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Insulin-like growth factor-1 receptor in mature osteoblasts is required for periosteal bone formation induced by reloading

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Abstract

Skeletal loading and unloading has a pronounced impact on bone remodeling, a process also regulated by insulin-like growth factor 1 (IGF-1) signaling. Skeletal unloading leads to resistance to the anabolic effect of IGF-1, while reloading after unloading restores responsiveness to IGF-1. However, a direct study of the importance of IGF-1 signaling in the skeletal response to mechanical loading remains to be tested. In this study, we assessed the skeletal response of osteoblast-specific *Igf-1 receptor* deficient (*Igf-1r*$^{-/-}$) mice to unloading and reloading. The mice were hindlimb unloaded for 14 days and then reloaded for 16 days. *Igf-1r*$^{-/-}$ mice displayed smaller cortical bone and diminished periosteal and endosteal bone formation at baseline. Periosteal and endosteal bone formation decreased with unloading in *Igf-1r*$^{+/+}$ mice. However, the recovery of periosteal bone formation with reloading was completely inhibited in *Igf-1r*$^{-/-}$ mice, although reloading-induced endosteal bone formation was not hampered. These changes in bone formation resulted in the abolishment of the expected increase in total cross-sectional area with reloading in *Igf-1r*$^{-/-}$ mice compared to the control mice. These results suggest that the Igf-1r in mature osteoblasts has a critical role in periosteal bone formation in the skeletal response to mechanical loading.

Keywords

Insulin-like growth factor 1 receptor; osteoblasts; unloading; reloading; periosteal bone formation

Introduction

Bone is a dynamic tissue that is responsive and sensitive to mechanical stimuli [1]. Regions undergoing increased mechanical loading augment bone mass [2]. Bone loss results from skeletal unloading during long-term spaceflight and prolonged bed rest caused by fractures,

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spinal injuries, and strokes [3, 4]. Astronauts dramatically lose bone mass in the hip at 1.5%/month on long-duration missions on the International Space Station [5]. Recovery of bone mineral density in astronauts after completing missions is much slower than the bone loss during spaceflight [6–8]. Skeletal unloading leads to decreased bone mass due to reduced bone formation and increased bone resorption [3, 9]. Despite identification of the cell types and signaling molecules responsible for skeletal unloading and loading [10, 11], the underlying mechanisms by which bone senses and responds to mechanical stimuli have not been fully elucidated.

Insulin-like growth factor-1 (IGF-1) is critical for bone homeostasis and required to achieve normal bone growth and bone mass [12–14]. IGF-1 and the IGF-1 receptor (IGF-1R) are expressed in osteoblasts [15, 16]. In vivo and in vitro studies have revealed that the most pronounced role of IGF-1 in osteoblasts is to stimulate osteoblast function and bone formation. Targeted over-expression of *Igf-1* in mature osteoblasts leads to increased bone formation [17]. Mice carrying *Igf-1 receptor* (*Igf-1r*) deletion in mature osteoblasts exhibit decreases in cortical thickness as well as bone formation [18, 19]. These lines of evidence demonstrate that the IGF-1/IGF-1R system in mature osteoblasts is indispensable for maintaining normal bone mass and bone formation.

Osteoblasts and osteocytes stimulated by mechanical loading produce IGF-1 [20, 21]. Skeletal unloading leads to resistance to the anabolic effect of IGF-1 on bone, while bone responsiveness to IGF-1 is restored with reloading following unloading [22, 23]. However, a direct study of the importance of IGF-1 signaling in the skeletal response to mechanical loading remains to be tested. In this study, we assessed the skeletal response of osteoblast-specific *Igf-1r* deficient mice to unloading and reloading.

Materials and Methods

Mice

Animal protocols were approved by the Institutional Animal Care and Use Committee at the Veterans Administration Medical Center, San Francisco. Homozygous conditional mice in which exon 3 of the *Igf-1r* gene was flanked by loxP sites [18] were bred with heterozygous mice in which cre recombinase was expressed under the control of the human osteocalcin promoter (gift from Dr. Thomas Clemens) [24] to generate osteoblast-specific *Igf-1r* deficient mice. The mice were maintained under pathogen-free conditions and fed ad libitum. We hindlimb-unloaded 12-week-old mice for 14 days, as described previously [25–27], and then reloaded them for 16 days.

Histomorphometry

Tibiae were fixed, dehydrated and embedded in methyl methacrylate. Ten-μm cross-sectional undecalcified sections from a region immediately proximal to the tibio-fibular junction at the tibial diaphysis were cut to assess bone formation measurements in cortical bone. Mosaic-tiled images were converted to a single image by the Axio Vision software (Carl Zeiss). Data were collected using the Bioquant Osteo software (Bioquant Image Analysis) and reported according to standard bone histomorphometry nomenclature [28]. In details, to obtain cortical bone formation measurements, alizarin complexone (30mg/kg, Sigma-Aldrich) and calcein (15 mg/kg, Sigma-Aldrich) were peritoneally given to mice 14 days and 2 days before euthanasia, respectively. The fluorochromes are incorporated into bone matrix and become fluorescent labels on bone surfaces only where and when bone formation occurs before being excreted by the kidney. Bone formation rate/bone surface (BFR/BS) ($\mu m^3/\mu m^2$/day) is calculated as the multiplication of mineralizing surface (MS)/BS and mineral apposition rate (MAR). MS is bone surface actively mineralized by

osteoblasts at a particular time when fluorescent labels are administered and calculated as the total extent of double labeled bone surfaces plus the half of single labeled bone surfaces. It correlates with the extent of bone surface covered with osteoblasts. MAR is the distance between two labels divided by the time (12 days in this study) between two labeling periods. MAR correlates with the activity of osteoblasts [19].

Micro–computed tomography (μCT)

Tibiae were scanned under anesthesia using a SCANO VivaCT 40 (Scano Medical AG) to obtain longitudinal 3D in vivo μCT imaging. A region of interest (ROI) for cortical bone analysis consisted of 0.42 mm proximal to the tibio-fibular junction at the tibial diaphysis. Data were reported according to standard μCT nomenclature [29]. Total cross-sectional area (Tt.Ar) is defined as total cross-sectional area inside the periosteal envelope in cortical bone.

Immunohistochemistry

Femurs were cleaned of adherent tissue, fixed overnight at 4 C in 4% paraformaldehyde in PBS, rinsed in PBS, dehydrated through an ethanol series, cleared in xylene, embedded in paraffin, and cut into 5-μm sections. Deparaffinized and rehydrated sections were incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase and with protein blocker (Abcam) to block the nonspecific binding of antibodies. Then, the sections were reacted with rabbit IGF-IR antibody (1:200) (Aviva Systems Biology) at 4 C overnight. After washing with PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (Abcam), then streptavidin peroxidase (Abcam), and visualized by 3,3′-diaminobenzidine.

Statistical analysis

Data are expressed as mean ± SE and analyzed by an unpaired Student's t-test, except using a paired Student's t-test for longitudinal measurements of μCT analysis.

Results

Deletion of the Igf-1r in mature osteoblasts and osteocytes in Igf-1r$^{-/-}$ mice

We evaluated mice in which the *insulin-like growth factor type-1 receptor* was deleted with osteocalcin-driven cre recombinase (*Igf-1r*$^{-/-}$) in order to determine the role of the IGF-1R in mature osteoblasts in the skeletal response to unloading and reloading. Before examining the skeletal response of *Igf-1r*$^{-/-}$ mice to unloading and reloading, we assessed the expression of Igf-1r in the growth plate, trabecular bone and cortical bone by immunohistochemistry (Fig 1). In *Igf-1r*$^{-/-}$ mice, very little expression of IGF-1R was identified in mature osteoblasts in the secondary spongiosa of trabecular bone as well as in osteocytes in trabecular and cortical bone (Fig. 1D, 1F, 1H), although IGF-1R was expressed in mature osteoblasts and osteocytes in *Igf-1r*$^{+/+}$ mice (Fig. 1C, 1E, 1G). IGF-1R expression in prehypertrophic and hypertrophic chondrocytes in the growth plate was comparable between *Igf-1r*$^{+/+}$ and *Igf-1r*$^{-/-}$ mice (Fig. 1A, 1B). These results demonstrate the specific deletion of the *Igf-1r* in mature osteoblasts and osteocytes in *Igf-1r*$^{-/-}$ mice.

Periosteal and endosteal bone formation in cortical bone is impaired in Igf-1r$^{-/-}$ mice

We assessed *Igf-1r*$^{-/-}$ mice at baseline by μCT and bone histomorphometry, before determining the role of the IGF-1R in mature osteoblasts in the skeletal response to unloading and reloading. μCT analysis revealed that total cross-sectional area (Tt.Ar) in cortical bone was decreased in *Igf-1r*$^{-/-}$ male mice compared to that in *Igf-1r*$^{+/+}$ littermates (Fig. 2A). Histomorphometry demonstrated that the bone formation rate (BFR/BS) was reduced in both periosteal and endosteal surfaces of cortical bone in *Igf-1r*$^{-/-}$ mice (Fig. 2B). The reduced BFR/BS was due to a decrease in MAR and MS/BS on both periosteal and

endosteal bone surfaces (data not shown). These results indicate that the IGF-1R in mature osteoblasts has a significant role in bone formation in cortical bone.

Periosteal bone formation is diminished in Igf-1r$^{-/-}$ mice in the response to skeletal unloading and reloading

We hindlimb-unloaded mice and then reloaded them in order to determine whether the IGF-1R in mature osteoblasts is associated with the skeletal response to unloading and/or mechanical loading. Cortical bone was longitudinally estimated by μCT at baseline and after unloading and reloading. Tt.Ar increased with reloading in *Igf-1r*$^{+/+}$ male mice compared to that after unloading in the same group (Fig. 3A). However, the response of Tt.Ar to reloading was diminished in *Igf-1r*$^{-/-}$ mice, indicating that periosteal bone expansion by reloading is inhibited in *Igf-1r*$^{-/-}$ mice. Histomorphometry showed that BFR/BS on periosteal surfaces of cortical bone declined with unloading in *Igf-1r*$^{+/+}$ mice and then increased with reloading (Fig. 3B.), along with a reduction and an increase in both MAR and MS/BS, respectively (data not shown). In contrast, this response to reloading was completely hindered in *Igf-1r*$^{-/-}$ mice. On the other hand, BFR/BS on endosteal surfaces of cortical bone fell with unloading and then increased with reloading in both *Igf-1r*$^{-/-}$ and *Igf-1r*$^{+/+}$ mice (Fig. 3B). A change of MAR and MS/BS with unloading and reloading on endosteal bone surfaces was in concordance with the changes of BFR/BS with unloading and reloading, respectively, in both *Igf-1r*$^{-/-}$ and *Igf-1r*$^{+/+}$ mice (data not shown). These results indicate that the IGF-1R in mature osteoblasts plays a crucial role in reloading-induced periosteal but not endosteal bone formation.

Discussion

We provide evidence that IGF-1R in mature osteoblasts is required for periosteal bone expansion and formation induced by reloading using osteoblast specific *Igf-1r* deficient mice that were hindlimb unloaded and then reloaded. Bone histomorphometry revealed that the augmentation in BFR on periosteal surfaces of cortical bone induced by reloading was completely blocked in *Igf-1r*$^{-/-}$ mice (Fig. 3B). μCT analysis showed that a reloading-induced increase in Tt.Ar in the cortical diaphysis was also completely blunted in *Igf-1r*$^{-/-}$ mice (Fig. 3A). Several studies have shown that mechanical loading enhances *Igf-1* expression in osteoblasts and osteocytes [20, 21]. However, a direct study determining whether the skeletal response to mechanical loading is mediated by IGF-1 signaling has not been previously demonstrated. We provide the evidence that the IGF-1 signaling in mature osteoblasts has a critical role in periosteal surfaces of cortical bone in response to reloading following unloading. In addition to our previous study [23], this study suggests that enhancing IGF-1 signaling in mature osteoblasts in an astronaut after returning to the earth could promote periosteal bone formation and expansion in cortical bone and mitigate the risk of fracture in the astronaut after completing their long-term spaceflight missions.

The response of endosteal bone formation to reloading was comparable between *Igf-1r*$^{-/-}$ and *Igf-1r*$^{+/+}$ mice (Figs. 3B). The *Igf-1r* is deleted only in the mature osteoblasts in our mouse model. Thus, the response to reloading in endosteal bone likely occurs in the less mature osteoblasts which express the IGF-1R normally, while the response to loading in periosteal bone requires the IGF-1R in mature osteoblasts. The BFR/BS in endosteal bone was much higher than that in periosteal bone at baseline in both *Igf-1r*$^{-/-}$ and *Igf-1r*$^{+/+}$ mice (data not shown), suggesting that less mature osteoblasts have a dominant role in the high turnover sites in the skeletal response to reloading. The deletion of the *Igf-1r* in less mature osteoblasts will be useful to test whether the IGF-1R is crucial for reloading-induced endosteal bone formation.

BFR/BS on periosteal bone surfaces declined with unloading in *Igf-1r*$^{+/+}$ mice and then increased with reloading (Fig. 3B), along with a reduction and an increase in both MAR and MS/BS, respectively. In contrast, this response of BFR/BS (Fig. 3B), MAR and MS/BS to reloading was completely inhibited in *Igf-1r*$^{-/-}$ mice, implying that IGF-1R in mature osteoblasts could affect the number of active osteoblasts and the activity osteoblasts of at baseline and in the skeletal response to mechanical load [28].

In conclusion, IGF-1 signaling in mature osteoblasts plays a critical role in periosteal bone formation in the skeletal response to reloading, suggesting that IGF-1 signaling is necessary for accelerating periosteal bone formation following the return to normal gravity and that approaches to enhance and maintain IGF-1 signaling in mature osteoblasts may be beneficial after long-term space missions and prolonged bed rest caused by medical conditions.

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Abbreviations

igf-1r	insulin-like growth factor 1 receptor
Base	baseline
Unl	unloading
Rel	reloading

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Highlights

- We tested the response of osteoblast-specific Igf-1r$^{-/-}$ mice to unloading/reloading.
- Increased periosteal bone formation with reloading is blocked in *Igf-1r*$^{-/-}$ mice.
- Increased cortical bone expansion with reloading is inhibited in *Igf-1r*$^{-/-}$ mice.
- Igf-1r in mature osteoblasts mediates reloading-induced periosteal bone formation.

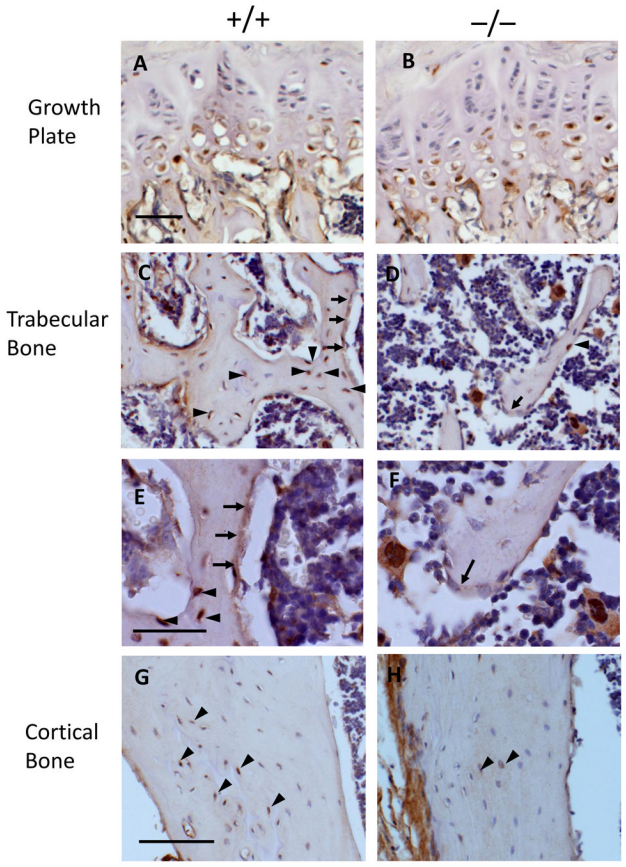

Fig 1. Expression of IGF-1R in *Igf-1r*$^{-/-}$ mice

Immunohistochemistry staining showed that in the growth plate (A & B), IGF-1R (brown) was expressed in the prehypertrophic and hypertrophic chondrocytes in the *Igf-1r*$^{+/+}$ (A) mice and the *Igf-1r*$^{-/-}$ (B) mice; in the trabecular bone of the secondary spongiosa, IGF-1R is expressed in the osteoblasts lining the bone surfaces (arrows) and osteocytes embedded in the bone matrix (arrowheads) in the *Igf-1r*$^{+/+}$ mice (C), but very little expression of IGF-1R was identified in the osteoblasts (arrows) and osteocytes (arrow heads) in the *Igf-1r*$^{-/-}$ mice (D). High magnification pictures of *Igf-1r*$^{+/+}$ (E) and *Igf-1r*$^{-/-}$ (F) mice are shown below. In the cortical bones, IGF-1R is expressed in the osteocytes (brown, arrow heads) in the *Igf-1r*$^{+/+}$ mice (G), but very few osteocytes (arrowheads) express IGF-1R in the *Igf-1r*$^{-/-}$ mice (H). 20 X in A–D, G & H; 40 X in E & F. Bars = 50 μm.

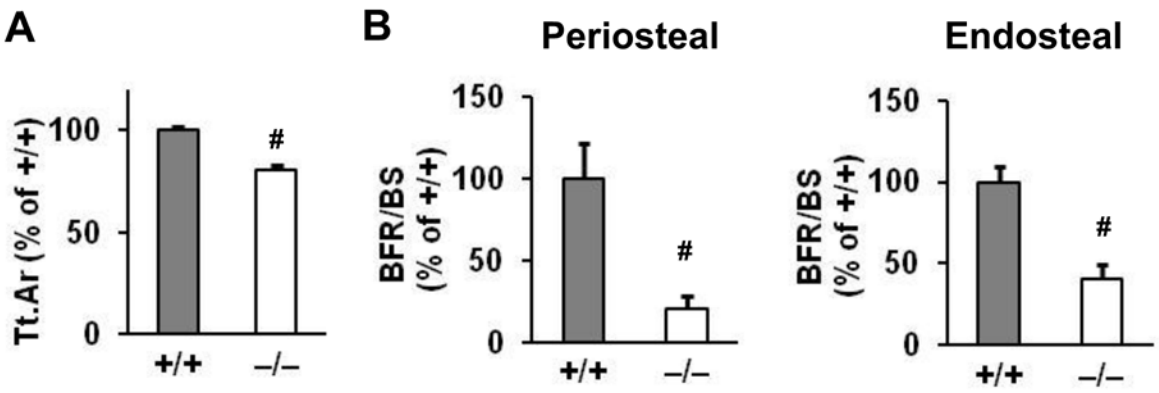

Fig 2. Periosteal and endosteal bone formation in cortical bone is impaired in *Igf-1r*$^{-/-}$ mice

(A) μCT analysis revealed that total cross-sectional area (Tt.Ar) of cortical bone was decreased in *Igf-1r*$^{-/-}$ male mice. n = 10 in the two groups. (B) Bone histomorphometry showed that bone formation rate (BFR/BS) was decreased in both periosteal and endosteal surfaces of cortical bone in *Igf-1r*$^{-/-}$ mice. n = 6 in *Igf-1r*$^{+/+}$ mice; n = 7 in *Igf-1r*$^{-/-}$ mice. Data are percent of the results of *Igf-1r*$^{+/+}$ mice and shown as mean ± SE. $^{\#}p < 0.01$.

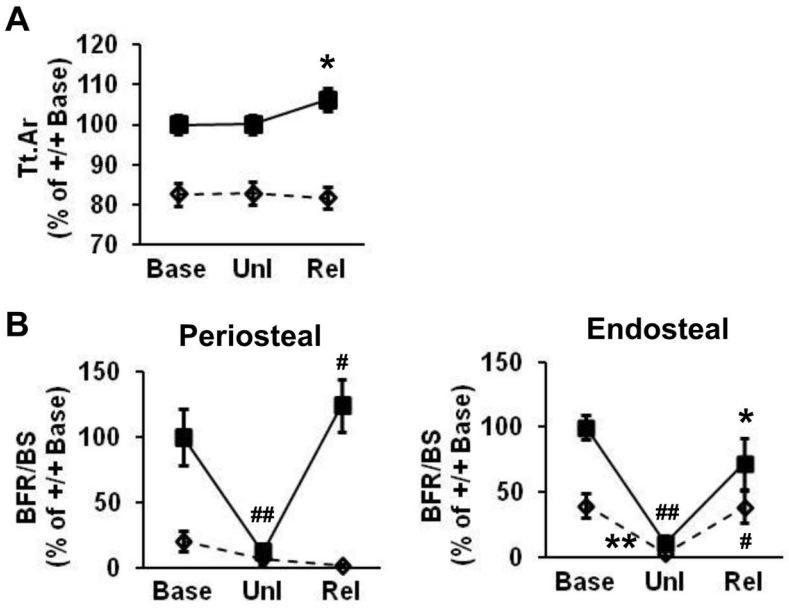

Fig 3. Periosteal bone formation is diminished in *Igf-1r*$^{-/-}$ mice in the response to skeletal unloading and reloading

(A) Total cross-sectional area (Tt.Ar) increased with reloading (Rel) in *Igf-1r*$^{+/+}$ mice (solid squares with solid lines) compared to that after unloading (Unl) in the same group. Note that the response in the Tt.Ar induced by reloading was inhibited in *Igf-1r*$^{-/-}$ mice (open diamonds with dashed lines). Base, baseline; n = 9 in the two groups. (B) The increased bone formation rate (BFR/BS) induced by reloading (Rel) seen in *Igf-1r*$^{+/+}$ mice was completely blocked on periosteal surfaces, but not on endosteal surfaces, of cortical bone in *Igf-1r*$^{-/-}$ mice. n = 6 in *Igf-1r*$^{+/+}$ mice at baseline, n = 8 in *Igf-1r*$^{+/+}$ mice with unloading, n = 9 in *Igf-1r*$^{+/+}$ mice with reloading, n = 9 in all the group of *Igf-1r*$^{-/-}$ mice. Solid lines, *Igf-1r*$^{+/+}$ mice; dashed, *Igf-1r*$^{-/-}$ mice. Data are percent of the results of *Igf-1r*$^{+/+}$ mice at baseline and shown as mean ± SE. $^{\#}$,*, Unl vs. Rel in the same genotype; $^{\#\#}$, Base vs. Unl in *Igf-1r*$^{+/+}$mice; **, Base vs. Unl in *Igf-1r*$^{-/-}$mice; $^{\#,\#\#,**}p < 0.01$, $^{*}p < 0.05$.