A Calcium-Deficient Diet Caused Decreased Bone Mineral Density and Secondary Elevation of Estrogen in Aged Male Rats—Effect of Menatetrenone and Elcatonin

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In view of the fact that a deficient calcium (Ca) intake results in osteoporosis in elderly males, we conducted an animal experiment on aged male Wistar rats given a Ca-deficient diet. The rats were divided into 2 groups according to diet: a Ca-deficient diet group (Ca content, 0.08% to 0.1%) and a regular diet group (Ca content, 0.8% to 1.2%). The Ca-deficient diet reduced bone mineral density (BMD) by approximately 12%. Administration of menatetrenone or elcatonin was able to reverse the reduction in BMD induced by Ca deficiency. The mean estradiol level in sera of rats fed the Ca-deficient diet was significantly increased to 4.3 times that in the regular diet group. However, the increased estradiol concentration was reduced after the administration of menatetrenone or elcatonin. The estrone concentrations in sera of menatetrenone- or elcatonin-treated rats fed the Ca-deficient diet decreased to a level lower than that of animals fed the regular diet. Testicular aromatase cytochrome P450 ($P450_{arom}$; estrogen synthetase) activity was significantly increased by 2.4-fold in the Ca-deficient diet group compared to that in the regular diet group, and the aromatase mRNA level was also significantly increased 1.45-fold. Testicular aromatase activity was strongly correlated with aromatase mRNA level and serum estradiol level. These data suggest that the change in testicular aromatase expression might be, in part, a compensatory mechanism for the bone mineral deficiency induced by the Ca-deficient diet in aged male rats.

FEMALE HORMONE DEFICIENCY is strongly implicated in the pathogenesis of osteoporosis. It is well known that the incidence of osteoporosis is high in postmenopausal women,[1] and that estrogen administration is effective in improving osteoporotic symptoms.[2,3] Gonadal hypofunction also occurs in males, and is believed to be one of the main causes of osteoporosis in elderly men.[4-6] Experimentally induced hypogonadism in male rats (testicular resection) has been shown to cause a decrease in femoral bone mineral density (BMD) at 1 year of age.[7] As men grow older, the concentrations of free testosterone and dehydroepiandosterone sulfate (DHE-S) decrease gradually, which contributes to the osteoporosis observed in elderly men. Interestingly, however, it has been reported that a man with an estrogen receptor mutation and a man with aromatase deficiency developed osteoporosis, although their serum testosterone concentrations were normal or high.[8-10] In addition, it has been reported in aging men that estrogen was the dominant sex steroid regulating bone resorption, whereas both estrogen and testosterone were important in maintaining bone formation.[11] These findings indicate that estrogen is important in bone metabolism in males as well as in females.[12-14] Moreover, epidemiologic studies have shown that estrogen deficiency contributes to bone loss in elderly men, as well as in postmenopausal women,[13,15] and it is clear that endogenous sex steroids help to maintain BMD in elderly men.[14,16] The testes secrete a small amount of estrogen as well as large amounts of androgens, mainly testosterone. The estrogen is produced from the aromatization of androgens[17] catalyzed by an aromatase cytochrome P450 ($P450_{arom}$; estrogen synthetase),[18] which functions as a rate-limiting enzyme in female hormone biosynthesis.

The purpose of the present study was to clarify the role of the testes in the etiology of osteoporosis in elderly males. As an experimental model of senile osteoporosis, we used aged male Wistar rats in which decreased BMD was induced using a calcium (Ca)-deficient diet. We measured serum estrogen levels, bone markers, testicular aromatase activity, and mRNA levels. Moreover, to treat the osteoporosis we evaluated the effects of menatetrenone (a vitamin K_2 homolog, and a cofactor for the γ-carboxylase, which synthesizes the γ-carboxyglutamic acid found in various proteins/peptides such as osteocalcin, which affects bone matrix synthesis),[19,20] and elcatonin,[21] a natural calcitonin analog derived from the eel, which inhibits bone resorption.

MATERIALS AND METHODS

Animal Treatment

Male Wistar rats, 54 weeks of age, were fed either a regular diet (Ca content, 0.8% to 1.2%; n= 6) or a Ca-deficient diet (Ca content, 0.08% to 0.1%; Clea Japan, Tokyo, Japan; n = 18) for 24 weeks. The rats on the Ca-deficient regimen were further divided into 3 subgroups: (1) an untreated subgroup (n = 6); (2) a subgroup given menatetrenone (donated by Eizai Pharmaceutical, Tokyo, Japan), administered orally at 30 mg/kg body weight/d for 8 weeks (n = 6); and (3) a subgroup given elcatonin (donated by Asahi Chemical Industry, Tokyo, Japan), administered intramuscularly at 10 IU/kg body weight, 3 times per week, for 8 weeks (n = 6). The drugs were administered from the 16th week after initiation of the Ca-deficient regimen. After 24 weeks maintenance on the diets, the animals were subjected to cardiocentesis, and then histologic specimens were collected. At the time of death, no differences in body weight were detected between the groups.

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Table 1. Changes in BMD, Serum Estrogen, and Testicular Aromatase in Aged Rats Fed a Regular or Low-Ca Diet

		Low-Ca Diet		
	Regular Diet	Not Treated	Menatetrenone-Treated	Elcatonin-Treated
BMD (g/cm^2)	0.176 ± 0.013 (0.181 ± 0.016*)	0.155 ± 0.009§ (0.161 ± 0.011†)	0.176 ± 0.027	0.175 ± 0.030
Estrone (pg/mL)	105.4 ± 5.7	86.4 ± 7.4	53.0 ± 14.2‡	53.5 ± 6.7‡¶
Estradiol (pg/mL)	1.32 ± 0.15	5.62 ± 0.94§	3.22 ± 1.40	3.09 ± 0.82
Aromatase activity (pmol ^3H_2O/h/mg microsome protein)	10.7 ± 1.2	26.2 ± 5.5‡	[19.9]‖	[8.2]‖
Aromatase mRNA (relative amount/μg total RNA)	2.38 ± 0.12	3.46 ± 0.28‡	[5.18]‖	[5.13]‖

NOTE. Each value represents the mean ± SE for 6 rats.

*Data from time 0, ie, is 54 weeks of age, and †data from 16 weeks into the low-Ca regimen.

‡$P = .04$, §$P = 0.01$: statistically significant compared with rats on a regular diet.

¶$P = .04$; statistically significant compared with untreated rats on a low-Ca diet.

‖Mean of 2 samples.

Measurement of Bone Mineral Content

A dual x-ray bone densitometer (Norland, XR-26, Fort Atkinson, WI) was used to measure the femoral bone mineral content.[22,23]

Measurement of Serum Estrogen and Other Bone Metabolic Factors

The serum concentrations of Ca, phosphorus, alkaline phosphatase (ALP), and albumin were measured using standard assays. Estrone was measured by means of a radioimmunoassay using an estrone-6 antibody (Wien Laboratories, Succasunna, NJ) (lowest detectable amount, 8.0 pg/mL; intra- and interassay coefficients of variation [CVs], 3.4% and 9.8%, respectively). Estradiol was measured using a double-antibody estradiol kit (Diagnostic Products, Los Angeles, CA) (lowest detectable amount, 1.0 pg/mL; intra- and interassay CVs, 6.2% and 5.8%, respectively). Parathyroid hormone (PTH) was measured by a rat RTH immunoradiometric assay (IRMA) kit (Immutopics, San Clemente, CA) (lowest detectable amount, 0.1 ng/mL; intra- and interassay CVs, 10% and 15%, respectively). 1,25-Dihydroxyvitamin D_3 was measured by a radioreceptor assay using bovine mammary gland receptor and non–high-performance liquid chromatographic purification (lowest detectable amount, 2.1 pg/mL; intra- and interassay CVs, 15% and 10%, respectively).[24]

Aromatase Assay

Testicular aromatase activity was determined by measuring the amount of ^3H_2O water produced in relation to estrogen in the aromatization reaction, i.e. the conversion of [1a-^{3}H] androstenedione substrate to estrogen.[18] NADPH at the final concentration of 0.4 mmol/L was added to the reaction mixture (total volume, 0.5 mL) containing a testicular microsomal fraction (30 μg each) as the enzyme source, 5 μmol/L [1a-^{3}H] androstenedione, 5 mmol/L $MgCl_2$, 1 μmol/L rotenone, and 50 mmol/L Tris-HCl (pH 7.5). The reaction mixture was stirred vigorously for a few minutes and then incubated at 37°C for 60 minutes under aerobic conditions. The substrate was removed by repeated extraction with $CHCl_3$. The scintillator, Scintisol EX-H (Dojun, Kumamato, Japan), was added to the aqueous layer and ^3H_2O was measured with a scintillation counter.

Measurement of the Aromatase mRNA Concentration

Total RNA was isolated from testicular tissue specimens by the method of Chirgwin et al.[25,26] Three micrograms of the total RNA fraction were treated with 7U avian myeloblastosis virus (AMV) reverse transcriptase in a mixture consisting of 50 pmol antisense primer (5' AACCACGATAGCACTTTCGT 3'), 20 U RNase inhibitor, 1.5 mmol/L each of deoxynucleotide triphosphate mixture (dATP, dGTP, dCTP, dTTP), 5 mmol/L dithiothreitol, 75 mmol/L KCl, 10 mmol/L $MgCl_2$, and 50 mmol/L Tris-HCl buffer (pH 8.3) for the reverse transcription reaction at 42°C for 30 minutes. After denaturation at 95°C for 3 minutes, polymerase chain reaction (PCR) was conducted using 2.5 U Taq-DNA polymerase, 15 pmol fluorescent dye, 5-carboxy-fluorescien (FAM) (Perkin Elmer, Foster City, CA)-labeled sense primer (5' TGTTAGAGGTGTCCAGCATG 3'), 15 pmol antisense primer (5' TACTACAACCGGGTATATGG 3'), 0.5 mg gelatin, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$ and 10 mmol/L Tris-HCl (pH 8.5). The PCR was performed with a reaction program of 94°C for 40 seconds, 57°C for 90 seconds, and 72°C for 40 seconds, for 20 cycles. The PCR products and fluorescent size markers were separated by agarose gel electrophoresis, and quantitatively analyzed with a Gene Scanner (Applied Biosystem, Inc, Foster City, CA) with a fluorescence detector. The PCR products were measured on the linear part (total RNA from 1 to 6 μg) of the experimental curve.

All data are expressed as means ± SE. Statistical analysis was performed by means of 1-way analysis of variance (ANOVA), followed by Bonferroni/Dunn test.

RESULTS

The BMD of aged male Wistar rats was 0.176 ± 0.013 g/cm^2 in the regular diet group. By comparison, BMD in the low-Ca diet group was significantly decreased, by approximately 12% ($P = .01$). In contrast, BMD in the drug-treated low-Ca diet groups did not differ from that in the regular diet group (Table 1).

Although the serum PTH and vitamin D_3 levels were slightly higher in all of the low-Ca diet groups than in the regular diet group, the levels of serum Ca, phosphorus, ALP, and albumin showed no statistically significant differences among the 4 groups (Table 2).

The serum estradiol concentrations were 1.32 ± 0.15 pg/mL in the regular diet group. The low-Ca diet group showed a significant increase in the serum estradiol concentration, which was approximately 4.3 times that of the regular diet group ($P = .01$). The increase in the serum estradiol concentration observed in the low-Ca diet group was attenuated by menatetrenone or elcatonin treatment (Table 1). The serum estrone concentrations were 105.4 ± 5.7 pg/mL in the regular diet group. There was a decrease in the serum estrone level in the low-Ca diet group when compared with that in the regular diet group, although this trend was not statistically significant. However, significant decreases in estrone levels were observed in the menatetrenone- or elcatonin-treated low-Ca diet groups (Table 1). Moreover, the estrone levels in the elcatonin-treated

Table 2. Levels of PTH, Vitamin D_3, Ca, Phosphorus, ALP, and Albumin in Sera From Aged Wistar Rats Fed the Regular Diet, Low-Ca Diet, or Low-Ca Diet With Menatetrenone or Elcatonin Treatment

Treatment	n	PTH (pg/mL)	1,25$(OH)_2$ Vitamin D_3 (pg/mL)	Ca (mg/dL)	P (mg/dL)	ALP (IU/L/37°C)	Albumin (g/dL)
Regular diet	6	18.3 ± 5.3	55.1 ± 10.6	8.4 ± 1.2	4.2 ± 0.2	315.8 ± 73.1	4.3 ± 0.2
Low-Ca diet	6	19.5 ± 3.0	97.3 ± 23.6	9.7 ± 0.1	4.7 ± 0.3	255.4 ± 53.5	4.4 ± 0.2
Low-Ca diet with menatetrenone treatment	6	23.8 ± 3.0	108.7 ± 14.8	10.2 ± 0.4	4.5 ± 0.4	298.2 ± 55.4	4.2 ± 0.2
Low-Ca diet with elcatonin treatment	6	23.5 ± 6.1	211.4 ± 82.4*	9.9 ± 0.1	4.2 ± 0.3	338.0 ± 43.0	3.9 ± 0.1

NOTE. Menatetrenone (30 mg/kg body weight/d) was administered orally and elcatonin (10 IU/kg body weight, 3 times/wk) was administered by intramuscular injection for 8 weeks. Each value represents the mean ± SE of 6 rats.

*P = .04; statistically significant compared with rats on a regular diet.

group were also significantly less (P = .04) than those in the untreated low-Ca diet group. Serum testosterone and gonadotropin levels varied within the normal range (data not shown).

From these data we determined the testicular aromatase activity and mRNA levels in these rats. The testicular aromatase activities were 10.7 ± 1.2 pmol ^3H_2O/h/mg microsome protein in the regular diet group. The results indicate a significant increase in the activity in the low-Ca diet group to a level approximately 2.4 times that of the regular diet group (P = .04) (Table 1). The amount of testicular aromatase mRNA also significantly increased in the low-Ca diet group, to a level approximately 1.45 times that of the regular diet group (P = .04) (Table 1).

The aromatase activities correlated significantly with the aromatase mRNA contents (Fig 1A) and the estradiol concentration (Fig 1B).

DISCUSSION

In view of the fact that a reduced calcium intake results in osteoporosis in elderly males,[27,28] we studied the relationship between BMD and endogenous sex steroids and their metabolism in a rat model of senile osteoporosis. BMD decreased by approximately 12% in aged rats given a low-Ca diet. In gonadectomy or postmenopausal models, serum estradiol levels decrease drastically, leading to a rapid decrease in BMD within about 4 weeks.[27] In our study on male rats, the testes were not removed. Consequently, the levels of estrone decreased only gradually and it took about 24 weeks for BMD to decrease significantly.

However, the aged male rats with decreased BMD due to a low-Ca diet showed a 4.3-fold increase in serum estradiol level compared to that in the regular diet group. Generally, deficient calcium intake leads to a decrease in the serum Ca concentration, acceleration of PTH secretion, and promotion of active vitamin D_3 synthesis in the kidneys. In women, the vitamin D receptor gene contributes to the determination of BMD[29] and long-term administration of calcium supplements reverses age-related increases in PTH level and bone resorption and decreases bone loss.[30] In the present study, the serum levels of PTH, 1,25$(OH)_2$vitamin D_3 and Ca tended to be increased, while the serum levels of phosphorus and ALP were not increased in the low-Ca diet groups. The fact that no significant increases in the serum levels of PTH and 1,25$(OH)_2$vitamin D_3 were observed in the untreated low-Ca diet group could suggest that these rats have mild secondary hyperparathyroidism. This may be induced because the development of the decreased BMD, osteoporosis, is slow, possibly because of the secondary elevation of estrogen level in response to the low calcium intake.

It is well known that menatetrenone[31,32] mainly promotes bone formation and elcatonin[33,34] inhibits bone resorption and alleviates the pain in osteoporosis. The mechanisms by which menatetrenone and calcitonin treatments elevate serum PTH and 1,25$(OH)_2$ vitamin D_3 levels are unknown. Calcitonin may

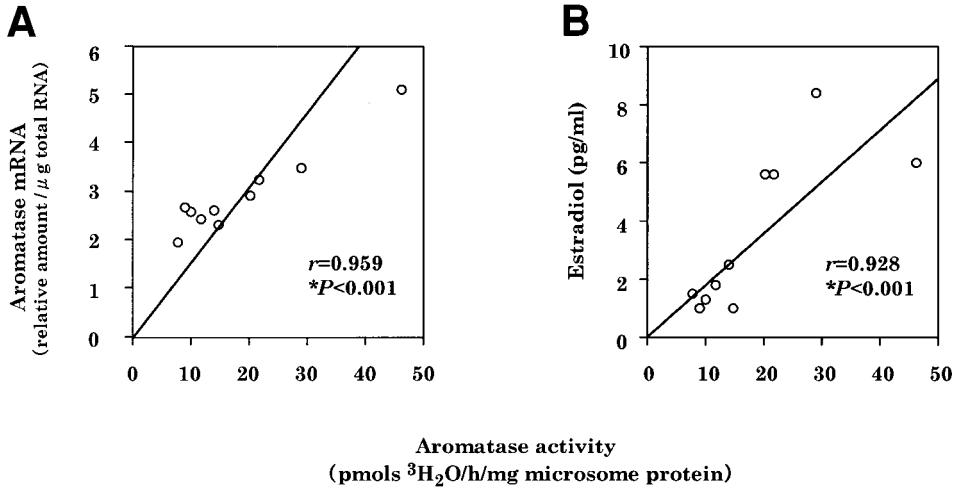

Fig 1. Correlations between (A) testicular aromatase activities and mRNA levels, and (B) serum estradiol levels and aromatase activities. *Statistically significant correlations.

reduce serum Ca levels, which then increase PTH secretion. The menatetrenone and elcatonin treatments, however, were effective in raising the low BMD induced by the low-Ca diet to the level of the regular diet group. The secondary increase in estradiol level was also attenuated by both drugs. Thus, when the BMD decreases, estradiol, which is known to act as an antiosteoporotic factor, seems to increase and play a compensatory role, although no significant changes of testosterone and gonadotropin were observed. Both drugs also ameliorated the measured estradiol levels and caused an exaggerated decrease in estrone levels. We do not fully understand the mechanism behind these results and further investigation will be required to clarify this point. There were good correlations between the serum estradiol and testicular aromatase activity levels, and between the testicular aromatase activity and testicular aromatase mRNA levels (Fig 1). As the aromatase activities we detected in other aromatase-containing tissues, such as the brain and adipose tissues,[35,36] were lower (corresponding to less than 0.1 pmol ^3H_2O/h/mg microsome protein), the compensatory increase in the serum estradiol level observed during the period of reduced BMD seems to be mediated mainly by the testes. The testes, which contain estrogen-producing cells, play an important role in aged male rats with decreased BMD due to a reduced calcium intake.

In conclusion, in aged male rats with declining gonadal function, sufficient calcium intake is necessary to maintain BMD. Moreover, decreases in BMD resulting from Ca deficiency can be abrogated by treatment with menatetrenone or elcatonin. This rat model may be useful in the study of male senile osteoporosis and the findings of this study may be of relevance to the clinical management of osteoporosis in elderly males.

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