Enhancement Effects of Vitamin K₁ (Phylloquinone) or Vitamin K₂ (Menaquinone-4) on Intestinal Alkaline Phosphatase Activity in Rats

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Summary Alkaline phosphatase (ALP) hydrolyzes a variety of monophosphate esters into inorganic phosphoric acid and alcohol at a high optimal pH, and is thought to play an important role in phosphate metabolism. Intestinal ALP, located at the brush border of intestinal epithelial cells, is known to be affected by several kinds of nutrients, but little is known about the physiological function of intestinal ALP. Vitamin K is an essential cofactor for the post-translational carboxylation of glutamate residues into gamma-carboxy glutamate (Gla). Recently, novel functions of vitamin K have been clarified, but no data exist on the relation between vitamin K and intestinal ALP. The aim of this study was to examine the effects of both vitamin K₅ (K₁: phylloquinone, and K₂: menaquinone) on ALP activity. Sprague-Dawley rats (6-wk-old) were divided into three groups: a control, phylloquinone (PK: 600 mg/kg diet), or menaquinone-4 (MK-4: 600 mg/kg diet) diet group. After 3 mo of feeding, we measured intestinal ALP activity by dividing it into five segments. In each segment, both PK and MK-4 increased intestinal ALP activity. The levels of intestinal ALP activity in the duodenum and proximal jejunum from the PK group were significantly higher than in the control group (p<0.05). Moreover, the levels of intestinal ALP activity from the proximal jejunum and distal ileum of the intestine in the MK group were significantly higher than in the control group (p<0.05). In this study, we clarified for the first time that both vitamin K₁ and K₂ as nutritional factors enhance intestinal ALP activity.

Key Words vitamin K, phylloquinone, menaquinone-4, intestinal alkaline phosphatase, rats

Alkaline phosphatase (ALP, EC 3.1.3.1) hydrolyzes a variety of monophosphate esters into inorganic phosphoric acid and alcohol at a high optimal pH (pH 8–10). In humans, studies on the genes of this enzyme have revealed that there are at least four ALP isozymes: tissue non-specific (liver/bone/kidney; TNSALP), intestinal, placental, and germ cell types (1–4). Based on studies of hypophosphatasia, which is a systemic skeletal disorder resulting from a TNSALP deficiency (5–12), TNSALP was suggested to be indispensable for bone mineralization.

In rats, ALP is classified into two types: TNSALP and intestinal ALP. The strong activity of intestinal ALP, which is located at the brush border of intestinal epithelial cells, appears to play an important role in active metabolism by hydrolyzing phospho-compounds to supply free inorganic phosphate, but little is known about the physiological function of intestinal ALP. It was well known that intestinal ALP is affected by several kinds of nutrients via fat feeding (13). Previously, we reported that intestinal ALP activity in rats was markedly increased after lactose feeding (14, 15).

Vitamin K is an essential cofactor for the post-translational modification of glutamic acid (Glu) residues to γ-carboxyglutamic acid (Gla) residues of vitamin K-dependent hepatic blood-coagulation proteins including prothrombin and factors VII, IX, and X (16). Vitamin K has been also suggested to play a role in the improvement of bone metabolism, because vitamin K-dependent proteins (osteocalcin and matrix Gla protein) are found in bone; osteocalcin (OC) is the most abundant (17–19). OC is produced in osteoblasts, and fully carboxylated osteocalcin binds the calcium ions of hydroxyapatite (20).

In nature, vitamin K is found in two forms that differ in the alkylic side chain at the 3-position of the common 2-methyl-1.4-napthoquinone ring group. Vitamin K₁ (phylloquinone: PK) contains the phytol group as the side chain, and is found in leafy, green vegetables. Vitamin K₂ (menaquinone: MK-n) has multi-isoprene as the side chain. Menaquinone-4 (MK-4), which is vitamin K₂ with four isoprene units, comprises a family of naphthoquinones and is contained in meat, eggs, and dairy products.

Recently, the pleiotropic functions of vitamin K were
clarified. Although the actions of PK and MK on γ-carboxylation are considered to be quite similar, PK and MK were suggested to have different effects on bone metabolism or other new functions (21). In a population-based study, dietary intake of MK, but not of PK, was associated with aortic calcification and coronary heart disease (22). Although the effects of PK and MK on bone metabolism have been reported, MK has a more powerful inhibitory effect on bone resorption and osteoclastogenesis than PK (23, 24).

It is reported that MK-4 increases bone-type TNSALP activity (25), but little is known about the influence of vitamin K on intestinal ALP. This is the first report to clarify the effect of vitamin K on intestinal ALP activity.

MATERIALS AND METHODS

Experimental animals. Twenty-six 6-wk-old female Sprague Dawley rats were allowed to acclimate for 8 d prior to any study procedure. Then, rats were separated into three groups: an AIN-93M diet (26) (control) group, vitamin K₁ (phylloquinone: PK) diet group, and vitamin K₂ (menaquinone-4: MK-4) diet group. The vitamin K-diets were modified from AIN-93M and contained PK or MK-4 600 mg/kg, respectively. PK and MK-4 were kindly supplied by Eisai Co. (Tokyo, Japan). Ca, P, protein, and lipid contents were identical in these diets. The animals were housed individually in wire cages with free access to ion-exchanged distilled water. Twelve-hour light/dark cycles, constant temperature (23 ± 1°C), and constant humidity (50 ± 5%) were maintained. All rats were observed each day. Their food intake was monitored and body weights were obtained every second day. The care and use of the rats in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals. Eighty-five days after the beginning of the experimental diet, the animals were fasted overnight and sacrificed by bleeding from the abdominal aorta under anesthesia.

Preparation and measurement of intestinal enzymes. We removed the small intestines from the pylorus side to the beginning of the cecum and divided it into five segments. From the pylorus, we took the first 3 cm as the duodenum, and then separated the remaining part into the jejunum and ileum. Moreover, we cut both the jejunum and ileum into two parts. The segments were slit open longitudinally, rinsed with ice-cold saline, and were directly homogenized under liquid nitrogen. The samples were stored at −30°C until being thawed for analyses. Each sample was homogenized with 10 mM Tris-buffered saline containing 1% Triton X-100 (pH 7.3). The supernatant obtained after centrifugation at 10,000 × g for 5 min was used for the enzyme assay. ALP activity was determined with 10 mM p-nitro-phenylphosphate as the substrate in 100 mM 2-amino-2-methyl-1,3-propanediol HCl buffer containing 5 mM MgCl₂, pH 10.0, at 37°C, as previously reported (13). To analyze the properties of ALP, an inhibitory assay using levamisole and l-phenylalanine, and a thermostability assay were performed, as previously described (13). Enzyme activity was determined by the rate of hydrolysis of p-nitrophenyl phosphate and expressed in units (U=μmol p-nitrophenol formed/min). Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gel (7.5%) electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out according to the method of Weber et al. (27). After electrophoresis, ALP isozymes separated in the gel were stained by the coupling of β-naphthylphosphoric acid monosodium salt with Fast Violet B salt (28).

Statistical analysis. Values are shown as means ± SE. Dunnet’s multiple comparison test was used after ANOVA to compare the significance of differences among control and PK, or MK groups, using SPSS13.0J (SPSS Inc., IL, USA).

RESULTS

Body weight gain and food intake

The initial body weight among the control, PK and MK groups was not significantly different (data not shown). As shown in Table 1, there was also no significant difference among the three groups in body weight gain, food intake, or food efficiency.

Table 1. Body weight gain, food intake, and food efficiency.

<table>
<thead>
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<th>Groups</th>
<th>n</th>
<th>Body weight gain (g/d)</th>
<th>Food intake (g/d)</th>
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<td>Cont.</td>
<td>9</td>
<td>1.45 ± 0.08</td>
<td>15.6 ± 0.4</td>
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Each value represents the mean ± SE.

Food efficiency = Body weight gain/Food intake.

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![Fig. 1. ALP-specific activity in duodenum. Results are the means ± SE. Cont.: control, PK: phylloquinone, MK: menaquinone-4. Significant difference between the PK and control groups (*p<0.05).](image-url)
Enhancement Effects of Vitamin Ks on Intestinal ALP

Fig. 2. ALP-specific activity in proximal jejunum. Results are the means±SE. Cont.: control. PK: phylloquinone. MK: menaquinone-4. Significant difference between the PK and control groups ("p<0.05). Significant difference between the MK and control groups ("p<0.05).

Fig. 3. ALP-specific activity in distal jejunum. Results are the means±SE. Cont.: control. PK: phylloquinone. MK: menaquinone-4.

ALP activities in the intestine

In each intestinal segment, ALP activities of both PK and MK groups were higher than those of the control group (Figs. 1–5). As shown in Fig. 1, ALP activity of the PK group in the duodenum was significantly higher than for the control group (p<0.05). In the proximal jejunum (Fig. 2), both PK and MK group values were significantly higher than for the control group (p<0.05, respectively). ALP activity in the distal ileum was significantly higher in the MK group than the control group (p<0.05, Fig. 5).

The results of the inhibition and thermal inactivation experiments in the duodenum are shown in Table 2. The inhibition and thermal inactivation experiments showed that L-phenylalanine was a strong inhibitor of ALP in the duodenum and that the enzyme was heat-stable (60°C, 10 min). This inhibition pattern is typical

Fig. 4. ALP-specific activity in proximal ileum. Results are the means±SE. Cont.: control. PK: phylloquinone. MK: menaquinone-4.

Fig. 5. ALP-specific activity in distal ileum. Results are the means±SE. Cont.: control. PK: phylloquinone. MK: menaquinone-4. Significant difference between the MK and control groups ("p<0.05).

Table 2. Inhibitory effects on duodenal alkaline phosphatase (%).

<table>
<thead>
<tr>
<th></th>
<th>L-Phenylalanine (20 mM)</th>
<th>Levamisole (1 mM)</th>
<th>Heat stability (60°C, 10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>25.5±0.9</td>
<td>95.6±1.2</td>
<td>55.7±2.6</td>
</tr>
<tr>
<td>PK</td>
<td>24.8±0.5</td>
<td>92.7±3.2</td>
<td>50.8±3.2</td>
</tr>
<tr>
<td>MK</td>
<td>24.2±1.8</td>
<td>95.7±0.9</td>
<td>51.2±4.3</td>
</tr>
</tbody>
</table>

ALP activity was assayed by the rate of hydrolysis of p-NPP. The effect of the inhibitor was determined in the presence of 5 mM MgCl₂ in the assay mixture. Activities of non-treated controls were designated as 100%. Data are expressed as means±SE.
of mammalian intestinal isozymes. There was no significant difference among the values of the three groups.

**SDS-PAGE**

Further identification of the enzyme types by SDS-PAGE was carried out (Fig. 6). In the duodenum (Fig. 6A), there was a main band of 110 kDa in the control, PK, and MK groups. In the proximal jejunum (Fig. 6B), there were two main bands of 110 kDa and 90 kDa detected among the three groups. ALP activity in the distal jejunum, proximal ileum, and distal ileum appeared as a main band of 90 kDa in the control, PK, and MK groups (Fig. 6C, D, E). There was no difference in the molecular size of ALP among the control, PK, and MK groups. In the PK group, the 110 kDa band for intestinal ALP in the duodenum was enhanced markedly (Fig. 6A). As shown in Fig. 6E, the 90 kDa bands for intestinal ALP in the distal ileum were intensified in the MK-4 group.

**DISCUSSION**

In this study, we examined the effect of two forms of vitamin K. phylloquinone (PK: vitamin K₁) and menaquinone-4 (MK-4: vitamin K₂), on intestinal ALP activity in vivo. We reported previously that intestinal ALP activity was increased by fat and lactose feeding (13–15). On the other hand, green tea powder decreased intestinal ALP activity (29). In this study, we clarified the novel action of vitamin K as a nutritional factor that affects intestinal ALP activities.

Both PK and MK-4 had effects of increasing ALP activities in each intestinal segment, and the ALP activities of both the PK and MK groups in the proximal jejunum were significantly higher than in the control group (p<0.05, respectively). Interestingly, PK also significantly increased ALP activity in the duodenum (p<0.05, Fig. 1), while MK significantly increased ALP activity in the distal ileum (p<0.05, Fig. 5).

The biochemical properties of the intestinal ALP preparations of both PK group and MK group, such as the inhibitory effects of levamisole and l-phenylalanine, and the heat stability were identical with those of the control and showed typical intestinal type ALP (Table 2). These results suggested that both PK and MK-4 enhanced intestinal type ALPs.

In a previous report, the cell-free translation of total rat intestinal RNA yielded two independently regulated intestinal ALP isoforms (30) that show different sugar contents of the glycosyl side chains. Two kinds of cDNA clones for rat intestinal ALP, rALP-1 and rALP-2, were isolated (31, 32). The size of rALP-1 mRNA is 2.7 kb and that of rALP-2 mRNA is 3.0 kb (33), and their cDNA sequences show 79% homology at the amino acid level. It was reported that the larger mRNA transcript, rALP-2, exists in the proximal intestine (34). In the proximal intestine, both rALP-1 and rALP-2 isoforms are expressed, while only the rALP-1 isozyme is expressed in the distal part of the intestine (35, 36). However, the physiological functions of the two isoforms have not been clarified.

By SDS-PAGE analysis (Fig. 6), the 110 kDa band was the major isozyme in the duodenum, and both 90 kDa and 110 kDa bands were detected in the proximal jejunum. In the distal part of the intestine, the main isozyme showed the 90 kDa band. We speculated that the 110 kDa isoform is rALP-2, and the 90 kDa isoform is rALP-1. As ALP contains about 20% (w/w) carbohydrates (37), it remains a possibility that even though it is encoded by the same mRNA, the isoforms may be glycosylated differently depending on the localization of the intestine. As shown in Fig. 6, PK enhanced the 110 kDa band for intestinal ALP in the duodenum mainly, while MK-4 enhanced the 90 kDa band for intestinal ALP in the distal ileum. These results may suggest that PK and MK-4 have effect on the enhancement of intestinal ALP activity by different mechanisms.

Although the physiological role of ALP is unknown, strong evidence for its role is provided by the rare genetic disease hypophosphatasia (HPP) (5–12), and TNSALP is thought to play a role in bone mineralization. Quite recently, we revealed a significant association between the TNSALP gene polymorphism 787T>C (Tyr246His) and BMD among 501 postmenopausal women. This result suggests that variations in TNSALP may be important determinants of age-related bone loss in humans, and that the phosphate metabolism pathway may provide a novel target for the prevention and treatment of osteoporosis (38).

As a result of studies on cDNA encoding ALP isoforms, it is known that the primary structure in the catalytic region is well conserved in the ALPs of humans, animals, and *Escherichia coli* (1), suggesting that both intestinal ALP and TNSALP play important roles in active metabolism by hydrolyzing phospho-compounds.

Recently, it was suggested that intestinal ALP may affect not only phosphate metabolism but also fat metabolism by an experiment using intestinal ALP knockout mice. Narisawa et al. reported that fat absorp-

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**Fig. 6.** Rat ALP isozymes separated by polyacrylamide gel electrophoresis. The gel was stained for ALP activity with a β-naphthyl-phosphoric acid monosodium salt, Fast Violet B salt. Std: molecular-size standard (Precision Plus Protein Standards, Bio-Rad Lab., Inc., CA, USA). Cont.: control; PK: phylloquinone; MK: menaquinone-4. A: duodenum; B: proximal jejunum; C: distal jejunum; D: proximal ileum; E: distal ileum.
tion was accelerated in intestinal ALP knockout mice and suggested that intestinal ALP participates in a rate-limiting step regulating fat absorption (39).

PK and MK-4 may have differences in uptake and plasma transport (40). Dietary PK is absorbed from the proximal intestine after solubilization into mixed micelles composed of bile salts (41), although the absorption of K-vitamins has not been clarified. On the other hand, the absorption of MK-4 also occurred at the distal small intestine, and colonic MK-4 absorption occurred even in the absence of bile salts (42, 43). Moreover, MK-4 was absorbed more effectively than PK when given in the same form (44). Transport of PK in the plasma takes place via the triacylglycerol-rich lipoprotein (45), while MK-4 is transported by both triacylglycerol-rich lipoprotein and low-density lipoprotein (LDL) (46).

In conclusion, we clarified that both PK and MK-4 enhanced intestinal ALP activities in rats. Further studies on the mechanism of intestinal ALP activity increased by PK and MK-4 intake would provide useful data on the physiological function of intestinal ALP.

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