Effects of vanadate and potassium depletion on renal H, K-ATPase protein expression

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Background: Vanadate (V) inhibits while potassium (K) depletion stimulates collecting tubule H, K-ATPase activity. In the presence of V, K depletion could not restore the decreased H,K-ATPase activity. The effects of V and K depletion on renal H,K-ATPase protein expression might explain such observation.

Objective: To examine the effects of V and K depletion on renal H,K-ATPase protein expression.

Methods: Rats treated with normal saline solution (NSS) or V (5 mg/kg body weight) received either normal potassium (NK) or low potassium (LK) diet for 10 days. Protein expressions of renal H,K-ATPase \( \alpha_1 \) and \( \alpha_2 \) isoforms were determined by immunohistochemistry.

Results: Both NK and LK animals treated with V had significantly increased vanadium levels in serum, urine, and renal tissues. LK diet caused hypokalemia. Animals treated with LK and V showed progressive hypokalemia. LK stimulated renal H,K-ATPase \( \alpha_1 \) protein expression in both cortex and medulla but enhanced H,K-ATPase \( \alpha_2 \) protein expression only in the cortex. Vanadate did not affect H,K-ATPase \( \alpha_1 \) protein expression in both NK and LK groups. Vanadate unaltered H,K-ATPase \( \alpha_2 \) protein expression in NK animals but could attenuate the increased expression in LK group.

Conclusion: The magnitude of direct inhibitory effect of V on renal H,K-ATPase activity with small suppressive effect on protein expression is greater than the stimulatory effect of K depletion on H,K-ATPase activity and protein expression.

Keywords: Hypokalemia, immunohistochemistry, protein expression, renal H, K-ATPase, vanadate.
Materials and methods  

Experimental animals  

The study was approved by the Ethics Committee of Research, Chulalongkorn University. Male Wistar rats, weighing 220 to 250 grams, were obtained from the National Center of Scientific Use of Animals (Mahidol University, Salaya, Thailand). The animals were housed with controlled temperature (23-25°C) and 12 hours of controlled light-dark time. The animals were given free access to a laboratory chow and water as assigned in the experimental protocols.

Experimental procedure  

The serum creatinine of each animal should be less than 1 mg/dL. The animals were divided into four groups (n = 8 / group) as follows.

Group 1: (NSS+NK) The animals received normal saline solution (NSS) (0.5 mL/kg body weight/day) by intraperitoneal injection (ip), and normal K diet (NK) (K = 150 mEq K/kg diet).

Group 2: (NSS+LK) The animals received NSS and low K diet (LK) (K = 1.5 mEq/kg diet, ICN Biomedicals, Irvine, USA).

Group 3: (V+NK) The animals received V (5 mg/kg body weight/day, ip, Sigma, St. Louis, USA) and NK diet.

Group 4: (V+LK) The animals received V and LK diet.

All groups received their respective treatment for 10 days.

One day before the experiment, the animals were placed in metabolic cages for twenty-four hour urine for collection. On the experimental date, they were anesthetized by sodium pentobarbital (60 mg/kg body weight, ip), and the abdomen was opened via a midline incision. Blood sample was collected from the abdominal aorta, and was centrifuged at 1,000 g for 15 minutes. Serum was stored at -80°C until use for measuring vanadium levels and blood chemistry. Arterial blood gas was determined by a blood gas analyzer (Osmetech OPT3™ CCA, Model No.OPTI3, Roche, USA).

In the mammalian kidney, there are at least two distinct H,K-ATPases: gastric (α₁) and colonic (α₂) isoforms [9]. Thus, the renal tissue samples were fixed in 10% paraformaldehyde overnight, and embedded in paraffin for immunohistochemical detection for H,K-ATPase α₁ and H,K-ATPase α₂ protein expression. Additional renal tissue samples (~200 mg wet weight.) were dried with heat for one day, and were measured for vanadium levels using an atomic absorption spectrophotometer (Model 4110ZL AA spectrometer, Perkin-Elmer Co., Ueberlingen, Germany).

Immunohistochemical study  

The paraffin-embedded kidney sections were cut at 4 µm in thickness. The slides were deparaffinized in xylene and alcohol, with endogenous peroxidase activity being quenched in 3% hydrogen peroxide for 10 minutes. The non-specific binding of the antibody was blocked by incubating tissue sections with 5% normal swine serum (Dako, Glostrup, Denmark) in phosphate buffer solution (PBS)-A (PBS + 1% BSA + 0.3% triton X-100) for 30 minutes at room temperature. Then, the section was incubated in monoclonal antibody against H,K-ATPase α₁ (MBL, Nagoya, Japan), or H,K-ATPase α₂ (generously provided by Dr. A. Smolka, University of South Carolina, Charleston, USA), at concentrations of 1:10 and 1:500, respectively (diluted in 3% normal horse serum) for over one hour at room temperature.

The sections were rinsed 3 x 10 minutes with PBS-B (PBS + 0.25% BSA+ triton X -100) and incubated with biotinylated swine anti-goat-mouse-rabbit immunoglobulin (Multi-Link; Dako, Denmark) diluted 1:50 in PBS-B for 60 minutes at room temperature. After incubation, tissue sections were rinsed 2 x 10 minutes with PBS-B and then 1 x 10 minutes in PBS. The tissue sections were reacted with ABC-streptavidin horseradish peroxidase complex (Vector, Burlingame, USA) for 60 minutes at room temperature. The sections were then rinsed 2 x 10 minutes in PBS and 10 minutes in 0.05 M Tris-HCl buffer (pH 7.6). The sections were reacted for peroxidative activity in a solution containing 0.025% 3, 3’-diaminobenzidine (DAB) and 0.01% H₂O₂ in 0.05 M Tris-HCl buffer (pH 7.6) for 30 minutes. Then, the sections were washed 2 x 5 minutes with distilled water, counterstained with hematoxylin (CV Laboratories, Bangkok, Thailand) and coverslipped with permount.

Areas of staining were identified and scored by three pathologists in a blinded fashion. The intensity of staining was scored from 0 to 3 (0 = no staining, 1 = weak positive, 2 = moderate staining, and 3 = strongly positive staining).

Statistical analysis  

The intensity scores of renal H,K-ATPase α₁ and H,K-ATPase α₂ protein expressions were present in
descriptive statistics by measuring central tendency (Mode). The results of blood and urine parameters were expressed as mean±SD. Statistical differences among groups were assessed by ANOVA (analysis of variance) with post hoc comparison by Tukey’s test where appropriate. A p-value of <0.05 was considered statistically significant. The statistical calculations were performed by means of the statistical package SPSS for Window 10.

Results

Metabolic parameters in rats treated with V or/and LK

As illustrated in Table 1, 10-day V administration significantly increased vanadium levels in serum, urine, and renal tissues in both NK and LK diet groups. Vanadium was deposited in the cortical region approximately two times when compared with the medulla area (p<0.001). In V+LK group, the vanadium accumulation was demonstrated in a greater extent in both renal cortex and medulla when compared with the V+NK group (p<0.001). The V+LK group had lesser vanadium concentrations in serum and urine when compared with the respective V+NK group (p<0.001, Table 1).

The values of serum potassium, serum bicarbonate, serum creatinine, blood pH, and fractional excretion of potassium (FEK) were illustrated in Table 2. LK caused marked hypokalemia with reduced FEK. V induced markedly kaliuresis with mild hypokalemia. Animals treated with LK and V exhibited progressive hypokalemia with tremendous kaliuresis. No significant changes in blood pH and serum bicarbonate were observed among all studied groups.

Table 1. Vanadium levels in rats treated with NK diet or LK diet (Mean ± SD, n = 8/group).

<table>
<thead>
<tr>
<th>Groups</th>
<th>NSS+NK</th>
<th>NSS+LK</th>
<th>V+NK</th>
<th>V+LK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (ng/mL)</td>
<td>0.25±0.01</td>
<td>0.15±0.02</td>
<td>678.15±44.62†</td>
<td>558.38±24.98‡*#</td>
</tr>
<tr>
<td>Urine (ng/mL)</td>
<td>0.38±0.05</td>
<td>0.42±0.04</td>
<td>2,658.20±78.15†</td>
<td>711.03±17.53‡*#</td>
</tr>
<tr>
<td>Renal cortex (μg/g dry wt)</td>
<td>0.20±0.04</td>
<td>0.18±0.04</td>
<td>34.98±0.84†</td>
<td>79.90±6.06‡*#</td>
</tr>
<tr>
<td>Renal medulla (μg/g dry wt)</td>
<td>0.16±0.06</td>
<td>0.11±0.03</td>
<td>17.73±3.66†</td>
<td>44.90±4.64‡*#</td>
</tr>
</tbody>
</table>

NSS = normal saline solution, NK = normal potassium diet, LK = low potassium diet, V = vanadate,
*p<0.001 vs. NSS+NK, †p<0.001 vs. NSS+LK, ‡p<0.001 vs. V+NK

Table 2. Metabolic parameters in rats treated with V or/and LK diet (Mean ± SD, n = 8/group).

<table>
<thead>
<tr>
<th>Groups</th>
<th>NSS+NK</th>
<th>NSS+LK</th>
<th>V+NK</th>
<th>V+LK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum K (mmol/L)</td>
<td>3.96±0.12</td>
<td>1.71±0.04”</td>
<td>3.25±0.13’</td>
<td>1.63±0.10”*</td>
</tr>
<tr>
<td>Serum HCO3 (mmol/L)</td>
<td>27.65±0.76</td>
<td>26.60±0.81</td>
<td>28.63±0.54</td>
<td>28.13±1.09</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.45±0.01</td>
<td>7.51±0.02</td>
<td>7.42±0.02</td>
<td>7.48±0.03</td>
</tr>
<tr>
<td>FEK (%)</td>
<td>63.34±3.50</td>
<td>10.08±0.93”</td>
<td>70.56±7.90</td>
<td>307.18±9.97”†</td>
</tr>
</tbody>
</table>

NSS = normal saline solution, NK = normal potassium diet, LK = low potassium diet, V = vanadate, Cr = Creatinine, FEK = fractional excretion of K. *p<0.05 vs. NSS+NK, †p<0.05 vs. NSS+LK, **p<0.001 vs. NSS+NK, ″p<0.001 vs. V+NK.
Renal H,K-ATPase $\alpha_1$ protein expression

The renal H,K-ATPase $\alpha_1$ protein expression detected by immunohistochemistry is shown in Fig. 1 (a-h). Most staining was apparent at the luminal membrane of the collecting ducts. No staining was noted in the glomeruli.

LK diet could enhance the expression from the scores of 1 to be 2 in the cortex and from 0 to be 1 in the medulla (see Fig. 1 (f, h) and Table 3). These occurred in both NSS- (Fig. 1b) and V-treated rats (see Fig. 1(d)). Ten days of V injection in NK and LK groups had no effect on the protein expression in both cortex and medulla when compared with the NSS group (see Fig. 1 (c, g) and Table 3). We note that the staining areas of H,K-ATPase $\alpha_1$ protein expression in the cortex were greater than the medulla.

Renal H,K-ATPase $\alpha_2$ protein expression

The expression of renal H,K-ATPase $\alpha_2$ protein is shown in Fig. 2 (a-h). Most staining was present at the luminal membrane of the cortical collecting duct, while there was no staining in the glomeruli.

LK diet increased the protein expression from the scores of 0-1 to be 2 in the cortex of NSS-treated rats (see Fig. 2(b) and Table 3). V did not alter the protein expression in the cortical region in NK rats (Fig. 2(c)), but could attenuate the increased protein expression in the LK group with the score of only 1 (see Fig. 2(d) and Table 3).

No expression of H,K-ATPase $\alpha_2$ protein was detected in the medulla in all studied groups (see Fig. 2(e-h) and Table 3).

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Table 3. The intensity scores of renal H,K-ATPase $\alpha_1$ and H,K-ATPase $\alpha_2$ in rats treated with V or/and LK diet (Mode, n = 8/group).

<table>
<thead>
<tr>
<th>Groups</th>
<th>NSS+NK</th>
<th>NSS+LK</th>
<th>V+NK</th>
<th>V+LK</th>
</tr>
</thead>
<tbody>
<tr>
<td>H,K-ATPase ($\alpha_1$)</td>
<td>cortex</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>H,K-ATPase ($\alpha_2$)</td>
<td>cortex</td>
<td>0-1</td>
<td>2</td>
<td>0-1</td>
</tr>
<tr>
<td></td>
<td>medulla</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NSS = normal saline solution, NK = normal potassium diet, LK = low potassium diet, V = vanadate.
Discussion

H,K-ATPase participates in renal K reabsorption and hydrogen excretion [1, 2, 9]. The present study is the first to examine the effects of K depletion, vanadate, and the combination of both on renal H,K-ATPase protein expression.

The present results have shown that 10-day intraperitoneal injection of vanadate significantly increased vanadium levels in serum, urine, and renal tissue (Table 1). In the kidney, vanadium was deposited in the cortex about twice as much when compared with the medulla. Adachi et al. [10] and Parker et al. [11] also demonstrated that the highest accumulation of vanadium occurred in renal tissue particularly in the cortical region.

In NSS+NK animals, renal H,K-ATPase $\alpha_1$ and $\alpha_2$ protein expressions were identified at the luminal membrane of the collecting duct as shown in Fig. 1, 2, and Table 3. However, there was no H,K-ATPase $\alpha_2$ protein expression in the medulla in all studied groups. Marsy et al. [12] also showed low renal H,K-ATPase $\alpha_2$ protein expression level [12].

Previous studies demonstrated that K depletion enhanced collecting tubule H,K-ATPase activity [13-15]. In NSS-treated group, the present study shows that in the cortex, LK diet for 10 days augmented H,K-ATPase $\alpha_1$ protein expression in both cortex and medulla (Fig. 1(b,f)). K depletion enhanced H,K-ATPase $\alpha_2$ protein expression only in the cortex (Fig. 2(b)) but had no effect in the medulla (Fig. 2(f)). Previous studies regarding the effect of K-depletion on H,K-ATPase protein expression had yielded varying results. An earlier study using Western blot analysis showed that 14-day LK diet did not alter H,K-ATPase $\alpha_1$ protein expression in either cortex or medulla, but enhanced H,K-ATPase $\alpha_2$ protein in membranes prepared from the renal medulla [7]. However, another study demonstrated that both protein subunits were stimulated by 14-day potassium depletion [8]. Studies regarding the effect of LK on renal H,K-ATPase mRNA synthesis also provided conflicting results reporting either increased, decreased, or unchanged expression [16-20]. The disparities of the results among these studies might be caused by the differences in animal species, study designs, and techniques.

Earlier studies illustrated that vanadate reduced H,K-ATPase activity in renal tubule segments [3, 5]. Vanadate could directly inhibit H,K-ATPase by competitive binding with ATP at catalytic site [21]. However, there were no available data regarding the effect of vanadate on H,K-ATPase protein expression. This is the first study to demonstrate that vanadate had no influence on renal H,K-ATPase $\alpha_1$ protein expression regardless of potassium status as shown in Fig. 1 and Table 3. The failure to inhibit H,K-ATPase $\alpha_1$ protein expression despite suppressing its activity implies that vanadate might
unalter the process of transcription as well as translation of renal H,K-ATPase α₁. Vanadate also had no effect on H,K-ATPase α₂ protein expression in NK diet group. However, vanadate could attenuate the increased H,K-ATPase α₁ protein expression mediated by K depletion in NK diet group (Fig. 2, Table 3). Therefore, vanadate had no effect on the number of renal H,K-ATPase pump in both normal and low potassium status, but had direct suppressive effect on renal H,K-ATPase activity.

Our important unique finding in the present study is that LK still plays a faithful stimulus on renal H,K-ATPase protein expression, irrespective of the presence of vanadate (Table 3). Therefore, LK has stimulatory effects on both isoform protein expressions and activity of H,K-ATPase. Thus, the decreased H,K-ATPase activity in the presence of both vanadate and K depletion [5] would indicate that the magnitude of direct inhibition of vanadate on H,K-ATPase activity with no effect on protein expression is greater than the stimulatory effect of K depletion on protein expression and activity of H,K-ATPase. This might be the underlying mechanism in developing hypokalemia distal renal tubular acidosis in Thailand. Q J Med. 1990; 14:289-301.

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More laboratory and clinical studies are required to prove this contention. 

**Conclusion**

Vanadate has no influence on renal H,K-ATPase α₁ protein expression irrespective of K status. Vanadate unalters renal H,K-ATPase α₂ protein expression in NK but elicits inhibitory effect in LK. Potassium depletion stimulates both renal H,K-ATPase isoform protein expressions regardless of the presence of vanadate. The suppressive effect of vanadate is greater than the stimulatory effect of K-depletion on renal HK-ATPase.

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