Effects of Ovariectomy on Intestinal Alkaline Phosphatase Expression in Rats

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Summary The aim of this study was to investigate the effects of ovariectomy (OVX) on intestinal alkaline phosphatase (ALP) activity in rats. The calcium (Ca) and phosphorus (P) contents and the mechanical strength of bone were decreased significantly by OVX. Two kinds of mRNAs of rat intestinal ALP (RTIN-1 and RTIN-2) were detected by reverse transcription-polymerase chain reaction (RT-PCR). In OVX rats, the level of RTIN-2 mRNA was lowered significantly, while that of RTIN-1 mRNA did not change. This result was compatible with the results of enzymatic activity. This finding suggests the possibility that OVX affects bone metabolism not only directly but also in an indirect way through an intestinal Ca and/or P metabolism via regulation of intestinal RTIN-2 ALP expression.

Key Words alkaline phosphatase (ALP), ovariectomy (OVX), reverse transcription-polymerase chain reaction (RT-PCR), intestine, calcium (Ca) metabolism

Alkaline phosphatase (ALP, EC 3.1.3.1) hydrolyzes a variety of monophosphate esters into inorganic phosphoric acid and alcohol at a high optimum pH (pH 8–10). Studies on the genes of this enzyme revealed that there are four ALP isozymes in the human body: tissue non-specific (liver/bone/kidney) (1), intestinal (2,3), placental (4) and placental-like (5). Based on the studies of hypophosphatasia (6), the tissue non-specific type ALP has been thought to be indispensable for bone mineralization.

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In rats, ALP is classified into two types: tissue non-specific (liver/bone/kidney/placenta) and intestinal (7). Two kinds of cDNA clones, RTIN-1 and RTIN-2, for rat intestinal ALP were isolated by Lowe et al (8) and Strom et al (9), respectively. Their cDNA sequences have 79% homology at the amino acid level.

The level of serum ALP activity is high in some systemic diseases such as rickets (10), calcium deficiency (11) and diabetes (12). In rats, serum ALP is reported to mainly originate from the intestine (13) and also from bone, especially when the animal is young (14). The high activity of intestinal ALP, which localizes at the brush border of the intestinal epithelial cells, suggests the participation of this enzyme in the transport of nutrients such as inorganic phosphate (P) and calcium (Ca) across the membrane (15, 16), but little is known about the physiological function of intestinal ALP.

The increased incidence of fractures accompanying osteoporosis is a serious problem in the aged population. A common type of osteoporosis is the post-menopausal bone loss associated with ovarian hormone deficiency. The balance between bone formation and resorption is regulated by the action of various hormones and growth factors. Therefore, it is important to investigate the effect of ovariectomy (OVX) on various factors related to the bone metabolism. Previously, we reported that OVX decreased osteogenetic activity and tissue non-specific ALP activity in rat bone (17). A decrease in Ca absorption by the intestine in OVX rats has been reported (18). Intestinal ALP seems to have an important role in active metabolism by hydrolyzing phospho-compounds to supply free inorganic phosphate; however, a very small number of reports have been published about the effect of OVX on intestinal ALP. In this study, we attempted to examine the expression of intestinal ALP mRNAs (RTIN-1 and RTIN-2) in OVX rats by reverse transcription-polymerase chain reaction (RT-PCR) analysis.

MATERIALS AND METHODS

Experimental animals. Twelve-month-old female Wistar rats weighing 300 g were used for the OVX study. The animals were divided into two groups of 6 each. Rats in one group were bilaterally ovariectomized under ether anesthesia. Those of the control group were sham-operated. The rats were then fed a low Ca diet (Ca: 0.01%, P: 0.3%, Table 1) for the first 42 days and then switched to a normal Ca diet (Ca: 0.3%, P: 0.3%) for 60 days. The diet and distilled water were given ad libitum to all animals, which were weighed periodically during the study. The animals were fasted overnight and sacrificed by bleeding from the abdominal aorta under anesthesia. In order to examine the effect of OVX on young animals, 6-week-old females of the Sprague-Dawley strain, weighing 140 g, were also studied. They were treated similarly; however, young rats were fed a low Ca diet for the first 28 days and then switched to a normal Ca diet for 28 days.

Determination of mechanical bone strength and Ca and P contents. The breaking-force of femurs dissected from the animals was determined with a

Table 1. Composition of the experimental diets (%).

Constituents	Low Ca diets	Normal Ca diets
	0.01% Ca 0.3% P	0.3% Ca 0.3% P
Glucose monohydrate	65.1	64.7
Casein (vitamin-free)	18.0	18.0
Cottonseed oil	10.0	10.0
Roughage	3.0	3.0
Ca- and P-free salt mixture ^a	2.0	2.0
Equimolar mixture of KH_2PO_4 and K_2HPO_4	1.39	1.03
CaCO_3	0.005	0.74
Cystine	0.2	0.2
Water-soluble vitamin mixture ^b	0.1	0.1
Fat-soluble vitamin mixture	^c	^c
Choline chloride	0.2	0.2

^a Ca- and P-free salt mixture (%): KCl, 57.7; NaCl, 20.9; MgSO_4 , 17.9; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 3.22; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.078; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.004; KI, 0.01; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.06; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.44; and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.005.

^b The water-soluble vitamin mixture (%): thiamine, 0.5; riboflavin, 0.5; pyridoxine, 0.5; calcium pantothenate, 2.8; nicotinamide, 2.0; inositol, 20.0; folic acid, 0.02; vitamin B_{12} , 0.5; biotin, 0.01; and glucose monohydrate, 73.7.

^c The rats received a supplement of the following fat-soluble vitamins in cottonseed oil three times a week: β -carotene, 70 μg ; 2-methyl-1,4-naphthoquinone, 105 μg ; tocopherol, 875 μg ; and vitamin D_3 , 515 IU.

Dynagraph (DYN-1255, Iio) (19). The femurs were dried to constant weight and extracted with 1 N nitric acid for Ca and P analysis. Ca content was measured with an atomic absorption spectrophotometer (type AA-640-12, Shimadzu) and P content by Fiske-Subbarow's method (20).

Preparation and measurement of intestinal enzyme. Rat duodenum segments were removed immediately after sacrifice, freed from adhering tissue and rinsed with ice-cold saline. The segments were slit open longitudinally, and the mucosa was scraped with a piece of slide glass after rinsing and stored at -80°C prior to use. The sample was homogenized with 10 mM Tris-buffered saline containing 1% Triton X-100 using a Teflon homogenizer. The supernatant of centrifugation at $16,000 \times g$ for 15 min was used for the enzyme assay. ALP activity was measured with 10 mM *p*-nitro-phenyl phosphate as the substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer (pH 10.0) containing 5 mM MgCl_2 . Absorption at 420 nm was measured after 10-min incubation at 37°C . Sucrase and trehalase activities were assayed by the Dahlqvist method (21).

RNA isolation and RT-PCR. Rat intestinal mucosa was scraped from the ileum, and the samples from the animals of one group were collected into one lot. Total RNA was extracted by the guanidium-cesium chloride density centrifugation

method (22). Single-strand cDNA was prepared from 1 µg of total RNA using the SuperScript pre-amplification system (Gibco BRL) and was used as a template for PCR. Two sets of specific primers were designed for the rat intestinal ALP sequences (23). For the RTIN-1 nucleotide sequence (8), PCR primers AL7 (1082–1102) and AL8 (1310–1330) were used. For the RTIN-2 nucleotide sequence (9), AL9 (1455–1475) and AL10 (1652–1672) were used. Amplification was performed by a 2-step incubation using a Perkin-Elmer/Cetus DNA thermal cycler (Type 9600) at 94°C (1 min), 50°C (1 min), 72°C (1 min) for 5 cycles and at 94°C (30 s), 55°C (30 s) and 72°C (30 s) for 25 cycles. The PCR products were electrophoresed on a 10% SDS-polyacrylamide gel.

Biochemical analysis of serum. Serum was separated by centrifugation and stored at –80°C prior to use. Ca concentration was measured by the OCPC method (24) and the P content by Machida's method (25). Protein was measured by the Lowry method (26).

Serum ALP activity was measured by the method of Bessey-Lowry (27). The inhibition experiment was carried out with L-homoarginine (L-HA, 0–10 mM), levamisole (LEV, 0–1 mM) and L-phenylalanine (L-PA, 0–20 mM). The inhibitor concentration in mM required for reduction of ALP activity to 50% was determined. In the thermostability test, the enzyme sample was pre-treated at 56°C or 60°C and the activity was measured. The time (in minutes) required for reduction of ALP activity to 50% was determined. In comparison with tissue isozymes, extracts from rat kidney, intestine, liver and calvarium were prepared. The tissue was homogenized in an equal volume of 10 mM Tris-buffered saline containing 1% Triton X-100. The homogenate was treated with 50% *n*-butanol, and the aqueous phase obtained was mixed with an equal volume of acetone. The precipitate was dried and used as a crude ALP preparation.

Polyacrylamide gel electrophoresis. Gradient gel electrophoresis (4–20%) was carried out as described previously (28). After electrophoresis, ALP isozymes separated in the gel were stained by the coupling method of β -naphthyl-phosphoric acid monosodium salt with Fast Violet B salt (29).

Statistics. The data were expressed as mean $\pm$ SD. Statistical significances were evaluated by Student's *t*-test. The difference was judged to be significant when *p* values were less than 0.05.

RESULTS

Effect of OVX on 12-month-old rat bone, serum and intestine

OVX caused a significant decrease in femoral bone strength as compared to the control group (Table 2). Also, bone Ca and P contents decreased significantly after OVX. However, there was no change in the serum Ca and P levels of the OVX and sham-operated rats. These data indicate that osteoporotic changes developed in OVX rats under our experimental conditions. No differences were observed in body weight gain or food efficiency between 12-month-old OVX and

Table 2. Body weight gain, food efficiency, bone breaking force, bone mineral and ALP activities of serum and intestine.

Group (n)	Body wt. gain (g/d)	Food efficiency (body wt. gain/ food intake)	Bone breaking force (10 ⁶ dyn/ 100 g body wt.)	Bone mineral		
				Ca (mg/dry femur wt.)	P (mg/dry femur wt.)	
Sham (6)	0.63 ± 0.13	0.04 ± 0.01	4.54 ± 0.51	0.23 ± 0.01	0.12 ± 0.005	
OVX (6)	0.68 ± 0.18	0.05 ± 0.01	3.87 ± 0.547*	0.21 ± 0.005**	0.11 ± 0.004**	
Group (n)	Serum					
	Ca (mg/dL)	P (mg/dL)	ALP (mU/mL)	ALP (U/protein)	Sucrase (U/protein)	Trehalase (U/protein)
Sham (6)	10.54 ± 0.41	2.77 ± 0.35	107.71 ± 14.1	4.06 ± 1.43	45.26 ± 13.83	77.04 ± 19.06
OVX (6)	10.11 ± 0.34	2.58 ± 0.37	186.83 ± 21.47***	3.82 ± 0.82	48.54 ± 11.96	80.44 ± 10.41

ALP U = μ mol *p*-nitrophenol formed/min, sucrase and trehalase U = μ mol disaccharide hydrolyzed/min under the conditions described in Materials and Methods.

Each value represents mean $\pm$ S.D.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when compared with the sham-operated group using Student's *t*-test.

sham-operated rats. The intestinal ALP activity tended to decrease in OVX rats but with no statistical significance (Table 2). Sucrase and trehalase activities did not change as a result of OVX. Serum ALP activity showed a tendency to increase due to OVX ($p < 0.001$).

Serum ALP isozymes

The results of inhibition and thermostability experiments on 12-month-old rat serum and tissue ALP activities are summarized in Table 3. Among the three inhibitors tested, L-homoarginine and levamisole did not inhibit serum or intestinal ALP activity, but L-phenylalanine inhibited both serum and intestinal ALP. In addition, the thermostability pattern of serum ALP seemed to be similar to that of liver ALP. These data suggest that serum ALP is a mixture of tissue isozymes.

Further identification of the enzyme types by polyacrylamide gradient gel electrophoresis was carried out on young rats (Fig. 1). There were two bands (indicated by arrows "a" and "b") in the rat serum. The upper band (a) is an isozyme corresponding to the bone type ALP which lost its activity as a result of the heat treatment. The activity of this isozyme in serum seemed to increase after OVX (Fig. 1, lane 6).

Table 3. Inhibitory pattern of ALPs in tissues and serum.

Inhibitor	Rat tissues				
	Calvarium	Liver	Kidney	Intestine	Serum
L-Homoarginine					
Activity % at 10 mM (%)	17	63	17	102	105
Concentration for 50% inhibition (mM)	1.47	10<<	1.63	10<<	10<<
Levamisole					
Activity % at 10 mM (%)	2	58	5	100	100
Concentration for 50% inhibition (mM)	0.02	1<<	0.03	1<<	1<<
L-Phenylalanine					
Activity % at 10 mM (%)	83	44	57	21	42
Concentration for 50% inhibition (mM)	20<<	14.7	20<<	3.2	15.0
56°C					
Activity % at 30 min (%)	8	12	1	60	12
Time for 50% inhibition (min)	2.36	4.39	2.84	30<<	5.20
60°C					
Activity % at 30 min (%)	14	7	1	40	10
Time for 50% inhibition (min)	0.99	2.03	0.90	8.70	2.30

Fig. 1. Rat serum and tissue ALP isozymes separated by polyacrylamide gel electrophoresis. Samples were electrophoresed on 4–20% gradient polyacrylamide gel. Serum samples of 15 μ L were applied onto one lane. ALP activity was stained with β -naphthyl-phosphoric acid monosodium salt, Fast Violet B salt. Lanes 1, 2, intestine; 3, 4, calvarium; 5, serum of sham-operated young rat; 6, 7, serum of OVX young rat. Lanes 2, 4 and 7 were pretreated at 56°C for 10 min.

RT-PCR analysis of intestinal ALP mRNA expression

Intestinal ALP mRNA expression was examined by RT-PCR analysis. A 249 bp product was detected by the primer set of AL7 and AL8 for RTIN-1, which was at a similar level in OVX and sham-operated rats (Fig. 2A, lanes 1, 2). On the other hand, the 218 bp band detected by the primer set of AL9 and AL10 for RTIN-2 decreased after OVX (Fig. 2A, lanes 3, 4). Because glyceraldehyde-phosphate dehydrogenase (GAPDH) bands did not change due to OVX (Fig. 2B), the decrease in RTIN-2 mRNA expression is specific for OVX. The same result was obtained in the experiment using young rats. These data coincide with the finding that the enzymatic activity of intestinal ALP tended to decrease in OVX rats. One of the two ALP messages, RTIN-2 expression, was suppressed by OVX, so that the total ALP activity did not dramatically decrease.

DISCUSSION

OVX significantly reduced the mechanical strength and the Ca and P contents of the femur in rats. Under the experimental conditions presented here, serum Ca and P showed no change. This may be because Ca-regulating hormones maintained serum Ca and P at normal levels even in the absence of estrogen. It was evident that OVX produced osteoporotic changes probably because of the altered bone and mineral metabolism in rats. Increase in body weight as a result of OVX has been reported in a previous paper (30). In our experiment, no differences were observed in body weight or food efficiency between OVX and sham-operated old (12-month-old) rats, although there was some body weight gain in young (6-week-old) OVX rats (2.05 ± 0.18 g/d) as compared to the sham-operated rats (1.39 ± 0.11 g/d). Intestinal ALP activity tended to decrease after OVX. There are two forms of ALP in the intestine: one is a membrane-bound type anchored to phosphatidylinositol and the other is a soluble type released from the membrane (31). In this study, the total activity of two types of ALP was determined by extraction with a surfactant, 1% Triton X-100 (32). Sucrase and trehalase are

Fig. 2. Detection of RTIN-1 and RTIN-2 ALP mRNA expression in rat intestine by RT-PCR. Samples were prepared from 12-month-old rat intestine and applied to PCR as described in Materials and Methods. PCR products were electrophoresed on a 10% polyacrylamide gel. A: PCR products generated by the primer set for RTIN-1 from sham-operated (lane 1) and OVX (lane 2) rat intestine, and generated by the primer set for RTIN-2 from sham-operated (lane 3), OVX (lane 4) rat intestine and molecular size marker *phi-X HaeIII* digest (lane 5). B: PCR products generated by the primer set for GAPDH from sham-operated (lane 2), OVX (lane 3) and molecular size marker *phi-X HaeIII* digest (lane 1).

digestive enzymes which are also anchored to the membrane, like ALP (33). Their enzymatic activities showed no change after OVX. Therefore, the decrease in activity caused by OVX is thought to be specific to intestinal ALP. Two types of mRNAs for intestinal ALP were examined by RT-PCR analysis. We confirmed the nucleotide sequences of the PCR products for RTIN-1 mRNA and RTIN-2 mRNA described previously (23). One of the transcripts, RTIN-2 mRNA, was found to be reduced

by OVX. Therefore, the decrease in intestinal ALP activity caused by OVX might result from the suppression of RTIN-2 mRNA transcription.

There is a report showing the elevation of intestinal ALP mRNA by $1\alpha,25(\text{OH})_2\text{D}_3$ treatment (34), and Strom et al (9) found that expression of RTIN-2 mRNA increased with $1\alpha,25(\text{OH})_2\text{D}_3$ administration. We also found that expression of RTIN-2 mRNA in the liver was enhanced by feeding fat (23). These findings support the possibility that the level of RTIN-2 is more variable than that of RTIN-1 in response to bioactive factors.

A decrease in Ca absorption by the intestine in OVX rats has been reported (18), and the possibility was suggested that Ca malabsorption may be induced by blocking $1\alpha,25(\text{OH})_2\text{D}_3$ action because there was a study (35) indicating that the intestinal $1\alpha,25(\text{OH})_2\text{D}_3$ receptor number decreased with estrogen deficiency. It is also believed that the decrease in RTIN-2 mRNA expression caused by OVX may be the result of the decrease in $1\alpha,25(\text{OH})_2\text{D}_3$ receptor expression. The active form of vitamin D enhances both intestinal ALP activity and the absorption rate of Ca and/or P in ligated intestine (36). However, we do not know whether there was a direct involvement of vitamin D in decreasing the ALP mRNA level in this study.

In contrast to the intestinal ALP, serum ALP activity was rather high in OVX rats in response to estrogen deficiency. Several studies suggest that rat serum ALP originates mainly from the intestine (13) and partly from bone, especially in young rats (14). The presence of two ALP bands was shown in the serum by electrophoresis (Fig. 1). The enzymatic activity of the upper band (band "a"), with the same mobility as that of the enzyme extracted from bone, was lost after heat treatment. In our previous study, serum ALP activity in the OVX group was higher than that in the sham-operated group, and heat-stable type ALP was not affected by OVX (17). Elevated serum ALP activity in OVX rats may be due to an increase in bone-derived ALP but not in the intestine-derived enzyme. In the case of human osteoporosis, there is a report on an increase in tissue non-specific type ALP activity (37).

It has been thought that OVX causes experimental osteoporosis by disturbed bone formation or enhanced bone resorption due to lack of estrogen. In addition, this study revealed that malabsorption mediated by the suppression of RTIN-2 ALP expression may occur in OVX rats. Therefore, there is a possibility that the Ca and P supply necessary for bone formation is not sufficient. The data presented here suggests that the nutritional effects of OVX on the intestine must be taken into account because its expression is also regulated by $1\alpha,25(\text{OH})_2\text{D}_3$. RTIN-2 is an important enzyme which may be involved in bone metabolism. Further study on the mechanism of estrogen action on RTIN-2 expression will be necessary in the future.

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