unchanged, suggesting a possible gut factor that may be able to sense K intake and is translated in the CCD to the appropriate response to maintain K homeostasis.

Our previous study has suggested that superoxide anions and related product play an important role in suppressing renal K excretion during K restriction (2) by activating MAPKs such as p38 and ERK (8) and stimulating Src family PTK (30). Stimulation of Src family PTK has been shown to inhibit ROMK-like SK channels while activating MAPK blocks both SK and BK channels in the CCD (1, 16, 32). Also, stimulation of ERK has been shown to inhibit epithelial Na channels (ENaC) (29), hereby decreasing the driving force for K secretion. Thus superoxide anion plays a key role in suppressing renal K excretion when K intake is restricted. The role of superoxide anions in mediating the effect of K depletion on SK channels and renal K secretion is best suggested by experiments in which decreasing superoxide anion levels with tempol treatment increased SK channel activity in the CCD and urinary K loss during K restriction (2).

The main finding of the present study that inhibiting AT1R significantly attenuated the K restriction-induced increase in superoxide production strongly suggests the role of AT1R in mediating the effect of K restriction on K excretion. This notion is also supported by more additional evidence: 1) inhibiting AT1R attenuated the effect of K restriction on c-Src expression and MAPK phosphorylation, 2) the K depletion-induced decrease in ROMK channel activity is largely abolished in rats treated with losartan, and 3) inhibiting AT1R increases urinary K loss and impairs the renal ability of K conservation. The role of ANG II in the regulation of renal K excretion is also supported by micropuncture study in which luminal ANG II inhibits K secretion in the distal nephron from the distal convoluted tubule to the initial CCD (31). The finding that losartan treatment increased ROMK-like SK channel activity suggests that activation of the ANG II pathway plays an important role...
in suppressing ROMK-like SK channel activity in the CCD during K restriction. However, it is not clear whether the effect of K restriction on SK channel activity is the result of increased renal ANG II level or AT1R expression in the CCD. Further experiments such as using inhibitor of angiotensin-converting enzyme (ACE) are required to determine whether ACE inhibitor could mimic the effect of losartan. However, K restriction has been shown to stimulates renin and ANG II signaling pathway, as evidenced by high plasma renin activity (25–27). It has been suggested that the renin-ANG II pathway may be involved in effecting K conservation and elimination (27). Thus increasing the renin-ANG II signaling pathway is expected to be at least partially responsible for decreasing ROMK-like SK channel activity in the CCD during K restriction. Although angiotensinogen is mainly produced in the liver, proximal tubules have been reported to express angiotensinogen mRNA and protein (13, 14). Moreover, renin has also been identified in the distal nephron, including the collecting duct (23). Because ACE activity is present in tubular fluid along the nephron (6), it is highly likely that angiotensinogen generated in the proximal tubule could be converted to ANG II in the renal tubule and affect the transport in the CCD. A large body of evidence indicates that ANG II plays an important role in the regulation renal membrane transport in the distal nephron segments (4, 21, 22, 34, 35). ANG II has been shown to stimulate ENaC (22) and increase α-ENaC protein abundance (4). Also, ANG II has been reported to stimulate Cl absorption in the CCD of mice through a pendria-dependent mechanism (21). Thus the present experiments provide another piece of evidence supporting the role of ANG II in the regulation of membrane transport in the CCD.

The mechanism by which K restriction stimulates AT1R is not completely understood; however, we speculate that K restriction-induced activation of the renin-angiotensin system is at least partially responsible for the effect. It is well known that dietary K intake regulates renin-ANG II signaling; high-K intake suppresses (26, 27) whereas low-K intake stimulates the renin and ANG II signaling pathway, as evidenced by high plasma renin activity (25–27). It has been suggested that the renin-ANG II pathway may be involved in effecting K conservation and elimination (27). Indeed, it has been reported that inhibiting ACE slightly increased renal K excretion in adrenalectomized rats fed on a low-K diet (18) and that inhibition of AT1R has been reported to decrease plasma K in patients with congestive heart failure (5). It is possible that a high ANG II level stimulates AT1R and increases superoxide production by activating NOX. The mechanism by which stimulation of AT1R increases superoxide production in the CCD is not explored. Stimulating AT1R has been demonstrated to stimulate NOX and increases superoxide production in vascular smooth muscle (24). It is generally believed that superoxide anions generated by NOX are a major source of reactive oxygen species in a variety of tissues (9, 12). A large body of evidence indicates that NOXII and NOXIV are expressed in the connecting tubule (CNT) and CCD, it is possible that NOXII could be activated by low-K intake and is partially responsible for increasing production of superoxide anions in the CNT and CCD in response to K restriction. This notion is suggested by experiments in which the K restriction-induced increase in superoxide anion level was significantly diminished in gp91phox null mice (3). Also, K restriction caused a significantly higher urinary K loss in gp91phox null mice than those in wild-type mice (3). This indicates that gp91phox-containing NOXII plays a role in mediating the effect of low-K intake on renal K excretion.

Although ANG II inhibits ROMK-like SK channels in the CCD, ANG II may not affect the net renal K excretion when dietary K intake is normal. ANG II has opposite effects on Na and K transport in the CCD. Thus ANG II-induced stimulation of ENaC would increase the driving force for K secretion and offset the ANG II-induced inhibition of SK channels in the CCD. On the other hand, inhibiting renin-ANG II is expected to decrease Na transport and the driving force for K secretion. Thus, although inhibiting AT1R is expected to stimulate ROMK-like SK channel activity, application of losartan in clinical practice is more likely to cause hyperkalemia rather than hypokalemia under normal K intake. However, the ANG II-induced inhibition of K secretion may play an important role in K conservation during K restriction. Therefore, application of losartan in patients with low-K intake could lead to hyperkalemia. Indeed, it has been reported that inhibiting ACE slightly increased renal K excretion in adrenalectomized rats fed on a low-K diet (18). In summary, we have demonstrated that inhibiting AT1R attenuates the effect of K restriction on superoxide generation, c-Src expression, and MAPK phosphorylation and that the renal ability of K conservation in response to K depletion is compromised in rats treated with losartan.
conclude that AT1R is involved in mediating the inhibitory effect of K restriction on renal K excretion by stimulating superoxide generation.

REFERENCES