Alkaline phosphatase (ALP, EC 3.1.3.1) is an enzyme containing zinc which hydrolyzes monophosphate esters into inorganic phosphoric acid and alcohol at a high optimum pH (pH 8–10). The enzyme is distributed widely throughout the living world from bacteria to animals, excluding plants, and it exists in various tissues such as the intestine, liver, kidney, bone, placenta, stomach, and leukocytes.

In humans, four kinds of ALP isozyme have been identified: tissue-nonspecific ALP (liver/bone/kidney: TNSALP), intestinal ALP (IAP), placental ALP, and germ cell ALP (1–4). The TNSALP gene is located on chromosome 1 and consists of 12 exons and 11 introns, with the coding sequence beginning in the second exon.

A single gene for human IAP has been isolated, and the multiple forms of mRNA encoding human IAP are due to differences in polyadenylation (2). Although most species express a single IAP, several kinds of IAP have been identified in three species: the mouse (5), rat (6, 7), and cow (8).

In rats, ALP is classified into two types: TNSALP and IAP. IAP is present in the membrane surrounding neutral fat droplets in the microvilli of the intestinal mucosa during fat absorption, and is thought to transport dietary lipids from the intestinal tract into the circulation as a component of unilamellar membranes called surfactant-like particles (SLPs) (9). Two different cDNA clones, IAP-I and IAP-II, for rat IAP were isolated by Lowe et al. (6) and Strom et al. (7), respectively. Strom et al. found that the expression of IAP-II mRNA was specifically enhanced by 1α,25(OH)₂D₃ administration. The two isoforms are products of two distinct genes and their cDNA sequences show 79% homology at the amino acid level. Functional differences between IAP-I and IAP-II were suggested by the differing regulation of the expression of the two mRNAs (10), as well as by structural and catalytic differences (11).

In mice, five different ALP loci have been identified: TNSALP, IAP, embryonic ALP (EAP), Akp6, and Akp-ps1. These ALP genes code for different proteins: Akp2 encodes TNSALP, Akp3 encodes IAP, Akp5 encodes EAP, Akp6 encodes a novel IAP-like isozyme expressed globally in the gut (thus called gIAP), and Akp-ps1 encodes...
the inactive pseudo-type ALP (5, 7).

Previously, we reported the enhanced effects of vitamin K on IAP activity in rats (12). Sprague-Dawley rats (6 wk old) were divided into three groups: a control (AIN-93M diet), phylloquinone (PK: 600 mg/kg diet), and menaquinone-4 (MK-4: 600 mg/kg diet) diet group. After 3 mo of feeding of vitamin K, the animals were fasted overnight. The small intestine was removed and divided into five segments. In each segment, both PK and MK-4 increased IAP activity (12).

Vitamin K acts as a cofactor for γ-glutamyl carboxylase (GGCX), and is well-known to participate in the activation of blood coagulation factors and bone mineralization (13). All forms of vitamin K have 1,4-naphthoquinone as a common ring structure, and natural vitamin K exists in two molecular forms, vitamin K1 (phylloquinone: PK) and vitamin K2 (menaquinone: MK-n). PK is abundant in green vegetables in a compound with a phytol side chain. Vitamin K2 is classified into MK-1–14 due to the repeat structure of the side chain, with isopren comprising the side chain. MK-4 shows marked physiological activities as a vitamin K, and is included in many animal-based foods such as meat. Recent studies have demonstrated the possibility that vitamin K regulates the expression of bone-related genes such as ALP through steroid X receptor (SXR, also termed pregnane X receptor: PXR) (14).

In the present study, we examined whether the enhancing effect of PK or MK-4 administration on IAP activity occurs via the intestinal mucosa directly, and we revealed the effects of the oral administration of PK or MK-4 on the expression of IAPs (Akp3 and Akp6) and PXR in the mouse intestine.

MATERIALS AND METHODS

Experimental animals. The care and use of mice in the present study followed the guidelines of governmental legislation in Japan on the proper use of laboratory animals, and the study protocol was approved by the Institutional Review Board of Japan Women’s University. All mice were 21 male ICR strain mice (7 wk old) were used (31.1±0.2 g). They were fasted overnight with free access to water. On the following day, the animals were given 0.1 mL of solution via an intragastric tube: vehicle (physiological saline) for the control group (Cont.), PK (3 mg/kg mouse) for the PK group, and MK-4 (3 mg/kg mouse) for the MK group. The molecular weights of PK (C_{3}H_{14}O_{2}: MW=450.7) and MK-4 (C_{1}H_{4}O_{2}: MW=444.7) are very similar. PK and MK-4 were kindly supplied by Eisai Co., Ltd. (Tokyo, Japan).

Serum and tissue sampling. Four hours after administration, blood was collected from the abdominal aorta under ether anesthesia, and perfusion with saline was performed until the liver was blanched, in order to minimize the blood contamination of tissue samples. The small intestine was removed and divided into three regions. From the pylorus, we took the first 1 cm as the duodenum, and then separated the remaining part into the jejunum and ileum. The segments were slit longitudinally, rinsed with ice-cold saline, and scraped from the mice just after dissection. Each sample was homogenized using a Polytron homogenizer (Kinematica, Switzerland) with 10 mM Tris-buffered saline containing 1% Triton X-100 (pH 7.3) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The supernatant obtained after centrifugation at 7,000 × g for 15 min was used as the enzyme extract.

Enzyme assay. ALP activity was determined with 10 mM p-nitro-phenolphosphate as a 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 (15). To analyze the biochemical properties of ALP, an inhibitory assay using levamisole (Lev) and L-phenylalanine (L-Phe) and a thermostability assay were performed, as previously described (15).

The enzyme activity was defined as the rate of hydrolysis of p-nitro-phenolphosphate and expressed in units (U=μmol p-nitro-phenol formed/min).

Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyacrylamide gel (7.5%) electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out according to the method of Weber et al. (16). After electrophoresis, ALP isozymes separated in the gel were stained by the coupling of β-naphthyl-phosphoric acid monosodium salt with Fast Violet B salt (17).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the intestinal mucosa was extracted employing the acid guanidinium thiocyanate-phenol-chloroform method (18). As a template for PCR, single-strand cDNA was prepared from 1 μg of total RNA using Ready-to-go You-Prime First-Strand Beads (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). PCR primers were used for Akp3 (19), Akp2 (20), Akp5 (21), and PXR (22). In order to detect Akp6, sense (Akp6-up) and anti-sense (Akp6-down) primers were designed on the basis of the Akp6 nucleotide sequence (GenBank: NCBI sequence data: AK0080000). Akp6-up spans nucleotide positions 882–902 and Akp6-down spans 1.333–1.354 (23). The PCR conditions were as follows: 5 cycles at 94°C (1 min), 50°C (1 min), and 72°C (1 min), and 25 cycles at 94°C (30 s), 55°C (30 s), and 72°C (30 s), followed by 10 min at 72°C. Negative controls were performed with each RT-PCR reaction, omitting the template. The efficiency of reverse transcription was verified by the detection of GAPDH (glyceraldehyde-3-phosphate dehydrogenase, forward: 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse: 5′-TCC ACC ACC AGT CGT TTG TG A-3′), as previously described (23).

The amplified samples were analyzed using 5.25% polyacrylamide gel electrophoresis (PAGE). The gels were stained with ethidium bromide and observed under UV light. The band intensity on PCR photographs was quantified by densitometry (AE6920M, ATTO, Tokyo, Japan). The PCR product was normalized to the intensity of the band for the house-keeping gene GAPDH, and is expressed as a ratio of the relative band intensity.
Statistical analyses. Values are shown as the mean ± standard error (SE).

Dunnet’s multiple comparison test was used after ANOVA to compare the significance of differences among the control and PK or MK. Differences were considered significant at \( p < 0.05 \). Analysis was conducted using SPSS 18.0J (SPSS, Inc., Chicago, IL, USA).

RESULTS

ALP activity
To examine whether IAP was secreted from the intestinal mucosa on the oral administration of PK or MK, we measured the levels of serum ALP activity (mU/mL) in the control, PK, and MK groups, being 13.7 ± 1.2, 10.3 ± 2.0, and 14.1 ± 1.8 (mean ± SE), respectively, showing no significant differences among these groups.

ALP-specific activities in the intestine are shown in Fig. 1. There were no significant differences in ALP activities among these groups in the duodenum (Fig. 1A) and ileum (Fig. 1C). As presented in Fig. 1B, ALP activity of the MK group in the jejunum was significantly higher compared with the control group (\( p < 0.05 \)).

Molecular weight determination by SDS-PAGE
The molecular weights of ALPs of each intestinal segment were estimated employing SDS-PAGE analysis. As shown in Fig. 1D, the 110-kDa band of the major ALP isoyme was detected in the duodenum among these groups. In the jejunum, ALP enzymes were separated into two bands of 110 and 90 kDa, and the intensity of their enzymatic activity increased markedly in both PK and MK groups, similarly to the results regarding the specific ALP activity in the jejunum. In the distal part of the intestine (ileum), the ALP isoyme showed a main band of 90 kDa among these groups. No additional band was observed in any intestinal samples among the groups.

Table 1. Inhibitory effects of levamisole, L-phenylalanine and heat inactivation of ALP preparations of the jejunum.

| Groups | Relative activity(%) |
|--------|----------------------|
|        | Levamisole (1 mM)    | L-Phenylalanine (20 mM) | Heat inactivation (60˚C 10 min) |
| Cont.  | 95.2±1.6             | 23.0±1.0                 | 42.4±3.4                     |
| PK     | 94.2±2.1             | 23.5±1.4                 | 46.1±2.3                     |
| MK     | 95.5±1.6             | 24.2±2.0                 | 45.9±2.5                     |

Each value represents mean ± SE (n=7).

The ALP activity was assayed based on the rate of p-NPP hydrolysis. The effect of the inhibitor was determined in the presence of 5 mM MgCl2 in the assay mixture. Remaining ALP activity with inhibitors or after heat treatment is expressed as a percent of non-treated controls. Results are the mean ± SE of 7 animals.

Properties of ALP in the intestine
The enzymatic properties of ALP preparations of the jejunum which increased significantly on PK or MK administration were investigated employing an inhibition experiment with levamisole (Lev) and L-phenylalanine (L-Phe) and through a thermo-stability test. It is well known that IAP activity is not inhibited by Lev and is more stable to L-Phe and more heat-stable than TNSALP. As shown in Table 1, there was no significant
Vitamin K Induces IAP Gene Expression

We confirmed that these jejunum ALP preparations were effectively inhibited by L-Phe but not by Lev, and were heat-stable (60°C, 10 min), corresponding to the property of other mammalian intestinal type ALPs.

**RT-PCR analysis of ALP mRNA expression**

RT-PCR-based detections of mRNA for *Akp3*, *Akp6*, and PXR in the duodenum, jejunum, and ileum are shown in Fig. 2. The PCR products of *Akp3* (335 bp) were detected in the duodenum and jejunum. The PCR products of *Akp6* (473 bp) were detected in the duodenum, jejunum, and ileum. The PCR products of PXR (602 bp) were detected in the duodenum, jejunum, and ileum. The intensities of mRNA expression of *Akp3* and *Akp6* were very similar both in the duodenum and ileum among the three groups (Cont., PK, and MK groups). The intensities of mRNA expression of *Akp3*, *Akp6*, and PXR were enhanced in both the PK and MK groups compared with the control group in the jejunum.

PCR products of *Akp2* (198 bp) and *Akp5* (500 bp) were not detected in any of these intestinal samples.

In order to compare these intensities of mRNA expression in the jejunum, we determined the relative density of the PCR products of the mouse jejunum. As shown in Fig. 3A, the intensities of *Akp3* expression increased significantly in the PK group compared with the control group (*p*<0.01). Furthermore, the intensities of *Akp6* expression were also increased in both the PK and MK groups compared with the control group (*p*<0.01, *p*<0.05, respectively) (Fig. 3B). Interestingly, the intensities of PXR expression in both the PK and MK groups were significantly higher than in the control group (*p*<0.05, *p*<0.01, respectively) (Fig. 3C).

**DISCUSSION**

Previously, we reported that several dietary factors such as fat-feeding, vitamin K, and lactose increased IAP activities in rats (12, 15, 24). The high-level activity of IAP, which localizes at the brush border of intestinal epithelium cells, suggests the participation of this enzyme in the transport of nutrients.

Recently, we reported that both long-term dietary PK and MK-4 supplementations enhance IAP activity in rats (12). After 3 mo of feeding, we measured IAP activity by dividing it into five segments. In each segment, both PK and MK-4 increased IAP, and the level of IAP activity in the proximal jejunum was significantly
higher than that in the control group (p<0.05) (12). To examine whether the enhancing effect of PK or MK-4 on IAP activity occurs via the intestinal mucosa directly, we performed an oral administration of PK or MK-4 using mice. In the present study, we discovered that the levels of mouse jejunum ALP activity were also significantly increased by the oral administration of PK or MK compared with the control group (Fig. 1B). In addition, we confirmed that the increased ALP isozymes induced by the oral administration of PK or MK showed similar biochemical properties to the typical intestinal type ALP, with no significant differences among these groups (Table 1).

By SDS-PAGE analysis, we detected a 110-kDa ALP enzyme in the duodenum and 90-kDa ALP enzyme in the ileum (Fig. 1D). Both the 110- and 90-kDa ALP enzymes were detected in the jejunum, and the enzymatic activities of these bands were enhanced by the oral administration of PK or MK. The product of the Akp3 gene was expressed specifically in the duodenum, and the product of Akp6 was expressed through the small intestine (5). Therefore, we considered that the 110- and 90-kDa ALP enzymes may correspond to the IAPs encoding Akp3 and Akp6, respectively.

We then performed RT-PCR analysis in order to examine the expression of IAPs (Akp3 and Akp6) in the mouse jejunum. PCR products for Akp3 and Akp6 mRNAs in the jejunum were detected, and a significant increase in the PCR products of Akp3 due to the oral administration of PK was observed (Fig. 3A). Moreover, a significant increase in the PCR products of Akp6 due to the oral administration of PK or MK-4 was also observed (Fig. 3B). These results suggest that the induction of Akp3 and Akp6 may be regulated by PK or MK-4.

As the results of RT-PCR, the expression of mRNA for PXR was detected in the duodenum, jejunum and ileum, and it was enhanced significantly in both the PK and MK groups in the jejunum compared with the control group (Fig. 3C). Interestingly, the intestinal segment where the expression of mRNA for PXR by vitamin K had been enhanced corresponded to a similar segment where the expression of mRNA for IAP was enhanced.

Recent studies have revealed that vitamin K functions as a ligand for nuclear steroid and xenobiotic receptor (SXR), as well as a cofactor for γ-carboxylase (25). SXR is expressed predominantly in the liver and intestine, and it regulates transcription such as of cytochrome P450 (CYP) 3A4, which is an enzyme involved in drug metabolism, and MDR1 (multidrug resistance protein 1) which is activated by a diverse array of pharmaceutical agents including taxol, rifampisin, and clorimazole (26, 27). Ichikawa et al. identified novel SXR target bone-related genes that were regulated by MK-4 in osteoblastic cells using microarray analysis (28). Among extracellular matrix-related genes, they demonstrated that a small leucin-rich repeat proteoglycan, tsukushi, contributes to collagen accumulation (28).

We demonstrated for the first time that the oral administration of vitamin K (both PK and MK-4) enhanced the level of IAP mRNA expression in the mouse intestine, and PXR mRNA expression also increased. Further studies on the physiological functions of ALP and transcriptional regulation of ALP induction will provide useful data on the novel effect of vitamin K.

Acknowledgments

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 20500725).

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