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VOLUNTARY EXERCISE INCREASES OSTEOGENETIC ACTIVITY IN RAT BONES

BY

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Abstract

The purpose of this study was to investigate the effect of voluntary exercise on osteoinductive activity in rat bone. Sprague-Dawley male and female rats were allowed to exercise freely by running on a treadmill or kept as controls without exercise for 53 days. Decalcified humeral diaphyses from experimental and control rats were implanted intraperitonealy into host rats and harvested after 33 days. A significant increase in bone formation was confirmed in the implanted bone matrices from the running group in comparison with those from control animals by soft X-ray photography and determination of alkaline phosphatase activity and mineral content. Alkaline phosphatase activity in bone and serum was increased by exercise in both male and female animals. The results suggest that osteoinductive activity in the bone was probably due to increased levels of bone morphogenetic protein following voluntary exercise.

Key words: Voluntary exercise, Bone mineral density, Bone morphogenetic protein.

Introduction

The increased incidence of fractures accompanying osteoporosis causes serious problems in elderly patients. The consensus among gerontolosits is that this disease can be effectively prevented by a regimen including physical exercise in addition to sufficient calcium intake and estrogen replacement in the postmenopausal women. Physical exercises have been demonstrated to retard bone loss and to increase bone mass in persons at risk for developing osteoporosis (Hatori et al. [1]; Shimkin et al. [2]; Smith et al. [3]).

It has been established that decalcified bone matrix, when implanted into extraskeletal sites, induces differentiation of cartilage and bone in the host tissues (Urist [4]; Urist et al. [5]). Several fractions of bone morphogenetic protein (BMP) responsible for bone induction have been detected (Urist et al. [6]; Harada et al. [7]; Bentz et al. [8]). Wozney et al. [9] reported the cloning of the full-length cDNA, and eight species of BMPs have been identified to date. BMP was also found to stimulate alkaline phosphatase (ALP; orthophosphoric monoester phosphohydrolase, alkaline optimum EC 3.1.3.1) activity in osteoblastic MC3T3-E1 cells *in vitro* (Vukicevic et al. [10]). A decrease in osteoinduction was reported in implants of vitamin D-deficient rat bone by Turner et

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al. [11].

The present study was undertaken to investigate the effect of voluntary exercise on osteoinductive activity in bone in an experimental animal model. Bone morphogenetic activity was determined by implanting into host animals, the decalcified matrix of bone from rats in the voluntary exercise or control groups.

A significant increase in bone formation was observed in terms of mineral content and radiophotography of the implanted bone matrix; these values were significantly increased for matrix from the running group when compared with those values from the non-exercise control animals. Elevated ALP activity was observed in bone and serum after voluntary exercise. The increased ALP in serum was identified as a heat-labile bone-type isozyme.

Materials and Methods

I, Animals.

Sprague-Dawley strain male and female rats (8 weeks old) were separated into two types of groups: voluntary exercise (running) groups and non-exercise (control) groups (7 rats/group). All the rats were fed a conventional solid diet containing 1.2% calcium and 0.96% phosphorus for 53 days. The animals were allowed food and water *ad libitum*. Animals in the running groups were each kept in a separate cage with a treadmill (27 × 35 × 35 cm) and revolution counter. The treadmill had a circumference of one meter. Rats were able to run freely on the treadmill. The control rats were each kept in a separate cage (15 × 25 × 19.5 cm). At the end of the experiment, all animals were deprived of food overnight.

II, Serum and bone sampling.

Blood was collected from the abdominal aorta under ether anesthesia, and serum was separated by centrifugation. Both humeri were dissected out and the diaphyses cleaned of soft tissue and bone marrow were rinsed with 0.9% NaCl. One humerus was mechanically crushed and homogenized with a Polytron homogenizer (Kinematica, Switzerland) in 10 mM Tris-HCl buffer containing 1% Triton X-100 (pH 7.7). After centrifugation at 10,000 × g for 5 min, the supernatant of the bone homogenate was collected and used for ALP assay. The other humerus was decalcified, and the bone induction activity of the matrix was assayed by the implantation experiment.

III, Preparation of bone matrix and implantation.

The humeral diaphysis was decalcified with 0.6 N HCl for 48 hours at 4°C, and excess acid was washed out exhaustively with distilled water at 4°C until the wash water measured pH 7.0. The matrix of a diaphysis was lyophilized and implanted intraperitonealy into a host rat (Wistar strain male, 7 weeks old, 225–250 g) and harvested after 33 days.

IV, Examination of the implant.

The implant was dissected out, and soft tissue was carefully removed. Radiophotography of the implant was done with a soft X-ray apparatus (M-40, Sofron Corp.) for 12 min at 38 KV and 45 mA on a Konica CS 100 E film. The density of the soft X-ray photograph in an area of 1 mm (width) × 15 mm (length) was determined with a densitometer (TIF-64, Immunomedica Co.) and calculated by using an aluminum step as reference. After measurement of the fresh weight, each implant was divided into three parts. One part was homogenized in Tris-HCl buffer containing 1% triton X-100 (pH 7.7) and centrifuged at 10,000 × g for 15 min. The ALP activity and protein content in the supernatant were determined as described below. A second part was used for histological examination. The last part was ashed at 550–600°C for 24 hours and

dissolved in 1 N nitric acid. Calcium and phosphorus contents were determined by the methods described below.

V, Measurement of bone mineral density.

The right tibial bone of an experimental or control animal was isolated and cleaned. The bone mineral density (BMD) of the whole tibia was measured by dual energy X-ray absorptiometry (DXA; Hologic QDR-1000 X-ray bone densitometer) as reported previously (Omi et al. [12]). A detector collimator with a single slit was affixed to on the X-ray generator, and the scan was performed in an ultra-high resolution mode (rat mode, version 2.0 software).

VI, Enzyme assay.

ALP activity was measured with 10 mM p-nitro-phenylphosphate as a substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer containing 5 mM $MgCl_2$, pH 10.0, at 37°C (Goseki et al. [13]). The hydrolysis rate of p-nitrophenyl-phosphate was determined and expressed in units ($U=\mu$ mol p-nitro-phenol formed/min).

VII, Biochemical determinations.

Protein concentration in the bone homogenate was determined by the method of Lowry et al. [14]. Serum calcium was measured by atomic absorption spectrophotometry (AA-640-12 atomic absorption spectrophotometer, Shimadzu). Phosphorus was determined by the Fiske-Subbarow method (Fiske and Subbarow [15]). Total protein in serum was measured by the biuret method (Gornal et al. [16]).

VIII, Gradient gel electrophoresis.

Cold acetone (−20°C) was added to serum or the bone homogenate in a final concentration of 60% (V/V). The precipitate was collected by centrifugation and dried to an acetone powder. The acetone powder sample was dissolved in 10 mM Tris-HCl buffer (pH 7.7) and electrophoresed with a 4–20% gradient polyacrylamide gel at 125 V for 3 hours. The bridge buffer was 0.1 M Tris-borate (pH 9.5), and gels were stained by the β-naphthyl phosphate method (Kurahashi and Yoshiki [17]).

VIV, Statistical methods.

Student's t-test was used to analyze the differences between the control and the exercise groups and between the male and the female control groups; $p<0.05$ was considered to be statistically significant.

Results

The average running distance of the voluntary exercise rats was 13,200 ± 1,200 m per day for females and 6,800 ± 2,100 m for males, indicating a significant

Table 1. Body weight gain, food intake and food efficiency during the experimental period

	Body weight gain (g/day)	Food intake (g/day)	Food efficiency[a]
Female			
Control	2.09 ± 0.11	18.1 ± 0.47	0.12 ± 0.00
Running	1.62 ± 0.11	20.4 ± 0.77	0.08 ± 0.01
Male			
Control	3.84 ± 0.25	23.9 ± 0.58	0.16 ± 0.01
Running	3.07 ± 0.40	26.2 ± 0.43	0.12 ± 0.02

[a]Food efficiency: Body weight gain/Food intake

Results are the means ± SE of 7 separate determinations.

sex difference. As shown in Table 1, there were also slight differences in food intake and body weight gain between voluntary exercise and control groups and also between males and females.

The results of bone mineral density measurements in tibia are summarized in Fig. 1. The BMD values of both males and females in the running groups were higher than those for both sexes in the control groups. The ALP activity in the humeral bones from the female running group was significantly higher ($P<0.05$) than that in the female control group but this effect was not so prominent for the male groups (Table 2).

Soft X-ray radiographs and the photographic densities of the decalcified hmeral matrices implanted into host animals and harvested after 33 days are shown in Fig. 2. Implants from the running group animals were significantly more radio-opaque than those from the control groups for both males and females. Histological examination revealed more prominent bone formation in the implant samples from the running groups than those from control animals (data not shown).

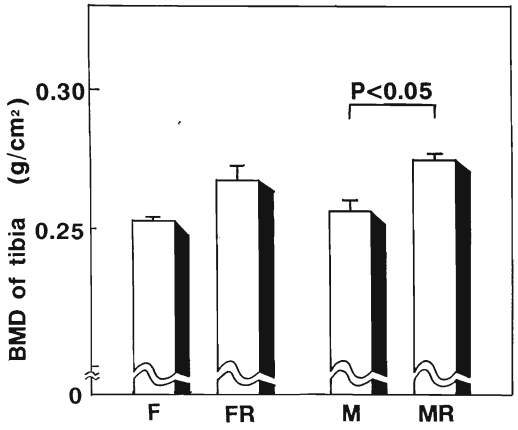

Fig. 1 Effect of voluntary exercise on BMD of tibia. Results are the means ± SE of 7 separate determinations.
F; Female control group, FR; Female running group
M; Male control group, MR; Male running group

The ALP activity (U/ml) and protein content (mg/ml) in the implants from the

Table 2. Effect of exercise on bone and serum alkaline phosphatase activity

	Female		Male	
	Control	Running	Control	Running
Bone ALP (U/mg protein)	0.40 ± 0.07	0.66 ± 0.09*	0.66 ± 0.06#	0.68 ± 0.06
Serum ALP (U/ml)	21.62 ± 1.87	42.55 ± 6.41*	37.39 ± 1.89#	48.82 ± 3.33*
Protein (g/dl)	6.99 ± 0.11	6.30 ± 0.18	6.56 ± 0.03	6.23 ± 0.13
Calcium (mg/dl)	10.20 ± 0.12	9.73 ± 0.09	10.19 ± 0.05	9.96 ± 0.15
Phosphorus (mg/dl)	4.89 ± 0.34	5.97 ± 0.26	6.87 ± 0.30	7.04 ± 0.22

U=μ mol p-nitrophenol formed/min under the conditions described in "Materials and Methods."
Results are the means ± SE of 7 separate determinations.
* Significant difference ($P<0.05$) between the values of running and control groups.
# Significant difference ($P<0.01$) between the values of female and male groups.

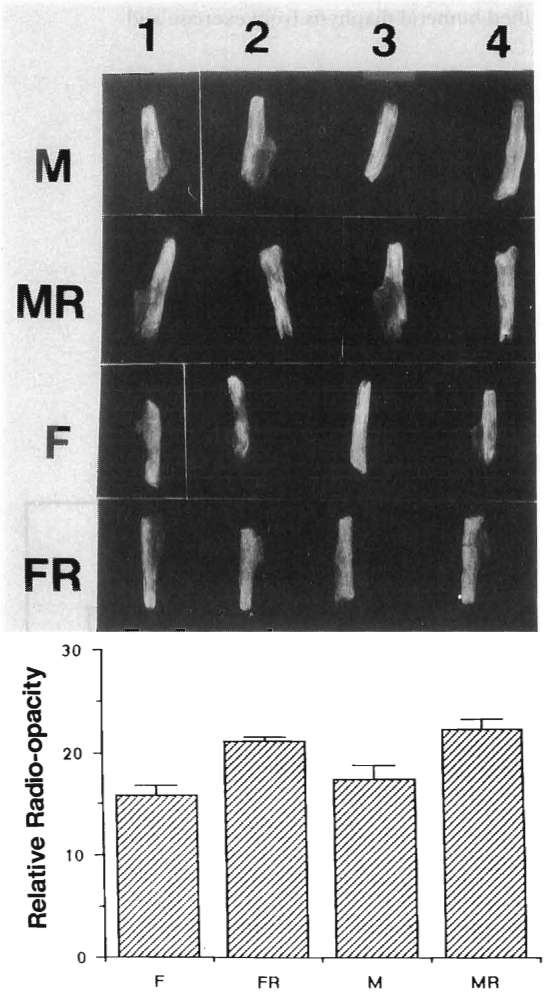

Fig. 2 Soft X-ray radiography of the decalcified humeral diaphyse implanted into host animals.

The upper photograph shows decalcified humeral diaphyse after implantation in the bone induction experiment. The lower histogram shows the relative radio-opacity of the soft X-ray radiographs. Results are the means ± SE of 4 separate determinations. Symbols are the same as in Fig. 1.

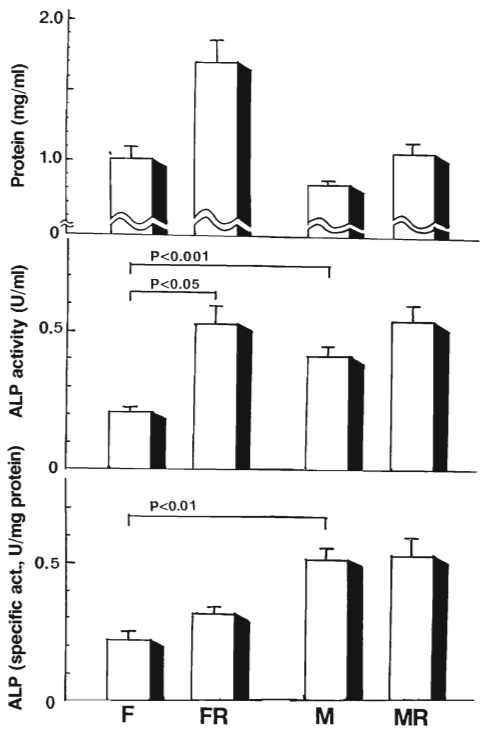

Fig. 3 ALP activity in units and as specific activity and total protein content in the implant of decalcified humoral diaphyse. Results are the means ± SE of 4 separate determinations. Symbols are the same as in Fig. 1.

running groups were higher than those from the control groups for both males and females (Fig. 3). Chemical analyses of the implants are summarized in Table 3. Calcium and phosphorus contents were also larger in the implants from the running groups. According to the differences in these parameters, the bone forming activity in the running groups was concluded to be higher than that in the in control groups.

The effects of exercise on bone and serum ALP values are shown in Table 2. The serum ALP levels in the running group were higher those in the control groups, although the effect was more obvious in the female group ($P<0.05$). Other serum parameters were within normal ranges, and no significant differences were found between running and control or between male and female groups.

The ALP activity in the experimental rat serum was separated into two bands by

Table 3. Mineral contents in implants of decalcified humeral diaphysis from exercise and control groups

	Female		Male	
	Control	Running	Control	Running
Calcium depostion (mg/g implanted bone matrix)	0.221 ± 0.041	0.239 ± 0.052	0.226 ± 0.046	0.242 ± 0.012*
Phosphorus deposition (mg/g implanted bone matrix)	0.112 ± 0.016	0.132 ± 0.032	0.126 ± 0.003	0.131 ± 0.009

Each value represents the mean ± SE of 4 separate determinations.
Calcium and phosphorus contents were calculated per 1 gram of implanted bone.
* Significant difference ($P<0.05$) between the values of running and control groups.

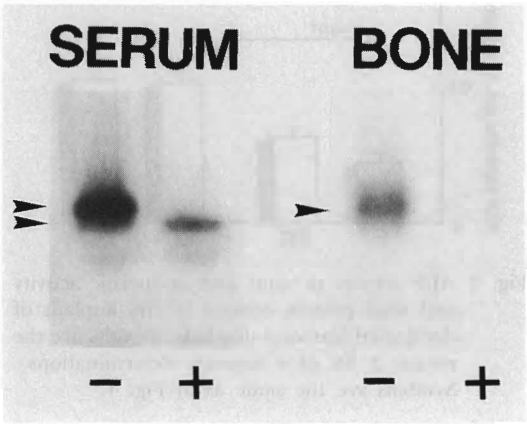

Fig. 4 Polyacrylamide gel (4–20% gradient) electrophoretic pattern of ALP preparations from bone and serum with (+) and without (−) heat treatment at 56°C for 10 min. Enzyme activity was stained by the β-naphthyl phosphate method [17].

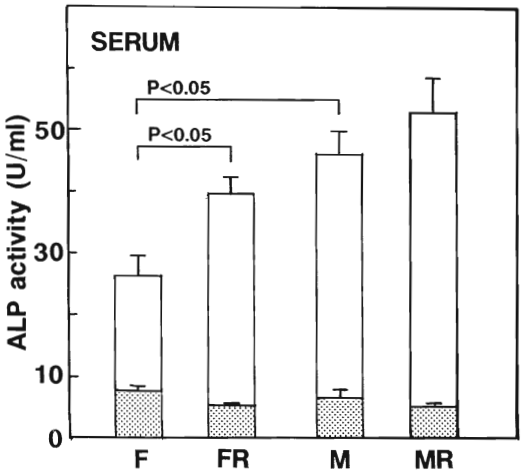

Fig. 5 Effect of voluntary exercise on rat serum ALP. Results are the means ± SE of 7 separate determinations. Open bars indicate heat-inactivated ALP activity (56°C, 10 min), and stippled bars heat-stable activity.
Symbols are the same as in Fig. 1.

4–20% gradient polyacrylamide gel electrophoresis (Fig. 4). The major ALP band was a slow one that showed the same mobility as ALP prepared from bone and was inactivated by heat treatment at 56°C for 10 min. The other ALP band was heat-stable indicating its non-bone origin. The activity of the heat-labile type ALP was found to be increased by voluntary exercise in both male and female rats, as shown in Fig. 5. The heat-stable type of ALP was not affected by exercise and showed no sex difference.

Discussion

There have been many reports about the effect of physical training to increase bone mass and density, and mineralization rate and to elevate ALP activity in bone and serum (Hatori et al. [1]). Severity of the exercise must be an important factor in the study on prevention of bone loss

(Swissa-Sivan et al. [18]). It is generally recognized that excessive training is rather disadvantageous for the protection of human bone. In experimental animals, it was reported that forced exercise in a rotating cage caused a decrease in serum ALP (Atland and Highman [19]). On the other hand, serum ALP was found to be higher in trained swimming rats than in controls (Swissa-Sivan et al. [18]). The experimental conditions used in this study are thought to be moderate and optimal because exercise was voluntary.

In this experiment, the bone mineral density in male and female rats in the exercise group was higher than that in animals in the control group (Fig. 1). The demineralized bone matrix from the running group was found to be more osteoinductive according to soft X-ray radiography, ALP activity (U/ml), and calcium and phosphorus contents in homograft implants (Figs. 2, 3; Table 3).

Several factors including BMP are known to stimulate osteogenesis and chondrogenesis. It was suggested that transforming growth factor-β (TGF-β) stimulated bone formation in embryonic development, whereas early fracture healing and ectopic bone formation were induced by BMP (Joyce et al. [20]; Bentsz et al. [8]; Finkelman et al. [21]). However, TGF-β *per se* cannot induce ectopic bone formation *in vivo*. To date, only BMPs are known to induce cartilage and bone formation at extraskeletal sites *in vivo*. The osteoinductive effect of the bone matrix implants from the voluntary exercise group in this study might reflect increased BMP content in the bone of the running group animals.

An effect of exercise on bone formation was also suggested by the elevation of bone in female rats and in serum ALP as shown in Table 2. In the rat, there are two types of genetically different ALP isozymes; one is liver/bone/kidney (L/B/K) or tissue-nonspecific type and the other intestinal type. Previous studies have suggested that rat serum ALP originated mainly from the intestine (Madsen and Tuba [22]; Saini and Posen [23]). In the present study, however, the main band in rat serum was a heat-labile, bone-type enzyme (Fig. 5). Intestinal type ALP was not dominant even under fasting conditions. The bone-type ALP activity in serum was elevated by voluntary exercise in male and female rat (Fig. 5).

The physiological role of ALP is unknown. However, Weiss et al. [24] have reported that a missense mutation in the human L/B/K ALP gene caused a lethal form of hypophosphatasia characterized by defective bone mineralization. Their report suggests a possible role of the bone ALP isozyme in skeletal mineralization.

Even though the effect of voluntary exercise might not be so dramatic as to increase BMP content in bone throughout the experimental period, it may, however, cause a coupling phenomenon in bone metabolism that leads to a gradual increase in bone mass over the long term.

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