High Phosphorus Diet Rapidly Induces Nephrocalcinosis and Proximal Tubular Injury in Rats

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Summary The development of nephrocalcinosis and the time course of changes in kidney function, especially proximal tubular function, were studied in young male rats fed a high-phosphorus diet. The animals were fed a purified diet with a phosphorus content of either 0.5% (normal phosphorus diet) or 1.5% (high-phosphorus diet). In the group fed the high-phosphorus diet, nephrocalcinosis was found in 4 of 42 rats after 1d of feeding and in all rats of this group at 3d. The degree of nephrocalcinosis gradually increased with time. Upon histological observation by electron microscopy, vacuoles, lysosomes and swelling of microvilli in the proximal tubules were observed in rats fed the high-phosphorus diet after 1d of feeding. Giant lysosomes with deposition of calcium and deposition of hydroxyapatite in mitochondria were observed in the proximal tubules of rats fed the high-phosphorus diet at 3d. Albumin concentration in the urine of these rats was significantly increased at 3d. The activity of N-acetyl-β-D-glucosaminidase in the urine was also significantly increased after 1d of feeding the high-phosphorus diet, and then reached a plateau. The β2-microglobulin concentration in the urine of rats fed the high-phosphorus diet was significantly increased at 14d, and increased more toward 21d. We concluded that nephrocalcinosis and injury to the proximal tubules are rapidly induced in rats fed a high-phosphorus diet.

Key Words nephrocalcinosis, rats, high-phosphorus diet, injury to the proximal tubules

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Dietary composition plays an important role in the etiology of nephrocalcinosis. Many dietary factors such as carbohydrate (1, 2), protein (3, 4), fat (1, 5) and minerals (6–11) are known to influence the frequency of nephrocalcinosis. Particularly, high phosphorus (8, 12, 13), high calcium (14) and low magnesium (15, 16) concentrations and a calcium:phosphorus ratio below one in the diet (17, 18) induce nephrocalcinosis, and increased magnesium intake prevents nephrocalcinosis (6, 19, 20).

During nephrocalcinosis, the deposition of calcium phosphates occurs in the entire corticomedullary junction of the kidney in rats (21). The mineral deposits are primarily composed of calcium and phosphorus as indicated by X-ray microanalysis (10). The ratio of these minerals in the deposits is similar to that found in hydroxyapatite (3, 22). The time course of changes involved in nephrocalcinosis has been examined after the administration of sodium phosphate intraperitoneally to rats, and the deposition of calcium was found within 6 d (23). A phosphate-supplemented diet induced intraluminal calcium deposits which were evident at 3 d (24).

Rats with nephrocalcinosis display impaired kidney functions. Plasma urea concentration and urinary albumin excretion are increased in rats with nephrocalcinosis (5, 13, 22, 25). A change in plasma creatinine concentration, however, is not observed in rats with nephrocalcinosis (26, 27). In a great number of studies, creatinine, urea and albumin have been used as indicators of kidney functions, but these are mainly indicators of glomerular function. On the other hand, we have reported that rats with nephrocalcinosis induced by a high-phosphorus diet display increased N-acetyl-β-D-glucosaminidase (NAG) activity in urine and urinary excretion of β2-microglobulin (28), which serves as an indicator of proximal tubular function.

A great deal of effort has been made to assess kidney functions in rats with nephrocalcinosis. What seems to be lacking, however, is proximal tubular function. In addition, the time course of changes in kidney functions in rats with nephrocalcinosis remains unclear. In this study, we investigated the time course of changes in kidney functions as well as the development of nephrocalcinosis in rats fed a high-phosphorus diet, with special reference to proximal tubular function, using biochemical indicators and histological examination.

MATERIALS AND METHODS

Diet. The compositions of the experimental diets are given in Table 1. The concentrations of phosphorus in the experimental diets were adjusted to 0.5% (normal phosphorus diet) and 1.5% (high-phosphorus diet) using potassium tripolyphosphate (Nacalai Tesque, Kyoto, Japan). The concentrations of calcium and magnesium in both experimental diets were adjusted to 0.5% and 0.05% using calcium carbonate (Kanto Chemical, Tokyo, Japan) and magnesium oxide (Wako Pure Chemical Industries, Osaka, Japan), respectively. The mineral mixture used was a modification of AIN-76 mineral mixture (29) without calcium, magnesium.
Table 1. Compositions of the experimental diets.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Normal phosphorus diet (0.5%)</th>
<th>High-phosphorus diet (1.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g/100 g diet)</td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>20.00</td>
<td>20.00</td>
</tr>
<tr>
<td>Corn starch</td>
<td>15.00</td>
<td>15.00</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Mineral mixture(^1)</td>
<td>1.66</td>
<td>1.66</td>
</tr>
<tr>
<td>Vitamin mixture(^2)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>K(_2)P(_3)O(_10)</td>
<td>1.92</td>
<td>6.75</td>
</tr>
<tr>
<td>MgO</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>CaCO(_3)</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>Sucrose</td>
<td>48.59</td>
<td>43.76</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.00</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>

\(^1\) The mineral mixture is a modification of AIN-76 mineral mixture without calcium, magnesium and phosphorus sources.

\(^2\) AIN-76A vitamin mixture.

and phosphorus sources, while the AIN-76A vitamin mixture (30) was used intact. The purified diets were stored at 4°C until use.

Animals and experimental design. Eighty-four male Wistar rats (four weeks old) (Clea Japan, Tokyo, Japan) were housed in individual stainless steel cages. During the experiment, the rat cages were located in a room with controlled lighting on a 12-h light : dark cycle (light, 0800–2000 h), temperature of 22 ± 1°C and relative humidity of 60–65%. The study was approved by the Tokyo University of Agriculture Animal Use Committee, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Tokyo University of Agriculture. All rats were given free access to a normal phosphorus diet and demineralized water for a 7-d pre-experimental period before initiation of the study. After the pre-experimental period, the rats were divided into two experimental groups of 42 rats each, having a similar mean body weight. Each group was assigned to one of the experimental diets. Rats fed the normal phosphorus diet were given an amount equivalent to that consumed by the rats fed the high-phosphorus diet throughout the experiment. Rats were given free access to demineralized water throughout the experiment. Food intake and body weight were recorded daily.

In each group, six rats were killed by decapitation at 0, 1, 3, 5, 7, 14 and 21 d.
Before dissection, the rats were housed individually in metabolism cages and urine was collected for 24 h. Blood was collected and centrifuged to separate the serum. Both kidneys were removed and weighed after the capsules were discarded. The right kidney was used for chemical analysis. All samples were stored at $-40^\circ C$ until analysis.

**Kidney calcium and phosphorus analysis.** The right kidney was dried overnight at $100^\circ C$, and the dry weight was measured. The kidney was ashed at $550^\circ C$ for 48 h and the minerals were extracted in 1 mol/L HCl solution for analysis. Calcium in the kidney was analyzed by means of atomic absorption spectrophotometry (Shimadzu AA-640-13) (31). Phosphorus in the kidney was analyzed according to the method of Gomori (32).

**Histological examination of the kidney**

*Light microscopy:* Immediately after collection, half of the left kidney was fixed in a 10% neutral formalin phosphate buffer. The tissue samples were embedded in paraffin wax, cut into sections 5$\mu$m thick, and the sections were stained with hematoxylin-eosin and Von Kossa’s. The degree of nephrocalcinosis at each time point was graded from 0 (nephrocalcinosis not detected) to 4 (severe nephrocalcinosis).

*Electron microscopy:* Immediately after collection, part of the left kidney was fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide. The tissue samples were dehydrated through a graded alcohol series and embedded in Epok 812. Ultrathin sections were cut on a Reihert-Nissei Ultracut N ultramicrotome with a diamond knife, and stained with uranyl acetate and lead citrate. The sections were examined using a Hitachi H-800 (75 kV) transmission electron microscope.

**Analysis of indicators of kidney functions.** Immediately after complete collection, 24-h urine volume and pH were measured. The creatinine in serum and urine was measured using Creatinine-TEST Wako (Wako Pure Chemical). Urea nitrogen in the serum was measured using Nyosotiso-TEST Wako (Wako Pure Chemical). Albumin and $\beta_2$-microglobulin in the urine were determined using PANATEST Rat Albumin and PANATEST Rat $\beta_2$-Microglobulin (Panapharm Laboratories, Kumamoto, Japan). The activity of NAG in the urine was determined using NAG TEST Shionogi (Shionogi, Osaka, Japan).

**Statistical analysis.** Data are presented as means $\pm$ SE. Statistical analysis was performed by one-way ANOVA (33). Differences between groups fed the normal and high-phosphorus diets were considered significant when the $p$ value was $<0.05$.

### RESULTS

**Kidney weight and water content, calcium and phosphorus concentrations in the kidney**

Kidney wet and dry weights were significantly increased in rats fed the high-phosphorus diet (Table 2). The water content, and calcium and phosphorus concentrations in the kidney were significantly higher in rats fed the high-phosphorus diet as compared to those in rats fed the normal phosphorus diet (Table 2).
Table 2. Kidney weight and water content, and calcium and phosphorus concentrations in the kidneys of rats fed normal and high-phosphorus diets for 21 d.1,2

<table>
<thead>
<tr>
<th></th>
<th>Normal phosphorus diet</th>
<th>High-phosphorus diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet weight (g)</td>
<td>0.77 ± 0.04</td>
<td>2.3 ± 0.1*</td>
</tr>
<tr>
<td>Dry weight (g)</td>
<td>0.18 ± 0.01</td>
<td>0.49 ± 0.02*</td>
</tr>
<tr>
<td>Water (g)</td>
<td>0.59 ± 0.03</td>
<td>1.8 ± 0.1*</td>
</tr>
<tr>
<td>Calcium (mmol/100 g dry weight)</td>
<td>0.87 ± 0.11</td>
<td>335 ± 13*</td>
</tr>
<tr>
<td>Phosphorus (mmol/100 g dry weight)</td>
<td>49.1 ± 2.7</td>
<td>250 ± 7*</td>
</tr>
</tbody>
</table>

1 Values are means ± SE (n=6).
2 One-way ANOVA was used to analyze the data. * represents a significant difference compared to the group fed the normal phosphorus diet (p<0.05).

**Histological examination of the kidney**

**Light microscopy.** The degree of nephrocalcinosis was graded according to the following criteria (Fig. 1): Grade 0, no nephrocalcinosis was evident (photomicrograph not shown); Grade 1, slight nephrocalcinosis was evident (Fig. 1A); Grade 2, mild nephrocalcinosis was evident (Fig. 1B); Grade 3, moderate nephrocalcinosis was evident (Fig. 1C); and Grade 4, severe nephrocalcinosis was evident (Fig. 1D).

Nephrocalcinosis was observed after 1 d of feeding the high-phosphorus diet (Table 3). The degree of nephrocalcinosis gradually increased over time (Table 3). In addition, the incidence of nephrocalcinosis in rats fed the high-phosphorus diet increased over time; it was observed in four rats of the group at 1 d, and in all rats of the group from 3 d to 21 d (Table 3). Nephrocalcinosis was not observed in any of the rats fed the normal phosphorus diet (data not shown).

**Electron microscopy.** After 1 d of feeding the high-phosphorus diet, vacuoles, lysosomes and swelling of microvilli were observed in the proximal tubules (Fig. 2). Giant lysosomes were observed in the proximal tubules at 3 d, and the deposition of calcium was evident (Fig. 3). In the proximal tubules, the deposition of hydroxyapatite was also observed in mitochondria at 3 days and in microvilli at 5 d (Figs. 3 and 4). Necrotic cells were observed in the proximal tubules at 21 d (Fig. 5). The fusion of foot processes in the epithelial cells, thickening of the glomerular basement membrane and increase in mesangial matrix were not observed in the glomeruli at 21 d (Fig. 6).

Otherwise, the deposition of hydroxyapatite was observed in the Henle’s loops and collecting ducts of rats fed the high-phosphorus diet. Interstitial edema was also observed in these rats. Furthermore, in the case of rats fed the normal phosphorus diet, the kidneys appeared normal (photomicrograph not shown).
Fig. 1. Degree of nephrocalcinosis graded by light microscopy. Von Kossa’s stained section. Bars: 500 μm. A: Grade 1 showing slight nephrocalcinosis. B: Grade 2 showing mild nephrocalcinosis. C: Grade 3 showing moderate nephrocalcinosis. D: Grade 4 showing severe nephrocalcinosis.
Table 3. Time course of changes in degree and incidence of nephrocalcinosis in rats fed a high-phosphorus diet.  

<table>
<thead>
<tr>
<th>Feeding period (d)</th>
<th>Degree of nephrocalcinosis</th>
<th>Incidence of nephrocalcinosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0/6</td>
</tr>
<tr>
<td>1</td>
<td>0.67±0.21</td>
<td>4/6</td>
</tr>
<tr>
<td>3</td>
<td>2.0±0.4</td>
<td>6/6</td>
</tr>
<tr>
<td>5</td>
<td>2.3±0.2</td>
<td>6/6</td>
</tr>
<tr>
<td>7</td>
<td>2.8±0.5</td>
<td>6/6</td>
</tr>
<tr>
<td>14</td>
<td>3.3±0.2</td>
<td>6/6</td>
</tr>
<tr>
<td>21</td>
<td>3.7±0.2</td>
<td>6/6</td>
</tr>
</tbody>
</table>

1 Values are means ± SE (n=6).

Fig. 2. Vacuoles (V), lysosomes (L) and swelling of microvilli (arrow head) are observed in the proximal tubule of a rat fed the high-phosphorus diet at 1 d.

**Indicators of kidney functions**

Urinary volume significantly increased and urinary pH significantly decreased in rats fed the high-phosphorus diet (Table 4). Creatinine clearance and serum urea nitrogen concentration significantly increased in these rats (Table 4). Albumin concentration in the urine of rats fed the high-phosphorus diet had significantly increased at 3d (Fig. 7). NAG activity in the urine had significantly increased
Fig. 3. Proximal tubule in a rat fed the high-phosphorus diet at 3d as shown by electron microscopy. A: Giant lysosomes with deposition of calcium (GL), deposition of hydroxyapatite in mitochondria (arrows), vacuoles (V) and swelling of microvilli (arrow heads) are seen. B: High-magnification electron micrograph showing a giant lysosome with deposition of calcium (GL). C: The deposition of hydroxyapatite in mitochondria (arrows) was observed.
Fig. 4. The deposition of hydroxyapatite was observed in the microvilli (arrow) of the proximal tubule of a rat fed the high-phosphorus diet at 5 d.

Fig. 5. A necrotic cell was observed in the proximal tubule of a rat fed the high-phosphorus diet at 21 d.
after 1 d of feeding the high-phosphorus diet, and thereafter reached a plateau level (Fig. 8). β2-Microglobulin concentration in the urine of rats fed the high-phosphorus diet had significantly increased at 14 d, and increased further toward 21 d (Fig. 9).

DISCUSSION

An increase in kidney calcium concentration and nephrocalcinosis were found in rats fed the high-phosphorus diet. This result is similar to reports from other investigators (8, 12, 34). With the development of high-phosphorus diet-induced nephrocalcinosis, large intraluminal calcium deposits were found in the inner strip of the outer medulla at 3 d (24). Haase (23) injected sodium phosphate intraperitoneally into rats at 2, 4, 6, 8 and 10 d, and observed calcium deposition in one rat of the group at 2 d, in two rats of the group at 4 d and in all rats of the group after 6 d of treatment. In our study, after feeding the high-phosphorus diet, nephrocalcinosis was observed in four rats of the group at 1 d and in all rats of the group at 3 d. The degree of nephrocalcinosis showed a rapid initial increase, and then further increased gradually over time. In rats fed the high-phosphorus diet, vacuoles, lysosomes and swelling of microvilli were observed in the proximal tubules after 1 d of feeding. These results suggest that
Table 4. Indicators of kidney functions in rats fed normal and high-phosphorus diets for 21 d.\textsuperscript{1,2}

<table>
<thead>
<tr>
<th></th>
<th>Normal phosphorus diet</th>
<th>High-phosphorus diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary volume (mL/d)</td>
<td>11.2 ± 2.4</td>
<td>20.7 ± 1.6*</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>7.6 ± 0.4</td>
<td>6.5 ± 0.2*</td>
</tr>
<tr>
<td>Serum creatinine concentration</td>
<td>58.1 ± 1.5</td>
<td>60.6 ± 2.2</td>
</tr>
<tr>
<td>(μmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>0.29 ± 0.01</td>
<td>0.54 ± 0.03*</td>
</tr>
<tr>
<td>(mL/min/100 g body weight)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum urea nitrogen concentration</td>
<td>3.5 ± 0.4</td>
<td>11.5 ± 1.0*</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} Values are means ± SE (n=6).

\textsuperscript{2} One-way ANOVA was used to analyze the data. * represents a significant difference compared to the group fed the normal phosphorus diet (p<0.05).

Fig. 7. Albumin concentration in the urine of rats fed normal (dotted line) and high- (solid line) phosphorus diets. Values are means ± SE (n=6). One-way ANOVA was used to analyze the data at each time point. * represents a significant difference compared to the group fed the normal phosphorus diet, at the same time (p<0.05).

A high-phosphorus diet rapidly induces nephrocalcinosis and ultrastructural changes in the proximal tubules. The onset time of this kidney damage may also depend on the phosphorus content in the diet.

Furthermore, an increase in phosphorus intake induces an increase in kidney weight (13, 28). In this study, kidney wet and dry weights increased in rats fed the high-phosphorus diet. The increase in calcinotic kidney weight was mainly due to an increase in calcium concentration in the kidney (22). In this study, an increase in kidney water content and interstitial edema were evident in rats fed the high-phosphorus diet, and this may be the cause of the increase in kidney wet weight.
An increase of urinary volume was found for the rats fed the high-phosphorus diet. A decrease of urinary pH was also found for the rats fed the high-phosphorus diet. The low urine pH in rats fed the high-phosphorus diet may cause an increased concentration of phosphate in the urine. Creatinine clearance and serum urea nitrogen concentration were increased in rats fed the high-phosphorus diet. These results agree with previous studies (13, 28). In this study, despite an increase in creatinine clearance in rats fed the high-phosphorus diet, serum urea nitrogen concentration also increased. Increased serum urea nitrogen concentration in this study may be due to tissue damage to the kidneys. In this study, tissue damage to the kidneys was found in rats fed the high-phosphorus diet, and this damage may have promoted the protein catabolism of these rats. Consequently, increased serum urea nitrogen concentration may be caused in rats fed a high-phosphorus diet.
A high-phosphorus diet has been reported to induce an increase in urinary albumin excretion \((22,35)\). In our study also, albumin concentration in the urine was increased for the rats fed the high-phosphorus diet. These results may indicate injury of the renal glomerular basement membrane, since an increase in urinary albumin excretion is known to be caused by a defect in the permeability of the renal glomerular basement membrane \((36)\). In our study, in rats fed the high-phosphorus diet, the fusion of foot processes in the epithelial cells and thickening of the glomerular basement membrane were not observed. Previously, we reported that increased urinary albumin excretion in rats fed a high-phosphorus diet may be due to depression of the proximal tubular function \((28)\). In this study, an increase in NAG activity in the urine and ultrastructural changes in the proximal tubules were evident in rats fed the high-phosphorus diet, which is indicative of proximal tubular injury. The proximal tubular injury may cause depression of reabsorption in the proximal tubules. In other words, the increased urinary albumin excretion in rats fed the high-phosphorus diet may be explained by the obstruction of albumin reabsorption in the proximal tubules. This finding supports the conclusions of our previous study \((28)\), and this study revealed that the obstruction of proximal tubular albumin reabsorption in rats fed a high-phosphorus diet occurs at 3d.

From the results concerning albumin concentration in the urine, NAG activity in the urine, and histological observations, we believe that a high-phosphorus diet induces depression of reabsorption in the proximal tubules at 3d. However, the \(\beta_2\)-microglobulin concentration in the urine is an indicator of proximal tubular function, and its levels did not change in rats during the initial 7d of feeding with either the normal or high-phosphorus diets. This result implies that, in rats fed a high-phosphorus diet, reabsorption in the proximal tubules is not depressed until after 7d of feeding. Further, the \(\beta_2\)-microglobulin concentration in the urine of rats fed the high-phosphorus diet increased rapidly during the initial 14d of feeding, indicating that depression of the proximal tubular function is induced after 14d of feeding. These results seem to introduce a contradiction with respect to the time of occurrence of depression of proximal tubular function. However, this contradiction may be explained as follows: a protein of high molecular weight causes obstruction of reabsorption in the proximal tubules in the early stages of kidney damage, and subsequently the obstruction of reabsorption in the proximal tubules by proteins of low molecular weight occurs in the later stages of kidney damage.

In conclusion, in rats fed a high-phosphorus diet, nephrocalcinosis was observed after 1d of feeding. In addition, ultrastructural changes in the proximal tubules were evident in these rats at this time. As indicators of kidney functions, albumin concentration in the urine significantly increased at 3d and NAG activity in the urine significantly increased after 1d of feeding a high-phosphorus diet. \(\beta_2\)-Microglobulin concentration in the urine of rats fed the high-phosphorus diet also significantly increased at 14d. These results suggest that a high-phosphorus diet induces nephrocalcinosis and proximal tubular injury after 1d.
of feeding, and obstruction of albumin reabsorption in the proximal tubules at 3d.

REFERENCES

Time Course of Change in Kidney Function


