The Effects of Dietary Restriction on Humeral and Mandibular Bone in SENCAR, C57BL/6, and DBA/2 Mice

Samuel S. Murray, Maria Eugenia L. Duarte, and Elsa J. Brochmann

Dietary restriction (DR) increases the life span and retards the development of age-related disorders. However, the low body mass that accompanies DR is associated with risk factors for fracture that may outweigh the beneficial effects of DR on cellular aging that are mediated, in part, by limiting free radical generation and oxidative damage. We tested the effects of DR in murine models that differ in free radical generation capacity (SENCAR > C57 > DBA). Male mice of each strain were killed at 10 weeks of age (t_0; time zero) or randomized to an ad libitum-fed (AL-fed) or 30% DR feeding regimen for 6 months. The food consumption of AL-fed mice was measured daily. DR mice received 70% of the amount of food consumed by their respective AL-fed mice the previous day. The DR diet was normalized with respect to calcium (Ca), phosphorus (P), and micronutrients. Lean body mass (LBM), bone mineral density (BMD), and bone mineral content (BMC) in the humerus and mandible were determined by PIXImus densitometry. The length and midshaft width of the humerus were determined by direct measurement. There were highly strain- and diet/time-dependent effects on LBM, humerus length, mandibular and humeral BMD, and humeral BMC. The interaction between diet/time and strain was more significant in the humerus than the mandible. All 30% DR mice had lower humeral BMDs and BMCs than their respective AL-fed controls. However, 30% DR C57 and DBA (but not SENCAR) mice had higher humeral BMD and BMC than their respective t_0 controls. There was a linear relationship between LBM and humeral BMD and BMC in both AL-fed and 30% DR mice, suggesting that the lower BMD and BMC in 30% DR mice, relative to AL-fed controls, reflects a physiologic adaptation to lower biomechanical loading. Mandibular BMC in 30% DR C57 (but not DBA or SENCAR) mice was lower than that observed in their AL-fed controls. Mandibular BMD and BMC increased versus t_0 values in 30% DR mice of all strains.

DIETARY RESTRICTION (DR) extends the maximum life span and retards the development of age-related diseases in many tissues.[1] The precise mechanisms by which DR does so remain controversial, but are thought to involve reduced energy expenditure and oxidative metabolism,[2] reduced free radical generation and downstream damage to proteins, lipids, and DNA,[3] reduced damage to major cellular organelles, including mitochondria,[4] and ultimately, maintenance of normal physiologic functions over the life span of the organism. To date, however, the effects of DR on bone have not been completely elucidated, because they reflect both the beneficial antioxidant effects of DR, as well as the complex interrelationships between diet, skeletal dynamics, and body mass or composition. For example, clinical and animal studies have demonstrated that body mass is positively correlated with bone mineral density (BMD) and bone mineral content (BMC) across the life span,[5,6] irrespective of sex or strain differences.[7] Mechanically-driven bone formation, a major component of total skeletal development, is directly related to body weight.[8] Conversely, calorie restriction (CR) in adults is associated with low body mass and reduced measures of skeletal mass, including BMD, BMC, calcium content, and bone strength, all of which are risk factors for fracture.[6]

The effects of DR on bone vary depending upon the animal model tested, the age at which DR is instituted, the composition of the diet, and the duration of DR. For example, long-term (11 years) CR was associated with low bone mass in rhesus monkeys (*Macaca mulatta*) that was accounted for by adjusting for age and body weight.[6] In contrast, short-term (12 months) CR had no effect on older rhesus monkeys (mean age, 19 years), while it was associated with slower gains in total body BMD and BMC in younger (mean age, 4 years) CR monkeys relative to ad libitum-fed controls.[6] Chronic (6 years) energy restriction (ER) reduced body and fat mass, but did not alter BMD, BMC, osteocalcin, or calcitropic and reproductive hormone levels in 7- to 27-year-old female rhesus monkeys.[9]

Disparate results of DR studies have also been obtained in rodents. For example, life-long DR with nutritionally balanced diets prevented senile osteopenia[10] and normalized calcitropic hormone status[11] in F344 rats, a model of age-related renal disease. In contrast, short-term (9 weeks) ER reduced body mass and decreased femoral BMD in mature (20-week-old) and aging (48-week-old) Sprague-Dawley rats, which are not prone to age-related kidney failure.[12] ER increased bone turnover in 10-month-old female rats[13] and reduced bone strength in 4- and 13-month-old F344 rats.[14]

To date, the effects of DR on bone have not been examined in comparative animal models of free radical generation. In the studies described here, the effects of DR on the humerus and mandible were tested in 3 murine models (SENCAR, C57BL/6, and DBA/2) of the extremes of free radical generation, oxidative metabolism, and aging. SENCAR mice are a model of

From the Geriatric Research, Education and Clinical Center, Veterans Affairs Greater Los Angeles Healthcare System, Sepulveda, CA; and the Department of Histology and Embriology and PABCAM/HUCFF, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

Submitted November 20, 2002; accepted March 5, 2003.

Supported in part by the Geriatric Research, Education, and Clinical Center of the VA Greater Los Angeles Healthcare System, Sepulveda, CA and the Office of Research and Development, Department of Veterans Affairs (S.S.M. and E.J.B.).

Presented in part at the annual meeting of the American Society for Bone and Mineral Research, September 20-24, 2002, San Antonio, TX.

Address reprint requests to Elsa J. Brochmann Murray, PhD, GRECC (11-E), Sepulveda Ambulatory Care Center and Nursing Home, VA Greater Los Angeles Healthcare System, 16111 Plummer St, Sepulveda, CA 91343.

0026-0495/03/5208-0021$30.00/0

doi:10.1016/S0026-0495(03)00164-1

elevated free radical generation,[15,16] high downstream oxidative damage,[17,18] and early onset metabolic bone disease[19] that can be partially ameliorated by DR.[20] C57BL/6 and DBA/2 mice exhibit low levels of free radical generation and oxidative damage, and they are frequently used as controls for SENCAR mice.[15,17,21-25] In addition, the C57BL/6 mouse is prone to age- and diet-induced obesity[26-31] and diabetes,[29] making it a good model of low free radical generation, as well as high diabetes-induced end-stage glycation and premature aging. Thus, the relative degree of susceptibility to free radical-mediated aging is SENCAR > C57BL/6 > DBA/2.

In these studies, the effects of 6 months of 30% DR were determined simultaneously in SENCAR, C57BL/6, and DBA/2 mice to minimize any confounding effects of age, dietary composition, and length of administration, housing, and analysis. We hypothesized that DR would have beneficial antioxidant effects on bone, particularly in the 2 strains (SENCAR and C57BL/6) that experience high levels of oxidant stress and downstream macromolecular damage. We also hypothesized that DR, which is associated with low body mass, would downregulate skeletal mass and density at weight-bearing sites, such as the humerus, particularly in the strain (DBA/2) that has the lowest body mass and free radical generation capacity.

MATERIALS AND METHODS

Mice and Dietary Treatments

The Sepulveda Animal Research Committee and the Research and Development Committee of the VA Greater Los Angeles Healthcare System approved this study. Weaned (4-week-old) male mice of each strain (CR:ORL SENCAR, C57BL/6NCR, and DBA/2NCR) were obtained from the NIH/NCI Mammalian Genetics and Animal Products Section, Cancer Research Facility (Frederick, MD). After a 1-week quarantine and acclimation period, all mice were housed individually in polycarbonate cages receiving HEPA-filtered air. Housing facilities were maintained at 22°C to 23°C and 50% to 60% relative humidity, with a 12-hour light/dark cycle. Deionized, distilled water was provided ad libitum. Mice were weighed weekly.

Certified rodent basal test diet #5755 (Purina Mills, Richmond, IN) containing 0.8% calcium (Ca), 0.6% phosphorus (P), 10% fat, 21.0% casein, 60.6% carbohydrate, and all of the vitamins, minerals, and micronutrients essential for growth, development, breeding, and maintenance was provided ad libitum to all mice from 6 to 10 weeks of age,[19,20] when randomly-selected time zero (t_0) control mice were killed. The remaining mice were randomized to the ad libitum (AL) or the long-term 30% DR feeding group for the next 6 months. The food consumption of the AL-fed mice was determined daily, and the 30% DR mice received 70% of the amount of food consumed by their respective AL-fed controls on the previous day.[20] AL-fed mice were fed basal test diet #5755, while DR mice received a nutritionally supplemented version of this diet normalized versus AL-fed mice with respect to dietary Ca, P, and micronutrient (methionine, vitamins, choline chloride, and mineral mix #10) intakes.[20] Thus, as in previous studies, DR and AL-fed mice consumed the same total amounts of dietary Ca and P.[20] The dietary fiber and dextrin contents of the DR diet were reduced to 1.3% and 41.2% (*v* 3.0% and 43.2% in the AL-fed diet), respectively, to compensate for the Ca, P, and micronutrient supplementation. The design conforms to that of previous studies in this laboratory, which demonstrated robust growth in SENCAR versus C57BL/6 mice,[32,33] as well as the development of early-onset metabolic bone disease with histologic features of osteoporosis (low mineral apposition rate and low osteoblast, and osteoclast cell number)[19] and its partial amelioration by short-term DR in SENCAR mice.[20]

Quantitation of Lean Body Mass and Bone Parameters

At the end of the experimental periods, the mice were killed by pentobarbital injection and exsanguination. Lean body mass (LBM) and humeral and mandibular BMD (g/cm^2) and BMC (g) were determined by densitometry using a PIXImus imager (GE Lunar, Madison, WI). Field calibration and calibration verus the quality control phantom were performed each day before mouse imaging. Each mouse, humerus, and mandible was positioned reproducibly on the imaging tray and scanned 3 times. The coefficient of variance (CV) for BMD and BMC were 0.5% and 0.6%, respectively, for in vitro measurements. The left humerus was removed by dissection, cleaned of soft tissue, and its length and midshaft width were measured with a microcaliper (Manostat #5921; Fisher Scientific, Tustin, CA).[20,33]

Statistical Analysis

Data are expressed as the mean ±SEM (n) for all groups. Data were subjected to multiple analysis of variance (ANOVA) using strain and diet/time as the main effects and testing for interactions. Where statistically significant interactions were observed, post hoc analysis consisting of 1-way ANOVAs for each strain looking at the effect of diet/time and *a posteriori* pair-wise tests using Tukey-Kramer adjustments for multiple comparisons were performed. Where significant interactions were not observed, we collapsed over strain to determine the differences between diet/time and collapsed over diet/time to determine the differences between strains. Results were considered significant at $P \leq .05$. In addition, the relationship between humeral BMD, humeral BMC, mandibular BMD, and mandibular BMC, and LBM was determined for all 8.5-month-old mice using the least squares linear regression method (SigmaPlot [version 8.0] Regression Wizard, Jandel Scientific, Corte Madera, CA) with fitting to the polynomial equation $f = y_0 + (a)(x)$, where f is the BMD ($g/cm^2 \times 100$) or BMC ($g \times 100$) and x is the lean body mass (g).

RESULTS

LBM

The effects of 6 months of aging and 30% DR on LBM in C57, DBA, and SENCAR mice are shown in Fig 1. Strain, diet/time, and the interaction between these 2 parameters all had significant effects on LBM (Fig 1). Throughout the study, the ranking of LBM was SENCAR (heaviest) > C57 > DBA (lightest) (Fig 1). Of interest is the observation that the lean body masses of the 30% DR C57 and DBA mice were not significantly different from those of their respective time-zero (t_0) controls (Fig 1). In contrast, 30% DR SENCAR mice had lean body masses that were lower than those of their respective t_0 controls, while 8.5-month-old AL-fed SENCAR mice exhibited a LBM similar to that of the t_0 controls (Fig 1). Unlike AL-fed SENCAR mice, AL-fed C57 and DBA mice had a higher LBM than their respective t_0 controls (Fig 1).

Humerus Length and Midshaft Width

The size of the humerus was estimated by measuring its length (Fig 2A) and midshaft width (Fig 2B). The length of the humerus was significantly affected by diet/time and strain, but there was no significant interaction between these parameters (Fig 2A). The length varied with strain, with SENCAR (longest) > C57 > DBA (shortest), and the differences in length were significant (Fig 2A). AL-fed and 30% DR C57, DBA, and SENCAR mice had longer humeri than their respective t_0 controls (Fig 2A). In contrast, 30% DR and AL-fed mice of

each strain had humeri of similar lengths (Fig 2A). Thus, DR was not sufficient to prevent the modest linear growth of the humeri observed between 2.5 and 8.5 months of age in SENCAR, DBA/2, or C57BL/6 mice, relative to their respective AL-fed controls (Fig 2A).

There were significant strain-dependent differences in the midshaft width of the humerus, with SENCAR (widest) > C57 > DBA (narrowest) (Fig 2B). Diet/time had no significant effect on this parameter, but a significant interaction between diet/time and strain was observed (Fig 2B). When the data for each strain were analyzed independently, no significant effects of diet/time were observed in C57 or SENCAR mice (Fig 2B). In contrast, AL-fed DBA mice had significantly higher midshaft humerus widths than their respective t_0 controls, while the widths of the 30% DR DBA mice were not different from those of the AL-fed or t_0 controls (Fig 2B). Thus, 6 months of AL or 30% DR feeding had no apparent effects on the humeral widths in C57 or SENCAR mice (Fig 2B). In contrast, 6 months of AL-feeding was associated with a modest increase in humeral width in DBA/2 mice, relative to their t_0 controls (Fig 2B).

Humerus BMD and BMC

Diet/time and strain, as well, as the interaction between these parameters, affected BMD in the humerus (Fig 3A). Humeral BMD tended to be higher in SENCAR mice than in C57 or DBA mice, while C57 and DBA mice had rather similar BMDs at this site (Fig 3A). Thirty percent DR mice of all strains had lower humeral BMDs than their respective AL-fed controls (Fig 3A). Thirty percent DR and AL-fed C57 and DBA mice had higher humeral BMDs than their respective t_0 controls (Fig 3A). In contrast, 2.5-month-old t_0 SENCAR mice had humeral

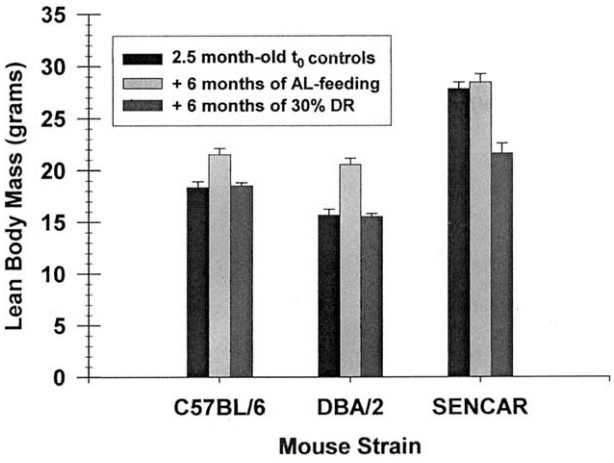

Fig 1. LBM of C57BL/6, DBA/2, and SENCAR mice at t_0 (2.5 months of age) and after 6 months of ad libitum feeding or 30% DR (8.5 months of age) (grams). Data are expressed as mean ± SEM (n = 10 to 12) and subjected to multiple ANOVA using diet/time and strain as the main effects and testing for interactions. The results of the ANOVA are: diet/time, $P < .0001$; mouse strain, $P < .0001$; interaction, $P < .0001$. Because the interaction between diet/time and strain was significant, a 1-way ANOVA was performed for each strain, looking at the effect of diet/time, and *a posteriori* pair-wise tests were performed using Tukey-Kramer adjustments for multiple comparisons: C57BL/6: t_0 *v* AL-fed ($P < .0001$), t_0 *v* 30% DR ($P = .9721$), 30% DR *v* AL-fed ($P = .0003$); DBA/2: t_0 *v* AL-fed ($P < .0001$), t_0 *v* 30% DR ($P = .9762$), 30% DR *v* AL-fed ($P < .0001$); SENCAR: t_0 *v* AL-fed ($P = .8538$), t_0 *v* 30% DR ($P < .0001$), 30% DR *v* AL-fed ($P < .0001$).

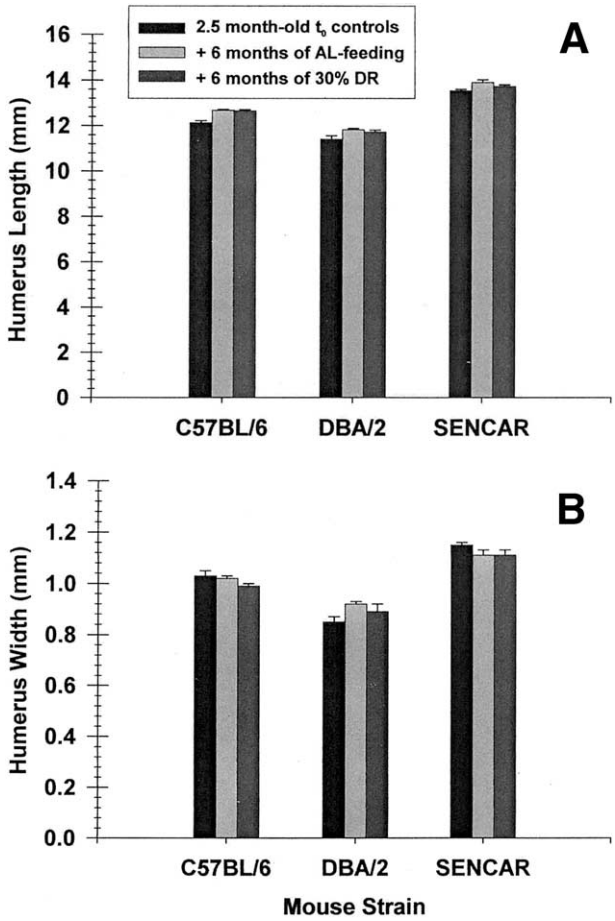

Fig 2. (A) Humerus length (millimeter) and (B) humerus width (millimeter) in C57BL/6, DBA/2, and SENCAR mice at t_0, (2.5 months or 10 weeks of age) and after 6 months of ad libitum feeding or 30% DR (8.5 months of age). Data are expressed as the mean ± SEM (n = 10 to 12) and subjected to multiple ANOVA using diet/time and strain as the main effects and testing for interactions. The results of the ANOVA are: diet/time humerus length ($P < .0001$), humus width ($P = .2091$); mouse strain humerus length ($P < .0001$, humerus width ($P < .0001$); interaction humerus length ($P = .47$), humerus width ($P = .009$). (A) Humerus length: Because the interaction between diet/time and strain was not significant for humerus length, we collapsed over strain to determine the differences between diet/time and collapsed over diet/time to determine differences between strains. *A posteriori* pair-wise tests using Tukey-Kramer adjustments for multiple comparisons were conducted. Diet/time comparisons: t_0 *v* AL-fed ($P < .0001$); t_0 *v* 30% DR ($P < .0001$); 30% DR *v* AL-fed ($P = .35$). Strain comparisons: C57BL/6 *v* DBA/2 ($P < .0001$), C57BL/6 *v* SENCAR ($P < .0001$), DBA/2 *v* SENCAR ($P < .0001$). (B) Humerus width: Because the interaction between strain and diet/time was significant, it means that the difference between strains depends on the level of diet/time and vice versa. Separate 1-way ANOVAs were performed for each strain, looking at the effect of diet/time, followed by *a posteriori* pair-wise tests using Tukey-Kramer adjustments for multiple comparisons. C57BL/6: comparison, all diet/time ($P = .0656$); DBA/2: comparison, t_0 *v* AL-fed ($P = .0183$); DBA/2: comparison, t_0 *v* 30% DR ($P = .2355$); DBA/2: comparison, 30% DR *v* AL-fed ($P = .4027$); SENCAR: comparison, all diet/time ($P = .2081$).

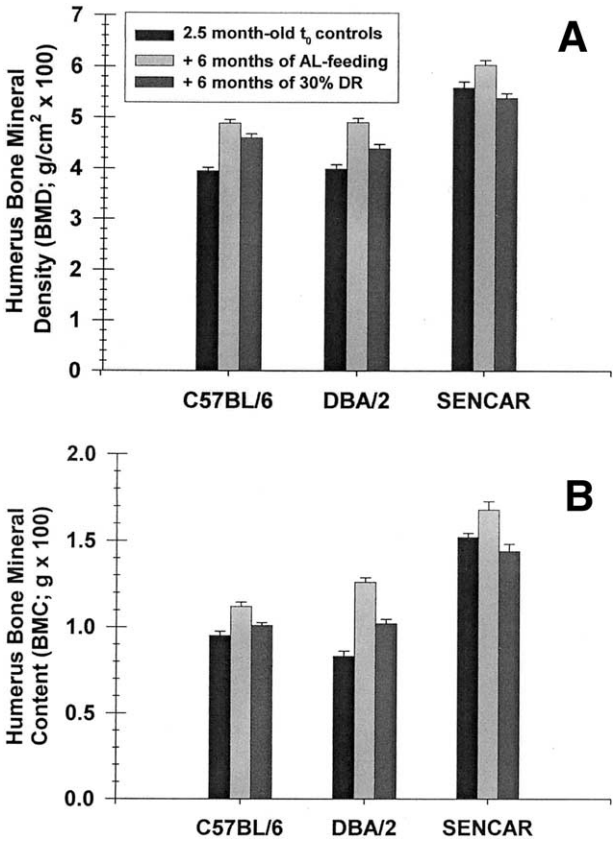

Fig 3. (A) Humeral BMD (g/cm^2 × 100) and (B) humeral BMC (g × 100) in C57BL/6, DBA/2, and SENCAR mice at t_0 (2.5 months or 10 weeks of age) and after 6 months of ad libitum feeding or 30% DR (8.5 months of age). Data are expressed as the mean ± SEM (n = 10 to 12) and subjected to multiple ANOVA using diet/time and strain as the main effects and testing for interactions. The results of the ANOVA are: diet/time: humeral BMD ($P < .0001$), humeral BMC ($P < .0001$); strain: humeral BMD ($P < .0001$), humeral BMC ($P < .0001$); interaction: humeral BMD ($P < .0001$), humeral BMC ($P < .0001$). (A) Humerus BMD: Because the interaction between diet/time and strain was significant, separate 1-way ANOVAs were conducted for each strain to determine where the differences between combinations of strain and diet/time lie. *A posteriori* pair-wise testing with Tukey-Kramer adjustments for multiple comparisons was then conducted. C57BL/6: t_0 *v* AL-fed ($P < .0001$), t_0 *v* 30% DR ($P < .0001$), 30% DR *v* AL-fed ($P = .0226$); DBA/2: t_0 *v* AL-fed ($P < .0001$), t_0 *v* 30% DR ($P = .0059$), 30% DR *v* AL-fed ($P = .0009$); SENCAR: t_0 *v* AL-fed ($P = .117$), t_0 *v* 30% DR ($P = .400$), 30% DR *v* AL-fed ($P = .0005$). (B) Humerus BMC: Because the interaction between diet/time and strain was significant, separate 1-way ANOVAs were conducted for each strain to determine where the differences between combinations of strain and diet/time lie. *A posteriori* pair-wise testing with Tukey-Kramer adjustments for multiple comparisons was then conducted. C57BL/6: t_0 *v* AL-fed ($P < .0001$), t_0 *v* 30% DR ($P = .1754$), 30% DR *v* AL-fed ($P = .005$); DBA/2: t_0 *v* AL-fed ($P < .0001$), t_0 *v* 30% DR ($P = .0002$), 30% DR *v* AL-fed ($P < .0001$); SENCAR: t_0 *v* AL-fed ($P = .0162$), t_0 *v* 30% DR ($P = .3038$), 30% DR *v* AL-fed ($P = .0004$).

BMDs that were not significantly different from strain-matched 8.5-month-old AL-fed or 30% DR mice (Fig 3A). Thus, BMD increased with age in AL-fed and 30% DR C57 and DBA, but not in SENCAR mice (Fig 3A).

Diet/time and strain, as well as the interaction between these parameters, affected humeral BMC (Fig 3B). Humeral BMC was higher in SENCAR mice than in C57 or DBA mice (Fig 3B). Time-zero control mice of all strains had lower humeral BMCs than their respective AL-fed controls (Fig 3B). In addition, 30% DR mice had lower humeral BMCs than their respective AL-fed controls (Fig 3B). However, 30% DR C57 and SENCAR mice had humeral BMCs that were not significantly different from those of their respective t_0 controls, while 30% DR DBA mice had higher humeral BMC than their t_0 controls (Fig 3B).

Mandibular BMD and BMC

Diet/time, strain, and the interaction between these parameters affected mandibular BMD (Fig 4A). The highest mandibular BMD was observed in 8.5-month-old AL-fed SENCAR mice, while the lowest values were observed in t_0 control C57 and DBA mice (Fig 4A). In all instances, mandibular BMD was higher in SENCAR mice than in C57 or DBA mice (Fig 4A). In addition, the mandibular BMD was lower in t_0 control mice than in their respective 8.5-month-old AL-fed or 30% DR groups (Fig 4A). Finally, 30% DR was associated with lower mandibular BMD, relative to AL-feeding, in C57 (but not DBA or SENCAR) mice (Fig 4A).

Diet/time and strain also affected mandibular BMC in C57, DBA, and SENCAR mice; however, there was no significant interaction between these parameters (Fig 4B). The values for all diet/time and strain comparisons were significantly different by post hoc analysis (Fig 4B). As in previous cases, the mandibular BMC was highest in SENCAR mice, followed by C57 and DBA mice (Fig 4B). Mandibular BMD increased with age, but was lower in 30% DR mice than in AL-fed mice of all strains (Fig 4B).

Relationships Between Humeral and Mandibular BMD and BMC and LBM

The relationships between LBM and humeral and mandibular BMD and BMC are shown in Fig 5 (humerus) and Fig 6 (mandible) for all 8.5-month-old mice. Regardless of the strain or feeding regimen, there were linear relationships between LBM and BMD and BMC at both the humerus (Fig 5) and mandible (Fig 5), as indicated by the regression analysis presented in Table 1. However, the *r* value for the mandibular BMD was lower than that observed for humeral BMD (Table 1), indicating that the linear regression model fits the weight-bearing site (humerus) better than the nonweight-bearing site (mandible).

DISCUSSION

The effects of DR on bone vary depending upon the animal model tested, the age at which DR is imposed, the duration and degree of DR, and the composition of the diet.[6,9-14] Therefore, the reported effects of DR on bone have ranged from deleterious (eg, reduced BMD, increased turnover, and lower strength in rats)[12-14] to neutral after adjusting for age and body weight in rhesus monkeys (*Macaca mulatta*)[6] to beneficial (eg, prevention of senile osteopenia in the F344 rat model of age-related renal failure).[10] One of the strengths of the present study is that it was conducted simultaneously in 3 well-defined mu-

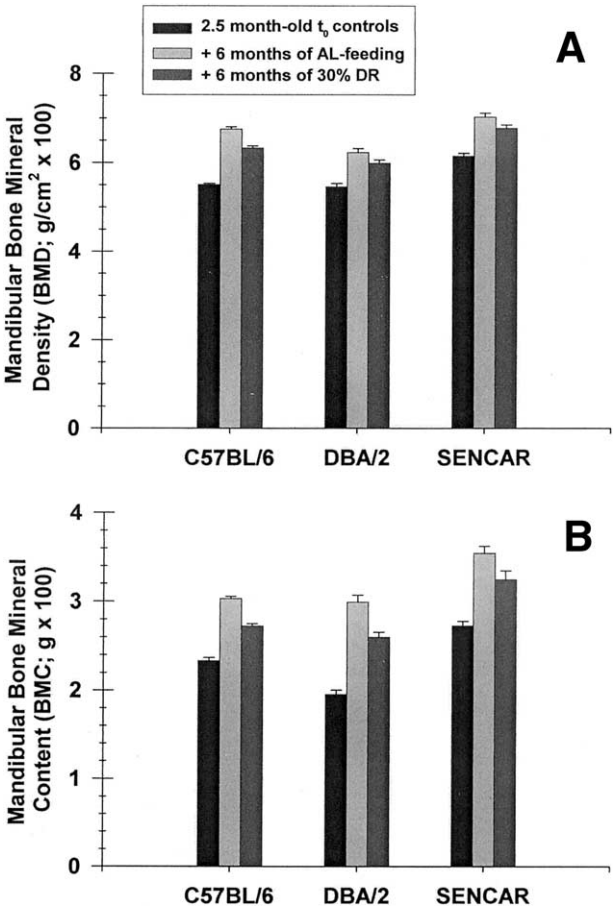

Fig 4. (A) BMD (g/cm^2 × 100) and (B) Mandibular BMC (g × 100) in C57BL/6, DBA/2, and SENCAR mice at t_0 (2.5 months or 10 weeks of age) and after 6 months of ad libitum feeding or 30% DR (8.5 months of age). Data are expressed as the mean ± SEM (n = 10 to 12) and subjected to multiple ANOVA using diet/time and strain as the main effects and testing for interactions. The results of the ANOVA are: diet/time: mandibular BMD ($P < .0001$), mandibular BMC ($P < .0001$); strain: mandibular BMD ($P < .0001$), mandibular BMC ($P < .0001$); interaction: mandibular BMD ($P < .0010$), mandibular BMC ($P = .06$). (A) Mandibular BMD: Because the interaction between diet/time and strain was significant, separate 1-way ANOVAs were conducted for each strain to determine where the differences between combinations of strain and diet/time lie. *A posteriori* analysis with Tukey-Kramer adjustments for multiple comparisons was then conducted. C57BL/6: t_0 *v* AL-fed ($P < .0001$), t_0 *v* 30% DR ($P < .0001$), 30% DR *v* AL-fed ($P < .0001$); DBA/2: t_0 *v* AL-fed ($P < .0001$), t_0 *v* 30% DR ($P = .0002$), 30% DR *v* AL-fed ($P = .1053$); SENCAR: t_0 *v* AL-fed ($P < .0001$), t_0 *v* 30% DR ($P < .0001$), 30% DR *v* AL-fed ($P = .0802$). (B) Mandibular BMC: Because the interaction between diet/time and strain was not significant, we collapsed over strain to discuss the differences between diet/time and collapsed over diet/time to discuss differences between strains using 1-way ANOVAs. *A posteriori* analysis with Tukey-Kramer adjustments for multiple comparisons was then conducted. Diet/time comparisons: t_0 *v* AL-fed ($P < .0001$); t_0 *v* 30% DR ($P < .0001$); 30% DR *v* AL-fed ($P < .0001$). Strain comparisons: C57BL/6 *v* DBA/2 ($P = .001$); C57BL/6 *v* SENCAR ($P < .0001$); DBA/2 *v* SENCAR ($P < .0001$).

rine models. Purchasing mice of identical ages and housing, feeding, and assaying them under identical conditions minimized the number of variables related to age, study duration, diet, and care that confounded previous studies.

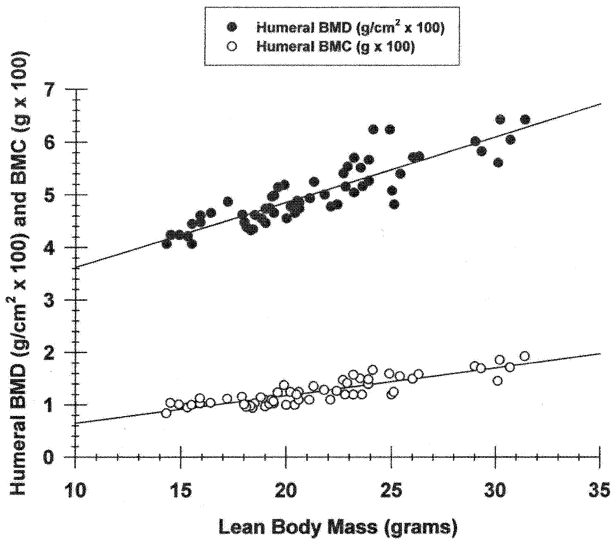

Fig 5. Relationships between LBM and humeral BMD or BMC for all 8.5-month-old AL-fed and 30% DR C57BL/6, DBA/2, and SENCAR mice. See Table 1 for the regression analysis.

We examined the effects of 6 months of 30% DR on NCI-supplied SENCAR, DBA/2, and C57BL/6 mice fed the well-defined semisynthetic test diet used in 2 earlier comparative studies of bone growth, development, and metabolism in young (10- to 16-week-old) SENCAR and C57 mice.[32-33] We previously demonstrated that the SENCAR mouse model of high free radical generation[15,16] and elevated downstream oxidative damage[17,18] grows more rapidly, weighs more, and has higher appendicular and axial bone size, mass, and mineral content than the C57BL/6 mouse.[32,33] However, 10- to 16-week-old SENCAR mice deposit mineral in vertebral and long bone

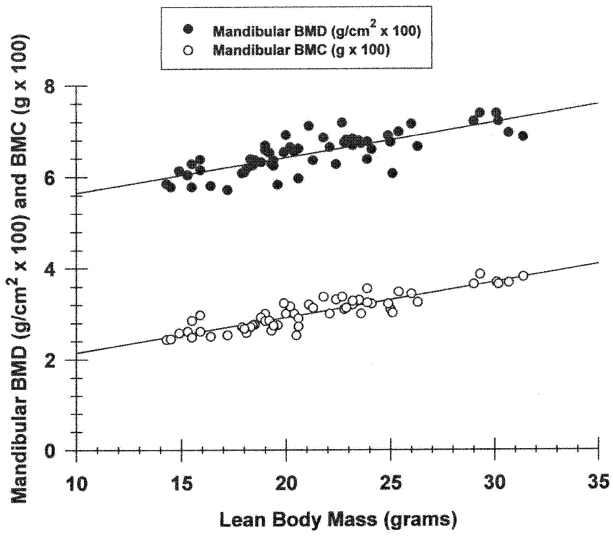

Fig 6. Relationships between LBM and mandibular BMD or BMC for all 8.5-month-old AL-fed and 30% DR C57BL/6, DBA/2, and SENCAR mice. See Table 1 for the regression analysis.

Table 1. Regression Analysis of the Relationship Between BMD (g/cm² × 100) and BMC (g × 100) and LBM (grams) in the Humeri and Mandibles in 8.5-Month-Old AL-Fed and 30% DR C57BL/6, DBA/2, and SENCAR Mice

Regression Parameter	Humeral BMD	Humeral BMC	Mandibular BMD	Mandibular BMC
r	0.884	0.873	0.779	0.896
y_0	2.3823	0.1183	4.8840	1.3627
a	0.1241	0.0531	0.0768	0.0779
P value	<.0001	<.0001	<.0001	<.0001

NOTE. The data (LBM and humeral or mandibular BMD or BMC) were plotted together for all mice and fitted to the regression line: $f = y_0 + ax$, where f = BMD or BMC at the skeletal site indicated, and x = lean body mass. The data were subjected to ANOVA, and the *P* values for the regression lines from Figs 5 and 6 are presented.

Abbreviations: BMD, bone mineral density; BMC, bone mineral content; LBM, lean body mass; ANOVA, analysis of variance.

more slowly than C57BL/6 mice, while the resorption rates in the 2 strains are similar, suggesting that the kinetics of skeletal growth and development differ in these mice.[32,33] The bone regeneration capacity of young SENCAR mice exceeds that of other inbred mice.[25] However, unlike other strains, SENCAR mice develop early-onset metabolic bone disease with characteristic features of aging, including low mineral apposition rate and low numbers of osteoblasts and osteoclasts.[19] DR corrects the underlying defect in protein kinase C signaling[34] that leads to high free radical generation and cellular oxidative damage in this strain and partially ameliorates their metabolic bone disease.[20]

In contrast, the DBA/2 and C57BL/6 are mouse models of low free radical generation and downstream oxidative damage.[15,17,21-25] They exhibit lower body weights, lower total and cortical BMDs, lower mineral contents, and lower bone volumes than other inbred mice.[35] Unlike DBA/2 mice, C57BL/6 mice are prone to gross diet- and age-related obesity, diabetes, and premature aging.[26-31] Thus, the effects of 30% DR on bone were tested in murine models of the extremes in free radical generation, body and bone mass, and BMD to better define the possible range of skeletal responses to DR and elucidate its potential mechanisms of action. Specifically, we tested the hypothesis that (1) DR has beneficial antioxidant effects on bone that are most significant in animal models of high oxidant stress (SENCAR mice) or downstream macromolecular damage (C57BL/6 mice) versus (2) DR downregulates LBM, thus reducing the mechanical load on the skeleton at weight-bearing sites and leading to lower absolute BMDs and BMCs than those observed in heavier AL-fed age-matched controls.

In agreement with studies in rats and rhesus monkeys,[1,6,9,12] one of the major effects of DR in our study was to normalize LBM at t_0 levels and prevent fat deposition in 2 of the strains tested (C57 and DBA mice). In contrast, DR was associated with a significant reduction in LBM, relative to t_0 control values, in SENCAR mice. In addition, unlike C57 and DBA mice, AL-fed SENCAR mice did not gain a significant amount of LBM over the 6-month course of the study. Thus, there is an intrinsic difference in body mass regulation in these 3 strains that determines LBM, and hence, the absolute and relative mechanical loads applied to the skeleton. Because LBM is one of the major determinants of BMD and BMC at many sites,[5,8] strain-dependent differences in LBM regulation may be one of the most important factors in determining how the skeleton responds to DR.[6]

The effects of 30% DR were examined at 2 distinct skeletal sites, including the weight-bearing humerus and the nonweight-bearing, but mechanically stressed, mandible. We observed consistent strain-dependent differences in the size of the humeri whether assessed as length or midshaft width, with SENCAR > C57 > DBA. DR did not restrict longitudinal growth in the humeri of these 3 strains, nor did it affect midshaft width in 2 of the 3 strains tested (C57 and SENCAR mice). Midshaft widths were stable between 2.5 and 8.5 months of age, with the exception of AL-fed DBA mice, which they increased relative to their respective t_0 controls. Thus, when 30% DR using a nutritionally-replete test diet was initiated at 10 weeks of age, it had little, if any, effect on the external size of the humeri in these 3 strains. In contrast, initiation of DR in more immature monkeys[36] and rats[10,11] is associated with reduced skeletal size, emphasizing the potentially confounding effects of the duration of DR and the age at which it is imposed. Thus, in our study the primary determinant of humeral dimensions was the genetic background of the test subject, not diet or age.

Humeral BMD and BMC were lower in 30% DR male mice than in their respective AL-fed controls in all strains tested. Similar results have been reported for rhesus monkeys subjected to long-term (11 years) CR, where statistical analysis indicated that lower LBM and age accounted for the decrease.[6] Similarly, when LBM of each of our 8.5-month old male mice was plotted versus humeral BMD or BMC, the relationships were found to be linear, suggesting that the lower humeral BMD and BMC observed in our studies can also be accounted for based on LBM. Although the lean body masses of our male C57 and DBA mice were different at t_0 and after 6 months of DR (C57 > DBA), the humeral BMDs tended to be very similar for the 2 strains. The results in primates[6] suggest that humeral BMD should have been lower in DBA mice than in C57BL/6 mice because DBA mice have a lower LBM. This apparent disparity in results emphasizes the potentially confounding effects of the animal model selected for DR studies, which our analyses suggest is a major determinant of all of the parameters examined in these studies. In addition, gender or sourcing differences may further confound the results of skeletal studies. For example, 12-month-old female DBA/2J mice have higher total and cortical femoral BMDs than age-matched female C57BL/6J mice,[35] an observation that does not agree with our results. When our study is compared with that of Beamer et al,[35] several potentially confounding variables, including gender (male *v* female), source of the mice (NCI *v*

Jackson Laboratories), diet (PMI semisynthetic, nutrient-enriched test diet *v* Teklad very low-fat [4%] autolaved rodent pellets), and age (2.5 and 8.5 months *v* 12 months) emerge that could potentially contribute to the differences observed.

The 30% DR C57 and DBA mice had higher humeral BMD than their respective t_0 controls, while SENCAR mice did not. Thus, the strains (C57 and DBA) that exhibited a stable LBM during 6 months of 30% DR also exhibited an increase in humeral BMD over the time course of the study. In contrast, the strain (SENCAR) that lost LBM relative to its respective t_0 when it was 30% diet restricted was the one that failed to gain humeral BMD between 2.5 and 8.5 months of age. This is additional validation of the hypothesis that DR, which is associated with low body mass, downregulates skeletal mass and density at weight-bearing sites. However, because there is a linear relationship between LBM and humeral BMD and BMC, the effects of DR may reflect physiologic adaptation to reduced levels of biomechanical stress (weight bearing), rather than an adverse nutritional effect of DR.

The combined influences of longitudinal bone growth and increased BMD are reflected in increased humeral BMC. AL-fed mice that had longer humeri (C57, DBA, and SENCAR) and/or higher humeral BMD (C57 and DBA) than their respective t_0 controls also exhibited greater humeral BMC at 8.5 months of age than at 2.5 months of age. Thirty percent DR mice that had longer humeri and higher humeral BMD (C57 and DBA) than their respective t_0 controls also exhibited greater humeral BMC at 8.5 months of age than at 2.5 months of age, although humeral BMC in DR mice of these strains remained lower than that observed in their respective AL-fed controls. Contrary to expectations, humeral BMC in 30% DR SENCAR mice, a model of elevated free radical generation and downstream oxidative damage, was actually lower than that observed at t_0 or in AL-fed SENCAR mice of the same age, reflecting the low humeral BMD observed in the DR group. Thus, we have failed to validate the hypothesis that DR, which mediates its effects, in part, by reducing free radical-mediated cellular aging, will have its most beneficial effects in animal models of high free radical generation and downstream oxidative damage, such as the SENCAR mouse.

We also determined the effects of DR on mandibular BMD and BMC in these mice. Although mandibular bone is subject to the biomechanical stresses induced by eating and chewing, it is not a weight-bearing bone. While LBM is a major mediator of the effects of DR on appendicular bone in higher animals,[6] its effects at nonweight bearing sites, such as the mandible, remain poorly defined. The data clearly indicate that mandibular BMD and BMC increase with age in AL-fed and 30% DR mice of all strains, relative to their respective t_0 controls. In addition, DR had no significant effects on mandibular BMD in DBA and SENCAR mice, when compared with AL-fed control levels, although mandibular BMC was lower in these groups. As with the humerus, there was a linear relationship between LBM and mandibular BMD and BMC in 8.5-month-old mice of all strains. This suggests that the systemic or hormonal regulators of BMD that also modulate LBM, such as insulin-like growth factors (IGFs), may play a role in regulating mandibular bone metabolism

In conclusion, much of our evidence supports one of our working hypotheses (eg, DR downregulates LBM, and hence, skeletal mass and density), while it fails to validate the other (eg, the cellular antioxidant effects of DR will be most beneficial in the SENCAR mouse model of high free radical generation and oxidative damage). Our data confirm the conclusion of Black et al,[6] who reported that the reduced skeletal mass observed in CR rhesus monkeys can be accounted for based on body mass. Taken together, these findings emphasize the importance of LBM in determining skeletal loading, which is a major modulator of BMD and BMC under all feeding conditions across the life span. In our studies, dissimilar results were obtained at the 2 sites analyzed in the 3 strains tested. Thus, even after controlling for the duration of DR, the composition of the diet, and the age at which DR is initiated, major potentially confounding variables related to the genetic background of the test animal remain. When designing DR studies, analysis of weight-bearing and nonweight-bearing skeletal sites in multiple animal models may provide valuable insights into the mechanisms that regulate bone mass under these conditions.

ACKNOWLEDGMENT

The authors thank Elizabeth C. Laird, Julian P. Barnett, and Cecil R. Carmack for their expert technical assistance throughout the course of this ongoing, long-term study. We also thank Judith Harker, PhD, and Susan Service for assistance with the statistical analyses.

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