Decreased mRNA Expression of the PTH/PTHrP Receptor and Type II Sodium-Dependent Phosphate Transporter in the Kidney of Rats Fed a High Phosphorus Diet Accompanied with a Decrease in Serum Calcium Concentration

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This study investigates the phosphorus (P) homeostasis in the process of an altered parathyroid hormone (PTH) action in the kidney of rats fed a high P diet. Four-week-old male Wistar strain rats were fed diets containing five different P levels (0.3, 0.6, 0.9, 1.2 and 1.5%) for 21 days. The serum PTH concentration and urinary excretion of P were elevated with increasing dietary P level. Compared to rats fed the 0.3% P diet, the serum calcium (Ca) concentration remained unchanged, while the serum 1,25(OH)2D3 concentration and urinary excretion of cAMP were elevated with increasing dietary P level in rats fed the high P diets containing 0.6–0.9% P. On the other hand, a lower serum Ca concentration was observed in rats fed the high P diets containing 1.2% or greater P. The serum 1,25(OH)2D3 concentration remained unchanged in rats fed the high P diets containing 1.2% or greater P. The serum 1,25(OH)2D3 concentration remained unchanged in rats fed the high P diets containing 1.2% or greater P, comparison with rats fed the 0.3% P diet. The urinary excretion of cAMP and PTH/PTH-related peptide (PTHrP) receptor and type II sodium-dependent phosphate transporter (NaPi-2) mRNA in the kidney were both decreased in rats fed the high P diets containing 1.2% or greater P. In conclusion, a high P diet with subsequent decrease in serum Ca concentration supressed the PTH action in the kidney due to PTH/PTHrP receptor mRNA down-regulation. Furthermore, an increase in the urinary excretion of P might have been caused by decreased NaPi-2 mRNA expression without the effects of PTH and 1,25(OH)2D3.

Key words: high P diet; PTH action; PTH/PTHrP receptor; type II sodium-dependent phosphate transporter; urinary excretion of P

Consuming a high phosphorus (P) diet elevates the serum parathyroid hormone (PTH) concentration in humans and experimental animals. Reiss et al.1 have reported that oral P administration increased the serum concentration of PTH in humans. Calvo et al.2 have reported that consuming high P and low calcium (Ca) diets increased the secretion of serum PTH in young adults. We have reported that a high P diet enhanced PTH secretion in rats.3,4 It is thought that a decrease in the serum Ca concentration and an increase in the serum P concentration, which would be induced by a high P diet, elevates PTH secretion.

PTH is one of the Ca regulating hormones, which maintain Ca and P homeostasis. In the kidney, PTH enhances Ca resorption and P excretion, and synthesizes 1,25-dihydroxyvitamin D3 (1,25(OH)2D3). PTH binds to the PTH/PTH-related peptide (PTHrP) receptor in the kidney, which causes an increase in the synthesis of 3’- cyclic adenosine monophosphate (cAMP), as a second messenger of PTH. The urinary excretion of cAMP is an index of PTH action, because cAMP increased by PTH signals leaks from the proximal tubular cells to urine.5–7 We have previously reported that a high P diet (1.5% P level) increased the urinary excretion of P, however, the urinary excretion of cAMP was decreased, although the serum PTH concentration was elevated.8 The dose-response effects of dietary P have not yet been examined, although our previous work suggested that there was a PTH-independent mechanism for P excretion in rats fed a high P diet. Recently, several P transporters have been discovered in the kidney. The type II sodium-dependent phosphate transporter (NaPi-2) has been identified in the brush border membrane of rat renal proximal tubules and is regulated by dietary P, PTH and 1,25(OH)2D3.8–10 These observations provide a more complex basis from which to explore the change in P metabolism by a high P diet.

The purpose of this study was to investigate the P homeostasis in the process of an altered PTH action in the kidney. We examined the renal PTH action by measuring the urinary cAMP excretion and PTH/PTHrP receptor and NaPi-2 mRNA expression in the kidney of rats fed a high P diet with different levels of P.

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Materials and Methods

Experimental design. Thirty 4-week-old male Wistar strain rats were purchased from Clea Japan (Tokyo, Japan) and individually housed in stainless-steel metabolic cages in a room maintained at 22 °C with a 12-hour light-dark cycle. The Tokyo University of Agriculture Animal Use Committee approved the study, and the animals were maintained in accordance with the guidelines of the university for the care and use of laboratory animals. The experimental diets were based on the AIN-93G diet,¹¹) five diets containing 0.3, 0.6, 0.9, 1.2 and 1.5% P being prepared. Each experimental diet contained 0.5% Ca (Table 1). All rats were fed the 0.3% P diet for a 7-day acclimatization period. After 7 days, the rats were randomly divided into five experimental groups (C, 2P, 3P, 4P and 5P) of 6 rats each and fed one of the five different P level diets for 21 days, respectively. The animals were allowed to eat ad libitum and given free access to distilled water. On the last day of the experimental period, their urine was collected for analyses. At the end of the experiment, all rats were sacrificed, and blood and kidney samples were collected for analyses.

Blood and urine analyses. The blood samples were centrifuged and the supernatants were used as serum samples. Each urine sample was diluted with H₂O to adjust the volume to a constant amount. Serum and urine were stored at −80 °C until needed for analyses. To measure the Ca and P contents, the urine was dried, ashed and then demineralized with 1 mol/l HCl solution. Ca was analyzed by atomic absorption spectrophotometry (Hitachi A-2000) according to the method of Gimblet et al.¹²) Serum P was assayed with a P-test Wako (Wako Pure Chemical Industries, Osaka, Japan). P in the urine was analyzed colorimetrically according to the method of Gomori.¹³) Serum PTH was assayed with an enzyme-linked immunosorbent assay kit (Immucor). Total RNA was isolated from a homogenized right kidney by using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s specifications. The amount and purity of the RNA were assessed by spectrophotometry. Samples of total RNA (20 μg) from each group of rats were denatured in 0.04 mol/l morpholinopropanesulfonic acid (pH 7.0), 10 mmol/l sodium acetate, 1 mmol/l EDTA, 2.2 mol/l formaldehyde, and 50% formamide at 65 °C for 15 min, separated by electrophoresis through 1% agarose/2.2 mmol/l formaldehyde gel, and transferred to a nylon membrane (Hybond-N, Amersham pharmacia biotech). The membrane was air-dried and the RNA samples crosslinked to the nylon membrane by UV irradiation. Migration of 28S and 18S ribosomal RNA was determined by ethidium bromide staining. To generate a probe, PTH/PTHrP receptor¹⁴) (sense, 5′-CCGGCTGT-CTTCGTGGCTGTC-3′; antisense, 5′-CCCTGGAAGG-AGTGGAGG-3′), NaPi-2 ¹⁵) (sense, 5′-ATGATGTC-CTACAGCAGAGAAG-3′; antisense, 5′-GACCAGAA-CAGACAGCCAGTTA-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)¹⁶) (sense, 5′-CCAGTAGTATTCTACCACTTCACT-3′; antisense, 5′-GAGGCCCATGCCAGTGAGCTTC-3′) primers were prepared and subjected to a reverse transcription-polymerase chain reaction (RT-PCR). Each probe was radiolabeled with [α-³²P]-dCTP (9.25 MBq/mmol; Amersham Pharmacia Biotech), and the RNA samples were crosslinked to the membrane by UV irradiation. Migration of 28S and 18S ribosomal RNA was determined by ethidium bromide staining. To generate a probe, PTH/PTHrP receptor (sense, 5′-CCGGCTGT-CTTCGTGGCTGTC-3′; antisense, 5′-CCCTGGAAGG-AGTGGAGG-3′), NaPi-2 (sense, 5′-ATGATGTC-CTACAGCAGAGAAG-3′; antisense, 5′-GACCAGAA-CAGACAGCCAGTTA-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense, 5′-CCAGTAGTATTCTACCACTTCACT-3′; antisense, 5′-GAGGCCCATGCCAGTGAGCTTC-3′) primers were prepared and subjected to a reverse transcription-polymerase chain reaction (RT-PCR). Each probe was radiolabeled with [α-³²P]-dCTP (9.25 MBq/mmol; Amersham Pharmacia Biotech), and the RNA samples were crosslinked to the membrane by UV irradiation.
StatView 5 software (SAS Institute, Cary, NC, USA). Whitney’s U test was used after the Kruskal–Wallis test. The homogeneity of variances was not equal, Mann–Whitney’s U test was used after the Kruskal–Wallis test.

### Results

Each result is expressed as the ratio of PTH/PTHrP receptor.

#### Table 2. Body Weight and Food Intake

<table>
<thead>
<tr>
<th>Group (P content)</th>
<th>Initial Body Weight (g)</th>
<th>Final Body Weight (g)</th>
<th>Weight Gain (g/21 days)</th>
<th>Food Intake (g/21 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (0.3% P)</td>
<td>116.4 ± 1.7</td>
<td>266.0 ± 5.8</td>
<td>149.7 ± 4.3</td>
<td>374.8 ± 10.5</td>
</tr>
<tr>
<td>2P (0.6% P)</td>
<td>116.5 ± 1.4</td>
<td>269.7 ± 5.5</td>
<td>153.1 ± 5.0</td>
<td>386.7 ± 11.4</td>
</tr>
<tr>
<td>3P (0.9% P)</td>
<td>116.8 ± 1.2</td>
<td>270.7 ± 3.1</td>
<td>153.8 ± 3.0</td>
<td>378.8 ± 4.4</td>
</tr>
<tr>
<td>4P (1.2% P)</td>
<td>116.8 ± 1.1</td>
<td>242.7 ± 3.8&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>125.9 ± 4.2&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>328.2 ± 9.4&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>5P (1.5% P)</td>
<td>116.9 ± 1.1</td>
<td>209.1 ± 3.2&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>92.2 ± 2.6&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>260.5 ± 4.3&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each data value is the mean ± S.E. of six rats.

- <sup>a</sup>P < 0.05 vs C group (Fisher’s PLSD test)
- <sup>b</sup>P < 0.05 vs 2P group (Fisher’s PLSD test)
- <sup>c</sup>P < 0.05 vs 3P group (Fisher’s PLSD test)
- <sup>d</sup>P < 0.05 vs 4P group (Fisher’s PLSD test)

#### Table 3. Serum Ca, P, PTH, and 1,25(OH)<sub>2</sub>D<sub>3</sub> Concentrations

<table>
<thead>
<tr>
<th>Group (P content)</th>
<th>Serum Ca (mg/dl)</th>
<th>Serum P (mg/dl)</th>
<th>Serum PTH (pg/ml)</th>
<th>Serum 1,25(OH)&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;3&lt;/sub&gt; (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (0.3% P)</td>
<td>12.24 ± 0.04</td>
<td>9.33 ± 0.12</td>
<td>58.96 ± 5.16</td>
<td>113.43 ± 1.77</td>
</tr>
<tr>
<td>2P (0.6% P)</td>
<td>12.43 ± 0.20</td>
<td>11.75 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>117.14 ± 8.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>140.35 ± 6.70&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3P (0.9% P)</td>
<td>12.43 ± 0.14</td>
<td>11.83 ± 0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>137.54 ± 19.87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>205.69 ± 6.31&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>4P (1.2% P)</td>
<td>11.12 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.47 ± 1.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>777.28 ± 71.17&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>128.27 ± 9.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5P (1.5% P)</td>
<td>9.44 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.87 ± 0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1558.84 ± 157.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>110.26 ± 5.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each data value is the mean ± S.E. of six rats.

- <sup>a</sup>P < 0.05 vs C group (Fisher’s PLSD test)
- <sup>b</sup>P < 0.05 vs 2P group (Fisher’s PLSD test)
- <sup>c</sup>P < 0.05 vs 3P group (Mann–Whitney’s U test after the Kruskal–Wallis test)
- <sup>d</sup>P < 0.05 vs 4P group (Mann–Whitney’s U test after the Kruskal–Wallis test)
- <sup>e</sup>P < 0.05 vs 5P group (Mann–Whitney’s U test after the Kruskal–Wallis test)

buffer containing 50% formamide, 6.6 × saline-sodium citrate (SSC), 0.1% SDS, 5 × Denhardt’s solution, 200 mg/ml salmon sperm DNA and 0.01 mol/l EDTA. The labeled probe was then added to the prehybridization buffer, and the membrane was incubated overnight (16–18 h) at 42 °C. The membrane was then washed twice, for 30 min at 42 °C in a buffer containing 1 × SSC and 0.1% SDS, and for 30 min at 65 °C in the same buffer. The relative abundance of the PTH/PTHrP receptor, NaPi-2 and GAPDH was quantitatively determined by a BAS-2000 II system (Fuji Photo Film, Tokyo, Japan). Each result is expressed as the ratio of PTH/PTHrP receptor or NaPi-2 mRNA to GAPDH mRNA.

**Statistical analyses.** Each data value is presented as the mean ± SE for each group of six rats. After one-way analysis of variance (ANOVA), Fisher’s PLSD was used to determine significant differences between groups. If the homogeneity of variances was not equal, Mann–Whitney’s U test was used after the Kruskal–Wallis test. Significant differences are considered for a p value of less than 0.05. Statistical analyses were performed by StatView 5 software (SAS Institute, Cary, NC, USA).

### Results

**Body weight and food intake**

The final body weight, weight gain and food intake were all significantly less in the 4P and 5P groups compared with the C, 2P and 3P groups, and being lower in the 5P group than in the 4P group (Table 2).

**Serum Ca, P, PTH and 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations**

A lower serum Ca concentration was observed in the 4P and 5P groups than in the C, 2P and 3P groups, and being lower in the 5P group than in the 4P group. A higher serum P concentration was observed in the 2P, 3P, 4P and 5P groups than in the C group, and being higher in the 5P group than in the 2P, 3P and 4P groups. The serum PTH concentration was significantly higher in the 2P, 3P, 4P and 5P groups than in the C group, being higher in the 4P and 5P groups than in the 2P and 3P groups, and in the 5P group than in the 4P group. The serum 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration was significantly higher in the 2P and 3P groups than in the C group, and did not differ among the C, 4P and 5P groups (Table 3).

**Urinary excretion of Ca, P and cAMP**

The urinary excretion of Ca was significantly higher in the 5P group than in the C group, and in the 3P, 4P and 5P groups than in the 2P group. The urinary excretion of P increased significantly with increasing dietary P level. The urinary excretion of cAMP was significantly higher in the 3P group than in the C group, and significantly lower in the 4P and 5P groups than in the C group, and significantly lower in the 4P and 5P groups than in the 2P and 3P groups (Table 4).
High Phosphorus Diet and Urinary Phosphorus Excretion

Table 4. Urinary Excretion of Ca, P, and cAMP

<table>
<thead>
<tr>
<th>Group (P content)</th>
<th>Urine Ca (mg/mmol Cre)</th>
<th>Urine P (mg/mmol Cre)</th>
<th>Urine cAMP (µmol/mmol Cre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (0.3% P)</td>
<td>16.02 ± 1.51</td>
<td>43.66 ± 9.57</td>
<td>15.32 ± 2.18</td>
</tr>
<tr>
<td>2P (0.6% P)</td>
<td>11.22 ± 2.00</td>
<td>949.87 ± 43.08</td>
<td>19.59 ± 2.88</td>
</tr>
<tr>
<td>3P (0.9% P)</td>
<td>18.05 ± 2.94</td>
<td>1884.51 ± 44.84</td>
<td>23.46 ± 1.71</td>
</tr>
<tr>
<td>4P (1.2% P)</td>
<td>17.95 ± 1.47</td>
<td>2412.59 ± 22.61</td>
<td>5.16 ± 0.84</td>
</tr>
<tr>
<td>5P (1.5% P)</td>
<td>21.81 ± 1.60</td>
<td>3317.31 ± 43.59</td>
<td>7.80 ± 2.74</td>
</tr>
</tbody>
</table>

Each data value is the mean ± S.E. of six rats.

Table 5. PTH/PTHrP Receptor and NaPi-2 mRNA Expression in the Kidney

<table>
<thead>
<tr>
<th>Group (P content)</th>
<th>PTH/PTHrP receptor/GAPDH</th>
<th>NaPi-2/GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (0.3% P)</td>
<td>0.41 ± 0.02</td>
<td>0.83 ± 0.05</td>
</tr>
<tr>
<td>2P (0.6% P)</td>
<td>0.39 ± 0.01</td>
<td>0.90 ± 0.06</td>
</tr>
<tr>
<td>3P (0.9% P)</td>
<td>0.40 ± 0.02</td>
<td>0.77 ± 0.06</td>
</tr>
<tr>
<td>4P (1.2% P)</td>
<td>0.27 ± 0.02</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>5P (1.5% P)</td>
<td>0.25 ± 0.04</td>
<td>0.51 ± 0.04</td>
</tr>
</tbody>
</table>

Each data value is the mean ± S.E. of six rats.

Discussion

In this study, we prepared five kinds of experimental diet containing different P levels to determine the dose-response effect of dietary P. We observed that the serum Ca concentration was not affected with high P diets containing less than or equal to 0.9% P, but the serum P and PTH concentrations were both increased in rats fed the 0.6% P diet. Previous reports have suggested that elevated PTH by a high P (1.5%) diet was due to changes in the serum concentrations of Ca and P. In this study, the high P diets (0.3–0.9%) elevated the serum PTH concentration without reducing the serum Ca concentration. Furthermore, the urinary excretion of cAMP, which transmits PTH signaling, and the serum 1,25(OH)2D3 concentration were increased in rats fed the high P diets containing 3 times as much as the control (0.3% P) diet. These results showed that PTH exhibited normal action on the kidneys of rats fed the 0.3–0.9% P diets while maintaining the normal level of serum Ca concentration.

In contrast, rats fed the high P diets containing 1.2% or greater P exhibited a decrease in serum Ca concentration and an increase in serum P concentration, which elevated the serum PTH concentration. Therefore, PTH secretion was greatly increased in rats fed the high P diets containing 1.2% or greater P compared to rats that maintained the normal level of serum Ca concentration in the 0.3–0.9% P diet groups. These results suggested that the decreased serum Ca concentration might have accelerated to increase secretion of PTH in rats fed the high P diets.

In our previous study, we observed an increased PTH secretion in seven week-old male Wistar-Imamich strain rats fed a 1.2% high P diet for 7 days without a decreased serum Ca concentration. In this study, a greater PTH secretion was apparent in five week-old Wistar strain male rats fed the 1.2% P diet for 21 days, but this was coupled with a decreased serum Ca concentration, in contrast to the effect on Ca in the previous study. This difference might have been due to differences in the age, strain and feeding term between the two studies.

Furthermore, in this study, when the serum PTH concentration was greatly increased, the urinary excretion of cAMP was decreased in rats fed the high P diets containing 1.2% or greater P in comparison with rats fed the 0.3% P diet. The serum 1,25(OH)2D3 concentration was unchanged in rats fed the high P diets containing 1.2% or greater P compared to rats fed the 0.3% P diet, although the serum PTH concentration was increased.
These results suggested that greater PTH secretion might decrease the PTH action in the kidney by a high P diet, in agreement with our previous results when using rats fed a 1.5% P diet. With regard to PTH/PTHrP receptor mRNA expression in the kidney, this was decreased in rats fed the high P diets containing 1.2% or greater P compared to the other three groups. Greater PTH secretion with a decreased serum Ca concentration by the high P diets containing 1.2% or greater P might have resulted in down-regulation of the PTH/PTHrP receptor mRNA expression, which in turn might suppress PTH action in the kidney. In addition, although we have previously reported that a high P diet (1.5% P level) decreased PTH/PTHrP receptor mRNA expression in the kidney, it was also apparent that the 1.2% P diet decreased such expression in this study.

PTH increases the urinary excretion of P and decreases the urinary excretion of Ca. PTH regulation of proximal tubule P reabsorption has also been well documented. On the other hand, in recent studies, several P transporters have been discovered in the proximal tubule of rats exposed to exogenous PTH to maintain the normal level in the serum (1.2% or greater) might have caused a decrease in NaPi-2 mRNA expression in the kidney, due to PTH/PTHrP receptor mRNA down-regulation. Furthermore, the urinary excretion of P was elevated with increasing dietary P level and might have been caused by decreased NaPi-2 mRNA expression in the kidney without alterations in PTH and 1,25(OH)2D3.

References

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4) Masuyama, R., Katai, K., Segawa, H., Haga, H., Morita, K., Arai, H., Shigematsu, T., Suzuki, K., and Goto, S., Chronic phosphorus supplementation decreases the expression of


