Endothelin A Receptor Blockade and Endothelin B Receptor Blockade Improve Hypokalemic Nephropathy by Different Mechanisms

SHIN-ICHI SUGA,* NAOMI YASUI,* FUMIKI YOSHIHARA,† TAKESHI HORIO,† YUHEI KAWANO,† KENJI KANGAWA,* and RICHARD J. JOHNSON‡

*National Cardiovascular Center Research Institute, †Department of Medicine, National Cardiovascular Center, Suita, Osaka, Japan; and ‡Baylor College of Medicine, Houston, Texas.

Abstract. Hypokalemia causes renal tubulointerstitial injury with an elevation in renal endothelin-1 (ET-1). It was hypothesized that hypokalemic tubulointerstitial injury is ameliorated by the blockade of ET-A receptors (ETA), whereas ET-B receptor (ETB) antagonism may exacerbate the injury, because ETB is thought to mediate vasodilation. Rats were fed a K⁺-deficient diet alone (LC) or with an ETA-selective antagonist ABT-627 (LA) or an ETB-selective antagonist A-192621 (LB) for 8 wk. Control rats were on a normal K⁺ diet alone or with the ETA-selective or ETB-selective antagonists. The severity of hypokalemia was not significantly different among LA, LB, and LC. LC developed tubulointerstitial injury with an elevation of renal preproET-1 mRNA level. There was an increase in tubular osteopontin expression, macrophage infiltration, collagen accumulation, and tubular cell hyperplasia. ETA blockade significantly ameliorated all parameters for renal injury in the cortex without suppressing local ET-1 and ETA expression. By contrast, ETB blockade significantly reduced local ET-1 and ETA expression and improved the injury to a similar extent in the cortex. In the medulla, ETA or ETB blockade only partially blocked renal injury. ETA blockade did not affect BP in normokalemic or hypokalemic rats. ETB blockade induced a BP elevation with a decrease in urinary Na⁺ excretion in normokalemic but not in hypokalemic rats. These results indicate that ET-1 can mediate hypokalemic renal injury in two different ways: by directly stimulating ETA and by locally promoting endogenous ET-1 production via ETB. Thus, ETA as well as ETB blockade may be renoprotective in hypokalemic nephropathy.

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide originally isolated from endothelial cells, but it has since been shown to also be produced by non-endothelial cells, including renal epithelial cells and mesangial cells. ET-1 exerts its biologic actions through the activation of two different receptor isoforms. Whereas ET-A receptors (ETA) mediate vasostriction, mononuclear cell infiltration, and production of matrix proteins (1), ET-B receptors (ETB) on endothelial cells mediate endothelium-dependent vasorelaxation via nitric oxide (NO) and prostacyclin formation (2). Several other functions are also attributed to ETB, including regulation of renal sodium (Na⁺) and water excretion (3) and stimulation of ET-1 gene expression (4). Taking these biologic actions into consideration, it is not surprising to regard ET-1 as one of the important mediators for the progression of chronic renal injury (5,6). Urinary excretion of ET-1 as well as preproET-1 mRNA expression in mononuclear cells increases in patients with glomerulonephritis (7,8). Renal ET-1 levels and/or preproET-1 mRNA levels are upregulated in several experimental renal disease models of immune and nonimmune origins (5). The significance of ET-1 has been strengthened by the observation that glomerular and tubulointerstitial injury develop in preproET-1 transgenic mice despite being normotensive (9).

Chronic hypokalemia is known to cause renal injury consisting of renal hypertrophy and tubulointerstitial fibrosis (10,11). Although the injury is often reversible, prolonged hypokalemia will result in irreversible damage that leads to end-stage renal disease, despite the correction of the potassium (K⁺)-deficiency (12). Recently, we demonstrated that chronic hypokalemia induces alterations in intrarenal vasoactive mediators, including an increase in renal ET-1 content (13). In the present study, therefore, we tested the hypothesis that chronic hypokalemia induces renal tubulointerstitial injury via the activation of ETA pathway, whereas the ETB pathway was hypothesized to provide renoprotective effects through its vasodilating properties.

Materials and Methods

Experimental Protocol

Studies were designed to examine roles of ET-1 in the pathogenesis of K⁺-deficient renal injury. Male Sprague-Dawley rats (230 to 270 g, n = 57; Keari Co., Osaka, Japan) were divided into six groups (n =...
8 to 11 per group) and fed either a K\(^+\)-deficient diet (0.01% K\(^+\), 0.26% NaCl; Oriental Bio Co., Kyoto, Japan) or a diet with normal K\(^+\) content (0.36% K\(^+\), 0.26% NaCl) for 8 wk. The content of nitrite/nitrate in the diets was essentially the same (0.071 μmol/g in the normal K\(^+\) diet and 0.073 μmol/g in the K\(^+\)-deficient diet).

**Group LA.** Rats on the K\(^+\)-deficient diet with an ETA-selective antagonist (ABT-627; Abbott Laboratories, Abbott Park, IL). ABT-627 was mixed with the K\(^+\)-deficient diet and administered at a dose of 10 mg/kg per day for 8 wk.

**Group LB.** Rats on the K\(^+\)-deficient diet with an ETB-selective antagonist (A-192621; Abbott). A-192621 was mixed with the K\(^+\)-deficient diet and administered at a dose of 30 mg/kg per day for 8 wk.

**Group LC.** Rats on the K\(^+\)-deficient diet alone.

**Group NA.** Rats on the normal K\(^+\) diet with ABT-627 at a dose of 10 mg/kg per day for 8 wk.

**Group NB.** Rats on the normal K\(^+\) diet with A-192621 at a dose of 30 mg/kg per day for 8 wk.

**Group NC.** Rats on the normal K\(^+\) diet alone.

The dose of ABT-627 was reported to inhibit ETA-mediated sustained vasoconstriction to ET-1 without affecting ETB-mediated transient vasodilation in vivo (14), whereas that of A-192621 was the smallest dose that completely blocked ET-1-induced transient vasodilation without affecting sustained vasoconstriction (15). Food intake was measured at least four times a week so that the dose of the antagonists could be adjusted. Chronic oral administration of ABT-627 and A-192621 has been shown effective in blocking ETA and ETB in vivo, respectively (16,17).

At weeks 4 and 8, animals were housed separately in metabolic cages and urine was collected for 16 h. Blood was drawn from the tail vein. At the end of week 8, rats were sacrificed and kidneys were excised.

**BP Measurements**

Systolic arterial BP and heart rate measurements were performed in conscious, restrained rats, using an automated system with a photoelectric sensor (BP-98A; Softron, Tokyo, Japan), the results from which have been shown to closely correlate with intraarterial measurements (18). After three times of preconditioning, rats were placed in pre-warmed chambers (35 to 36°C) for 5 to 10 min, and pressure and pulse were measured by an automatic tail cuff inflator and a built-in transducer. The mean of three BP readings was used (13).

**Renal Histologic Studies**

Kidneys were fixed with Methyl Carnoy or 10% buffered formalin, 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 (13): osteopontin with OP 199, a goat anti-rat osteopontin antibody (gift of C. Giachelli, University of Washington, Seattle, WA); macrophages with ED-1, a monoclonal IgG1 to rat macrophages (Harlan Bioproducts, Indianapolis, IN); type III collagen with a goat anti-human type III collagen antibody (Southern Biotechnology Associates, Birmingham, AL); and proliferating cell nuclear antigen (PCNA) with a monoclonal IgG1 to rat PCNA (MBL, Nagoya, Japan).

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 hypercellularity, basement membrane thickening, dilatation, atrophy, sloughing, or interstitial widening as follows (13): grade 0, no changes present; grade 1, <10% tubulointerstitial changes present; grade 2, 10 to 25% tubulointerstitial involvement; grade 3, 25 to 50% tubulointerstitial involvement; grade 4, 50 to 75% tubulointerstitial involvement; and grade 5, 75 to 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 (19). Using computer-assisted image analysis software (Optimas, v. 6.2; Media Cybernetics, Silver Spring, MD) 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 mm\(^2\)) at 25x, 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 25x. In addition, the number of macrophages (ED-1–positive cells/mm\(^2\)) in the cortex and medulla was quantified at 50x.

To evaluate the implication of ET-1 in hypokalemia-induced renal tubular hyperplasia, the number of PCNA-positive tubular cells in the cortex and outer medulla was quantified at 200x.

**Determination of Renal Endothelin Concentration**

Renal ET-1 level was measured as we reported (13). In brief, cortices and medullae were boiled in 10 volumes of 1 M acetic acid for 7 min and homogenized with a polytron homogenizer (Kinematica AG, Luzern, Switzerland). The homogenate was centrifuged at 10,000 x g for 10 min at 4°C. Measurement of ET-1 concentration in the supernatants was performed using a specific RIA for ET-1 (20). The cross-reactivity to ET-2, ET-3, and big ET-1 was 80%, 20%, and 80% on a molar basis, respectively.

**Determination of PreproET-1 and ET Receptor mRNA Levels**

Total RNA was isolated from dissected renal cortices and medullae from six rats of each group using Trizol (Invitrogen Co., Carlsbad, CA). Two micrograms of total RNA was reverse-transcribed using Ready-To-Go (Amer sham Pharmacia Biotech Inc., Piscataway, NJ), and the resulting cDNA was subjected to real-time quantitative RT-PCR using a Roche LightCycler System (Roche Diagnostics Co., Indianapolis, IN). The following primers for rat ETA, ETB, preproET-1, and 18S rRNA were designed: for ETA, forward 5’-GCTGTTCCCTCTACTTCA-3’, reverse 5’-GGTTCGTCTCCTGTTCTTC-3’; for ETB, forward 5’-GATGCTCAGAAGAAGATGT-3’, reverse 5’-CCAATGTAGTCCTCCCCAG-3’, for preproET-1, forward 5’-GCCATACGAACACGACG-3’, reverse 5’-GGAGGACACTCCTTCTCTCT-3’; and for 18S rRNA, forward 5’-GCTGCTCTCCATCTTCT-3’, reverse 5’-GCCATACGAAAGAAGATGT-3’, reverse 5’-CCAATGTAGTCCTCCCCAG-3’, for preproET-1, forward 5’-GCCATACGAACACGACG-3’, reverse 5’-GGAGGACACTCCTTCTCTCT-3’. The relative quantification of 18S rRNA and rat ETA, ETB, and preproET-1 mRNA was calculated using the comparative threshold cycle number for each sample fitted to a five point standard curve (LightCycler Software 3.0, Roche Diagnostics). ETA, ETB, and preproET-1 mRNA levels were normalized to 18S rRNA and related to samples derived from the cortex of normal K\(^+\) controls (group NC). Data quoted are the mean of at least two separate analyses performed in duplicate.

**Urinary Nitrite/Nitrate Assay**

The urinary concentration of nitrite/nitrate, stable end products of NO, was measured as previously reported (13). Briefly, urine samples were first incubated with 100 mU/ml Aspergillus nitrate reductase (Sigma Chemical Co., St. Louis, MO) in the presence of 20 μM
NADPH for 1 h to convert nitrate in the samples to nitrite. After the incubation, the total nitrite content was measured using the 2,3-diaminonaphthalene fluorometric assay kit following the manufacturer’s instruction (Dojin Chemical Co., Kumamoto, Japan).

Additional Measurements

Serum and urinary creatinine, Na⁺, K⁺, and protein concentrations were measured by Hitachi 7170 autoanalyzer (Hitachi Ltd., Hitachi, Japan). Urinary albumin concentration was measured with a rat albumin enzyme-linked immunosorbent assay kit (Exocell Inc., Philadelphia, PA).

Statistical Analyses

Values are expressed as mean ± SEM. A comparison among groups was made by ANOVA with the Fisher protected least significant difference test for multiple comparisons.

Results

K⁺-Deficiency Stimulated Renal Production of ET-1

Hypokalemia was induced in rats by the K⁺-deficient diet alone (LC) within 2 wk and was maintained until the end of week 8 (Table 1). Food intake was smaller in LC than in NC at week 8 (Table 1), but there was no significant difference in food intake per body weight between LC and NC (data not shown). LC gained less body weight than NC, but the kidney weights were markedly greater in LC than NC (Table 1), being consistent with the existence of hypokalemic renal hypertrophy as previously reported (11). To obtain the rationale for the consistent with the existence of hypokalemic renal hypertrophy, we first examined renal expression of preproET-1 mRNA was significantly upregulated in both the cortex and medulla of ET-1 and ETB mRNA levels in the cortex (Figure 1, A and B). Particularly, medullary ET-1 content was increased in both the cortex and medulla of LC (Figure 1, C and D), indicating an increase in local ET-1 synthesis. There was no significant difference in the expression of ETA and ETB mRNA between NC and LC in the cortex (Figure 2, A and C). However, ETA and ETB mRNA levels were significantly elevated in the medulla of LC (Figure 2, B and D).

ET Receptor Blockade Improved Hypokalemic Renal Hypertrophy

Administration of ABT-627, the ETA-selective antagonist, or A-192621, the ETB-selective antagonist, had no significant effect on the amount food intake and the severity of hypokalemia during the study period (Table 1). However, chronic administration of ABT-627 and A-192621 to the rats on the K⁺-deficient diet (LA and LB) resulted in a significant reduction of the kidney weight (Table 1). ABT-627 and A-192621 had no significant effect on the kidney weight in rats on the normal K⁺ diet (NA and NB). Renal function, as assessed by endogenous creatinine clearance, decreased significantly in LC but not in LA or LB, when compared with NC (Table 1).

Table 1. Systemic parameters in rats on the K⁺-deficient diet

<table>
<thead>
<tr>
<th>Group</th>
<th>NC</th>
<th>NA</th>
<th>NB</th>
<th>LC</th>
<th>LA</th>
<th>LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum K⁺ at 8 wk (mEq/L)</td>
<td>4.38 ± 0.13</td>
<td>4.70 ± 0.46</td>
<td>4.49 ± 0.18</td>
<td>2.15 ± 0.08ᵇ</td>
<td>2.33 ± 0.12ᵇ</td>
<td>2.55 ± 0.11ᵇ</td>
</tr>
<tr>
<td>Food intake at 8 wk (g/d)</td>
<td>25 ± 2</td>
<td>23 ± 1</td>
<td>25 ± 1</td>
<td>18 ± 1ᵇ</td>
<td>18 ± 1ᵇ</td>
<td>19 ± 1ᵇ</td>
</tr>
<tr>
<td>Body weight at 0 wk (g)</td>
<td>232 ± 4</td>
<td>238 ± 3</td>
<td>231 ± 3</td>
<td>233 ± 2</td>
<td>241 ± 3</td>
<td>237 ± 4</td>
</tr>
<tr>
<td>Body weight at 8 wk (g)</td>
<td>541 ± 26</td>
<td>487 ± 25</td>
<td>508 ± 18</td>
<td>320 ± 12ᵇ</td>
<td>298 ± 12ᵇ</td>
<td>324 ± 17ᵇ</td>
</tr>
<tr>
<td>Kidney weight at 8 wk (g)</td>
<td>1.42 ± 0.06</td>
<td>1.38 ± 0.10</td>
<td>1.34 ± 0.05</td>
<td>1.87 ± 0.06ᵇ</td>
<td>1.54 ± 0.07ᶜ</td>
<td>1.43 ± 0.06ᶜ</td>
</tr>
<tr>
<td>Ccr at 8 wk (µl/100 g body weight per min)</td>
<td>630 ± 30</td>
<td>590 ± 70</td>
<td>530 ± 40</td>
<td>450 ± 30ᵇ</td>
<td>530 ± 80</td>
<td>600 ± 50ᶜ</td>
</tr>
<tr>
<td>Urinary protein at 8 wk (mg/d)</td>
<td>7.4 ± 1.4</td>
<td>11.2 ± 3.9</td>
<td>8.5 ± 2.3</td>
<td>10.1 ± 3.5</td>
<td>4.8 ± 2.1</td>
<td>5.9 ± 1.3</td>
</tr>
<tr>
<td>Urinary albumin at 8 wk (mg/d)</td>
<td>0.40 ± 0.17</td>
<td>0.54 ± 0.31</td>
<td>0.47 ± 0.09</td>
<td>1.79 ± 0.78ᵇ</td>
<td>0.93 ± 0.36</td>
<td>1.05 ± 0.45</td>
</tr>
</tbody>
</table>

ᵃ Data are expressed as mean ± SE. NC, rats on the normal K⁺ diet; NA, rats on the normal K⁺ diet with the ETA antagonist; NB, rats on the normal K⁺ diet with the ETB antagonist; LC, rats on the K⁺-deficient diet; LA, rats on the K⁺-deficient diet with the ETA antagonist; LB, rats on the K⁺-deficient diet with the ETB antagonist; Ccr, creatinine clearance.

ᵇ P < 0.05 versus NC.
ᶜ P < 0.05 versus LC.
of ABT-627 or A-192621 had no beneficial effect on the tubulointerstitial injury score in rats on the normal K\(^+\) diet.

Immunohistologic studies documented increased expression of osteopontin, a sensitive marker of injured tubules, in LC. Treatment with ABT-627 or A-192621 significantly suppressed the increase in tubular osteopontin expression in the cortex to a similar extent (Figure 3, A to D; Table 2). ABT-627 and A-192621 also inhibited the elevation of osteopontin expression in the medulla (Figure 3, E to H; Table 2).

The number of macrophages (ED-1-positive cells) was elevated in the cortex and medulla of LC. Similar to the suppression of tubular osteopontin expression, the accumulation of macrophages reduced in the cortex of LA and LB (Table 2). Neither ABT-627 nor A-192621 provided significant suppression of the macrophage infiltration in the medulla.

Deposition of type III collagen increased in the cortical and medullary interstitium of LC. Administration of ABT-627 or A-192621 ameliorated the collagen deposition significantly in the cortex (Figure 4, A to D) but had no significant effect at all in the medulla (Table 2).

Renal tubular cell proliferation (PCNA-positive cells), another typical renal injury in hypokalemic nephropathy, was prominent in the cortex and medulla of LC, and treatment with ABT-627 or A-192621 inhibited the increase in the number of PCNA-positive tubular cells in rats on the K\(^+\)-deficient diet (Figure 4, E to H; Table 2).

**Effects of ET Receptor Blockade on Renal ET System**

**ET-1.** In the rats on the normal K\(^+\) diet, selective ETA blockade by ABT-627 (NA) induced a mild elevation of ET-1 level in the cortex and preproET-1 mRNA levels in the medulla (Figure 5, A and D). It did not affect medullary ET-1 or cortical preproET-1 mRNA levels significantly (Figure 5, B and C). In K\(^+\)-deficient rats, ABT-627 (LA) slightly stimulated ET-1 peptide level in the medulla (Figure 5B), but it did not change cortical ET-1 level or cortical and medullary preproET-1 mRNA levels significantly (Figure 5, A, C, and D).

Selective ETB blockade by A-196261 (NB) had no significant effect on ET-1 peptide and preproET-1 mRNA levels in the rats on the normal K\(^+\) diet (Figure 5, A to D). By contrast,
in K⁺-deficient rats, ETB blockade (LB) potently suppressed ET-1 peptide and preproET-1 mRNA levels in the cortex (Figure 5, A and C) and markedly inhibited the elevation of ET-1 in the medulla (Figure 5B). ETB blockade also had a tendency to reduce preproET-1 mRNA level in the medulla of K⁺-deficient rats, but the difference was not statistically significant (Figure 5D; 2.4 ± 0.4 versus 1.7 ± 0.2, LC versus LB; P = 0.095).

**ET Receptors.** ETA inhibition by ABT-627 had no significant effect on ETA and ETB mRNA expression in the cortex and medulla of normal K⁺ and K⁺-deficient rats (Figure 6, A to D). ETB inhibition by A-192621 in normal K⁺ rats (NB) did not affect renal ETA mRNA expression (Figure 6, A and B). However, ETB inhibition in K⁺-deficient rats (LB) suppressed ETA mRNA levels in the cortex and medulla (Figure 6, A and B). Renal ETB mRNA levels were not affected significantly by A-192621 in normal K⁺ and K⁺-deficient rats (Figure 6, C and D).

**Effects of ET Receptor Blockade on Urinary Protein and Albumin Excretion**

We did not observe a significant increase in urinary protein excretion in LC when compared with NC. Treatment with ABT-627 or A-192621 did not affect urinary protein excretion significantly in rats on the K⁺-deficient diet (Table 1). By contrast, urinary albumin excretion was significantly higher in LC than NC. Administration of ABT-627 or A-192621 tended

---

**Table 2. Renal histologic findings in rats on the K⁺-deficient diet**

<table>
<thead>
<tr>
<th>Group</th>
<th>NC</th>
<th>NA</th>
<th>NB</th>
<th>LC</th>
<th>LA</th>
<th>LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical TII score</td>
<td>0.04 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>0.66 ± 0.11</td>
<td>0.22 ± 0.07</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>Medullary TII score</td>
<td>0.02 ± 0.02</td>
<td>0.08 ± 0.06</td>
<td>0.09 ± 0.05</td>
<td>4.2 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Cortical osteopontin (%)</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td>0.16 ± 0.11</td>
<td>2.44 ± 0.31</td>
<td>0.83 ± 0.23</td>
<td>0.82 ± 0.21</td>
</tr>
<tr>
<td>Medullary osteopontin (%)</td>
<td>0.37 ± 0.10</td>
<td>0.26 ± 0.08</td>
<td>0.47 ± 0.09</td>
<td>2.50 ± 0.32</td>
<td>1.49 ± 0.24</td>
<td>1.82 ± 0.36</td>
</tr>
<tr>
<td>Cortical macrophages (cells/mm²)</td>
<td>21 ± 1</td>
<td>20 ± 2</td>
<td>23 ± 3</td>
<td>64 ± 7</td>
<td>37 ± 5</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>Medullary macrophages (cells/mm²)</td>
<td>18 ± 2</td>
<td>24 ± 5</td>
<td>18 ± 3</td>
<td>83 ± 20</td>
<td>63 ± 15</td>
<td>58 ± 16</td>
</tr>
<tr>
<td>Cortical type III collagen deposition (%)</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.14</td>
<td>1.1 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Medullary type III collagen deposition (%)</td>
<td>2.9 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>2.7 ± 0.4</td>
<td>4.4 ± 0.4</td>
<td>4.7 ± 0.7</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>PCNA-positive cells in the cortex (cells/mm²)</td>
<td>0.6 ± 0.2</td>
<td>1.4 ± 0.4</td>
<td>1.2 ± 0.5</td>
<td>6.5 ± 1.5</td>
<td>2.9 ± 1.2</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td>PCNA-positive cells in the medulla (cells/mm²)</td>
<td>21 ± 1</td>
<td>20 ± 2</td>
<td>23 ± 3</td>
<td>70 ± 7</td>
<td>37 ± 5</td>
<td>40 ± 2</td>
</tr>
</tbody>
</table>

* Data are expressed as mean ± SE. TII, tubulointerstitial injury; PCNA, proliferating cell nuclear antigen.

* P < 0.05 versus NC.

* P < 0.05 versus LC.
to decrease urinary albumin excretion in rats on the K⁺-deficient diet, although the difference was NS (Table 1).

**ET Receptor Blockade Restored a Decrease in Urinary Excretion of Nitrite/Nitrate**

Urinary nitrite/nitrate excretion significantly decreased in LC, when compared with NC at week 4. Although there was a difference of 7 g in the amount of food intake between NC and LC at week 4 (data not shown), its influence on urinary nitrite/nitrate excretion could be small because the difference in dietary intake of nitrite/nitrate was less than one tenth of the difference in urinary nitrite/nitrate excretion. Administration of ABT-627 or A-192621 restored the decrease in urinary nitrite/nitrate excretion in rats on the K⁺/H₁₁₀₀₁-deficient diet (Table 3).

**ETB Blockade Induced Hypertension in Normal K⁺ Rats, But Not in K⁺-Deficient Rats**

BP was not significantly different between LC and NC at week 4, but LC had lower BP (Figure 7) and heart rate (310 ± 11 versus 360 ± 7; *P* < 0.05) than NC at week 8 as previously reported (21,22). ETA blockade had no significant effect on BP in normal K⁺ and K⁺-deficient rats. Administration of the ETB antagonist resulted in a BP elevation in normokalemic rats, being consistent with previous reports that ETB-deficient animals developed hypertension (23,24). The elevation of BP was accompanied by a reduction of fractional excretion of sodium (FENa) at week 4 (Table 3). By contrast, ETB block-
Table 3. Urinary nitrite/nitrate excretion and fractional excretion of Na⁺ in rats on the K⁺-deficient diet

<table>
<thead>
<tr>
<th>Group</th>
<th>NC</th>
<th>NA</th>
<th>NB</th>
<th>LC</th>
<th>LA</th>
<th>LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>FENa at 4 wk (%)</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.02</td>
<td>0.03 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.13 ± 0.03</td>
<td>0.14 ± 0.03</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>FENa at 8 wk (%)</td>
<td>0.07 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.09 ± 0.03</td>
<td>0.23 ± 0.08&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are expressed as mean ± SE. FENa, fractional excretion of sodium.
<sup>b</sup> P < 0.05 versus NC.
<sup>c</sup> P < 0.05 versus LC.

Discussion

Chronic hypokalemia is associated with renal tubulointerstitial injury consisting of tubular cell hyperplasia, tubular atrophy, macrophage infiltration, and interstitial fibrosis that is a final common pathway of progressive renal diseases (10–12). Although precise mechanisms by which hypokalemia induces nephropathy remain to be resolved, recent studies by our groups and others suggest that intrarenal hypoxia, as a result of microvascular injury and loss or alterations in local vasoactive mediators, could be responsible for the tubulointerstitial injury (25,26). By analyzing chronic K⁺-deficient rats, we previously reported the existence of intrarenal hypoxia and an elevation of renal vasoconstrictive peptides, including ET-1 and angiotensin II (AngII) (13). We also documented that AngII can mediate hypokalemic tubulointerstitial injury by activating AngII type 1 receptor (27). In this study, we observed an elevation of preproET-1 mRNA expression in the kidney of K⁺-deficient rats and demonstrated an upregulation of local ET-1 production. Therefore, we examined the effects of blocking ET-1 with an ETA-selective antagonist or an ETB-selective antagonist in the chronic hypokalemia model.

Our first finding is the amelioration of tubulointerstitial injury by ETA blockade. We observed significant suppression of tubular osteopontin expression, macrophage infiltration, and collagen deposition in the cortex. ET-1 is the most potent vasoconstrictive peptide ever found; as reported by Linas et al. (22), chronic hypokalemia induces an increase in intrarenal vascular resistance that likely predisposes to tubulointerstitial fibrosis. Accordingly, ETA blockade may have improved the renal injury by inhibiting vasoconstriction and restoring intrarenal blood flow. The renoprotective effects are less likely to be ascribed to the effect on systemic BP because there was no significant difference in BP between LA and LC. However, we cannot preclude systemic hemodynamic effects of ETA antagonism in hypokalemic rats, because ambulatory BP monitoring such as measurements with radiotelemetry devices might reveal subtle changes in BP. In addition to the hemodynamic effects, ET-1 exerts a variety of nonhemodynamic actions via ETA, including stimulation of endothelial production of MCP-1 (28), cytokine secretion by macrophages (29), the synthesis of matrix proteins and matrix-degrading protease inhibitors by myofibroblasts (1), and tubular epithelial cell and fibroblast proliferation (30). Thus, another possible mechanism of renoprotection by ETA blockade could be the inhibition of nonhemodynamic, profibrotic actions of ET-1.

On the basis of the observation that treatment with a selective ETA antagonists ameliorates renal injury in several models of proteinuric renal diseases, including remnant kidney, passive Heymann nephritis, lupus nephritis, and diabetic nephropathy (5,6), most people agree that the ET-1-ETA pathway participates in the development of chronic renal injury. However, implication of ETB in the development of renal injury is controversial. ETB on endothelial cells mediates vasodilation through the activation of NO and prostacyclin production (2), and ETB on renal tubular epithelial cells stimulates natriuresis (3); therefore, chronic ETB blockade was anticipated to exacerbate renal injury. Indeed, ETB-deficient rats are more susceptible to tubulointerstitial injury in a model of thrombotic microangiopathy (31), and selective blockade of ETB exacerbates renal injury in the remnant kidney model and deoxycorticosterone acetate-salt-induced hypertension in rats (17,32). On the other hand, blocking ETB may be beneficial as well, because ETB is reported to mediate proliferation of tubular...
epithelial cells (33), and ETB located on vascular smooth muscle cells has been shown to mediate vasoconstriction (3,34).

The second major finding was the observation that ETB blockade was renoprotective in hypokalemic tubulointerstitial injury. To the best of our knowledge, this is the first report that clarifies beneficial effects of ETB blockade in vivo in renal injury. Interestingly, ETB blockade improved all of the histologic parameters we examined to a level comparable to that obtained by ETA blockade in the cortex. ETB blockade as well as ETA blockade had a tendency to decrease urinary albumin excretion to a similar extent. The next question addressed was the mechanism for the improvement of the injury. In this regard, a major finding was that ETB blockade suppressed the increase in local ET-1 peptide and mRNA levels. These results are consistent with the existence of the autoinduction of ET-1 generation via ETB in the kidney in vivo, as previously reported in several types of culture cells (4,35). In addition, ETB blockade suppressed ETA expression. Thus, although we did not evaluate ETA activity in this study, we propose that the reduction in renal injury in ETB inhibited rats was due to the reduction of ET-1 as well as functional ETA, whereas in ETA blocked rats the ET-1 levels were high but the ETA functions were blocked. Hence, we would propose that the ETB blockade also protected rats by inhibiting the ET-1/ETA ligand/receptor pathway.

Yang et al. (36) reported that hypokalemia can attenuate endothelium-dependent vasorelaxation, possibly by inactivating NO released from endothelial cells. In addition, we previously showed that chronic hypokalemia resulted in a marked reduction of urinary nitrite/nitrate excretion (13). Interestingly, urinary nitrite/nitrate excretion was significantly increased in the hypokalemic rats by blocking either ETA or ETB. Normally one would expect blockade of ETB to reduce NO generation, because ETB on vascular endothelium are thought to mediate vasodilation. However, it is likely that the improvement in urinary nitrite/nitrate (NO metabolites) in the treated groups reflects blockade of ETA (in the group receiving the ETA blocker) and a reduction in ET-1 and ETA (in the group receiving the ETB blocker).

A pathophysiologic role of ETB stimulation has also been suggested in patients with essential hypertension and atherosclerosis who could have endothelial dysfunction (37,38). In patients with essential hypertension, nonselective ETA/ETB blockade was reported to induce a greater vasodilatory effect than selective ETA blockade (38), suggesting a vasoconstrictive role of ETB stimulation under the existence of endothelial dysfunction. Thus, although we do not have direct evidence, it is possible that ETB blockade could no longer induce vasoconstriction in this study as the result of hypokalemia-induced endothelial dysfunction, and rather ETB blockade was primarily protective by inhibiting ET-1 autoinduction and ETB-mediated constriction of vascular smooth muscle cells in hypokalemic nephropathy.

Consistent with previous reports (23,24), pharmacologic disruption of ETB induced BP elevation with a decrease in FENa in normokalemic rats, but BP was not elevated in hypokalemic rats. Gariepy et al. (24) utilized ETB-deficient rats to show that the absence of ETB resulted in an increase in Na⁺ reabsorption and hypertension that was mediated by stimulation of the renal epithelial sodium channel (ENaC). As previously reported (39), chronic K⁺ deficiency suppressed plasma aldosterone level, which could result in a downregulation of ENaC and reduction of fractional reabsorption of Na⁺ by collecting ducts in comparison with normokalemia. Accordingly, the absence of ETB-mediated inhibition of ENaC may stimulate Na⁺ reabsorption less in chronic K⁺ deficiency. We should also address the activation of Na⁺/H⁺ exchanger subtype 3 (NHE3) via ETB (40). Metabolic alkalosis usually suppresses NHE3 in proximal tubules, but chronic K⁺ deficiency does not, despite the existence of metabolic alkalosis (41). Thus, in chronic K⁺ deficiency, Na⁺ reabsorption and H⁺ excretion (HCO₃⁻ reabsorption) is preserved or relatively upregulated, and fractional reabsorption of Na⁺ and water increases in proximal tubules (39). Under such a condition, NHE3 inactivation by ETB blockade may result in suppression of Na⁺ reabsorption and H⁺ excretion. This possibility is supported by the increase in FENa in K⁺-deficient rats with ETB blockade. Thus, ETB blockade could not stimulate Na⁺ reabsorption so that BP elevation did not occur in chronic K⁺ deficiency. On the other hand, Ohuchi et al. (23) speculated, by analyzing ETB-deficient mice expressing less than one eighth of ETB than wild-type mice, that ETB deficiency elicits hypertension via inhibition of tonic prostacyclin production by endothelial cells. If this mechanism occurs in normokalemic animals, then endothelial dysfunction induced by hypokalemia may also contribute to the lack of BP elevation in hypokalemic rats administered the ETB antagonist. In addition, Murray et al. (21) reported that the systemic vasculature is resistant to the pressor effect of AngII and vasopressin in chronic hypokalemia. Such pressor resistance to vasoconstrictors may also contribute to the absence of BP elevation in hypokalemic rats with ETB inhibition.

Compared with the cortex, improvement of the tubulointerstitial injury was less in the medulla of K⁺-deficient rats with ABT-627 or A-192621. Although the dose of ABT-627 was the same that was used to prevent hypertension, vascular hypertrophy and renal injury in deoxycorticosterone acetate-salt-induced hypertensive rats (17), we cannot rule out the possibility that higher doses might have prevented medullary injury more effectively, because the medullary ET-1 level was 30-fold higher in K⁺-deficient rats than controls. Similarly, the dose of A-192621 might not be sufficient because the medullary content of ET-1 was not normalized. Alternatively, locally generated ET-1 might not play an important role in the development of tubulointerstitial fibrosis in the medulla.

In various models of proteinuric renal diseases, one of the mechanisms for the elevation of renal ET-1 production is augmented tubular ET-1 production by urinary protein (5). Albumin stimulates ET-1 production by proximal tubular cells in vitro (42); therefore, elevated urinary albumin excretion in K⁺-deficient rats might be responsible for the increase in renal ET-1 production. However, this possibility...
could be inconsistent with the elevated renal ET-1 production in K⁺-deficient rats with the ETA antagonist whose albumin excretion had a tendency to decrease. Although we did not address the mechanism of increased ET-1 production directly in this study, there are potential mechanisms that link chronic hypokalemia to stimulation of ET-1. Despite the existence of metabolic alkalosis, renal intracellular pH decreases in chronic hypokalemia (43) and acidosis is reported to elevate ET-1 production at least in renal microvascular endothelial cells (44). Thus, hypokalemia can directly stimulate renal ET-1 production. Hypokalemia is associated with intrarenal hypoxia and an upregulation of renal cortical AngII generation (13). The former is known to stimulate ET-1 production, possibly through a hypoxia-inducible factor-1 binding site (45), and the latter is a potent stimulator for renal ET-1 generation (46). AngII-mediated ET-1 production may actually have some pathogenic roles in hypokalemic nephropathy, because we previously reported amelioration of hypokalemia-induced renal injury by an AngII type 1 receptor antagonist, losartan (27), with a ported amelioration of hypokalemia-induced renal injury by ET-1 production may actually have some pathogenic roles of ET-1 as a mediator of progressive renal diseases, the mechanisms of ET-1 upregulation should deserve further investigation.

In conclusion, renal production of ET-1 increases with chronic hypokalemia and contributes to the development of tubulointerstitial injury. ETA may mediate renal injury either hemodynamically (by inducing vasoconstriction and ischemia) or nonhemodynamically (by mediating the profibrotic effects of ET-1). In contrast, ETB is likely to mediate renal injury by stimulating ET-1 autoinduction. These studies suggest that both ETA and ETB blockade may be beneficial in the prevention of hypokalemic tubulointerstitial injury.

Acknowledgments

Support for this study was provided in part by the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan, Grants-in-aid for Scientific Research (14571044) from Japan Society for the Promotion of the Science, and United States Public Health Service Grants DK-52121 and HL-68607. We thank Drs. Jerry L. Wessale and John Groff of Abbott Laboratories for supplying ABT-627 and A-192621. We also thank Dr. C. Giachelli, University of Washington, Seattle, WA, for donating an antibody for osteopontin and Dr. Y. Saito, Nara Medical University, Nara, Japan, for providing an antibody for endothelin-1.

References


