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Role of Inducible Nitric Oxide Synthase in Skeletal Adaptation to Acute Increases in Mechanical Loading*

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ABSTRACT

To clarify the role of nitric oxide (NO) in regulation of bone metabolism in response to skeletal loading, we examined inducible NO synthase (iNOS) gene knockout mice in the tail-suspension model. Histomorphometric analyses of proximal tibias revealed that 7 days of tail suspension decreased the bone volume (BV/TV) and bone formation rate (BFR/BS) and increased the osteoclast surface (Oc.S/BS) in mice with all iNOS genotypes. Both $iNOS^{+/+}$ and $iNOS^{+/-}$ mice responded to subsequent 14-day reloading, with increases in BV/TV and BFR/BS and a decrease in Oc.S/BS, whereas these responses were abolished in $iNOS^{-/-}$ mice. The osteoblasts flattened after tail suspension appeared cuboidal during subsequent reloading. Immunoreactivity for iNOS was detected in these osteoblasts and osteocytes by immunohistochemistry. These defective responses after reloading were rescued in $iNOS^{-/-}$ mice by treatment with an NO donor nitroglycerine (NG). Conversely, the responses in $iNOS^{+/+}$ mice were inhibited by treatment with an NOS inhibitor aminoguanidine (AG). In bone marrow cell cultures, mineralized nodules derived from $iNOS^{-/-}$ mice after reloading were significantly reduced. Taken together, our results suggest that NO generated by iNOS in osteoblasts plays a critical role in adjusting bone turnover and increasing osteogenic activity in response to the acute increase in mechanical loading after tail suspension. (J Bone Miner Res 2002;17:1015–1025)

Key words: **inducible nitric oxide synthase knockout mice, tail suspension, osteoblast, mineralized nodule formation, nitroglycerine**

INTRODUCTION

MECHANICAL STRESS is an important determinant of the structural and functional integrity of the skeletal system.[1–4] Skeletal unloading such as weightlessness during space flight or bed rest causes osteopenia in weight-bearing bones in humans.[5–7] However, the molecular mechanisms mediating the effects of loading and unloading on bone are unknown.

Tail-suspended rats[8–10] and mice,[11,12] simulating skeletal unloading for hindlimbs, provide a useful model to investigate the regulation of bone turnover by mechanical stress. Unloading by tail suspension results in diminished bone formation and accelerated bone resorption in the animals, consequently leading to bone loss in the lower extremities.[8–12] Furthermore, proliferation and differentiation of osteoprogenitor cells are reduced in the bone marrow,[13,14] and expression levels of messenger RNAs (mRNAs) for insulin-like growth factors (IGFs), transform-

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ing growth factor (TGF) β2, osteocalcin, and osteopontin are reduced in these animals.[15–17] Conversely, subsequent normal weight bearing after a period of unloading results in increased bone formation to the level observed at baseline.[13,18] Previous studies using the rat model showed a rise in c-*fos* mRNA expression in periosteal cells within 20 minutes after normal reloading and a subsequent increase in the expression of cyclo-oxygenase 2 mRNA after 2 h in intracortical bone cells.[19]

Nitric oxide (NO) is a short-lived free radical synthesized from L-arginine by NO synthases (NOSs), which has three isoforms including constitutively expressed forms of neural NOS (nNOS) and endothelial NOS (eNOS) and an inducible form of NOS (iNOS). In vitro, NO is produced by osteoblasts[20,21] and stimulates their proliferation.[22] NO production in bone cells is enhanced in response to mechanical stimulation such as fluid shear stress and bending.[23,24] eNOS gene knockout mice exhibit retarded bone formation during the period of rapid growth[25] and a blunted response to high-dose estrogen after ovariectomy.[26] Previous in vitro studies provided clear evidence for NO production and iNOS expression by bone cells in response to cytokine stimulation.[20–22] However, the role of iNOS in bone cells in vivo has not yet been well established.

We hypothesized that iNOS in bone cells contributes to increased bone formation in response to increased mechanical loading. In this study, iNOS gene knockout mice underwent tail suspension, and bone and marrow cells in the hindlimbs were analyzed after unloading and subsequent normal reloading. We also examined the effects of aminoguanidine (AG), an iNOS inhibitor, in $iNOS^{+/+}$ mice and nitroglycerine (NG), an NO donor, in $iNOS^{-/-}$ mice, during the period of normal reloading after tail suspension.

MATERIALS AND METHODS

Animals and tail suspension

Mice lacking the iNOS gene (B6.129P2-*Nos2*^{tm1lau}), obtained from The Jackson Laboratory (Bar Harbor, ME, USA),[27] were bred and housed in the facility in the Center for Laboratory Animal Care at the Product Research Laboratory (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan). Mice of three genotypes, including wild-type $iNOS^{+/+}$, heterozygous type $iNOS^{+/-}$, and homozygous type $iNOS^{-/-}$ were delivered to the Center for Laboratory Animal Care unit at the University of Occupational and Environmental Health at the age of 7 weeks. Mice were allowed to acclimatize for 1 week before the experiments. All mice were housed individually in cages in an air-conditioned environment (temperature, 24 ± 1°C; humidity, 55 ± 5%) with illumination from 07:00 to 19:00. Mice were allowed free access to water and food, a standard rodent chow containing 1.25% calcium, 1.06% phosphorus, and 2.0 IU/g vitamin D_3 (CE-2; Clea Japan, Inc., Tokyo, Japan) during the acclimatization period. Mice body weight ranged from 20 to 24 g. The mice were divided randomly into body weight–matched groups. Tail suspension of mice was performed as described previously by our laboratory.[13] Tail-suspended mice were allowed free access to food (CE-2) and water, and the average amounts of food taken by tail-suspended mice were fed also to the ground control mice with normal loading. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the University of Occupational and Environmental Health.

Experimental design and group assignments

The effects of the iNOS gene on bone during unloading and normal reloading were examined using histomorphometry. Mice were divided into four groups based on the experimental protocol: group 1, unloading group, subjected to hindlimb unloading by tail suspension for 7 days and then killed; group 2, normal-loading control group (without suspension), killed at 7 days; group 3, unloading-reloading group, subjected to unloading for 7 days followed by normal reloading for 14 days and then killed on day 21; and group 4, normal-loading control group (without suspension), killed on day 21. Groups 1–4, respectively, comprised three genotype groups of the $iNOS^{+/+}$, $iNOS^{+/-}$, and $iNOS^{-/-}$ mice. Six mice from each genotype group were included in the study.

The effects of AG administration on normal reloading after suspension in $iNOS^{+/+}$ mice and that of NG in $iNOS^{-/-}$ mice were examined also. Groups 5 and 6 each consisted of five $iNOS^{+/+}$ mice. Group 5 mice underwent tail suspension for 7 days followed by normal reloading for 14 days and then were killed on day 21 of the experiment. During the period of normal reloading, mice were treated with AG (Nacalai, Kyoto, Japan) dissolved in drinking water at a concentration of 4 mg/ml.[28] Group 6 served as the nonsuspended normal-loading control group, and mice from this group were treated in a manner similar to group 5. The average amount of AG taken by mice was 20 mg/day and did not differ between groups 5 and 6. Groups 7 and 8 each consisted of five $iNOS^{-/-}$ mice. Group 7 mice underwent tail suspension for 7 days followed by normal reloading for 14 days and then were killed. During the period of normal reloading, mice were treated with NG twice daily by dermal application of 0.1 mg of NG with 2% NG tape (Nippon Kayaku, Tokyo, Japan).[29] Group 8 served as the normal-loading ground control group and was treated similarly to group 7.

The impact of the iNOS gene on unloading and reloading on bone marrow capacity for bone formation was examined by the nodule formation assay. Sixteen mice including eight $iNOS^{+/+}$ mice and eight $iNOS^{-/-}$ mice underwent tail suspension for 7 days, and then four mice of each genotype were killed. The remaining mice were allowed normal reloading for 14 days and then were killed on day 21 of the experiment. For the normal-loading ground controls, eight mice including four $iNOS^{+/+}$ mice and four $iNOS^{-/-}$ mice were killed at baseline, and on days 7 and 21 of the experiment, respectively. For immunohistochemistry of iNOS and enzyme histochemistry of alkaline phosphatase (ALP), bone specimens were obtained from groups of mice comprising five $iNOS^{+/+}$ mice each killed 7 days after tail suspension and at periods of 12 h and 1, 2, 3, 5, 7, and 14 days after subsequent normal reloading. Two normal-

loading iNOS$^{-/-}$ mice were killed also on day 7 of the experiment.

Anesthesia and tissue samples

For histomorphometry specimens, mice underwent bone labeling using injections of calcein (6 mg/kg body weight) intraperitoneally at 6 days and 2 days, respectively, before death. At the time of death, mice were anesthetized with ether and exsanguinated, and then both tibias were harvested. The left femur was used for size measurement and genotyping. For bone marrow cell culture specimens, both femurs and tibias were harvested from mice in which their bones were not labeled with calcein. For immunohistochemistry specimens for iNOS and enzyme histochemistry for ALP, mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body weight) and perfused via the ventricle with physiological saline and then by a solution of 2% paraformaldehyde (PFA) in 0.1 M of phosphate buffer (PB; pH 7.2), followed by isolation of bilateral tibias.

Body weight and size of femur

Body weight of mice was measured weekly from the start of the experiment. At the end of the experiment, femur sizes were measured with a digital caliper (Digimatic Caliper; Mitsutoyo, Kanagawa, Japan). The length of the femur represented the distance between the top of the greater trochanter to the distal end of the lateral femoral condyle. The anteroposterior diameter was measured at the midportion of the femur.

Genotyping

Genotyping for the iNOS gene was performed by reverse-transcription polymerase chain reaction (RT-PCR) amplification of RNA isolated from the normal-loading control mice. Total RNA was extracted from the femur using the RNEasy kit (Qiagen, Hilden, Germany). First-strand complementary DNA (cDNA) was reverse-transcribed from total RNA (3 μg) using Moloney murine leukemia reverse transcriptase (SuperScript; Life Technologies, Inc., Rockville, MD, USA) and oligo(dT)$_{12-18}$ primer (Life Technologies, Inc.). PCR amplification was performed using gene-specific PCR primers and *Taq* polymerase (EX *Taq*; Takara Biomedicals Shuzo Co., Shiga, Japan). The cycling conditions were set as follows: 28 cycles of denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. For amplification of iNOS, sense primer 5′-ACCTACTTCCTGGACATTACGACCCC-3′ and antisense primer 5′-AAGGGAGCAATGCCCG-TACCAGGCCC-3′ were used.[30] β-actin (sense primer, 5′-TCTTCATGGTGCTAGGAGCCA-3′, antisense primer, 5′-CCTAAGGCCAACCGTGAAAAG-3′) was used as an internal control.[31] The expected sizes were 456 base pairs (bp) for iNOS and 632 bp for β-actin. The PCR products were electrophoresed on a 1% agarose gel containing 0.5 μg/ml of ethidium bromide.

Histomorphometry

Right tibias were fixed with 10% buffered formalin and embedded in methylmethacrylate (MMA) resin after Villanueva's bone staining. Serial nondecalcified 5-μm-thick frontal sections were obtained using a microtome (model 2050 Supercut; Reichert-Jung, Heidelberg, Germany). Left tibias were fixed with ice-cold 5% PFA in 0.1 M of PB containing 2% sucrose, pH 7.4. Samples were then embedded in a mixture of MMA, hydroxyglycol methacrylate, and 2-hydroxyethylacrylate.[32] Polymerization was performed at 4°C. The specimens were sectioned in the center to yield 5-μm frontal nondecalcified sections, which were stained for tartrate-resistant acid phosphatase (TRAP).

We measured the secondary spongiosa in the tibial specimens as described previously.[32] MMA sections were measured at 100-fold magnification to determine the percentage of trabecular bone volume to tissue volume (BV/TV, %) and trabecular bone surface (BS, mm). Double-labeled surfaces to total labeled surfaces (dLS, mm) in MMA sections were obtained from measurement of the trabecular perimeter at 100-fold magnification and the interlabel thickness (Ir.L.Th, μm) at 200-fold magnification. Percentages of dLS to bone surface (dLS/BS, %) were obtained. The mineral apposition rate (MAR, μm/day) was calculated as $\pi/4 \times$ Ir.L.Th/3 (μm/day). The ratio of bone formation to bone surface (BFR/BS, %-μm/day) was calculated by the formula (MAR $\times$ dLS/BS). The percentage of trabecular osteoclast surface to bone surface (Oc.S/BS, %) was obtained from measurement of TRAP-stained sections at 200-fold magnification. TRAP$^+$ cells that formed resorption lacunae at the surface of trabeculae and contained one or more nuclei were designated as osteoclasts.

Bone marrow cell cultures for nodule formation

Bone marrow cells were obtained from both tibias and femurs after cleaning of soft tissues. Marrow cell cultures were prepared using the method reported previously.[32] Bone marrow cells were disseminated into 6-well plates (Corning, New York, NY, USA) at a concentration of 5×10^6 cells/ml in 2 ml of α-modified Eagle's medium (Flow Laboratories, Irvine, UK). The medium was supplemented with 15% fetal calf serum (FCS; Life Technologies, Inc.), 2.0 g/liter of $NaHCO_3$, 100 μg/ml of streptomycin, 100 U/ml of penicillin, 1.25 U/ml of nystatin (Sigma Chemical Co., St. Louis, MO, USA), 50 μg/ml of ascorbic acid (Wako, Osaka, Japan), 10 mM of sodium β-glycerophosphate (Sigma Chemical Co.), and 10 nM of dexamethasone (Wako). On day 21 of culture, cells were fixed for 24 h in a 1:1:1.5 solution of 10% formalin, methanol, and water, stained for 15 minutes with a saturated solution of alizarin red S at pH 4.0 (Sigma Chemical Co.), washed with water, and air-dried. The surface area covered with dark red stain, representing mineralized nodules, was measured using an imaging software program (National Institutes of Health NIH Image 1.62) on a Macintosh computer (Apple Computer, Inc., Cupertino, CA, USA).[33,34]

Immunohistochemistry and enzyme histochemistry

Both tibias were fixed in a solution of 4% PFA in 0.1 M of PB, decalcified with 0.2 M of EDTA in 0.1 M of PB containing 10% sucrose at 4°C, dehydrated through a graded series of alcohol baths and embedded in paraffin. Five-micrometer-thick serial sections were cut on a microtome. For immunohistochemistry of iNOS, deparaffinized and hydrated sections were briefly rinsed in phosphate-buffered saline (PBS) at room temperature and incubated with methanol containing 0.3% H_2O_2 for 20 minutes at room temperature to remove endogenous peroxidase activity. Nonspecific immunoglobulin binding was blocked by incubation with PBS containing 5% normal goat serum for 15 minutes at room temperature. Them, sections were incubated with rabbit anti-iNOS polyclonal antibody (Wako) at a dilution of 1:500 for 12 h at 4°C. After a brief rinse with PBS, sections were incubated with the biotinylated secondary antibody, coupled to the labeled streptavidin-biotin complex (LSAB Kit; Dako, Carpinteria, CA, USA). The peroxidase complex was visualized by treatment with a freshly prepared diaminobenzidine tetrahydrochloride (0.1 mg/ml) solution with 0.01% H_2O_2 for 5 minutes.[35] For ALP activity, after deparaffinization and hydration, sections were rinsed briefly in Tris-buffered saline (pH 7.4) for 5 minutes at room temperature and stained using a bromochloro indolylphosphate-Nitro blue tetrazolium (BCIP-NBT) solution kit for ALP stain (Nacalai) for 60 minutes at room temperature.[35]

Statistical analysis

Results were expressed as the mean ± SEM. At each time point, differences between the treatment group and the control group in the same genotype were examined for statistical significance using the Student's *t*-test and Mann-Whitney's *U* test. Additionally, to compare the changes between different time points in the treatment scheme, Tukey-Kramer post hoc test after one-way analysis of variance (ANOVA) and Mann-Whitney's *U* test were used. A value of $p < 0.05$ denoted the presence of a statistically significant difference.

RESULTS

iNOS mRNA expression

Expression of iNOS mRNA was not detected in RNA extracted from the femur of $iNOS^{-/-}$ mice. iNOS mRNA expression level in femurs from $iNOS^{+/-}$ mice was intermediate between those of $iNOS^{+/+}$ and $iNOS^{-/-}$ mice (Fig. 1).

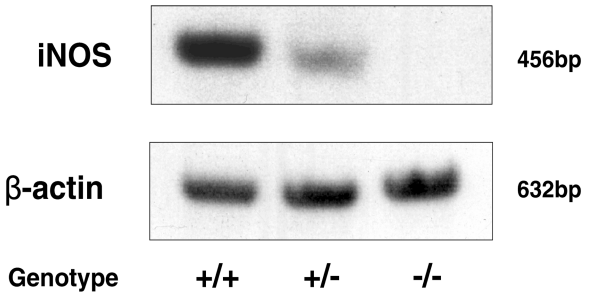

FIG. 1. RT-PCR expression of iNOS mRNA extracted from the femur in $iNOS^{+/+}$, $iNOS^{+/-}$, and $iNOS^{-/-}$ mice. β-actin mRNA was used as an internal control.

Body weight and size of femur

No significant differences were observed in body weight gain or length of femur among mice of the three genotypes in the unloading group and the normal-loading control group at 7 days or in those of the subsequent reloading group after unloading and the normal-loading control group at 21 days. In addition, the mean values were not different among mice of the AG-treatment group and the NG-treatment group (Tables 1, 2, and 3).

Trabecular BV and local turnover of proximal tibia during unloading and reloading

In all three genotypes, trabecular BV (BV/TV) after tail suspension was significantly lower than that of normal-loading control on day 7 of the experiment (Table 1 and Fig. 2). dLS/BS, MAR, and BFR/BS, after tail suspension, were significantly lower, and trabecular Oc.S/BS was significantly greater than the respective parameters of normal-loading control.

On day 21 of the experiment, BV/TV, MAR, BFR/BS, and Oc.S/BS of $iNOS^{+/+}$ mice, after reloading, were similar to those of the normal-loading control (Table 2), whereas dLS/BS was significantly higher than the control group. Furthermore, BV/TV of $iNOS^{+/-}$ mice after reloading was significantly smaller than that of the normal-loading control. Other parameters were not significantly different between the two groups. In $iNOS^{-/-}$ mice, BV/TV, dLS/BS, MAR, and BFR/BS were significantly lower and Oc.S/BS was significantly greater than the respective parameters of the control group.

BV/TV and BFR/BS of $iNOS^{+/+}$ mice significantly increased on day 21 compared with day 7 (Figs. 3A and 3B). In $iNOS^{-/-}$ mice, BV/TV and BFR/BS values on day 7 and day 21 were not significantly different (Figs. 3D and 3E). Compared with values at 7 days, Oc.S/BS of $iNOS^{+/+}$ mice were significantly lower on day 21 of the experiment (Fig. 3C). However, Oc.S/BS on day 21 in $iNOS^{-/-}$ mice was not different from that on day 7 (Fig. 3F).

Effects of AG in $iNOS^{+/+}$ mice and NG in $iNOS^{-/-}$ mice on reloading

In $iNOS^{+/+}$ mice treated with AG on day 21, BV/TV and MAR of the unloading-reloading group were significantly smaller, whereas Oc.S/BS was significantly larger, than the respective parameters of the normal-loading control group (Table 3). Treatment of $iNOS^{-/-}$ mice with NG did not result in any significant change between the reloading group and the control group.

TABLE 1. BODY WEIGHTS AND HISTOMORPHOMETRIC PARAMETERS OF TRABECULAR BONE IN THE PROXIMAL TIBIA FOR iNOS$^{+/+}$, iNOS$^{+/-}$, AND iNOS$^{-/-}$ MICE 7 DAYS AFTER TAIL SUSPENSION

	iNOS$^{+/+}$ mice		*iNOS$^{+/-}$ mice*		*iNOS$^{-/-}$ mice*	
	Group 1 Unloading by tail suspension	*Group 2 Normal loading control*	*Group 1 Unloading by tail suspension*	*Group 2 Normal loading control*	*Group 1 Unloading by tail suspension*	*Group 2 Normal loading control*
Weight at day 0 (g)	21.28 ± 0.41	21.92 ± 0.46	23.40 ± 0.38	22.15 ± 0.82	22.62 ± 0.43	23.38 ± 0.95
Weight at day 7 (g)	21.03 ± 0.63	22.18 ± 0.46	22.82 ± 0.49	22.43 ± 0.52	21.27 ± 0.62	24.08 ± 0.90
BV/TV (%)	6.62 ± 0.53**,††	12.85 ± 0.58	08.00 ± 1.17*,†	12.55 ± 1.04	6.32 ± 0.89**,††	12.66 ± 0.65
dLS/BS (%)	10.27 ± 1.46**,††	19.41 ± 0.44	10.98 ± 1.30**,††	20.35 ± 0.30	11.73 ± 0.76**,††	16.35 ± 1.11
MAR (μm/day)	1.01 ± 0.06**,††	1.45 ± 0.04	1.09 ± 0.02**,††	1.39 ± 0.01	1.11 ± 0.07**,††	1.49 ± 0.06
BFR/BS (%-μm/day)	10.79 ± 1.94**,††	28.04 ± 1.09	11.96 ± 1.54**,††	28.26 ± 0.52	12.94 ± 0.86**,††	24.59 ± 2.34
Oc.S/BS (%)	21.64 ± 0.35**,††	15.12 ± 0.74	19.42 ± 1.80*,†	13.13 ± 1.32	20.61 ± 1.03**,††	13.22 ± 0.91

Data are mean ± SEM values of six mice.

* $p < 0.05$; ** $p < 0.01$; significantly different from group 2 in the same genotype mice (Student's *t*-test); † $p < 0.05$; †† $p < 0.01$; significantly different from group 2 in the same genotype mice (Mann-Whitney *U* tests).

TABLE 2. BODY WEIGHTS AND HISTOMORPHOMETRIC PARAMETERS OF TRABECULAR BONE IN THE PROXIMAL TIBIA FOR iNOS$^{+/+}$, iNOS$^{+/-}$, AND iNOS$^{-/-}$ MICE AT 14 DAYS OF NORMAL RELOADING AFTER TAIL SUSPENSION

	iNOS$^{+/+}$ mice		*iNOS$^{+/-}$ mice*		*iNOS$^{-/-}$ mice*	
	Group 3 Normal reloading after tail suspension	*Group 4 Normal loading control*	*Group 3 Normal reloading after tail suspension*	*Group 4 Normal loading control*	*Group 3 Normal reloading after tail suspension*	*Group 4 Normal loading control*
Weight at day 0 (g)	23.92 ± 0.47	23.23 ± 0.37	23.28 ± 0.46	24.00 ± 0.57	23.68 ± 0.39	22.83 ± 0.59
Weight at day 21 (g)	26.28 ± 0.62	24.50 ± 0.24	25.26 ± 1.03	25.35 ± 0.78	26.20 ± 0.32	24.52 ± 1.26
BV/TV (%)	11.68 ± 1.12	12.56 ± 0.90	10.56 ± 0.57*,†	12.94 ± 0.70	7.12 ± 0.48**,††	13.03 ± 1.15
dLS/BS (%)	23.23 ± 0.73*	19.77 ± 1.04	19.08 ± 1.59	18.37 ± 0.92	10.85 ± 0.66**,††	18.17 ± 0.88
MAR (μm/day)	1.55 ± 0.10	1.52 ± 0.06	1.34 ± 0.05	1.40 ± 0.03	1.06 ± 0.04**,††	1.50 ± 0.08
BFR/BS (%-μm/day)	36.07 ± 2.41	30.31 ± 2.66	25.82 ± 2.74	25.77 ± 1.47	11.49 ± 0.79**,††	27.40 ± 2.30
Oc.S/BS (%)	14.79 ± 0.84	14.81 ± 0.73	13.85 ± 0.98	13.50 ± 1.44	20.15 ± 1.53*,†	15.18 ± 1.32

Data are mean ± SEM values of six mice.

* $p < 0.05$; ** $p < 0.01$; significantly different from group 4 in the same genotype mice (Student's *t*-test); † $p < 0.05$; †† $p < 0.01$; significantly different from group 4 in the same genotype mice (Mann-Whitney *U*-tests).

TABLE 3. EFFECTS OF AG IN iNOS$^{+/+}$ MICE AND THOSE OF NG IN iNOS$^{-/-}$ MICE ON THE HISTOMORPHOMETRIC PARAMETERS OF TRABECULAR BONE IN THE PROXIMAL TIBIA

	AG effects in iNOS$^{+/+}$ mice		*NG effects in iNOS$^{-/-}$ mice*	
	Group 5 Normal reloading after tail suspension	*Group 6 Normal loading control*	*Group 7 Normal reloading after tail suspension*	*Group 8 Normal loading control*
Weight at day 0 (g)	22.56 ± 0.27	22.18 ± 0.19	22.36 ± 0.62	22.42 ± 0.49
Weight at day 21 (g)	23.64 ± 0.37	23.84 ± 0.32	24.00 ± 0.85	24.88 ± 0.56
BV/TV (%)	8.21 ± 0.50**,†	12.97 ± 0.87	14.76 ± 0.65	15.97 ± 0.77
dLS/BS (%)	15.29 ± 1.48	11.77 ± 1.77	21.96 ± 2.57	22.64 ± 1.87
MAR (μm/day)	1.11 ± 0.05*,†	1.41 ± 0.08	1.72 ± 0.10	1.75 ± 0.03
BFR/BS (%-μm/day)	14.53 ± 0.85	16.46 ± 2.15	37.00 ± 3.04	39.52 ± 3.20
Oc.S/BS (%)	19.21 ± 1.11**,†	13.86 ± 0.71	12.67 ± 1.40	13.07 ± 0.91

Data are mean ± SEM values of six mice.

* $p < 0.05$; ** $p < 0.01$; significantly different from group 6 in the same genotype mice (Student's *t*-test); † $p < 0.01$; significantly different from group 6 in the same genotype mice (Mann-Whitney *U* tests).

Because of the treatment with AG in iNOS$^{+/+}$ reloading mice, BV/TV, BFR/BS, and Oc.S/BS did not significantly differ from those of the unloading group (Figs. 3A–3C). These BV/TV and BFR/BS values were significantly lower, and the mean Oc.S/BS value was significantly higher than the corresponding values of the AG untreated reloading group. Because of the treatment with NG in iNOS$^{-/-}$ reloading mice, BV/TV and BFR/BS were significantly higher, whereas Oc.S/BS was significantly lower compared with those of the unloading group (Figs. 3D–3F). The same parameters also were significantly different from those of the NG untreated reloading group.

Bone marrow cell cultures for nodule formation

In iNOS$^{+/+}$ mice, the bone nodule area after tail-suspension was significantly smaller compared with baseline value and normal-loading ground controls at 7 days (Fig. 4A). However, the corresponding values after reloading were not significantly different from controls on day 21 or the baseline values. In iNOS$^{-/-}$ mice, the bone nodule area after tail suspension also was lower on day 7 relative to the baseline (Fig. 4B). However, the area after reloading remained lower compared with the control on day 21 and baseline value. When the bone nodule areas of tail-suspended mice of the two genotypes were compared, the value on day 21 of iNOS$^{+/+}$ mice was significantly greater than that of iNOS$^{-/-}$ mice (Figs. 4C and 4D).

Immunoreactivity for iNOS and ALP enzyme activity

In iNOS$^{+/+}$ mice, immunoreactivity for iNOS was detected in osteoblasts and osteocytes in the trabecular and endocortical surfaces in all specimens throughout the experimental period. The osteoblasts were apparently flattened and their immunoreactivity for iNOS was positive in specimens harvested on day 7 after tail suspension (Fig. 5A). Twelve hours after subsequent reloading, the osteoblasts were cuboidal and densely immunopositive for iNOS (Fig. 5B). Immunoreactivity for iNOS was negative in specimens from iNOS$^{-/-}$ mice (Fig. 5C). ALP activity was identified in the osteoblastic layers in all specimens (Figs. 5D and 5E). Thickening of the ALP^{+} layer was noted at the trabecular and endocortical surfaces of mice killed 12 h after reloading (Fig. 5E). ALP activities and height of cuboidal osteoblasts subsequently decreased to normal levels in specimens from later periods after reloading.

DISCUSSION

Our study indicated that trabecular bone mass in the proximal tibia of iNOS$^{+/+}$ mice diminished during a 7-day period of tail suspension and then recovered to the initial level during subsequent 14-day normal reloading. The increase in BV induced by reloading was associated with increased BFRs and reduced Oc.S. Mineralized nodule formation from bone marrow cells recovered to the initial levels. However, in iNOS$^{-/-}$ mice trabecular BV, reduced by tail suspension, failed to recover. Bone formation did not increase and neither did the mineralized nodule formation. In iNOS$^{+/-}$ mice, the parameters of bone turnover and volume partially improved during reloading. Administration of AG in iNOS$^{+/+}$ mice inhibited the increase in BV, and suppressed recovery of bone formation. Furthermore, dermal application of NG tape in iNOS$^{-/-}$ mice increased BV, improving turnover to the level of normal-loading controls.

Unloading by tail suspension reduced bone formation and increased bone resorption, consequently leading to decreased trabecular bone mass in both iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. The changes in bone turnover and mineralized nodule formation from bone marrow cells in these C57BL/6J mice were compatible with previous observations in ddy mice.[13] However, in iNOS$^{-/-}$ mice, the response of bone and bone marrow cells to subsequent reloading was blunted, resulting in persistently reduced bone

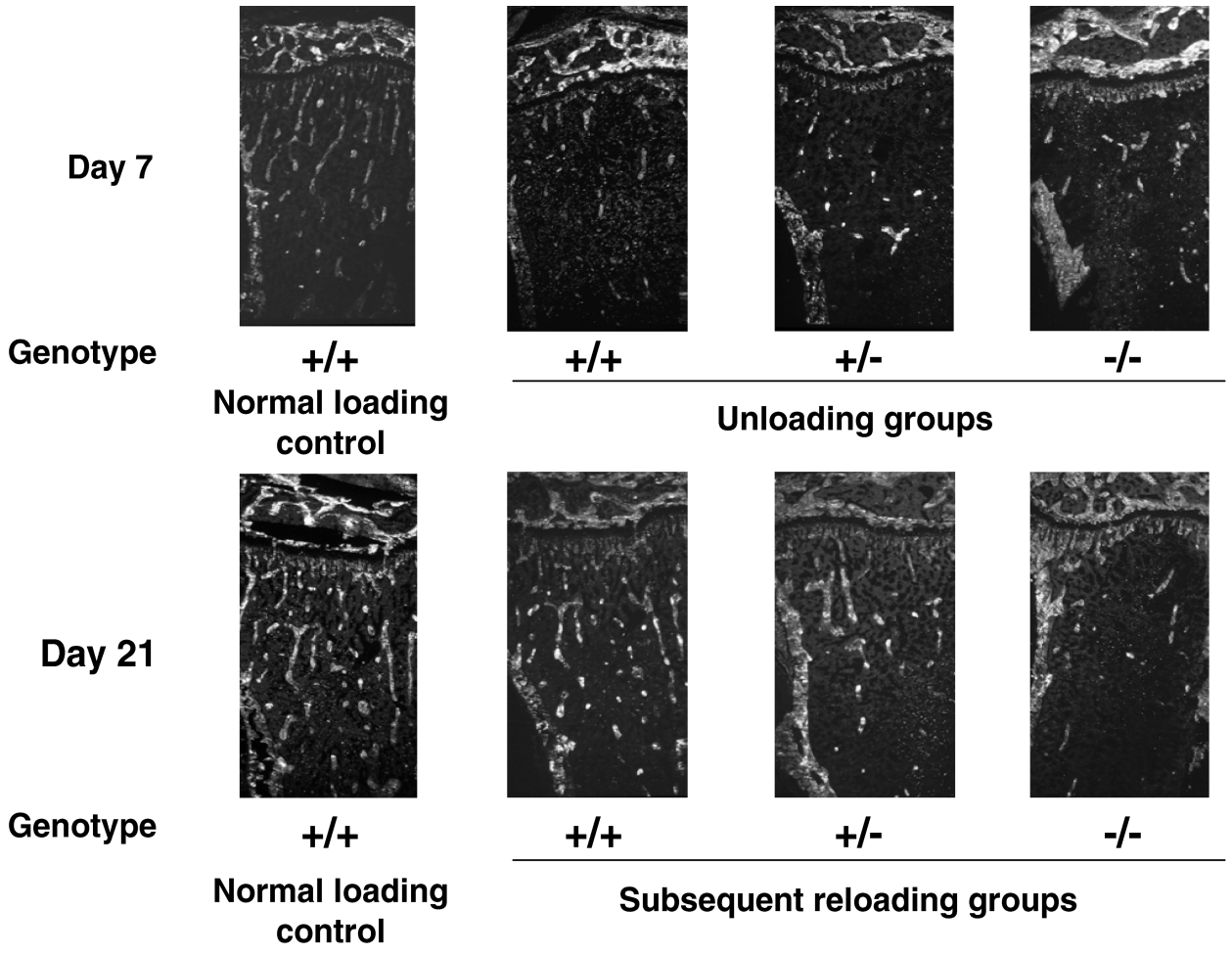

FIG. 2. Trabecular bone in the proximal tibia of iNOS$^{+/+}$, iNOS$^{+/-}$, and iNOS$^{-/-}$ mice. Undecalcified sections under dark-field illumination (magnification ×10).

formation and increased bone resorption. The responses of bone formation and resorption to reloading were blunted also in iNOS$^{+/+}$ mice that were treated with AG, which exerts inhibitory effects on all NOS isoforms and other L-arginine–dependent metabolic pathways distinct from NOS.[36] In iNOS$^{-/-}$ mice, trabecular bone formation apparently recovered after administration of NG, a donor of NO through a metabolic pathway independent of NOS.[37] These data indicate that NO generated through iNOS played a critical role in recovery of bone turnover and bone mass during the period of reloading after tail suspension.

The role of NO in stimulating bone formation induced by mechanical loading has been observed in rats.[37,38] Chow et al.[37] showed that acute compression of the coccygeal vertebral body increased the mineralized surface and trabecular bone formation; both parameters further increased by administration of an NO donor, *S*-nitroso-*N*-acetyl-D, L-penicillamine, or *S*-nitroso-glutathione. In this rat model, the increase in trabecular bone formation was suppressed by pretreatment with L-N^G-monomethyl-arginine, an NO inhibitor, 15 minutes before application of the compressive force to the bone.[38] In this study, we confirmed the rapid increase in ALP activity of osteoblasts after 12 h of reloading in iNOS$^{+/+}$ mice. Immunoreactivity to iNOS was densely positive in osteoblasts and osteocytes. Although the reductions in Oc.S were similar in iNOS$^{+/+}$ and iNOS$^{+/-}$ mice, trabecular bone formation and BV in iNOS$^{+/-}$ mice was at an intermediate level between that observed in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. These results suggest that the degree of increase in bone formation depends on the amount of NO generated in bone cells by acute overloading, such as mechanical compression and reloading after bone mass was decreased by tail suspension.

Recently, we have shown that the numbers of adherent stromal cells and colony-forming unit fibroblastic (CFU-f) colonies were similar in tail-suspended mice and control mice during 7-day tail suspension.[13] In contrast, reloading for 14 days increased total bone marrow cells, adherent stromal cells, and mineralized nodule formation in tibias. These findings indicated that the effect of unloading on osteogenic cells in bone marrow apparently differed from that of subsequent reloading. This study suggested that iNOS had no impact on reduction of bone formation or increased bone resorption during tail suspension. Because

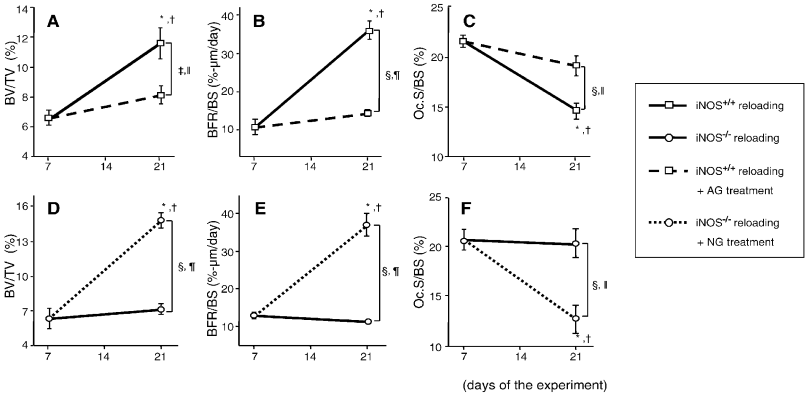

FIG. 3. Changes in iNOS genotypes and effects of AG in iNOS$^{+/+}$ mice and NG in iNOS$^{-/-}$ mice on histomorphometric parameters of trabecular bone in the proximal tibia during normal reloading after tail suspension. (A and D) BV/TV, (B and E) BFR/BS, and (C and F) Oc.S/BS. Data are presented as mean ± SEM. $^{*}p < 0.01$, compared with day 7 unloading mice in same genotype by Tukey-Kramer post hoc test after one-way ANOVA and $^{\dagger}p < 0.01$ by Mann-Whitney U test, respectively. $^{\ddagger}p < 0.05$; $^{\S}p < 0.01$, between AG or NG-treated and untreated reloading mice by Tukey-Kramer post hoc test after one-way ANOVA and $^{\|}p < 0.05$ and $^{\P}p < 0.01$ by Mann-Whitney U test.

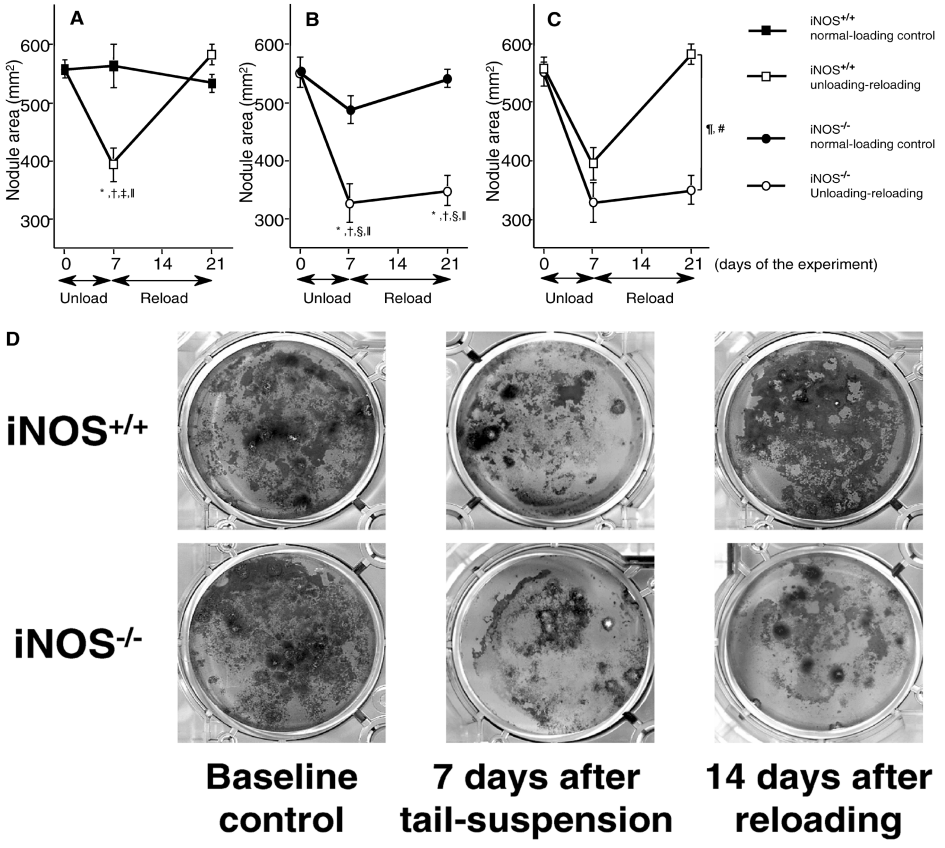

FIG. 4. Changes in bone nodule area in bone marrow cultures in (A) iNOS$^{+/+}$ and (B) iNOS$^{-/-}$ mice during unloading and subsequent reloading experiments. The values of bone nodules were compared between unloading-reloading iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (C). Data are presented as mean ± SEM ($n = 4$). $^{*}p < 0.01$ and $^{\dagger}p < 0.05$ compared with the controls of the same genotype at baseline by Tukey-Kramer post hoc test after one-way ANOVA and Mann-Whitney U test, respectively. $^{\ddagger}p < 0.05$ and $^{\S}p < 0.01$ compared with the controls in the same genotype during the same experimental period by Student's t-test, and $^{\|}p < 0.05$ by Mann-Whitney U test. $^{\P}p < 0.01$ and $^{\#}p < 0.05$ between unloading-reloading iNOS$^{+/+}$ mice and iNOS$^{-/-}$ mice by Student's t-test and Mann-Whitney U test, respectively. (D) Dark red–stained areas represent mineralized nodules.

the development of osteoclasts is regulated by osteogenic cells,[39] it is reasonable to suggest that the increase in bone resorption during unloading and its reduction during subsequent reloading are both secondary to the effects on the development of osteoblasts.

Previous studies have indicated that although immunolocalization of eNOS was detected in flat bone lining cells, osteocytes,[40] and cuboidal osteoblasts,[41] iNOS expression was not detected in any bone cells of osteoblastic lineage in normal adult rats.[40,41] We have detected iNOS

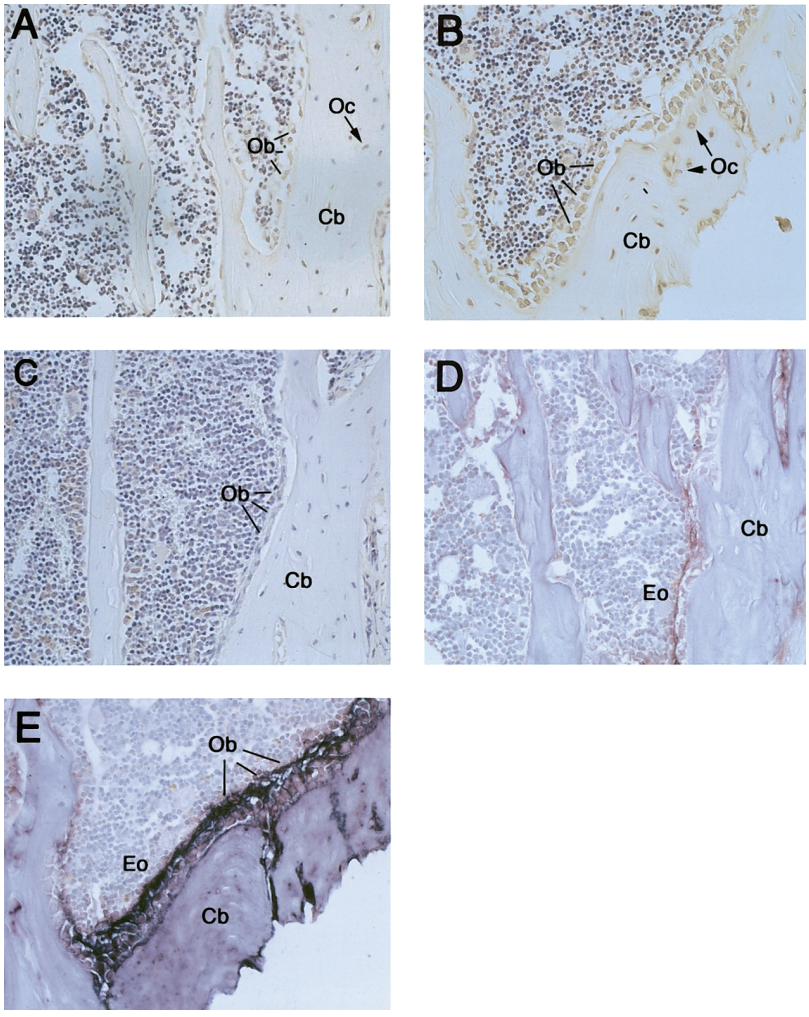

FIG. 5. Immunoreactivity for iNOS and enzyme activity of ALP in the proximal tibia during reloading after tail suspension. Adjacent sections (magnification ×65) obtained after (A and D) 7 days of unloading and (B and E) 12 h after subsequent reloading were (A and B) immunostained with an anti-iNOS polyclonal antibody (1:500 dilution) and (D and E) stained with ALP. iNOS was localized in osteocytes and osteoblasts. (C) Immunoreactivity for iNOS was negative in specimens from $iNOS^{-/-}$ mice. ALP was localized in the endosteum and osteoblasts. Note that endosteal osteoblasts exhibited cuboidal shapes after reloading. Specific regions are indicated as follows: Eo, endosteum; Cb, cortical bone; Ob, osteoblast; Oc, osteocyte.

protein in osteoblasts and osteocytes during the unloading-reloading experimental period. In our study, it should be noted that iNOS expression was observed in the limited conditions of unloading and reloading but not in the normal-loading state. Some studies have observed iNOS expression in bone cells by cytokine stimulation,[40] with experimental colitis[41] and in neonates.[42] Furthermore, bone turnover in $iNOS^{-/-}$ mice is apparently different from that in $eNOS^{-/-}$ mice. $eNOS^{-/-}$ mice exhibit marked reduction in trabecular BV in the distal femur until the age of 20 weeks,[26] compared with values in $eNOS^{+/+}$ mice.[25] Trabecular BFRs and mineralized surfaces measured by fluorescence labeling are retarded until the age of 8 weeks.[25] Mineralized nodule formation from bone marrow cells of the hindlimbs was reduced also. However, in this study in $iNOS^{-/-}$ mice, the normal-loading controls at the ages of 9–11 weeks did not exhibit any reduction in trabecular BV or bone formation. In addition, mineralized nodule formation at 8 weeks of age was not reduced. These may be speculated that although eNOS contributes to the physiological control of bone turnover such as bone growth[25] and the bone-conserving action of estrogen,[26] iNOS activity plays a critical role in adjusting bone turnover to acute increases in mechanical loading. Because of normal bone growth in $iNOS^{-/-}$ mice, we expect that this suppressed bone formation will recover gradually if allowed to reload for a longer period.

In conclusion, in this study we showed that unloading by tail suspension reduced bone formation and increased bone resorption, consequently leading to decreased trabecular bone mass in both $iNOS^{+/+}$ and $iNOS^{-/-}$ mice. However, in $iNOS^{-/-}$ mice the response of bone and bone marrow cells to subsequent reloading was blunted, resulting in persistent reduction in bone formation and increased bone resorption. Administration of an NO donor to $iNOS^{-/-}$ mice and of an NO inhibitor to $iNOS^{+/+}$ mice simulated the changes in bone mass and turnover during reloading. iNOS plays a critical role in adjusting bone turnover in response to an acute increase in mechanical loading.

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