Insulin-like growth factor I stimulates recovery of bone lost after a period of skeletal unloading

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SKELETAL UNLOADING INDUCES osteopenia (3, 4, 10, 19, 26, 29 –31). The progressive loss of bone that occurs during unloading is largely a consequence of a decrease in osteoblast recruitment and activity (8, 9, 11, 18, 30).

Insulin-like growth factor (IGF)-I, a potent anabolic agent for bone, promotes osteoprogenitor proliferation, osteoblast formation, and osteoblast survival, and stimulates bone formation (6, 7, 25). The decline in osteoblast activity and loss of bone induced by skeletal unloading are associated with a loss of bone responsiveness to the anabolic actions of IGF-I (5, 12, 20, 22). Skeletal unloading using the rat hindlimb elevation model impairs IGF-I receptor activation, disrupts IGF-I signal transduction, and inhibits osteoblast formation and activity (5, 20, 22).

Although skeletal unloading and the mechanisms responsible for loss of bone have been well studied, what happens to bone when the skeleton is reloaded after a period of unloading has received less attention. Bone lost during spaceflight or in models mimicking the weightlessness of spaceflight is at least partially recovered after return to normal loading or weight bearing, but the process is extremely slow (1, 2, 14, 15, 23, 28). Complete recovery may never occur. In spaceflight missions of 14-day duration, skeletal mass in the rat decreases. Fourteen days after return to normal ambulation, bone formation is reported to be increased, but total recovery of bone mass has not yet occurred (14, 32). In human bed rest studies, bone is also lost, and recovery may require 6 mo or more of normal ambulation (16). These studies show that the bone lost during periods of skeletal unloading may be restored, at least in part, with return to normal weight bearing but the process is slow, and the risk of fracture during this period may be increased.

Based on the observation that skeletal unloading induces resistance to IGF-I, we hypothesized that reloading after a period of unloading will increase bone responsiveness to IGF-I and promote skeletal recovery. To test this hypothesis, we examined bone structure and formation in response to IGF-I in loaded (normal ambulatory), unloaded (hindlimb elevation), and unloaded/reloaded rats.

MATERIALS AND METHODS

Animals

Forty-eight male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA; 3 mo of age) were used for this study. Skeletal unloading was induced using the hindlimb elevation or tail suspension model (10, 19, 31). Animals were divided into six groups of eight animals each. They were either normally loaded for 4 wk, unloaded for 4 wk, or unloaded for 2 wk and then reloaded for 2 wk. Each group was treated with either vehicle or IGF-I (2.5 mg·kg⁻¹·day⁻¹) using osmotic minipumps (Durect, Cupertino, CA) for the last 2 wk. The time intervals chosen for unloading and reloading were based on previous studies showing that 2 wk of unloading produce a clear decrease in bone formation rate and loss of cancellous bone volume and that 2 wk of reloading initiate an increase in bone formation and restoration of bone volume (23). Animals were pair fed and weighed daily. To measure bone formation, 2 wk and 2 days before euthanasia,

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rats were injected subcutaneously with calcein (15 mg/kg) and demeclocycline (20 mg/kg), respectively, to label mineralizing surfaces.

At the time of euthanasia, blood was collected from the abdomen of the aorta, and serum was harvested for determination of IGF-I. The left tibia and the second lumbar vertebrae were obtained for measurement of fat-free weight and for micro-computed tomography. The right tibia was obtained for measurement of periosteal bone formation rate. These studies were approved by the Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center.

Micro-Computed Tomography Scanning Procedure

The tibiae and vertebrae were cleaned of adherent soft tissue and extracted in ethanol (24 h) and diethyl ether (24 h) using a soxhlet apparatus (Fisher Scientific, Pittsburg, PA). The bones were dried at 100°C, and they were weighed to determine the fat-free weight. The same bones were analyzed using micro-computed tomography (Scanco Medical, Zurich, Switzerland).

Primary spongiosa. The proximal ends of the tibiae were scanned from the top of the epiphysis distally to the middle of the secondary spongiosa to include the whole primary spongiosa. The isotropic voxel (volumetric pixel) size was nominally 10.5 μm. The X-ray energy was 55 kV. The inner structure of the primary spongiosa is a complex mix of mineralized tissue, marrow, and cartilage. To better discriminate between these different structures, we set the integration time to 1 s to optimize the signal-to-noise ratio.

Cross sections of the bone were reformatted into longitudinal sections to allow the user to visually recognize and isolate the primary spongiosa, the region of interest (ROI). The limits of the ROI were the growth plate at the proximal surface and the end of the bone spiculae at the distal side. The cortices were the side limits.

Secondary spongiosa. The region of analysis for the proximal tibia was defined as follows. Three hundred slices (16-μm voxels) were scanned from the bottom of the growth plate distally. The first 100 slices (1.6 mm) were discarded, and the region from 1.6 to 4.8 mm below the growth plate was analyzed. The region of analysis for the vertebrae included the entire vertebral body excluding the cortical bone.

Micro-Computed Tomography Scanning Analysis

A global threshold was applied to segment mineralized from soft tissue (lean and fat marrow). In the secondary spongiosa, the threshold was determined as 22% of the maximal possible value corresponding to a threshold of 220 in the “per mille” unit. The trabecular number (Tb.N; 1/mm), thickness (Tb.Th; μm), and spacing (Tb.Sp; μm); connectivity density (Conn-dens; 1/mm²), structure model index (SMI; ranges from 0 to 3 with 0 = platelike and 3 = rodlike) and bone mineral density (BMD; mg hydroxyapatite/cm³) were calculated using software provided by Scanco (Scanco Medical). BMD is the average density of the segmented fraction of the ROI (bone) not including the marrow fraction. BMD is derived from the linear attenuation coefficient of the X-rays after calibration with a phantom of known hydroxyapatite densities.

The average density of the mineralized tissue in the primary spongiosa is on average lower than the density of cancellous bone in the secondary spongiosa and covers a wide range from the lowest in the vicinity of the hypertrophic chondrocyte zone to the highest in the bone spiculae. To accommodate the lower densities in the primary spongiosa, the threshold was fixed as 20% of the maximal possible value corresponding to a threshold of 200 in the per mille unit. The predefined threshold was also checked by the operator to match the value corresponding to a threshold of 200 in the per mille unit. The trabecular number was determined as 22% of the maximal possible value corresponding to a threshold of 220 in the “per mille” unit. The trabecular number and trabecular spacing were derived using either two-way analysis of variance or one-way repeated-measures analysis of variance (body weight). The Holm-Sidac post hoc test was applied when significance was observed. All analyses were performed using SigmaStat 3.0 software (SPSS, Chicago, IL).

RESULTS

Body weights among the groups at the beginning of the experiment were not significantly different. At the end of the experiment (day 28), the vehicle-treated loaded, unloaded, and reloaded animals weighed (mean ± SD) 403 ± 20, 381 ± 20, and 415 ± 25 g, respectively, and weights within and between vehicle-treated groups did not change during the course of the 4-wk experiment. IGF-I-treated loaded, unloaded and reloaded animals weighed 340 ± 25, 434 ± 25, and 432 ± 25 g, respectively, and weights between these groups did not differ during the course of the experiment (4 wk) (repeated-measures analysis of variance). At day 28, when taken collectively (all IGF-I treated vs. all vehicle treated), there was a significant increase in body weight (P < 0.01) in the IGF-I-treated animals.

The serum concentrations of IGF-I in vehicle- and IGF-I-treated animals are shown in Fig. 1. In normally loaded, vehicle-treated animals, the serum concentration of IGF-I was 43 ng/ml in the vehicle-treated animals to 1,024 ± 41 ng/ml in the IGF-I-treated animals. A two-way ANOVA was performed using SigmaStat 3.0 software (SPSS, Chicago, IL).

![Fig. 1. Serum concentrations of insulin-like growth factor I (IGF-I) at day 28. Values are means ± SD. IGF-I treatment increased serum concentrations from 599 ± 43 ng/ml in the vehicle-treated animals to 1,024 ± 41 ng/ml in the IGF-I-treated animals. §P < 0.01 (2-way ANOVA).](image-url)
563 ± 71 ng/ml. The serum concentrations in unloaded and reloaded animals treated with vehicle were similar. In animals receiving IGF-I, serum concentrations increased by ~70% and did not differ among loading groups.

Tibial fat-free weight (normalized to body weight) did not differ among the groups (Fig. 2), although there was a trend for levels to be lower in vehicle-treated, unloaded animals ($P = 0.06$). Fat-free weights were normalized to correct for the small differential in body weights at day 28.

Periosteal bone formation rates at the TFJ and midhumerus are shown in Fig. 3, A and B, respectively. In vehicle-treated animals, skeletal unloading decreased formation at the TFJ from $17 \pm 6$ to $6 \pm 3 \times 10^3 \mu m^2/day$ ($P < 0.01$). IGF-I treatment in normally loaded animals increased the formation rate at the TFJ from $17 \pm 6$ to $25 \pm 5 \times 10^3 \mu m^2/day$ ($P < 0.01$). No difference was observed between vehicle- and IGF-I-treated unloaded animals. Within the IGF-I-treated animals, unloading produced a decrease in bone formation similar in magnitude to that seen in vehicle-treated animals. Reloading increased the formation rate from $7 \pm 2$ to $23 \pm 2 \times 10^3 \mu m^2/day$ or 238% ($P < 0.001$). The differential between the increase in vehicle treated animals ($2 \times 10^3 \mu m^2/day$) and IGF-I-treated animals ($16 \times 10^3 \mu m^2/day$) was >700%.

Fluorochrome labeling of the periosteal surface is shown in Fig. 3, C and D, for reloaded vehicle (C) and reloaded IGF-I-treated animals (D).
Effects of IGF-I treatment and skeletal unloading on cancellous bone structure in the proximal tibia

Fig. 4. Effects of IGF-I treatment and skeletal unloading on cancellous bone volume-to-total tissue volume ratio (BV/TV) in the proximal tibia (A) and the lumbar vertebra (B). Values are means ± SD. Bone volume was assessed by micro-computed tomography analysis as described in MATERIALS AND METHODS. *P < 0.05 compared with respective load control. †P < 0.01 compared with respective unload (1-way ANOVA within-treatment groups).

Skeletal unloading resulted in a loss of cancellous bone volume in the proximal tibia (Fig. 4A) and vertebra (Fig. 4B) in both vehicle- and IGF-I-treated animals. In the tibia, reloading for 2 wk increased cancellous bone volume in the IGF-I-treated but not vehicle-treated animals. In the vertebrae, reloading did not produce a significant increase in bone volumes in either vehicle- or IGF-I-treated animals.

The changes in bone volume induced by IGF-I treatment and skeletal unloading were associated with specific changes in bone structure (Tables 1 and 2). In the secondary spongiosa of the tibia, Tb.Th decreased with unloading and then increased with reloading in the IGF-I-treated animals (Table 1). Tb.Th was not significantly altered with unloading in the vehicle-treated animals, but with reloading it increased significantly compared with the unloaded state. In the vertebrae, Tb.Th decreased in both vehicle- and IGF-I-treated animals, but it did not increase with reloading (Table 2). Connectivity density decreased with unloading in the tibia but not in the vertebrae. The SMI increased (trabeculae became more rodlike in structure) in both the tibiae and vertebrae of vehicle- and IGF-I-treated animals during unloading and returned to a more plate-like structure during reloading with the exception of the vertebrae in vehicle-treated animals. No changes in segmented BMD were observed.

The primary spongiosa also responded to skeletal unloading and IGF-I treatment. Examples of the micro-computed tomography images of the primary spongiosa are shown in Fig. 5. Skeletal unloading decreased the total volume and the calcified volume of the primary spongiosa in vehicle-treated animals (Fig. 6). IGF-I treatment prevented the decrease induced by unloading in both total and calcified volume. An increase in the total and calcified volumes was observed in reloaded animals treated with vehicle as well as for the calcified volume of IGF-I-treated animals.

**DISCUSSION**

The similarity in body weights within the vehicle-treated and separately within the IGF-I-treated groups suggests that the changes in bone associated with unloading were not a consequence of changes in body weight. Animals receiving IGF-I taken collectively were significantly heavier than vehicle-treated animals. These findings are consistent with the rise in serum IGF-I in treated animals and the expected response to the anabolic actions of IGF-I.

Although there was no significant change in fat-free weight among the groups, unloading tended to result in a small (9%) loss of bone mass. This has also been observed in 6-mo-old rats where skeletal unloading for 4 wk reduced bone mass by

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**Table 1. Effects of IGF-I treatment and skeletal unloading on cancellous bone structure in the proximal tibia**

<table>
<thead>
<tr>
<th>Treatment/Condition</th>
<th>Tb.N 1/mm</th>
<th>Tb.Th, μm</th>
<th>Tb.Sp, μm</th>
<th>Conn-dens, 1/mm³</th>
<th>SMI</th>
<th>Segmented BMD, mg HA/cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Load</td>
<td>2.3 ± 0.6</td>
<td>67 ± 5</td>
<td>465 ± 146</td>
<td>40 ± 10</td>
<td>2.5 ± 0.1</td>
<td>940 ± 11</td>
</tr>
<tr>
<td>Unload</td>
<td>2.1 ± 0.7</td>
<td>63 ± 5</td>
<td>541 ± 179</td>
<td>22 ± 18*</td>
<td>2.9 ± 0.2*</td>
<td>925 ± 14</td>
</tr>
<tr>
<td>Reload</td>
<td>1.5 ± 0.6*</td>
<td>70 ± 3†</td>
<td>733 ± 252*</td>
<td>25 ± 9</td>
<td>2.6 ± 0.2†</td>
<td>926 ± 29</td>
</tr>
<tr>
<td><strong>IGF-I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Load</td>
<td>2.0 ± 0.4</td>
<td>69 ± 5</td>
<td>516 ± 113</td>
<td>41 ± 16</td>
<td>2.5 ± 0.1</td>
<td>947 ± 14</td>
</tr>
<tr>
<td>Unload</td>
<td>1.5 ± 0.6</td>
<td>61 ± 3*</td>
<td>727 ± 268</td>
<td>11 ± 7*</td>
<td>3.0 ± 0.1*</td>
<td>922 ± 19</td>
</tr>
<tr>
<td>Reload</td>
<td>1.5 ± 0.4</td>
<td>72 ± 8†</td>
<td>718 ± 191*</td>
<td>25 ± 7†</td>
<td>2.6 ± 0.1†</td>
<td>917 ± 24</td>
</tr>
</tbody>
</table>

Values are means ± SD. Load, normally loaded for 4 wk; Unload, unloaded for 4 wk; Reload, unloaded for 2 wk and reloaded for 2 wk. Treatment was administered by subcutaneous minipumps for the last 2 wk. [Vehicle or insulin-like growth factor I (IGF-I)] Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular spacing; Conn-dens, connectivity density; SMI, structure model index; segmented BMD, average density of the trabeculae; HA, hydroxyapatite. Significant differences were assessed with a 1 way analysis of variance followed by a Holm-Sidak post-hoc test. *Significantly different from its respective load control, P < 0.05. †Significantly different from its respective unload, P < 0.05.
In studies in young rats (6 wk old), skeletal unloading produces a much greater deficit in bone (12–25%) in a much shorter time (2 wk) (10, 23). This may be explained by the high metabolic rate and rapid turnover of bone in young animals. High bone turnover would be expected to provide a greater opportunity to lose or gain bone mass, and it may explain why older animals respond more sluggishly to unloading with respect to changes in fat-free weight and cancellous bone volume.

Periosteal bone formation at the TFJ decreased dramatically during skeletal unloading in the vehicle-treated animals as expected. Our laboratory and others have shown this to occur in both young (6 wk old) and adult (6 mo old) animals (9, 23, 27). With reloading the rate of formation increased but the difference did not reach statistical significance. In previous studies (23), our laboratory has shown that in young rats reloading after a 2-wk period of unloading increases bone formation by roughly 35% ($P < 0.01$). The discrepancy between young and mature animals in response to reloading is likely again a consequence of the more rapid rate of bone turnover in the young animal. As expected, IGF-I treatment increased bone formation at the TFJ in normally loaded animals and at the midhumerus in all animals. That IGF-I did not prevent the fall in formation accompanying unloading is consistent with our laboratory’s observations that skeletal unloading induces resistance to the anabolic actions of IGF-I on bone (13, 21, 22). Our laboratory’s previous studies have shown that this resistance is linked to deficient IGF-I receptor activation and downstream signaling in response to IGF-I binding, a process that may be mediated through integrin signaling (22). Interestingly, skeletal unloading also attenuates the anabolic response of bone to parathyroid hormone (27).

The remarkable finding in our studies is the effect of IGF-I on bone formation during reloading. Skeletal reloading in the vehicle-treated animals produced a small (37%) insignificant increase in formation rate, whereas reloading in the IGF-I-treated animals produced a 238% increase in formation rate. This suggests that bone responsiveness to IGF-I increases when bone is reloaded after a period of

Table 2. Effects of IGF-I treatment and skeletal unloading on cancellous bone structure in the second lumbar vertebrae

<table>
<thead>
<tr>
<th>Treatment/Condition</th>
<th>Tb.N, 1/mm³</th>
<th>Tb.Th, μm</th>
<th>Tb.Sp, μm</th>
<th>Conn-dens, 1/mm³</th>
<th>SMI</th>
<th>Segmented BMD, mg HA/cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Load</td>
<td>4.1±0.4</td>
<td>75±4</td>
<td>236±27</td>
<td>110±15</td>
<td>0.4±0.4</td>
<td>990±23</td>
</tr>
<tr>
<td>Unload</td>
<td>3.9±0.4</td>
<td>66±6*</td>
<td>252±27</td>
<td>101±19</td>
<td>1.2±0.4*</td>
<td>996±13</td>
</tr>
<tr>
<td>Reload</td>
<td>3.9±0.2</td>
<td>66±4*</td>
<td>249±16</td>
<td>104±14</td>
<td>1.1±0.3*</td>
<td>980±17</td>
</tr>
<tr>
<td>IGF-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Load</td>
<td>4.0±0.4</td>
<td>72±6</td>
<td>242±25</td>
<td>107±17</td>
<td>0.8±0.2</td>
<td>979±25</td>
</tr>
<tr>
<td>Unload</td>
<td>3.9±0.3</td>
<td>61±6*</td>
<td>249±24</td>
<td>93±19</td>
<td>1.6±0.4*</td>
<td>983±11</td>
</tr>
<tr>
<td>Reload</td>
<td>3.9±0.3</td>
<td>67±4</td>
<td>250±24</td>
<td>98±9</td>
<td>1.1±0.3†</td>
<td>986±21</td>
</tr>
</tbody>
</table>

Values are means ± SD. Significant differences were assessed with a 1-way analysis of variance followed by a Holm-Sidak post-hoc test. *Significantly different from its respective load control, $P < 0.05$. †Significantly different from its respective unload, $P < 0.05$.

Fig. 5. Micro-computed tomography tridimensional representation of the primary spongiosa of the proximal tibia (A) and two-dimensional micro-computed tomography section of the proximal tibia (B). The growth plate is not closed (arrowheads) and the primary spongiosa is significantly larger (arrows) in the IGF-I-treated reloaded rats.
unloading. These findings support our hypothesis and are consistent with the reciprocal loss of responsiveness observed during unloading. Studies are now underway to determine whether reloading increases receptor activation with ligand binding.

The load and IGF-I-induced changes in bone formation in cortical bone were accompanied by changes in trabecular bone volume and structure (Fig. 4, A and B, Tables 1 and 2). The loss of bone volume in the tibia induced by unloading was associated with a tendency for decreases in Tb.N and Tb.Th. The increase in Tb.Sp very likely reflected these changes. Similar findings have been reported by other investigators (27). Re-loading, which had little effect on BV/TV, increased Tb.Th but not Tb.N, and spacing increased further. The data suggest that these changes, at least in part, are mediated through alterations in bone responsiveness to IGF-I. Unloading and reloading cannot restore Tb.N. Once the interconnectedness of the trabecular network is compromised, new trabeculae cannot be generated. To meet the new demands of normal weight bearing, Tb.Th increases but cannot sufficiently offset the loss in number to restore bone volume, at least after 2 wk of reloading. The initial loss and sustained deficit in Conn-dens reflects the loss of Tb.N. The changes in SMI with unloading are consistent with trabecular thinning. With loss of number and thickness, the trabeculae become more rodlike, a finding similar to that reported by David et al. (8). With reloading, Tb.Th increases and the trabeculae return to a more platelike structure. That the density of the bone was unaffected by unloading and reloading suggests that over the course of our experiment these do not alter the composition of the mineral phase.

The vertebral response to unloading and reloading, and to treatment with IGF-I, was similar to that observed in the secondary spongiosa of the tibia with the exceptions of Tb.N, Tb.Sp and Conn-dens. Only Tb.Th decreased and the changes in the SMI reflected this loss. That Conn-dens remained unchanged reflected the maintenance of Tb.N. The differences in how the tibiae and vertebrae responded to loading may reflect differences in the pattern of loading between these two bones. The bones are structurally different and the muscular forces applied to the bones are different. There may also be site-specific differences with respect to how the bone responses to load.

The measurement of the primary spongiosa by micro-computed tomography is a novel approach that permitted us to examine the effects of IGF-I during unloading and reloading on its three-dimensional structure. At 4 mo of age rats are considered young adults, and their growth rate is slowing. They are, however, still growing. The growth plate in the male rat does not close until after 8 mo of age (17). Thus in our animals the tibia was continuing to grow longitudinally (albeit very slowly), and new bone was being formed in the primary spongiosa. Although we did not measure longitudinal bone growth, previous studies have shown that unloading has no effect on longitudinal growth regardless of age, duration of unloading, and method of unloading (24). Thus the changes in the primary spongiosa in the vehicle treated animals induced by unloading and reloading cannot be attributed to changes in growth. It appears that the primary spongiosa has the potential to adjust its total and calcified volumes to meet the loading conditions.

IGF-I prevented the loss of bone in the primary spongiosa during unloading and enhanced the restoration of bone during reloading, suggesting that the primary spongiosa, unlike the secondary, retains at least some responsiveness to IGF-I. Whether responsiveness depends on longitudinal growth is not clear.

In the early stages of skeletal unloading or loss of weight bearing, bone mass exceeds mechanical demands and mechanisms come into play to reduce bone formation and stimulate resorption. After adaptation to the unloaded state, reloading or return to normal weight bearing increases mechanical demands and lost bone is replaced. Our data suggest that these changes, at least in part, are mediated through alterations in bone responsiveness to IGF-I. Unloading reduces while overloading (reloading in our model) increases responsiveness to IGF-I. The loss and gain of bone associated with mechanical loading are linked to changes in the responsiveness of bone to IGF-I. That IGF-I can stimulate replacement of bone lost during periods of skeletal unloading or disuse suggests that IGF-I may be of therapeutic use in patients who have lost bone as a consequence of prolonged skeletal disuse.

Fig. 6. Effect of IGF-I treatment and skeletal unloading on the total (A) and calcified (B) volumes of the primary spongiosa. Values are means ± SD. Volumes were assessed by micro-computed tomography as described in MATERIALS AND METHODS. *P < 0.05 compared with respective load control. †P < 0.05 compared with respective vehicle control. ‡P < 0.01 compared with respective unload [1-way (within-treatment group) and 2-way ANOVA].

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REFERENCES


GRANTS

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