JOURNAL OF BONE AND MINERAL RESEARCH
Volume 23, Number 5, 2008
Published online on February 4, 2008; doi: 10.1359/JBMR.080107

Genetic Disruption of All NO Synthase Isoforms Enhances BMD and Bone Turnover in Mice In Vivo: Involvement of the Renin-Angiotensin System

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ABSTRACT:

Introduction: NO is synthesized by three different NO synthase (NOS) isoforms, including neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS). The roles of NO in bone metabolism have been extensively investigated in pharmacological studies and in studies with NOS isoform–deficient mice. However, because of the nonspecificity of agents and compensation among the NOS isoforms, the ultimate roles of endogenous NO are still poorly understood. To address this point, we successfully generated mice in which all three NOS genes are completely disrupted. In this study, we examined whether bone metabolism is abnormal in those mice.
Materials and Methods: Experiments were performed in 12-wk-old male wildtype, singly $nNOS^{-/-}$, $iNOS^{-/-}$, and $eNOS^{-/-}$ and triply $n/i/eNOS^{-/-}$ mice. BMD was assessed by DXA. The kinetics of osteoblastic bone formation and those of osteoclastic bone resorption were evaluated by measurements of morphological and biochemical markers.
Results: BMD was significantly higher only in the triply $NOS^{-/-}$ mice but not in any singly $NOS^{-/-}$ mice compared with the wildtype mice. Markers of osteoblastic bone formation, including bone formation rate, mineral apposition rate, and serum alkaline phosphatase concentration, were also significantly larger only in the triply $NOS^{-/-}$ mice compared with wildtype mice. Furthermore, markers of osteoclastic bone resorption, including osteoclast number, osteoclast surface, and urinary deoxypyridinoline excretion, were again significantly greater only in the triply $NOS^{-/-}$ mice. Importantly, the renin-angiotensin system in bone was significantly activated in the triply $NOS^{-/-}$ mice, and long-term oral treatment with an angiotensin II type 1 (AT_1) receptor blocker normalized this pathological bone remodeling in those mice.
Conclusions: These results provide the first direct evidence that genetic disruption of the whole NOS system enhances BMD and bone turnover in mice in vivo through the AT_1 receptor pathway, showing the critical role of the endogenous NO/NOS system in maintaining bone homeostasis.
J Bone Miner Res 2008;23:633–643. Published online on February 4, 2008; doi: 10.1359/JBMR.080107

Key words: NO synthase, bone formation, bone resorption, bone metabolism, angiotensin II

INTRODUCTION

NO, an important intracellular messenger, plays important roles in maintaining homeostasis.[1–5] NO is formed from its precursor L-arginine by a family of NO synthases (NOSs) with stoichiometric production of L-citrulline. NO is a gaseous free radical, freely passes through plasma membranes, and elicits multiple actions without receptor coupling either directly or through the soluble guanylate cyclase/cGMP-mediated pathway. Three distinct NOS isoforms exist, and they are encoded by three distinct genes: neuronal (nNOS, NOS1), inducible (iNOS, NOS2), and endothelial NOS (eNOS, NOS3).[1–5] nNOS and eNOS are constitutively expressed mainly in the nervous system and the vascular endothelium, respectively, synthesizing a small amount of NO in a calcium-dependent manner both under basal conditions and on stimulation. In contrast, iNOS is induced when stimulated by microbial endotoxins or certain proinflammatory cytokines, producing a greater amount of NO in a calcium-independent manner.[1–5]

Bone is a vital dynamic connective tissue that possesses

The authors state that they have no conflicts of interest.

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two major functions: provision of mechanical integrity for locomotion and modulation of mineral homeostasis.[6] New bone is formed by osteoblasts, and mineralized bone is continuously resorbed by osteoclasts. This process is highly regulated by a variety of regulatory factors, including calcitonin, PTH, and sex steroids.[7] NO is also noted as a candidate for the regulatory factors,[8] and the roles of NO in bone metabolism have thus far been extensively examined in pharmacological studies with NOS inhibitors[9,10] and in studies with mice lacking each NOS isoform.[11–13] However, in the pharmacological studies, the specificity of NOS inhibitors continues to be an issue of debate,[14] whereas in the studies with mice lacking each NOS isoform, compensation by other NOSs seems to be involved.[15] Thus, the ultimate roles of endogenous NO derived from the whole NOS system in bone metabolism still remain to be fully elucidated. To address this issue, we have recently developed mice in which all three NOS isoforms are completely deficient.[16,17]

Our preliminary study has shown that the tissue renin-angiotensin system is activated in the triply $NOS^{-/-}$ mice,[17] which condition could cause pathological bone remodeling.[18,19] Thus, in this study, we examined whether abnormal bone phenotypes are noted in the triply $NOS^{-/-}$ mice, and if so, whether activation of the renin-angiotensin system is involved.

MATERIALS AND METHODS

Animal preparation

Experiments were performed in 12-wk-old male wildtype C57BL/6 and 129SV (Charles River, Yokohama, Japan), singly $nNOS^{-/-}$, $iNOS^{-/-}$, and $eNOS^{-/-}$ and triply n/i/$eNOS^{-/-}$ mice[16] maintained on a standard diet containing 1.25% calcium and 1.06% phosphorus (CE-2; Clea, Tokyo, Japan).

BMD

The animals were killed by inhalation of an overdose of diethyl ether (Wako Pure Chemical Industries, Osaka, Japan). The right femur and lumbar spine (L_2–L_4) were isolated, and the specimens were stored in 70% ethanol. BMD was measured by DXA (DCS-600; Aloka, Tokyo, Japan).

Bone histomorphometry

The unilateral left proximal tibia was stained with a Villanueva solution. The specimen was embedded in methyl methacrylate without decalcification and cut in 5-μm-thick coronal slices by a microtome (RM 2125 RT; Leica, Nussloch, Germany). The structural parameters of the proximal tibia were analyzed, as we previously reported.[20] In each section, the area of the secondary spongiosa was determined with a semiautomatic image analysis system linked to a light microscope (Histometry-RT; System Supply, Nagano, Japan).

The contralateral right proximal tibia was decalcified with a 10% EDTA buffer and embedded in paraffin. The specimen was cut in 3-μm-thick coronal slices by a microtome and stained with a TRACP solution. Osteoclasts were identified as $TRACP^+$ cells that formed resorption lacunae on the surface of trabeculae and that contained two or more nuclei. In calcein double labeling, calcein was injected subcutaneously at days 3 and 7 before death.

3D μCT

3D analysis of femurs was performed with a μCT system (mCT40; Scano Medical, Bassersdorf, Switzerland), as we previously reported.[21]

Biochemical markers

Blood was collected after 15 h of fasting. Sampling of urine was performed with metabolic cages. Urinary deoxypyridinoline excretion was assessed by an ELISA kit (Osteolinks; DS Pharma Biomedical, Osaka, Japan). Angiotensin-converting enzyme (ACE) activity in the femur was determined by an ACE activity assay kit (Life Laboratory, Yamagata, Japan)

Quantitative RT-PCR

Total RNA was extracted by crushing and homogenizing the femur with 0.5 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was reverse-transcribed from 1 μg of total RNA using a Moloney murine leukemia virus reverse transcriptase and an oligo (dT) 12–18 primer (Invitrogen). Real-time RT-PCR was performed with an iCycler apparatus (Bio-Rad Laboratories, Hercules, CA, USA) as we previously reported.[20] mRNA levels of the angiotensin II type 1a (AT_{1a}) receptor, bone morphogenic protein 2 (BMP2), Runx2 (Cbfa1), Osterix, Osteocalcin, RANKL, c-*fos*, and β-actin were examined. We used the comparative cycle threshold method for the real-time RT-PCR data analysis, and the mRNA expression levels were normalized with the β-actin mRNA expression level.[22]

Immunostaining

Sections of paraffin-embedded tissue were incubated with a rabbit polyclonal AT_1 receptor antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), as we previously reported.[14]

Drug treatment

Olmesartan medoxomil was mixed in pellets and administered orally to the triply n/i/$eNOS^{-/-}$ mice (5 mg/kg/d; Sankyo Pharmaceutical, Tokyo, Japan). The treatment was performed for 8 wk, from 4 to 12 wk of age.

Statistical analysis

Results are expressed as mean ± SD. Statistical analysis was performed by unpaired *t*-test, or ANOVA followed by Tukey-Kramer posthoc test for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

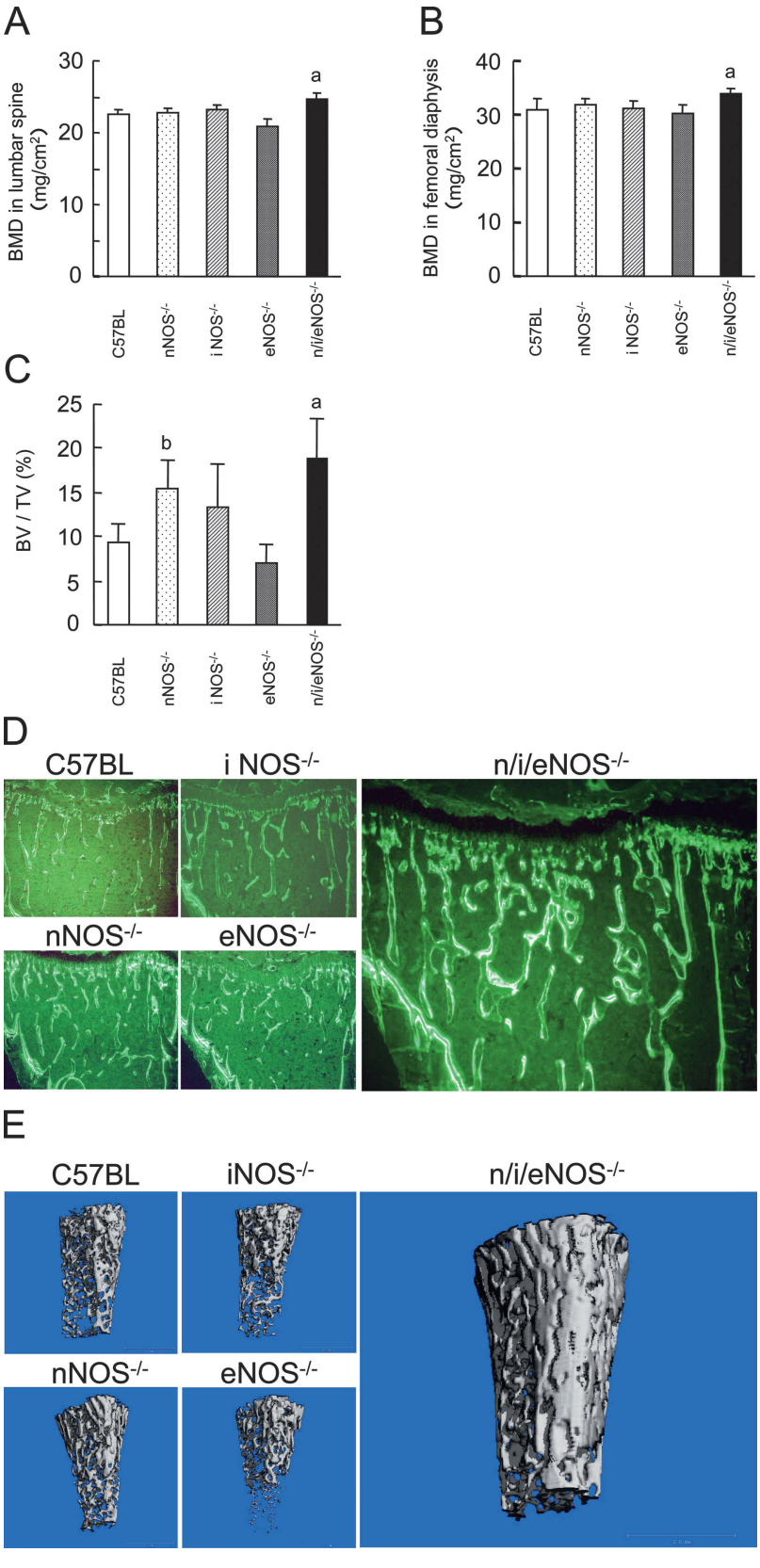

FIG. 1. Increased BMD, enhanced trabecular bone volume (BV/TV), and abnormal trabecular bone microstructure in the triply n/i/eNOS$^{-/-}$ mice. All experiments were performed in 12-wk-old male mice. (A and B) BMD values in the lumbar spine and femoral diaphysis (as assessed by DXA; $n = 7–10$). (C) Trabecular BV/TV ($n = 6–8$). (D and E) Calcein double labeling in the proximal tibia and 3D μCT of the femur. In both analyses, trabecular bone thickness and density were increased in the triply n/i/eNOS$^{-/-}$ mice. [a]$p < 0.01$, [b]$p < 0.05$ vs. wildtype C57BL.

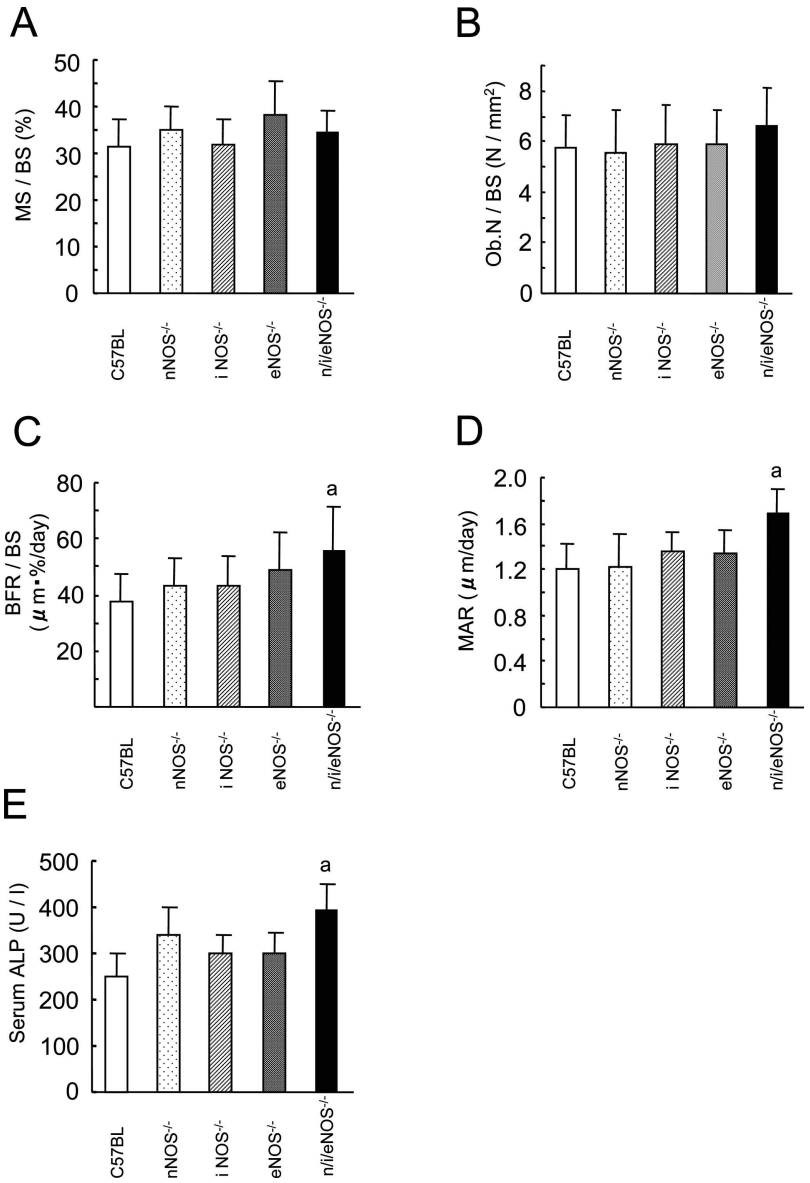

FIG. 2. Accelerated osteoblastic bone formation in the triply n/i/eNOS$^{-/-}$ mice. (A–D) The mineralizing surface (MS/BS) (A), the osteoblast number (Ob.N/BS) (B), the bone formation rate (BFR/BS) (C), and the mineral apposition rate (MAR) (D) in the proximal tibia (n = 6–8). (E) The serum alkaline phosphatase (ALP) concentration (n = 6). [a]$p < 0.01$ vs. wildtype C57BL.

RESULTS

Increased BMD associated with trabecular bone microstructural changes in triply n/i/eNOS$^{-/-}$ mice

The BMD values in both the lumbar spine (Fig. 1A) and femoral diaphysis (Fig. 1B), as assessed by DXA, were slightly but significantly higher only in the triply n/i/eNOS$^{-/-}$ mice, but not in any singly NOS$^{-/-}$ mice, compared with the wildtype C57BL mice. The trabecular bone volume (BV/TV) in the proximal tibia was also significantly larger in the triply n/i/eNOS$^{-/-}$ mice than in the wildtype mice (Fig. 1C). Furthermore, both calcein double labeling in the proximal tibia (Fig. 1D) and 3D μCT analysis of the femur (Fig. 1E) showed that thickness and density of the trabecular bone were increased only in the triply n/i/eNOS$^{-/-}$ mice. On the other hand, the singly nNOS$^{-/-}$ mice also had a significantly greater BV/TV value compared with wildtype mice (Fig. 1C). We previously reported that there was no significant difference in body weight among the five genotypes studied.[16]

Accelerated osteoblastic bone formation in triply n/i/eNOS$^{-/-}$ mice

The mineralizing surface (MS/BS) and the osteoblast number (Ob.N/BS) in the proximal tibia, both of which are morphological markers of osteoblastic cell number, tended to be increased in the triply n/i/eNOS$^{-/-}$ than in the wildtype mice, although the differences were not statistically significant (Figs. 2A and 2B). The bone formation rate (BFR/BS) and the mineral apposition rate (MAR), both of which are morphological markers of osteoblastic cell function, were significantly higher only in the triply n/i/eNOS$^{-/-}$ mice compared with the wildtype mice (Figs. 2C and 2D). Furthermore, the serum alkaline phosphatase concentra-

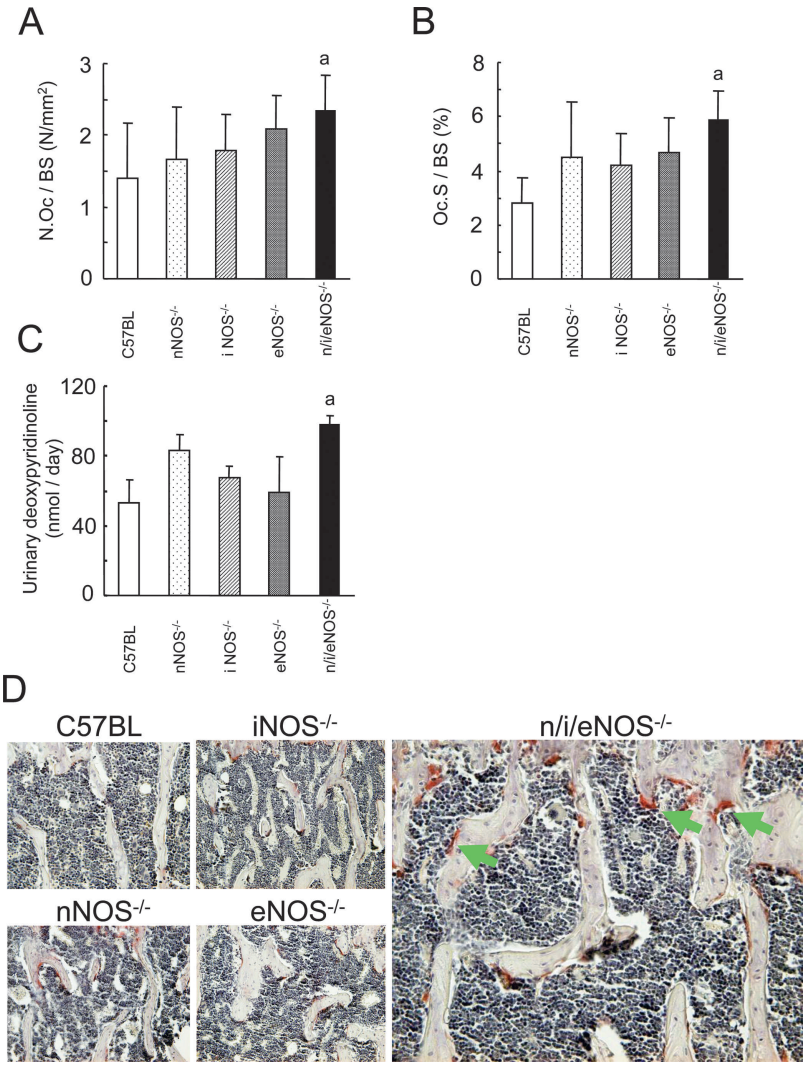

FIG. 3. Enhanced osteoclastic bone resorption in the triply n/i/eNOS$^{-/-}$ mice. (A) The osteoclast number (N.Oc/BS) in the proximal tibia (n = 5–6). (B) The osteoclast surface (Oc.S/BS) in the proximal tibia (n = 5–6). (C) The urinary deoxypyridinoline excretion (n = 5–6). (D) TRACP staining in the proximal tibia. Green arrows indicate TRACP^{+} osteoclasts (red). $^{a}p < 0.05$ vs. wildtype C57BL.

tion, which is a biochemical marker of osteoblastic cell function, was also significantly elevated only in the triply n/i/eNOS$^{-/-}$ mice (Fig. 2E).

Enhanced osteoblastic bone resorption in triply n/i/eNOS$^{-/-}$ mice

The osteoclast number (N.Oc/BS) in the proximal tibia, which is a morphological marker of osteoclastic cell number, and the osteoclast surface (Oc.S/BS) in the proximal tibia, which is a morphological marker of osteoclastic cell function, were both significantly larger only in the triply n/i/eNOS$^{-/-}$ mice compared with wildtype mice (Figs. 3A and 3B). Urinary deoxypyridinoline excretion, which is a biochemical marker of osteoclastic cell function, was similarly elevated only in the triply n/i/eNOS$^{-/-}$ mice (Fig. 3C). Furthermore, the number of TRACP^{+} osteoclasts (red) was also greater in the triply n/i/eNOS$^{-/-}$ mice (Fig. 3D).

In this study, in addition to C57BL mice, we also used 129SV mice as a wildtype control. No significant differences in the parameters of bone histomorphometry or BMD were noted between the two normal genotypes (data not shown).

Time course, sex difference, and effect on growth in bone phenotypes of triply n/i/eNOS$^{-/-}$ mice

In regard to age, significant differences in the BMD values in the lumbar spine and the femoral diaphysis between the wildtype and triply n/i/eNOS$^{-/-}$ mice were noted at 12 and 20 wk, but not at 4 wk of age (Figs. 4A and 4B). On the other hand, significant differences in BFR/BS and MAR, markers of osteoblastic cell function, and N.Oc/BS and Oc.S/BS, markers of osteoclastic cell number and function, were seen at only 12 wk of age (Figs. 4C–4F). In regard to sex difference, there was no significant difference in the BMD in the lumbar spine or the femoral diaphysis between 12-wk-old male and female triply n/i/eNOS$^{-/-}$ mice (Figs. 4G and 4H), whereas BFR/BS, MAR, N.Oc/BS, and Oc.S/BS were all significantly higher in the female than in the male triply n/i/eNOS$^{-/-}$ mice (Figs. 4I–4L). In regard to effect on growth, no significant difference in the length of the femur or the tibia between the 12-wk-old wildtype and triply n/i/eNOS$^{-/-}$ mice was seen (data not shown).

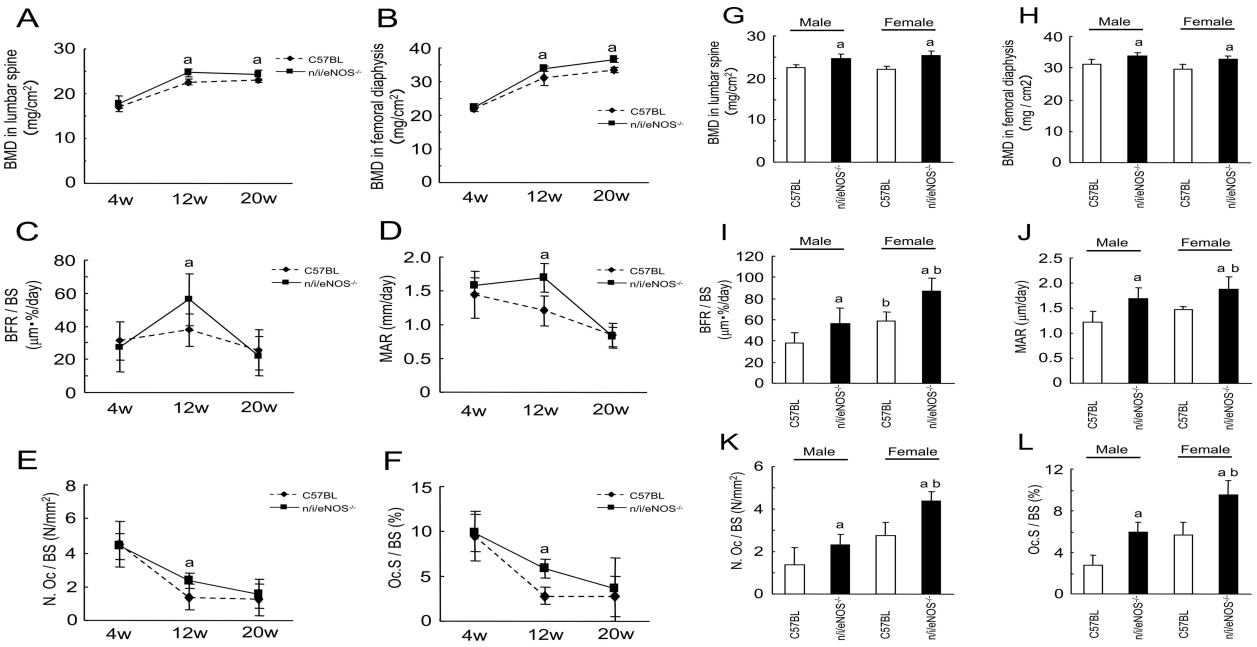

FIG. 4. Time course and sex difference of bone phenotypes in triply n/i/eNOS$^{-/-}$ mice. (A–F) Time course of the BMD values in the lumbar spine (A) and femoral diaphysis (B) and of the BFR/BS (C), MAR (D), N.Oc/BS (E), and Oc.S/BS (F) in the proximal tibia (n = 6–7). (G–L) Sex difference of the BMD values in the lumbar spine (G) and femoral diaphysis (H) and of the BFR/BS (I), MAR (J), N.Oc/BS (K), and Oc.S/BS (L) in the proximal tibia (n = 6–7). [a]$p < 0.05$ vs. wildtype C57BL, [b]$p < 0.05$ vs. male triply n/i/eNOS$^{-/-}$.

TABLE 1. mRNA EXPRESSION LEVELS OF BMP2, RUNX2, OSTERIX, OSTEOCALCIN, RANKL, AND c-*fos* IN THE FEMUR OF THE WILDTYPE C57BL AND THE TRIPLY n/i/eNOS$^{-/-}$ MICE

	C57BL (β-actin)	*Triply n/i/eNOS$^{-/-}$ (β-actin)*	*p*
BMP2	1.03 ± 0.33	1.18 ± 0.29	NS
Runx2	1.06 ± 0.41	1.38 ± 0.52	NS
Osterix	0.97 ± 0.06	1.05 ± 0.23	NS
Osteocalcin	1.01 ± 0.15	1.21 ± 0.14	NS
RANKL	1.11 ± 0.49	0.90 ± 0.53	NS
c-*fos*	1.25 ± 0.88	1.34 ± 1.07	NS

Data are expressed as mean ± SD, n = 6–7.
NS, not significant; BMP2, bone morphogenic protein 2.

Expression levels of BMP2, Runx2, Osterix, osteocalcin, RANKL, or c-fos in bone of wildtype and triply n/i/eNOS$^{-/-}$ mice

The mRNA expression levels of BMP2, Runx2, Osterix, osteocalcin, and c-*fos* in the femur tended to be more increased in the triply n/i/eNOS$^{-/-}$ than in the wildtype mice, although the difference did not reach statistically significant levels (Table 1). There was no significant difference in the expression level of RANKL in the two genotypes (Table 1).

Activation of the renin-angiotensin system in bone of triply n/i/eNOS$^{-/-}$ mice

We next studied the involvement of the renin-angiotensin system in the development of bone abnormalities in the triply n/i/eNOS$^{-/-}$ mice. The ACE activity in the femur was significantly higher only in the triply n/i/eNOS$^{-/-}$ mice, but not in any singly NOS$^{-/-}$ mice, compared with wildtype mice (Fig. 5A). The mRNA expression of the angiotensin II type 1 (AT_1) receptor in the femur was significantly more enhanced in the singly nNOS$^{-/-}$, iNOS$^{-/-}$, and eNOS$^{-/-}$ and the triply n/i/eNOS$^{-/-}$ mice than in the wildtype mice, and it was highest in the triply n/i/eNOS$^{-/-}$ mice (Fig. 5B). Immunostaining for the AT_1 receptor showed that the protein (brown) was upregulated predominantly in the hypertrophic chondrocytes of the proximal tibia compared with wildtype mice (Fig. 5C).

Alleviation of bone abnormalities of triply n/i/eNOS$^{-/-}$ mice by long-term treatment with an AT_1 receptor blocker

Long-term oral treatment with olmesartan, a selective and potent AT_1 receptor blocker (ARB),[23] for 8 wk significantly prevented increases in the BMD in the lumbar spine and femoral diaphysis and an increase in the BV/TV in the proximal tibia in the triply n/i/eNOS$^{-/-}$ mice (Figs 6A–6C). Thicker and denser trabecular bone microstructural alterations in the triply n/i/eNOS$^{-/-}$ mice were also reversed by long-term treatment with olmesartan (Figs. 6D and 6E). Furthermore, long-term treatment with olmesartan in the triply n/i/eNOS$^{-/-}$ mice significantly reduced the values in the BFR/BS, MAR, and serum alkaline phosphatase level, markers of osteoblastic cell function, and values in the Oc.N/BS, Oc.S/BS, and urinary deoxypyridinoline level, markers of osteoclastic cell number and function, to the levels of the wildtype mice (Fig. 7).

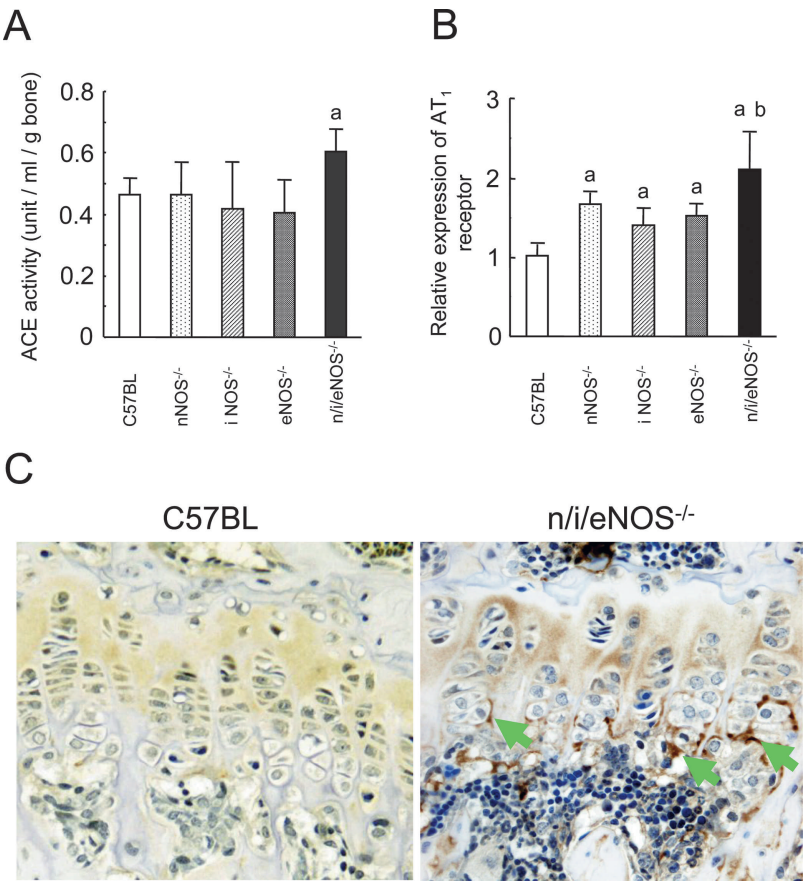

FIG. 5. Activation of the renin-angiotensin system in the bone of the triply n/i/eNOS$^{-/-}$ mice. (A) Angiotensin-converting enzyme (ACE) activity in the femur (n = 5–6). (B) The mRNA levels of the angiotensin II type 1 (AT_1) receptor in the femur (n = 6). (C) Immunostaining for the AT_1 receptor in the proximal tibia. Green arrows indicate positive immunoreactivity (brown). [a]$p < 0.05$ vs. wildtype C57BL.

DISCUSSION

The major novel findings of this study were that the triply NOS$^{-/-}$ mice manifested increased BMD and enhanced bone turnover and that long-term treatment with an ARB normalized all the bone abnormalities. These results provide the first direct evidence that complete deletion of the whole NOS system accelerates BMD and bone turnover in mice in vivo through the AT_1 receptor pathway, showing the critical role of the endogenous NOS system in maintaining bone homeostasis.

It has been shown that bone cells produce NO in response to various stimuli, including mechanical loading, estrogen, fluid flow, and pro-inflammatory cytokines.[8] It has also been reported that all three NOS isoforms are expressed in bone cells under physiological conditions: nNOS is present in osteocytes[24]; iNOS is located in osteoclasts and osteocytes[24,25]; and eNOS is detected in osteoblasts, osteoclasts, and osteocytes.[24,26] To study the roles of the whole NOS system in bone cell biology, nonselective NOS inhibitors have been widely used. However, we and others have shown that NOS inhibitors, such as N^{ω}-nitro-L-arginine (L-NNA), N^{ω}-nitro-L-arginine methyl ester (L-NAME), and N^{G}-monomethyl-L-arginine (L-NMMA), possess multiple nonspecific actions other than simple inhibition of NO synthesis.[14,27] This problem has caused conflicting results among previous studies with the NOS inhibitors, such that NO has been suggested to be stimulatory[28] or nonessential[29] for osteoblast function and to be stimulatory[25] or inhibitory[30] for osteoclast function. Thus, the authentic roles of the whole NOS system in bone cell biology remain to be fully elucidated. Our triply NOS$^{-/-}$ animal is a useful experimental tool to solve this problem and to investigate the role of the endogenous NOS system.[16,17]

In the triply NOS$^{-/-}$ mice, markers of osteoblastic cell number (MS/BS and Ob.N/BS) showed tendencies to increase, and markers of osteoblastic cell function (BFR/BS, MAR, and serum alkaline phosphatase level) were increased. Thus, it is possible that osteoblastic cell function is augmented in the triply NOS$^{-/-}$ mice. In the triply NOS$^{-/-}$ genotype, markers of osteoclastic cell number (TRACP^{+} osteoclast number and N.Oc/BS) and markers of osteoclastic cell function (Oc.S/BS and urinary deoxypyridinoline excretion) were also augmented, suggesting that the osteoclastic cell number and function are enhanced in the triply NOS$^{-/-}$ mice. Taken together, it is conceivable that both osteoblastic bone formation and osteoclastic bone resorption are accelerated in the triply NOS$^{-/-}$ mice. Because the triply NOS$^{-/-}$ mice showed higher BMD, it is likely that the extent of accelerated bone formation is relatively greater than that of accelerated bone resorption in those mice.

The triply NOS$^{-/-}$ mice exhibit hypertension,[16] dyslipidemia, and impaired glucose tolerance (unpublished data, 2008), which might affect bone turnover.[31–33] However, those factors have been reported to be associated with de-

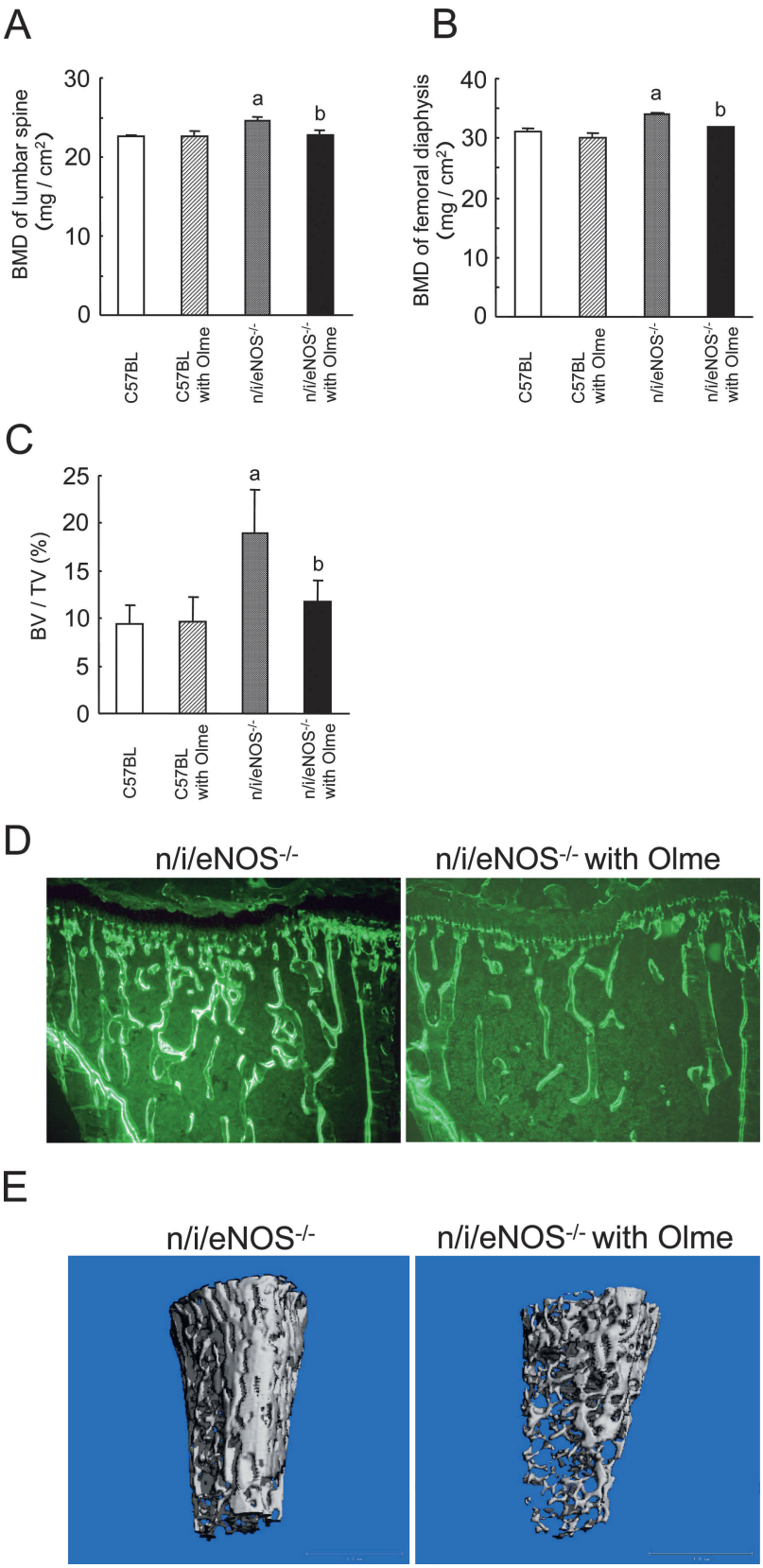

FIG. 6. Reversal of abnormal BMD, BV/TV, and trabecular bone microstructure in triply n/i/eNOS$^{-/-}$ mice by long-term treatment with an AT_1 receptor blocker. (A–E) Effects of long-term oral treatment with olmesartan, an AT_1 receptor blocker (ARB), for 8 wk on the BMD in the lumbar spine (A), the BMD in the femoral diaphysis (B), the BV/TV in the proximal tibia (C), trabecular bone microstructure in the proximal tibia (calcein double labeling) (D), and trabecular bone microstructure in the femur (3D μCT) (E) in the triply n/i/eNOS$^{-/-}$ mice. Olme, olmesartan treatment. $^{a}p < 0.05$ vs. wildtype C57BL. $^{b}p < 0.05$ vs. untreated triply n/i/eNOS$^{-/-}$.

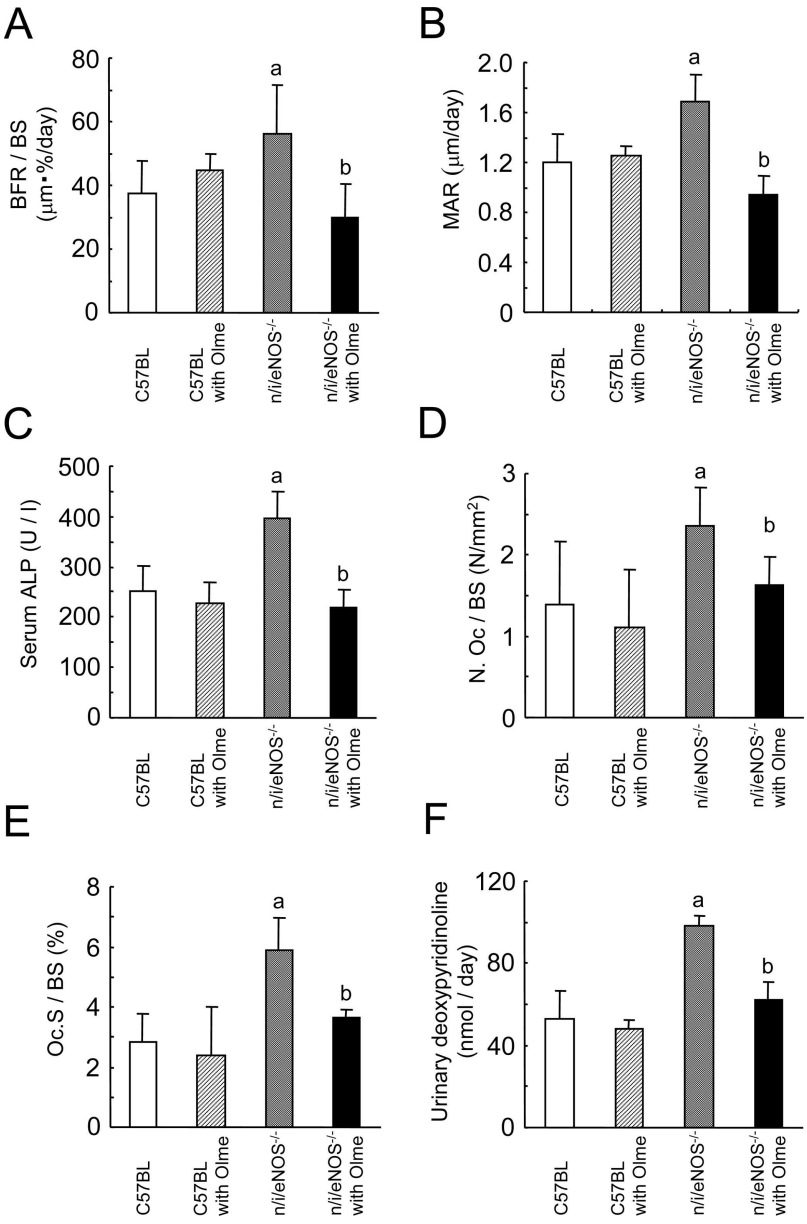

FIG. 7. Reversal of abnormal osteoblastic bone formation and osteoclastic bone resorption in the triply n/i/eNOS$^{-/-}$ mice by long-term treatment with an ARB. (A–F) Effects of long-term oral treatment with olmesartan for 8 wk on BFR/BS in the proximal tibia (A), MAR in the proximal tibia (B), serum ALP concentration (C), N.Oc/BS in the proximal tibia (D), Oc.S/BS in the proximal tibia (E), and urinary deoxypyridinoline excretion (F) in the triply n/i/eNOS$^{-/-}$ mice. Olme, olmesartan treatment. [a]$p < 0.05$ vs. wildtype C57BL. [b]$p < 0.05$ vs. untreated triply n/i/eNOS$^{-/-}$.

creases in BMD and bone formation.[31–33] Thus, it is unlikely that those abnormalities are involved in the increases in BMD and bone turnover seen in the triply NOS$^{-/-}$ mice.

Bone phenotypes in each singly NOS$^{-/-}$ mice have been reported. In the male eNOS$^{-/-}$ mice, the BMD, BV/TV, and bone formation are reduced at 8 wk of age, but they are normalized at 12 wk of age.[11] The female iNOS$^{-/-}$ mice show 30% higher BMD compared with the wildtype mice at 4 wk of age, but the difference becomes a marginal 9% at 9 wk of age.[12] These results suggest age-related bone abnormalities in the eNOS$^{-/-}$ and iNOS$^{-/-}$ mice.[11,12] We used 12-wk-old male mice in this study, and our results of normal bone metabolism in the eNOS$^{-/-}$ and iNOS$^{-/-}$ mice seem to be in agreement with those findings. The 10-wk-old female nNOS$^{-/-}$ mice have been reported to have enhanced BMD and BV/TV.[13] Consistent with the evidence, our nNOS$^{-/-}$ mice also exhibited a significant increase in the BV/TV. In the singly NOS$^{-/-}$ mice, a compensatory mechanism by other NOSs that are not genetically disrupted seems to be operative. Indeed, we have found that in all singly NOS$^{-/-}$ mice, the remaining NOSs are expressed, and in the eNOS$^{-/-}$ mice, nNOS expression was upregulated (unpublished data, 2008). Furthermore, we have indicated that in all singly NOS$^{-/-}$ mice, total NOS activity and systemic NO production are considerably well preserved.[16] Therefore, only the triply NOS$^{-/-}$ mice represent bone phenotypes that reflect the impact of endogenous NO derived from the whole NOS system.

In the triply NOS$^{-/-}$ mice, both bone formation and bone resorption were increased at 12 wk of age and normalized at 20 wk of age. These results suggest that the triply NOS$^{-/-}$ mice also exhibit age-related bone abnormalities as do the

singly eNOS$^{-/-}$ and iNOS$^{-/-}$ mice.[11,12] On the other hand, bone formation and bone resorption were more enhanced in the female than in the male triply NOS$^{-/-}$ mice, indicating the presence of a sex difference in bone turnover of the triply NOS$^{-/-}$ genotype.

We studied the molecular mechanism(s) for the abnormal bone remodeling in the triply NOS$^{-/-}$ mice. Although the expression levels of regulatory factors for osteoblast or osteoclast differentiation, including BMP2, Runx2, Osterix, osteocalcin, and c-*fos*, all showed tendencies to increase in the bone of the triply NOS$^{-/-}$ mice, the differences were not statistically significant. Our preliminary study has shown that the triply NOS$^{-/-}$ mice develop coronary arteriosclerotic lesion formation spontaneously and that activation of the renin-angiotensin system may play an important role in the pathogenesis of the coronary arteriosclerosis.[17] Thus, we next tested our hypothesis that activation of the renin-angiotensin system is also involved in the bone abnormalities of the triply NOS$^{-/-}$ mice. In the activated renin-angiotensin system, angiotensin II is synthesized from angiotensin I by ACE and exerts a variety of deleterious effects, including vasoconstriction, sodium retention, and cell growth.[18,19] There are two different types of angiotensin II receptor, the AT_1 and the AT_2, of which the former type mostly mediates those deleterious actions of angiotensin II.[18,19] In this study, activation of ACE and upregulation of the AT_1 receptor were noted in the bone of the triply NOS$^{-/-}$ mice, and both of them correlated with the bone phenotype in the singly and triply NOS$^{-/-}$ mice. Expression of the AT_1 receptor was abundant in the hypertrophied chondrocytes where endochondral bone formation occurs.

To further prove the involvement of the AT_1 receptor pathway, we studied the effects of long-term oral treatment with olmesartan, an ARB, on the bone phenotypes in the triply NOS$^{-/-}$ mice. Importantly, the treatment with olmesartan prevented the progression of increases in the BMD and BV/TV, along with the correction of abnormal trabecular bone microstructural changes. Furthermore, the treatment with olmesartan ameliorated augmented osteoblastic bone formation and enhanced osteoclastic bone resorption in those mice. A previous study has reported that the plasma concentration of olmesartan achieved by olmesartan treatment that we used in this study inhibits the binding of the AT_1 receptor almost completely without affecting the AT_2 receptor,[23] indicating that olmesartan antagonizes the AT_1 receptor both selectively and potently under our experimental conditions. Thus, it is evident that the AT_1 receptor pathway plays an important role in the development of abnormal bone phenotypes in our triply NOS$^{-/-}$ mice. Consistent with our findings, it has been indicated that the AT_1 receptor is expressed in adult and fetal bone in humans.[34] In addition, it has also been shown that angiotensin II is generated by ACE in bone cells and that this peptide stimulates both osteoblastic bone formation and osteoclastic bone resorption in vitro.[18,19] To the best of our knowledge, this study provides the first evidence for the involvement of the AT_1 receptor pathway in pathological bone remodeling in vivo.

In conclusion, we were able to show that genetic disruption of the whole endogenous NOS system causes increased BMD and enhanced bone turnover through the AT_1 receptor–mediated mechanism in mice in vivo, showing the critical role of the endogenous NOS system in maintaining bone homeostasis. Our findings should contribute to a better understanding of the role of the NO/NOS system in the regulation of bone metabolism.

ACKNOWLEDGMENTS

The authors thank Erika Kobayashi, Keiko Kohno, Nao Terada, and Ryoko Utanohara at the University of Occupational and Environmental Health, Kitakyushu, Japan for their excellent technical assistance. This work was supported in part by the Grants-in-Aid for Scientific Research 17390071 from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan, and grants from the Sankyo Pharmaceutical Co Ltd, Tokyo, Japan, and the University of Occupational and Environmental Health for Advanced Research, Kitakyushu, Japan. The first author of this manuscript received the Young Investigator Award at the American Society for Bone and Mineral Research Annual Meeting 2006 for this study.

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Received in original form August 31, 2007; revised form December 29, 2007; accepted January 28, 2008.