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

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Jin Y, Wang Y, Wang Z, Lin D, Wang W. Inhibition of angiotensin type 1 receptor impairs renal ability of K conservation in response to K restriction. Am J Physiol Renal Physiol 296: F1179–F1184, 2009. First published February 11, 2009; doi:10.1152/ajprenal.90725.2008.—We previously demonstrated that ANG II inhibits ROMK-like small-conductance K channels (SK) in the cortical collecting duct from rats on a K-deficient diet (KD) (35). In the present study, we examined the role of angiotensin type 1 receptor (AT1R) in mediating the effect of K restriction on K secretion. We confirmed the previous finding that K restriction increased the superoxide anion level, c-Src expression, and the phosphorylation of both p38 and extracellular signal-regulated kinase mitogen-activated protein kinase (MAPK) in renal cortex and outer medulla. However, the effect of K restriction on superoxide anion generation, c-Src expression, and MAPK phosphorylation was significantly attenuated in rats receiving losartan, an inhibitor of AT1R. In contrast, losartan treatment had no effect on superoxide anion level, c-Src expression, and MAPK phosphorylation in animals on a normal K diet (NK). K restriction decreased SK channel activity and increased the tyrosine phosphorylation of ROMK. However, inhibiting AT1R abolished the effect of K restriction on SK channels and tyrosine phosphorylation of ROMK channels. The notion that AT1R is involved in regulating renal K excretion was also supported by the experiments with metabolic cages showing that losartan treatment significantly enhanced urinary K loss in rats on a KD diet while it had no effect in animals on a NK diet. Consequently, losartan-treated animals had severe hypokalemia in response to K restriction compared with rats without losartan intake. We conclude that AT1R is involved in mediating the effect of K restriction on superoxide generation, c-Src, and MAPK and that inhibiting AT1R impairs renal ability of K conservation in response to K depletion.

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equilibrated MOPS-sucrose buffer (pH = 7.4) containing 5 μM lucigenin. The chemiluminescence elicited in the presence of lucigenin was measured in a liquid scintillation counter with a single active photomultiplier tube positioned in out-of-coincidence mode. Blanks were subtracted from the average level of chemiluminescence signal.

**Immunoprecipitation and Western blot.** The renal cortex and the OM were suspended in RIPA buffer solution (1:8 ratio, wt/vol) containing 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxylolate, 0.1% SDS, 10 μl of phenylmethylsulfonyl fluoride (PMSF; 10 mg/ml stock solution in isopropanol), and 10 μl of a cocktail of protease inhibitors (Sigma, St. Louis, MO) were added per ml of buffer at the time of lysis. The samples were homogenized on ice for 15 min with a mortar and pestle. The suspension was incubated at 4°C for 1 h in the presence of DNase (5 μg/ml) followed by centrifugation at 1,800 revolutions/min (rpm) for 10 min, and the resultant supernatant was collected. Protein concentrations were measured in duplicate using a Bio-Rad D, protein assay kit.

For immunoprecipitation, the corresponding antibody was added to the protein samples (500 μg) at a ratio of 5 μl/ml of solution. The mixture was gently rotated at 4°C overnight, followed by incubation with 25 μl protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 2 h at 4°C. The tube containing the mixture was centrifuged at 3,000 rpm and washed twice with PBS containing 10 μl/ml PMSF and 10 μl of protease inhibitor cocktail per milliliter. The agarose was resuspended in 25 μl 2× SDS sample buffer containing 4% SDS, 100 mM Tris·HCl (pH 6.8), 20% glycerol, 200 mM dithiothreitol, and 0.2% bromphenol blue. After the sample was boiled for 5 min, proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). For Western blot, the membrane was blocked with Odyssey blocking buffer and incubated with the primary antibody at 4°C for 12 h. The membrane was washed four times for 5 min with PBS containing 0.1% Tween 20 and followed by incubation with the secondary antibody for an additional 30 min. The membrane was then washed several times and scanned by an Odyssey infrared imaging system (LI-COR, Lincoln, NE) at a wavelength of 700–800 channel.

**Preparation of CCDs for patch clamping.** Single CCDs were isolated, placed on a 5 × 5 mm cover glass coated with polylysine and transferred to a chamber (1,000 μl) and open probability (P_o) channel number (N) from data samples of 60 s duration in the steady state as follows:

\[ N P_o = \sum (t_1 + t_2 + \cdots + t) \]

where \( t_i \) is the fractional open time spent at each of the observed current levels.

**Experimental materials and statistics.** Antibodies to phospho-p38, p38, phospho-ERK, ERK, and c-Src were purchased from Santa Cruz. The antibody of 4G10 and ROMK was obtained from Upstae (Lake Placid, NY) and Alomone Laboratories (Jerusalem, Israel), respectively. The data are presented as means ± SE. We used paired Student’s t-test to determine the statistical significance. If the P value is <0.05, the difference is considered to be significant.

**RESULTS**

To determine the role of AT1R in mediating the effect of K restriction on superoxide anion generation, we used lucigenin methods (2) to measure the superoxide anion level in renal cortex and OM from rats on a NK (1.1%) 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 on a NK diet (Fig. 1) (2). 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 expression of c-Src and enhances the phosphorylation of p38 and ERK MAPK 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% \((n = 3)\), 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% \((n = 3)\) compared with those on a NK diet. However, 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 $NP_o$ from 1.25 ± 0.3 to 0.55 ± 0.16 ($n = 17$ patches). However, the $NP_o$ of ROMK-like SK channels in the CCD from rats treated with losartan was 1.14 ± 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 restriction on SK channel activity.

![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.](http://ajprenal.physiology.org/)

![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.](http://ajprenal.physiology.org/)

![Effect of losartan treatment on ROMK-like small-conductance K (SK) channel activity defined by $NP_o$, 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.](http://ajprenal.physiology.org/)
Thus inhibiting AT$_1$R impairs the renal ability for K conservation. This was examined by K excretion in rats on a NK diet but significantly increased urinary 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 AT$_1$R significantly attenuated the K restriction-induced increase in superoxide production strongly suggests the role of AT$_1$R in mediating the effect of K restriction on K excretion. This notion is also supported by more additional evidence: 1) inhibiting AT$_1$R 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 AT$_1$R 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 regulating renal K conservation.
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 kidney (7, 28). Because NOXII is 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 gp91 phox 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 hypokalemia. 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.

GRANTS

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REFERENCES


