Effects of Dietary Restriction on Appendicular Bone in the SENCAR Mouse

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Peptide hormones, cytokines, and growth factors regulate cellular metabolism by stimulating second messenger signal transduction cascades in target tissues. A mutation in the regulatory domain of protein kinase C (PKC) in SENCAR (*sen*sitive to *car*cinogenesis) mice renders them extremely sensitive to diacylglycerol and phorbol esters, resulting in rapid growth, high free radical generation, carcinogenesis, and metabolic bone disease. Dietary restriction (DR) normalizes PKC and ameliorates adverse downstream effects, including carcinogenesis, in SENCAR mice. We hypothesized that DR sufficient to ameliorate carcinogenesis would prevent or delay the early onset of metabolic bone disease in SENCAR mice. Male mice were assigned to 1 of 4 feeding groups from 10 to 16 weeks of age (the critical period when metabolic bone disease develops): ad libitum (AL)-fed; AL antioxidant (0.07% thioproline)-fed; 40% DR; or 40% DR antioxidant-fed. Femoral bone mass was determined gravimetrically. Tibial total, cortical, and trabecular bone mineral density (BMD) were determined by quantitative computed tomography. Body weight, femoral bone mass, and tibial cortical BMD were lower in DR than in AL mice. However, tibial total and trabecular BMD were higher in DR than in AL mice. Serum calcitonin, the hormone that inhibits the osteoclastic bone resorption that is most notable in trabecular bone, was 2-fold higher in DR than in AL-fed mice. Dietary thioproline had no major effects. Thus, DR sufficient to ameliorate carcinogenesis in SENCAR mice did not prevent early-onset metabolic bone disease, but it had a beneficial effect on tibial trabecular BMD that occurred at the apparent expense of cortical BMD. DR in SENCAR mice was also associated with elevated serum calcitonin, which may inhibit osteoclastic resorption and account for trabecular bone conservation in this model. In conclusion, PKC or the downstream metabolic processes regulated by it appear to play previously unrecognized roles in the regulation of tibial trabecular BMD and serum calcitonin in SENCAR mice.

INBRED AND MUTANT mice, with their defined genetic backgrounds and short life spans, are useful models for determining the effects of diet and heredity on bone and mineral metabolism.[1,2] The SENCAR (*sen*sitive to *car*cinogenesis) mouse is a model of elevated free radical generation,[3-6] oxidative damage,[7-9] and carcinogenesis[10-12] that can be ameliorated by defined total dietary or calorie (fat or carbohydrate) restriction and exacerbated by high dietary fat intake.[13-21] The primary genetic defect in SENCAR mice is a mutation in the regulatory domain of a protein kinase C (PKC) isozyme that renders it exquisitely sensitive to diacylglycerol and phorbol ester stimulation.[22,23] Enhanced PKC signal transduction induces 8-lipoxygenase activity and arachidonic acid synthesis in SENCAR mice, resulting in elevated hydroperoxide and superoxide anion production by many cells, including peritoneal macrophages and peripheral blood leukocytes,[5,6,8,9] and a 5- to 10-fold increase in free radical–mediated oxidative DNA damage.[10] Phorbol esters induce high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF),[24,25] epidermal growth factor receptor, transforming growth factor-α and -β (TGF-α and -β),[26,27] and interleukin-1 (IL-1)[28,29] mRNA, protein expression, and activity in SENCAR mice.

Previously, we reported that SENCAR mice rapidly grow to a large size and have higher vertebral and long bone and body mass at sexual maturity[30,31] than most other mouse strains.[2] However, sexually mature SENCAR mice develop histologic features of metabolic bone disease (low numbers of osteoblasts and osteoclasts, fatty infiltration of the marrow cavity, low mineral apposition rate, and low osteoid volume) by 14 weeks of age,[1] while similar characteristics are not observed in control strains until about 2 years of age.[32,33] These results suggest that the mutation in the regulatory domain of PKC that enhances oxidative metabolism, arachidonic acid synthesis, and cytokine and growth factor protein and receptor levels in SENCAR mice may also have important downstream effects on bone metabolism.

Since defined total dietary or calorie restriction regimens can normalize PKC activity or ameliorate its adverse downstream biologic consequences, such as carcinogenesis, in SENCAR mice,[15,17-19,34] we tested the hypothesis that dietary restriction (DR), alone or in combination with antioxidant feeding, will prevent the development of metabolic bone disease in this unique, well-defined murine model of elevated free radical generation, oxidative damage, and growth factor induction. During the critical period from about 10 to 16 weeks of age, when metabolic bone disease develops,[1,30,31] SENCAR mice were subjected to 40% DR,[15] antioxidant feeding, or 40% DR plus antioxidant feeding. The effects of dietary treatment on the appendicular skeleton were assessed by classical gravimetric

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and morphometric methods, as well as by high-resolution peripheral quantitative computed tomography (pQCT). In addition, 2 serum parameters (calcitonin and osteocalcin) germane to mineral metabolism were measured by radioimmunoassay.

MATERIALS AND METHODS

Mice and Dietary Treatments

All experiments were approved by the Animal Subjects Subcommittee and the Research and Development Committee. A SENCAR mouse colony was maintained as outlined in the Guide for the Care and Use of Laboratory Animals (DHEW Publication 86-23). Deionized, distilled water and Richmond standard 9F mouse breeding chow (Diet #5020, Purina Mills, St Louis, MO) were provided ad libitum to the breeding colony. Housing facilities were maintained at 20° to 23°C and 50% to 60% relative humidity with a 12-hour light:12-hour dark cycle.

To permit comparison with previously published studies of bone metabolism in SENCAR mice,[1,30,31] experimental mice were fed modified rodent basal test diet #5755 (Purina Mills, Richmond, IN) from 10 to 16 weeks of age. Modified test diet #5755 for AL-fed experimental mice contained 0.8% calcium and 0.6% phosphorus. Modified test diet #5755 for DR mice contained 1.33% calcium, and 1.0% phosphorus, so that the total dietary calcium and phosphorus intakes of AL-fed and 40% DR mice were equal. Where indicated, modified basal test diet #5755 also contained 0.07% (wt/wt) thioproline (Sigma, St Louis, MO), a natural intracellular antioxidant derivative of the amino acid proline.[35]

At 10 weeks of age, 51 male mice were randomly assigned to 1 of 5 groups: time-zero (t_0) control; AL/−T (ad libitum-fed, no thioproline); AL/+T (ad libitum fed, 0.07% thioproline); DR/−T (40% dietary restriction, no thioproline); or DR/+T (40% dietary restriction, 0.07% thioproline). Time-zero control mice were killed at 10 weeks of age, and the remaining AL-fed or DR mice were killed at 16 weeks of age. The mice in each of the AL-fed and DR groups were weighed weekly from 10 to 16 weeks of age. Food consumption was determined on a daily basis for the AL-fed (0.00% or 0.07% thioproline) mice, and the mice in the 40% DR groups received 60% of the amount of food consumed by their respective AL-fed control groups on the previous day[15] beginning on day 2 of the 6-week dietary treatment.

Quantification of Bone Mass and Density

At the end of the experimental period, the mice were killed by carbon dioxide inhalation followed by exsanguination. The femurs were removed and defleshed, and their maximum lengths and widths were measured with a #5921 micro caliper (Manostat, Geneva, Switzerland).[31] The dry, fat-free and ash weights of the femur were determined as previously reported.[31] Briefly, the femurs were defatted for 48 hours in diethylether:ethanol (1:1; vol/vol), dried at 105°C for 18 hours to obtain constant dry, fat-free weights, and heated at 550°C for 18 hours to obtain constant ash weights.[31] The tibias were excised, lightly defleshed, and stored at 4°C in 95% ethanol until subjected to pQCT with a Stratec XCT 960M (Norland Medical Systems, Ft Atkinson, WI) specifically modified to measure bone mineral and volume in small specimens.[2] Briefly, isolated intact tibias were scanned at 1-mm intervals beginning approximately 0.6 mm from the proximal end. The unit volume within which mineral was measured was set at 0.1 mm^3.[2] Attenuation data for scans through the spongiosal region at the proximal end of the marrow cavity were used to generate values for bone mineral content, volume, and density using XMICE software (version 1.3; Norland).[2] Data are presented for total bone parameters defined by an attenuation threshold $\geq$ 500 and for cortical bone parameters defined by an attenuation threshold $\geq$ 2,000.[2] Total bone density values were calculated by dividing the total mineral content by the total volume.[2] The precision for midshaft total cortical bone mineral density is 1.2%.[2] The resolution of detection is 0.05 mm.[2] The total mass of mineral per femur calculated from pQCT data using the XMICE software algorithms ranged from 15.3 mg to 27.4 mg in 11 strains of inbred mice, which is in excellent agreement with the range of 16.9 mg to 27.4 mg obtained by directly ashing and weighing bone from mice of similar ages.[2]

Serum Osteocalcin and Calcitonin Assays

Blood samples were collected by heart puncture between 8 AM and noon on the day the mice were killed. Serum was obtained by centrifuging heparinized unhemolyzed whole blood in a microfuge for 3 minutes at 4°C. The serum was stored at −20°C until assayed for calcitonin and mouse osteocalcin using commercial radioimmunoassay (RIA) test kits and reagents (Incstar, Stillwater, MN, and Biomedical Technologies, Stoughton, MA, respectively).[30] All assays were conducted in triplicate in multiple dilutions of serum, as well as in control serum containing high or low levels of osteocalcin or calcitonin.[30] The osteocalcin antibody is specific for highly purified intact murine osteocalcin, and the RIA has an intra-assay coefficient of variation (CV) of 7% and inter-assay CV of 12%. The antibody to synthetic human calcitonin (1-32) (a highly conserved mammalian sequence) exhibits excellent cross-reactivity ($\geq$99%) with rodent calcitonins. The calcitonin RIA has an intra-assay CV of 11% and an inter-assay CV of 15%. Assay results were calculated using the four-parameter spline fit option of RIACALC software (Wallac, Turku, Finland).[30]

Statistical Analysis

Data are expressed as the mean ± SEM (n) for all groups. Data were subjected to 2-way multiple ANOVA using dietary food intake and antioxidant feeding as the main effects and testing for interaction using StatView 512$^+$ software (BrainPower Software, Agoura Hills, CA). Where indicated, data for individual groups were analyzed by 2-tailed Student's *t* test for parametric data or Mann-Whitney test for nonparametric data. Results were considered significant at $P \leq .05$.

RESULTS

Food Consumption and Body Mass

Altering the calcium and phosphorus content or adding 0.07% thioproline to Purina test diet #5755 did not affect its palatability, assessed as food consumption (Fig 1), or the apparent health status and activity levels of 10- to 16-week-old SENCAR mice, relative to that observed in previous studies of bone metabolism in this mouse strain.[1,30,31] No consistent statistically significant differences in food consumption were observed when AL/−T mice were compared with AL/+T mice (Fig 1). Since the palatability of the thioproline-supplemented diet had not been determined for AL-fed mice prior to these experiments, the mice in the 40% DR/−T group were fed 60% of the amount of food consumed by the mice in the AL/−T group,[15] while the mice in 40% DR/+T group were fed 60% of the amount of food consumed by the mice in the AL/+T group. The mice in both of the 40% DR groups (DR/−T and DR/+T) consumed all the food supplied to them each day, and the total food intake was essentially the same for both DR groups (DR/−T and DR/+T) for the 6-week test period from 10 to 16 weeks of age. It should be noted that such a 40% total dietary restriction regimen reduced the incidence and number of skin tumors in SENCAR mice seen 16 and 20 weeks after treatment with the tumor initiator 7,12-dimethylbenzanthracene and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate.[15] Therefore, this dietary regimen was specifically chosen

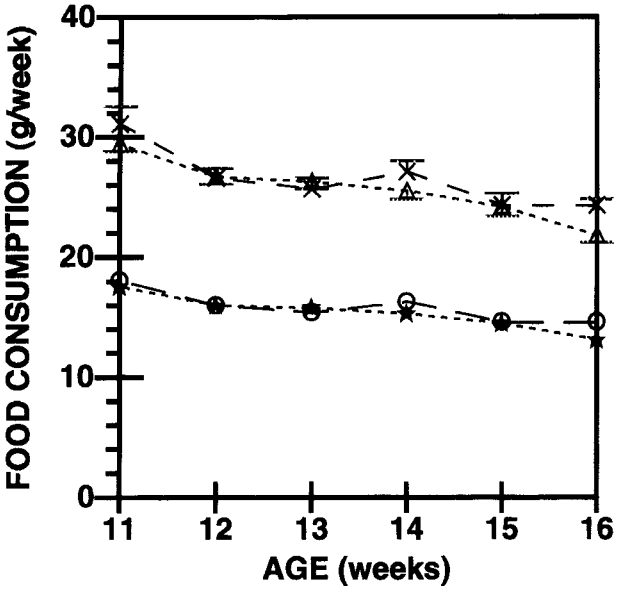

Fig 1. Food consumption by SENCAR mice. Mice were randomly assigned to 1 of 4 experimental feeding groups from 10 to 16 weeks of age, and food consumption was recorded daily. AL/−T (X); AL/+T (△); 40% DR/−T (○); 40% DR/+T (★). Data are the mean ± SEM (n = 10).

for the present studies because it was associated with a statistically significant improvement in a downstream biologic effect (carcinogenesis) of PKC stimulation in SENCAR mice.[15]

AL-fed (AL/−T and AL/+T) SENCAR mice weighed more than 40% DR (DR/−T and DR/+T) mice from 11 to 16 weeks of age ($P < .001$) (Fig 2). The average weight of SENCAR mice in the AL/−T group was not significantly different from that of mice in the AL/+T group. Similarly, the average weight of mice in the 40% DR/−T group was not different from that of 40% DR/+T group, confirming that this natural intracellular antioxidant has no overt adverse effects on food consumption or energy utilization in SENCAR mice.

Femoral Bone Size, Mass, and Ash Content

Six weeks of DR and antioxidant feeding had no statistically significant effects on the maximum external length or width of the femur (Table 1). As previously reported,[31] the dry, fat-free and ash weights of femurs from 10-week old t_0 control mice were equal to or greater than those observed in older mice. In addition, the dry, fat-free and ash weights of the femurs of 16-week-old AL-fed mice were significantly greater ($P = .0008$ and $P = .001$, respectively) than those of DR mice by ANOVA (Table 1). The data demonstrating that AL-fed mice had heavier femurs than DR mice are consistent with preliminary observations that the cortical width (eg, the width of both cortices measured at 7 equidistant sites) of the femurs was 20.7 μm (−T) to 28.2 μm (+T) greater in AL-fed mice than it was in the corresponding group of DR mice. This corresponds to a cortical thickness that was 17% (−T) to 25% (+T) greater in AL-fed mice than in the respective group of DR mice. In addition, the cortical width of the femur was lower in 16-week-old DR mice than in 10-week-old t_0 control mice, while the cortical widths of the femurs were similar in t_0 and AL-fed mice (data not shown). Since the cortices were thicker in AL-fed mice, but the exterior dimensions of the femur were similar in AL-fed and DR mice, the higher dry, fat-free and ash weights observed in AL-fed mice can be attributed to reduced endosteal remodeling, enhanced endosteal deposition, or a combination of both in AL-fed mice, relative to DR mice. DR did not prevent the fatty infiltration of the marrow cavity that develops in sexually mature SENCAR mice[1] (data not shown).

The dry, fat-free and ash weights of the femurs of the DR mice that received no thioproline (DR/−T) were 13.9% (dry, fat-free weight) and 12.6% (ash weight) lower than those of AL/−T control mice (Table 1). In contrast, when thioproline was added to the diet, the femoral bone masses of 40% DR/+T SENCAR mice were only 4.3% (dry, fat-free weight) to 4.5% (ash weight) lower than those of the respective AL/+T control group (Table 1). However, although it appears that there was a trend toward higher femoral bone dry, fat-free and ash weights in thioproline-fed DR mice than in DR mice that did not receive dietary thioproline supplementation (DR/−T), compared with their respective AL-fed control groups, the trend was not statistically significant. Finally, the degree of mineralization, assessed as the percentage ash, was not significantly affected by DR or antioxidant feeding (Table 1).

Tibial Bone Density

When 16-week-old AL-fed and DR SENCAR mice were compared, DR mice had significantly greater tibial trabecular bone density than AL-fed mice ($P = .0002$ by ANOVA) (Table 2). In contrast, 16-week-old AL-fed SENCAR mice had significantly higher tibial cortical bone density than DR mice ($P =$

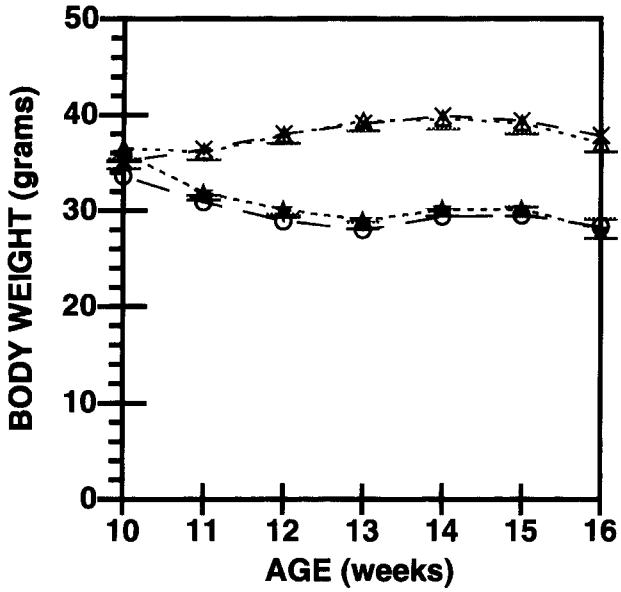

Fig 2. Body weights of 10- to 16-week old SENCAR mice. AL/−T (X); AL/+T (△); 40% DR/−T (○); 40% DR/+T (★). Data are the mean ± SEM (n = 10). Addition of the dietary antioxidant thioproline had no effect on body mass over the course of the experiment.

Table 1. Femoral Bone Length, Width, Mass, and Ash Content in 10- and 16-Week-Old SENCAR Mice

Parameter*	Dietary Group†					ANOVA (*P* values)‡		
	t_0	AL/−T	AL/+T	DR/−T	DR/+T	Effect of DR	Thioproline	Interaction
Age (wk)	10	16	16	16	16			
Length (mm)	17.2 ± 0.11 (11)	17.6 ± 0.06 (9)§	17.4 ± 0.09 (10)	17.1 ± 0.05 (8)§	17.5 ± 0.12 (10)	NS	NS	NS
Width (mm)	2.59 ± 0.03 (11)	2.38 ± 0.04 (9)§	2.43 ± 0.04 (10)	2.38 ± 0.06 (8)§	2.44 ± 0.04 (10)	NS	NS	NS
Dry, fat-free weight (mg)	49.1 ± 1.2 (11)	48.8 ± 0.8 (10)	46.2 ± 1.3 (10)	42.0 ± 1.7 (9)¶	44.2 ± 0.9 (10)	.0008	NS	NS
Ash weight (mg)	32.8 ± 0.8 (11)	31.8 ± 0.4 (10)	30.8 ± 0.9 (10)	27.8 ± 1.03 (9)¶	29.4 ± 0.6 (10)	.001	NS	NS
% Ash‖	66.7 ± 0.2 (11)	65.3 ± 0.7 (10)	66.7 ± 0.4 (10)	66.3 ± 0.3 (9)¶	66.5 ± 0.4 (10)	NS	NS	NS

Abbreviation: NS, not significant.

*All values are expressed as the mean ± SEM (n).

†All mice received Richmond standard 9F mouse breeding chow ad libitum from weaning to 10 weeks of age (t_0). To permit comparison to previous studies of bone metabolism,[1,30,31] DR,[15] and thioproline,[35] AL-fed mice received Purina basal test diet #5755 containing 0.8% Ca and 0.6% Ca; 40% DR mice received test diet #5755 containing 1.33% Ca and 1.0% P; and thioproline-fed (+T) mice received 0.07% (wt/w) thioproline from 10 to 16 weeks of age.

‡All data for 16-week-old mice were subjected to ANOVA, with DR and antioxidant feeding as the major variables.

§The lengths and widths of intact unbroken femurs are reported.

¶The bone from 1 mouse fragmented during cleaning and was lost from the study.

‖% Ash = (Ash weight)/(Dry, fat-free weight) × 100.

.0001). The net result was that total tibial bone density was greater in DR mice than in AL-fed mice ($P = .047$).

When 10-week-old t_0 control mice were compared with 16-week-old AL-fed and DR SENCAR mice, total and trabecular tibial bone density were preserved in 40% DR mice, relative to 10-week-old t_0 controls, while 16-week-old AL-fed SENCAR mice had 40% to 42% lower tibial trabecular bone density than t_0 controls (Table 2). In contrast, tibial cortical bone density in 16-week-old AL-fed mice was conserved, relative to 10-week-old t_0 control values, and the lowest tibial cortical bone densities were observed in 40% DR mice. In summary, there was conservation of tibial total and trabecular bone density in 40% DR SENCAR mice, relative to 10-week-old t_0 control mice, that occurred at the apparent expense of cortical density, while trabecular bone density declined significantly in AL-fed mice. The greatest change in tibial bone density that occurred between 10 and 16 weeks of age in SENCAR mice was the decline observed in the trabecular bone of AL-fed mice, and addition of dietary thioproline had no protective effect on this parameter. Finally, the higher cortical bone density observed in the tibias of AL-fed SENCAR mice (Table 2) is consistent with the greater cortical width (data not

Table 2. Tibial Bone Densities in 10- and 16-Week-Old SENCAR Mice

Parameter*	Dietary Group†					ANOVA (p values)‡		
	t_0	AL/−T	AL/+T	DR/−T	DR/+T	DR	Thioproline	Interaction
Age (wk)	10	16	16	16	16			
Total density (mg/mm^3)	0.439 ± 0.014 (10)	0.413 ± 0.011 (10)	0.411 ± 0.017 (10)	0.429 ± 0.015 (10)	0.453 ± 0.011 (10)	.047	NS	NS
Cortical density (mg/mm^3)	0.515 ± 0.006§¶ (10)	0.538 ± 0.012 (10)	0.524 ± 0.005 (10)	0.497 ± 0.005§ (10)	0.495 ± 0.005¶ (10)	.0001	NS	NS
Trabecular density (mg/mm^3)	0.233 ± 0.030‖,# (10)	0.135 ± 0.017‖ (10)	0.140 ± 0.018# (10)	0.198 ± 0.025 (9)**	0.240 ± 0.018 (8)**	.0002	NS	NS

*Experimental values are expressed as the mean ± SEM (n).

†The diets are described in Table 1.

‡All data for 16-week-old mice were subjected to ANOVA, with DR and antioxidant feeding as the major variables.

¶Cortical bone density in 10-week-old t_0 control mice was significantly higher than that observed in 16-week-old 40% DR mice at § $P = .0272$ (DR/−T) and ¶ $P = .0197$ (DR/+T) by Student's *t* test.

‖,#Trabecular bone density in 10-week-old t_0 t_0 control mice was significantly greater than that observed in 16-week-old AL-fed mice at ‖ $P = .0108$ (AL/−T) and # $P = .0151$ (AL/+T) by Student's *t* test.

**The attenuation by trabecular bone could not be distinguished in 3 samples.

shown) and mass observed in their femurs (Table 1), when compared with DR mice.

Serum Osteocalcin and Calcitonin

When 16-week-old AL and DR mice were compared, there was no statistically significant difference in serum osteocalcin (one of the major noncollagenous proteins of bone synthesized and secreted by the osteoblast) (Table 3).[36] Calcitonin, the systemic peptide hormone that inhibits osteoclastic bone resorption,[37] was significantly higher ($P = .0001$) in the serum of 16-week-old 40% DR SENCAR mice than in 16-week-old AL-fed controls (Table 3).

DISCUSSION

The SENCAR mouse is a novel, well-defined model of 2-stage carcinogenesis characterized by enhanced PKC activation[22,23] and the downstream consequences of this activation, including elevated free radical generation,[5,6,8,9] oxidative damage,[10] and growth factor (GM-CSF, TGF-α and -β, and IL-1) and growth factor receptor transcription, synthesis, and activity in epidermal cells, monocytes, and macrophages.[24-29] In addition, the metabolic regulation of bone development, growth, and maintenance is disordered in SENCAR mice. Specifically, the SENCAR mouse rapidly grows to a large size, and its vertebrae and long bones are larger and heavier at sexual maturity than those of 10 other strains of mice.[2,30,31] Although the biochemical mechanisms underlying this robust bone growth have not been elucidated, it is consistent with the high levels of growth factor and growth factor receptor synthesis and activity observed in the nonskeletal tissues of SENCAR mice.[24-29] For example, osteoblasts secrete TGF-β, which is deposited in the extracellular matrix during bone formation.[37] TGF-β, which has previously been shown to be upregulated by phorbol esters in SENCAR mouse skin,[26,27] is an autocrine/paracrine upregulator of osteoblast anabolism when it is subsequently released from the bone matrix by osteoclastic resorption.[37] Enhanced expression and activity of local growth factors that affect bone, such as TGF-β, in immature SENCAR mice would favor the development of high peak bone mass.

The vertebrae[30] and femurs[1,31] of SENCAR mice stop growing significantly by the time they are sexually mature ($\geq$10 weeks of age). By 14 weeks of age, SENCAR mice exhibit severe histologic abnormalities in bone and cartilage, including the development of architectural disarray and focal discontinuities in the growth plate, low bone and osteoid volume, low mineral apposition rate, fatty infiltration of the marrow cavity, and very low numbers and biosynthetic activity of osteoblasts and osteoclasts.[1] Although the pathophysiologic mechanisms of bone loss in SENCAR mice have not been elucidated, the perforation of structural elements, increased marrow cavity size, and discontinuities in bone structure that are observed[1] are consistent with an osteoclast-mediated imbalance in focal remodeling.[38] GM-CSF and IL-1 (which are upregulated in phorbol ester-stimulated macrophages and skin in SENCAR mice[24,25,28,29] and induce osteoclastogenesis in bone[39]) may initiate cycles of osteoclast-mediated remodeling[38] that result in the unique histologic features of metabolic bone disease that are observed in this model.[1] In addition, reactive oxygen species, including superoxide and peroxide (which are produced at high levels by the leukocytes and macrophages of phorbol ester-treated SENCAR mice[5,6,8,9,11]) stimulate osteoclastogenesis in bone marrow stem cell cultures.[40] Since the dietary antioxidant thioproline had no statistically significant beneficial effects on femoral bone mass or basic morphometry or on tibial bone density in 16-week-old SENCAR mice (Tables 1 and 2), the results presented here suggest that autocrine and paracrine cytokines and growth factors, such as IL-1 and GM-CSF, probably play a more important role in the development of bone and cartilage abnormalities in mature SENCAR mice than do free radicals. Earlier implementation of antioxidant feeding or the substitution of other antioxidants for thioproline may have greater effects, but this remains to be determined empirically.

Since defined total dietary or calorie (fat or carbohydrate) restriction regimens are sufficient to normalize PKC activity and ameliorate its downstream biologic consequences in SENCAR mice,[15,17-19,34] the present studies were undertaken to test the hypothesis that DR would prevent the development of metabolic bone disease if it were instituted during the critical period of the life span when metabolic bone disease developed. Long bones were analyzed by classical gravimetric and morphometric methods,[30,31] as well as by pQCT, which is extremely sensitive to regional changes in cortical and trabecular bone mass, volume, and density.[2] The results obtained in 16-week-old AL-fed SENCAR mice confirmed previous reports that bone growth slows or stops at about 10 weeks of age (Table 1) and that early-onset metabolic bone disease develops within weeks of achieving sexual maturity (Table 2).[1,31] When

Table 3. Serum Osteocalcin and Calcitonin Levels in 16-Week-Old SENCAR Mice

Parameter*	Dietary Group				ANOVA (P value),† DR Effect
	AL/−T	AL/+T	DR/−T	DR/+T	
Serum osteocalcin (ng/mL)	3.19 ± 0.40 (9)‡	3.46 ± 0.33 (9)‡	4.87 ± 0.71 (10)	3.82 ± 1.07 (10)	NS
Serum calcitonin (pg/mL)	83.9 ± 5.5 (9)‡	91.4 ± 14.3 (9)‡	214.9 ± 25.8 (9)§	175.9 ± 18.5 (8)§	.0001

*All experimental results are expressed as the mean ± SEM (n).

†All data for AL-fed and DR mice were subjected to ANOVA, with DR and antioxidant feeding as the major variables. No significant ($P < .05$) antioxidant effects or interactive effects between DR and antioxidant feeding were observed.

‡Hemolyzed samples were not assayed.

§There was not enough serum available to assay calcitonin in multiple dilutions in 3 mice.

assessed by pQCT, 16-week-old AL-fed SENCAR mice were found to have lower tibial total and trabecular bone mineral density (BMD) than 10-week-old t_0 controls (Table 2). Trabecular bone has a much larger surface area to volume ratio than cortical bone.[37-39] Since osteoclastic resorption occurs on the surfaces of bone, trabecular bone is much more labile to osteoclast-mediated imbalances in remodeling and bone loss than is cortical bone.[37-39] The novel observation that decrements in trabecular BMD, but not cortical BMD, are observed at an early age in SENCAR mice (Table 2) supports a major role for the osteoclast in the evolution of metabolic bone disease in this model.

Our findings also suggest that the effects of DR appear to vary depending on the type of bone (cortical *v* trabecular) examined and the sensitivity and selectivity of the method of analysis (gravimetric or morphometric *v* pQCT) employed (Tables 1 and 2). Specifically, 40% DR did not affect gross femoral morphometry (maximum external length and width) (Table 1), but it was associated with reduced cortical width, relative to 10-week-old control and 16-week-old AL-fed mice. Although the external dimensions of the femur were unchanged, the decrease in cortical width in DR mice resulted in lower femoral bone mass in DR mice than in t_0 control and AL-fed mice (Table 1). In conclusion, the observations that 40% DR did not prevent femoral bone loss (assessed gravimetrically as dry, fat-free and ash weights, or morphometically as cortical width) (Table 1) between 10 and 16 weeks of age in SENCAR mice appear to refute the initial hypothesis that DR sufficient to correct the underlying biochemical defect would also prevent bone loss. However, when the bones were analyzed by pQCT, 40% DR was associated with a relative conservation of tibial total and trabecular BMD that occurred at the apparent expense of cortical BMD (Table 2). The mechanisms by which DR effects the preservation of trabecular bone, relative to cortical bone, in SENCAR mice have not been determined. Diacylglycerol-regulated growth factor or growth factor receptor synthesis may be reduced by DR in SENCAR mice, thus reducing the rates of osteoclastogenesis and bone resorption, and preferentially preserving trabecular bone. This hypothesis could be tested in several ways. For example, the rate of bone metabolism (anabolism + catabolism) in AL-fed and DR SENCAR mice could be assessed by measuring bone resorption and formation markers in the blood and urine. The levels, rates of synthesis, and turnover of specific growth factors and growth factor receptors could be measured in vitro in osteoblast-enriched cultures or osteoblast and osteoclast cocultures derived from AL-fed and DR control (eg, C57BL/6 or DBA/2) and SENCAR mice. The rates of bone resorption in calvariae or long bones from AL-fed and DR control and SENCAR mice could be tested ex vivo in the absence or presence of vehicle, phorbol esters, diacylglycerol, or regulatory hormones, such as calcitonin or PTH.

DR was also associated with 2-fold increase in serum calcitonin levels (Table 3). Calcitonin is the calcitropic hormone that inhibits osteoclastic bone resorption and enhances urinary calcium reabsorption.[37] If PKC-mediated increases in GM-CSF,[24,25] IL-1,[28,29] superoxide and hydroperoxide[3-6] documented in nonskeletal tissues in SENCAR mice also induce osteoclastogenesis and osteoclast activation in bone, as suggested above, then the increased circulating calcitonin levels observed in DR mice may account for preservation of tibial total and trabecular BMD. However, further research will be required to determine the physiologic mechanism responsible for increased circulating calcitonin levels in DR SENCAR mice. In conclusion, more detailed and longer-term studies of bone and mineral metabolism, bone cell biology, and the hormonal regulation of bone remodeling in SENCAR mice will be required to determine the mechanistic bases for the development of metabolic bone disease and the beneficial effects of DR on total and trabecular BMD.

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