Effect of Polyphenols on Calcium Content and Alkaline Phosphatase Activity in Rat Femoral Tissues in Vitro

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The effect of various polyphenols on calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues of young rats in vitro was investigated. Bone tissues were cultured for 24 h in serum-free Dulbecco’s modified Eagle’s medium containing either vehicle or various polyphenols (10⁻⁷—10⁻⁴ M). The presence of genistein (10⁻⁴—10⁻³ M) caused a significant increase in calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues. Resveratrol (10⁻³ M) decreased metaphyseal calcium content significantly, and it (10⁻⁴—10⁻³ M) had a significant inhibitory effect on diaphyseal enzyme activity. Epigallocatechin gallate (EGCg; 10⁻⁴ M) significantly inhibited alkaline phosphatase activity in the diaphyseal and metaphyseal tissues. EGCg (10⁻⁷—10⁻⁴ M) had no effect on bone calcium content. Meanwhile, glycitein, quercetin, or catechin in the range of 10⁻⁷ to 10⁻⁴ M did not have an effect on calcium content and alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues. The present study suggests that a phytoestrogen genistein has a unique anabolic effect on bone calcification in vitro.

Key words genistein; resveratrol; quercetin; epigallocatechin gallate; bone calcification; rat femur

Bone loss with increasing age induces osteoporosis. This loss may be due to increased bone resorption and decreased bone formation. Osteoporosis with a decrease in bone mass is widely recognized as a major public health problem. The most dramatic expression of this disease is represented by fractures of the proximal femur. Pharmacological and nutritional factors may prevent bone loss with increasing age. The chemical compounds in food that act on bone metabolism, however, are poorly understood.

Genistein is a natural isoflavonoid phytoestrogen found in Leguminosae. The isoflavonoid has been shown to have a strong inhibitory effect on protein tyrosine kinase, and it can produce cell cycle arrest and apoptosis in leukemic cells. The biological effect of genistein, however, has not been fully clarified. Recently, genistein has been demonstrated to have an anabolic effect on bone metabolism, suggesting its role in the prevention of osteoporosis. Genistein has a stimulatory effect on bone formation and mineralization in tissue culture systems in vitro, and it can stimulate protein synthesis in osteoblastic cells. Moreover, genistein has been shown to inhibit osteoclastic bone resorption by preventing the formation and differentiation of osteoclast-like cells from bone marrow cells, and the apoptosis of mature osteoclasts is induced by genistein through the Ca²⁺ signaling mechanism. Thus genistein may be of significance in the prevention of bone loss with increasing age.

The effect of various polyphenols found in food and plants on bone metabolism, however, has not yet been clarified. The present study was undertaken to determine the effect of polyphenols (glycitein, resveratrol, quercetin, catechin, and (−)-epigallocatechin gallate (EGCg) on bone calcification in vitro. Of various polyphenols used, genistein was found to have a unique anabolic effect on bone calcification.

MATERIALS AND METHODS

Chemicals Dulbecco’s modified Eagle’s medium (MEM) (high glucose) and a penicillin–streptomycin solution (5000 units/mg penicillin and 5000 µg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Bovine serum albumin (BSA), genistein, glycitein, resveratrol, quercetin, (+)-catechin, and EGCg were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). All other chemicals were reagent grade from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass-distilled.

Animals Male Wistar rats (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). For 7 d the animals were fed commercial laboratory chow (solid) containing 57.4% carbohydrate, 1.1% Ca, and 1.1% P at a room temperature of 25°C, and were given distilled water freely.

Bone Culture The femurs were removed aseptically after exsanguination and soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. The femoral-diaphyseal and -metaphyseal tissues were cut into small pieces. Femoral-diaphyseal or -metaphyseal fragments were cultured for 24 h in a 35-mm dish in 2.0 ml medium consisting of Dulbecco’s MEM (high glucose, 4.5 g/dl) supplemented with 0.25% BSA plus antibiotics (100 units of penicillin and 100 µg of streptomycin/ml of medium). In experiments, bone tissues were cultured for 24 h in a medium containing either vehicle (including 0.1% ethanol) or various polyphenols (diluted with 0.1% ethanol). Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO₂ and 95% air.

Analytical Procedures The diaphyseal and metaphyseal tissues were dried for 16 h at 110°C and weighed. Bone tissues were digested for 24 h at 110°C. Calcium was determined by atomic absorption spectrophotometry. Calcium content in bone tissue was expressed as milligrams per gram of dry bone.

To assay alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml of ice-cold 6.5 mM barbital (pH 7.4), cut into small pieces, homogenized with a Physcotron homogenizer, and disrupted for 60 s with an ultrasonic device. The supernatant centrifuged at 600×g for 5 min was used to measure enzyme activity. Enzyme
assay was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt. Enzyme activity was expressed as μmol of p-nitrophenol liberated per minute per milligram of protein. Protein concentration was determined by the method of Lowry et al.

**Statistical Analysis** The significance of the difference between values was estimated by Student’s t-test. *p* values of less than 0.05 were considered to indicate statistically significant differences.

**RESULTS**

The effect of various polyphenols on calcium content in the femoral-diaphyseal and -metaphyseal tissues of rats in vitro is shown in Fig. 1. Bone tissues were cultured for 24 h in a serum-free medium containing either vehicle or polyphenol (10^{-7}—10^{-4} M). The presence of genistein caused a significant increase in calcium content in the femoral-diaphyseal and -metaphyseal tissues. Femoral-metaphyseal calcium content was significantly decreased in the presence of resveratrol (10^{-4} M). The presence of glycitein, quercetin, catechin, or EGCg in the range of 10^{-7}—10^{-4} M did not have an appreciable effect on calcium content in the femoral-diaphyseal and -metaphyseal tissues.

The effect of various polyphenols on alkaline phosphatase activity, which is related to bone calcification, in the femoral-diaphyseal and -metaphyseal tissues of rats in vitro is shown in Fig. 2. The presence of genistein (10^{-7}—10^{-3} M) caused a significant increase in alkaline phosphatase activity in the femoral-metaphyseal tissues. The enzyme activity in the diaphyseal tissues was significantly increased by genistein in the range of 10^{-6} to 10^{-4} M. Femoral-diaphyseal alkaline phosphatase activity was significantly reduced in the presence of resveratrol (10^{-6}—10^{-3} M). No such effect was seen in the metaphyseal tissues. Glycitein, quercetin, or catechin in the range of 10^{-7}—10^{-4} M had no effect on alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues. EGCg (10^{-4} M) caused a significant inhibitory effect on the enzyme activity in the femoral-diaphyseal and -metaphyseal tissues.

**DISCUSSION**

Of various polyphenols used, genistein had a stimulatory effect on calcium content and alkaline phosphatase activity in rat femoral-diaphyseal and -metaphyseal tissues in vitro. Glycitein, resveratrol, quercetin, catechin, and EGCg did not have an anabolic effect on bone calcification. Genistein was found to reveal a unique anabolic effect on bone calcification in vitro.

Osteoporosis is widely recognized as a major public health problem. Nutritional factors may play a role in the prevention of bone loss with increasing age. Polyphenols are present in great quantities in food and plant. Resveratrol, quercetin, catechin, and EGCg did not have an anabolic effect on bone calcification in vitro, suggesting that these chemical compounds do not have a role in the conservation of bone mass.

Resveratrol revealed a significant inhibitory effect on femoral-metaphyseal calcium content, whereas it did not cause a significant decrease in the metaphyseal alkaline phosphatase activity. Also, EGCg did not decrease femoral calcium content, although it significantly inhibited femoral alkaline phosphatase activity. The effect of resveratrol and EGCg on bone calcium content and alkaline phosphatase activity did not have a corresponding inhibition. Alternatively, resveratrol and EGCg did not have an anabolic effect on bone calcification in vitro.

Soybeans contain great quantities of saponin and glycitein in addition to genistin, genistein, daidzin, and daidzein. Genistin and daidzin are hydrolyzed to genistein and...
Genistein may partly be mediated through the mechanism of the isoflavone has a potent inhibitory effect on osteoblastic bone resorption in vitro.22—24 This action may allow genistein to increase bone calcium content. A phytoestrogen genistein may partly be mediated through the mechanism of estrogen action.15,16

Genistein is a 4’,5,7-trihydroxyisoflavone, and daidzein is a 4’,7-dihydroxyisoflavone. Glycitein is a 4’,7-dihydroxy-6-methoxyisoflavone, while quercetin is a 3,3’,4’,5,7-pentahydroxyflavone. Glycitein and quercetin had no effect on bone calcification. The anabolic effect of genistein and daidzein on bone calcification is weakened by the phosphorylation of the aminoacyl-tRNA synthetase in osteoblastic cells,18,20 and the isoflavone has a potent inhibitory effect on osteoblastic bone resorption in vitro.19,28 This action may allow genistein to increase bone calcium content. A phytoestrogen genistein may partly be mediated through the mechanism of estrogen action.15,16

In conclusion, of various isoflavones and polyphenols in food, the isoflavone genistein had a potent anabolic effect on bone calcification in the femoral-diaphyseal and -metaphyseal tissues of rats in vitro. Presumably, genistein is important as a nutritional factor in the prevention of osteoporosis with increasing age.

REFERENCES