

## Oral Administration of Phytocomponent *p*-Hydroxycinnamic Acid Has Anabolic Effects on Bone Calcification in Femoral Tissues of Rats *in Vivo*

Ying Ling Lai and Masayoshi Yamaguchi\*

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan  
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The effects of phytocomponent *p*-hydroxycinnamic acid (HCA) on biochemical components in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of rats *in vivo* were investigated. Rats were orally administered HCA 1, 2, or 5 mg/100 g body weight once daily for 7 days. The administration of HCA did not cause a significant change in body weight or serum calcium and inorganic phosphorus levels. Calcium content, alkaline phosphatase activity, and DNA content in the diaphyseal and metaphyseal tissues were significantly increased with the administration of HCA 2 or 5 mg/100 g. Diaphyseal calcium and metaphyseal DNA contents were significantly increased with the dose of HCA 1 mg/100 g. The activity of tartrate-resistant acid phosphatase, which is a marker enzyme in osteoclastic bone resorption, was significantly decreased with the administration of HCA 1, 2, or 5 mg/100 g. This study demonstrates that the administration of HCA has anabolic effects on bone calcification in the femoral tissues of rats *in vivo*.

**Key words** — *p*-hydroxycinnamic acid, bone formation, bone resorption, rat femur

### INTRODUCTION

Bone loss with aging induces osteoporosis, which is widely recognized as a major public health problem.<sup>1–3</sup> A decrease in bone mass leads to bone

fracture. Bone loss may be due to decreased bone formation and increased bone resorption. Pharmacologic and nutritional supplements may prevent bone loss with increasing age.<sup>4,5</sup>

Micronutrients and phytochemicals are found in vegetables and fruit. Recent studies have shown that isoflavones (including genistein and daidzein), which are contained in soybeans,<sup>5–7</sup> and menaquinone-7, an analogue of vitamin K<sub>2</sub> which is abundant in fermented soybeans,<sup>8–10</sup> have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption *in vitro*, thereby increasing bone mass in animal models of osteoporosis.<sup>11,12</sup> Moreover,  $\beta$ -cryptoxanthin, a carotenoid that is abundant in Satsuma mandarin oranges (*Citrus unshiu* MARC.) has been shown to have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption *in vitro*.<sup>13–15</sup> Lutein, lycopene, and  $\beta$ -carotene, which are carotenoids, do not affect bone calcification *in vitro*.<sup>16</sup> The intake of  $\beta$ -cryptoxanthin-supplemented juice has stimulatory effects on bone formation and inhibitory effects on bone resorption in healthy individuals as estimated based on serum bone metabolic markers.<sup>17</sup> Thus food chemical factors may have a useful role in the prevention of osteoporosis with aging.

*p*-Hydroxycinnamic acid (HCA) is present in many plants and synthesized from tyrosine. The biologic effect of HCA on bone metabolism has not yet been clarified. This study was undertaken to determine the effects of oral administration of HCA on bone components in the femoral tissues on rats *in vivo*.

### MATERIALS AND METHODS

**Chemicals** — HCA and other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan) and Sigma Chemical (St. Louis, MO, U.S.A.). All water used was glass-distilled.

**Animals** — Male Wistar rats (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 57.4% calcium and 1.1% phosphorus for 7 days at room temperature of 25°C and were allowed distilled water *ad libitum*.

**Administration Procedures** — HCA was dissolved in 99.5% ethanol (1.0 ml) and added to 99 ml of corn oil (Sigma Chemical) at a concentration of 1, 2, or 5 mg/ml. HCA 1 ml/100 g body weight was

\*To whom correspondence should be addressed: Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan. Tel. & Fax: +81-54-264-5580; E-mail: yamaguch@u-shizuoka-ken.ac.jp

**Table 1.** Body Weight, Serum Calcium and Inorganic Phosphorus Levels in Rats Orally Administered HCA

Treatment	Body Weight (g)	Serum level (mg/100 ml)	
		Calcium	Inorganic phosphorus
Control	115.7 ± 6.0	10.71 ± 0.05	9.08 ± 0.15
HCA (mg/100 g body weight)			
1	118.7 ± 2.0	10.94 ± 0.13	9.44 ± 0.22
2	109.6 ± 1.8	10.80 ± 0.10	8.97 ± 0.18
5	108.2 ± 1.9	10.63 ± 0.23	9.05 ± 0.16

Rats were orally administered HCA once daily for 7 days, and 24 hr after the last administration they were killed by cardiac puncture. Each value is the mean ± SEM of 6 rats. Data were not significantly different among groups.

orally administered to rats through a stomach tube once daily for 7 days. Control rats received corn oil orally. The animals were killed 24 hr after the final administration by cardiac puncture under light ether anesthesia, and the blood and femur were immediately removed.

**Analytical Procedures** — Blood samples obtained by cardiac puncture were centrifuged 30 min after collection, and the serum was separated. Serum was kept frozen at  $-80^{\circ}\text{C}$  until assay. Serum calcium and inorganic phosphorus concentrations were determined using an assay kit (Wako Pure Chemical Industries).

The diaphyseal or metaphyseal tissues were dried for 16 hr at  $110^{\circ}\text{C}$ . Calcium was determined using atomic absorption spectrophotometry.<sup>18)</sup> The calcium content in bone tissues was expressed as milligrams per gram of dry bone.

To assay alkaline phosphatase activity, the diaphyseal or metaphyseal tissues were immersed in 3.0 ml of ice-cold barbital buffer 6.6 mM (pH 7.4), cut into small pieces, and disrupted for 60 sec with an ultrasonic device. The supernatant centrifuged at  $600 \times g$  for 5 min was used to measure enzyme activity. Enzyme assays were carried out under optimal conditions. Alkaline phosphatase activity was determined using the method of Walter and Schutt.<sup>19)</sup> Enzyme activity was expressed as micromole of *p*-nitrophenol liberated per minute per milligram of protein. Protein concentrations were determined using the method of Lowry *et al.*<sup>20)</sup>

To measure bone DNA content, the diaphyseal or metaphyseal tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 hr after the homogenization of the bone tissues.<sup>21)</sup> After alkaline extraction, the samples were centrifuged at  $10000 \times g$  for 5 min, and the supernatant was collected. DNA content in the supernatant was determined using the method of Ceriotti<sup>22)</sup> and expressed as the amount

of DNA milligrams per gram of wet weight of bone tissue.

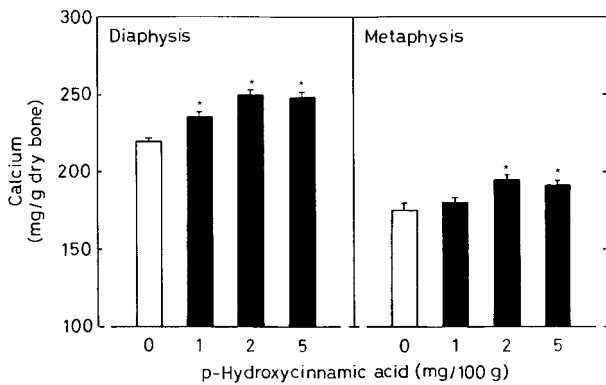
Tartrate-resistant acid phosphatase (TRACP) activity in the extracts from the diaphyseal or metaphyseal tissues was assayed using *p*-nitrophenylphosphate (pNPP) as a substrate in an incubation medium (1.2 ml) containing the following: 1.0 mM pNPP, 0.1 M sodium acetate (pH 5.8), 0.15 M KCl, 10 mM sodium tartrate, 1 mM ascorbic acid, and 0.1 mM  $\text{FeCl}_3$ .<sup>23)</sup> The enzyme reaction mixture was incubated for 60 min at  $37^{\circ}\text{C}$ . After incubation, the reaction was stopped by the addition of 0.1 N NaOH (4.0 ml). The absorbance of *p*-nitrophenol liberated was immediately measured at 405 nm. TRACP activity was expressed as nmoles of *p*-nitrophenol liberated per minute per milligram of protein.

**Statistical Analysis** — The significance of differences between values was estimated using Student's *t*-test. We also used a multiple analysis of variance (ANOVA) to compare the treatment groups. A *p*-value of less 0.05 was considered to indicate a statistically significant difference.

## RESULTS

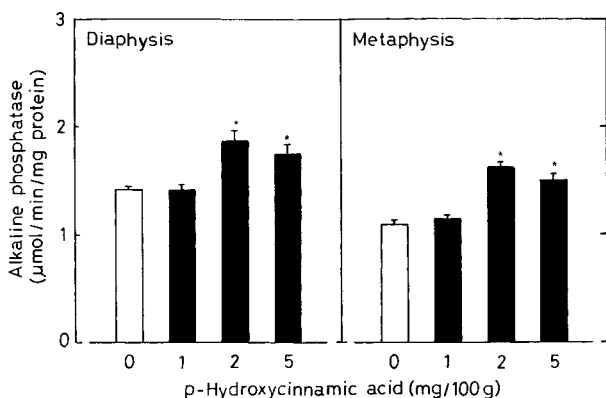
After the rats were orally administered HCA 1, 2, or 5 mg/100 g body weight for 7 days, body weight or serum calcium and inorganic phosphorus levels did not change significantly (Table 1). Calcium content in the femoral-diaphyseal tissues was significantly increased with the administration of HCA 1, 2, or 5 mg/100 g (Fig. 1). Metaphyseal calcium content was significantly increased with the administration of HCA 2 or 5 mg/100 g (Fig. 1).

Alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues was significantly increased with the administration of HCA 2 or 5 mg/100 g (Fig. 2). DNA content in the femoral-diaphy-



**Fig. 1.** Effects of HCA on Calcium Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vivo*

Rats were orally administered HCA 1, 2, or 5 mg/100 g body weight once daily for 7 days. The animals were killed 24 hr after the last administration. Each value is the mean  $\pm$  SEM of 6 rats. \* $p < 0.01$  compared with the control (none) value.



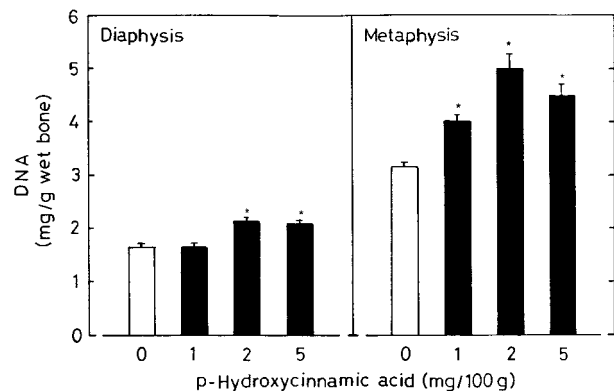
**Fig. 2.** Effects of HCA on Alkaline Phosphatase Activity in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vivo*

The procedure of administration is described in the legend to Fig. 1. Each value is the mean  $\pm$  SEM of 6 rats. \* $p < 0.01$  compared with the control (none) value.

seal tissues was significantly increased with the administration of HCA 2 or 5 mg/100 g (Fig. 3). Metaphyseal DNA content was significantly increased with the administration of HCA 1, 2, or 5 mg/100 g (Fig. 3). TRACP activity in the femoral-diaphyseal and -metaphyseal tissues was significantly decreased with the administration of HCA 1, 2, or 5 mg/100 g (Fig. 4).

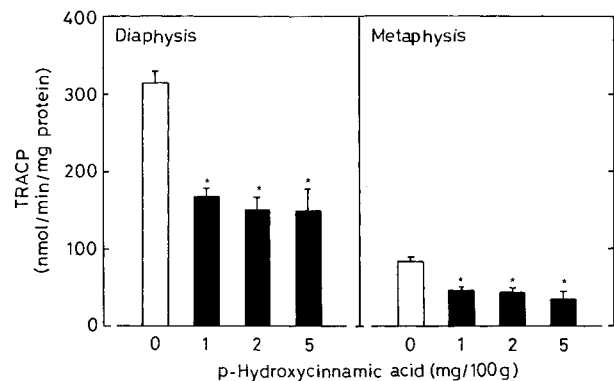
## DISCUSSION

Oral administration of HCA, a component in



**Fig. 3.** Effects of HCA on DNA Content in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vivo*

The procedure of administration is described in the legend to Fig. 1. Each value is the mean  $\pm$  SEM of 6 rats. \* $p < 0.01$  compared with the control (none) value.



**Fig. 4.** Effects of HCA on TRACP Activity in the Femoral-Diaphyseal and -Metaphyseal Tissues of Rats *in Vivo*

The procedure of administration is described in the legend to Fig. 1. Each value is the mean  $\pm$  SEM of 6 rats. \* $p < 0.01$  compared with the control (none) value.

plants, was found to cause a significant increase in calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of rats *in vivo*. Alkaline phosphatase is a marker enzyme of osteoblast and participates in bone mineralization.<sup>24)</sup> DNA content in bone tissues is an index of the number of bone cells.<sup>25)</sup> The present findings suggest that the intake of HCA induces anabolic effects on bone calcification in normal growing rats.

TRACP is a marker enzyme of osteoclasts in bone tissues, and the enzyme activity is enhanced by bone-resorbing factors.<sup>23)</sup> Oral administration of HCA caused a significant decrease in TRACP activity in the femoral-diaphyseal and -metaphyseal

tissues of rats. This result suggests that the administration of HCA induces a decrease in bone-resorbing activity in the femoral tissues of rats *in vivo*.

Culture with HCA ( $10^{-5}$  or  $10^{-4}$  M) has been shown to have stimulatory effects on bone formation and inhibitory effects on bone resorption in rat femoral-diaphyseal and -metaphyseal tissues *in vitro*.<sup>26)</sup> This study may support the view that HCA has anabolic effects on bone metabolism. Supplemental intake of dietary HCA may have preventive effects on bone loss with increasing age. This, however, remains to be elucidated in animal models of osteoporosis.

In conclusion, it was demonstrated that oral administration of the phytochemical HCA has anabolic effects on bone calcification *in vivo*.

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