The Influences of Casein Phosphopeptides on Metabolism of Ectopic Bone Induced by Decalcified Bone Matrix Implantation in Rats

Tohru Matsui, Hideo Yano, Takako Awano, Tadashi Harimoto, and Yasuhiro Saito

Department of Animal Science, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan
1Department of Applied Biological Science, Faculty of Agriculture, Shimane University, Matsue 690, Japan
2Physiological Chemistry Division, Bioscience Laboratories, Meiji Seika Kaisha, Sakado 350-02, Japan

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Summary The effect of casein phosphopeptides (CPP) on bone metabolism was studied in the ectopic bone induced by the implantation of decalcified bone matrix in rats. Forty-two Wistar male rats of 7 weeks old were fed low calcium diets (0.39% of calcium) with or without supplying 0.50% of CPP, or a control diet (0.91% of calcium) without CPP supplementation. After a 1-week preliminary period, each rat was subcutaneously implanted with 30 mg of demineralized bone matrix powder. Fourteen and 21 days after the implantation, the implants were harvested from 7 rats of each group. Calcium content in the graft was not significantly different among all groups on day 14. Subsequently, the content of calcium rapidly increased in the grafts irrespective of diets given. However, the graft of the CPP− group contained less calcium than the other groups and the calcium content was more in the control rats compared to the CPP+ animals on day 21. Alkaline phosphatase activity (an index of bone and cartilage calcification) was lower in the control group than in the CPP+ group on day 14. The enzyme activity subsequently decreased in the control group but the activity was not changed in the other groups. As a result, the activity of alkaline phosphatase was lower in the control animals than in the other rats on day 21. Tartrate-resistant acid phosphatase activity (an index of bone resorption) was higher in the CPP− group compared to the control on day 14. On day 21, the activity was higher in the CPP− group compared to the others. Histological study indicated that the number of osteoclastic cells was larger in the CPP− rats than in the other animals on day 21. These results suggest that CPP supplementation mitigates the reduction of calcium content in bone of rats fed a low calcium diet and that this action
of CPP is due to the suppression of bone resorption, which is involved in the reduction of osteoclast differentiation.

**Key Words** casein phosphopeptides, bone metabolism, ectopic bone induction, rat

Casein phosphopeptides (CPP) have been reported to stimulate calcium absorption *in vitro* (1) and *in situ* (1-3). The stimulative action was suggested to be owing to its ability to increase solubility of calcium in the digestive tract (2). Lee et al. (4) indicated that CPP increased the apparent absorption of calcium and its retention in rats. On the other hand, there are controversial reports about the action of CPP *in vivo*. Scholz-Ahrens et al. (5) reported that CPP did not affect apparent calcium absorption and its retention in rats and pigs. In addition, Shah et al. (6) reported that a synthetic analogue of CPP did not affect apparent calcium absorption in rats. Because almost all of calcium is deposited to bone and bone calcium is easily exchangeable to calcium in the extracellular fluid, calcium in bone is thought to reflect calcium bioavailability. It was reported that the supplementation of CPP to a low calcium diet increased calcium content in the femur (4). In addition, CPP was suggested to enhance the deposition of radioactive calcium into the femur of rats (2) and the tibia of chicks (1) when the ligated ileal segment was perfused with radioactive calcium and CPP. However, Yuan and Kitts (7) showed that CPP did not affect the deposition of radioactive calcium in rats using the same method. Furthermore, it was reported that the supplementation of CPP did not affect bone mineralization in rats and pigs (5).

It is not clear why these discrepancies in CPP actions occur. However, the apparent absorption of calcium is not a sensitive method to assess bioavailability of calcium because the homeostatic mechanisms affect calcium absorption and an apparent absorption of calcium is affected by endogenous calcium excretion in feces. In addition, as Sinha et al. (8) suggested, detectable changes in skeletal integrity usually require a long period because of the relative stability of bone. It is possible that the stimulative action of CPP on calcium absorption is so slight that the changes in apparent absorption of calcium and bone metabolism were not detected in some cases.

It has been reported that subcutaneous implantation of demineralized bone matrix induces bone within 2 weeks (9) and the bone induction is used to study the effects of nutritional factors on bone metabolism (8, 10-12). In the present report, the effect of CPP administration on bone metabolism was examined using the ectopic bone induced by implantation of demineralized bone matrix in rats.

**EXPERIMENTAL**

Demineralized bone matrix powder was prepared by the method of Huggins et al. (13), i.e., the diaphysis of long bones of Wistar rats were cleaned of the bone...
marrow and the adherent soft tissue. The bones were washed with water and then crushed and sieved to obtain particles of 200–400 μm. The powder was demineralized by 0.5 mol/liter HCl for 3 h then washed in distilled water for 2 h. The demineralized bone powder was subsequently defatted with ethyl alcohol and diethyl ether.

Forty-two male Wistar rats of 7 weeks old were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). These animals were housed individually in wire stainless cages. They were divided into 3 dietary groups of 14 rats each; a control diet, a low calcium diet with the supplementation of CPP (CPP+) and low calcium diet without the supplementation of CPP (CPP−). The diets mainly consisted of soybean protein and corn starch. Casein phosphopeptides was added to 0.5% of CPP+ diet. To adjust the contents of nitrogen, calcium, and phosphorus, the contents of soy protein, calcium carbonate, and monopotassium phosphate were changed among the diets (Table 1). The analyzed contents of calcium were 9.1 and 3.9 g/kg in the control diet and the low calcium diets, respectively, and the phosphorus content was 8.0 g/kg in all diets. According to National Research Council (14), calcium and phosphorus requirements of growing rats are 5 and 4 g/kg, respectively and the ratio of calcium and phosphorus between 1:1 and 1.5:1 is optimal. The low calcium diets used in the present experiment contained less calcium than its requirement for growing rats.

Table 1. Ingredients of diets (g/100 g).

|                | Control | CPP− | CPP+ |
|----------------|---------|------|------|
| Soybean protein | 20      | 20   | 19.59|
| CPP            | 0       | 0    | 0.5  |
| Starch         | 63.53   | 64.78| 64.8 |
| Corn oil       | 5       | 5    | 5    |
| Cellulose powder | 0.5    | 0.5  | 0.5  |
| Mineral mixture| 4       | 4    | 4    |
| Vitamin mixture| 2       | 2    | 2    |
| CaCO3          | 1.86    | 0.61 | 0.55 |
| KH2PO4         | 3.01    | 3.01 | 2.96 |
| Choline chloride | 0.1   | 0.1  | 0.1  |

1Diet does not contain casein phosphopeptides. 2Diet contains casein phosphopeptides. 3Fujipro R, Fuji Oil Co., Osaka, Japan. 4Casein phosphopeptides (CPP–3), Meiji Seika Kaisha, Tokyo, Japan. 5Oriental Yeast Co., Tokyo, Japan. 6The mineral mixture had the following composition: NaCl, 250 g/kg; MgSO4·7H2O, 100 g/kg; CaHPO4·2H2O, 135.7 g/kg; FeSO4·7H2O, 5.6 g/kg; CuSO4·5H2O, 2 g/kg; ZnSO4·7H2O, 1.5 g/kg; MnSO4·4H2O, 1.4 g/kg; Ca(IO3)2, 14 mg/kg; CoSO4·7H2O, 5 mg/kg. 7The vitamin mixture had the following composition: retinyl acetate, 466 IU/g; cholecalciferol, 233 IU/g; dl-α-tocopheryl acetate, 12 mg/g; thiamine HCl, 0.59 mg/g; riboflavin, 0.59 mg/g; pyridoxine HCl, 0.29 mg/g; cyanocobalamin, 2 mg/kg; ascorbic acid, 5.88 mg/g; dl-biotin, 10 mg/kg; folic acid, 20 mg/kg; Ca pantothenate, 2.35 mg/g; nicotinic acid 2.94 mg/g; myo-inositol, 11.76 mg/g.
One week after the initiation of feeding the experimental diets, each animal was subcutaneously implanted with 3 gelatin capsules (No. 5, Eli Lilly, Indianapolis, IN) containing approximately 30 mg of the demineralized bone matrix powder under diethyl ether anesthesia.

Fourteen and 21 days after the implantation, the implants were harvested from 7 rats of each dietary group. Two grafts from each rat were free of adherent tissue for measuring calcium content and enzyme activities. For measuring enzyme activities, a cleaned graft in each rat was homogenized in 0.15 mol/liter NaCl containing 3 mmol/liter NaHCO₃ at 4°C by Ultra disperser (Yamato Scientific Co., Tokyo, Japan) with 32G generator. The homogenates were centrifuged for 30 min at 4,500×g at 4°C and the activities of alkaline (EC 3.1.3.1) and tartrate-resistant acid (EC 3.1.3.2) phosphatase, and protein concentration in the supernatants were determined. Protein concentration was measured by the method of Lowry et al. (15). The activity of the enzymes was determined by the amount of p-nitrophenol production from p-nitrophenylphosphate and one unit of enzyme activity was defined as 1 µmol of p-nitrophenol production for 30 min at 37°C. The solution used in the assay of alkaline phosphatase activity (ALP) was 6.7 mmol/liter disodium p-nitrophenylphosphate (Wako Pure Chemical Industries Co., Osaka, Japan) and 100 mmol/liter sodium carbonate buffer (pH 9.8). The solution for the assay of tartrate-resistant acid phosphatase activity (TR-ACP) was 6.7 mmol/liter disodium p-nitrophenylphosphate, 50 mmol/liter sodium citrate buffer (pH 4.9) with 20 mmol/liter sodium tartrate. One graft from each rat was ashed by nitric and perchloric acids and calcium content in the solution was measured by an atomic absorption spectrophotometry. The other grafts were fixed in Bouin's fluid and five 3-µm sections were stained with hematoxylin and eosin. The number of multinucleated osteoclastic cells attached to the implanted powder was counted in about 15 contiguous fields per section (area per field was 0.67 mm²) in the grafts harvested on day 21. All data other than histological measurements were analyzed by analysis of variance and Duncan's multiple range test (16). A p value of less than 0.05 was considered significant.

RESULTS

Table 2 shows calcium contents and enzyme activities in the grafts. Calcium content was not different among all the groups on day 14. Then the content of calcium rapidly increased irrespective of diets given. However, the graft contained significantly less calcium in the CPP+ group than in the control on day 21. Meanwhile the calcium content was significantly more in the CPP+ rats compared to the CPP− animals. Alkaline phosphatase activity was significantly higher in the CPP+ group than in the control group on day 14. Subsequently, ALP activity decreased in the control group. The activity, however, did not change in the other groups. As a result, the activity of ALP was significantly lower in the control group than in the other groups on day 21. The activity of TR-ACP was significantly lower

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Table 2. Effect of casein phosphopeptides supplementation on calcium contents and activities of enzymes in the graft induced by the subcutaneous implantation of bone matrix powder.

|                | Calcium (mg/dry weight) | Alkaline phosphatase (unit/mg protein) | Tartrate-resistant acid phosphatase (unit/mg protein) |
|----------------|-------------------------|----------------------------------------|---------------------------------------------------|
| Day 14         |                         |                                        |                                                   |
| Control        | 12.6^a                  | 5.72^a                                 | 1.55^a                                            |
| CPP−           | 9.9^a                   | 6.71^ad                                | 1.77^bc                                           |
| CPP+           | 11.7^a                  | 7.84^ad                                | 1.71^abc                                           |
| Day 21         |                         |                                        |                                                   |
| Control        | 29.7^b                  | 3.48^b                                 | 1.42^a                                            |
| CPP−           | 21.4^c                  | 7.43^ad                                | 1.98^b                                            |
| CPP+           | 25.9^d                  | 8.22^e                                 | 1.60^ce                                           |
| SE             | 0.9                     | 0.28                                   | 0.07                                               |

Values are means for 7 rats. One unit of enzyme activity was defined as 1 µmol of p-nitrophenol production for 30 min at 37°C. ^a Rats fed the control diet. ^b Rats fed the low calcium diet without casein phosphopeptides. ^c Rats fed the low calcium diet with casein phosphopeptides. ^d Means in the same row with unlike superscripts significantly (p<0.05) differ.

in the control group than in the CPP− group on day 14, and there was no difference between the CPP+ and the other groups. On day 21, TR-ACP activity was significantly higher in the CPP− group compared to the other groups.

The histological study indicated that calcified cartilage was prominent and some basophilic osteoblasts appeared near the implanted bone matrix in all groups on day 14 (figures not shown). There were not clear differences among the three dietary groups. On day 21, calcified cartilage disappeared and many osteoblasts existed in each group (Fig. 1a-c). Some multinucleated osteoclastic cells also appeared near the implanted bone matrix in every group. The numbers of these cells were 0.78±0.42, 1.38±0.62, and 0.83±0.23 (M±SD, number/mm²) in the control, the CPP− and the CPP+ rats, respectively.

DISCUSSION

It has been indicated that cartilage differentiation occurred 7 days after the implantation of demineralized bone matrix, which was followed by its calcification on day 10 and eventually bone formation and bone marrow development were shown between day 14 and day 21 (9). The sequences after the implantation were not apparently different among all groups in the present experiment. However, some quantitative results were different among the groups.

The activity of TR-ACP was higher in the CPP− group than in the control during the experiment. In addition, histological observation indicated that the number of osteoclastic cells was larger in the CPP− group compared to the control.
Fig. 1

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Fig. 1. Photographs of the plaques 21 days after the subcutaneous implantation of bone matrix powder in a control rat (a), a rat fed the low calcium diet not supplying casein phosphopeptides (b), and a rat fed the low calcium diet supplying casein phosphopeptides (c). Each scale bar represents 50 μm. The basophilic osteoblasts (†) and multinucleated osteoclastic cells (♀) appeared near the implanted bone matrix (BM) in all groups.

on day 21. It has been accepted that TR-ACP activity is a marker of osteoclast (17, 18). Because osteoclasts secrete TR-ACP during bone resorption, this enzyme activity is suggested to indicate osteoclast function (17). These results show that feeding the low calcium diet increases osteoclastic bone resorption, which is due to the stimulation of osteoclastic cell differentiation. Tanimoto et al. (19) reported that calcium depletion increased TR-ACP activity in the tibia of rats. And Liu et al. (20) indicated that calcium deficiency increased the number of osteoclasts in the tibia of rats. These results from in vivo researches are consistent with the present experiment using the ectopic bone. Compared with the control group, calcium content was lower in the CPP− group on day 21. The stimulation of bone resorption is likely to decrease the content of calcium in the CPP− rats.

The activities of TR-ACP were not different between the control and the CPP+ groups during the experiment and CPP supplementation reduced the activity of TR-ACP in the rats fed the low calcium diet on day 21. Histological observation indicated that the number of osteoclastic cells was not different between the control and the CPP+ rats, and the number was larger in the CPP− group than in the CPP+ group on day 21. In addition, CPP supplementation
increased calcium content on day 21. These results indicate that CPP suppresses
the increased osteoclastic bone resorption by feeding the low calcium diet and that
the suppression is owing to the reduction of the number of osteoclastic cells.
Furthermore, it is suggested the suppression of osteoclastic bone resorption in-
creases bone calcium content in the rats fed CPP. Because CPP has been reported
to stimulate calcium absorption (1-4), bone resorption appears to be reduced by the
improved calcium absorption in the rats fed CPP.

The present results showed that activity of ALP decreased during the experi-
ment in the control rats. Schwartz and Reddi (12) showed that ALP activity
reached the maximum value 11 days after the implantation and the activity
gradually decreased thereafter. The present results from the control group were in
agreement with their report. Meanwhile, the activity of ALP was not changed in
CPP+ during the experiment and it was higher in CPP− animals than in controls
on day 21. Calcium deficiency is known to increase the activity of ALP in the tibia
of rats (19), which accounts for the difference of the enzyme activities between the
control and the CPP− animals.

CPP supplementation did not affect ALP activity and the activity in the CPP+
group was higher than in the control during the experiment. Because calcium
content in the CPP+ group was lower than in the control, it is possible that the
improved calcium absorption by CPP is not enough to decrease ALP activity.
However, there is another possibility that the increased ALP activity by CPP
supplementation is not due to its action on calcium absorption. Kusuhara et al.
(21) reported that CPP supplementation stimulated calcification of epiphyseal
cartilage in chicks fed enough amount of calcium. In addition, an in vitro study
showed that the administration of CPP into the medium stimulated calcification of
cultured embryonic rat bone (22). It is well known that ALP is involved in the
calcification of bone and cartilage (23). It might be possible that CPP itself or its
fragments are absorbed and stimulate bone and/or cartilage calcification, which
increases ALP activity.

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