A Cysteine Protease Inhibitor Prevents Suspension-Induced Declines in Bone Weight and Strength in Rats

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Abstract In this study, we examined the effects of a potent cysteine protease inhibitor, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucine-4-aminobutylamide (E-64a), on bone weight and strength in tail-suspended rats. We first administered a vehicle or 4 or 8 mg/rat of E-64a to rats fed with a low calcium diet for 7 wks to determine effective doses of E-64a on bone resorption *in vivo*. Femoral cathepsin K-like activity and serum hydroxyproline level in rats fed with a low calcium diet were significantly higher than those in rats fed with a standard diet. The intraperitoneal injection of 8 mg/rat of E-64a to rats decreased their serum calcium and hydroxyproline concentrations after 3 to 6 hrs in parallel with changes in femoral cathepsin K-like activity, while 4 mg/rat of E-64a had weaker effects on these parameters. Based on these results, we injected 8 mg/rat of E-64a to tail-suspended rats twice a day for 2 wks and compared the results with those of treatment with 1 mg/rat of etidronate, a bisphosphonate, twice a week. In tail-suspended rats, femoral weight and strength, assessed by three-point bending test, significantly decreased from Day 5 to 21, while femoral cathepsin K-like activity and serum calcium and hydroxyproline concentrations did not change. E-64a inhibited femoral cathepsin K-like activity in tail-suspended rats, but etidronate did not. E-64a as well as etidronate significantly prevented the suspension-induced declines in bone weight and strength. However, more frequent injection and higher doses were required for E-64a to exhibit significant efficacy of antiresorption, compared with those of etidronate. Our results suggest that a cysteine protease inhibitor could improve suspension-induced osteopenia by inhibiting cathepsin K-like activity in bone; however, it

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Introduction

Skeletal bones are vulnerable to rapid and marked loss of their density under microgravity conditions. To prevent the microgravity-induced bone loss, several countermeasures were performed. Norman et al. (2000) reported that moderate aerobic exercise on a treadmill did decrease bone loss caused by hindlimb suspension, however its effectiveness differed by the investigated tissue, anatomical site and parameter. Negative pressure on the lower body, calcitonin, and phosphorus did not prevent negative calcium balance associated with bed-rest (Schneider and McDonald, 1984). In contrast, bisphosphonates such as risedronate, alendronate and etidronate have been reported to prevent immobilization- or bed-rest-induced bone loss (Mosekilde et al., 2000; Schneider et al., 1993), suggesting that potent antiresorptive agents may effectively prevent microgravity-induced bone loss.

Lysosomal cysteine proteases from osteoclasts degrade the organic matrix (mainly collagen fibers) prior to release of minerals from bone (Baron, 1989; Delaissé et al., 1984). Unlike cathepsins B and L, cathepsin K is predominantly expressed in osteoclasts (Inaoka et al., 1995), suggesting that this enzyme may play a major role

in this osteoclast-mediated degradation of collagen fibers. Recently, Saftig et al. (1998) reported that in osteoclasts isolated from cathepsin K-knockout mice, the resorptive activity was severely impaired. Along with other investigators, we also reported that a potent cysteine protease inhibitor, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butan (E-64), could markedly inhibit the pit formation of primary cultured rat osteoclasts and decrease the serum calcium level in rats fed with a low calcium diet (Delaissé et al., 1984; Kakegawa et al., 1993), suggesting that E-64 acts as a new antiresorptive agent.

In this study, we examined the effects of a derivative of the potent cysteine protease inhibitor, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucine-4-aminobutylamide (E-64a), on the femoral weight, strength, and cathepsin K-like activity of tail-suspended rats, to elucidate whether cysteine protease inhibitors are effective for microgravity-induced osteopenia.

Methods

Feeding a low calcium diet, tail-suspension, and E-64a administration

Administration of E-64a, provided from Taisho Pharmaceutical Co., Saitama, Japan, did not change the serum calcium level in rats fed with a non-purified standard diet*; the serum calcium levels in rats treated with a vehicle and E-64a were 11.3 ± 0.6 and 11.0 ± 0.5 mg/dl, respectively. Therefore, we prepared rats fed with a low calcium diet according to the method of Delaissé et al. (1984) and administered E-64a to them. Briefly, 6-wk-old male Wistar rats (Japan SLC, Shizuoka, Japan) were allowed free access to a low calcium diet (calcium concentration, less than 0.05%; Oriental Yeast, Osaka, Japan) and distilled water for 7 wks. E-64a was intraperitoneally injected into the rats at 4 or 8 mg/rat. Blood samples were collected from the tail vein at indicated times under anesthesia. Rats were also killed by cervical dislocation under anesthesia at the indicated times after E-64 injection, and the femur and blood were collected.

Six-wk-old male Wistar rats were also subjected to tail-suspension as described previously (Ikemoto et al., 2001). Their tails were suspended to keep their rear legs off the ground. They were allowed free access to a 20% casein diet** and water. The amount of food intake of tail-suspended rats decreased to about 80% of that of non-suspended rats. Pair-fed control rats without suspension with the same duration were prepared. E-64a (4 and 8

mg/rat) was intraperitoneally injected into tail-suspended rats twice a day for 2 wks. As a vehicle, phosphate-buffered saline (PBS) was injected into suspended or non-suspended rats for the same period. We also injected 1 mg/rat of a bisphosphonate, etidronate, kindly provided from Sumitomo Pharmaceutical Co., to tail-suspended rats twice a week for 14 days as described previously (Ongphiphadhanakul et al., 1993). The rats were killed at the indicated times, and the samples were prepared as described below.

All of the treatments described here were performed according to the Guide for the Care and Use of Laboratory Animals (1985) and approved by the Animal Care Committee of National Space Development Agency of Japan (NASDA) counterpart.

Protease activities in bone

Frozen femur of the right leg was pulverized and then homogenized for 1 min on ice with a Polytron homogenizer in three times in volume of ice-cold 50 mM sodium acetate buffer, pH 5.5, containing 1 mM EDTA, 0.2 M NaCl, and 0.1% Triton-X 100. After unbroken tissue was separated, the homogenate was centrifuged at $13,000 \times g$ for 30 min at 4°C and the supernatant was used for measurement of protease activities in bone. Cathepsin K and collagenase-like activities were determined by measuring hydrolysis of their synthetic amidocoumaryl substrates, benzylmoxycarbonyl-Phe-Arg-4-methylcoumaryl-7-amide (Z-Phe-Arg-NH-Mec) and succinyl-Gly-Pro-Leu-Gly-Pro-NH-Mec, respectively, as described previously (Brömme et al., 1996; Kojima et al., 1979; Nikawa et al., 1989).

Mechanical testing of femoral middiaphysis

The femora were placed in a testing jig constructed for three-point bending test according to the method of Turner and Burr (1993). Briefly, the left femur was subjected to a material testing machine (RE-3305, Yamaden Co., Tokyo, Japan) at a constant deformation rate of 1 mm/sec with a rod at the upper midpoint of the femur. The distance between the supporting rods was fixed at 18 mm. During the three-point bending test, load-deformation data were recorded and subsequently analyzed by a PC with an in-house computer program specifically made for the analysis of biomechanical parameters. We determined the maximal force to break the femur as bone strength.

Other biochemical and statistical analyses

Serum calcium concentration was measured with o-cresolphthalein complexone assay reagent (Wako, Osaka, Japan) according to the method of Connerty and Briggs (1966). Protein concentrations were measured by Lowry's method with bovine serum albumin as a standard. All data were expressed as mean $\pm$ SD and were

*A non-purified standard MF diet (Oriental Yeast) contains the following nutrients (g/kg): water, 7.5; protein, 246; lipid, 56; carbohydrate, 528; minerals, 63; vitamins, 7.

**A 20% casein diet contains the following nutrients (g/kg): casein (Oriental Yeast), 237; lipid, 50; α -corn starch, 432; sucrose, 216; minerals, 35; vitamins, 10; cellulose, 20.

statistically evaluated by analysis of variance (ANOVA) with SPSS software (release 6.1; SPSS Japan Inc., Tokyo, Japan). One-way ANOVA was used to determine the significant effects of E-64a or a low calcium diet on the measured variables such as bone weight, serum calcium level and bone strength. Individual differences between groups were assessed using Duncan's multiple range test. Differences were considered significant at $P < 0.05$.

Results

E-64a inhibited bone resorption in vivo

In rats fed with a low calcium diet for 7 wks, right femoral weight (0.9 ± 0.2 g, $n=5$) significantly decreased compared with that (1.2 ± 0.1 g, $n=5$) in rats fed with a standard diet for 7 wks. Femoral cathepsin K-like activity and serum hydroxyproline level in rats fed with a low calcium diet were significantly higher than those in rats fed with a standard diet, whereas femoral collagenase-like activity were not changed by a low calcium diet (Fig. 1). The intraperitoneal injection of E-64a to rats caused a significant decrease in their serum calcium level after 3 to 6 hrs (Fig. 1A). Serum calcium level reached the minimum 6 hrs after injection in rats treated with a high dose (8 mg/rat) of E-64a, while it returned to the control level at the same time point in those treated with a low dose (4 mg/rat) of E-64a. After treatment with 8 mg/rat of E-64a, femoral cathepsin K-like activity changed in parallel with the serum calcium level. To confirm whether the decrease in serum calcium level was due to the inhibition of cathepsin K-mediated bone resorption, we also measured collagenase-like activity in bone and serum hydroxyproline concentration. Serum hydroxyproline level significantly decreased 6 hrs after injection of 8 mg/rat of E-64a, while collagenase-like activity in bone did not change (Fig. 1B). Urinary excretion of calcium did not change after the E-64 treatment (data not shown).

Effects of tail-suspension on weight and cathepsin K-like activity in femur

Tail-suspension also caused a significant bone weight loss from Day 5 to Day 21 (Fig 2). Unlike rats fed with a low calcium diet, femoral cathepsin K-like activity in tail suspended rats was constant (Fig. 2), and tail-suspension did not change serum calcium and hydroxyproline levels (data not shown). In tail-suspended rats, femoral strength determined by three-point bending test significantly decreased by 20% of that of control rats on Day 14 and 21 (Fig. 2).

E-64 prevents suspension-induced decline in bone strength

Since E-64a was effective to bone cathepsin K-like activity within 12 hrs after its intraperitoneal injection

Fig. 1 Effects of E-64a on serum calcium and hydroxyproline concentrations, and bone cathepsin K- and collagenase-like activities in rats fed with a low calcium diet. (A) Rats fed with a low calcium diet for 7 wks were intraperitoneally injected with a vehicle or 4 or 8 mg/rat of E-64a. The rats were killed under anesthesia before ($n=5$) or at 3 ($n=5$), 6 ($n=5$), 12 ($n=5$) or 24 hrs ($n=5$) after E-64a injection. Femora for examination of cathepsin K-like activity were prepared from rats injected with 8 mg/rat of E-64a. Blood samples for examination of calcium concentration were collected from the tail vein of other rats injected with a vehicle ($n=5$) or 4 ($n=5$) or 8 mg/rat ($n=5$) of E-64a at the indicated times. Values are mean $\pm$ SD ($n=5$). *Indicates significant difference, compared with a group before injection ($P < 0.05$). Cath. K: cathepsin K. (B) Protease activities and serum hydroxyproline (Hyp) concentration in rats fed with a low calcium diet were measured at 6 hrs after injection of a vehicle or 8 mg/rat of E-64a. Rats fed with a non-purified standard diet for 7 wks were also prepared as a normal diet group. Values are mean $\pm$ SD ($n=5$). Means with different superscripts are significantly different ($P < 0.05$).

(Fig. 1A), we injected E-64a to tail-suspended rats twice a day to prevent suspension-induced bone loss and compared the effects with those of a bisphosphonate. E-64a inhibited femoral cathepsin K-like activity (Fig. 3A).

Fig. 2 Time-dependent changes in weight, cathepsin K-like activity and strength in the femur of tail-suspended rats. Rats were killed before or 2, 5, 10, 14 or 24 day after tail-suspension. Wet weight, cathepsin K-like activity and strength of femur were measured as described in "Materials and Methods". Values are mean $\pm$ SD (n=5). *Indicates significant difference, compared with a group before injection ($P < 0.05$).

The inhibition of cathepsin K-like activity by 4 mg/rat of E-64a was significantly less than that by 8 mg/rat of E-64a. Etidronate administration did not affect bone cathepsin K-like activity. Daily treatment with 8 mg/rat of E-64a as well as twice treatment per week with 1 mg/rat of etidronate significantly prevented declines in bone weight and strength caused by tail-suspension, but treatment with 4 mg/rat of E-64a did not (Fig. 3B and C).

Discussion

E-64a significantly decreased concentrations of serum calcium and hydroxyproline as well as femoral cathepsin K-like activity in rats fed with a low calcium diet, suggesting that E-64a could function as an antiresorptive agent possibly by inhibiting cathepsin K activity released from osteoclast. In this study, we investigated the effects of this antiresorptive agent on bone mass weight and strength in tail-suspended rats and compared them with those of etidronate.

In tail-suspended rats, serum hydroxyproline level and femoral cathepsin K-like activity did not increase. These findings are consistent with the previous report showing that bone formation decreased and bone resorption was unchanged under microgravity conditions; bone resorption was relatively high compared with bone

Fig. 3 Effects of E-64a on (A) cathepsin K-like activity, (B) weight, and (C) strength in bone of tail-suspended rats. Rats were suspended and daily injected with a vehicle or E-64a (4 or 8 mg/rat) for 2 wks. We also injected 1 mg/rat of etidronate to tail-suspended rats twice a week for 14 days. Control rats were maintained without tail-suspension. Rats were killed at 6 hrs after the last injection. Cathepsin K-like activity (A), wet weight (B) and strength (C) of femur were measured as described in "Materials and Methods". Values are mean $\pm$ SD (n=5). Means with different superscripts are significantly different ($P < 0.05$).

Fig. 4 Design of a novel antiresorptive agent from E-64 and bisphosphonate.

formation (Arnaud et al., 1991; Holick, 1998; Lueken et al., 1993). Bone resorption decreased by treatment with E-64a may improve the imbalance of bone formation and resorption under microgravity-simulated conditions, leading to preventing declines in bone weight and strength in rats. In fact, several investigations have already reported that decreased bone resorption by treatment with bisphosphonates resulted in increased femoral strength in hindlimb-immobilized rats (Lockwood et al., 1990; Mosekilde et al., 2000).

Suppressive effects of E-64a on femoral cathepsin K-like activity and serum calcium level were cancelled 12 hrs after its injection *in vivo*, whereas E-64a irreversibly inhibited cysteine proteases *in vitro*, suggesting that E-64 may be easily washed out by blood flow. These results were consistent with our previous report showing that when ^3H -labeled E-64 was injected into rats intraperitoneally, the high radioactivity in the serum decreased in a short time (Hashida et al., 1980). Therefore, injections of a high dose (more than 8 mg/rat) of E-64a twice a day were required to prevent a decline in bone strength caused by tail-suspension. In contrast, twice a week of bisphosphonate effectively prevented suspension-induced decline in femoral strength, whereas it did not change femoral cathepsin K-like activity. Bisphosphonates are known to be easily incorporated into bone matrix. Therefore, they could remain in the bone and persist suppressive effects on bone resorption for a long time *in vivo*, compared with E-64a.

Murakami et al. (1995) reported that bisphosphonates should be incorporated into osteoclasts to inhibit bone resorption. However, the detailed mechanism of antiresorptive effect of incorporated bisphosphonate is still unknown. In addition, even pamidronate, which had 100-fold potent antiresorptive effects of etidronate, have been reported to be tentatively effective for severe hypercalcemia associated with carcinoma and its metastasis (Body et al., 2000; Brincker et al., 1998). Therefore, developing novel clinical drugs possessing higher antiresorptive efficacy is the next important objective. E-64 is a compound available for easily developing its derivatives. We have already developed

various E-64 derivatives such as cathepsin B-specific inhibitors by changing side chains of L-*trans*-epoxysuccinyl structure of E-64 (Towatari et al., 1991). In contrast, basic structure (P-C-P structure) of bisphosphonate has a unique characteristic of binding it to bone matrix preferentially (Sato et al., 1991). Therefore, we are constructing a novel antiresorptive agent by conjugation of E-64 and a bisphosphonate, as shown in Fig. 4. This conjugation could target E-64 to the bone and retain its antiresorptive effect there for a long time.

E-64 and its derivatives have originally been developed as drugs against cysteine protease-associated muscular degenerative diseases, such as Duchenne-type muscular dystrophy (Sugita et al., 1980). About 10 years ago a double-blind test of E-64d (EST; *trans*-epoxysuccinyl-L-leucylamide-3-methyl-butane ethyl ester) was performed to patients bearing Duchenne-type muscular dystrophy (Ishihara, 1991). Unfortunately, E-64d did not significantly prevent the advance of this disease. We considered that the negative effect of E-64d was mainly due to its quick turnover by blood flow *in vivo*, as described above. Since E-64 and its derivatives have potent inhibitory activities against cysteine proteases *in vitro*, they may be available as effective drugs against cysteine protease-associated muscular diseases as well as suspension-induced osteopenia, if they could be retained in skeletal muscle for a long time.

In this study, we showed a Z-Phe-Arg-NH-Mec hydrolytic activity in the solution extracted from whole femur as a femoral cathepsin K-like activity, although it contained not only cathepsin K activity but also activities of cathepsins B and L from bone marrow cells. Interestingly, in rats fed with a low calcium diet, the femoral cathepsin K-like activity and serum hydroxyproline level were significantly higher than those in rats fed with a standard diet, whereas femoral collagenase-like activity were not changed by a low calcium diet (Fig. 1B). In addition, the femoral cathepsin K-like activity changed in parallel with serum calcium and hydroxyproline levels after treatment with E-64a, but femoral collagenase activity did not respond to E-64a treatment (Fig. 1A). Our results suggest that the cathepsin K-like activity measured with the present method could be a useful marker for bone resorption *in vivo*.

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