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Role of endothelial nitric oxide synthase in estrogen-induced osteogenesis

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Abstract

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It is well recognized that high-dose estrogen induces a marked osteogenic response in long bones of female mice. In light of evidence which suggests that nitric oxide synthase (NOS) plays a role in regulation of osteoblast activity, we analyzed whether NOS is involved in mediating this response. Intact female mice were administered 17β-estradiol (E_2) either alone or in combination with N^G-nitro-L-arginine methylester (L-NAME) or aminoguanidine (AG), over 24 days. The former inhibits both constitutive and inducible isoforms of NOS, whereas the latter is a selective inhibitor of inducible NOS. Bone mineral density (BMD) of the femur was subsequently measured by dual-energy X-ray absorptiometry (DXA), and histomorphometry performed at the proximal metaphysis on longitudinal tibial sections. As expected, E_2 given alone led to a marked accumulation of cancellous bone at the proximal tibial metaphysis, associated with a significant gain in femoral BMD, and an increase in cancellous mineralizing surfaces as assessed by histomorphometry. Neither L-NAME nor AG affected cancellous histomorphometric indices when given alone. However, when administered in combination with L-NAME, the magnitude of the skeletal response to E_2 was significantly reduced. The tendency for L-NAME to reduce estrogen-induced bone formation within the proximal tibial metaphysis was more marked distally compared with proximally. In contrast, AG showed no tendency to suppress the osteogenic response to E_2. Subsequently, we examined the effect of E_2 administration on expression within mouse femoral bone marrow of endothelial NOS (eNOS), which is the predominant constitutive isoform of NOS within bone. No change in eNOS mRNA levels was observed following E_2 administration, as assessed by reverse transcription-polymerase chain reaction (RT-PCR). Taken together, our results suggest that eNOS plays a role in mediating estrogen-induced bone formation in intact female mice, possibly as a consequence of posttranscriptional

regulation of eNOS activity by estrogen.

Keywords

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Estrogen; Mouse; Bone formation; nitric oxide

Introduction

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Loss of estrogen's protective effect on the skeleton is thought to play an important role in the development of postmenopausal osteoporosis. Although estrogen is known to prevent bone loss by suppressing bone resorption, 5,30 5,30 direct stimulation of osteoblast function has also been implicated, particularly when estrogen is administered at doses within the upper physiological range. 22 In the mouse, an exaggerated effect of high-dose estrogen on osteoblast function is observed, characterized by the rapid deposition of new cancellous bone within the medullary cavity of long bones. 20 In subsequent studies intended to identify mediators of this response, the latter was found to be significantly inhibited by coadministration of indomethacin, 21 suggesting that prostaglandin synthesis may play a role.

Because estrogen-induced osteogenesis is only partially prevented by indomethacin, other regulatory factors may also be involved. One likely candidate is nitric oxide (NO), which is known to be produced in bone by both constitutive and inducible forms of NO synthase (NOS). 6 Although initial investigations have focused on the role of cytokine-mediated NO release in excessive bone resorption associated with inflammatory disorders, NO is also believed to act as an important regulator of bone formation. For example, in vitro studies suggest that low-dose NO acts to stimulate osteoblast proliferation, 6 whereas NO has been shown to act as an important mediator of mechanically induced bone formation as assessed in vivo. 7,25 7,25

Studies in which NO donors have been found to prevent or restore ovariectomy-induced bone loss provide more direct evidence to support a possible role of NO in mediating skeletal actions of estrogen on bone. 28,29 28,29 Although these observations may reflect a role of NO in mediating estrogen's inhibitory effect on bone resorption, involvement in estrogen's stimulatory effects on osteoblast function may also be responsible. Consistent with this possibility, estrogen has previously been found to upregulate NOS expression in human osteoblast-like cells. 2 In the latter study, the response was specific for endothelial NOS (eNOS), which is the predominant constitutive isoform of NOS expressed in bone, suggesting that eNOS may be preferentially involved in mediating estrogen's effects on osteoblasts.

In the present investigation, we aimed to determine whether NO production plays a significant role in mediating estrogen-induced bone formation in vivo, as assessed in intact female mice. To compare the contributions of constitutive and inducible NOS isoforms, we examined to what extent this response is suppressed by concurrent administration of N^G-nitro-L-arginine methylester (L-NAME), which inhibits both constitutive and inducible forms of NOS, 13 or aminoguanidine (AG), which is a relatively selective inhibitor of inducible NOS (iNOS). 12

Methods

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Experimental design

Ten-week-old intact female CBA-1 mice from the University of Bristol breeding colony (mean pretreatment body weight 20.1 g) were divided into six weight-matched groups (eight animals per group), and administered the following by subcutaneous injection: vehicle; AG (10 mg/animal per day; Sigma Chemical Co., Poole, Dorset, UK); L-NAME (10 mg/animal per day; Sigma) E_2, (500 µg/animal per week; Sigma); E_2 plus AG; or E_2 plus L-NAME. AG and L-NAME were dissolved in saline, and E_2 in corn oil. The doses of AG and L-NAME were selected as being similar to those previously found to influence skeletal metabolism in the rat. 25,29 25,29 Tetracycline hydrochloride (25 mg/kg; Sigma) and calcein (30 mg/kg; Sigma) were injected intraperitoneally 4 and 1 days before killing, which was 24 days after the first subcutaneous (s.c.) injection.

Following termination of the experiment, animals were killed by cervical dislocation, body weights recorded, and femurs and tibiae removed for bone mineral density (BMD) measurement and histomorphometric analysis, respectively. Vena caval blood samples were centrifuged at 4000 rpm for 10 min, and serum stored in aliquots at -20°C for subsequent assessment of nitrate/nitrite levels (see later). Throughout the study, animals received a standard diet (Rat and Mouse Standard Diet; B&K Ltd., Humberside, UK) and water and libitum, and were maintained on a cycle of 12 h light/12 h dark. All experimental procedures complied with the guiding principles of *The Care and Use of Animals*.

A second experiment was also performed to evaluate the effect of E_2 administration on eNOS expression in bone marrow from mouse long bones, as assessed by RT-PCR. Eight-week-old intact female mice were killed before or 1, 2, 4, 8, 12, and 16 days after treatment with E_2 500 μg/animal per week (four animals per timepoint). Bone marrow was subsequently flushed from femor using 0.5 mL 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4, and pooled between animals.

Femoral bone mineral density measurement

Left femurs were cleaned and fixed in 70% ethanol at 4°C for 2 weeks. Femoral BMD was subsequently measured by dual-energy X-ray absorptiometry (DXA) using a PIXImus scanner (Lunar Corp., Madison, WI) with small animal software. The accuracy of this technique in measuring calcium content was confirmed in preliminary studies in which a highly significant correlation between femoral total bone mineral content and ash weight ($r = 0.86$, $p < 0.0001$) was found. The coefficient of variation for femoral BMD, obtained after scanning 30 mouse femurs five times each with repositioning between scans, was 2.7%.

Histomorphometry

To quantify the osteogenic response to estrogen, tibiae were subsequently processed for histomorphometric analysis. Tibiae were removed of soft tissue, fixed in 70% ethanol for 48 h, then dehydrated through a graded series of alcohols: 80% ethanol; 90% ethanol; then three changes of 100% ethanol for 24 h each. Tibiae were then cleared in chloroform for 24 h, placed for a further 24 h in 100% ethanol, and embedded without decalcification in LR White Hard Grade (London Resin Co., Reading, UK). Longitudinal sections of the tibial metaphyses were cut on a Reichert–Jung (Model 2050) microtome with a "D" profile tungsten carbide knife; 7 μm sections were stained with 1% toluidine blue in 0.01 mol/L citrate phosphate buffer; 14 μm sections were mounted unstained in fluoromount (BDH, Laboratory Supplies, Poole, UK) for assessment by fluorescent microscopy.

Histomorphometric analysis of the proximal tibial metaphysis was performed using transmitted and epifluorescent microscopy linked to a computer-assisted image analyzer (Osteomeasure, Osteometrics, Atlanta, GA). All sections were examined in a blinded manner. Two sampling sites, each with a standard area of 0.36 mm^2, were analyzed; the proximal border of the proximal sampling site was situated 0.3 mm below the growth plate to exclude primary spongiosa; the second sampling site was immediately distal to the first sampling site. Cancellous bone parameters were measured at ×200 magnification. Cancellous bone volume was assessed on two nonconsecutive sections stained with toluidine blue for each animal, and expressed as a percentage of total tissue volume (BV/TV).

Fluorochrome measurements were made on two nonconsecutive 14 μm sections per animal. The amount of trabecular bone surface covered by double label was expressed with reference to the total tissue volume (tissue volume referent [dLS/TV]), and as a percentage of cancellous bone surface (cancellous surface [dLS/BS]). Mineral apposition rate (MAR) was determined by dividing the mean distance between the tetracycline and calcein labels by the time interval between the administration of the two labels (values were not corrected for the obliquity of the plane of section). The bone formation rate was obtained from the product of MAR and either dLS/TV or dLS/BS, giving BFR/TV and BFR/BS, respectively. Longitudinal growth rate (LGR) was determined at ×400 by measuring the distance between the tetracycline and calcein bands lying distal to the epiphyseal growth plate and dividing by the time interval between the administration of the two labels.

Serum nitrate/nitrite assay

To analyze the effect of NOS inhibitors on serum nitrite/nitrate levels, serum was first ultrafiltered through a 30 kDa molecular weight filter (Millipore, Herts, UK). Total serum nitrate/nitrite levels were subsequently assessed using a nitrate/nitrite colorimetric assay kit according to the supplier's instructions (Cayman Chemicals/Alexis Corp., Nottingham UK). Analyses were performed on serum samples pooled from two or three animals.

RT-PCR

RT-PCR was used to analyze changes in expression of eNOS mRNA in mouse bone marrow following E_2 administration, following preliminary studies in which expression could not be detected by northern blot analysis. Total RNA was prepared from pooled marrow samples using Trizol (Gibco BRL) and 400 ng aliquots were reverse transcribed into cDNA using the GeneAmp RNA PCR kit and oligo-dT primers (Perkin Elmer Corp., San Diego, CA) according to the supplier's instructions. eNOS expression was compared with that of β-actin for each sample using the Advantage 2 PCR system (Clontec).

To enable semiquantitative data to be obtained, we developed a PCR protocol by which products for eNOS and β-actin are generated simultaneously within the same tube at logarithmic rates. Initially, 50 μL reactions were prepared containing eNOS-specific primers (5' eNOS: CTCAGACCCACTGGTATCCTCTT; 3' eNOS: CTGCTGTTCGCTGGACTCTTCT), 9 which were denatured at 95°C for 5 min then subjected to 13 cycles of 95°C 30 sec, 56°C 30 sec, 72°C 2 min. Primers specific for β-actin (5' β-actin ACCAAGGTGTGATGGTGGGAAT; 3' β-actin ATCGGAACCGCTCGTTGCCAAT) were then added to the reactions before a further 11 cycles and 5 min incubation at 72°C. Five microliter samples were removed at three cycle intervals after 24, 27, 30, and 33 cycles and applied to a 1% agarose gel containing ethidium bromide. Both products were found to be generated at logarithmic rates at 30 cycles, which was employed in subsequent analyses. Data presented are representative of triplicate experiments.

Analysis of results

Results are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to analyze differences in body weight, nitrate/nitrite levels, DXA, and histomorphometric parameters between treatment groups. In cases in which the ANOVA was significant, Tukey's posttest was used to compare pairs of group means. The cut-off for statistical significance was taken as $p < 0.05$.

Results

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When given alone, E_2 L-NAME, and AG exerted a small inhibitory influence on body weight, which failed to reach statistical significance (**Table 1**). However, in the combined E_2 and L-NAME group, a significant decrease in body weight was observed compared with animals given vehicle or E_2 in combination with AG. A consistent trend was observed toward lower nitrate/nitrite levels in animals receiving NOS inhibitors, which reached significance by Student's *t*-test (Table 1).

Table 1. Effect of 17β-estradiol (E_2) and/or nitric oxide synthase inhibitors on body weight, serum nitrate/nitrite, and longitudinal growth rate legend

Group	Final body weight (g)	Serum nitrate/nitrite (μmol/L)	Longitudinal growth rate (μm/day)
VEH	21.2 ± 0.2	18.2 ± 1.4	11.4 ± 1.3
AG	20.3 ± 0.5	13.4 ± 0.9	9.2 ± 1.0
L	20.3 ± 0.5	14.0 ± 3.0	12.8 ± 0.7
E_2	20.3 ± 0.5	18.2 ± 2.9	1.8 ± 0.4 [b]
E_2 + AG	21.4 ± 1.0	13.6 ± 1.0	1.7 ± 0.4 [b]
E_2 + L	19.3 ± 0.5 [a]	13.2 ± 2.7	1.1 ± 0.1 [b]

[legend]Data expressed as mean ± SEM, following treatment with vehicle (VEH), aminoguanidine (AG), L-NAME (L), E_2, E_2 plus AG, and E_2 plus L, for 24 days in female mice (eight animals per group). No statistical differences in nitrate/nitrite levels were found by analysis of variance. However, unpaired Student's *t*-test revealed a statistically significant difference when pooled data from groups receiving NOS inhibitor were compared with those from animals receiving vehicle/E_2 ($p = 0.01$).

[a]$p < 0.05$ vs. VEH, E_2 plus AG.

[b]$p < 0.001$ vs. VEH, AG, and L (Tukey's posttest).

E_2 treatment led to the deposition of cancellous bone within the tibial metaphysis (**Figure 1**). This response was associated with an increase in long bone BMD, as assessed by measurement of femoral BMD (**Figure 2**). Subsequent histomorphometric analysis revealed a striking increase in cancellous bone volume in estrogen-treated mice, in both the proximal and distal regions of the proximal tibial metaphysis (**Figure 3**). This gain in bone was associated with an equivalent increase in absolute extent of mineralizing surfaces within the metaphysis, as reflected by dLS/TV (**Figure 4**), as previously reported. 20 In contrast, E_2 treatment had no effect on the proportion of cancellous surfaces that showed mineralizing surface as assessed by dLS/BS, or on other fluorochrome-based indices such as MAR (**Table 2**).

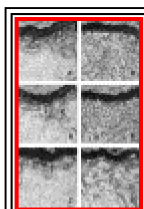

Figure 1. Effect of 17β-estradiol (E_2) and/or nitric oxide synthase inhibitors on morphology of the proximal tibial metaphysis. Photomicrographs show longitudinal sections of the proximal tibial metaphysis of female mice stained with toluidine blue, after 24 day treatment with vehicle (A), aminoguanidine (B), L-NAME (C), E_2 (D), E_2 + aminoguanidine (E), and E_2 + L-NAME (F). Magnification × 100.

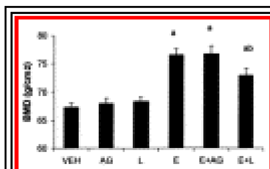

Figure 2. Effect of 17β-estradiol (E) and/or nitric oxide synthase inhibitors on bone mineral density (BMD). Results show mean ± SEM femoral BMD, following treatment with vehicle (VEH), aminoguanidine (AG), L-NAME (L), E, E plus AG, and E plus L, for 24 days in female mice (eight animals per group). One-way ANOVA revealed a significant difference between treatment groups ($p < 0.0001$). $^a p < 0.001$ vs. VEH, L, and AG; $^b p < 0.05$ vs. E (Tukey's posttest).

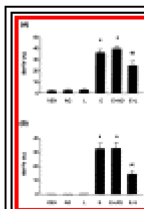

Figure 3. Effect of 17β-estradiol (E) and/or nitric oxide synthase inhibitors on cancellous bone volume (BV/TV). Results show mean ± SEM BV/TV at the proximal (A) and distal (B) regions of the proximal tibial metaphysis, following treatment with vehicle (VEH), aminoguanidine (AG), L-NAME (L), E, E plus AG, and E plus L, for 24 days in female mice (eight animals per group). One-way ANOVA revealed a significant difference between treatment groups ($p < 0.0001$). $^a p < 0.001$ vs. VEH, L, and AG; $^b p < 0.05$ vs. E; $^c p < 0.001$ vs. E (Tukey's posttest).

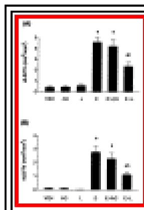

Figure 4. Effect of 17β-estradiol (E) and/or nitric oxide synthase inhibitors on cancellous bone mineralizing perimeter, tissue volume referent (dLS/TV). Results show mean ± SEM (dLS/TV) at the proximal (A) and distal (B) regions of the proximal tibial metaphysis following treatment with vehicle (VEH), aminoguanidine (AG), L-NAME (L), E, E plus AG, and E plus L, for 24 days in female mice (eight animals per group). One-way ANOVA revealed a significant difference between treatment groups ($p < 0.0001$). $^a p < 0.001$ vs. VEH, L, and AG; $^b p < 0.001$ vs. E (Tukey's posttest).

Table 2. Effect of 17β-estradiol (E_2) and/or nitric oxide synthase inhibitors on histomorphometric measurements at the proximal and distal regions of the proximal tibial metaphysis legend

Group	Proximal region				Distal region			
	dLS/BS	MAR	BFR/BS	BFR/TV	dLS/BS	MAR	BFR/BS	BFR/TV
VEH	24.3 ± 4.7	2.1 ± 0.1	0.51 ± 0.10	0.98 ± 0.25	20.5 ± 7.8	1.8 ± 0.4	0.33 ± 0.12	0.14 ± 0.09

AG	21.6 ± 4.3	1.7 ± 0.1	0.37 ± 0.07	0.83 ± 0.20	19.4 ± 7.3	1.8 ± 0.2	0.37 ± 0.15	0.15 ± 0.07
L	26.0 ± 5.0	2.0 ± 0.1	0.52 ± 0.10	1.13 ± 0.28	4.0 ± 2.7	1.2 ± 0.2	0.05 ± 0.04	0.02 ± 0.01
E_2	23.6 ± 1.6	2.0 ± 0.1	0.47 ± 0.03	9.02 ± 0.79 [a]	16.0 ± 2.07	2.0 ± 0.1	0.32 ± 0.05	5.57 ± 0.01 [a]
E_2 + AG	20.6 ± 2.6	1.7 ± 0.1	0.35 ± 0.05	3.89 ± 0.55 [a]	12.7 ± 2.0	1.8 ± 0.1	0.23 ± 0.04	4.15 ± 0.83 [a]
E_2 + L	18.0 ± 3.0	1.7 ± 0.1	0.30 ± 0.05	7.17 ± 1.20 [a,b]	18.5 ± 7.3	1.8 ± 0.1	0.32 ± 0.12	1.96 ± 0.36 [a,b]

[legend]Data show mean ± SEM following treatment with vehicle (VEH), aminoguanidine (AG), L-NAME (L), E_2, E_2 plus AG, and E_2 plus L-NAME for 24 days in female mice. Measurements made at each region were: cancellous double-labeled surface; bone surface referent (dLS/BS; %); mineral apposition rate (MAR; μm/day); bone formation rate; cancellous surface referent (BFR/BS; mm^3/mm^2 per day); and bone formation rate, tissue volume referent (BFR/TV; mm^3/mm^3 per day).

[a]$p < 0.001$ vs. VEH, L, and AG.

[b]$p < 0.001$ vs. E_2 (Tukey's posttest).

When given alone, neither L-NAME nor AG significantly influenced any of these skeletal indices. However, in mice treated with both L-NAME and E_2, a significantly reduced response to E_2 was observed. If anything, the tendency of L-NAME to reduce BV/TV and dLS/TV in the presence of E_2 was more marked in the distal sampling site. In contrast, concurrent treatment with AG was not found to significantly affect response to E_2. Although E_2 was found to suppress longitudinal bone growth as previously described, L-NAME and AG had no effect, either when given alone or in combination with E_2 (Table 1). Administration of E_2 did not appear to increase the rate of eNOS mRNA transcription in mouse femoral bone marrow, as assessed by RT-PCR (**Figure 5**).

Figure 5. Effect of 17β-estradiol (E_2) on expression of endothelial nitric oxide synthase (eNOS) in mouse femoral bone marrow, as assessed by RT-PCR. Marrow was obtained immediately prior to, and 1, 2, 4, 8, 12, and 16 days after, commencing subcutaneous injections of E_2 (four animals per timepoint). The photomicrograph shows RT-PCR products from different timepoints, using primers for eNOS. β-actin expression was also analyzed to provide a loading control.

Discussion

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Estrogen-induced bone formation in intact female mice appeared to be partially suppressed by coadministration of L-NAME, which is a relatively nonspecific inhibitor of NOS activity. In contrast, AG, which selectively inhibits iNOS, had no effect on this response. Therefore, our findings suggest that constitutive isoforms of NOS are involved in mediating estrogen-induced osteogenesis in female mice. Of the two constitutive isoforms of NOS that have previously been identified (i.e., neuronal NOS and endothelial NOS 6), previous studies indicate that eNOS is the isoform predominantly expressed in skeletal tissues. 3,8 3,8 Taken together, these observations suggest that eNOS is involved in mediating the stimulatory action of estrogen on bone formation in female mice.

The suggestion from our results that NOS is involved in mediating estrogen-induced stimulation of bone formation is consistent with previous findings that NO production plays an important role in regulation of osteoblast function as assessed in vitro 14,17 14,17 and in vivo. 7,25 7,25 Our results are also in line with

recent evidence which suggests that nitrates protect from bone loss induced by estrogen deficiency through stimulation of bone formation as well as suppression of resorption. 28 The possibility that estrogen-induced bone formation preferentially involves eNOS is also consistent with preliminary reports which suggest that this response is suppressed in transgenic mice rendered deficient in eNOS. 1 It is possible that eNOS is predominantly involved in regulation of bone formation processes in general, in light of previous findings which suggest that skeletal production of constitutive NOS also mediates mechanically induced bone formation. 7

Although LNAME and AG were found to suppress systemic NO production as judged by measurement of serum nitrate/nitrite concentration, the latter was not affected by E_2 administration. Therefore, any tendency for estrogen to stimulate bone formation via induction of NO production is likely to reflect local stimulation within the skeleton. Consistent with this possibility, E_2 has been shown to upregulate the expression of eNOS in a variety of cell types including osteoblasts. 2,10,27 2,10,27 2,10,27 This may reflect direct transcriptional control of NOS production by estrogen, in view of the fact that the eNOS promoter has been reported to possess an estrogen response element. 26 However, in the present study, estrogen was not found to stimulate eNOS mRNA expression in mouse bone marrow, suggesting that any tendency for estrogen to influence eNOS levels in bone occurs at the posttranscriptional level. Alternatively, it is possible that estrogen alters eNOS mRNA levels within bone as opposed to bone marrow as analyzed here.

Although our observation that L-NAME partially inhibited estrogen-induced bone formation suggests that constitutive NOS plays a role in mediating this response, L-NAME is also known to exert effects that are independent of the NO pathway. 16,18 16,18 Further evidence in support of a role for NO synthesis in mediating estrogen-induced bone formation would be provided by investigating whether L-NAME's inhibitory effect on this response is reversed by adding L-arginine. 7 In addition, we are unable to exclude a nonspecific inhibitory effect on bone formation in the E_2 plus L-NAME group, in light of our observation that this treatment combination appeared to cause weight loss. The latter finding is consistent with previous reports of weight loss following L-NAME administration in rodents. 24,29 24,29

In our previous time-course study, estrogen-induced bone formation was found to commence within the proximal portion of the proximal tibial metaphysis, before extending distally toward the diaphysis. 20 Because L-NAME appeared to cause greater suppression of bone formation distally compared with proximally, this suggests that L-NAME preferentially inhibits later stages of the osteogenic response. Interestingly, administration of the prostaglandin synthesis inhibitor, indomethacin, was recently found to cause equivalent effects, 21 suggesting that prostaglandins and NO may play similar roles in amplifying later phases of the osteogenic response to estrogen. Prostaglandins and NO are also both required in mechanically induced osteogenesis, 4 and in vitro studies suggest that prostaglandin production following mechanical stimulation may be dependent on NO. 11 Taken together, these observations suggest that NO and prostaglandins represent part of a final common pathway by which osteogenic stimuli influence osteoblast function.

In the present study, osteoblast function was assessed by measurement of fluorochrome-based indices of bone formation, as previously used to characterize the time-course and dose response of estrogen-induced osteogenesis in female mice. 19,20 19,20 Although this gives an accurate reflection of the extent of cancellous bone surfaces undergoing active bone formation, further studies are required to distinguish the relative roles of alterations in osteoblast survival or generation. These two aspects of osteoblast function may have been affected, in view of evidence that estrogen influences both osteoblast apoptosis and osteoprogenitor formation. 15,23 15,23 Although estrogen is also known to suppress osteoclastic bone resorption, 5,30 5,30 which was not assessed in the present study, our previous time-course study indicated that enhanced osteoblast function is largely responsible for the gain in bone observed following estrogen administration. 20

In conclusion, we found that estrogen-induced osteogenesis in intact female mice was partially, but significantly, inhibited by coadministration of the nonspecific NOS inhibitor, L-NAME. In contrast, cotreatment with the iNOS inhibitor, AG, had no discernible effect on this response, suggesting that estrogen-induced osteogenesis preferentially involves eNOS, which is the predominant constitutive NOS isoform expressed in bone. However, RT-PCR analysis performed on RNA extracted from mouse bone marrow revealed little effect of estrogen administration on eNOS mRNA levels. Further studies are justified to examine functional relationships between eNOS and other local regulators of bone formation, such as prostaglandins, and to study the role of posttranscriptional regulation of eNOS activity within the skeleton.

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