Effects of a High Protein Diet on Bone Formation and Calcium Metabolism in Rats

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Summary The effects of a high protein diet on bone formation and calcium (Ca) metabolism were evaluated in rats using an ectopic endochondral bone induction model. A control diet (18% casein) or a high protein diet (18% casein + 20% lactalbumin) was given to 50-day-old rats. Ten days after the feeding of the experimental diet, rats were intramuscularly implanted with demineralized bone powder (day 0). On day 14 and day 21, the implanted bone powder was harvested, and blood and urine samples were also obtained. Urinary Ca excretion was not increased on day 12-14; however, it was elevated on day 19-21 in rats fed the high protein diet compared with rats fed the control diet. The high protein diet remarkably stimulated urinary sulfate excretion in both sampling periods, which reflected dietary sulfur-containing amino acids contents. Also, rats fed the high protein diet exhibited a decrease in serum Ca concentrations. There was little difference in Ca contents and the activities of alkaline phosphatase and acid phosphatase in the implants between control group and high protein diet group on day 14 and day 21. Histological examination in the implanted demineralized bone powder on day 14 indicated only cartilage in rats fed the high protein diet in contrast to the occurrences of osteogenesis and remodeling in those fed the control diet. Retarded bone formation in rats fed the high protein diet might be owing to, in part at least, a restricted amount of Ca utilized at the stage of cartilage calcification.

Key Words high protein diet, ectopic bone formation, urinary calcium excretion, rat

Since a report by Wachman and Bernstein in 1968 (1) that an increase in protein intake caused hypercalciuria, leading to a body calcium (Ca) loss and an

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induction of osteoporosis, a number of studies have been conducted to observe the effect of a high protein diet on bone and Ca metabolism (2–9). In our previous report (9), it was clarified that urinary Ca excretion was increased and apparent Ca absorption was decreased when growing rats were fed a high protein diet. Reduced retention of Ca might induce impaired bone formation and resorption. Draper and co-workers reported that a high protein diet did not increase the bone resorption using $^{45}$Ca kinetics (3, 6, 8) but radiographic evidence of a reduction in metaphyseal bone density indicating the depression of bone formation was found in rats fed a high sulfur-containing amino acids diet (7).

Using a demineralized bone matrix-induced endochondral bone forming system, Weiss et al. showed that a high protein diet decreased the incorporation of $^{45}$Ca into the bone matrix at the stage of osteogenesis and suggested that the decrease in bone formation was induced by the restricted availability of Ca at the site of mineralization (5). Weiss et al. used an 80% casein-included diet as a high protein diet (5), while other investigators commonly used 35–40% protein diet as a high protein diet (3, 8–10). It was reported that a dietary protein level changed the degree of hypercalciuria (10). Therefore, there is a possibility that a remarkably high level of dietary protein may influence bone formation.

This study was designed to determine the effect of a high protein diet, including 38% of protein, on bone formation and Ca metabolism in rats using an ectopic demineralized bone forming model.

MATERIALS AND METHODS

Twenty Wistar-strain male rats of 50 days old were used in this study. They were individually housed in metabolic cages with a room temperature at 24°C and a 12-h light-dark cycle. They were divided into 2 groups: a control diet group and a high protein diet group, which were similar to those of a previous study (9). The experimental diets are shown in Table 1.

The protein source in the high protein diet was lactalbumin in this study, since it has been reported that high lactalbumin intake caused remarkable hypercalciuria (9, 10) and lactalbumin contains more sulfur-containing amino acids than casein, egg white, and gelatin (10). Each animal from both groups was offered 20.0 g/day of diet and had free access to distilled water.

Demineralized bone powder (10 mg) was intramuscularly implanted into 3 abdominal sites of a rat 10 days after being placed on the experimental diet. The day of implantation was designated as day 0, and all surgical procedures and autopsies were performed between 8:00 and 10:00 h. The implants and blood from the abdominal artery were sampled on day 14 and day 21. Seventy-two-hour urine samples were collected on day 12–14 and day 19–21.

Demineralized bone powder was prepared by the method of Reddi and Huggins (11). The femur and the tibia of Wistar-strain male adult rats were used. The diaphyses without bone marrow were washed with water for 2 h, with absolute
ethanol for 1 h, and with ethyl ether for 0.5 h, in this order. Dried diaphyses were crushed with a hammer and sieved. The powder having particle size of 74–420 μm was demineralized in 0.5 N HCl solution for 3 h at 4°C. Then they were also repeatedly washed with water, absolute ethanol, and diethyl ether (11).

To take histological pictures, the implants of day 14 were stained with hematoxylin-eosin and those of day 21 with the method of von Kossa (12). In a process to measure enzyme activity, the implants were homogenized in saline with a homogenizer (Biotron, Biotrona, Switzerland). After centrifugation of the homogenate, alkaline phosphatase (Alp) activities in supernatant were analyzed by the method of Bessey et al. (13), acid phosphatase (Acp) activities by that of Lowry et al. (14), and protein contents by that of Lowry et al. (15). Ca contents in the implants were measured by atomic absorption spectrophotometry after being wet-ashed.

Ca concentrations in blood serum and urine were analyzed and inorganic phosphorus (P_i) concentrations in these samples were measured by the method of Gomori (16). Blood urea nitrogen concentrations were measured using a commercial kit (Urea N B-test kit, Wako Junyaku, Tokyo) and serum protein concentrations were analyzed by the method of Lowry et al. (15). Urinary sulfate excretion was measured by the method of Swaroop (17).

Data were analyzed by analysis of variance using SAS (Statistical Analysis System) after calculating least squares mean (18). Data were expressed as least squares mean ± SE.

### Table 1. Composition and mineral contents of experimental diets.

|                | Control (g/kg diet) | High protein (g/kg diet) |
|----------------|--------------------|--------------------------|
| Casein         | 180                | 180                      |
| Lactalbumin    | 201                | 201                      |
| Dextrose       | 555                | 359                      |
| Lard           | 150                | 150                      |
| Corn oil       | 50                 | 50                       |
| Cellulose      | 25                 | 25                       |
| Vitamin mix^a  | 6.0                | 6.0                      |
| Mineral mix^b  | 14.7               | 14.7                     |
| CaCO_3         | 12.1               | 10.2                     |
| Ca(H_2PO_4)_2·H_2O | 6.8      | 3.8                      |

*One gram of vitamin mixture contains 500 IU of vitamin A, 100 IU of vitamin D_3, 5 mg of vitamin E acetate, 5.2 mg of vitamin K, 1.2 mg of vitamin B_6, HCl, 4 mg of vitamin B_12, 0.8 mg of vitamin B_12, HCl, 500 mg of vitamin B_12, 30 mg of vitamin C, 20 μg of D-biotin, 200 μg of folic acid, 5 mg of Ca-pantothenate, 5 mg of p-aminobenzoic acid, 6 mg of nicotinic acid, 6 mg of inositol, and 200 mg of choline chloride. Supplying (mg/kg diet): MgCO_3, 6,900; ZnCO_3, 96; FeSO_4·7H_2O, 124; CuSO_4·5H_2O, 0.20; MnSO_4·H_2O, 150; KI, 1.3; NaCl, 2,300; Na_2CO_3, 1,300; K_2CO_3, 3,530; Na_2SeO_3, 0.22.
RESULTS

Serum concentrations of Ca, Pi, BUN, and total protein are shown in Table 2. When the considered factors by analysis of variance were the dietary treatment (control or high protein) and the sampling time (day 14 or day 21) on serum data,

Table 2. Effects of a protein diet on serum values.

| Diet          | Control (mg/100 ml) | High protein (mg/100 ml) |
|--------------|---------------------|--------------------------|
| Ca           | 10.00±0.07          | 9.70±0.11*               |
| Pi           | 6.30±0.18           | 6.54±0.19                |
| BUN          | 16.00±1.01          | 22.93±1.40**             |
| Protein      | 8.40±0.09           | 8.68±0.11                |

Values are least squares mean±SE. *Significantly different from control group (p<0.05, 0.01, respectively).

Table 3. Effects of a high protein diet on urinary excretion (mg/24 h).

| Diet          | Control (day 12–14) | High protein (day 12–14) |
|--------------|---------------------|--------------------------|
| Ca           | 0.70±0.19           | 0.78±0.25                |
| Pi           | 2.63±0.58           | 10.42±1.16**             |
| Sulfate      | 1.50±0.29           | 46.42±4.96**             |

Values are least squares mean±SE. **Significantly different from control value (p<0.05, 0.01, respectively).

Table 4. Effects of a high protein diet on Ca contents and enzyme activities in implanted demineralized bone powder.

| Diet          | Control (day 14) | High protein (day 21) |
|--------------|------------------|-----------------------|
| Ca           | 8.84±1.03        | 20.91±3.93            |
| Alp*         | 1.407±0.250      | 0.957±0.187           |
| Acp*         | 1.503±0.276      | 1.850±0.302           |

Values are least squares mean±SE. *mmol substrate utilized per mg soluble protein per hour at 37°C.
Fig. 1. Histological observation of the implanted demineralized bone powder in rats on day 14. (×100) Paraffin sections stained with hematoxylin and eosin. (a): Rats fed the control diet. Note bone matrix (M) and multinucleated osteoclast (arrows). (b): Rats fed the high protein diet. Note prominent chondrocytes (arrow) and the absence of bone formation.

The effect of the sampling time was not significant. Therefore, Table 2 shows the least squares mean concentrations of serum samples obtained from both day 14 and day 21. A decrease in serum Ca concentration and an increase in serum urea nitrogen concentration were observed in rats fed the high protein diet. Serum P_
and protein concentrations were not affected by the dietary treatment.

Urinary metabolites excretion on day 12–14 and day 19–21 are shown in Table 3. Although the high protein diet increased urinary Ca excretion on day 19–21, the increase in urinary Ca excretion was not found on day 12–14. Urinary P, excretion was remarkably accelerated in rats fed the high protein diet both on day 12–14 and on day 19–21. Rats fed the high protein diet excreted more sulfate, which appeared to be proportional to dietary sulfur-containing amino acids contents. According to a calculation on the basis of the formula (7), the high protein diet contains 1.47% sulfur-containing amino acids (1.28% cystine + 0.19% methionine).

Biochemical properties in the implanted demineralized bone powder are shown in Table 4. Since Ca contents in the implant had a wide variation in the same dietary group on day 14 and day 21, the difference between the control diet and the high protein diet group was not observed. Also, both Alp and Acp activities appeared not to be affected by the dietary treatment on day 14 and day 21.

Figure 1 shows a histological observation in the implanted demineralized bone powder on day 14. Reddi and Anderson observed the maximal osteogenesis and remodeling on day 14 and the maximal hematopoietic bone marrow differentiation in the newly formed ossicle on day 21 (19). The implant in rats fed the control diet exhibited active osteoblasts and a small number of osteoclasts, indicating osteogenesis and remodeling (Fig. 1a). On the contrary, rats fed the high protein diet did not induce osteogenesis but exhibited only cartilage at the same time (Fig. 1b). The histological picture on day 14 indicated that rats fed the high protein diet retarded the former stage(s) of cartilage calcification of endochondral bone formation as compared to rats fed the control diet. On day 21, there was no apparent difference in Ca deposition surrounding the bone marrow between the two dietary groups.

**DISCUSSION**

Rats fed the high protein diet excreted more Ca in urine on day 19–21, while any difference in urinary Ca excretion was not found between the control diet group and the high protein diet group on day 12–14. Whiting and Draper have reported the similar result by using a similar dietary composition in their study (10). Urinary Ca excretion in rats fed the high protein diet was rapidly increased within 2 days, then steadily decreased to near control values after 3 to 4 weeks, followed by a moderate stimulation (10).

Although the reason(s) why a high protein diet increases urinary Ca excretion has been investigated in men (20–25), rats (3, 6–8, 9, 26–28), and sheep (29), it is not yet completely elucidated. Schuette et al. suggested that mild metabolic acidosis resulting from the oxidation of excess amino acids would be responsible for a depressed Ca reabsorption by renal tubule cells, leading to hypercalciuria in men (21, 24). However, in our previous study, a high protein diet did not increase urinary net acid excretion in rats (9) and sheep (29).
Whiting and Draper studied a relationship between dietary sulfur-containing amino acids contents and urinary Ca excretion and suggested that, in renal tubule fluid, CaSO₄-complexes which would be poorly reabsorbed by renal tubule cells were formed and urinary Ca excretion was increased (10). However, Zemel et al. showed that urinary Ca excretion in men fed sulfur-containing amino acids was not as much as that in men fed a high protein diet (25). It was also reported that sulfur-containing amino acids supplementation to a control diet failed to increase urinary Ca excretion (20). Rats fed the high protein diet excreted more sulfate in urine than those fed the control diet both on day 12–14 and on day 19–21. The high protein diet, however, did not increase urinary Ca excretion on day 12–14. Accordingly, a close relationship between urinary Ca excretion and urinary sulfate excretion could not be found in rats fed the high protein diet. The elevation of urinary Ca excretion in rats fed the high protein diet on day 19–21 was thought to have little relationship to urinary sulfate excretion.

Since protein-induced hypercalciuria appeared not to result from stimulated Ca absorption from the gastrointestinal tract (2, 4, 9), a high protein intake might induce to a body Ca loss. A slight depression of serum Ca level in rats fed the high protein diet might reflect an increase in Ca excretion via urine and feces. The histological examination of implanted demineralized bone powder showed the retardation of bone formation at the stage(s) of cartilage calcification in rats fed the high protein diet, although the Ca content and the enzyme activities of implants were unaffected by the dietary treatment. The restricted amount of Ca utilized at cartilage calcification was thought to cause, in part at least, the retardation and the failure of bone formation.

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