Bone and hormonal changes induced by skeletal unloading in the mature male rat

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Dehorary, Walter, Bernard P. Halloran, Daniel D. Bikle, Tracy Curren, Paul J. Kostenuik, Thomas J. Wronski, Ying Shen, Brian Rabkin, Abderrahman Bouraoui, and Emily Morey-Holton. Bone and hormonal changes induced by skeletal unloading in the mature male rat. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E62–E69, 1999.—To determine whether the rat hindlimb elevation model can be used to study the effects of spaceflight and loss of gravitational loading on bone in the adult animal, and to examine the effects of age on bone responsiveness to mechanical loading, we studied 6-mo-old rats subjected to hindlimb elevation for up to 5 wk. Loss of weight bearing in the adult induced a mild hypercalcemia, diminished serum 1,25-dihydroxyvitamin D, decreased vertebral bone mass, and blunted the otherwise normal increase in femoral mass associated with bone maturation. Unloading decreased osteoblast numbers and reduced periosteal and cancellous bone formation. However, there were no changes in bone resorption. Mineralizing surface, mineral apposition rate, and bone formation rate decreased during unloading. Our results demonstrate the utility of the adult rat hindlimb elevation model as a means of simulating the loss of gravitational loading on the skeleton, and they show that the effects of nonweight bearing are prolonged and have a greater relative effect on bone formation in the adult than in the young growing animal.

Spaceflight is accompanied by a decrease in bone mass in humans (25, 32, 35, 37). Urinary calcium increases early and tends to remain elevated throughout flight (29, 39). The serum concentration of 1,25-dihydroxyvitamin D [1,25(OH)2D], although elevated on the first day, decreases during flight (24). Serum parathyroid hormone (PTH) remains unchanged (24). On long-duration missions, calcium balance becomes negative and bone mineral deficits can reach as high as 1.4%/mo in the calcaneus (37).

Ground-based bed-rest studies in humans mimic these findings (1, 17, 18, 22, 26, 35). Loss of gravitational loading as effects by head-down antorthostatic (to induce the cephalad fluid shift encountered during spaceflight) or horizontal bed rest in normal adult volunteers induces selective loss of bone mineral in those segments of the skeleton that normally experience the greatest physical loads. Mineral apposition rate decreases and resorption surfaces increase (35). Intestinal calcium absorption decreases, and urinary and fecal calcium increases, resulting in a negative calcium balance (18). Serum ionized calcium tends to increase, PTH decreases or remains unchanged, and serum 1,25(OH)2D decreases (1, 18).

To study the influence of gravitational loading on bone and mineral metabolism, we developed a ground-based animal model in which the hindlimbs of young growing rats are unweighted (5, 7, 9, 42). This reduces mechanical loading on the rear limbs and produces a cephalad fluid shift similar to that encountered during spaceflight. In the young growing animal, hindlimb unloading decreases serum 1,25(OH)2D, reduces bone formation in the tibia, and induces a bone mineral deficit in unloaded regions of the skeleton, thus mimicking the effects of spaceflight on mineral metabolism. This model uses young growing rats, however, and its relevance for the study of gravitational loading in the adult is not clear. To examine the appropriateness of hindlimb elevation as a model for diminished mechanical loading in adults, and to determine the influence of age on bone responsiveness to skeletal unloading, we studied the effects of hindlimb unloading in adult (6-mo-old) rats and compared our results with previous skeletal unloading studies in young growing animals. The results suggest that hindlimb unloading in the adult rat closely resembles the effects of diminished gravitational loading and spaceflight on mineral metabolism in adult humans. The data also illustrate unique age-related differences in the responsiveness of bone and mineral metabolism to skeletal unloading.

MATERIALS AND METHODS

Animal protocols. Fifty-six 6-mo-old virgin male Sprague-Dawley rats weighing 400–450 g (Simonsen Laboratories, Gilroy, CA) were fed standard laboratory rat chow (Purina Rodent Diet 5012) containing 1.01% calcium and 0.74% phosphorus and were maintained on a 12:12-h light-dark cycle. After a 6-day equilibration period in the Animal Care Facility, the rats were divided into seven groups of eight animals each: a baseline control group and groups unloaded or pair-fed for 1, 3, and 5 wk. The study was conducted twice over the course of 12 mo to ensure that the results were reproducible.

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Skeletal unloading was achieved using the hindlimb elevation model (5, 42). In this model, the forelimbs remain weight bearing and thus act as an internal control. There is no increase in loading of the forelimbs in this model. The tension on the unloading apparatus is adjusted so that the angle between the floor and an imaginary horizontal line through the long axis of the rat is 30°. This ensures that loading on the forelimbs is maintained at a normal level (10). Normal levels of feeding, grooming, and activity were maintained throughout the experiment. The protocol for hindlimb elevation was approved by the Animal Care and Use Committee at National Aeronautics and Space Administration-Ames Research Center, where the experiments were conducted.

Animals were weighed on Monday and Friday of each week, and normally loaded control groups for the 1-, 3-, and 5-wk unloaded groups were pair-fed to maintain the average body weights between groups as equal as possible. To measure bone formation rate, the rats were injected subcutaneously with calcein (15 mg/kg) (1-, 3-, and 5-wk groups) at the time of hindlimb elevation (day 1) and with demeclocycline (15 mg/kg) on days 14 and 15 (3-wk group only) or on days 28 and 29 (5-wk group only).

On days 7, 21, and 35, control and unloaded rats in the 1-, 3-, and 5-wk groups were euthanized while under isoflurane anesthesia by exsanguination from the abdominal aorta. Blood was withdrawn into calcium-titrated heparinized syringes, and whole blood ionized calcium (Ca²⁺) and pH were immediately measured (≤60 s) using a calcium ion/pH analyzer (Ciba-Corning 234 Ca²⁺/pH analyzer, Ciba-Corning Diagnostics, Medfield, MA). Plasma was harvested from the remaining blood and stored at −80°C for determination of PTH and 1,25(OH)₂D₃ concentrations.

The soleus, gastrocnemius, and plantaris muscles and the thymus were dissected free of other tissues and weighed. The soleus muscle is important in maintaining posture under normal gravitational loading and, as such, should respond to loss of weight bearing. The gastrocnemius, a muscle activated during normal ambulation, would also be expected to respond to diminished loading. The plantaris, a nonpostural, fast-twitch muscle, would be expected to respond less to unloading and was used as a control. Thymus weight was measured as a means of assessing glucocorticoid excess. Chronic hypersecretion of glucocorticoids induces thymic atrophy (14).

The right tibia (cut into proximal and midshaft/distal regions), right and left humer, right femur, and lumbar vertebrae (L₁, L₂, and L₃) were removed, cleaned of adherent tissue, and processed for bone histomorphometry or fat-free weight.

Bone histomorphometry. Tibial and humeral diaphyseal segments were dehydrated, defatted in acetone followed by ether, and embedded undecalified in polyester casting resin (Chemco, San Leandro, CA). Cross sections (80 µm) of the embedded bones were cut using a Gillings Hamco thin sectioning machine (Rochester, NY), mounted on slides, and examined using fluorescence microscopy. The first section proximal to the complete detachment of the fibula from the tibia was analyzed, and this site is referred to as the tibiofibular junction (TFJ). Additional sections from a site 4 mm proximal to the TFJ (midshaft) and sections from the midshaft of the humerus were also cut and analyzed. The area of bone between the calcine and demeclocycline labels was determined using a modification of the NIH Image program and was divided by the time interval between administration of the labels to determine the periosteal bone formation rate (12).

The proximal tibia was shaved across the anterior face (to expose the marrow cavity and permit penetration of fixative) and placed in 10% neutral phosphate-buffered Formalin for 24 h. The bones were dehydrated in ethanol and embedded undecalified in methyl methacrylate (3). Longitudinal sections (4 and 8 µm) were cut with an AO Autocut DJ ung 1150 microtome and either stained (4 µm sections) according to the Von Kossa method with a tetrachrome counterstain (Poly sciences, Warrington, PA) or left unstained (8 µm sections) for fluorochrome-based measurements. Cancellous measurements were performed in an area beginning 1.0 mm distal to the growth plate-metaphyseal junction to exclude primary spongia and ending 4 mm distal to the growth plate. Two sections were examined from each animal, resulting in histomorphometric measurements along 30–40 mm of cancellous bone perimeter.

All cancellous bone measurements were performed using the Bioquant Bone Morphometry System (R & M Biometrics, Nashville, TN) as previously described (40, 41). Cancellous bone volume as a percentage of bone tissue area and osteoblast and osteoclast surfaces as percentages of total cancellous perimeter were measured in 4-µm-thick stained sections. Fluorochrome-based indexes of bone formation, including percentages of cancellous bone surfaces with double fluorochrome labels (mineralizing surfaces, MS) and mineral apposition rate (MAR) were measured in 8-µm-thick, unstained sections from the animals euthanized after 3 wk of skeletal unloading only. Labels in the animals euthanized at 1 wk were insufficiently separated, and the calcine label (given at the time of unloading) in the animals euthanized at 5 wk was too diffuse to permit measurement of cancellous MS or MAR in these groups of animals. Bone formation rate (total surface referent, BFR/BS) was calculated by multiplying MS by MAR (uncorrected for obliquity of the plane of section) (4).

Bone fat-free weight. The right femur, right humerus, and lumbar vertebrae (L₁, L₂, and L₃) were extracted in ethanol followed by diethyl ether by use of a Soxhlet apparatus, dried overnight at 100°C, and weighed to determine the fat-free weight.

Clinical laboratory analyses. The serum concentration of PTH was determined in duplicate using a commercially available immunoradiometric assay kit (Nichols Institute Diagnostics/Immunotopics, San Juan Capistrano, CA), which measures intact rat PTH (1–84). Intra-assay and interassay coefficients of variation for this assay are 6.9 and 12.4%, respectively, at a serum concentration of 19.6 pg/ml. The minimum detectable concentration (B/B₀ = 0.8) is 6 pg/ml. The serum concentration of 1,25(OH)₂D₃ was measured using the method of Reinhardt et al. (30).

Statistical analyses. For consistency, the results of experiment 1 are presented unless otherwise indicated. Data are reported as means ± SD. Statistical analysis was performed using Student's t-test, the Mann-Whitney rank-sum test for nonparametric populations, and two-way analysis of variance and the Newman-Keuls test where appropriate (Sigma-Stat, Jandel Scientific, San Rafael, CA). Linear regression analysis was used to estimate the slope of the relationship between bone fat-free weight and time of unloading for both the control and unloaded rats.

RESULTS

Results of the two experiments were virtually identical. Body weight was below basal levels (−5.7%, P < 0.05) after 1 wk in both control and unloaded animals (Fig. 1). By 3 and 5 wk, body weight had returned to the basal level in control but not unloaded animals. At 1, 3, and 5 wk, body weights tended to be lower in unloaded compared with normally loaded rats (448 ± 18 vs. 463 ± 18 g, unloaded vs. loaded, P < 0.05).
Thymus weight was not different in normally loaded and unloaded animals and did not change during the experiment (Fig. 2). Ca\textsuperscript{2+} was also not different in normally loaded and unloaded animals at all time points (Fig. 3). Whole blood pH was unaffected by skeletal unloading.

The serum concentration of 1,25(OH)\textsubscript{2}D remained unchanged in normally loaded animals but decreased sharply from 31 ± 8 to 13 ± 4 pg/ml (P < 0.001) after 1 wk of skeletal unloading (Fig. 4). By 3 and 5 wk, the serum concentration of 1,25(OH)\textsubscript{2}D in hindlimb-elevated animals had increased from its nadir toward normal but tended to remain below control levels even after 5 wk. The serum concentration of PTH remained constant and did not differ between loaded and unloaded animals at any time during the 5-wk experiment (data not shown). Overall mean serum PTH levels in control and unloaded animals were 36 ± 10 and 31 ± 4 pg/ml, respectively.

Muscle weights in the hindlimbs decreased significantly during skeletal unloading (Fig. 5). After 1 wk of hindlimb elevation, the weights of the soleus and gastrocnemius muscles were reduced by 35 and 16%, and by 5 wk by 48 and 27%, respectively, from control animals (P < 0.001). Plantaris weight was also signifi-
cantly lower (−10%, P < 0.002) in unloaded animals (data not shown).

Cortical bone changes in the skeletally unloaded animals were striking. Mean periosteal MARs between the time of unloading and 2 and 4 wk were reduced by 85 and 81%, respectively, in the unloaded compared with the normally loaded animals (data not shown). Mean periosteal BFR at the TFJ, tibial midshaft, and humeral midshaft are summarized in Fig. 6. Mean formation rates, measured between 0 and 2 wk of unloading in the animals hindlimb elevated for 3 wk and between 0 and 4 wk of unloading in the animals hindlimb elevated for 5 wk at both the TFJ and tibial midshaft, were reduced by ~80% (P < 0.001). No change in mean periosteal BFR was observed in the humerus, a normally loaded bone in our model. The results from experiment 2 were similar. Mean BFR at the TFJ during unloading decreased by −72 ± 6%.

Cancellous bone measurements from the proximal tibia are summarized in Fig. 7, A-C, and Table 1. The percent surface of cancellous bone occupied by osteoblasts decreased by 66% (P < 0.05) within 1 wk of skeletal unloading and remained below control levels for the duration of the experiment. In the second experiment, osteoblast numbers were decreased by −50, −28, and −48% at 1, 3, and 5 wk, respectively. Neither osteoclast surface nor cancellous bone volume, however, was significantly altered by skeletal unloading. Cancellous MS, MAR, and BFR decreased by 54, 33, and 69% (P < 0.05), respectively, during unloading (weeks 0–2) (Table 1).

Femoral, vertebral, and humeral fat-free weights were compared in normally loaded and unloaded animals using regression analysis (Fig. 8). Significant differences in the regression slopes between loaded and unloaded animals were observed for the femur and vertebrae but not the humerus. The deficits in bone mass induced by unloading, although small, were reproducible. At 5 wk, unloading produced deficits in vertebral mass of −13 ± 10 and −9.3 ± 4.3% (P < 0.05) in experiments 1 and 2, respectively. Hindlimb elevation decreased mass (vertebrae) or blunted the otherwise normal increase in mass (femur) associated with bone maturation in the rat.

DISCUSSION

Our data demonstrate that skeletal unloading by use of the adult rat hindlimb elevation model closely mimics the effects of spaceflight on bone and mineral metabolism in the human. The small but significant decrease in body weight (5.7%) during unloading is consistent with observations that some but not all astronauts lose weight during flight (34). The loss of weight, which occurs acutely after unloading and then
stabilizes, probably represents a combination of diminished appetite and loss of fluids as a consequence of the cephalad fluid shift induced by head-down tilt in our model (10). The reason for the acute loss of weight in the control animals after 1 wk is not clear but may reflect the influence of pair-feeding and diminished food intake in the unloaded animals.

Glucocorticoid excess is known to induce thymic atrophy in rats (14). Because thymus weight did not change in unloaded rats, stress and excessive glucocorticoid secretion are not likely to play a major role in the bone and mineral changes induced by hindlimb elevation. That stress and increased circulating corticosterone are not factors in the bone changes induced by hindlimb elevation is further supported by the observation that normally loaded bones in our model, such as the humerus, show no evidence of glucocorticoid excess, and direct measurements of serum corticosterone throughout the day in young hindlimb-elevated animals show no elevation in hormone levels (7).

Ca\textsuperscript{2+} was modestly elevated in hindlimb-unloaded animals, a finding consistent with previous spaceflight and ground-based immobilization studies in humans (1, 11, 15, 23, 24, 31). The mild hypercalcemia associated with loss of weight bearing frequently does not reach significance, may be transient, and probably arises from the abrupt decrease in bone formation and/or increase in bone resorption induced by unloading.

Spaceflight, as well as disuse induced by bed rest or immobilization, is routinely accompanied by a decrease in the serum concentration of 1,25(OH)\textsubscript{2}D (1, 8, 9, 23, 24, 31). In our adult rat model, hindlimb elevation induced a 52% decrease in serum 1,25(OH)\textsubscript{2}D after 1 wk of unloading. Thereafter, serum 1,25(OH)\textsubscript{2}D increased, reaching an apparent steady state by 3 wk. At 3 and 5 wk, serum 1,25(OH)\textsubscript{2}D remained below control levels, but the difference did not reach significance. The pattern in serum 1,25(OH)\textsubscript{2}D after hindlimb elevation

\begin{table}[h]
\centering
\begin{tabular}{lccc}
\hline
 & MS, % & MAR, µm/day & BFR/BS \\
\hline
Control & 16.2 ± 2.0 & 0.9 ± 0.2 & 14.8 ± 9.2 \\
Unloaded & 7.4 ± 4.1* & 0.6 ± 0.1* & 4.6 ± 3.1* \\
\hline
\end{tabular}
\caption{Fluorochrome-based indexes of bone formation, including percentages of cancellous bone surfaces with double fluorochrome labels (MS, MAR, and BFR/BS), in proximal tibia of normally loaded (control) and unloaded rats.}
\end{table}

Fig. 7. Cancellous bone volume (A), osteoblast surface (B), and osteoclast surface (C) in proximal tibia of unloaded and pair-fed control rats after 0 (basal), 1, 3, and 5 wk of hindlimb elevation. Data are reported as means ± SD, n = 8, and are analyzed using two-way analysis of variance and the Newman-Keuls test. *P < 0.05, unloaded vs. pair-fed control. **P < 0.05, wk 5 control compared with basal.
in the adult rat is virtually identical to that seen in other models of skeletal unloading and is similar to that previously observed in the young growing hindlimb-elevated animal (8, 9) between 0 and 2 wk of unloading. Previous studies in the young rat indicate that the decrease in serum 1,25(OH)₂D induced by skeletal unloading is a consequence of a decrease in synthesis of the hormone and not an increase in metabolic clearance rate (8). Whether the decrease in synthesis is linked to changes in renal hemodynamics (27), a decrease in the demand for calcium by the bone, the mild hypercalcemia associated with skeletal unloading, or other metabolic changes, is not clear.

Serum PTH did not change during unloading. This is consistent with the findings during spaceflight (24) but differs from the results in bed-rest studies (1, 31), in which serum PTH tends to decrease. Previous studies with the hindlimb elevation model in young growing animals also did not reveal a change in serum PTH (8). The absence of a consistent decrease in serum PTH in our model despite the modest increase in serum calcium is not clear. It is possible that basal serum PTH is almost maximally suppressed to begin with and cannot be further reduced, or that our assays are not sufficiently sensitive to detect the magnitude of change associated with unloading.

The decrements in soleus, gastrocnemius, and plantaris muscle weights after 5 wk of hindlimb elevation in the adult rat (48, 27, and 10%, respectively) are consistent with those reported by other investigators (20, 33). Changes in muscle mass in response to decreased use appear to occur more rapidly and reach a new steady state before changes in bone. Conceivably, part of the change in bone mass is due to the reduction in muscle mass.

MS, MAR, and BFR on the periosteal surface decreased by as much as 80% during unloading. These same parameters in the humerus, a normally loaded bone in our model, were unaffected by hindlimb elevation, suggesting that the mechanisms effecting bone loss are primarily local in origin. The decreases in MS and MAR are consistent with the observed decrease in osteoblast number and also suggest a decrease in bone-forming activity per cell. Similar changes in cancellous bone were observed, along with a decrease in fluorochrome-labeled surface. These data clearly demonstrate that skeletal unloading effected by hindlimb elevation can decrease both osteoblast number and activity.

Osteoclast surface did not change significantly during unloading, suggesting that the decrease in bone mass induced by hindlimb elevation is primarily a consequence of a decrease in bone formation. However, it is possible that unloading induced an increase in osteoclastic activity (without a change in cell number). Indeed, previous studies using the taped-hindlimb rat model to effect skeletal disuse indicate that eroded surfaces can increase during unloading (20). Furthermore, spaceflight data indicate that urinary hydroxyproline is increased during flight, suggesting that bone resorption can increase during loss of gravitational loading (16, 28).
That cancellous bone volume did not decrease significantly during 5 wk of unloading despite a decrease in bone formation may be a consequence of the relatively large bone mass to begin with and the relatively short duration of our experiment. In long-term studies of hindlimb taping, cancellous bone volume has been shown to decrease and reach a new steady state after ~20 wk (20). The total deficit approaches 60%. Although cancellous bone volume did not decrease, total bone mass (fat-free weight) in unloaded skeletal regions (femur, lumbar vertebrae) was less in the hindlimb-elevated animals, a consequence presumably of the dramatic decrease in cortical bone formation.

The bone changes induced by hindlimb elevation in our adult rat model of skeletal unloading are similar to the bone changes associated with bed rest in adult humans (1, 22, 26, 35) and limb immobilization in adult animals (2, 13, 19–21). They also resemble the bone changes induced by hindlimb elevation in young growing rats but with a few notable exceptions (5–7, 42). In the adult, unloading is associated with a dramatic decrease in periosteal bone formation rate (~80%), a sustained decrease in the cancellous osteoblast population, and a continued loss of bone through ~5 wk. Indeed, the bone deficit probably continues to accumulate through 20 wk before reaching a new steady state, after which bone histomorphometric parameters return to normal (20). In the young growing rat, the decrease in periosteal formation is less (~40%). Osteoblast surface decreases initially in the young growing rat but by 2 wk returns to normal. MS, MAR, and BFR also return to normal by 2 wk in the young rat, and the deficit in bone mineral reaches a steady state. In the growing animal, bone formation appears to be driven largely by growth of the animal, whereas in the adult, the relative rate of bone formation is more dependent on mechanical loading. The effects of unloading on bone in the adult are also prolonged. In both aged models, however, osteoblast surfaces are not significantly affected by unloading, suggesting that bone resorption in the rat is relatively insensitive to weight bearing, at least under the experimental conditions used in our studies.

In summary, our results demonstrate the utility of the adult rat hindlimb elevation model as a means of simulating both the loss of gravitational loading on the skeleton and the cephalad fluid shift induced in adult humans by spaceflight. They also show that the effects of nonweight bearing are prolonged and have a greater relative effect on bone formation in the adult than in the young growing animal.

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