Stimulatory Effect of Calcitonin on Bone Formation in Tissue Culture

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The present investigation was undertaken to clarify the *in vitro* effect of synthetic [Asu¹⁻⁷]eel calcitonin (CT) on bone metabolism in tissue culture. Calvaria were removed from weanling rats (3-week-old male) and cultured for periods up to 96 h in Dulbecco's Modified Eagle Medium supplemented with antibiotics and bovine serum albumin. The experimental cultures contained 1.0 to 100 ng/ml CT. All cultures were incubated at 37 °C in 5% CO₂-95% air. Bone calcium content was increased significantly by the presence of 10 and 100 ng/ml CT. This increase was blocked by the presence of 10⁻⁶ M cycloheximide or 10⁻⁷ M actinomycin D. Bone alkaline phosphatase activity was significantly increased by the presence of 100 ng/ml CT for 48 and 96 h. Bone acid phosphatase activity was not altered significantly by CT (1—100 ng/ml). The incorporation of [³H]proline into the acid-insoluble residues of bone tissue was significantly increased by the presence of CT (1—100 ng/ml) for 96 h. This increase was completely blocked by the presence of 10⁻⁷ M cycloheximide. Bone DNA content was significantly raised by the presence of 10 and 100 ng/ml CT for 96 h. Furthermore, the culture with CT (10 and 100 ng/ml) produced a significant decrease in glucose concentration in the medium. Also, CT (10 and 100 ng/ml) stimulated the production of pyruvic acid from bone tissue. These results suggest that CT had a direct stimulatory effect on bone formation and mineralization *in vitro*, and that the hormone stimulates energy metabolism in bone cells.

Keywords — calcitonin; bone formation; calcium; protein synthesis; DNA; glucose consumption; rat calvaria

Introduction

It has been established that calcitonin (CT), a calcitropic hormone, can inhibit osteoclastic bone resorption stimulated by bone resorptive factors.¹⁾ The role of CT in osteoblastic bone formation, however, is controversial, although the hormone has been demonstrated to stimulate the growth and maturation of cartilage.²⁾ Inhibitory effect,³⁾ stimulatory effect,⁴⁾ and no effect^{5,6)} of CT on bone formation have been reported. Interestingly, Weiss *et al.*⁷⁾ have reported the effect of CT on calcification at various stages of bone formation by using a matrix-induced endochondral bone-forming system; CT can stimulate bone formation when administered prior to initiation of osteogenesis. It has also been shown that CT stimulates Type I collagen synthesis of chondrocytes in culture.⁸⁾

Recently, there is growing evidence of CT action to stimulate osteoblastic bone formation. Rao *et al.*⁹⁾ have reported that osteoblasts have specific CT binding sites. CT has been shown to increase alkaline phosphatase activity at a specif-

ic cell density or differentiation stage in osteoblastic clonal cell line (MC3T3-E1).¹⁰⁾ More recently, it has been reported that CT can act *in vitro* to directly increase bone formation and bone cell proliferation in the tibiae and calvaria from chicken embryos, and MC-3T3-E1 mouse calvarial cells.¹¹⁾ These reports strongly suggest that osteoblasts respond to CT directly.

It has not been established, however, whether CT can reveal a direct stimulatory effect on bone formation and mineralization in a tissue culture system. Therefore, the present study was undertaken to evaluate the effect of CT on bone metabolism in tissue culture using the calvaria from weanling rats. The results showed that synthetic [Asu¹⁻⁷]eel CT has an anabolic effect on bone metabolism. The present study may support the view that CT can stimulate bone formation and mineralization *in vitro*.

Materials and Methods

Chemicals — Dulbecco's Modified Eagle Medium (high glucose) was obtained from

Gibco Laboratories (Grand Island, NY, U.S.A.). Penicillin-streptomycin solution (5000 units/ml penicillin; 5000 μ g/ml streptomycin) was obtained from Gibco Laboratories. Bovine serum albumin (Fraction V), cycloheximide, and actinomycin D were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Synthetic [Asu^{1,7}]eel calcitonin (CT; 4000 MRC U/mg) was kindly supplied by Toyo Jozo Research Laboratory (Shizuoka, Japan). L-[2,3-³H]Proline (34.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were reagent grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All water used was glass distilled.

Animals — Weanling male Wistar rats weighing 60–65 g (3-week-old) were obtained from the Japan SLC Inc. (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium and 1.1% phosphorus, and distilled water. The rats were killed by decapitation.

Bone Culture — Calvaria from 3-week-old male rats were removed aseptically and cut along the sagittal suture into left and right halves. One-half of each calvarium served as a control for its paired, treated half. Each half-calvarium (17–23 mg wet weight) was cultured in a 35-mm dish in 2.0 ml of medium (serum-free) consisting of Dulbecco's Modified Medium (high glucose) supplemented with 0.25% bovine serum albumin (Fraction V) plus antibiotics, with either vehicle (distilled water) or CT (1.0 to 100 ng/ml).¹²⁾ Cultures were maintained at 37 °C in a water-saturated atmosphere containing 5% CO₂ and 95% air for 96 h. The respective media, containing either vehicle (control) or CT, were changed at 48 h, and cultures were maintained for an additional 48 h. In the separate experiments, the respective media contained cycloheximide or actinomycin D.

Analytical Procedures — Calvaria were cultured in the medium containing CT for 48 or 96 h at 37 °C. After culture, the bone was removed and washed with ice-cold 0.25M sucrose solution, blotted, and weighed.

The bone tissues were ashed for 24 h at 640 °C, weighed, and then dissolved in 6 N HCl

solution.¹²⁾ Calcium was determined by atomic absorption spectrophotometry. Calcium content in bone tissues was expressed as mg of calcium per g bone ash.

Alkaline and acid phosphatases activities in the bone tissues were determined by the method of Walter and Schutt.¹³⁾ The bone tissues were immersed in 3.0 ml of ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle, and disrupted for 50 s with an ultrasonic device. The supernatant fraction, centrifuged at 600 × *g* for 5 min, was used for measurement of the enzyme activity. The enzyme assay was carried out under optimal conditions. Enzyme activity was expressed as nmol of *p*-nitrophenol liberated per min per mg protein. Protein was determined by the method of Lowry *et al.*¹⁴⁾

The effect of CT on newly synthesized bone total protein was determined by studying the incorporation of [³H]proline.^{15,16)} The calvaria was pulsed with [³H]proline (2.5 μ Ci/ml of medium) at the culture period indicated in the legends to the figures, and cultured for 2 h. At the end of the culture, the bone was removed and washed with ice-cold 0.25 M sucrose. The calvariae were extracted with ice-cold 5% trichloroacetic acid (TCA), acetone and ether, and then rinsed in ice-cold 0.25 M sucrose. The bones were dried and weighed. For determination of the amount of [³H]proline incorporated into bone total protein, the dried bone residues were dissolved in 1.0 ml of 0.2 N NaOH and an aliquot was removed and placed in a vial for measurement of the disintegrations per minute by scintillation counting. Data are expressed as disintegrations per min per mg dry weight of acid-insoluble residues.

To measure deoxyribonucleic acid (DNA) content, bone tissues were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 h.¹⁷⁾ After alkali extraction, samples were centrifuged at 10000 × *g* for 5 min, and the supernatant fraction was collected. DNA content in the supernatant was determined by the method of Ceriotti.¹⁸⁾ and expressed as the amount of DNA (mg) per g of wet bone tissue.

The concentration of glucose in the medium

TABLE I. Effect of Calcitonin (CT) on Calcium Content in Rat Calvaria *in Vitro*

Treatment	Calcium content (mg/g bone ash)	
	48 h	96 h
Control	376.3 ± 10.9	426.2 ± 17.0
CT (1.0 ng/ml)	404.7 ± 11.7	442.8 ± 4.4
CT (10 ng/ml)	505.4 ± 17.0 ^{a)}	498.8 ± 9.4 ^{a)}
CT (100 ng/ml)	537.7 ± 13.4 ^{a)}	491.1 ± 10.8 ^{a)}

Each value is the mean ± SEM of five calvaria. The bones were cultured in the presence of 1.0 to 100 ng/ml of medium for 48 and 96 h. ^{a)} $p < 0.01$, as compared with the control value.

cultured with calvaria up to 96 h was determined by a colorimetric method using *o*-toluidine.¹⁹⁾ The dry weight of the bone tissue was measured after extraction with 5.0% TCA, acetone and ether, and ranged from 3.0 to 4.5 mg. Medium glucose was expressed as per mg dry acid-insoluble residues. Likewise, the medium pyruvic acid was measured by an enzymatic method.²⁰⁾

Statistical Analysis — Data are expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. *p* values of less than 0.05 were considered to indicate statistically significant differences.

Results

Effect of CT on Calcium Content in Bone Tissue

The alteration of calcium content in rat calvaria cultured for 48 and 96 h is shown in Table I. Calcium content in bone cultured for 96 h in control medium without CT was increased appreciably. The presence of 10 and 100 ng/ml CT, but not 1.0 ng/ml CT, significantly increased calcium content in the bone cultured for 48 and 96 h. When the bone cultured for 48 h in the presence of 100 ng/ml CT and/or 10^{-6} M cycloheximide or 10^{-7} M actinomycin D, no increase in bone calcium content occurred (Fig. 1).

Effect of CT on Enzyme Activity in Bone Tissue

The effects of increasing concentrations of CT (1.0 to 100 ng/ml) on the activities of alkaline and acid phosphatases in rat calvaria cul-

tured for 48 and 96 h are shown in Table II. Bone alkaline phosphatase activity was significantly increased by the presence of CT (10 and 100 ng/ml) for 48 h. Bone acid phosphatase activity was not significantly altered by the presence of CT (1.0 to 100 ng/ml) for 48 and 96 h.

Effect of CT on Protein Synthesis in Bone Tissue

The effect of CT on the incorporation of

Fig. 1. Effect of Cycloheximide or Actinomycin D on Calcitonin (CT)-Increased Calcium Content in Rat Calvaria *in Vitro*

Calvaria were cultured in the presence of either vehicle, CT (100 ng/ml), 10^{-6} M cycloheximide or CT (100 ng/ml) plus 10^{-6} M cycloheximide, and 10^{-7} M actinomycin D or CT (100 ng/ml) plus 10^{-7} M actinomycin D for 48 h. Each bar represents the mean of five calvaria. Vertical lines give the SEM. ^{a)} $p < 0.01$, as compared with the control value. □, control; ■, CT.

TABLE II. Effect of Calcitonin (CT) on Alkaline and Acid Phosphatases Activities in Rat Calvaria *in Vitro*

Treatment	Alkaline phosphatase (nmol/min/mg protein)		Acid phosphatase (nmol/min/mg protein)	
	48 h	96 h	48 h	96 h
Control	854 ± 10	398 ± 11	54.1 ± 1.6	65.2 ± 9.2
CT (1.0 ng/ml)	882 ± 10	434 ± 20	51.4 ± 2.0	68.4 ± 6.0
CT (10 ng/ml)	939 ± 12 ^{b)}	440 ± 25	65.0 ± 2.8	61.6 ± 2.8
CT (100 ng/ml)	961 ± 24 ^{b)}	443 ± 14 ^{a)}	64.6 ± 6.0	61.1 ± 6.0

Each value is the mean ± SEM of five calvaria. The bones were cultured in the presence of 1.0 to 100 ng/ml of medium for 48 and 96 h. ^{a)} $p < 0.05$ and ^{b)} $p < 0.01$, as compared with the control value.

[³H]proline into the acid-insoluble residues of rat calvaria is shown in Fig. 2. Calvaria were incubated for up to 96 h in the presence of 100 ng/ml CT. When the bones were pulsed with [³H]proline (2.5 μCi/ml of medium) at 22, 46 and 70 h of the culture and 2 h later the bones were removed from the medium, the incorporation of [³H]proline into the acid-insoluble

residues of bones was enhanced slightly, but not significantly, by the presence of CT. With the pulse at 94 h of culture, [³H]proline incorporation was significantly increased by the presence of CT.

The effect of increasing concentrations of CT (1–100 ng/ml) on the incorporation of [³H]proline into the acid-insoluble residues of bone is shown in Fig. 3. The bones were incubated for 96 h in the presence of CT and the bones were pulsed with [³H]proline (2.5 μCi/ml). In the

Fig. 2. Effect of Calcitonin (CT) on [³H]Proline Incorporation into the Acid-Insoluble Residues of Rat Calvaria *in Vitro*

Calvaria were cultured for 96 h in the presence of CT (100 ng/ml). Calvaria were pulsed with [³H]proline (2.5 μCi/ml of medium) 2 h before removing the bone. Calvaria were removed from the medium at each time point indicated in figure. Each bar represents the mean of five calvaria. Vertical lines give the SEM. ^{a)} $p < 0.01$, as compared with the control value. □, control; ■, CT.

Fig. 3. Effect of Increasing Concentrations of Calcitonin (CT) on the Incorporation of [³H]Proline into the Acid-Insoluble Residues of Rat Calvaria *in Vitro*

Calvaria were cultured for 96 h in the presence of 1–100 ng/ml CT. The bones were pulsed with [³H]proline (2.5 μCi/ml of medium), and 2 h later the bones were removed from the medium. Each bar represents the mean of five calvaria. Vertical lines give the SEM. ^{a)} $p < 0.05$ and ^{b)} $p < 0.01$, as compared with the control value.

Fig. 4. Effect of Cycloheximide on Calcitonin (CT)-Stimulated $[^3\text{H}]$ Proline Incorporation into the Acid-Insoluble Residues of Rat Calvaria *in Vitro*

Calvaria were cultured for 96 h in medium containing either: vehicle alone; CT (100 ng/ml); 10^{-7} M cycloheximide; or CT (100 ng/ml) plus 10^{-7} M cycloheximide. The bones were pulsed with $[^3\text{H}]$ proline (2.5 $\mu\text{Ci}/\text{ml}$ of medium), and 2 h later the bones were removed from the medium. Each bar represents the mean of five calvaria. The vertical lines give the SEM. a) $p < 0.01$, as compared with the control value. $\square$, control; $\blacksquare$, CT.

presence of 1.0 ng/ml CT, the incorporation of $[^3\text{H}]$ proline into the acid-insoluble residues was enhanced significantly. The presence of 10 and 100 ng/ml CT produced a significant increase of $[^3\text{H}]$ proline incorporation.

The effect of cycloheximide, an inhibitor of protein synthesis, on CT-stimulated $[^3\text{H}]$ proline incorporation into the acid-insoluble residues of bone is shown in Fig. 4. Calvaria were incubated

Fig. 5. Effect of Calcitonin (CT) on DNA Content in Rat Calvaria *in Vitro*

Calvaria were cultured in the presence of 1.0 to 100 ng/ml of medium for 48 and 96 h. Each point represents the mean of five calvaria. Vertical lines give the SEM. a) $p < 0.01$, as compared with the control value.

for 96 h in the presence of either 100 ng/ml CT or 10^{-7} M cycloheximide plus 100 ng/ml CT. The increase in $[^3\text{H}]$ proline incorporation into the acid-insoluble residues of bone caused by the presence of 100 ng/ml CT was completely blocked by the presence of 10^{-7} M cycloheximide.

The radioactivity of $[^3\text{H}]$ proline in the acid (10% TCA)-insoluble residues of culture medium was not significantly different between the control group and the 100 ng/ml CT-treated

TABLE III. Change of Glucose Concentration in the Medium Cultured with Rat Calvaria in the Presence of Calcitonin (CT) *in Vitro*

Treatment	Medium glucose (mg/mg dry acid-insoluble residues)	
	48 h	96 h
Control	2.763 ± 0.114	3.399 ± 0.205
CT (1.0 ng/ml)	2.814 ± 0.213	2.997 ± 0.361
CT (10 ng/ml)	2.518 ± 0.100	2.818 ± 0.160 a)
CT (100 ng/ml)	2.307 ± 0.114 b)	2.222 ± 0.113 c)

Each value is the mean $\pm$ SEM of five calvaria. The bones were cultured in the presence of 1.0 to 100 ng/ml of medium for 48 and 96 h. a) $p < 0.05$, b) $p < 0.025$ and c) $p < 0.01$, as compared with the control value.

Fig. 6. Time Course of Glucose Consumption by Rat Calvaria *in Vitro*

Calvaria were cultured in the presence of either vehicle or CT (100 ng/ml) for 12, 24 and 48 h. Each bar represents the mean of five calvaria. Vertical lines give the SEM. a) $p < 0.025$ and b) $p < 0.01$, as compared with the control value. □, control; ■, CT.

group (data not shown). This indicates that the release of newly synthesized protein from the cultured bone to the medium was not prevented by the presence of CT.

Effect of CT on DNA Content in Bone Tissue

The effect of CT on DNA content in rat calvaria cultured for 48 and 96 h is shown in Fig. 5. DNA content in the bone was not significantly altered by the culture of 48 h with 10 ng/ml CT. At 96 h of culture, DNA content in the bone was significantly increased by the presence of 10 ng/ml CT. With culture for 48 and 96 in the presence of 100 ng/ml CT, bone DNA content increased significantly. Thus, with greater CT concentrations, the hormonal effect enhanced.

Effect of CT on Glucose Consumption in Bone Tissue

The alteration of glucose concentration in the medium cultured with rat calvaria is shown in Table III. Glucose concentration in the medium was not significantly altered by the culture of 48 h with 10 ng/ml CT. At 96 h of culture, the medium glucose concentration was significantly decreased by the presence of 10 ng/ml CT. The presence of 100 ng/ml CT caused a significant

Fig. 7. Time Course of Pyruvic Acid Production by Rat Calvaria *in Vitro*

Calvaria were cultured in the presence of CT (100 ng/ml) for 12, 24 and 48 h. Each bar represents the mean of five calvaria. Vertical lines give the SEM. a) $p < 0.01$, as compared with the control value. □, control; ■, CT.

decrease in glucose concentration in the medium cultured for 48 and 96 h. A significant decrease in the medium glucose concentration in the presence of 100 ng/ml CT was seen at 12 h (Fig. 6). Thus, at earlier time of the culture with CT, an appreciable decrease in the medium glucose concentration occurred.

Effect of CT on Pyruvic Acid Production in Bone Tissue

The effect of CT on pyruvic acid concentration in the medium cultured with rat calvaria for 12, 24 and 48 h is shown in Fig. 7. At culture of 12 h, the concentration of pyruvic acid in the medium was markedly increased by the presence of CT (100 ng/ml). This increase was seen at the culture of 48 h with 100 ng/ml CT. Also, the medium pyruvic acid concentration with the culture periods of 96 h was significantly increased by the presence of CT (100 ng/ml) (data not shown).

Discussion

Although it is well known that calcitonin (CT) inhibits bone resorption,¹⁾ the hormonal

action on bone formation is not established. However, there is growing evidence that CT has an effect on bone formation and mineralization in osteoblastic cells.⁸⁻¹¹⁾ The present investigation was therefore undertaken to clarify the effect of synthetic eel CT on bone formation in a tissue culture system using the calvaria from weanling rats. It was found that the presence of CT (10 and 100 ng/ml) causes a significant increase of calcium content in calvaria cultured in the presence of the hormone for 48 and 96 h. This increase was completely blocked by the presence of 10^{-6} M cycloheximide and 10^{-7} M actinomycin D, suggesting that bone response to CT requires the protein components which is newly synthesized. Bone alkaline phosphatase activity, which is related to bone mineralization, was increased by the presence of CT (10 and 100 ng/ml) for 48 and 96 h. Meanwhile, bone acid phosphatase activity was not altered by CT (10 and 100 ng/ml), although it is well known that the enzyme activity is increased by stimulation of bone resorption. These results suggest that the effect of CT to increase bone calcium may be related to the stimulation of bone mineralization rather than the inhibition of bone resorption. CT may stimulate bone mineralization in tissue culture *in vitro*.

CT increased the incorporation of [³H]proline into the acid-insoluble residues of calvaria, when the bones were cultured with the 100 ng/ml CT for up to 96 h. The hormone may stimulate total protein (noncollagen and collagen) synthesis in the bone cells. The effect of CT was remarkable with 96-h culture and the lowest dose of CT (1.0 ng/ml) used had an appreciable effect. Although CT-stimulated increase in bone protein synthesis was slight at 24-h culture, it can not exclude the possibility that the hormone-induced bone mineralization may be partly based on the stimulated bone protein synthesis. The increase in bone calcium content by CT was completely blocked by inhibitors of protein synthesis (Fig. 1).

Bone DNA content was significantly increased by the culture with CT (100 ng/ml) for 48 and 96 h. This result suggests the possibility that CT stimulates proliferation of bone cells, although the present study does not show whether

the hormone increases DNA synthesis. The significant effect of CT to increase bone DNA content was not seen by the culture with 10 ng/ml dose for 48 h. In these culture conditions, bone calcium content increased significantly. However, a slight increase in bone DNA content caused by CT may make a contribution to the elevation of bone calcium content, because of relative increase of proteins in bone tissue. More recently, it has been reported that salmon CT increases calvarial cell proliferation ([³H]thymidine incorporation into DNA) and bone matrix synthesis ([³H]proline incorporation into collagen) in intact calvaria and tibiae derived from embryonic chickens.¹¹⁾

CT produced a significant decrease in glucose concentration in the medium cultured with rat calvaria, indicating that the hormone stimulates glucose utilization by bone cells. The hormonal effect was seen with 100 ng/ml of CT for 48 and 96 h; this CT dose coincides with the dose to reveal the hormonal effect on bone DNA content. Meanwhile, the presence of 10 ng/ml CT for 48 and 96 h caused a significant increase in bone calcium content (Table I). However, the culture with 10 ng/ml CT for 96 h caused a significant decrease in the medium glucose concentration. From these results, it is possible that CT-stimulated glucose utilization plays a role in the development of bone mineralization. CT also increased the production of pyruvic acid from bone cells, supporting the view that CT enhances the utilization of glucose in bone cells. It has been shown that glucose appears to be an important nutrient for bone tissue,²¹⁾ and that glucose metabolism constitutes an important pathway in bone tissue.²¹⁾ This novel finding, that CT can enhance the utilization of glucose in bone cells, suggests that the hormone stimulates energy metabolism in bone cells.

There have been previous reports that CT stimulated the growth and maturation of embryonic chicken cartilage *in vitro* and acutely increased bone formation indices in humans.^{2,7,22,23)} The current studies extend those observations. CT has direct effects on the number and/or activity of osteoblast line cells that result in increased bone formation.¹¹⁾ However, we used calvaria obtained from weanling

rats to clarify the effect of CT on bone formation and mineralization *in vitro*. It is not clear whether the hormonal effect is due to one or more of the bone cells (preosteoblasts, osteoblasts, osteocytes, and/or osteoclasts). Calvaria used in this experiment may richly contain osteoblasts, because the bone tissue had a specifically high activity of alkaline phosphatase, which is a marker enzyme of osteoblasts, as compared with other enzymes.¹²⁾ It is likely that CT acts largely on osteoblasts in calvaria tissue. The cellular mechanism of CT action remains to be elucidated.

In conclusion, the present investigation clearly demonstrates that CT can increase calcium content, alkaline phosphatase activity, protein synthesis, DNA content and glucose utilization in rat calvaria *in vitro*. CT may play a physiologic role in the stimulation of bone formation and mineralization.

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