Effects of Copper on Bone Component in the Femoral Tissues of Rats: Anabolic Effect of Zinc Is Weakened by Copper

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The effects of copper on biochemical components in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of rats in vivo and in vitro were investigated. Rats were orally administered copper sulfate (50, 100, or 200 μg Cu/100 g body weight) once daily for 7 d. Calcium content in the diaphyseal and metaphyseal tissues was significantly decreased with the administration of copper (200 μg/100 g), while alkaline phosphatase activity in these tissues was not significantly changed by copper administration. The diaphyseal DNA content was significantly decreased with the administration of copper (50, 100, or 200 μg/100 g). Moreover, the femoral-diaphyseal and -metaphyseal tissues were cultured for 48 h in serum-free medium containing either vehicle or copper (10⁻⁷—10⁻⁴ M). Culture with copper (10⁻⁷—10⁻⁴ M) caused a significant decrease in alkaline phosphatase activity in the diaphyseal and metaphyseal tissues, while calcium and DNA contents in these tissues were not significantly changed. Culture with parathyroid hormone [PTH (1-34); 10⁻⁷ M], a bone-resorbing factor, caused a significant decrease in calcium content in the diaphyseal and metaphyseal tissues. This decrease was completely inhibited in the presence of copper (10⁻⁶ or 10⁻⁵ M). Culture with zinc sulfate (10⁻⁴ M) caused a significant increase in calcium content and alkaline phosphatase activity in the diaphyseal and metaphyseal tissues. The effects of zinc (10⁻⁴ M) in increasing femoral calcium content and alkaline phosphatase activity were not seen in the presence of cycloheximide (10⁻⁴ M), an inhibitor of protein synthesis, suggesting that the effects of zinc are involved in newly synthesized protein components. The effects of zinc in increasing calcium content and alkaline phosphatase activity in the diaphyseal and metaphyseal tissues were significantly weakened in the presence of copper (10⁻⁴ M). The inhibitory effects of copper were further enhanced in the presence of cycloheximide. This study demonstrates that supplementation with copper in adequate copper nutrition does not have anabolic effects on bone components in vivo and in vitro and that copper weakens the anabolic effects of zinc in vitro.

Key words copper; zinc; bone calcification; bone resorption; rat femur

MATERIALS AND METHODS

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% Ca and 1.1% P for 7 d at room temperature of 25 °C and were allowed distilled ad libitum.

Administration Procedures The amount of copper in the diet was 820 μg per 100 g of diet. Daily intake of copper from the diet in each rat was in the range of 41 to 82 μg. Copper sulfate was dissolved in distilled water at a concentration of 50, 100, or 200 μg of copper per milliliter. Copper (1 ml/100 g body weight/d) was orally administered to rats through a stomach tube once daily for 7 d. Control rats received distilled water orally. The animals were killed 24 h after the final administration by cardiac puncture under light ether anesthesia, and the blood and femur were immediately removed.

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 48 h in 35
mm dishes in 2.0 ml of medium consisting of DMEM (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 48 h in a medium containing either vehicle, copper (10^{-5}—10^{-4} M) or zinc (10^{-5} or 10^{-4} M) in the presence or absence of cycloheximide (10^{-6} M). Cultures were maintained at 37 °C in a water-saturated atmosphere containing 5% CO2 and 95% air.

**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 °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 h at 110 °C. Calcium was determined using atomic absorption spectrophotometry. 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 s with an ultrasonic device. The supernatant centrifuged at 600×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. Enzyme activity was expressed as micromole of p-nitrophenol liberated/min/mg of protein. Protein concentration was determined using the method of Lowry et al.

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 h after the homogenization of the bone tissues. After alkaline extraction, the samples were centrifuged at 10000×g for 5 min, and the supernatant was collected. DNA content in the supernatant was determined using the method of Ceriotti and expressed as the amount of DNA (milligram)/gram wet weight of bone tissue.

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

RESULTS

**Effects of Copper Administration on Bone Components in Vivo** Rats were orally administered copper (50, 100, or 200 μg/100 g body weight) for 7 d. Body weight or serum calcium and inorganic phosphorus levels were not significantly changed with the administration of copper (50, 100, or 200 μg/100 g) (Table 1).

Calcium content in the femoral-diaphyseal and -metaphyseal tissues was significantly decreased with the administration of copper (200 μg/100 g) (Fig. 1). Alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues was not significantly changed with the administration of copper (50, 100, or 200 μg/100 g) (Fig. 2). Diaphyseal DNA content was significantly decreased with the administration of copper (50, 100, or 200 μg/100 g), while metaphyseal DNA content was not significantly changed (Fig. 3).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Serum level (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Calcium</td>
</tr>
<tr>
<td>Control</td>
<td>118.2±2.7</td>
<td>10.78±0.16</td>
</tr>
<tr>
<td>Copper (μg/100 g body weight)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>122.5±1.2</td>
<td>10.82±0.32</td>
</tr>
<tr>
<td>100</td>
<td>118.2±3.5</td>
<td>10.33±0.09</td>
</tr>
<tr>
<td>200</td>
<td>121.2±2.4</td>
<td>11.39±0.30</td>
</tr>
</tbody>
</table>

Rats were orally administered copper sulfate once daily for 7 d and 24 h later after the last administration they were killed by cardiac puncture. Each value is the mean±S.E.M. of six rats. Data were not significantly different between groups.

**Effects of Copper Addition on Bone Components in Vitro** Femoral-diaphyseal or -metaphyseal tissues were cultured for 48 h in serum-free medium containing either vehicle or copper (10^{-5}—10^{-4} M). The change in bone components is shown in Fig. 4. Culture with copper (10^{-7}—10^{-5} M) did not cause a significant decrease in diaphyseal calcium content. Alkaline phosphatase activity in the diaphyseal or metaphyseal tissues was significantly decreased in the presence of copper (10^{-7}—10^{-5} M). On the other hand, culture...
Effects of Copper on PTH-Induced Decrease in Bone Calcium Content

Femoral-diaphyseal or -metaphyseal tissues were cultured for 48 h in serum-free medium containing either vehicle or PTH (10\(^{-7}\) M), which induces bone resorption. Culture with PTH caused a significant decrease in calcium content in the diaphyseal and metaphyseal tissues (Fig. 5). This decrease was completely inhibited in the presence of copper (10\(^{-6}\) M). Calcium content in the diaphyseal and metaphyseal tissues did not significantly change during culture with copper (10\(^{-5}\) or 10\(^{-4}\) M), or copper (10\(^{-5}\) or 10\(^{-4}\) M) plus zinc (10\(^{-5}\) or 10\(^{-4}\) M). Calcium content in the diaphyseal and metaphyseal tissues did not significantly change during culture with copper (10\(^{-5}\) or 10\(^{-4}\) M) (Fig. 6). The presence of zinc (10\(^{-5}\) or 10\(^{-4}\) M) caused a significant increase in diaphyseal calcium content. The effect of zinc (10\(^{-5}\) or 10\(^{-4}\) M) in increasing diaphyseal calcium content was also observed in the presence of copper (10\(^{-4}\) M). However, the effect of zinc (10\(^{-4}\) M) in increasing diaphyseal calcium content was significantly inhibited in the presence of copper (10\(^{-4}\) M). Metaphyseal calcium content was significantly increased after culture with zinc (10\(^{-5}\) or 10\(^{-4}\) M). This effect was not seen in the presence of copper (10\(^{-5}\) or 10\(^{-4}\) M).

Effects of the Interaction of Copper and Zinc on Bone Components in Vitro

Femoral-diaphyseal or -metaphyseal tissues were cultured for 48 h in serum-free medium containing either vehicle, copper (10\(^{-5}\) or 10\(^{-4}\) M), zinc (10\(^{-5}\) or 10\(^{-4}\) M), or copper (10\(^{-5}\) or 10\(^{-4}\) M) plus zinc (10\(^{-5}\) or 10\(^{-4}\) M). Calcium content in the diaphyseal and metaphyseal tissues did not significantly change during culture with copper (10\(^{-5}\) or 10\(^{-4}\) M), while the enzyme activity was significantly increased in the presence of zinc (10\(^{-5}\) M). Metaphyseal calcium content was significantly increased after culture with zinc (10\(^{-5}\) or 10\(^{-4}\) M). This effect was not seen in the presence of copper (10\(^{-5}\) or 10\(^{-4}\) M).
DISCUSSION

The effects of copper deficiency on bone growth are well established.1—4 The enzyme lysyl oxidase, which mediates the final step in the synthesis of collagen, a constituent of bone and connective tissue, is a copper-dependent enzyme.6,7 The effects of adequate copper nutrient on bone metabolism have not been fully clarified. The effects of oral administration of copper (50 to 200 μg/100 g body weight) to growing rats were examined in this study. The administration of copper (200 μg/100 g) for 7 d decreased the calcium content in the femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues. Moreover, the diaphyseal DNA content was significantly decreased with the administration of copper (50 to 200 μg/100 g); DNA content in bone tissues is an index of the number of bone cells.15 The present findings suggest that the supplementation of copper induces a significant decrease in bone components in normal growing rats.

The effects of copper addition in vitro were examined using femoral-diaphyseal and -metaphyseal tissues obtained from growing rats. The effects of copper on bone tissue culture in vitro have not been fully clarified. We found that culture with copper (10−7—10−5 M) caused a significant decrease in alkaline phosphatase activity in the femoral-diaphyseal and -metaphyseal tissues. Alkaline phosphatase is an enzyme marker of osteoblasts, and the enzyme participates in bone mineralization.16 Presumably copper has an inhibitory effect on osteoblastic cell function. On the other hand, the presence of copper (10−7—10−5 M) for 48 h did not induce a significant alteration in calcium and DNA contents in femoral-diaphyseal and -metaphyseal tissues in vitro. However, oral administration of copper caused a significant decrease in calcium and DNA contents in rat femoral tissues in vivo. Thus the effects of copper on bone components in vivo differed from the effects in vitro. The effects of copper in decreasing bone components in vivo may result from the direct and indirect action of copper on bone metabolism in rats.

Osteoblasts are derived from mesenchymal stem cells present in bone marrow stroma, which also differentiate into bone, adipocytes, and other cell phenotypes.17 It has been shown that copper modifies both the differentiation and proliferative activity of mesenchymal stem cells in vitro.18 Copper (5×10−5 M) diminishes the proliferation rate of mesenchymal stem cells in vitro.
enchymal stem cells, increasing their ability to differentiate into the osteogenic and the adipogenic lineages.\textsuperscript{18} Our finding that culture with copper (10\textsuperscript{−7}−10\textsuperscript{−5} M) induces a marked decrease in alkaline phosphatase activity in bone tissue culture may support the view that copper inhibits osteoblastic differentiation and induces an impairment of osteoblastic cell function in bone tissues.

Culture with PTH, a bone-resorbing factor, caused a significant decrease in the calcium content of rat femoral-diaphyseal and -metaphyseal tissues \textit{in vitro}. This decrease was completely inhibited in the presence of copper (10\textsuperscript{−5} M), indicating that the element has inhibitory effects on PTH-induced bone resorption \textit{in vitro}. Copper has been shown to inhibit active bone resorption in cultured explanted mouse calvaria bones \textit{in vitro}.\textsuperscript{19} Copper may have a suppressive effect on bone resorption. In addition, copper has been shown to inhibit osteoclastogenesis in a bone marrow culture system \textit{in vitro}.\textsuperscript{20}

Osteoclastogenesis has been induced by the production of the receptor activator of NF-κB ligand (RANKL), an essential factor that promotes differentiation from bone marrow cells to osteoclasts, in osteoblasts.\textsuperscript{21} The action of PTH on bone resorption is related to RANKL. If copper has an inhibitory effect on osteoclastic bone resorption, the element may cause a preventive effect on PTH-induced bone resorption.

The interaction between cadmium and copper in the osseification of embryonic chick bone in tissue culture \textit{in vitro} has been reported.\textsuperscript{22} The addition of cadmium (2×10\textsuperscript{−6} M) and copper (10\textsuperscript{−5} M) causes severe interactive damage to osteogenic mesenchymal cells in the periosteum and severe degenerative changes in osteoblasts around the trabecula, resulting in severe impairment of ossification in the diaphysis.\textsuperscript{22} We examined the interaction of copper and zinc in rat femoral-diaphyseal and -metaphyseal tissues in our culture system \textit{in vitro}. Zinc has been demonstrated to have a stimulatory effect on osteoblastic bone formation,\textsuperscript{23−26} and an inhibitory effect on osteoclastic bone resorption.\textsuperscript{20,27,28} Culture with zinc (10\textsuperscript{−4} M) caused a significant increase in the calcium content and alkaline phosphatase activity in rat femoral-diaphyseal and -metaphyseal tissues \textit{in vitro}. Copper (10\textsuperscript{−5} M) had no effect on diaphyseal and metaphyseal calcium content and caused a marked decrease in alkaline phosphatase activity in the bone tissues. The effects of zinc on bone components were weakened in the presence of copper.

The effects of zinc in increasing the calcium content and alkaline phosphatase activity in rat femoral-diaphyseal and metaphyseal tissues \textit{in vitro} were completely inhibited in the presence of cycloheximide, an inhibitor of protein synthesis. The effect of copper in decreasing bone alkaline phosphatase activity \textit{in vitro} was not changed in the presence of cycloheximide. It is speculated that copper has an inhibitory effect on protein synthesis in bone tissues \textit{in vitro}. Moreover, the inhibitory effects of copper on the zinc-induced increase in alkaline phosphatase activity in the diaphyseal and metaphyseal tissues were further enhanced in the presence of cycloheximide. The effects of copper in inhibiting the anabolic effects of zinc on bone may involve in the inhibition of protein synthesis.

Copper plays important functional roles in bone metabolism and turnover and is essential for the normal growth and development of the skeleton in humans and animals.\textsuperscript{29} Bone abnormalities are a feature of severe copper deficiency. Nutritional copper deficiency results in marked changes in the crosslinking of collagen and elastin, presumably in relationship to the role of copper as a cofactor for lysyl oxidase, which controls one of the initial steps in the crosslinking of collagen and elastin.\textsuperscript{21} In adequate copper nutrition, however, supplementation of dietary copper may induce an impairment of bone metabolism. Moreover, the anabolic effects of zinc on bone components may be weakened by supplementation of copper at a relatively high level. Copper supplementation may have beneficial effects in copper deficiency. In adequate copper nutrition, however, the supplemental intake of copper may cause impairment of bone metabolism.

In conclusion, it has been demonstrated that the oral administration of copper induces a significant decrease in bone components in the femoral tissues of rats \textit{in vivo}, and that copper addition causes a marked decrease in alkaline phosphatase activity \textit{in vitro}. Copper at a relatively high level reduces the anabolic effects of zinc on bone components \textit{in vitro}.

REFERENCES


