Effects of disrupted β1-integrin function on the skeletal response to short-term hindlimb unloading in mice

U. T. Iwaniec,1 T. J. Wronski,1 D. Amblard,2 Y. Nishimura,2 M. C. H. van der Meulen,3 C. E. Wade,2 M. A. Bourgeois,1 C. D. Damsky,4 and R. K. Globus2,4

1Department of Physiological Sciences, University of Florida, Gainesville, Florida; 2Life Sciences Division, National Aeronautics and Space Administration-Ames Research Center, Moffett Field, California; 3Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, New York; and 4Department of Stomatology, University of California, San Francisco, California

Submitted 2 July 2004; accepted in final form 24 September 2004

Bone loss is a potentially serious consequence of long-term spaceflight. This phenomenon was first documented in Skylab astronauts subjected to 1–3 mo of weightlessness (40). More recently, long-term occupants of the Mir and International Space Stations also exhibited osteopenic changes (29, 50). In view of obvious limits to human experimentation, animal models have been employed for more detailed studies of the skeletal effects of spaceflight. Growing rats placed in orbit for 1–3 wk were characterized by a pronounced inhibition of periosteal bone formation (35, 54, 55) and cancellous osteopenia associated primarily with decreased bone formation (26, 47, 49, 56).

The limited opportunities for rodent experiments in space emphasized the need for the development of a ground-based model for spaceflight. Fortunately, hindlimb unloading (HU) of rodents via tail suspension has been shown to mimic important aspects of weightlessness (36). Similar to spaceflight, HU of rodents induces an inhibition of periosteal bone formation (5, 8, 14, 20) as well as cancellous bone loss and a decline in osteoblast numbers and activity (1, 9, 13, 14, 42, 52). Although these skeletal responses to mechanical unloading have been studied in detail at the tissue level, the underlying molecular mechanisms by which bone cells sense and respond to changes in mechanical loads remain obscure. However, recent findings implicate integrins as possible mediators for the response of bone to mechanical forces.

Integrins are a major class of cell surface receptors responsible for cell adhesion to extracellular matrix proteins (12, 31). They also mediate critical functions such as migration, differentiation, and mechanotransduction in cultured cells (12, 21, 22, 28, 31). Osteoblasts, the bone-forming cells, express β1-integrin on their cell surface (19), which binds to major proteins in the extracellular matrix of bone, such as type I collagen and fibronectin (6). In cultured osteoblast-like cells, interference with the binding of β1-integrin to collagen and fibronectin impairs cell differentiation (27, 38), mechanical stimulation causes redistribution of β1-integrin at the cell surface (11), and antibodies to β1-integrin inhibit mechanosensitive ion channel activity (43). These in vitro findings support the hypothesis that β1-integrin may mediate the skeletal effects of altered mechanical loading.

One strategy for testing this hypothesis in vivo is to perform studies with genetically modified mice. Deletion of β1-integrin by homologous recombination results in embryonic lethality (46). Therefore, transgenic mice were generated to express a β1-integrin receptor with dominant negative activity targeted to mature osteoblasts and osteocytes (58). These mice were found to have a relatively mild skeletal phenotype, but an abnormal scaling of bone size, morphology, and strength to body size was detected during growth (18, 58). This finding suggested that integrin signaling influences the adaptation of skeletal
mass, structure, and strength to the mechanical loads generated by weight bearing. Elucidation of the mechanism by which bone cells sense changes in mechanical forces may contribute to a rationale for the design of molecular-based therapies for disuse osteoporosis. The purpose of the present study was to determine the role of $\beta_1$-integrin in the acute skeletal response to HU by utilizing transgenic mice with altered integrin function.

**MATERIALS AND METHODS**

DBA/2 × C56BL/6 F1 mice were used to generate wild-type (WT) and transgenic (TG) lines as previously described (58). Expression of a $\beta_1$-integrin fragment consisting of the transmembrane and cytoplasmic domains was driven by the rat osteocalcin promoter, which targeted the transgene to mature osteoblasts (3). Confirmation of transgene expression was achieved by RT-PCR of mRNA extracted from long bones and calvariae, by Western blot analysis of protein lysates from long bones, and by immunostaining for the epitope tag (18). Furthermore, cultured osteocyte-like cells from TG mice were characterized by impaired adhesion to collagen I, a ligand for $\beta_1$-integrin (18).

The experimental animals were female WT and TG mice that were 63 days of age at the beginning of the study. The experiment was performed in relatively young mice to avoid the complicating variable of age-related cancellous osteopenia in the long bones of older mice. WT and TG mice were subjected to HU by standard tail suspension techniques (37). Other groups of WT and TG mice were housed individually in cages of the same size as cages for HU mice, but these control animals ambulated normally. Each of the 4 groups of mice had a sample size of 10. All mice were fed standard mouse chow (Picolab Mouse Diet 20, Purina, St. Louis, MO). The food consumption of the control mice was matched to the mean daily food intake of the HU mice (pair feeding) to minimize differences in body weight between groups. Each mouse was injected subcutaneously at 6 and 2 days before euthanasia with calcium at a dose of 15 mg/kg body wt to label actively mineralizing bone surfaces. The duration of HU was chosen to be 7 days because previous studies have shown that bone formation is inhibited transiently by HU and returns to normal by 2 wk of HU (1, 17). At the end of the 7-day period, all mice were anesthetized by isoflurane inhalation and subjected to cardiac puncture for collection of blood samples within 2–3 min of exposure to the anesthetic agent. The mice were then euthanized by cervical dislocation. All procedures were approved by the Institutional Animal Care and Use Committee at National Aeronautics and Space Administration-Ames Research Center (Moffett Field, CA).

Plasma samples were stored at −80°C for future analyses. The right soleus muscle was carefully dissected free from other leg muscles and weighed with a Sartorius balance (model BA1105, Goettingen, Germany). The left femur was wrapped in saline-soaked gauze and stored at −20°C for biomechanical testing. The distal half of the right femur and the first and second lumbar vertebrae were placed in 10% phosphate-buffered formalin for 24 h and then stored in 70% ethanol at 4°C for bone histological processing.

Plasma corticosterone levels were measured in duplicate with a radioimmunoassay specific for rats and mice (ICN Biomedical, Costa Mesa, CA). Inter- and intra-assay coefficients of variation were 9.0 ± 2.2% and 4.0 ± 1.1%, respectively.

The right tibia and humerus from each mouse were dried for 24 h at 100°C under vacuum (model 5851, National Appliance) and then weighed to measure dry mass. The bone samples were burned in a muffle furnace (Sybron/Thermolyne, Dubuque, IA) at 500°C for 6 h and then weighed to measure ash mass. Ash fraction was calculated as ash mass/dry mass.

The left tibia from each mouse was microradiographed in the frontal plane with a Faxitron (Hewlett Packard 43805N, Faxitron X-Ray, Wheeling, IL) for 30 s at 35 kV. These microradiographs were then used for measurements of the proximal tibial curvature as previously described (7, 18, 34). Briefly, a chord line was drawn along the length of each bone, bisecting lines drawn at the epiphyses. A line perpendicular to the chord line was drawn at the point of maximum deflection on the tibial crest. This line extended from the midpoint of the tibia to the chord line. Tibial curvature was defined as the ratio between the length of this perpendicular line to the length of the chord line between the epiphyses. This calculation for curvature normalizes for different bone sizes (7, 34).

The right distal femur and first and second lumbar vertebral bodies from each mouse were dehydrated in graded ethanol solutions and xylene and then embedded undecalified in modified methyl methacrylate (4). These bone samples were sectioned longitudinally with Leica/Jung 2065 and 2165 microtomes at indicated thicknesses of 4 and 8 μm. The 4-μm-thick sections were stained according to the Von Kossa method with a tetrachrome counterstain (Polysciences, War- rington, PA), whereas the 8-μm-thick sections remained unstained for collection of fluorochrome-based data under UV illumination.

Bone histomorphometric measurements were performed in cancellous bone tissue of the distal femoral metaphysis in a sample area beginning 0.5 mm proximal to the growth plate-metaphyseal junction, which excluded the primary spongiosa. Bone measurements were performed in two sections per animal with a Bioquant Bone Morphometry System (R&M Biometrics, Nashville, TN) and an Os- teoMeasure System (Osteometrics, Atlanta, GA). Cancellous bone volume, as a percentage of bone tissue area, and osteoblast and osteoclast surfaces, as percentages of total cancellous perimeter, were measured in the stained sections at magnifications of ×20 and ×200, respectively. Fluorochrome-based indexes of bone formation, including the percentage of cancellous bone perimeter with a double fluorochrome label (mineralizing surface) and mineral apposition rate, were measured at a magnification of ×200 in the thicker, unstained sections. In addition, bone formation rate (total surface referent) was calculated by multiplying mineralizing surface by mineral apposition rate (16).

Some bone histomorphometric measurements were also performed in cancellous bone tissue of the lumbar vertebral body within a sample area consisting of secondary spongiosa at distances >0.5 mm from the cranial and caudal growth plates. Cancellous bone volume and mineral apposition rate were measured in two vertebral sections per animal as described above. The lack of HU-induced changes in these variables in mice of both genotypes (see below) precluded additional histomorphometric analysis at this sample site.

The whole bone strength of the left femur was evaluated in torsion as previously described (48). The proximal and distal ends of the femur were embedded in polymethylmethacrylate. The gauge length (distance between potted surfaces) was measured before testing. The femur was then loaded to failure at 1%/s. Torque and angular displacement were recorded at 10 Hz during each test. The failure torque (N-mm) and torsional stiffness were calculated from torsional load (N-mm) and displacement (degrees). Torsional stiffness is a composite measure reflecting material (shear modulus) and geometric properties (torsional constant).

Data are expressed as means ± SE. The statistical analysis consisted of a two-way ANOVA with the factors of genotype (WT or TG) and loading status (control or HU). P values of <0.05 were considered to be significant.

**RESULTS**

Mean values for body weight, serum corticosterone, and soleus mass for the four groups of mice are listed in Table 1. A significant difference in initial body weight was observed due to genotype at the beginning of the study. In addition, final body weight was affected by both genotype and loading status. TG mice weighed 8–10% less than WT mice regardless of loading status. The pair feeding protocol did not result in
equivalent body weights for normally loaded and unloaded mice because 1 wk of HU induced weight loss of 8–10% in both genotypes.

Serum corticosterone was affected by loading status but not by genotype. HU induced 120 and 25% increases in serum levels of corticosterone in WT and TG mice, respectively.

Soleus mass was not affected by genotype. However, WT and TG mice subjected to HU exhibited a decrease of nearly 20% in soleus mass compared with normally loaded WT and TG mice.

Mean values for bone mass, curvature, strength, and stiffness for the four groups of mice are shown in Table 2. Tibial ash fraction was not affected by genotype, but it decreased as a consequence of HU in both WT and TG mice. In contrast, humeral ash fraction was significantly lower in TG mice compared with WT mice regardless of loading status, but it was not significantly affected by HU. Proximal tibial curvature was affected by both genotype and loading status. TG mice were characterized by lower tibial curvature compared with WT mice, and this variable decreased in response to HU in mice of both genotypes. Femoral failure torque and torsional stiffness were affected by genotype but not loading status. TG mice exhibited lower bone strength and stiffness compared with WT mice regardless of loading status.

Bone histomorphometric data from the distal femoral metaphysis are shown in Fig. 1. Significant differences in cancellous bone volume (Fig. 1A) were observed according to both genotype and loading status. TG mice were characterized by lower cancellous bone mass regardless of loading status, and HU induced cancellous bone loss regardless of genotype (Fig. 2).

Osteoclast surface (Fig. 1B) was significantly affected by loading status. The observed increase in this index of bone resorption was greater in unloaded TG mice (92%) than in unloaded WT mice (52%). In contrast, osteoblast surface (Fig. 1C), a static index of bone formation, was not significantly affected by genotype or loading status.

Cancellous bone formation rate (Fig. 1D) was significantly affected by loading status but not genotype. Both WT and TG mice exhibited a decline in bone formation rate in response to 1 wk of HU. This adverse effect of HU was due primarily to a decrease in mineral apposition rate, which was significantly affected by loading status but not genotype. For example, the mean values for unloaded and normally loaded WT mice are 2.6 ± 0.3 and 3.1 ± 0.4 μm/day, respectively. This index of osteoblast activity also tended to be lower in unloaded TG mice compared with normally loaded TG mice (2.7 ± 0.5 vs. 3.0 ± 0.4 μm/day). Mineralizing surface (data not shown) was not affected by genotype or loading status.

The lumbar vertebral body was found to have lower cancellous bone volume in TG mice compared with WT mice, but it did not exhibit cancellous bone loss in response to HU in both genotypes (Table 3). Vertebral mineral apposition rate was similar in the four groups of mice with no significant differences due to genotype or loading status.

**DISCUSSION**

The present study suggests that disruption of integrin function in mature osteoblasts does not have a major effect on cancellous bone loss induced by short-term HU in growing mice. Although the skeletal phenotype of normally loaded TG mice was characterized by lower cancellous bone mass and proximal tibial curvature compared with normally loaded WT mice, both genotypes exhibited cancellous bone loss and decreased tibial curvature after only 1 wk of skeletal unloading. Furthermore, the observed loss of cancellous bone appeared to be due to a combination of increased bone resorption and decreased bone formation in both genotypes. Therefore, the adverse effects of skeletal unloading were, for the most part, similar in WT and TG mice. Nevertheless, there was some indication of an altered skeletal response to HU in TG mice. Osteoelastic surface, a well-established index of bone resorption, was increased to a greater extent in unloaded TG mice com-

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**Table 1. Effects of genotype and loading status on body weight, serum corticosterone, and soleus muscle mass**

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT CT</th>
<th>WT HU</th>
<th>TG CT</th>
<th>TG HU</th>
<th>ANOVA Effects (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight, g</td>
<td>19.5±0.4</td>
<td>19.4±0.5</td>
<td>17.3±0.4</td>
<td>17.4±0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>20.5±0.4</td>
<td>18.7±0.6</td>
<td>18.8±0.3</td>
<td>16.8±0.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serum corticosterone, mg/dl</td>
<td>106.7±29.2</td>
<td>235.5±31.1</td>
<td>124.9±18.2</td>
<td>155.6±22.7</td>
<td>NS (0.24) 0.0037 NS (0.06)</td>
</tr>
<tr>
<td>Soleus muscle mass, mg</td>
<td>5.7±0.4</td>
<td>4.7±0.4</td>
<td>5.0±0.3</td>
<td>4.1±0.5</td>
<td>NS (0.15) 0.0296 NS (0.82)</td>
</tr>
</tbody>
</table>

Values are means ± SE. WT CT, wild-type control; WT HU, wild-type hindlimb unloaded; TG CT, transgenic control; TG HU, transgenic hindlimb unloaded; NS, not significant.

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**Table 2. Effects of genotype and loading status on bone mass, curvature, strength, and stiffness**

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT CT</th>
<th>WT HU</th>
<th>TG CT</th>
<th>TG HU</th>
<th>ANOVA Effects (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tibial ash fraction</td>
<td>0.585±0.004</td>
<td>0.572±0.008</td>
<td>0.577±0.004</td>
<td>0.565±0.004</td>
<td>NS (0.17) 0.0165 NS (0.94)</td>
</tr>
<tr>
<td>Humeral ash fraction</td>
<td>0.593±0.005</td>
<td>0.579±0.006</td>
<td>0.576±0.008</td>
<td>0.567±0.006</td>
<td>0.0307 NS (0.09) NS (0.69)</td>
</tr>
<tr>
<td>Tibial curvature, %</td>
<td>7.6±0.3</td>
<td>6.7±0.2</td>
<td>6.1±0.3</td>
<td>5.6±0.2</td>
<td>&lt;0.0001          NS (0.45)</td>
</tr>
<tr>
<td>Femoral failure torque, N-mm</td>
<td>21.5±1.4</td>
<td>22.8±0.8</td>
<td>17.3±0.8</td>
<td>18.5±0.8</td>
<td>&lt;0.0001 NS (0.21) NS (0.93)</td>
</tr>
<tr>
<td>Femoral torsional stiffness, N/rad</td>
<td>770.9±63.3</td>
<td>865.3±76.5</td>
<td>595.6±53.0</td>
<td>659.7±53.4</td>
<td>0.0047 NS (0.21) NS (0.81)</td>
</tr>
</tbody>
</table>

Values are means ± SE.
pared with unloaded WT mice. In contrast, the inhibitory effects of HU on cancellous bone formation rate did not appear to be affected by genotype. The minimal influence of the transgene on the response of bone formation to HU may be due to the fact that β1-integrin function appears to be inhibited, but not abrogated, in mature bone cells from TG mice (18). It is also possible that β1-integrin may be important for sensing mechanical loads in bone but not as important for sensing lack of mechanical loading.

The significant interaction of genotype and load on osteoclast surface is somewhat surprising in that inhibition of β1-integrin function was expected to affect primarily bone formation rather than bone resorption. However, a recent in vitro study revealed a relationship between β1-integrin signaling by osteoblasts, receptor activator of nuclear factor-κB ligand (RANKL) expression by these cells, and osteoclast formation (39). Therefore, β1-integrin signaling may be important for communication between osteoblasts and osteoclasts and, consequently, the regulation of bone resorption by osteoblasts, which may explain, at least in part, the greater increase in bone resorption in unloaded TG mice compared with unloaded WT mice.

HU has been shown previously to induce an increase in serum corticosterone in mice (2, 15). In the current study, serum levels of this hormone were also found to be increased in both WT and TG mice subjected to HU. This finding may be considered a complicating variable because corticosteroid excess has adverse skeletal effects, some of which are similar to those of skeletal unloading. For example, exogenous treatment with corticosteroids is known to inhibit bone formation in mice and rats (30, 32, 44, 53). However, these hormones have also been reported to inhibit bone resorption in rodents (30, 44, 53), which is not consistent with our finding of increased bone resorption in unloaded WT and TG mice. Furthermore, corticosteroid excess would be expected to induce systemic skeletal changes. In the current study, cancellous bone volume and mineral apposition rate, an index of osteoblast activity, were significantly decreased in unloaded mice with high serum corticosterone levels in the distal femur but not in the lumbar vertebral body. During spaceflight, bone changes in flight rats with adrenal hypertrophy were not found to differ from those of adrenalectomized flight rats with serum corticosterone maintained at physiological levels (56). Therefore, corticosteroid excess does not appear to play a major role in the pathogenesis of the adverse skeletal effects of actual and simulated spaceflight. Nevertheless, the possibility that mice may have a greater stress response to HU than rats and, consequently, a greater contribution of corticosteroid excess to their bone changes cannot be eliminated.

The skeletal phenotype of TG mice with altered integrin function at 12–35 days of age was originally reported to include increased cortical porosity of long bones, thinner parietal bones in the skull, and decreased bone formation at the endocortical surface (58). However, no differences in cancel-
Fig. 2. Histological sections of the distal femur from CT (A) and HU (B) wild-type mice and CT (C) and HU (D) transgenic mice. Note the decreased amount of black-stained cancellous bone in the metaphysis of the HU wild-type and transgenic mice compared with their respective CT wild-type and transgenic mice. Note also that the CT transgenic mouse has less cancellous bone than the CT wild-type mouse. Von Kossa and tetrachrome stain, magnification ×20.

Table 3. Bone histomorphometric variables in cancellous bone tissue of the lumbar vertebral body

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>ANOVA Effects (P Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT CT</td>
<td>WT HU</td>
</tr>
<tr>
<td>Cancellous bone volume, %</td>
<td>11.1 ± 0.6</td>
<td>10.5 ± 0.8</td>
</tr>
<tr>
<td>Mineral apposition rate, μm/day</td>
<td>2.2 ± 0.06</td>
<td>2.3 ± 0.07</td>
</tr>
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</table>

Values are means ± SE.
lous bone mass and bone formation were observed in TG mice compared with WT mice at this early stage of skeletal development. A more recent study showed that, in older mice (90 days of age), tibial dry mass, ash mass, and femoral strength in torsion, when normalized to body mass, were decreased in TG mice compared with WT mice (18). Furthermore, these TG mice had lower proximal tibial curvature than WT mice at 35–365 days of age. However, cancellous bone volume in the distal femur was not affected by genotype. In the current study, TG mice were characterized at 70 days of age by lower tibial curvature and decreased cancellous bone mass even though these animals had normal levels of cancellous bone formation. A possible explanation for the difference in cancellous bone mass in TG mice in the previous (18, 58) and current studies may be genetic drift because the TG mouse lines are not inbred. Nevertheless, despite low cancellous bone mass at the beginning of the study, TG mice with altered integrin function still lost cancellous bone as a consequence of HU.

Muscle atrophy and cancellous bone loss have been consistent findings in the unloaded hindlimbs of rats. With increased use of genetically modified mice for biomedical research, it is interesting to compare the musculoskeletal effects of hindlimb unloading in the two rodent species. In the current study, atrophy of the soleus muscle was documented to confirm the occurrence of disuse in mice subjected to HU. This finding is consistent with previous reports of atrophied soleus muscles in the unloaded hindlimbs of mice and rats (10, 33, 37). The consensus of bone histromorphic analyses of rats subjected to HU is that cancellous bone loss in these animals is due primarily to decreased bone formation (9, 13, 14, 51). Osteoclast surface is most often found to be unaffected by HU in rats (9, 13, 14), although some investigators have reported an increase in this index of bone resorption (51). In contrast, with few exceptions (1), HU in mice has nearly always been reported to stimulate bone resorption as well as inhibit bone formation in cancellous bone (24, 25, 41, 42, 52). Therefore, these previous findings and the results of the present study indicate that increased bone resorption is a stronger component of the skeletal response to mechanical unloading in mice than in rats. The question arises as to whether the observed stimulation of bone resorption in the unloaded hindlimbs of mice is consistent with the skeletal effects of actual and simulated spaceflight in humans. Although results from human studies are often limited by small sample sizes and reliance on noninvasive techniques, the consensus of these studies is that lack of mechanical loading stimulates bone resorption and inhibits bone formation in humans subjected to spaceflight and prolonged bed rest (23, 45, 57). Therefore, disuse appears to induce similar bone changes in mice and humans.

In summary, the current study suggests that short-term skeletal unloading induces cancellous bone loss in growing mice despite altered integrin function in osteoblasts. The observed loss of cancellous bone was found to be associated with a combination of increased bone resorption and decreased bone formation regardless of genotype. Although disruption of $\beta_1$-integrin in mature cells of the osteoblast lineage may enhance the osteoclastic response to mechanical unloading, it does not appear to have a major effect on the development of cancellous osteopenia in mice during the early stages of HU.

ACKNOWLEDGMENTS

The authors are grateful to Dongxia Yuan and Laura Donovan from the University of Florida and Nathan Netaervali from Cornell University for technical assistance. We thank Dr. Emily Morey-Holton from National Aeronautics and Space Administration-Ames Research Center for helpful discussions.

GRANTS

This research was supported by National Aeronautics and Space Administration Grant 99-HEDS-062 and National Institute of Dental Research Grant P60 DE-13058.

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