Effects of Dietary Restriction on Total Body, Femoral, and Vertebral Bone in SENCAR, C57BL/6, and DBA/2 Mice

Elsa J. Brochmann, Maria Eugenia Duarte, Hasan A. Zaidi, and Samuel S. Murray

Dietary restriction (DR) increases the life span and retards aging, in part, by limiting free radical generation and oxidative damage. DR also reduces body mass, a major determinant of bone mass across the life span. We tested the hypothesis that DR has its most beneficial effects on bone in mouse strains with high free radical generation (sensitive to carcinogenesis [SENCAR] > C57 > DBA) versus the hypothesis that bone mass at weight-bearing sites is determined by body mass in DR and ad libitum (AL)-fed mice. Male mice of each strain were killed at 10 weeks of age (t_0) or randomized to an AL-fed or 30% DR feeding regimen for 6 months. Food consumption by AL-fed mice was measured daily, and DR mice received 70% of the amount of food consumed by their respective AL-fed mice the previous day. Body fat (%) and bone mineral density (BMD) and content (BMC) were determined by PIXImus densitometry. There were strain-dependent effects on body mass, crown-to-rump length, percent body fat, and total body, femoral, and vertebral BMD and BMC under all conditions. SENCAR mice were heavier, longer, had larger bones, and generally exhibited higher total body, femoral, and vertebral BMC and BMD than C57 and DBA mice. DR had beneficial effects on BMD and BMC in the vertebrae of the SENCAR mouse model of high free radical generation and in the obese, diabetes-prone C57 mouse model of high end-stage protein glycation. DR DBA and SENCAR mice had lower femoral BMDs and BMCs than their respective AL-fed controls. Regression analysis confirmed linear relationships between total and lean body mass and total body and femoral BMDs and BMCs, suggesting that physiologic adaptation to a lower body mass accounts for the lower femoral bone mineral values observed in DR versus AL-fed mice. Thus, both hypotheses are, at least, partially valid. DR is beneficial in the trabeculae-rich vertebrae of animal models of high oxidant stress, and total/lean body mass determines BMD and BMC in the weight-bearing femur in DR and AL-fed mice.

LONG-TERM DIETARY restriction (DR) increases the life span by up to 50% and delays the development of or reduces the severity of age-related disorders of the central nervous, cardiovascular, renal, endocrine, and musculoskeletal systems.[1] The biochemical bases for the beneficial effects of DR remain controversial, but are thought to involve reduced rates of metabolic fuel utilization, free radical generation, and downstream oxidative damage to lipids, proteins, and DNA.[2] To date, the effects of DR on bone have not been compared in established models of the extremes of free radical generation or downstream oxidative damage.

The SENCAR (*sen*sitive to *car*cinogenesis) mouse is a well-defined model of elevated free radical generation and downstream oxidative damage[3-9] that can be ameliorated by dietary restriction[10] and exacerbated by high dietary fat intake.[11] The SENCAR mouse has a mutation in the regulatory domain of a protein kinase C (PKC) isozyme that renders it exquisitely sensitive to diacylglycerol and phorbol ester stimulation.[12,13] Enhanced PKC signal transduction induces 8-lipoxygenase activity and arachidonic acid synthesis, resulting in elevated free radical production by leukocytes and peritoneal macrophages.[4,5,7,8] This induces a 5-fold to 10-fold increase in downstream oxidative damage.[9] Treatment with phorbol esters also induces high levels of growth factor and growth factor receptor expression and activity in SENCAR mice.[14-16] As a result, SENCAR mice exhibit robust bone growth[17,18] and regeneration,[19] but develop early-onset metabolic bone disease histologically similar to age-related osteoporosis[20] that is partially ameliorated by dietary restriction.[21]

Conversely, other murine strains, such as the DBA/2 and C57BL/6, are models of lower free radical generation and more limited downstream responsiveness or oxidative damage that often serve as controls for SENCAR mice.[3,5,7,9,13,14,19] The DBA mouse is a long-lived strain[22] that exhibits low levels of prostaglandin production,[23] as well as low hydroxylase and oxidase activities.[24,25] Unlike SENCAR mice, DBA mice are hyporesponsive to high-fat diets,[26] chemically-induced carcinogenesis,[27] and ozone damage.[28] The DBA/2 mouse also has a low preference for sweets[29] and does not exhibit the tendency toward obesity of C57BL/6 mice.[30] Thus, the DBA/2 mouse is a model of low free radical generation and metabolic fuel utilization. Although it too is a low free radical generation control for the SENCAR mouse, the C57BL/6 mouse is prone to diet[30-34] and age-related obesity[35] and the development of high carcass fat content[36] and diabetes,[33] making it a good model of low free radical generation, but high end-stage protein glycation and premature aging. The obese, diabetes-prone middle-aged C57 mouse has an impaired response to leptin,[35] an important central regulator of bone formation.[37] This concept of the C57BL/6 mouse as a model of premature aging is consistent with the observation that mature (12-month-old) female C57BL/6J mice have lower values for bone parameters

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

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

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Table 1. Total Body Mass (g) of C57BL/6, DBA/2, and SENCAR Mice at Time Zero (t_0 2.5 months or 10 weeks of age) and After 6 Months of Ad Libitum Feeding or 30% Dietary Restriction (8.5 months of age)

Age (mo)	Treatment	Strain		
		C57BL/6	DBA/2	SENCAR
2.5	t_0	24.29 ± 0.83 (12)	22.77 ± 1.07 (11)	39.84 ± 1.26 (11)
8.5	AL fed	44.26 ± 1.59 (11)	31.36 ± 1.43 (11)	51.24 ± 1.94 (11)
8.5	30% DR	25.28 ± 0.62 (11)	21.63 ± 0.41 (11)	33.63 ± 1.97 (12)

NOTE. Data are expressed as the mean ± SEM (n).

Effect by ANOVA: Diet/time, $P < .0001$; 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* pairwise tests were performed, using Tukey-Kramer adjustments for multiple comparisons, to look at differences between diet-time separately by strain. CB7BL/6: t_0 *v* AL, $P < .0001$; t_0 *v* DR, $P = .7738$; AL *v* DR, $P < .0001$. DBA/2: t_0 *v* AL, $P < .0001$; t_0 *v* DR, $P = .7278$; AL *v* DR, $P < .0001$. SENCAR: t_0 *v* AL, $P = .0003$; t_0 *v* DR, $P = .0463$; AL *v* DR, $P < .0001$.

than 10 other inbred strains of mice, including the DBA, which exhibits a similar body mass.[38] In conclusion, the relative degree of genetic susceptibility to free radical-induced aging and end-stage protein glycation would be predicted to range from SENCAR (highest) > C57 > DBA (lowest). We hypothesize that the most beneficial effects of DR in bone will be observed in the most extreme models of elevated free radical generation or downstream oxidative damage.

Conversely, calorie restriction (CR) in adults is associated with low body mass and reduced measures of skeletal mass, including bone mineral density (BMD), bone mineral content (BMC), and calcium content.[39] Clinical and animal studies have demonstrated that body mass is positively correlated with BMD and BMC across the life span[39,40] irrespective of strain or sex differences.[41] Mechanically driven bone formation, a major component of total skeletal development, is directly related to body weight.[42] Two major studies in rhesus monkeys (*Macaca mulatta*)[39] and Lobund-Wistar rats[43] have demonstrated that CR is associated with a low bone mass that is accounted for by adjusting for body weight and age. In the studies summarized here, we tested the alternate hypothesis that bone mass at weight-bearing sites is determined by body mass in both DR and ad libitum (AL)-fed mice. In order to do so, high-resolution small animal densitometry was conducted at time zero (10 weeks of age) and after 6 months of AL feeding or 30% DR to assess body composition, BMD, and BMC.

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 all studies. 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 high-efficiency particle air (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, 0.2% sodium (Na), 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,[17,18,20,21] when randomly selected time zero (t_0) control mice were killed. The remaining mice were randomized to the AL- or the 30% diet-restricted (DR) feeding group for the next 6 months. The food consumption of the AL-fed mice was determined on a daily basis, and the 30% DR mice received 70% of the amount of food consumed by their respective AL-fed controls on the previous day.[21] 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.[21] Thus, as in previous studies, DR and AL-fed mice consumed the same total amounts of dietary Ca and P.[21] 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.[21] The design conforms to that of previous studies in this laboratory, which demonstrated robust growth in SENCAR versus C57BL/6 mice,[17,18] as well as the development of early-onset metabolic bone disease in the SENCAR mouse[20] and its partial amelioration by short-term dietary restriction.[21]

Table 2. Total Fat (%; [fat mass]/[body mass] × 100) of 10-Week-Old and 8.5-Month-Old C57BL/6, DBA/2, and SENCAR Mice

Age (mo)	Treatment	Strain		
		C57BL/6	DBA/2	SENCAR
2.5	t_0	19.7 ± 1.5 (12)	27.6 ± 1.5 (12)	21.1 ± 1.5 (11)
8.5	AL fed	48.1 ± 1.6 (10)	30.2 ± 1.6 (10)	37.0 ± 1.5 (11)
8.5	30% DR	23.8 ± 1.5 (11)	22.6 ± 1.6 (10)	26.4 ± 1.5 (12)

NOTE. Data are expressed as the mean ± SEM (n).

Effect by ANOVA: Diet/time, $P < .0001$; strain, $P = .0140$; interaction, $P < .0001$.

Post hoc analysis for comparisons between strains was as follows. C57BL/6 *v* DBA/2: t_0, $P = .0003$; AL fed, $P < .0001$; 30% DR, $P = .5959$. C57BL/6 *v* SENCAR: t_0, $P = .5039$; AL fed, $P < .0001$; 30% DR, $P = .2241$. DBA/2 *v* SENCAR: t_0, $P = .0031$; AL fed, $P = .0030$; 30% DR, $P = .0859$.

Table 3. Crown to Rump Length (cm) of 10-Week-Old and 8.5-Month-Old C57BL/6, DBA/2, and SENCAR Mice

Age (mo)	Treatment	Strain		
		C57BL/6	DBA/2	SENCAR
2.5	t_0	8.23 ± 0.14 (12)	8.04 ± 0.12 (12)	10.07 ± 0.17 (11)
8.5	AL fed	9.71 ± 0.13 (11)	9.48 ± 0.12 (11)	11.01 ± 0.10 (11)
8.5	30% DR	8.52 ± 0.06 (11)	8.81 ± 0.09 (10)	10.14 ± 0.09 (12)

NOTE. Data are expressed as the mean ± SEM (n).

Effect by ANOVA: Diet/time, $P < .0001$; strain, $P < .0001$; interaction, $P = .0042$.

Post hoc analysis for comparisons between age and feeding groups was as follows. C57BL/6: t_0 *v* AL, $P < .0001$; t_0 *v* DR, $P = .1250$; AL *v* DR, $P < .0001$. DBA/2: t_0 *v* AL, $P = .0001$; t_0 *v* DR, $P = .0020$; AL *v* DR, $P = .0013$. SENCAR: t_0 *v* AL, $P < .0001$; t_0 *v* DR, $P = .9111$; AL *v* DR, $P < .0001$.

Quantification of Body Fat, BMD, and BMC

At the end of the experimental periods, the mice were killed by pentobarbital injection and exsanguination. The crown to rump lengths of the mice were determined with a microcaliper (Manostat #5921, Fisher Scientific, Tustin, CA). Body mass was determined gravimetrically on an XL-300 balance (Denver Instruments, Denver, CO). Body fat (%) and total, vertebral, and femoral BMD (g/cm^2) and BMC (g) were determined by densitometry using a PIXImus imager (GE Lunar, Madison, WI). Field calibration and calibration versus the quality control phantom were performed each day before mouse imaging. Each mouse was positioned reproducibly in a prone position on the imaging tray and scanned 3 times. The coefficients of variance (CV) for fat, BMD, and BMC were 1.6%, 0.5%, and 0.6%, respectively, for in vitro measurements.

Statistical Analyses

All data are expressed as the mean ± SEM (n). Data were subjected to multiple analysis of variance (ANOVA) using strain and time/diet as the main effects and testing for interactions. Where statistically significant interactions were observed, post hoc analysis consisting of 1-way ANOVA for each strain looking at the effect of diet-time and a posteriori pairwise tests using Tukey-Kramer adjustments for multiple comparisons were performed. Results were considered significant at $P < .05$. In addition, the relationships between total, femoral, and vertebral BMC or BMC and body mass (total, lean, or fat) were determined for all 8.5-month-old mice using the least squares linear regression method [SigmaPlot (version 8.0) Regression Wizard] 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 mass indicated (gram).

RESULTS

Body Mass

The total body masses of C57, DBA, and SENCAR mice at time-zero (t_0; 10 weeks or 2.5 months of age) and after 6 months of DR or AL feeding (8.5 months of age) are shown in Table 1. Throughout the study, the ranking of body mass was SENCAR (heaviest) > C57 > DBA (lightest). Of interest is the observation that DR did not greatly affect the body masses of C57 and DBA mice, relative to their respective t_0 controls (Table 1). In contrast, SENCAR mice weighed less after 6 months of DR than they did at the beginning of the experiment (Table 1). The greatest absolute increase in body mass relative (grams) to t_0 controls was observed in 8.5-month-old AL-fed C57 mice (+17.62 g or +72%), followed by AL-fed SENCAR (+11.4 g or +28.6%) and DBA (+8.9 g or +35%) mice (Table 1).

Body Fat

At dissection, it was readily apparent that the amount of white adipose tissue had dramatically increased in 8.5-month-old C57 and SENCAR mice, relative to t_0 controls. This was quantitatively assessed as the percentage of the total body mass that was present as fat (%; [fat mass]/[total mass] × 100) (Table 2). Body fat (%) was affected by strain, diet/time, and the interaction between these parameters (Table 2). At 10 weeks of age, DBA mice had a higher percentage body fat than the 2 other strains (Table 2). However, by 8.5 months of age, the percent body fat was much higher in AL-fed C57BL mice than in the other 2 strains (Table 2). The body fat (%) in AL-fed 8.5-month old C57BL mice was almost 50%, and the relative ranking of the strains under AL feeding conditions at 8.5 months of age was C57BL/6 > SENCAR > DBA/2 (Table 2). All 30% DR mice had less body fat than age-matched AL-fed mice of the same strain, but the body fat (%) in DR C57 and SENCAR mice was higher than it was in their respective t_0 controls (Table 2). Thirty percent DR prevented the development of gross obesity in 8.5-month-old C57 mice (Table 2).

Table 4. Total Bone Mineral Density ($g/cm^2 \times 100$) of 10-Week-Old and 8.5-Month-Old C57BL/6, DBA/2, and SENCAR Mice

Age (mo)	Treatment	Strain		
		C57BL/6	DBA/2	SENCAR
2.5	t_0	4.71 ± 0.10 (12)	4.37 ± 0.10 (12)	5.98 ± 0.10 (11)
8.5	AL fed	5.33 ± 0.11 (10)	5.76 ± 0.11 (10)	6.20 ± 0.10 (11)
8.5	30% DR	5.45 ± 0.10 (11)	5.26 ± 0.10 (10)	5.79 ± 0.10 (12)

NOTE. Data are expressed as the mean ± SEM (n).

Effect by ANOVA: Diet/time, $P < .0001$; strain, $P < .0001$; interaction, $P < .0001$.

Post hoc analysis for comparisons between strains was as follows. C57BL/6 *v* DBA/2: t_0, $P = .0150$; AL fed, $P = .0063$; 30% DR, $P = .2084$. C57BL/6 *v* SENCAR: t_0, $P < .0001$; AL fed, $P < .0001$; 30% DR, $P = .0178$. DBA/2 *v* SENCAR: t_0, $P < .0001$; AL fed, $P = .0041$; 30% DR, $P = .0004$.

Table 5. Total Bone Mineral Content (g × 100) of 10-Week-Old and 8.5-Month-Old C57BL/6, DBA/2, and SENCAR Mice

Age (mo)	Treatment	Strain		
		C57BL/6	DBA/2	SENCAR
2.5	t_0	35.6 ± 1.20 (12)	28.3 ± 1.20 (12)	51.4 ± 1.25 (11)
8.5	AL fed	40.3 ± 1.31 (10)	39.2 ± 1.31 (10)	46.1 ± 1.25 (11)
8.5	30% DR	39.5 ± 1.25 (11)	37.8 ± 1.31 (10)	48.2 ± 1.20 (12)

NOTE. Data are expressed as the mean ± SEM (n).

Effect by ANOVA: Diet/time, $P = .0009$; strain, $P < .0001$; interaction, $P < .0001$.

Post hoc analysis for comparisons between strains was as follows. C57BL/6 *v* DBA/2: t_0, $P < .0001$; AL fed, $P = .5659$; 30% DR, $P = .3506$. C57BL/6 *v* SENCAR: t_0, $P < .0001$; AL fed, $P = .0018$; 30% DR, $P < .0001$. DBA/2 *v* SENCAR: t_0, $P < .0001$; AL fed, $P = .0003$; 30% DR, $P < .0001$.

Crown to Rump Length

The crown to rump lengths of the mice are listed in Table 3. All parameters (diet/time, and strain and the interaction between diet/time and strain) had significant effects on crown to rump length (Table 3). At both t_0 and 8.5 months of age, SENCAR mice were longer than C57 and DBA mice, which tended to be of relatively similar length (Table 3). The mice in the AL-fed groups grew more than 1.0 cm in length from 2.5 to 8.5 months of age (Table 3). In addition, there was a modest increase in length in 30% DR DBA mice, when compared with their respective t_0 controls, while 30% DR C57 and SENCAR mice were similar in length to their t_0 controls (Table 3). Although 30% DR DBA mice weighed less and had a lower percentage of body fat than DR C57 mice, they grew more in length (0.77 cm *v* 0.29 cm) over the 6-month period from 2.5 to 8.5 months of age than C57 mice did (Table 3). The 8.5-month-old AL-fed SENCAR mice were almost 1 cm longer than the t_0 control mice of this strain, but there was no significant increase in the length of 30% DR SENCAR mice when compared with t_0 controls (Table 3).

Total BMD and BMC

Diet/time, strain, and the interaction between diet/time and strain all affected total BMD (Table 4) and BMC (Table 5). Total body BMD (Table 4) and BMC (Table 5) were much higher in SENCAR mice than in the other 2 strains. Thirty percent DR DBA and SENCAR mice had lower total BMDs than those observed in their respective AL-fed control groups (Table 4). In contrast, 30% DR and AL-fed C57 mice had similar total body BMDs (Table 4). The lowest total BMD in 8.5-month-old AL-fed mice was observed in the grossly obese C57 mice (Table 4) with over 48% total body fat (Table 2). Total BMC, which is a function of both the density and size of bones, was different in all strains at t_0, with SENCAR > C57 > DBA (Table 5). By 8.5 months of age, there was no difference in total BMC observed when AL-fed or 30% DR C57 and DBA mice were compared, although both strains had lower BMCs than SENCAR mice in each of the respective feeding groups (Table 5).

Vertebral BMD and BMC

Vertebral BMD (Table 6) and BMC (Table 7) were affected by all variables tested (diet/time, strain, and the interaction between these variables). At t_0, vertebral BMD (Table 6) and BMC (Table 7) were significantly greater in SENCAR mice than in C57 or DBA mice. Vertebral BMD (Table 6) and BMC (Table 7) were lower in AL-fed SENCAR mice than in their respective t_0 controls. C57 and DBA mice had similar vertebral BMDs (Table 6) and BMCs (Table 7) at t_0 and after 6 months of DR feeding. The grossly obese 8.5-month-old AL-fed C57 mice had lower vertebral BMD (Table 6) and BMC (Table 7) than AL-fed DBA or SENCAR mice. Finally, DR C57 and SENCAR mice had vertebral BMDs (Table 6) and BMCs (Table 7) greater than those of their respective AL-fed controls.

Femoral BMD and BMC

Femoral BMD (Table 8) and BMC (Table 9) were significantly affected by all variables tested (diet/time, strain, and the interaction between these variables). At t_0 and after 8.5 months of AL feeding, SENCAR mice had higher femoral BMDs (Table 8) and BMCs (Table 9) than C57 or DBA mice. Femoral BMD (Table 8) and BMC (Table 9) were similar in AL-fed C57 and DBA mice. However, 30% DR DBA mice had lower femoral BMD than DR C57 mice (Table 8), although the femoral BMC (a function of BMD and bone size) was similar in these 2strains (Table 9). Femoral BMD and BMC were much

Table 6. Vertebral Bone Mineral Density (g/cm² × 100) of 10-Week-Old and 8.5-Month-Old C57BL/6, DBA/2, and SENCAR Mice

Age (mo)	Treatment	Strain		
		C57BL/6	DBA/2	SENCAR
2.5	t_0	4.54 ± 0.15 (12)	4.41 ± 0.15 (12)	5.95 ± 0.15 (11)
8.5	AL fed	4.68 ± 0.16 (10)	5.56 ± 0.16 (10)	5.24 ± 0.15 (11)
8.5	30% DR	5.52 ± 0.15 (11)	5.43 ± 0.16 (10)	5.73 ± 0.15 (12)

NOTE. Data are expressed as the mean ± SEM (n).

Effect by ANOVA: Diet/time, $P = .0001$; strain, $P < .0001$; interaction, $P < .0001$.

Post hoc analysis for comparisons between strains was as follows. C57BL/6 *v* DBA/2: t_0, $P = .5113$; AL fed, $P = .0002$; 30% DR, $P = .6828$. C57BL/6 *v* SENCAR: t_0, $P < .0001$; AL fed, $P = .0140$; 30% DR, $P = .3346$. DBA/2 *v* SENCAR: t_0, $P < .0001$; AL fed, $P = .1517$; 30% DR, $P = .1759$.

Table 7. Vertebral Bone Mineral Content (g × 100) of 10-Week-Old and 8.5-Month-Old C57BL/6, DBA/2, and SENCAR Mice

Age (mo)	Treatment	Strain		
		C57BL/6	DBA/2	SENCAR
2.5	t_0	6.23 ± 0.37 (12)	5.78 ± 0.37 (12)	10.48 ± 0.39 (11)
8.5	AL fed	6.87 ± 0.41 (10)	8.37 ± 0.41 (10)	9.23 ± 0.39 (11)
8.5	30% DR	8.29 ± 0.39 (11)	8.90 ± 0.41 (10)	11.00 ± 0.37 (12)

NOTE. Data are expressed as the mean ± SEM (n).

Effect by ANOVA: Diet/time, $P < .0001$; strain, $P < .0001$; interaction, $P < .0001$.

Post hoc analysis for comparisons between strains was as follows. C57BL/6 *v* DBA/2: t_0, $P = .3902$; AL fed, $P = .0107$; 30% DR, $P = .2802$. C57BL/6 *v* SENCAR: t_0, $P < .0001$; AL fed, $P < .0001$; 30% DR, $P = .0001$. DBA/2 *v* SENCAR: t_0, $P < .0001$; AL fed, $P = .1302$; 30% DR, $P = .0002$.

lower in 30% DR SENCAR mice than in their t_0 controls (Tables 8 and 9).

Regression Analysis of Body Weights Versus Total Body, Vertebral, and Femoral BMD and BMC

The relationships between body mass (total, lean, and fat) and total body, vertebral, and femoral BMD and BMC were assessed by linear regression. The statistically significant results ($P \leq .05$) are summarized in Table 10. Briefly, the most significant linear relationships that were found were between lean body mass and total body and femoral BMD and BMC (Fig 1A and B, Table 10). In addition, there were similarly significant linear relationships between total body mass and total body and femoral BMC and BMD (Fig 2A and B, Table 10). As might be expected for a non–weight-bearing site, vertebral BMD or BMC were not strongly correlated with total or lean body mass (Table 10). There were positive correlations between total body fat mass and total body, vertebral and femoral BMD, as well as femoral BMC (Table 10).

DISCUSSION

DR is thought to mediate its antiaging effects, in part, by limiting free radical generation and downstream damage to lipids, proteins, and DNA.[1,2] Therefore, we hypothesized that DR would have its most beneficial effects on bone in animal models of high free radical generation and oxidative stress. This hypothesis was tested in 2 well-characterized murine models of high (SENCAR)[3-9] and low (DBA/2)[23-25] free radical generation, as well as in a model of intrinsically low free radical generation,[3,5,7,9] obesity,[30-35] and diabetes,[33] with its attendant high end-stage protein glycation and premature aging (C57BL/6). The existing literature clearly established a predictable gradient of free radical generation capacity, oxidative stress, and end-stage protein glycation, with SENCAR >>> C57 >> DBA.[3-9,13,14,23-28,30-33] By undertaking these studies simultaneously in each of the 3 strains, we minimized or eliminated several confounding variables, such as the age at which DR was instituted, the duration of DR, variations in the composition of the diet, and the conditions under which the animals were housed and handled. Using this design, we have partially validated the hypothesis that the most beneficial effects of DR would be observed in the most extreme models of free radical generation by demonstrating that vertebral BMD and BMC are higher in DR C57 and SENCAR mice than in their respective AL-fed controls (Tables 6 and 7). In contrast, DR had no major effect on vertebral BMC and BMD in DBA mice, which experience low levels of oxidative stress (Tables 6 and 7). We also observed that femoral BMD and BMC were lower in DR SENCAR mice than in their age-matched AL-fed controls (Tables 8 and 9). We previously subjected 10- to 16-week-old SENCAR mice to 40% DR and demonstrated that tibial trabecular density was conserved, while tibial cortical density declined, relative to t_0 controls.[21] We had concluded that tibial trabecular bone was conserved at the apparent expense of cortical bone.[21] The present studies showing that DR has a relatively beneficial effect at a highly trabecularized site in the SENCAR mouse (vertebrae) (Tables 6 and 7), while it is associated with lower BMD and BMC in a site relatively rich in cortical bone (femur) (Tables 8 and 9) are consistent with these previous observations.[21] Femoral BMD and BMC also tended to be lower in DR DBA mice than in their age-matched AL-fed controls (Tables 8 and 9). High levels of reactive oxygen species, such as superoxide anions, are generated by osteoclasts during bone remodeling. The relatively beneficial effects of DR on the vertebrae versus the femur in the SENCAR mouse model of high free radical generation and oxidant

Table 8. Femoral Bone Mineral Density (g/cm^2 × 100) of 10-Week-Old and 8.5-Month-Old C57BL/6, DBA/2, and SENCAR Mice

Age (mo)	Treatment	Strain		
		C57BL/6	DBA/2	SENCAR
2.5	t_0	6.83 ± 0.28 (12)	6.42 ± 0.28 (12)	9.81 ± 0.29 (11)
8.5	AL fed	8.11 ± 0.30 (10)	8.41 ± 0.30 (10)	9.37 ± 0.29 (11)
8.5	30% DR	8.29 ± 0.29 (11)	7.35 ± 0.30 (10)	8.06 ± 0.28 (12)

NOTE. Data are expressed as the mean ± SEM (n).

Effect by ANOVA: Diet/time, $P < .0004$; strain, $P < .0001$; interaction, $P < .0001$.

Post hoc analysis for comparisons between strains was as follows. C57BL/6 *v* DBA/2: t_0, $P = .3042$; AL fed, $P = .4838$; 30% DR, $P = .0267$. C57BL/6 *v* SENCAR: t_0, $P < .0001$; AL fed, $P = .0032$; 30% DR, $P = .5639$. DBA/2 *v* SENCAR: t_0, $P < .0001$; AL fed, $P = .0234$; 30% DR, $P = .0862$.

Table 9. Femoral Bone Mineral Content (g × 100) of 10-Week-Old and 8.5-Month-Old C57BL/6, DBA/2, and SENCAR Mice

Age (mo)	Treatment	Strain		
		C57BL/6	DBA/2	SENCAR
2.5	t_0	2.81 ± 0.11 (12)	2.22 ± 0.11 (12)	4.78 ± 0.11 (11)
8.5	AL fed	3.10 ± 0.12 (10)	2.90 ± 0.12 (10)	4.56 ± 0.11 (11)
8.5	30% DR	2.76 ± 0.11 (11)	2.62 ± 0.12 (10)	3.78 ± 0.11 (12)

NOTE. Data are expressed as the mean ± SEM (n).

Effect by ANOVA: Diet/time, $P < .0001$; strain, $P < .0001$; interaction, $P < .0001$.

Post hoc analysis for comparisons between strains was as follows. C57BL/6 *v* DBA/2: t_0, $P = .0001$; AL fed, $P = .2402$; 30% DR, $P = .3801$. C57BL/6 *v* SENCAR: t_0, $P < .0001$; AL fed, $P < .0001$; 30% DR, $P < .0001$. DBA/2 *v* SENCAR: t_0, $P < .0001$; AL fed, $P < .0001$; 30% DR, $P < .0001$.

stress may reflect the fact that the free radical-dependent surface-active process of osteoclastic bone resorption is more prominent in highly trabecularized bones, such as the vertebrae, with their large surface area to volume ratios, than in the cortical bone that comprises much of the femur.

The data for the femur provide support for the second hypothesis tested. Briefly, caloric restriction is associated with low body mass, one of the major determinants of BMD and BMC across the life span.[39-42] Previous investigators have demonstrated that CR is associated with low bone mass in rhesus monkeys[39] and rats,[43] and that low bone mass can be accounted for by adjusting for body weight and age. However, comparative studies of the relationships between body mass (total, lean, or fat) and BMD or BMC at weight-bearing and nonweight-bearing sites in DR and AL-fed mice with well-defined genetically determined differences in body and bone mass[38] had not been undertaken. Because bone responds to the mechanical stress imposed by body mass, a mathematical relationship between mass and BMD or BMC would be expected in both DR and AL-fed mice. How each of the strains respond to DR, however, could well vary depending on their intrinsic rates of bone formation or remodeling, which are known to differ in SENCAR, C57, and DBA mice.[17-19] Therefore, we tested our second hypothesis that bone mass, particularly at weight-bearing sites, is determined by body mass in DR and AL-fed mice of all strains. We validated the hypothesis, in part, by demonstrating that total and lean body mass were positively correlated with total body BMD, total body BMC, and femoral BMD and BMC in all strains (Figs 1 and 2, Table 10). Interestingly, a single regression line was sufficient to confirm these results for all strains after 6 months of AL or DR feeding (Figs 1 and 2, Table 10). These data support the conclusions previously reached in strain-limited studies of rats and monkeys.[39,43]

Finally, previous investigators have characterized the C57BL/6 mouse as a genetic model of low bone regeneration capacity[19] and low adult peak bone mass or density at multiple sites.[38] Conversely, the SENCAR mouse has been characterized as a genetic model of high bone regeneration capacity.[19] Development of congenic strains and mapping quantitative trait loci for bone density or regenerative capacity have been undertaken[44] or suggested for these strains.[19] While this approach is generally useful for identifying genetic factors that are biologically relevant, the specific metabolic defects that these 2 strains exhibit are generally overlooked, even though they may play major roles in mediating the differences in bone metabolism that have been reported. Thus, metabolic abnormalities in these 2 strains may limit their general utility in identifying more general genetic factors responsible for extremes in bone development. For example, C57 mice are the parental strain from which the classic *ob/ob* and *db/db* mice were derived. The *ob/ob* mouse is leptin deficient,[45] while the *db/db* mouse does not express the long form of the leptin receptor.[46] The parental

Table 10. Regression Analysis of the Relationships Between Body Mass (total, fat, or lean [g]) and Total Body, Vertebral, and Femoral BMD [g/cm^2 × 100] or BMC [g × 100]

Variables	P Value	*r*	v_0	a
Total body mass × total body BMD	.0011	.419	5.059	0.018
Total body mass × total body BMC	< .0001	.519	32.704	0.2473
Total body mass × femoral BMD	< .0001	.492	6.710	0.045
Total body mass × femoral BMC	< .0001	.757	1.485	0.052
Fat mass × total body BMD	.0172	.3118	38.484	0.259
Fat mass × vertebral BMD	.0011	.418	5.810	−0.043
Fat mass × femoral BMC	.0002	.466	2.694	0.056
Fat mass × femoral BMD	.0139	.321	7.714	0.051
Lean body mass × total body BMD	< .0001	.613	4.274	0.066
Lean body mass × total body BMC	< .0001	.592	25.987	0.723
Lean body mass × femoral BMD	< .0001	.598	5.311	0.140
Lean body mass × femoral BMC	< .0001	.879	0.002	0.156

NOTE. The data were plotted together for all strains at 8.5 months of age and fitted to the regression line: $f = y_0 + (a)(x)$, where f = BMD or BMC at the site indicated, and x = mass. The data were subjected to ANOVA, and the *P* values for the regression lines are presented only when $P \leq .05$.

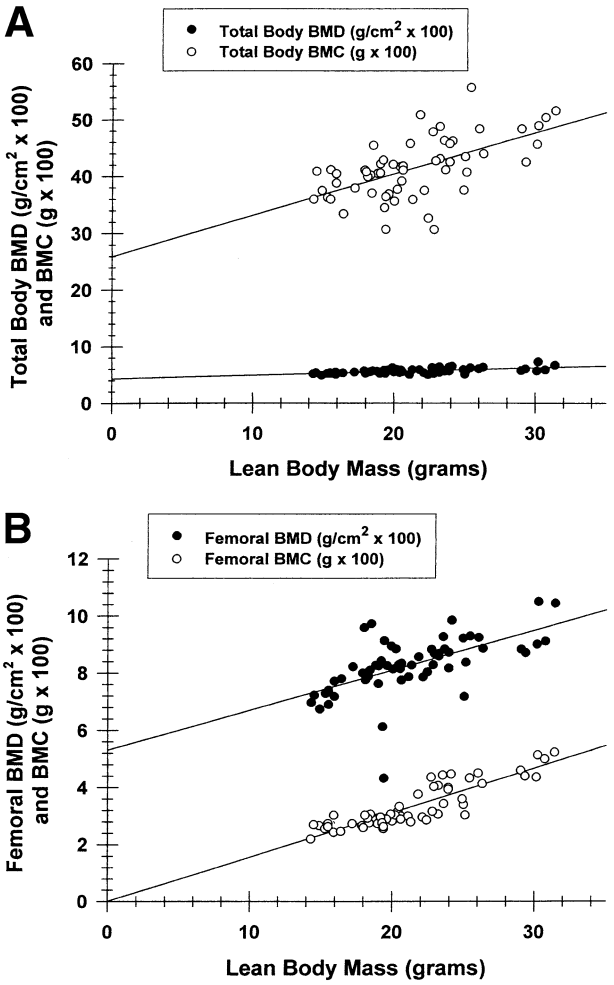

Fig 1. Relationships between (A) lean body mass (g) and total body BMD and BMC or (B) femoral BMD and BMC in all 8.5-month old AL-fed and 30% DR C57BL/6, DBA/2, and SENCAR mice. See Table 10 for the regression analyses of the lines.

C57 line spontaneously exhibits age- and diet-related obesity,[30-35] diabetes,[33] and impaired responsiveness to leptin,[35] the major central regulator of bone metabolism.[37] In addition, the highly-inbred C57 parental line exhibits heterogeneous metabolic responses to experimental regimens, such as adaptation to a high-fat diet.[32] For example, when C57 mice are fed a high-fat, carbohydrate-free diet for 9 months, 50% become obese and diabetic, 10% become lean and diabetic, 10% remain lean and nondiabetic, and 30% display an intermediate phenotype. Genetic analysis of how bone mass is regulated in C57-derived strains may identify specific molecules, such as leptin or its receptors, that simultaneously account for the metabolic abnormalities of this parental strain and contribute to bone mass regulation across the life span in the C57 mouse. The positive correlation between fat mass and vertebral BMD observed in these studies (Table 10) suggests that adipose cell derived-factors, such as leptin, play an important role in regulating bone mass in these 3 strains. However, molecules, such as leptin, or its receptors may only account for a portion of the genetic differences in bone mass development and regulation that are observed across most murine strains.

Similarly, the SENCAR mouse exhibits a basic defect in PKC that affects a wide variety of downstream processes in addition to free radical generation and downstream oxidation.[3-8,13] For example, SENCAR mice exhibit elevated levels of growth factor and growth factor receptor expression. Many of these molecules, such as granulocyte-macrophage colony-stimulating factor,[14] epidermal growth factor,[15] and transforming growth factor,[16] are highly relevant in bone. Many transcription factors and their receptors, including E2F transcription factor[47] and peroxisome proliferator-activated receptor,[48] as well as cytokines and their downstream signaling pathways, such as NF-κB,[49-50] are activated after stimulation of PKC in SENCAR mice. Thus, it is feasible that an examination of the genetics of bone formation and regeneration in SENCAR mice may provide valuable insights into the role of PKCs in these processes. However, the general utility of the strain in elucidating the

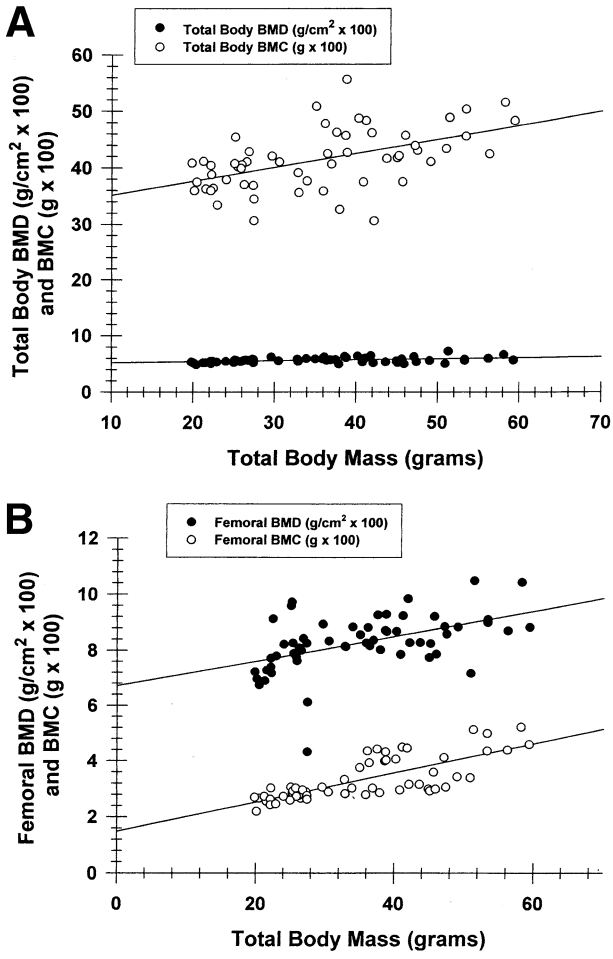

Fig 2. Relationships between (A) total body mass (g) and total body BMD and BMC or (B) femoral BMD and BMC in all 8.5-month old AL-fed and 30% DR C57BL/6, DBA/2, and SENCAR mice. See Table 10 for the regression analyses of the lines.

genetic basis for strain-dependent differences in bone mass development and maintenance may be somewhat limited. In conclusion, one of the most interesting results of the present studies is that it appears that the bone mechanostat is set at a similar level in all strains tested under DR and AL feeding conditions. In addition, DR appears to be beneficial in the vertebrae of strains prone to high levels of oxidative stress. Downregulation of surface-active processes, such as bursts of free-radical production by osteoclasts during bone resorption, may play a role in mediating the beneficial effects of DR at this skeletal site, but further research will be required to confirm this mechanism.

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