

Bone Re/Modeling Is More Dynamic in the Endothelial Nitric Oxide Synthase^(-/-) Mouse

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Nitric oxide is a ubiquitous estrogen-regulated signaling molecule that has been implicated in the regulation of bone maturation and remodeling. To better understand the role that bone-cell-secreted nitric oxide plays in ovariectomy-induced modifications of bone turnover, we examined the expression of endothelial NO synthase (eNOS) in bone cells and bone progenitor cells at regular intervals up to 10 wk after acute estrogen deprivation. Ovariectomy led to an anticipated initial decline in bone cell eNOS production, but surprisingly, 17 d after ovariectomy, eNOS expression by bone and marrow stromal cells dramatically rebounded and was maintained at high levels for at least 10 wk after surgery. We examined the long-term consequences of eNOS in the process of ovariectomy-induced bone loss by prospectively analyzing bone min-

eral density in wild-type and eNOS^(-/-) mice for 10 wk after ovariectomy. Ovariectomized eNOS^(-/-) mice were observed to undergo an exaggerated state of estrogen-deficiency-induced bone remodeling compared with wild-type controls, suggesting that eNOS may act to mitigate this process. Furthermore, we found that whereas bone formation in estrogen-replete wild-type mice slowed between 14 and 20 wk of age, eNOS knockout mice continued to accrue basal bone mass at a high rate and showed no sign of entering a remodeling stage. Our data suggest that eNOS may play an important role in limiting ovariectomy-induced bone remodeling as well as regulating the transition from basal modeling to remodeling. (*Endocrinology* 147: 4392–4399, 2006)

MODELING IS THE process whereby the skeleton is sculpted to achieve its shape and size by the removal of bone from one site and deposition at a different one (1). In mice, peak bone density typically occurs at around 4 months of age (2) with peak bone strength being achieved by 5 months (3). Once the skeleton has reached maturity, the periodic replacement of old bone with new occurs through a process of remodeling. The signals that regulate the transition from modeling to remodeling are not well understood. Bone resorption and formation occur simultaneously, and new osteoblasts assemble only at sites where osteoclasts have recently completed resorption, a phenomenon referred to as coupling (1). Pathological conditions can lead to a loss of coupling between osteoclasts and osteoblasts. One such condition is postmenopausal osteoporosis, which is induced by the loss of circulating estrogen. Estrogen deficiency leads to an acceleration of bone remodeling where osteoclastic bone resorption outpaces the anabolic activity of osteoblasts. The mechanisms that regulate accelerated bone turnover during estrogen deficiency are complex and multifactorial.

Nitric oxide is a ubiquitous signaling molecule that has

been implicated in the regulation of both bone modeling and remodeling (4–6). NO levels *in vivo* are believed to be, at least in part, under estrogenic control because endothelial nitric oxide synthase (eNOS), the predominant isoform in bone (7), is directly activated by estrogen in a variety of cell types via a MAPK-dependent mechanism (4, 5). Moreover, estrogen can up-regulate eNOS expression in human osteoblastic cells (6), suggesting that increased eNOS levels could be part of the anabolic osteoblast response to estrogen (8). In keeping with this hypothesis, several laboratories have shown that eNOS^(-/-) transgenic mice have marked abnormalities in bone formation during skeletal development (9–11). As well, interactions between estrogen and NO have been shown in bone remodeling; long-term use of NO donors prevents bone loss in both postmenopausal women (12) as well as in ovariectomized rats (13). Women treated with nitrates on a daily basis have greater hip and heel bone mineral density (BMD) (14). Thus, NO appears to be able to modulate responses to estrogen in the skeleton.

NO has pleiotropic effects on bone cells *in vitro*. In osteoblast and osteoblast-like cells, NO promotes differentiation (15) and increases proliferation (16). As well, NO inhibits both osteoclast formation (17) and activity (18). The source of NO production in bone cells is largely due to eNOS, but inflammatory NOS and neuronal NOS (nNOS) are found during fetal development and fracture repair (7, 19, 20). A study of multiple human osteoblast cell lines suggested that eNOS but not nNOS was constitutively expressed and that inflammatory NOS was expressed only after cytokine stimulation (21). Nevertheless, nNOS has been shown to play a

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Abbreviations: BMD, Bone mineral density; μ CT, microcomputed tomography; 3D, three-dimensional; DEXA, dual-energy x-ray absorptiometry; eNOS, endothelial nitric oxide synthase; KO, knockout; nNOS, neuronal NOS; TRAP5b, tartrate-resistant acid phosphatase 5b; WT, wild type.

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role as a stimulator of bone turnover *in vivo* potentially through a neurogenic relay (22).

Osteoblasts taken from eNOS^(-/-) transgenic animals show reduced proliferation, differentiation, and mineralization that may contribute to formation deficits (9, 10). Although histomorphometric analysis of femurs taken from young eNOS^(-/-) animals demonstrates decreased bone volume and mineralization, the differences have disappeared by 8–12 wk (9, 11). Importantly, this is a time when peak bone density is typically achieved in wild-type (WT) mice (2) and bone modeling is transitioning to a remodeling phase. The ability of the eNOS-deficient animal to recover from an early deficit in bone mineral suggests that eNOS may support different functions during bone development than during adult skeletal remodeling.

In this study, we examined the expression of eNOS in bone cells and bone progenitor cells at regular intervals up to 10 wk after acute estrogen deprivation. Although estrogen deficiency in WT mice led to an initial decline in eNOS expression, 17 d after ovariectomy, eNOS expression dramatically rebounded and was maintained at high levels for at least 10 wk. Furthermore, eNOS knockout (KO) mice underwent an exaggerated bone remodeling after ovariectomy, leading to a larger net difference in bone mass than measured in WT controls. Interestingly, we found that whereas the rate of bone accumulation reached plateau in estrogen-replete WT mice at the time of the transition of modeling to remodeling, between 12 and 14 wk of age, eNOS^(-/-) mice continued to accrue bone mass well into the predicted remodeling phase, resulting in an enhanced basal BMD relative to WT controls. Our data suggest that eNOS may play a relevant role in regulating the transition from physiological bone modeling to remodeling as well as moderating the magnitude of stimulated bone remodeling after estrogen deprivation.

Materials and Methods

Materials

Culture media, antibiotics, reverse transcriptase, and *Taq* polymerase were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Hyclone (Logan, UT). RNA isolation kits and DNase I were purchased from QIAGEN (Valencia, CA) and random primers from Ambion (Austin, TX). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise.

Animals

All procedures were approved and in compliance with the Institutional Animal Care and Use Committee at Emory University and the Veterans Affairs Medical Center Atlanta. eNOS^(-/-) female mice (23) purchased from the Jackson Laboratory (Bar Harbor, ME) had been backcrossed for at least 10 generations onto the C57BL/6 background. Genetically matched WT C57BL/6 female mice were purchased from the same source. At 10 wk of age, WT and eNOS^(-/-) mice were ovariectomized or sham operated. For intermediate endpoints, three to four mice per group were killed to collect bone marrow and serum. For BMD and microcomputed tomography (μ CT) measurements, groups of 20 mice from each genotype were either ovariectomized or sham operated and BMD measured at 2-wk intervals up to 10 wk after surgery. Ten weeks after surgery, mice were killed to collect bones for μ CT and serum for quantitation of biochemical markers of formation and resorption.

Cell culture

Bone marrow cells were collected from tibias and femurs of female C57BL/6 WT or eNOS^(-/-) mice. Primary stromal cell cultures were plated as previously described (24). Cells were cultured in α -MEM/10% fetal bovine serum for 5 d before extraction of total RNA.

RNA isolation and real-time PCR

For stromal cell cultures, total RNA was extracted followed by the QIAGEN RNeasy mini-kit protocol. All bone samples were stored in RNAlater solution (Ambion). Total RNA was isolated from bone using Trizol reagent (Invitrogen). All samples were treated with DNase I to remove any contamination by genomic DNA. For RT, 1 μ g total RNA was used in a 20- μ l reaction. Real-time PCR was performed using the Bio-Rad iCycler (Hercules, CA). Amplification reactions were performed in 25 μ l containing 0.5 μ M primers, dNTPs (0.2 mM each) in PCR buffer, and 0.03 U *Taq* polymerase along with SYBR-green (Molecular Probes, Eugene, OR) at 1:150,000. Aliquots of cDNA were diluted 10- to 10,000-fold to generate relative standard curves to which sample cDNA was compared. Standards and samples were run in triplicate. eNOS and 18S primers were used as described previously (25, 26). PCR products from all species were normalized for the amount of 18S in the same RT sample, which was also standardized on a dilution curve from the RT sample.

Bone densitometry

Total body and femoral BMD was determined *in vivo* in 10-wk-old mice (baseline) and 4, 6, 8, and 10 wk after surgery using a PIXImus2 bone densitometry instrument (GE Medical Systems, Madison, WI), as described in (27). The short-term reproducibility of this technique is 0.9% (28). Data points represent the average of 10 mice per group.

μ CT analysis

Changes in bone structure and morphology were evaluated from three-dimensional (3D) core images from whole bones reconstructed from individual μ CT slices, as described with modifications (29–31). Briefly, after careful dissection, the right distal femur was fixed in 10% neutral buffered formalin overnight and stored in 70% ethanol at 4 C until analysis. μ CT analysis was performed using a μ CT 40 scanner (Scanco Medical, Bassersdorf, Switzerland) by an operator blinded as to the nature of the specimens. Bones were scanned at a resolution of 12 μ m. Histomorphometric indices were calculated using 50 slices proximal to the growth plate (600 μ m). A representative sample from each group was used for cross-sectional 3D image reconstruction based on 250 slices for a total 3 mm in length. Trabecular static measurements were made using a cylindrical core sample that excluded cortical bone, with contouring for all subsequent slices. Cortical bone was measured at the femoral mid-diaphysis by taking 80 slices at a resolution of 12 μ m. Histomorphometric data were calculated using the Image Processing Language package provided by the manufacturer. One representative sample from each group clustering closest to the average indices in the histomorphometric analysis was selected and a longitudinal section virtually cut through the 3D image to generate a visual representation.

Osteocalcin assay

Osteocalcin was quantitated in mouse serum using a specific ELISA (Biomedical Technologies Inc., Stoughton, MA) according to manufacturer's instructions.

Tartrate-resistant acid phosphatase 5b (TRAP5b) assay

Mouse TRAP5b was assayed with the TRAP5b ELISA kit (SBA Sciences, Turku, Finland) according to the manufacturer's protocol.

Statistical analysis

All cross-sectional data were analyzed by ANOVA and Fisher protected test or the Kruskal-Wallis test for data not normally distributed. Prospective data were analyzed by ANOVA for repeated measures. Multiple comparison tests were performed by the Fisher protected test.

Simple comparisons were made by using a two-tail unpaired Student's *t* test.

Results

eNOS is up-regulated in bone marrow stromal cells as a late response to ovariectomy

To better understand the role that bone-cell-secreted nitric oxide plays in ovariectomy-induced bone turnover, we examined the expression of eNOS in bone cells and bone progenitor cells after acute estrogen deprivation. We measured eNOS mRNA expression in cortical bone cells, comprised largely of osteocytes, and in bone marrow stromal cells at both early (4 d) and late (17 d) time points after ovariectomy (Fig. 1). eNOS expression was significantly down-regulated in cortical bone shortly after ovariectomy (4 d), as shown in Fig. 1A, consistent with evidence that estrogen positively regulates eNOS expression (6, 32, 33). Expression of eNOS by stromal cells, studied 4 d after collection to allow for separation from other marrow elements, did not show a similar decrease. At this time of collection, cells from both genotypes were plated at similar densities and when counted 4 d later, showed $225,000 \pm 18,000$ and $244,000 \pm 17,000$ for the WT and KO stromal cells, respectively ($n = 8$). However, at 17 d after ovariectomy, a time when formation is expected to be activated as a coupled response to the acute resorptive phase, eNOS was strongly up-regulated by about 3-fold both in cortical bone cells and in the marrow stromal cell population (Fig. 1B). These data suggest that eNOS is involved in the

coupled bone formation as a late response set into motion by the resorption induced by estrogen deprivation.

To test whether up-regulation of eNOS was a sustained response to ovariectomy, we analyzed eNOS expression in bone marrow stromal cells by a careful time course. WT mice were operated at 10 wk of age and eNOS mRNA expression measured in stromal cells harvested from bone for up to 10 wk after surgery (final at 20 wk of age). Consistent with our first observations, eNOS mRNA expression was strongly up-regulated in ovariectomized compared with sham mice at all time points after surgery (Fig. 2). This response peaked at 4 wk at 4-fold in ovariectomized compared with sham-operated mice and remained elevated for at least 10 wk. Thus, up-regulation of eNOS may be part of a compensatory mechanism by which a bone deficit is attenuated after estrogen depletion.

eNOS deficiency leads to exaggerated differences in BMD after ovariectomy and promotes enhanced basal bone density

Given the extensive rebound in eNOS production by 17 d after ovariectomy, we further examined BMD prospectively for 10 wk after ovariectomy in WT and eNOS^(-/-) mice. Animals were subjected to ovariectomy at 10 wk of age and BMD analyzed every 2 wk by dual-energy x-ray absorptiometry (DEXA) for an additional 10 wk. The data show (Fig. 3) that ovariectomy completely halted the acquisition of total body BMD in both WT and eNOS^(-/-) animals for at least 10 wk after surgery. As previously reported (10), eNOS^(-/-) mice mirrored WT mice in their response to ovariectomy for the first 4 wk after surgery, leading to an expected approximately 5% deficit in BMD in both ovariectomized groups relative to WT controls at that time (Fig. 3A); estrogen deprivation was observed to completely stall acquisition of BMD over this period. The data are represented as a percent change from sham-operated animals in Fig. 3B, where the decrement due to ovariectomy is more pronounced in the eNOS^(-/-) animal. Data for the total-body BMD were confirmed in the femur as well, as shown in Fig. 3, C and D; in

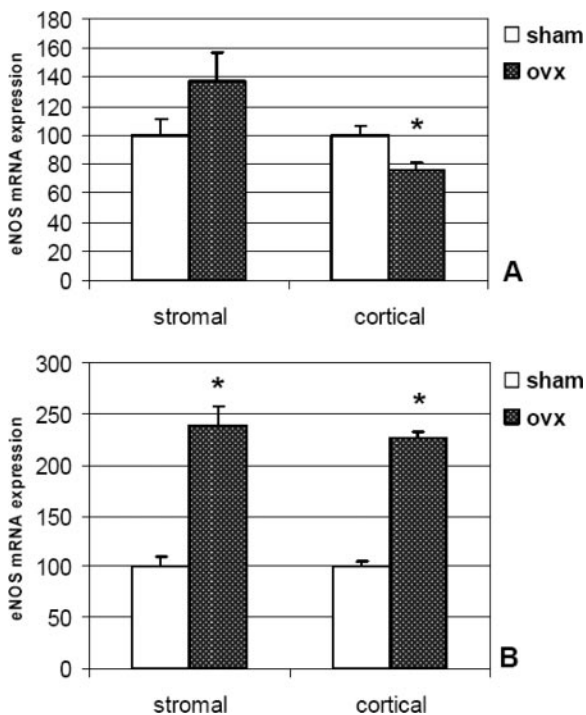


FIG. 1. eNOS mRNA expression in cortical bone cells from sham-operated or ovariectomized (ovx) WT mice at early (4 d) (A) and late (17 d) (B) time points after surgery in bone marrow stromal cells and cortical bone cells. Data are expressed as percent change from 100% in the cells taken from sham-operated animals. The values represent average \pm SEM, with $n = 8$ –11 for all. *, $P < 0.01$ vs. sham.

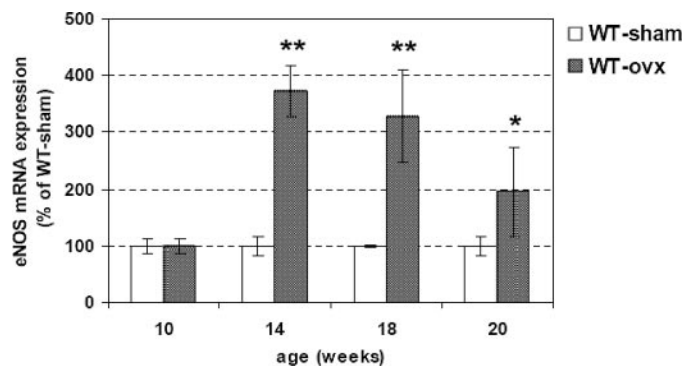


FIG. 2. eNOS mRNA expression in bone marrow stromal cells from ovariectomized (ovx) mice (percent increase compared with eNOS mRNA expression by sham mice) at the indicated time points after surgery. Mice were operated at 10 wk of age. Data are expressed as percent change from 100% in the cells taken from sham-operated animals. The values represent average \pm SEM, with $n = 3$ –4 for each group. *, $P < 0.05$ vs. sham; **, $P < 0.01$ vs. sham mice.

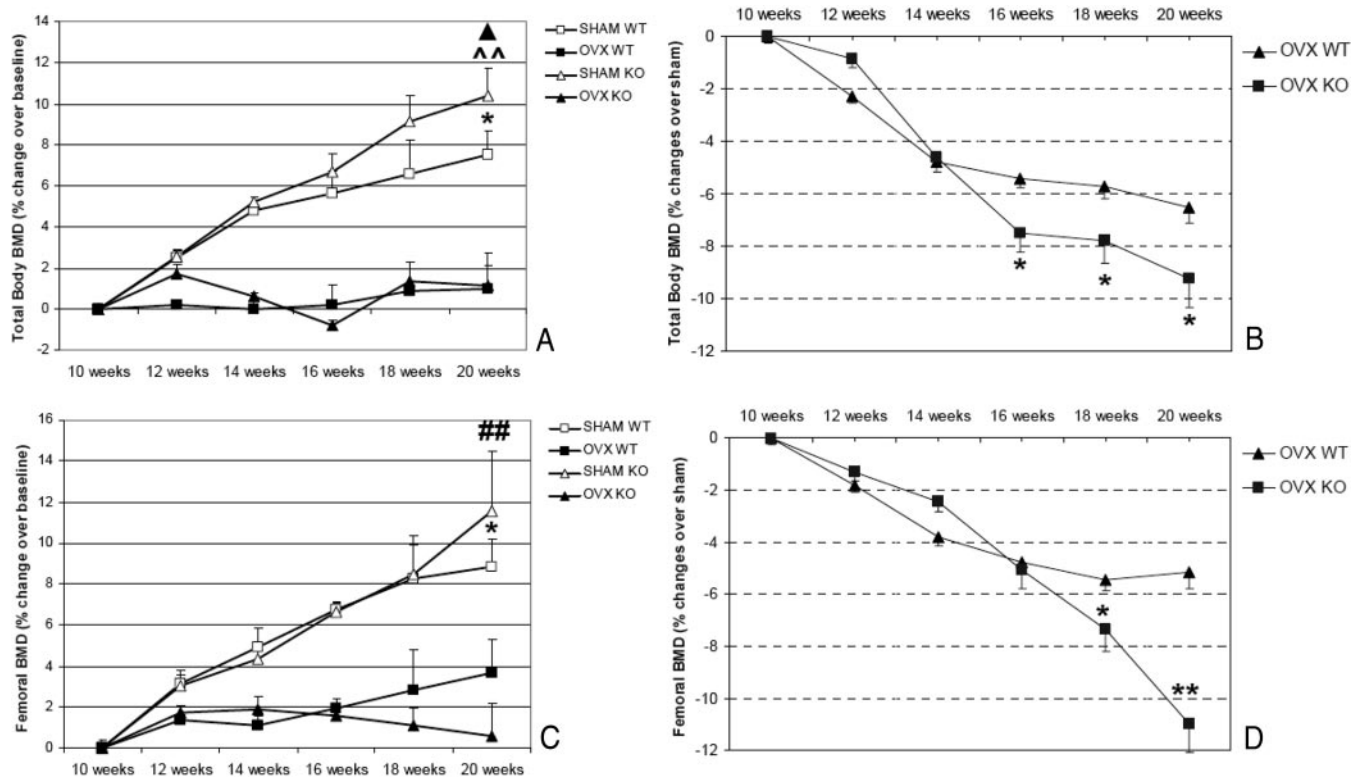


FIG. 3. Effect of ovariectomy (OVX) in WT and eNOS^(-/-) (KO) mice. DEXA measurement of BMD in total body at the indicated time points after surgery. A and B, Data for total-body BMD; C and D, femoral BMD. Data are expressed as percent change over baseline (A and C) or percent change over sham mice BMD (B and D). The values represent average \pm SEM, with $n = 6$ –10 for each group. *, $P < 0.05$ vs. WT OVX mice; ▲, $P < 0.01$ vs. eNOS^(-/-) OVX mice; ▲▲, $P < 0.05$ vs. eNOS^(-/-) sham mice; ##, $P < 0.05$ vs. sham mice.

sham-operated animals, the femoral BMD continued to rise after 10 wk but was nearly flat in ovariectomized mice.

Between 14 and 16 wk of age, a time when maximal BMD is attained in WT mice (2) and bone modeling transitions to remodeling, BMD in the estrogen-replete WT animals was seen to plateau. In contrast, eNOS^(-/-) sham-operated animals continued to accrue BMD at a high rate between 10 and 20 wk of age, leading to a significant difference in BMD between estrogen-replete WT and eNOS KO mice by 10 wk. These data suggest that loss of eNOS prevented or delayed the transition from modeling to remodeling. Moreover, the absence of eNOS compounded the skeletal deficit caused by estrogen deficiency, leading to a net increase in the deficit of bone mineral measured as total or femoral BMD (Fig. 3, B and D).

Because changes in body weight can impact BMD, we also assessed body weight in both mice strains at each time point. Body weights in WT and eNOS^(-/-) groups remained within 2.5% of each other between 14 and 20 wk (Table 1), the time points at which maximal differences in BMD were observed between WT and eNOS^(-/-) mice.

eNOS deficiency leads to an exaggerated decrease in bone volume after ovariectomy and promotes enhanced basal bone acquisition

To confirm our findings and allow discrimination between cortical and trabecular bone compartments, we further analyzed bone volume and cortical thickness by μ CT at 10 wk after surgery. As shown in Fig. 4, ovariectomized eNOS^(-/-) mice displayed a more statistically significant deficit in both trabecular volume (BV/TV, Fig. 4A) and trabecular thickness (Fig. 4B) than WT mice, compared with the respective sham groups. Importantly, cortical thickness (Fig. 4C) was also significantly altered in the absence of eNOS, because ovariectomized eNOS^(-/-) mice showed nearly twice as great a deficit in cortical thickness than WT when compared with sham mice of the same strain. Interestingly, we also observed a significantly greater ($P < 0.05$) cortical thickness in eNOS^(-/-) sham-operated mice compared with sham wild-type animals. Representative 3D reconstructions of the μ CT images for each group are shown in Fig. 4D.

Our μ CT data confirm the results from the DEXA studies

TABLE 1. Mouse body weight at the indicated time points

	Body weight (g) at baseline (10 wk)	Body weight (g) at 14 wk		Body weight (g) at 20 wk	
		Sham	OVX	Sham	OVX
WT	15.94 \pm 1.26	17.77 \pm 1.3	19.06 \pm 1.35	18.78 \pm 1.22	20.43 \pm 1.39
eNOS ^(-/-)	15.28 \pm 0.76	17.39 \pm 0.95	19.49 \pm 1.56	19.26 \pm 1.05	20.97 \pm 1.74

Data are expressed as mean \pm SD. There was no significant difference in any group. OVX, Ovariectomized.

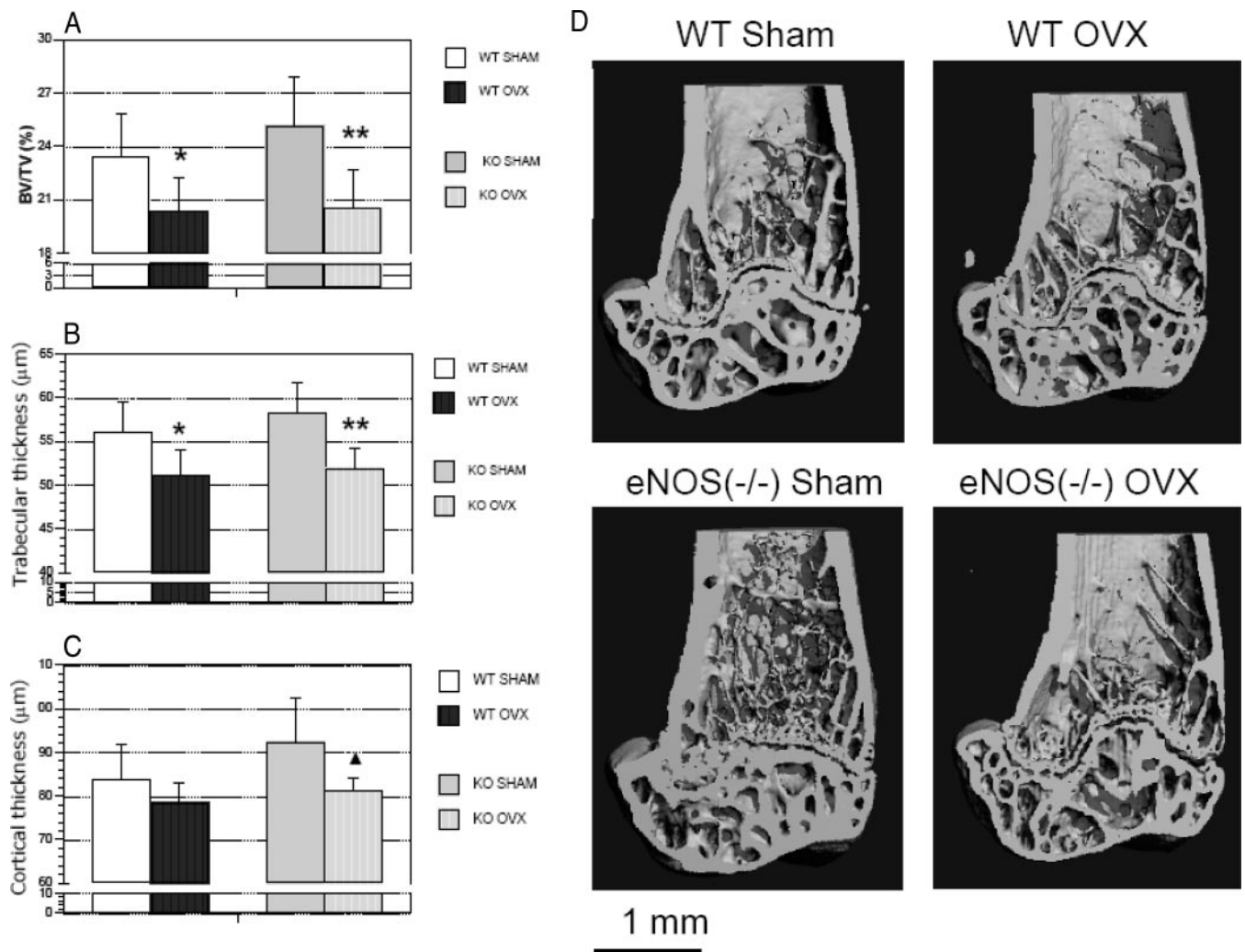


FIG. 4. μ CT analysis of femoral bone volume at 10 wk after surgery. A, Trabecular volume (BV/TV); B, trabecular thickness; C, cortical thickness in each experimental group. *, $P < 0.05$ vs. WT sham mice; ** = $P < 0.01$ vs. eNOS^(-/-) sham; ▲, $P < 0.05$ vs. eNOS^(-/-) sham. D, 3D images of representative bones from each group of WT and eNOS^(-/-) mice. The values represent average \pm SEM, with $n = 6$ –10 for each group. OVX, Ovariectomized.

and demonstrate increased basal cortical bone mass in eNOS^(-/-) mice. In addition, in the absence of ovarian estrogen, the enhanced rate of bone density accumulation in eNOS^(-/-) mice was completely stalled, leading to a more pronounced reduction in cortical bone mass relative to ovariectomized WT mice. These data suggest that eNOS plays an important role in regulating the accelerated remodeling set into motion by estrogen deficiency.

eNOS deficiency leads to an exaggerated bone turnover after ovariectomy and promotes enhanced basal bone turnover

To further investigate basal and ovariectomy-induced bone turnover in the absence of eNOS, we analyzed serum osteocalcin and TRAP5b, specific *in vivo* metabolic markers of bone formation and resorption, respectively. Groups of estrogen-replete (sham-operated) and estrogen-deficient (ovariectomized) WT and eNOS^(-/-) mice were killed every 2 wk from 10 wk of age up to 20 wk. Serum osteocalcin levels were significantly higher in eNOS^(-/-) sham compared with

their WT matched groups at baseline and at 14 wk and remained higher for the duration of the experiment (Fig. 5). These findings support our *in vivo* data indicating an enhanced rate of bone acquisition as a result of the loss of eNOS. Moreover, although estrogen-replete eNOS^(-/-) mice showed no significant change in osteocalcin levels throughout the experiment, ovariectomized eNOS^(-/-) mice showed a significant increase in osteocalcin levels 4 wk after surgery, at 14 wk, which declined to baseline by the next sampling at 18 wk. Therefore, a deficiency of eNOS in adult mice appears to result in increased osteoblast activity consistent with accelerated growth that responds more dynamically to the imposition of estrogen deficiency on bone remodeling.

Similar to osteocalcin concentrations, levels of serum TRAP5b, a marker of osteoclastic activity (34), in estrogen-replete (sham) eNOS^(-/-) mice were consistently higher than in estrogen-replete WT mice at all time points and showed no significant change during the experiment (Fig. 6). However, ovariectomized eNOS^(-/-) mice showed a robust in-

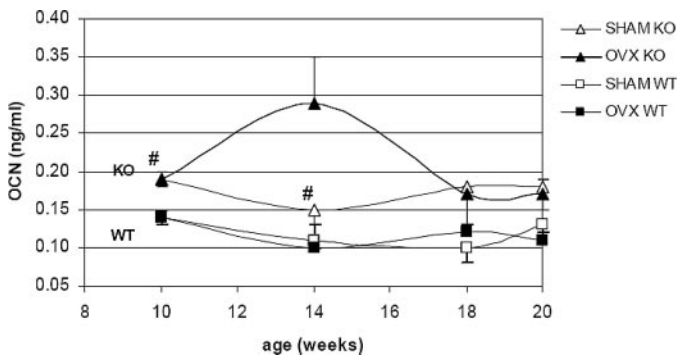


FIG. 5. Serum osteocalcin concentrations. At each time point, mice were killed and osteocalcin was determined in serum. Osteocalcin (OCN) was significantly different, with eNOS^(-/-) greater than WT at baseline and 14 wk (#, $P < 0.05$) but did not reach significance thereafter. Within genotypes, there was a trend toward increase after ovariectomy (OVX) at 14 wk only within the eNOS^(-/-) group. The values represent average \pm SEM, with $n = 3-4$ for each group.

crease in TRAP5b in response to ovariectomy early after surgery (age 14 wk), which steadily declined during the next 6 wk to be significantly below intact eNOS^(-/-) animals at 18 and 20 wk.

Taken together, the response of the metabolic markers support the notion that basal bone turnover is significantly elevated in eNOS^(-/-) mice and that an exaggerated bone turnover is elicited by estrogen deprivation.

Discussion

Although it is clear that nitric oxide has the ability to regulate aspects of bone remodeling, a specific role for this ubiquitous molecule in bone biology is not defined. Previous work has suggested that nitric oxide might be critical for a full anabolic response of bone cells to estrogen (6, 10). Our study investigated further the role of eNOS in the transition from modeling to remodeling in growing mice as well as the long-term remodeling response to estrogen deficiency.

The mechanisms that regulate the transition from modeling to remodeling are poorly characterized. Both our DEXA

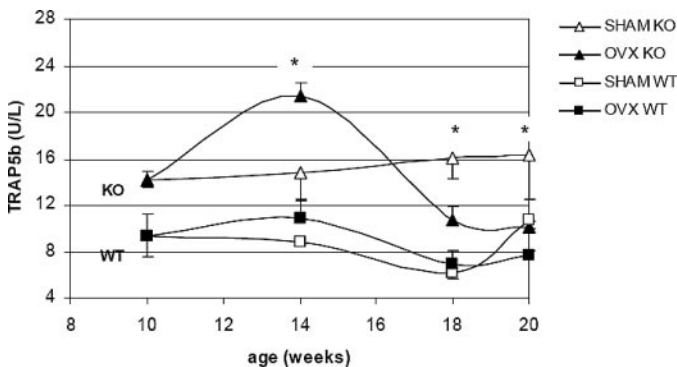


FIG. 6. Serum TRAP5b concentrations at the indicated time points. At each time point, mice were killed and TRAP5b was determined in serum. TRAP5b was significantly higher in all groups of eNOS^(-/-) compared with WT animals at all time points ($P < 0.05$). Within the eNOS^(-/-) group, ovariectomy (OVX) caused a significant increase in TRAP5b at 14 wk and a significant decrease compared with intact animals at 18 and 20 wk as shown (*, $P < 0.05$). The values represent average \pm SEM, with $n = 3-4$ for each group.

and cortical μ CT data suggest that estrogen-replete eNOS^(-/-) mice continue to accumulate bone mass at essentially the same rate up to at least 5 months of age. In contrast, bone accrual in WT C57BL/6 mice slows around 14–16 wk of age as modeling transitions to remodeling. Although we did not perform histomorphometric analysis to confirm this in the eNOS^(-/-) mouse, the continued growth supports our contention that this mouse has not transitioned because it shows no evidence of slowed skeletal growth up to 20 wk. This suggests that eNOS may play an important role in regulating the slowing of bone turnover during this conversion. It should be pointed out that in mice, bone accumulation plateaus around 4–5 months but that homeostasis is never actually achieved because mice continue to accrue bone mass, albeit at a slower rate, throughout life.

Histomorphometric and DEXA analysis of bones from young eNOS^(-/-) animals show decreased bone volume and mineralization. These age-related bone abnormalities in the femora and spine have been shown to catch up to WT animals by 8–12 wk of age (9, 11). Our observations that BMD in eNOS^(-/-) mice has equilibrated with that of the WT by 10 wk of age is consistent with these studies and in good agreement with bone density measurements by DEXA at the same site as others (9) in 12-wk-old mice. In contrast, Armour *et al.* (10) reported a significantly lower total bone density and cortical thickness at 20 wk of age in eNOS^(-/-) mice compared with WT mice. The reason for this difference is not clear; however, those measurements were taken by analyzing mice tibias by peripheral quantitative computed tomography, whereas our data were obtained by μ CT analysis of the femoral bone as well as by DEXA. It is also possible that the different strategy used for deleting the eNOS gene in the Armour study (10) compared with the strain we used (23) may further account for the difference observed at this late time point. There are at least three strains of eNOS^(-/-) mice available made with different exon deletion strategies: 1) that used in our studies (23), 2) one made by Huang *et al.* (35) and used in Afzal *et al.* (11), and 3) that of Godecke *et al.* (36), which was used in Armour *et al.* (10). These transgenic mice do not express eNOS and have similar lesions across organ systems. It is possible that there are skeletal variations in the colonies available for skeletal research.

Estrogen deficiency results in an early, accelerated bone loss that is followed by a plateau phase in humans (37) as well as in rodents (38). In the early response, increased resorption by osteoclasts outpaces new bone formation, but eventually bone cells reach a new steady state where formation more equally balances resorption. We have shown that eNOS expression, the isoform most prevalent in bone (7), is strongly up-regulated in marrow stromal cells by 2 wk after estrogen depletion and is sustained for at least several weeks. This increase in eNOS expression contrasts with an early significant decrease in eNOS expression in the cortical bone cells, which is consistent with the known ability of estrogen to increase levels of eNOS (6, 8). This suggested that the later increase in eNOS levels in the marrow compartment containing new osteoblastic progenitors might be part of a repair process initiated in response to the osteoclastic bone resorption. Indeed, nitric oxide is implicated in the regulation of bone homeostasis (9, 10, 39) as well as in the response of bone

cells to mechanical stress (40–42). The ability of nitric oxide to stimulate osteoblast function and to alleviate ovariectomy-induced bone loss (13, 43, 44) is an indication that NO might be an important part of the repair response taking place in bone late after ovariectomy-induced bone resorption.

To further elucidate the effect of eNOS deletion on skeletal modeling and estrogen-deficiency remodeling, we performed measurement of serum osteocalcin to assess osteoblast activity and serum TRAP5b, a validated marker of bone resorption (34), throughout the experiment. In keeping with the analysis of bone content by DEXA and μ CT showing greater bone formation during 10–20 wk of age, both markers were higher in eNOS^(-/-) mice than WT mice at all time points during the experiment. Our data support an increased bone modeling in the eNOS^(-/-) animal preceding ovariectomy, which is sustained during the next 10 wk of adult life. Furthermore, the exaggerated increase in both osteocalcin and TRAP5b observed after ovariectomy in the eNOS^(-/-) animal relative to WT suggests that there may be an increased rate of bone turnover induced by estrogen deficiency in the absence of eNOS. Although our measurements of these two bone markers were not sensitive enough to show significant changes at 4 wk after ovariectomy in the WT group, the exaggerated compensatory response triggered in the eNOS KO mice was readily detectable, indicating a more dynamic underlying modeling in this animal.

In summary, when estrogen deficiency is induced, up-regulation of eNOS in bone cells appears to be part of a compensatory mechanism that follows the acute resorptive phase. Estrogen depletion entirely halts bone acquisition for at least 10 wk in both eNOS-deficient and WT animals. Because the eNOS-deficient mouse has a higher rate of basal skeletal modeling during the adult life of the animal, estrogen depletion provides a greater insult. The early skeletal retardation in the immature eNOS^(-/-) animal, combined with its inability to compensate for the absence of eNOS in the secondary absence of estrogen in the adult animal suggests that eNOS and estrogen actions in bone are linked.

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