Angiotensin II type 1 receptor blockade ameliorates tubulointerstitial injury induced by chronic potassium deficiency

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Potassium (K$^+$) deficiency is one of the commonly encountered electrolyte abnormalities, occurring most frequently in patients who use diuretics, or who have vomiting, diarrhea or undergo nasogastric suction. The renal manifestations of K$^+$ deficiency are diverse and include functional changes such as increased ammoniagenesis, metabolic alkalosis, nephrogenic diabetes insipidus, chloride wasting, sodium retention [1], and structural changes consisting of renal hypertrophy and tubulointerstitial injury [2, 3]. K$^+$ deficiency also is associated with intrarenal hemodynamic changes, including decreased renal blood flow and increased renal vasoconstriction with only a minor reduction in glomerular filtration rate (GFR), for which angiotensin (Ang) II and thromboxane A$_2$ have been postulated as possible mediators [4, 5].

Despite the frequency with which K$^+$ deficiency occurs, little is known about the intrarenal events that lead to these structural changes. Since the existence of chronic hypoxia and/or ischemia is regarded as one of the key events in the progression of chronic tubulointerstitial injury [6], we recently hypothesized that chronic K$^+$ deficiency could induce alterations in local vasoactive mediators favoring vasoconstriction that could result in intrarenal ischemia and subsequent tubulointerstitial injury [7]. By analyzing chronic hypokalemic rats, we documented alterations in vasoactive mediators including increased cortical angiotensin-converting enzyme (ACE) expression and unsuppressed cortical Ang II generation despite systemic suppression of the renin-angiotensin system (RAS), suggesting the activation of RAS in the cortex, an increase in renal endothelin-1, a decrease in renal kallikrein, and a decrease in urinary nitrate/nitrate [7]. In the present study, we tested the hypothesis that K$^+$ deficiency–induced renal injury could be mediated by Ang II or a reduction in nitric oxide (NO).
METHODS

Experimental protocol

Studies were designed to examine the role of Ang II and NO in the pathogenesis of hypokalemic tubulointerstitial injury. Male Sprague-Dawley rats (230 to 270 g, N = 24; Simonsen Labs, Gilroy, CA, USA) were divided into four groups (N = 6, in each) and fed either a K+-deficient diet (0.01% K+, 0.26% NaCl; Zeigler Brothers, Gardners, PA, USA) or a diet with normal K+ content (0.36% K+, 0.26% NaCl) for 10 weeks.

Group I, LK. Rats on a K+-deficient diet alone with no additives in the drinking water.

Group II, LK + losartan (Los). Rats on a K+-deficient diet were treated with the Ang II type 1 receptor (AT1) antagonist, losartan (Merck and Co., Inc., West Point, PA, USA), at a dose of 10 mg/kg per day in the drinking water from the beginning of the K+-deficient diet for 10 weeks. Water intake was measured three times a week so that the concentration of losartan could be adjusted.

Group III, LK + Arg. Rats on a K+-deficient diet were treated with 1% of L-arginine (L-Arg; Sigma Chemical Co., St. Louis, MO, USA) in the drinking water from the beginning of the K+-deficient diet for 10 weeks.

Group IV, NK. Rats on a normal K+ diet with no additives in the drinking water. Since we previously showed that losartan and L-Arg treatment in control rats did not induce significant renal histological changes [8], these control groups were omitted in the present study.

At week 10, animals were housed separately in metabolic cages and urine was collected for 16 hours. Blood was drawn from the tail vein on a different day of the same week. At the end of week 10, rats were sacrificed and kidneys were excised.

Renal histologic studies

Methyl Carnoy’s fixed tissue was processed and paraffin embedded, and 4-μm sections were stained with the periodic acid-Schiff reagent (PAS). An indirect immunoperoxidase method was used to identify the following antigens [7]: osteopontin with OP 199, a goat anti-rat osteopontin antibody (gift of C. Giachelli, University of Washington, Seattle, WA, USA); macrophages with ED-1, a monoclonal IgG1 to rat macrophages (Harlan Bioproducts, Indianapolis, IN, USA) and type III collagen with a goat anti-human type III collagen antibody (Southern Biotechnology Associates, Birmingham, AL, USA).

Several parameters were used to evaluate tubulointerstitial injury. The first method was a blinded semiquantitative scoring system (0 through 5) of PAS-stained sections based on the presence of tubular cellularity, basement membrane thickening, dilation, atrophy, sloughing or interstitial widening as follows [7]: 0, no changes present; grade 1, <10% tubulointerstitial changes present; grade 2, 10–25% tubulointerstitial involvement; grade 3, 25–50% tubulointerstitial involvement; grade 4, 50–75% tubulointerstitial involvement; and grade 5, 75–100% tubulointerstitial involvement. For each biopsy, the entire cortical and outer medullary regions were evaluated and a mean score per biopsy was calculated. The second method was to measure a percent area occupied by osteopontin-positive tubules, based on observations that osteopontin expression by injured tubules is a sensitive marker of tubulointerstitial injury [9]. Using computer-assisted image analysis software (Optimas, v 6.2; Media Cybernetics, Silver Springs, MD, USA) and digitized images, the percent area occupied by osteopontin-positive tubules (including the entire cortical and outer medullary regions, exclusive of glomeruli) was measured per field (4 mm²) at ×25 and the mean percent area was calculated for each biopsy. We also measured interstitial fibrosis by the percent area of type III collagen positive interstitium, obtained by the same analytical method at ×25. In addition, the number of macrophages (ED-1 positive cells/mm²) in the cortex and medulla was quantified at ×50.

Urinary nitrite/nitrate assay

The urinary concentration of nitrite/nitrate, stable end products of NO, was measured as previously reported [7]. Briefly, urine samples were first incubated with Aspergillus nitrate reductase (Sigma Chemical Co.) in the presence of NADPH for one hour to convert nitrate in the samples to nitrite. After the incubation, the total nitrite content was measured using the Griess reagent following the manufacturer’s instruction (Clontech, Palo Alto, CA, USA).

EIA for PGE₂

The urinary concentration of prostaglandin E₂ (PGE₂) was measured using commercial enzyme immunoassay kit (EIA kit; Cayman Chemical, Ann Arbor, MI, USA). The cross-reactivity to PGE₃, PGE₁ and 6-keto-PGF₁α was 43%, 19% and 1%, on a molar basis, respectively.

Additional measurements

Serum and urinary creatinine and K+ concentrations were measured by Cobas autoanalyzer (Roche Diagnostics, Nutley, NJ, USA).

Statistical methods

Values are expressed as mean ± SE. A comparison between groups was made by ANOVA with the Fisher’s protected least significant difference test for multiple comparisons.
RESULTS

General features

Marked hypokalemia was induced by a K⁺-deficient diet alone (2.34 ± 0.16 mEq/L). Neither losartan nor L-Arg affected the severity of hypokalemia (Table 1). K⁺-deficient rats (groups I to III) gained less body weight than control rats on a normal K⁺ diet (group IV). In contrast, kidney weights were markedly greater in K⁺-deficient rats than in controls, as already described [3]. K⁺-deficient rats had a less increase in kidney weight compared to the other two groups on the K⁺-deficient diet (groups I and III). Renal function, as assessed by creatinine clearance, decreased significantly in all three groups administered the K⁺-deficient diet. Although the difference was not significant, the K⁺-deficient rats gained a higher mean creatinine clearance than the K⁺-deficient rats (P = 0.080; Table 1).

Effects of losartan and L-Arg on hypokalemic tubulointerstitial injury

Hypokalemic rats developed prominent tubulointerstitial injury with no apparent glomerular injury. There was focal atrophy or dilation of tubules with mild interstitial accumulation of mononuclear cells and interstitial expansion in both the cortex and medulla (Fig. 1A). In the outer medulla, there was also diffuse swelling and interstitial injury with no apparent glomerular injury. There was focal atrophy or dilation of tubules with mild interstitial expansion in both the cortex and medulla (Fig. 1C). Administration of losartan significantly suppressed the increase in tubular osteopontin expression both in the cortex and medulla (Fig. 2B). The striped pattern of osteopontin positive tubules that radiated into the cortex of control K⁺-deficient rats was completely prevented by losartan. L-Arg did not improve but rather increased tubular osteopontin expression in the cortex. However, it inhibited the increase in osteopontin expression mildly but significantly in the medulla (Fig. 2C and Table 2).

The number of macrophages (ED-1 positive cells) was elevated in the cortex and medulla of hypokalemic rats (Table 2). Similar to the suppression of tubular osteopontin expression, losartan prevented the cortical macrophage infiltration and reduced the accumulation of macrophages in the medulla. Macrophage infiltration was not affected in the cortex of L-Arg treated rats, although some reduction was observed in the medulla (Table 2).

An increase in the deposition of type III collagen was present in the cortical and medullary interstitium of hypokalemic rats (Fig. 3A and Table 2). Losartan ameliorated the collagen deposition significantly in both the cortex and medulla (Fig. 3B), whereas L-Arg had no significant effect (Fig. 3C and Table 2).

Effects of losartan and L-Arg on hypokalemia-induced alteration in vasoactive mediators

Urinary nitrite/nitrate excretion was reduced by 70% in K⁺-deficient rats at week 10 (Table 3). The decrease in urinary nitrite/nitrate was partially recovered by the administration of losartan and fully restored by L-Arg. We also measured urinary excretion of PGE₂, a potent vasodilator produced in the outer medulla. A reduction of urinary PGE₂ (Table 3) was observed at week 10, being consistent with previous reports of chronic K⁺-deficiency [7, 10]. Neither losartan nor L-Arg had any significant effect on the decrease in urinary PGE₂ excretion (Table 3).

DISCUSSION

Chronic K⁺ deficiency in both experimental animals and humans causes renal enlargement consisting of hypertrophy and hyperplasia of tubular cells in the outer
medulla as well as tubular injury, mononuclear cell infiltration and interstitial fibrosis [2, 3]. Although attention has been paid to the mechanisms of renal hypertrophy [11, 12], tubulointerstitial fibrosis may be more important clinically, because the severity of tubulointerstitial fibrosis strongly correlates with renal function in various forms of human chronic renal diseases including hypokalemic nephropathy [2].

The pathogenesis of hypokalemic nephropathy is not completely understood. Tolins, Hostetter and Hostetter suggested that injury might be secondary to intrarenal complement activation from amidation of C3 as a consequence of increased ammonium generation [13]. However, the significance of intrarenal ammonium generation in hypokalemia has been debated [14]. Recently we reported that chronic hypokalemia alters intrarenal vasoactive mediators favoring renal vasoconstriction [7]. While renin is stimulated with acute hypokalemia [15, 16], we found that prolonged hypokalemia is associated with suppression of plasma and medullary Ang II [7]. However, in the cortex there is continued Ang II generation in association with up-regulation of ACE at sites of tubulointerstitial injury. In addition to persistent generation of cortical Ang II, there was increased production of endothelin-1 in both the cortex and medulla, a reduction in renal kallikrein, and a reduction in urinary nitrite/nitrate (NO metabolites) and prostaglandin E2 excretion.

This study examined the effects of blocking Ang II with an AT1 antagonist in the chronic hypokalemia model. We also examined whether stimulating nitric oxide production with L-Arg would confer histologic benefit.

Rats on a K⁺-deficient diet for 10 weeks developed typical renal enlargement in the outer medulla and tubulointerstitial injury with tubular atrophy and dilation, interstitial widening, accumulation of type III collagen and an infiltration of ED-1 positive cells. The first major finding was that AT1 blockade by losartan significantly blunted renal injury, as reflected by a reduction in tubular osteopontin expression, macrophage infiltration and type III collagen deposition in the cortex and medulla. Tubulointerstitial injury was almost completely prevented in the cortex, whereas the improvement was less in the medulla. We cannot rule out the possibility that higher doses of losartan might prevent medullary injury more effectively, because it is postulated that Ang II antagonists in excess of antihypertensive doses may provide additional renoprotective effects [17]. However, the dose of losartan we selected was the same that was used to protect against renal injury in previous studies of the SHR and in rats treated with cyclosporine A [8, 18]. Thus, our finding suggests that locally generated Ang II could be a key factor in the development of cortical tubulointerstitial injury, while other factors also may play an important role in the medulla. This possibility is supported by our previous observation that ACE expression is up-regulated and Ang II generation maintained in the cortex of hypokalemic rats, whereas Ang II levels fall in the outer medulla and plasma [7].

There are several potential mechanisms by which losartan could prevent tubulointerstitial injury in chronic K⁺-deficiency. First, losartan can protect against intrarenal hypoxia that has been postulated as a key factor for the progression of chronic tubulointerstitial injury [6]. Linas and Dickmann reported that ACE inhibitors block hypokalemia-induced renal vasoconstriction [4], and losartan could prevent tubulointerstitial injury in chronic K⁺-deficiency.

**Table 2. Renal histologic findings in hypokalemic rats**

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<tr>
<td></td>
<td>LK</td>
<td>LK + Los</td>
<td>LK + Arg</td>
<td>NK</td>
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<tr>
<td>Cortical tubulointerstitial injury score</td>
<td>0.55 ± 0.1⁺</td>
<td>0.32 ± 0.03⁺</td>
<td>0.75 ± 0.1⁺</td>
<td>0.19 ± 0.05</td>
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<td>Medullary tubulointerstitial injury score</td>
<td>4.1 ± 0.2⁺</td>
<td>3.6 ± 0.2⁺</td>
<td>4.1 ± 0.2⁺</td>
<td>1.2 ± 0.2</td>
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<td>Cortical osteopontin %</td>
<td>1.7 ± 0.3⁺</td>
<td>0.49 ± 0.15⁺</td>
<td>2.8 ± 0.4⁺</td>
<td>0.33 ± 0.05</td>
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<td>Medullary osteopontin %</td>
<td>7.4 ± 0.5⁺</td>
<td>3.4 ± 0.8⁺</td>
<td>5.3 ± 0.7⁺</td>
<td>1.6 ± 0.1</td>
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<td>Cortical macrophages (ED-1 positive cells) mm²</td>
<td>77 ± 9°</td>
<td>43 ± 13°</td>
<td>73 ± 10°</td>
<td>36 ± 5</td>
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<tr>
<td>Medullary macrophages (ED-1 positive cells) mm²</td>
<td>317 ± 26°</td>
<td>206 ± 22°</td>
<td>247 ± 24°</td>
<td>46 ± 9</td>
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<td>% Area of type III collagen deposition in the cortex</td>
<td>3.4 ± 0.7⁺</td>
<td>2.0 ± 0.4⁺</td>
<td>2.9 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>% Area of type III collagen deposition in the medulla</td>
<td>5.0 ± 1.0⁺</td>
<td>2.8 ± 0.6⁺</td>
<td>3.7 ± 0.6</td>
<td>2.7 ± 0.8</td>
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⁺ P < 0.05 vs. NK

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**Fig. 1. Effects of losartan and L-arginine (L-Arg) on hypokalemic tubulointerstitial injury.** In contrast to control rats on a normal K⁺ diet (D), K⁺-deficient rats developed focal tubular atrophy, dilation, mononuclear cell infiltration and interstitial widening (arrow) in the cortex (A), which was mostly abolished in K⁺-deficient rats with losartan (B), but not in K⁺-deficient rats with L-Arg (C). There was no apparent difference in the severity of collecting duct hyperplasia, interstitial widening, and eosinophilic granule deposition in the medulla among K⁺-deficient rats (E), K⁺-deficient rats with losartan (F), and K⁺-deficient rats with L-Arg (G). Rats on a normal K⁺ diet showed no apparent tubulointerstitial injury (H). PAS staining, ×100.
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Fig. 2. Effects of losartan and L-Arg on tubular expression of osteopontin. In the cortex of K⁺-deficient rats, osteopontin was detected in tubules at the site of injury and often appeared as a ‘striped’ pattern radiating into the cortex from the medullary ray. Tubular expression of osteopontin also increased in the medulla (A, ×25). In the outer medulla, osteopontin was mainly detected in non-hyperplastic tubules. Losartan significantly suppressed elevation of osteopontin expression in the cortex and medulla (B, ×25). L-Arg did not ameliorate osteopontin expression in the cortex. However, it inhibited tubular osteopontin expression in the medulla (C, ×25). Rats on a normal K⁺ diet only showed weak focal tubular staining of osteopontin (D, ×25).

sartan may act similarly to increase renal blood flow under low K⁺ dietary conditions. Since AT1 blockade can restore impaired endothelial NO-mediated renal vasodilation in rats with experimental congestive heart failure [19], losartan might also improve renal blood flow by the same mechanism. The recovery of urinary nitrite/nitrate excretion in losartan treated rats is consistent with this possibility. Besides hemodynamic effects, losartan may antagonize nonhemodynamic effects of Ang II, such as the stimulation of tubular expression of osteopontin, which is a chemoattractant for macrophages [9], or the stimulation of transforming growth factor-β (TGF-β) synthesis that could promote local fibrosis and tubular hypertrophy [20]. Recently, Hsu et al reported that TGF-β was up-regulated in hypertrophied TALH in chronic K⁺-deficiency [21]. Interestingly, this is the segment where osteopontin expression also is increased.

Since TGF-β stimulates osteopontin production in tubular epithelial cells [22], K⁺-deficiency might stimulate osteopontin expression by an Ang II–TGF-β cascade. Conversely, osteopontin mediates macrophage recruitment that can lead to increased local TGF-β expression. Indeed, OPN knockout mice have less TGF-β expression and fibrosis in the ureteral obstruction model [23].

We also examined the effect of L-Arg on hypokalemic tubulointerstitial injury. Although L-Arg administration completely restored the decrease in urinary nitrite/nitrate excretion in hypokalemic rats, the improvement of tubulointerstitial injury was less than that observed with losartan. While there was mild but significant suppression of the tubular osteopontin expression and macrophage infiltration in the medulla, no significant improvement of parameters was observed in the cortex and the tubular osteopontin expression was actually exacer-
Fig. 3. Effects of losartan and L-Arg on type III collagen deposition. There was an increase in interstitial accumulation of type III collagen in K⁺-deficient rats (A, ×25). Losartan inhibited the collagen accumulation (B, ×25), but the effect of L-Arg was not significant (C, ×25). Panel D shows the result of rats on a normal K⁺ diet (×25).

Table 3. Hypokalemia-induced alterations in vasoactive mediators

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<td>LK + Arg</td>
<td>NK</td>
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<tr>
<td>Urinary nitrite/nitrate nmol/day</td>
<td>0.77 ± 0.16ᵃ</td>
<td>1.6 ± 0.3ᵇ</td>
<td>2.6 ± 0.4ᵇ</td>
<td>2.7 ± 0.8</td>
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<tr>
<td>Urinary prostaglandin E₂ ng/day</td>
<td>22 ± 3ᵃ</td>
<td>20 ± 4ᵇ</td>
<td>17 ± 4ᵇ</td>
<td>33 ± 4</td>
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ᵃ P < 0.05 vs. NK  
b P < 0.05 vs. LK

bated. The inability of L-Arg to improve tubulointerstitial injury in the cortex of hypokalemic rats may reflect dichotomous effects of NO on renal diseases [24]. Thus, studies suggest that stimulating NO synthesis with L-Arg can ameliorate renal injury in several experimental models, including the remnant kidney model [25, 26] and a model of thrombotic microangiopathy [27]. In these models it is thought that the benefit relates to stimulation of endothelial nitric oxide synthase (eNOS) with its pro-survival effects on the vascular endothelium and stimulation of blood flow [24]. In contrast, stimulation of the inducible NOS (iNOS) in macrophages is thought to increase local cytotoxicity and may augment renal injury, such as in the Thy 1 model [28]. Taken together with our observation that some of the parameters of tubulointerstitial injury improved in the medulla, but were exacerbated in the cortex of hypokalemic rats with L-Arg, it is tempting to speculate that L-Arg administration
might stimulate iNOS more in the cortex and lead to tubular injury, while an eNOS-dependent, tissue protective pathway may be predominant in the medulla in K⁺-deficient rats. Alternatively, stimulation of NO may serve to antagonize hypokalemia-induced intrarenal vasoconstriction preferentially in the renal medulla, resulting in improvement of renal injury in the medulla of hypokalemic rats treated with L-Arg. Further studies with selective activation or inhibition of each NOS isoform are necessary to clarify the roles of NO in hypokalemic nephropathy.

In conclusion, chronic K⁺-deficiency–induced tubulointerstitial injury is mediated, at least in part, by Ang II via the AT1 receptor. NO production may have some protective role in the medulla of hypokalemic rats. These studies suggest that AT1 blockade may be beneficial in the prevention of hypokalemic tubulointerstitial injury.

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