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# Life-Long Caloric Restriction Reveals Biphasic and Dimorphic Effects on Bone Metabolism in Rodents

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**Caloric restriction (CR) extends the lifespan of various organisms and slows the onset of age-related disorders; however, little is known about the long-term effects of CR *per se* on bone. In the present study, we have examined the effects of life-long CR *vs. ad libitum* (AD) feeding, mainly on the trabecular bone of proximal tibiae in male C57BL/6 mice and F344 rats. Micro-computed tomography scanning of tibiae revealed that CR for 3–9 months caused a substantial decrease in three-dimensional bone volume with structural derangements. Bone histomorphometry revealed the reduced bone mass was due mainly to suppression of bone formation. In *db/db* mice with defective leptin receptor, CR was unable to decrease bone mass and suppress bone formation. The effect of CR on bone mass was inhibited by administration of a β-adrenergic blocker, propranolol. Thus, CR may regulate bone formation through leptin signaling and elevated sympathetic nervous tone. Interestingly, the difference in bone volume between the CR and AD groups disappeared after 1 yr of age, and mice and rats on an additional extension of CR to natural death maintained higher bone mass than the AD groups, with reduced bone turnover, suggesting that CR slows skeletal aging by regulating the rate of bone turnover. This is the first report, to our knowledge, that has examined the effects of lifelong CR on bone metabolism and trabecular microstructure and documents its contrasting effects during maturation *vs.* the postmaturational, involutional period. (*Endocrinology* 149: 634–641, 2008)**

FRAGILITY FRACTURES DUE to osteoporosis is a serious cause of morbidity and mortality and imposes a major economic burden in aged societies (1). Osteoporosis is a multifactorial disease, and lifestyle as well as genetic factors contribute to age-related bone loss and fragility (2, 3). In addition to low bone mineral density, clinical factors including age, female sex, premature menopause, previous fracture, a family history of fracture, low body mass index, glucocorticoid use, excessive alcohol consumption, and cigarette smoking constitute risk factors for bone fragility and fracture (3).

Caloric restriction (CR) extends the lifespan of various organisms as diverse as yeast, worms, flies, and mammals and delays the progression of geriatric disorders, such as malignancy, neurodegenerative disease, renal disease, cataract, and immune disease (4). How CR affects bone metabolism is controversial, and little is known of the long-term effects of CR *per se*. It has been reported that CR ameliorates age-related bone loss in male Fisher 344 (F344) rats through a prevention of secondary hyperparathyroidism (5). On the other hand, others report that CR results in bone loss in aging male rats (6) and rhesus monkeys (7). Little is known about the effects of CR on bone cell activity, and the mechanism by which CR affects bone remodeling remains to be clarified.

In the present study, we have examined the effects of life-long CR in C57BL/6 mice and F344 rats on bone mass and trabecular microstructure, using micro-computed tomography (micro-CT). The results indicate that CR exerts biphasic effects on bone depending on the duration of the restriction; until 1 yr of age, CR decreases bone mass, mainly through a suppression of bone formation, which activity involves leptin signaling and elevated sympathetic nervous tone. In contrast, CR for more than 2 yr has a protective effect against age-related bone loss through reducing the rate of bone turnover. This is the first report, to our knowledge, that clarifies the effects of CR on bone physiology throughout life and may have implications for optimizing nutritional programs for human bone health.

## Materials and Methods

### *Reagents*

Tetracycline hydrochloride and calcein were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

### *Animal experiments*

Six-week-old male C57BL/6J mice and F344 rats were purchased from CLEA Japan, Inc. (Tokyo, Japan) and raised at Oriental Bio-service (Kyoto, Japan). Animals were maintained under standard laboratory conditions at 24 ± 2 C and 50–60% humidity and allowed free access to tap water and commercial standard rodent chow (CE-2) containing 1.20% calcium, 1.08% phosphate, and 240 IU/100 g vitamin $D_3$ (CLEA Japan, Inc.). CR was started at the age of 3 months by alternate-day feeding; mice and rats were fed three times per week (Monday, Wednesday, and Friday), a commonly used method in aging research (8). An-

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Abbreviations: AD, *Ad libitum*; BFR, bone formation rate; BS, bone surface; BV, bone volume; Conn.Dens., connectivity density; CR, calorie restriction; CT, computed tomography; 3D, three-dimensional; MAR, mineral apposition rate; NMU, neuromedin U; Ob.S, osteoblast surface; Oc.S, osteoclast surface; SMI, structure model index; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TV, tissue volume.

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imals maintained on this regimen consumed approximately 40% less than those maintained on the *ad libitum* (AD) regimen. To adjust the calcium consumed by CR mice to that of AD mice, chow with 67% increases in calcium and phosphate was fed, so that 40% less consumption of this high-calcium diet would result in the same amount of calcium.

The *db/db* mice used in this study were originally from the Jackson Laboratory (Bar Harbor, ME) (9) and are on the C57BLKS/J background, estimated to derive from 71% C57BL/6J and 25% DBA/2J and 4% from a combination of some other strains. *m* (for misty) is the coat color mutation closely linked to *db*, and mating repulsion double heterozygotes, *m* +/+ *Lepr* $^{db}$, produced 1/4 diabetics (black color and obese), 1/2 wild-type repulsion double heterozygotes (black and lean), and 1/4 misty mice (gray color and lean).

To achieve a continuous CR state in certain cohorts of F344 rats, the amount of chow was adjusted to 40% and reduced by measuring the amount consumed by AD rats.

The animal experiments were carried out in accordance with the ethical guidelines for animal care of NCGG, and the experimental protocols were approved by the animal care committee.

### *RNA extraction and RT-PCR*

Total RNA was isolated from the mouse hypothalamus with TRIzol reagent (Invitrogen, San Diego, CA). For quantitative RT-PCR, total RNA (1 $\mu$g) was reverse transcribed using Superscript III (Invitrogen), and samples were analyzed using a Light Cycler system (Roche Diagnostics, Basel, Switzerland). The primers included 5′-GTTCCTGAGGCTTCTGGGAAAT-3′ and 5′-TCAAAGATTGCAGCCAGAACAA-3′ for NMU and 5′-AGCTTGTCATCAACGGGAAG-3′ and 5′-TTTGATGTTAGTGGGGTCTCG-3′ for GAPDH.

### *Biochemical analysis*

Serum leptin, insulin, and IGF-I concentrations were determined by using rat leptin RIA and rat insulin RIA kits (Linco Research, Inc., St. Louis, MO) and Quantikine mouse IGF-1 immunoassay kit (R&D Systems Inc., Minneapolis, MN), respectively. Serum albumin and calcium and urinary calcium concentrations were determined by using an autoanalyzer (Hitachi 7170, Japan).

### *Micro-CT scanning*

Micro-CT scanning was performed on proximal tibiae using a $\mu$CT-40 scanner (SCANCO Medical AG, Bassersdorf, Switzerland) with a resolution of 12 $\mu$m, and microstructure parameters were calculated three-dimensionally as described previously (10). The proximal tibia was positioned to be scanned craniocaudally using 320 slices with 12$\mu$m increments at 55 kVp and 72 $\mu$A. On the original three-dimensional (3D) image, morphometric indices, including bone volume (BV), tissue volume (TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N), were directly determined from the binarized volume of interest. Nonmetric parameters, such as structure model index (SMI) and connectivity density (Conn-Dens.), were also obtained as described previously (10); for SMI, the characteristic form of a three-dimensionally described structure in terms of the amount of plates and rods composing the structure was quantified (11), and Conn-Dens was determined as the number of trabecular connections per cubic millimeter.

### *Bone analysis*

Tetracycline hydrochloride (30 mg/kg body weight) and calcein (6 mg/kg body weight) were injected with a 2-d interval for the fluorescent labeling of bone. Tibiae were fixed in 70% ethanol and embedded in glycol-methacrylate without decalcification. Sections were prepared and stained with Villanueva Goldner to discriminate between mineralized and unmineralized bone and to identify cellular components. Histomorphometric parameters were measured at the Ito Bone Science Institute (Niigata, Japan). Nomenclature and units were used according to the recommendation of the nomenclature committee of the American Society for Bone and Mineral Research.

Bone strength was assessed in femurs by the four-point bending test, according to the previously reported methods (12, 13). Mechanical tests were performed at the Japan Fine Ceramics Center (Nagoya, Japan) using a materials testing machine (type 5582; Instron, Norwood, MA). Specimens were loaded in the anterior-posterior plane at a constant displacement rate of 0.5 mm/min. The distances between the upper (loading) and lower (support) points were 4 and 8 mm, respectively. A 100-N load cell was used to measure the load applied to the specimens, and displacement was measured with a linear variable differential transducer. Load and displacement data were collected using Merlin software (Instron).

### *Statistical analysis*

Data are expressed as means ± SEM. Statistical analysis was performed using unpaired Student's *t* test or ANOVA followed by Dunnett's test or Student-Newman-Keuls test. Values were considered statistically significant at $P < 0.05$.

## Results

### *CR decreases bone mass in young adult mice and rats*

To examine the effects of chronic CR on bone metabolism, a cohort of male C57BL/6J mice and F344 rats were raised under alternate-day feeding, whereas an age- and sex-matched AD feeding group served as the control. CR was started when animals reached 3 months of age. As shown in Fig. 1, both CR mice (A) and rats (B) gained body weight, but more slowly than control AD animals, and after 1 yr, CR mice and rats weighed approximately 20 and 30% less, respectively, than age-matched AD controls.

Figure 2, A and B, shows representative 3D micro-CT images of trabecular bone at the metaphysis of the proximal tibiae of C57BL/6 mice and F344 rats, respectively, after 6 months of CR (*i.e.* at the age of 9 months). Chronic CR caused marked osteopenia, and the 3D bone volume fraction (BV/

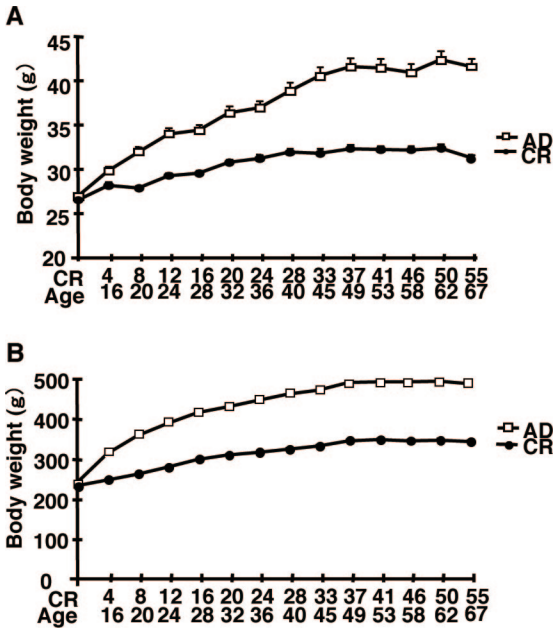


FIG. 1. Body weight changes in CR mice and rats. Cohorts of C57BL/6 male mice (A) and F344 male rats (B) were divided into two groups at the age of 3 months and raised under CR or AD feeding for 1 yr (n = 13–14 each group). Body weight was measured every week until 28 wk and every 4–5 wk thereafter. The time scale is provided as both time after the start of CR (*upper values*) and the age of animals (*lower values*) in weeks.

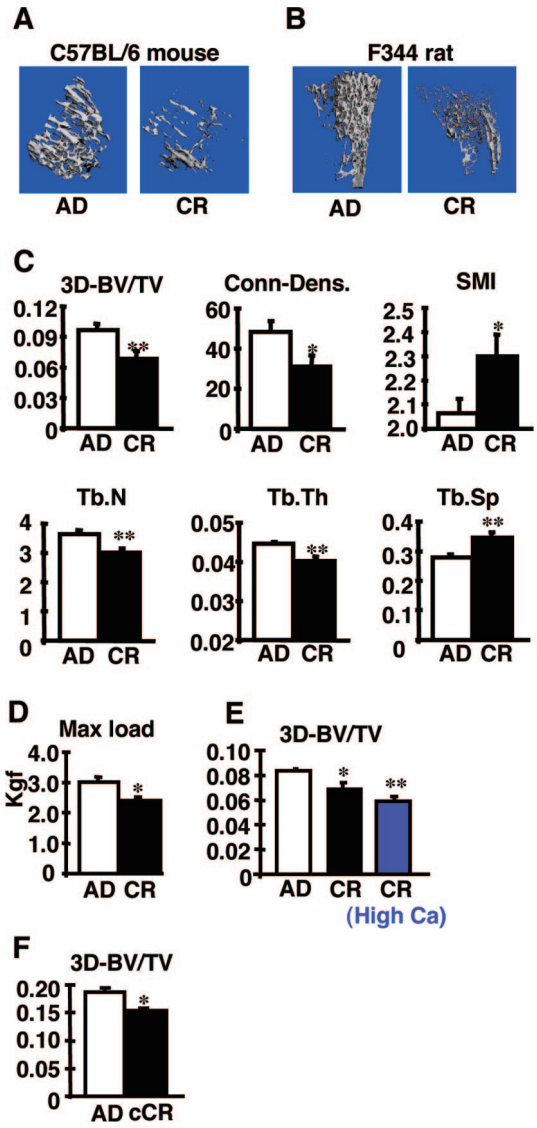


FIG. 2. Chronic CR induces reduced bone mass, deteriorated trabecular structure, and decreased bone strength. A and B, Representative micro-CT images of trabecular bone at the proximal tibiae of AD feeding *vs.* CR mice (A) and AD *vs.* CR rats (B) at the age of 9 months (*i.e.* after 6 months of CR). C, Microstructural parameters derived from micro-CT analysis of trabecular bone at the proximal tibiae of AD *vs.* CR mice. *, $P < 0.05$; **, $P < 0.01$ (n = 18–19). D, Bone strength after 6 months of CR was determined by maximal load at the femur by the four-point bending test. *, $P < 0.05$ (n = 4). E, Reduced bone mass by CR is not due to a concomitant reduction in calcium intake. Mice were maintained for 6 months on ordinary CR (both calorie and calcium reduced by 40%) or CR with 67% higher calcium content in chow (high Ca) so that only the caloric intake was reduced while the calcium consumption was kept at the same level as the AD group. The 3D BV/TV was determined at the proximal tibia by micro-CT. *, $P < 0.05$; **, $P < 0.01$ (n = 3–5). F, F344 male rats were fed a 40% decreased daily diet (cCR, continuous CR) for 6 months, and 3D BV/TV was determined at the proximal tibia by micro-CT. *, $P < 0.05$ (n = 5 in each group).

TV) decreased in both CR mice (Fig. 2C) and CR rats (data not shown) by 29 and 25%, respectively, compared with the AD groups. In addition, detailed microstructure analysis of trabecular bone of mice and rats revealed that after 6 months of CR, Tb.N and Tb.Th as well as Conn.Dens. decreased significantly, whereas SMI and Tb.Sp increased (Fig. 2C and data not shown), suggesting that CR makes trabeculae thinner and more of a fragile rod-like (rather than plate-like) structure with less connectivity. Assessment of maximal load by the four-point bending test indicated that bone strength in the femurs of CR group was indeed significantly weaker than that of the control AD group (Fig. 2D).

The average food intake of CR mice under alternate-day feeding was nearly 40% less than that of AD mice (1.22 ± 0.02 g/d in CR *vs.* 2.06 ± 0.12 g/d in AD), indicating that calcium intake was accordingly decreased by 40%. To examine whether bone mass decreased as a result of the reduction in total calorie intake or, rather, a concomitant reduction in calcium intake, another cohort of C57BL/6 mice was fed a chow with 67% higher calcium content so that the 40% reduction of total food intake resulted in no net decrease in calcium intake. As shown in Fig. 2E, mice on CR with the high-calcium content exhibited lower bone mass than the AD group, just like ordinary CR mice, suggesting that decreased bone mass by CR is not the result of relative calcium deficiency but due to reduced caloric intake.

Also, to rule out the possibility that the alternate-day feeding regimen *per se* affected bone metabolism, a cohort of F344 rats were fed every day with 40% reduced chow of the amount consumed by AD rats (continuous CR). Micro-CT scanning of proximal tibia again revealed the 3D BV decreased by this continuous CR for 6 months, as with the CR by alternate-day feeding (Fig. 2F), suggesting that reduced total calorie intake causes osteopenia with microstructural deterioration and fragility and that the effects of CR on bone takes place irrespectively of the methods employed for reducing food intake.

### *CR suppresses bone formation*

To examine whether the decreased bone mass after chronic CR was due to suppressed bone formation, accelerated bone resorption, or a combination of both mechanisms, histomorphometric analyses were performed at the tibial metaphysis. As shown in Fig. 3A, bone formation rate (BFR)/bone surface (BS) substantially decreased after 6 months of CR, with a reduction in mineral apposition rate (MAR), suggesting that bone formation, especially the mineralizing function of mature osteoblasts, is inhibited by CR. When CR mice (aged 9 months) were switched to AD feeding, the suppression of the BFR was reversed (Fig. 3B), indicating that the inhibitory effect of CR on bone formation is reversible.

The suppressive effect of CR on bone formation was observed as early as 1 month after the initiation of CR and consistently for at least 9 months (Fig. 3C). On the other hand, a consistent change in bone resorption was not observed; osteoclast surface (Oc.S)/BS increased transiently at 6 months of CR, whereas there was no significant change in the eroded surface (ES)/BS (Fig. 3A). Collectively, these data suggest that the decreased bone mass after chronic CR is mainly due to suppressed bone formation, although a transient increase in bone resorption may have helped enhance the effect.

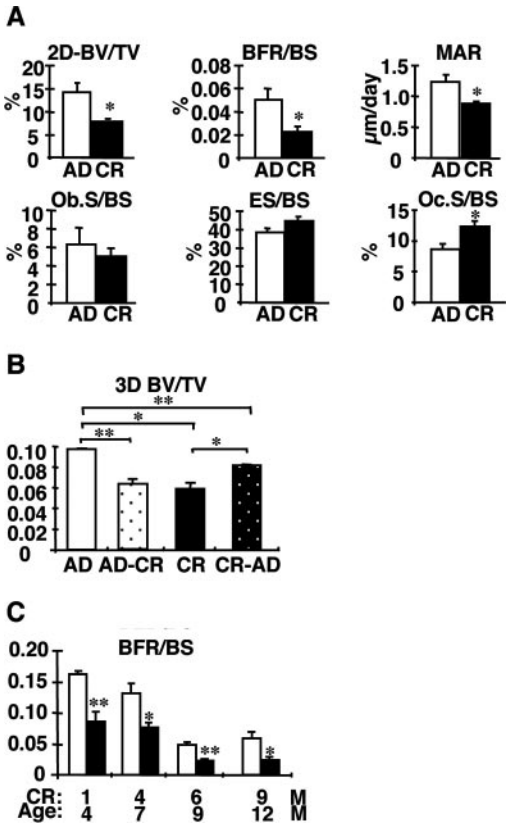


FIG. 3. CR causes suppression of bone formation with transiently elevated bone resorption. A, Results of histomorphometric analysis at the proximal tibiae of AD *vs.* CR mice after 6 months of CR. Data are normalized for the BS. *, $P < 0.05$ (n = 5). B, Reversibility of the effects of CR on bone mass. After 6 months of CR or AD, mice were switched to AD (CR-AD) or CR (AD-CR), respectively, for 1 month, and 3D BV/TV was determined at the proximal tibia by micro-CT. *, $P < 0.05$; **, $P < 0.01$ (n = 4). C, Continuous suppression of bone formation by CR. After cohorts of C57BL/6 male mice were raised under CR or AD feeding for the indicated times, the BFR (corrected for BS) was determined. The time scale is provided as both the time after the start of CR (*upper values*) and the age of the animals (*lower values*) in months (M). *, $P < 0.05$; **, $P < 0.01$ (n = 4–5).

### *CR does not suppress bone formation in db/db mice*

Biochemical markers of energy metabolism were assessed after 6 months of CR in the blood samples of rats due to the availability of a larger volume of samples. As shown in Fig. 4, serum albumin concentrations did not differ between the CR and AD groups, indicating that chronic CR did not induce malnutrition. There was no significant difference in serum calcium concentrations between the AD and CR groups (Fig. 4), indicating that CR did not affect systemic calcium homeostasis. Along with a 20–30% reduction in body weight (Fig. 1), CR was associated with a substantial decrease in adipose tissue, compared with the AD group (epididymal fat pad: 5.2 ± 0.6 g in CR *vs.* 11.3 ± 1.3 g in AD rats). Accordingly, serum leptin concentrations as well as insulin, IGF-I, and blood sugar levels were significantly reduced after long-term CR (Fig. 4). It is conceivable, therefore, that an altered signaling of these hormones may have affected bone metabolism under CR. In light of the emerging understanding that leptin links food intake and bone metabolism (14), we focused on leptin signaling and examined the effects of CR on bone in *db/db* mice lacking functional leptin receptors.

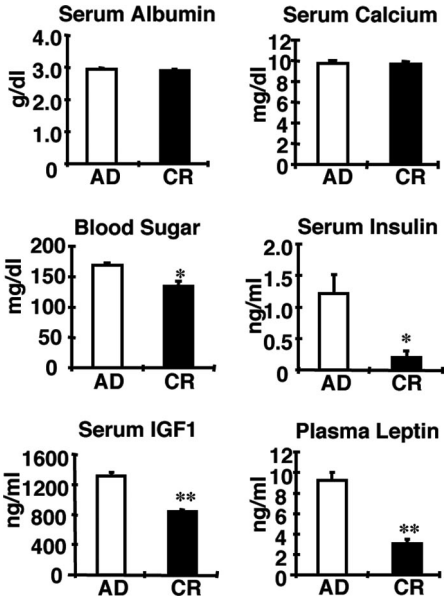


FIG. 4. Blood biochemical characteristics under long-term CR. The results of serum, whole blood, and plasma biochemistry in AD (*white bars*) *vs.* CR (*black bars*) rats is shown. Blood was collected at the age of 9 months (*i.e.* after 6 months of CR). *, $P < 0.05$; **, $P < 0.01$ (n = 5–7).

First, *db/db* mice were raised under AD or CR conditions, and trabecular bone at the tibial metaphysis was analyzed by micro-CT. As shown in Fig. 5A, CR for 4 months caused a substantial reduction in the 3D BV in control (*m+/m+*) mice, whereas CR failed to do so in *db/db* mice. In fact, CR caused a modest but significant increase in bone mass in *db/db* mice.

The results of histomorphometry at the proximal tibiae revealed that the BFR/BS decreased substantially after 4 months of CR in control *m+/m+* mice, whereas in *db/db* mice, suppression of bone formation by CR did not take place (Fig. 5C), suggesting that leptin signaling may be involved in the suppressive effect of CR on bone formation. Consistent with this notion are the results that the expression of neuromedin U (NMU), a mediator of the antiosteogenic action of leptin in the hypothalamus (15), was increased in the hypothalamus of CR mice in the face of reduced circulating leptin concentrations, compared with the AD group (Fig. 5D).

At baseline, the BV of *db/db* mice was lower than that of control *m+/m+* mice in the proximal tibia (Fig. 5A), whereas *db/db* mice exhibited a high bone mass phenotype in the lumbar vertebrae (Fig. 5B), as reported previously (16). CR caused a slight but significant reduction in BV in the lumbar vertebrae of control but not *db/db* mice (Fig. 5B).

### *Reduced BV by CR is mediated through increased sympathetic nervous system tone*

It has been demonstrated that the suppression of bone formation induced by leptin is mediated through an increased sympathetic nervous tone and signaling through the β2-adrenergic receptor expressed on osteoblasts (17). To examine the involvement of the sympathetic nervous system in the suppressive effect of CR on bone, the effect of blocking

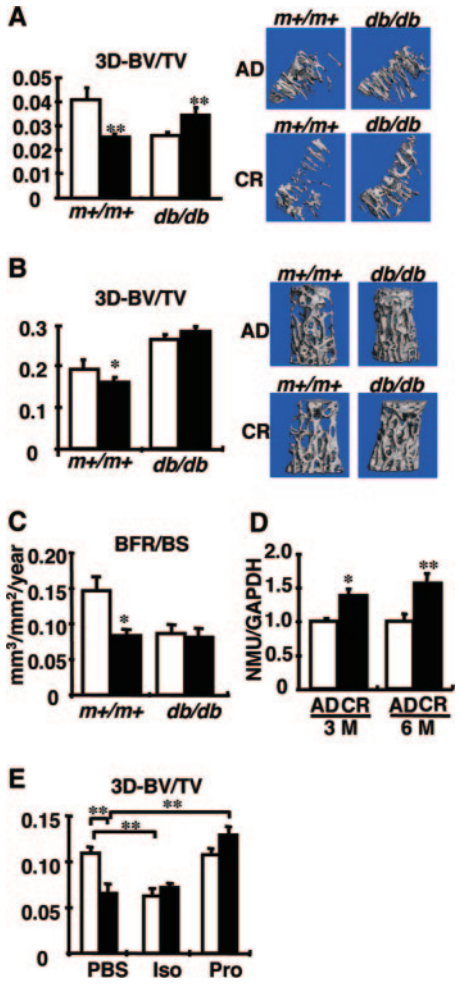


FIG. 5. CR reduces bone mass through leptin signaling and sympathetic nervous tone. A and B, 3D BV/TV was determined at the proximal tibiae (A) or second lumbar vertebrae (B) by micro-CT scanning in *db/db* mice on an AD (*white bar*) *vs.* CR (*black bar*) regimen for 4 months. *m+/m+* mice served as the control. * in B, $P < 0.05$ (n = 5); ** in A, $P < 0.01$ (n = 9–10). Representative micro-CT images are shown to the *right*. C, Suppression of bone formation by CR was not observed in *db/db* mice. BFR was determined at the proximal tibiae of control (*m+/m+*) and *db/db* mice under AD feeding (*white bars*) or CR (*black bars*) for 4 months. *, $P < 0.05$ (n = 4–5). D, Expression of NMU in the hypothalamus of CR *vs.* AD mice. RNA was extracted from the hypothalamus after CR for the indicated periods (in months, M) and subjected to quantitative RT-PCR. The ratio of NMU/GAPDH mRNA is shown. *, $P < 0.05$; **, $P < 0.01$ (n = 4). E, Involvement of the sympathetic nervous system. The decrease in bone mass by CR was prevented by propranolol (Pro), a β-blocker, whereas isoproterenol (Iso), a β-stimulant, reduced BV in AD mice. The 3D BV/TV was determined at the proximal tibiae by micro-CT after 6 months of CR (*black bars*). **, $P < 0.01$ (n = 3–5).

adrenergic tone on bone mass under the CR regimen was examined. When wild-type C57BL/6 mice were treated orally with propranolol, a β-adrenergic blocker, CR failed to cause a reduction in BV (Fig. 5E). Conversely, when mice were treated with isoproterenol, a β-stimulant, BV decreased, even in the AD group, down to the CR level (Fig. 5E). Treatment with isoproterenol did not cause a further reduction in BV in CR mice (Fig. 5E). These results are consistent with our concept that the reduced BV by CR is mediated through increased activity of the sympathetic nervous system.

*CR prevents age-related decline in bone mass*

We examined the time course of the effects of CR on 3D BV. After CR was initiated at the age of 3 months, there was a significant decrease in bone mass at 3 months of CR (*i.e.* at the age of 6 months), and a clear-cut reduction in BV was also observed after 6–9 months of CR (*i.e.* at the age of 9–12 months) (Fig. 6A). However, the effect of CR on BV became more and more obscure thereafter, and after 13–17 months of CR (*i.e.* at the age of 16–20 months), BV was indistinguishable between CR and AD mice (Fig. 6A), implying that CR mice are relatively resistant to age-related bone loss. In fact, after 24 months of CR (*i.e.* at the age of 27 months), CR mice

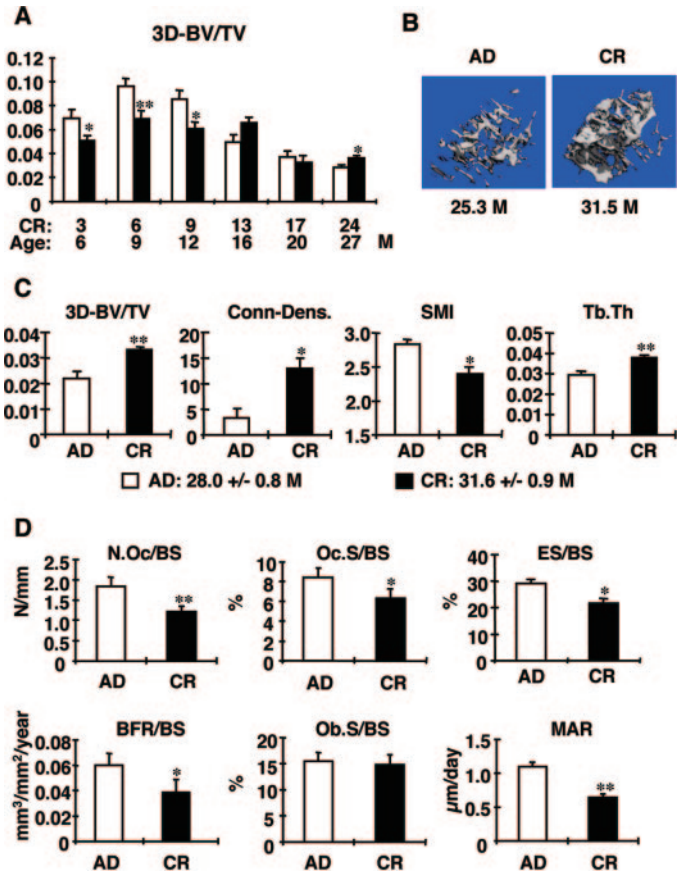


FIG. 6. CR protects against age-related bone loss by reducing bone turnover. A, Changes in the 3D BV fraction at the proximal tibiae of AD (*white bars*) *vs.* CR (*black bars*) mice, as determined by micro-CT, were followed at the indicated times. The time scale is provided as both the time after the start of CR (*upper values*) and the age of mice in months (*lower values*). *, $P < 0.05$; **, $P < 0.01$ (n = 4–8). B, Representative micro-CT images of trabecular bone in the proximal tibiae of AD *vs.* CR mice at natural death at the indicated age in months (M). C, Microstructural parameters derived from micro-CT analysis of trabecular bone at the proximal tibiae of life-long CR mice (*black bars*). The age at death is provided below in months (M) as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ (n = 6–8). D, Results of histomorphometric analysis of the proximal tibiae of AD (*white bars*) *vs.* CR (*black bars*) mice after 24 months of CR. N.Oc, Number of osteoclasts. Data are normalized for the BS. *, $P < 0.05$; **, $P < 0.01$ (n = 7–9).

exhibited a modestly but significantly higher bone mass than the AD control group (Fig. 6A).

To confirm this phenomenon and to see whether a further extension of the CR period would reverse the effect on bone mass, bone was collected from a cohort of C57BL/6 mice on life-long CR, either on the day of natural death or within 24 h of death. Because CR extends the maximal lifespan, the average age of CR mice at death was higher than that of the control AD group (31.6 ± 0.9 months in CR mice *vs.* 28.0 ± 0.8 months in AD group). As shown in Fig. 6, B and C, despite the fact that CR mice had attained lower bone mass as young adults than AD controls and were older at the time of death, aged CR mice maintained a significantly higher BV than AD group, suggesting that lifelong CR delays age-related bone loss. Detailed microstructure analysis indicates that the higher bone mass in CR mice was associated with increased Tb.Th and connectivity and decreased SMI (Fig. 6C), suggesting that CR maintains the thickness of trabeculae and their plate-like structures. The protective effects of lifelong CR on age-related bone loss and microstructural deterioration were also confirmed in F344 rats (Fig. 7).

Finally, to gain some insight into the mechanism by which CR countered age-related bone loss, histomorphometric analysis was performed at the tibiae at the age of 27 months (*i.e.* after 2 yr of CR). The results indicate that the number of osteoclasts (N.Oc/BS), the bone surface covered by osteoclasts (Oc.S/BS), and the ES/BS were all significantly reduced in CR mice (Fig. 6D). With respect to indices of bone formation, the osteoblast surface (Ob.S/BS) remained unchanged, whereas the MAR and BFR declined in CR mice, compared with the age-matched AD group (Fig. 6D). These data suggest that the higher bone turnover rate in aged AD animals is mitigated by CR and that CR counters the aging-related bone loss by reducing bone turnover. Thus, regulation of bone turnover appears to be a protective strategy deployed by CR against skeletal aging.

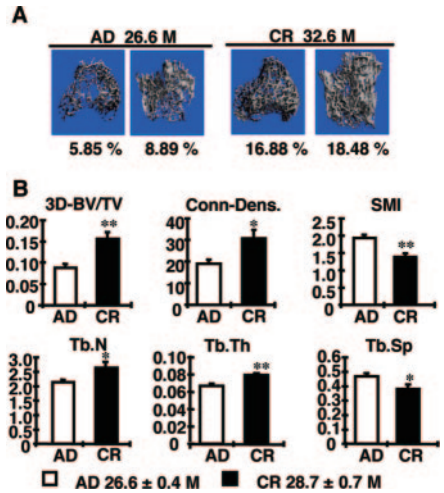


FIG. 7. CR protects against age-related bone loss and microstructural deterioration in F344 rats. A, Representative micro-CT images of trabecular bone at the proximal tibiae of AD *vs.* CR F344 rats at natural death at the indicated age in months (M). B, Microstructural parameters derived from micro-CT analysis of trabecular bone at the proximal tibiae of AD (*white bars*) *vs.* CR (*black bars*) F344 rats. The age at death is provided *below* in months (M) as mean ± SEM for each group. *, $P < 0.05$; **, $P < 0.01$ (n = 10–11).

## Discussion

Nutrition, especially the intake of calcium and vitamin D, is an important remedy for maintaining bone health. The present study demonstrates that under physiological conditions, the amount of total caloric intake *per se* has a profound impact on bone remodeling. It is widely recognized that the negative calcium balance seen in calcium/vitamin D deficiency causes secondary hyperparathyroidism with tonic PTH hypersecretion (18). Under these conditions, bone resorption and subsequently bone formation are stimulated, with a net balance such that the former exceeds the latter, resulting in bone loss and structural deterioration. Bone remodeling under chronic CR is clearly distinct from these catabolic changes in calcium/vitamin D deficiency and is characterized by persistent and profound suppression of bone formation, *i.e.* anti-anabolism. CR with relatively increased calcium content failed to reverse the reduction in bone mass, which lends further support to our conclusion that a reduction in caloric, not calcium intake, is responsible for the decreased bone formation and bone mass induced by CR.

Bone remodeling, performed by bone-resorbing osteoclasts and bone-building osteoblasts, functions under hormonal (19, 20), neuronal (21), immunological (22), and mechanical (23, 24) control. Recently, much attention has been focused on the central control of bone remodeling (14). Mice harboring genetic mutations in leptin signaling and the sympathetic nervous system have provided powerful tools in dissecting molecular pathways that link energy homeostasis to bone remodeling (16, 17). However, the physiological conditions under which the pathway operates have been elusive. Our data suggest that changes in leptin signaling may be involved in the suppression of bone formation and osteopenia after chronic CR.

The effects of leptin on bone metabolism appear to be complex and to depend on the site and type of bone analyzed. The leptin-deficient *ob/ob* mouse was originally reported to exhibit a high bone mass phenotype in the trabecular bone of vertebrae (16). In contrast, it has been shown that the bone mineral density of *ob/ob* mice was decreased in the femur with a reduced cortical thickness (25) and that continuous leptin infusion increased bone mineral density in *ob/ob* mice but not in wild-type mice (26). In addition, leptin treatment partially prevents the bone loss induced by ovariectomy in the trabecular bone of the proximal tibia in rats (27) and counters the inhibition of the longitudinal effects of calorie deprivation in young mice (28). Leptin can affect bone metabolism not only through the central nervous system (16) but also through a peripheral pathway by acting directly on the cells in bone (29, 30). The results that the suppressive effect of CR on bone is impaired in *db/db* mice in the current study are consistent with our view that the skeletal adaptation to CR involves leptin signaling, although there is no direct evidence for enhanced or reduced leptin signaling by CR. A recent study demonstrates that NMU-deficient mice exhibit high bone mass with increased bone formation and

are resistant to the antiosteogenic effects of leptin and isoproterenol, suggesting that NMU is a mediator of the antiosteogenic action of the leptin-sympathetic nervous system (15). Taken together with the findings that NMU expression is reduced in the leptin-deficient *ob/ob* mice (31) and that leptin stimulates the release of NMU from hypothalamic explants (32), the current results that the expression of NMU in the hypothalamus is significantly increased after CR in the face of reduced circulating leptin concentrations support the notion that the antiosteogenic action of leptin is increased under CR, which may be involved in the suppressive effect of CR on bone formation. We cannot, however, rule out the possibility that other factors, such as IGF-I, insulin, and GH, which are reduced during CR (33), are involved in the skeletal response or that the improvement of diabetes by CR may have modulated the bone phenotype of *db/db* mice.

The data show that treatment with propranolol blocked the suppressive effect of CR on bone. Taken together with the link between central leptin signaling and the peripheral sympathetic nervous system (17), one plausible hypothesis is that leptin signaling regulates bone metabolism in response to CR through increased activity of the sympathetic nervous system. However, the data cannot rule out the possibility that the adrenergic receptor responds to CR independently of leptin signaling.

Importantly, this study discloses another and unexpected aspect of CR, namely a protective effect against age-related bone loss during the latter half of life. The trabecular BV at the proximal tibia of C57BL/6 mice peaked at around 9 months of age and decreased progressively thereafter (Fig. 6A). Histomorphometric analysis of 1- *vs.* 2-yr-old mice suggested that the age-related bone loss is due to sustained osteoclastic bone resorption with insufficient bone formation (data not shown). Although CR animals had gained less bone during young adulthood, they maintained a higher bone mass after 27 months of age. This protection from skeletal aging is likely to be attained by reducing bone turnover, because reduced osteoclast number and activity with a reduced bone formation rate was observed in aged CR mice compared with the AD group. At present, there is no evidence for the involvement of leptin signaling in the slower age-related bone loss in the CR group, and more studies are required to identify which factor(s) specifically elicited by the CR regimen provides protection against skeletal aging by reducing bone turnover.

In conclusion, if the present results were to extrapolate to humans, it would follow that excessive dieting during young adulthood would be discouraged, whereas a mild reduction in calorie intake after middle age would be encouraged to help slow the aging of the skeleton. It remains to be determined whether CR during the latter half of life alone has a protective effect or a whether combination of AD and CR, *i.e.* AD feeding until 1 yr of age followed by CR thereafter, would be even more effective in maintaining skeletal health in rodents, both by increasing peak bone mass and by slowing the rate of age-related bone loss.

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The American Neuroendocrine Society is sponsoring a Neuroendocrine Workshop on Stress June 12-15, 2008, at the Embassy Suites Hotel in San Rafael, CA. This Workshop is just prior to the Endocrinology meetings. Major areas to be addressed include stress and epigenetics, neuroadaptation and plasticity induced by stress, and sex-related differences in stress responsivity. In addition, a session will celebrate Dr. Mary Dallman's contributions to the neuroendocrinology of stress on the occasion of her retirement from UCSF.

During the 2008 Workshop, Dr. Bruce McEwen will deliver a keynote address and a session will be devoted to new approaches for the study of stress neuroendocrinology. There will also be an open poster session for other scientific contributions.

Information for joining the society and the workshop can be found at www.neuroendocrine.org. Other inquires should be directed to Dr. Jim Koenig (jkoenig@mprc.umaryland.edu).