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Unloading-Induced Bone Loss was Suppressed in Gold-Thioglucose Treated Mice

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Abstract Loss of mechanical stress causes bone loss. However, the mechanisms underlying the unloading-induced bone loss are largely unknown. Here, we examined the effects of gold-thioglucose (GTG) treatment, which destroys ventromedial hypothalamus (VMH), on unloading-induced bone loss. Unloading reduced bone volume in control (saline-treated) mice. Treatment with GTG-reduced bone mass and in these GTG-treated mice, unloading-induced reduction in bone mass levels was not observed. Unloading reduced the levels of bone formation rate (BFR) and mineral apposition rate (MAR). GTG treatment also reduced these parameters and under this condition, unloading did not further reduce the levels of BFR and MAR. Unloading increased the levels of osteoclast number (Oc.N/BS) and osteoclast surface (Oc.S/BS). GTG treatment did not alter the basal levels of these bone resorption parameters. In contrast to control, GTG treatment suppressed unloading-induced increase in the levels of Oc.N/BS and Oc.S/BS. Unloading reduced the levels of mRNA expression of the genes encoding osteocalcin, type I collagen and Cbfa1 in bone. In contrast, GTG treatment suppressed such unloading-induced reduction of mRNA expression. Unloading also enhanced the levels of fat mass in bone marrow and mRNA expression of the genes encoding PPARgamma2, C/EBPalpha, and C/EBPbeta in bone. In GTG-treated mice, unloading did not increase fat mass and the levels of fat-related mRNA expression. These results indicated that GTG treatment suppressed unloading-induced alteration in bone loss. J. Cell. Biochem. 99: 845–852, 2006.

Key words: unloading; sympathetic system; central control; osteoblasts; osteoclasts; fat tissue

Mechanical stimuli influence body skeleton in most of the animals as one of the determination factors for bone growth in the process of skeletal development and maintenance. Skeletal loading is essential for homeostasis of bone in vertebrates [Ehrlich and Lanyon, 2002]. Disuse osteoporosis is caused by skeletal unloading due to weightlessness during long-term bed rest and space flight [Bikle and Halloran, 1999]. Unloading of bone based on tail suspension has been used as a model in rodents to simulate conditions of loss of mechanical stimuli [Bikle and Halloran, 1999]. Such skeletal unloading suppresses osteoblastic activities and enhances osteoclastic activities. Both of these features lead to bone loss [Simske et al., 1992]. However, the mechanisms underlying this phenomenon have not been fully elucidated.

Bone volume is maintained by the balance in bone remodeling, which is based on two

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processes including bone formation by osteoblasts and bone resorption by osteoclasts [Boyle et al., 2003; Harada and Rodan, 2003]. These activities of osteoblasts and osteoclasts are under the control of systemic hormones and local cytokines. Recent reports suggest treatment with gold-thioglucose (GTG), which impairs ventromedial hypothalamus (VMH) [Debons et al., 1962; Powley and Prechtl, 1986], results in modulation of central control of bone metabolism [Takeda et al., 2002]. However, it is not known whether GTG treatment affects unloading-induced bone loss. We, therefore, examined the effect of GTG treatment on the mice with respect to unloading-induced bone loss and found that GTG treatment suppressed unloading-induced bone loss.

MATERIALS AND METHODS

Animals

C57BL6/J male mice were obtained from Sankyo Labo service. The mice were housed under controlled conditions at 24°C on a 12-h light/12-h dark cycle and fed with standard laboratory chow containing normal calcium and given tap water. Four-week-old C57BL6/J male mice were given a single intraperitoneal injection with GTG (Research Diagnostics, Inc., NJ) to destroy VMH at 0.5 mg/g of body weight. Control mice were treated with saline. Then, the mice were allowed to grow for 6 weeks before they were subjected to experiments. Tail suspension was conducted at the age of 10-week-old (6 weeks after GTG treatment) to avoid acute effects of GTG treatment. Tail suspension period was conducted for 2 weeks for the analysis of bone mass and bone formation parameters and 1 week for the analysis of bone resorption parameters. For tail suspension; a tape was applied to the surface of the tail to set a metal clip. The other end of the clip was fixed to an overhead bar and the height of the bar was adjusted to maintain the mice at 30° head down tilt with the hind limbs elevated above the floor of the cage. Loaded control mice were also housed individually under the same condition except for tail suspension for the same duration ($n = 12$ per group). After tail suspension, these mice were anesthetized with pentobarbital. All experiments were conducted according to the institutional guidelines for animal welfare.

Micro-CT Analysis of Bone

2D-μCT analysis was carried out by using Musashi (Nittetsu-ELEX, Osaka, Japan). For quantification of cancellous bone of femora, images of μCT slices were made within a midsagittal plane in the metaphyseal region of the bones. The bone volume was quantified in a 1.12 mm^2 square area (0.7×1.6 mm) located at 0.5 mm away from the growth plate. The image data were subsequently quantified using Luzex-F automated image analysis system (Nireco, Tokyo, Japan). Threshold for the measurements was set at 110 for the analyses.

Histomorphometric Analysis of Bone

The mice were injected intraperitoneally with calcein at 4 mg/kg, 7 and 2 days before sacrifice at the end of unloading. For undecalcified section, femora were prestained with Villanueva osteochrome (bone stain), and embedded in methylmethacrylate. Undecalcified sagittal sections were used to examine cancellous bone formation parameters (MAR, MS/BS, BFR) and were also used to examine bone marrow adipose tissue volume in the sections stained with toluidine blue. The area of adipose tissue per total marrow tissue was measured by using Luzex-F automated image analyzer (Nireco). For decalcified section, tibiae from mice subjected to 1-week unloading were decalcified in EDTA, embedded in paraffin, and sectioned. The sections were stained for tartrate-resistant acid phosphatase (TRAP) activity. Osteoclast number was quantified in the metaphyses of the tibiae.

Bone Marrow Cell Cultures

Bone marrow cells were flushed out from femora. For mineralized nodule formation, bone marrow cells were plated in 12-well plates (2.0 cm^2 per well) at a density of 2×10^6 cells/well and cultured in alpha-MEM supplemented with 10% fetal bovine serum, 100 μg/ml antibiotics-antimycotics mixture, 50 μg/ml ascorbic acid and 10 mM sodium beta-glycerophosphate. The cultures were fixed on day 21 and were stained in a saturated solution of alizarin red. The area of mineralized nodules was measured by using the Luzex-F automated image analyzer (Nireco) and the values were normalized against total well surface.

RNA Extraction, cDNA Synthesis, and RT-PCR Analysis

Total RNA from whole femora was extracted according to acid guanidiumthiocyanate phenol chloroform method. First-strand cDNA was synthesized using 1 μg of the total RNA and Molony murine leukemia virus reverse transcriptase. Primers were synthesized on the basis of the reported mouse cDNA sequences. Amplification was performed at 21–40 cycles within a linear range. Each reaction mixture was subjected to electrophoresis to be analyzed on 1% agarose gel. The bands were visualized by ethidium bromide staining. The bands were quantified and were normalized against those of GAPDH. Sequences of the primers were as follows; GAPDH, forward, 5′-ACC ACA GTC CAT GCC ATC AC-3 and reverse, 5′-TCC ACC ACC CTG TTG CTG TA-3′; Osteocalcin, forward, 5′-CTC TGT CTC TCT GAC CTC ACA G-3′ and reverse, 5′-CAG GTC CTA AAT AGT GAT ACC G-3′; Type I Collagen, forward, 5′-TTT GTG GAC CTC CGG CTC-3′ and reverse, 5′-AAG CAG AGC ACT CGC CCT-3′; Cbfa1, forward, 5′-GAA CCA AGA AGG CAC AGA CA-3′ and reverse, 5′-AAC TGC CTG GGG TCT GAA AA-3′; PPARgamma2, forward, 5′-AAC ACC GAG ATT TCC TTC AA-3′ and reverse, 5′-TCA CGC CTT TCA TAA CAC AT-3′; C/EBPalpha, forward, 5′-TGG ACA AGA ACA GCA ACG AG-3′ and reverse, 5′-TCA CTG GTC AAC TCC AGC AC-3′; C/EBPbeta, forward, 5′-AAG GCC AAG GCC AAG AAG GC-3′ and reverse, 5′-TGA ACA AGT TCC GCA GCG TG-3′.

Statistical Evaluations

The results were presented as mean values ± SEM. Statistical analysis was performed based on ANOVA and followed by post hoc test for multiple comparisons. *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

When control (saline-treated) mice were subjected to 2-weeks unloading, bone volume (BV/TV) levels in femora were reduced (Fig. 1a,b; column 1 vs. 2). GTG treatment alone reduced bone volume compared to saline-treated control mice (Fig. 1a,b; column 1 vs. 3). In contrast to control mice, unloading-induced reduction in bone mass was not observed in GTG-treated mice and BV/TV levels were similar to those in loaded GTG-treated group (Fig. 1a,b; column 3 vs. 4).

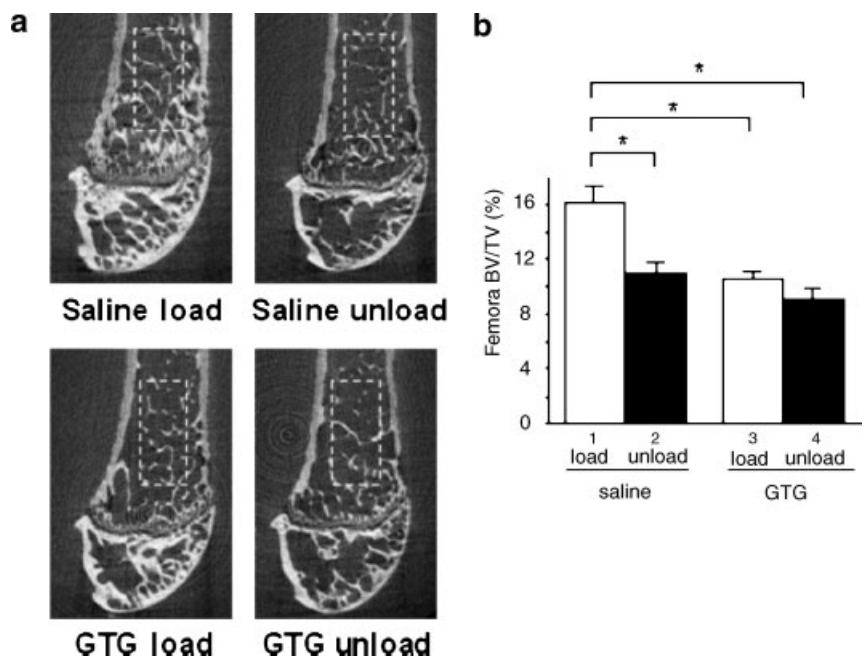

Fig. 1. Unloading-induced bone loss was suppressed by GTG treatment. **a**: 2D-μCT analysis of the midsagittal planes of femora after 2-weeks unloading. **b**: Fractional trabecular BV/TV was quantified based on the image analysis of 2D-μCT. Analyses were conducted in the rectangular area shown in (a). Asterisks indicate statistically significant difference (*$P < 0.05$).

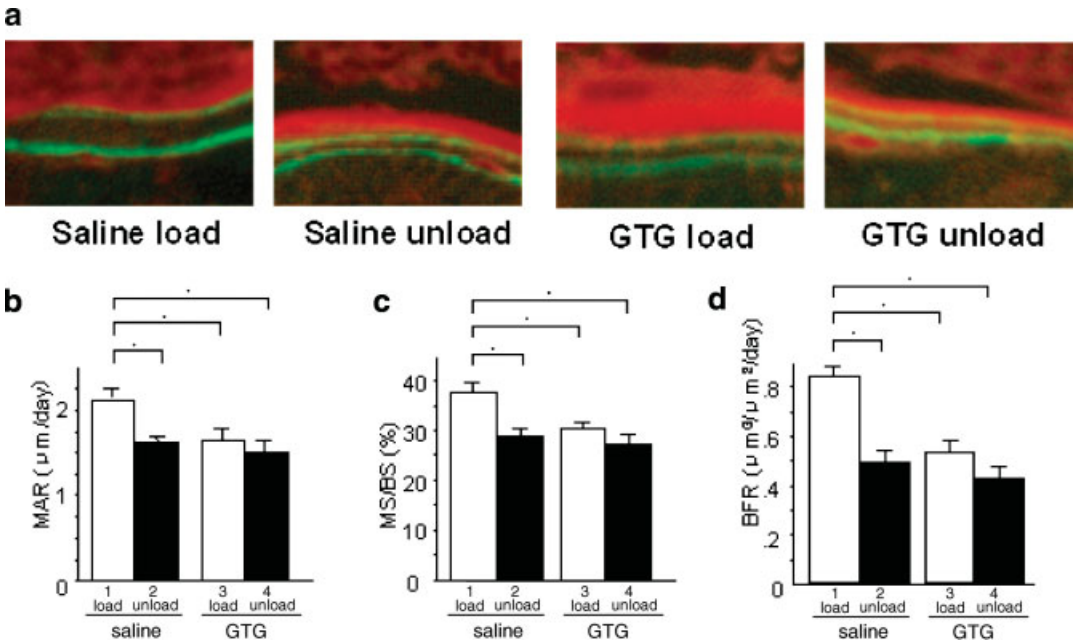

Fig. 2. GTG treatment suppressed unloading-induced reduction in bone formation. Histomorphometric analysis in control (saline-treated) and GTG-treated mice after unloading. **a**: Calcein double-labeled surfaces in the cancellous bone of femora. **b**: Quantification of mineral apposition rate (MAR). **c**: Quantification of mineralized surface per bone surface (MS/BS). **d**: Quantification of bone formation rate (BFR). Asterisks indicate statistically significant difference (*$P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

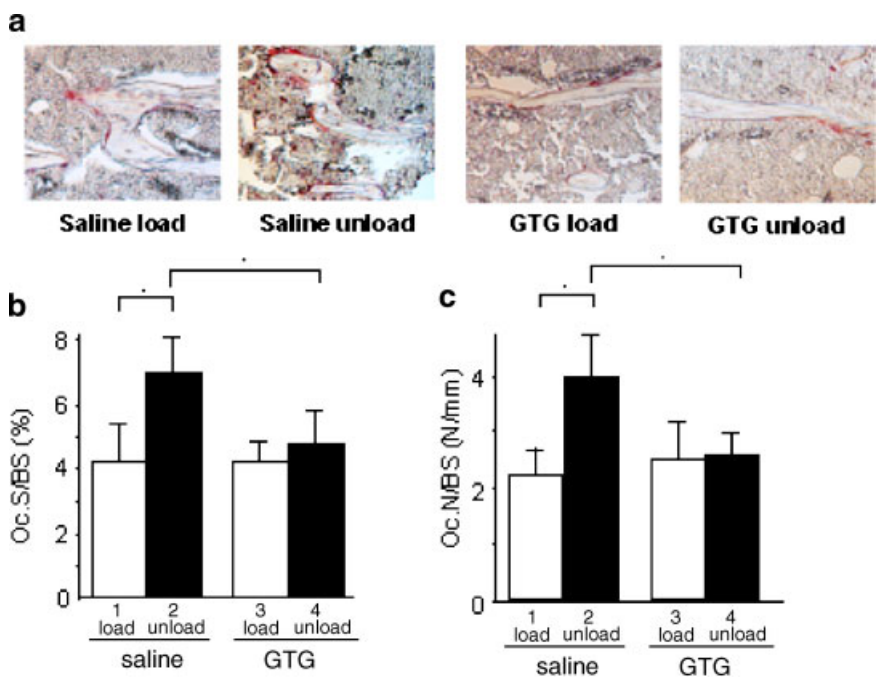

Fig. 3. GTG treatment suppressed unloading-induced increase in the levels of TRAP cells in vivo. **a**: Sections of the tibia were subjected to TRAP staining after 1-week unloading. **b**: Quantification of osteoclast surface per bone surface (Oc.S/BS) in the trabecular bone of the tibia. **c**: Quantification of osteoclast number per bone surface (Oc.N/BS) in the trabecular bone of the tibia. Asterisks indicate statistically significant difference (*$P < 0.05$).

To understand the dynamics of the GTG effects on bone turnover, we conducted histomorphometric analysis. Unloading reduced the levels of bone formation parameters (MAR, MS/BS, BFR) (Fig. 2a–d; column 1 vs. 2). In contrast, GTG treatment suppressed unloading-induced reduction in MAR, MS/BS, and BFR (Fig. 2b–d; column 3 vs. 4). Unloading enhanced osteoclast surface (Oc.S/BS) and the osteoclast number (Oc.N/BS) in control mice (Fig. 3a–c; column 1 vs. 2). In contrast, GTG treatment suppressed such unloading-induced increase in the levels of Oc.N/BS and Oc.S/BS (Fig. 3b,c; column 3 vs. 4). These observations indicated that GTG treatment suppresses unloading-induced enhancement in osteoclastic activity. Thus, GTG treatment affected two major unloading-induced phenomena on both osteoblastic and osteoclastic sides in vivo.

Cellular mechanisms underlying these observations were examined further. We conducted bone marrow cell cultures after unloading in vivo in the presence of ascorbate and beta-glycerophosphate. Unloading reduced the formation of alizarin red-positive nodules in control group (Fig. 4a,b, column 1 vs. 2). GTG treatment alone reduced the nodule formation (Fig. 4a,b; column 1 vs. 3). In contrast to control group, unloading did not reduce nodule formation levels in GTG-treated mice (Fig. 4a,b; column 3 vs. 4). These observations indicated that GTG treatment suppressed unloading-induced events in the osteoblastic cell activities in the marrow cells.

To elucidate molecular basses underlying the effects of GTG treatment on unloading-induced reduction in bone formation, RNA expression in the femora was analyzed. In control mice, mRNA expression levels of the genes encoding bone formation-related proteins such as osteocalcin, type I collagen and Cbfa1 were reduced after unloading. In contrast, such unloading-induced reduction was suppressed in GTG-treated mice (Fig. 5a). Interestingly, in control mice, the expression levels of mRNAs of the genes encoding fat-related proteins such as PPARgamma2, C/EBPalpha, and C/EBPbeta were enhanced by unloading. In contrast, unloading did not enhance the levels of fat-related mRNA expression in GTG-treated mice (Fig. 5b).

In order to see whether the observations in the expression levels of genes related to adipogenesis could be reflected in morphology, histological

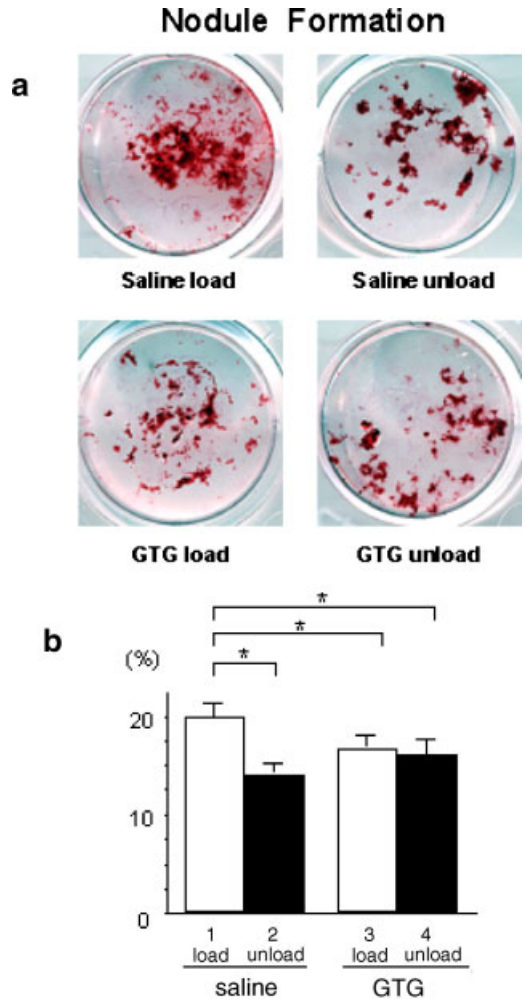

Fig. 4. GTG treatment in vivo suppressed unloading-induced decrease in mineralized nodule formation in culture. **a**: Bone marrow cells were cultured in the presence of ascorbate and beta-glycerophosphate after 2-weeks unloading. Mineralized nodules were visualized by alizarin red-S staining. **b**: Quantification of the area of mineralized nodules per total dish surface. Asterisks indicate statistically significant difference ($*P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

analysis was conducted. Adipose tissue volume in the bone marrow was increased after unloading in control mice (Fig 6a,b; column 1 vs. 2). GTG treatment per se also enhanced the levels of adipose tissue volume (Fig. 6a,b; column 1 vs. 3). In contrast to control, unloading did not increase adipose tissue volume in the marrow in GTG-treated mice (Fig. 6a,b; column 3 vs. 4).

DISCUSSION

We showed that GTG treatment suppressed unloading-induced bone loss. As these mice were treated with GTG at 4-week-old and tail suspension was conducted much later in the

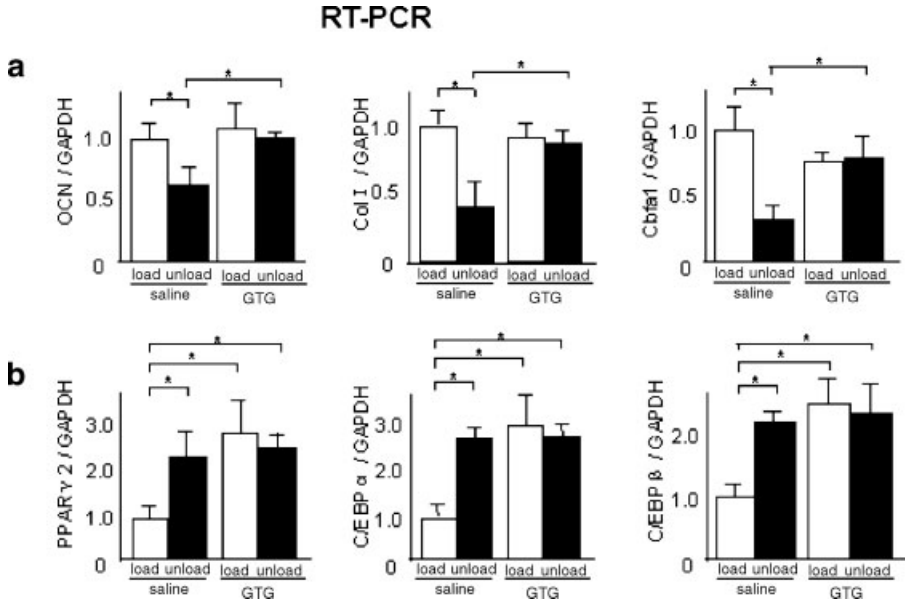

Fig. 5. Unloading-induced changes in the levels of osteoblast-related and adipocyte-related mRNA expression were suppressed in GTG-treated mice. RT-PCR analysis in saline and GTG-treated mice after 2-weeks unloading. mRNA expression in the whole femora was performed using primer sets for murine (**a**) Osteocalcin, type I collagen and Cbfa1. **b**: PPARgamma2, C/EBPalpha, and C/EBPbeta. Asterisks indicate statistically significant difference ($*P < 0.05$).

adult 10-week-old mice, observed effects of GTG treatment on unloading-induced would be due to GTG destruction of VMH. The effects of GTG treatment in the regulation of bone subjected to unloading were further analyzed in detail based on histomorphometry. Our data indicated that the levels of osteoblastic bone formation parameters (MAR, BFR) were reduced by unloading and GTG treatment suppressed such reduction. These observations indicate that GTG treatment affects the function of osteoblastic cells subjected to unloading. The reduction in nodule formation in the cultures of the bone marrow cells subjected to unloading was also prevented by GTG treatment. Therefore, GTG treatment could influence the effects of unloading on the levels of osteoprogenitor population. Moreover, our RT-PCR data indicate that GTG treatment influenced mRNA expression levels of osteoblastic bone formation related genes.

We also examined the effect of GTG treatment on bone loss induced by unloading on the side of bone resorption. Unloading increased osteoclastic parameters in control mice. On the other hand, GTG treatment suppressed such unloading-induced increase in osteoclastic parameters. These observations indicate that GTG treatment suppressed unloading-induced changes in both osteoblastic and osteoclastic activities. These observations supported the notion that GTG treatment interfered signaling upstream of osteoblasts and osteoclasts.

GTG treatment impairs VMH, which is responsible for central control of bone metabolism [Takeda et al., 2002]. In fact, GTG-treated mice fail to display changes in sympathetic activity in response to nutritional stimuli and cold exposure [Young and Landsberg, 1980]. Although downstream inhibitors such as propranolol could prevent bone loss due to unloading condition [Kondo et al., 2005], the role of upstream central nervous system in unloading-induced bone loss was not known. Our data indicate that GTG treatment, which would destroy hypothalamic neuron in VMH, modulates central control regarding unloading-induced bone loss.

Osteoblasts and adipocytes have been suggested to share common progenitors [Pittenger et al., 1999]. Reciprocal relationship between these cell lineages of osteoblasts and adipocytes has been suggested to exist [Beresford et al., 1992; Nuttall et al., 1998]. Clinically, age-related osteoporosis in aged population is accompanied by an increase in bone marrow adipose tissue [Meunier et al., 1971; Rozman

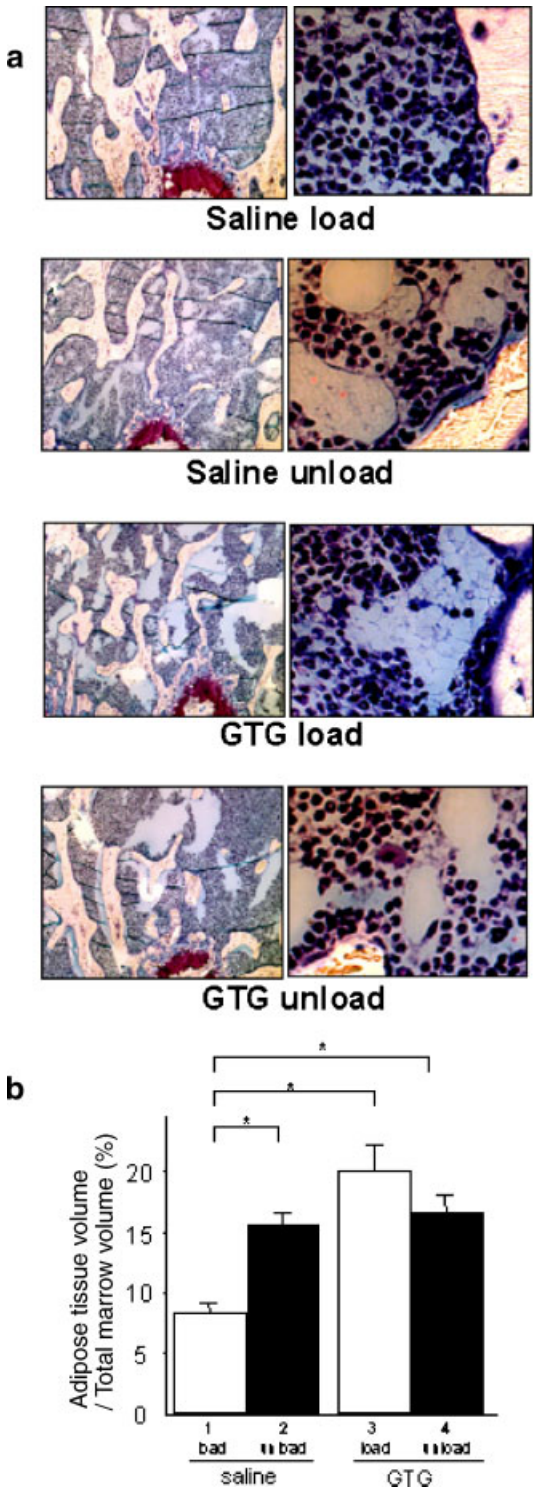

Fig. 6. Unloading increases bone marrow fat mass in control but not in GTG-treated mice. **a**: Sections of the femora were subjected to toluidine blue staining after 2-weeks unloading. **Left panel**: 100×, **Right panel**: 400×. **b**: Quantification of adipose tissue volume per total marrow volume of femora in saline and GTG-treated mice after 2-weeks unloading. Asterisks indicate statistically significant difference ($*P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 1989]. Exercise strengthens skeletons by increasing their bone volume while it also contributes to reduce fat tissue mass [Bass et al., 1998; Mori et al., 2003; Wu et al., 2004]. Thus, the regulation in the direction of differentiation into osteoblasts or adipocytes could be a critical component in the process of reduction in osteogenesis during unloading. Our observations revealed that in the presence of GTG treatment, unloading did not alter fat mass levels. Whether this could mean that GTG treatment affects determination of the fate of the cells to differentiate into osteoblasts and adipocytes in marrow in response to unloading is still to be determined. In conclusion, GTG treatment affects unloading-induced alteration in bone loss.

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