Oestadiol can stimulate osteoblast activity. Osteo-
blast function is thought to be regulated by nitric ox-
ide (NO). We hypothesised that the effect of 17β-
oestradiol (17β-E₂) on osteoblast activity is mediated
by NO. This hypothesis was tested using osteoblasts
isolated from human trabecular bone, calvariae of
rats, endothelial NO synthase (eNOS) gene-deficient
mice, and their wild-type counterparts. Our results
show that 17β-E₂ dose-dependently stimulated prolif-
eration and differentiation of primary human, rat
and wild-type osteoblasts. The presence of N⁰-
monomethyl-L-arginine (10⁻⁷ M), an inhibitor of NOS activ-
ity, blocked the 17β-E₂-(10⁻⁷ M)-induced increases in
thymidine incorporation (P < 0.01), alkaline phospha-
tase activity (P < 0.01) and bone nodule formation (P <
0.01) of wild-type, human and rat osteoblasts, respec-
tively. Moreover, 17β-E₂ did not induce a response in
eNOS gene-deficient osteoblasts. 17β-E₂ also increased
total eNOS enzyme expression in rat osteoblasts.
These findings indicate 17β-E₂ modulates osteoblast
function by NO-dependent mechanisms mediated via
the eNOS isoform.

Key Words: nitric oxide; endothelial nitric oxide syn-
thase; osteoblast; knock-out mice; 17β-oestradiol.

Oestrogens influence the skeleton, as evidenced by re-
duced bone density in oestrogen-depleted women and
the preventive action of exogenous oestrogens on this reduc-
tion in post-menopausal women (1, 2). While the major
effect of oestrogen is thought to be an inhibition of oste-
dlastic bone resorption, oestrogen also has direct effects
on osteoblast-like cells (3). The presence of functional
oestrogen receptors in cells of the osteoblast lineage (4)
support the hypothesis that 17β-oestradiol (17β-E₂) stim-
ulates bone formation in vivo by a direct action on osteo-
blasts. It has been shown that bone mineral loss in oes-
trogen depletion-induced osteoporosis can be prevented
by administration of nitroglycerin, a nitric oxide (NO)
donor (5). NO is a free radical synthesised from
L-arginine by NO synthase (NOS) (6). Three different
isoforms have been identified, endothelial NOS (eNOS)
(7), neuronal NOS (nNOS) (8), and inducible NOS (iNOS)
(9). Both eNOS and nNOS are expressed constitutively,
whereas iNOS is generally only expressed in response to
cytokine stimulation (10, 11).

Increasing evidence indicates that nitric oxide (NO)
contributes significantly to bone homeostasis and pre-
vious reports have demonstrated that NO exerts a
primary suppressive effect on osteoclastic bone resorp-
tion and promotes osteoblastic bone formation (12–19).
Osteoblasts are responsible for the synthesis, organi-
sation, and mineralisation of the extracellular matrix
of bone and osteoblast cultures can be used as an in
vitro model to investigate bone formation (20–22). Ar-
mour and Ralston (23) have provided evidence that
NOS expression can be upregulated by oestrogen in a
human osteoblast-like cell line. We hypothesised that
the eNOS isoform is implicated in bone formation and
osteoblast function, and also that the stimulatory effect
of 17β-E₂ on osteoblast function is mediated by eNOS.

MATERIALS AND METHODS

Human osteoblast-enriched cell cultures. Osteoblasts were iso-
lated from trabecular bone of femoral heads taken during hip arthro-
plasty, from female patients, age range 50–71 years. According to
the method of Bereford et al. (20), bone tissue was cut and minced to
fragments less than 3 mm. The bone tissue fragments were rinsed
several times in Dulbecco’s modified Eagle media (DMEM) and then
placed in DMEM supplemented with 10% w/v foetal bovine serum
(FBS), 2 mM L-glutamine and 500 U/ml penicillin G, 500 μg/ml streptomycin,
and 0.3 μg/ml amphotericin B (antibiotics/
antimycotics) at 37°C, 95% air humidity, and 5% CO₂ until subcon-
fluent for experiments. For 48 h prior to experimentation, cultures
were placed in 0.1% v/v FBS and phenol red-free MEM. After 48 h the cells were placed in phenol red-free MEM supplemented with 10% v/v dextran-charcoal stripped FBS. The cells used in these experiments were treated with 17β-E₂ (10⁻⁹–10⁻⁷ M) to determine the optimal dose, which was used throughout the succeeding experiments. Cells were also treated with the inactive stereoisomer 17α-oestradiol (10⁻⁷ M). In addition, cells were treated with 17β-E₂ (10⁻⁷ M) in the presence and absence of N⁶-monomethyl-L-arginine (L-NMMA) (10⁻³ M). Additionally, the inactive stereoisomer N⁶-monomethyl-D-arginine (D-NMMA) (10⁻³ M) was administered to these cells alone and in combination with 17β-E₂ (10⁻⁷ M).

Rodent osteoblast-enriched cell cultures. Primary rat and mouse osteoblast-enriched cultures were isolated by sequential enzymatic digestion of neonatal calvariae, as previously described (21, 22). The calvariae were dissected from either 50 female neonatal (1) Wistar rats, (2) eNOS gene-deficient (knock-out) mice (eNOS KO) or 3) wild-type control mice (WT), and digested with collagenase for periods of 2 x 10 and 3 x 20 min sequentially. The cells isolated from the final three digests were cultured in modified minimum essential media (α-MEM) with nucleotides supplemented with 15% v/v FBS, antibiotics/antimycotics until sub-confluent for experiments at 37°C, in 95% air humidity and 5% CO₂. The cells were treated as described above. Cultures used to grow bone nodules were kept in 4 cm wells in modified α-MEM with nucleotides supplemented with 15% v/v FBS, 10 mM sodium β-glycerophosphate, 50 μg/ml ascorbic acid, antibiotics/antimycotics for 21 days.

Genotyping of eNOS gene-deficient mice and wild-type controls. Homozygous eNOS mutant mice were generated by homologous recombination (24). They were backcrossed for 10 generations to the C57BL/6 strain. Wild-type C57Bl/6 mice were used as controls. Loss of the wild-type allele was confirmed in each littermate by polymerase chain reaction (PCR) to confirm presence or absence of the eNOS gene. Samples were placed in 0.4 ml of lysis buffer (10 mM Tris, pH 8.0, 50 mM EDTA, 100 mM NaCl, 0.5% SDS, and 500 μl/ml proteinase K) at 55°C with agitation, overnight. The DNA was precipitated by absolute ethanol, washed with 70% ethanol and resuspended in 30 μl of distilled water. DNA products were amplified by subjecting 2–4 μl of resuspended DNA to 35 cycles of PCR (94°C for 30 s; 60°C for 30 s, and 72°C for 1 min) and then to 1 cycle of PCR (72°C for 10 min) in the presence of dNTPs (50 μM) and 2.5 units of Taq polymerase (Promega, Madison, WI) in a standard buffer containing 1.8 mM MgCl₂. The sense oligonucleotide 5′GGGCTCCCTTCCGGCTGCCACC′ and the antisense oligonucleotide 5′GGATCCCTGGAAAAGGCGGTGAGG′, which amplify a 900 bp PCR product of eNOS cDNA, identified WT mice. To identify the eNOS KO mice, the sense oligonucleotide 5′ATGAACCTGACAGGAGGACGCCG′ and the antisense 5′GGCGATAAGGCGGATGCCTG′, which amplify a 603 bp PCR product, corresponding to the neo resistance gene.

For each experiment, positive and negative control samples were always run. The amplified products were electrophoresed in 1% agarose gel with 50 μl/ml ethidium bromide.

FIG. 1. (A) Dose-dependent effect of 17β-E₂ on thymidine incorporation human and rat osteoblasts treated for 24 h. (B) Effect of various treatments on NO metabolite levels in rat osteoblasts over 24 h. (C) Thymidine incorporation in human and rat osteoblasts treated with 17β-E₂ in the presence and absence of an NO inhibitor, L-NMMA, for 24 h. (D) Dose-dependent effect of 17β-E₂ on thymidine incorporation eNOS KO and WT osteoblasts treated for 24 h. *P < 0.05, **P < 0.01, ***P < 0.001. Results are mean ± SEM, n = 3.
Thymidine incorporation assay. Tritiated thymidine incorporation was used to assess cell proliferation. Osteoblasts were plated in 1 cm wells at a density of 5 x 10^4 cells/well and left to adhere in DMEM containing 10% FBS. After 24 h, the cells were placed in 0.1% dextran-charcoal stripped FBS and phenol red-free MEM for 48 h prior to treatment. During the 24-h treatment period (1^H)-thymidine (1 μCi/ml; Amersham, Little Chalfont, UK) was included in the media. Following treatment, cells were washed with phosphate buffered saline (3 x 5 min) and then 10% trichloroacetic acid (1 x 30 min), before 0.5 ml of 0.2 M NaOH was added and left overnight at 4°C. The resulting lysate was used to determine the thymidine incorporation by scintillation counting (Packard Tri-Carb 2200 CA, Packard Bioscience, Berkshire, UK). Each sample was counted for 2 min and radioactivity was expressed as disintegration per minute (dpm).

Assessment of mature osteoblastic activity. Determination of total alkaline phosphatase activity was performed after 24 h of treatment after extraction in cell digestion buffer (0.1% lauryl sulfate (SDS), 0.5% sodium deoxycholate, phenylmethylsulfonyl fluoride 10 μg/ml, aprotinin 30 μg/ml, sodium orthovanadate 10 μg/ml). The enzyme activity was determined colorimetrically using p-nitrophenylphosphate as substrate and reading the optical density at 405 nm using a Multiscan RC plate reader (Labsystems, Life Sciences International Ltd., Hampshire, UK). A standard curve was constructed using p-nitrophenol as a standard (25). The results were normalised for total protein concentration in the lysates as measured by the Bradford dye-binding method (26).

Bone nodule assay. Cells were grown in 4 cm wells for 21 days with an initial seeding density of 5 x 10^4 cells/well, in which the media was changed every 3 days. Cultures were fixed in 10% v/v formal saline for 5 min. Bone nodules were detected using a calcium specific dye Alizarin Red (pH 7.4) (27). Bone nodules per well were counted macroscopically.

Measurement of nitric oxide metabolism. NO metabolites (nitrate and nitrite) in the medium of cell cultures after 24 h of treatment were measured using the chemiluminescence assay (28). Samples were injected into a purge vessel where nitrate and nitrite was converted to NO in a reducing mixture of 0.1 M VCl2, in 1 M HCl at 90°C. NO was detected by its reaction with ozone generated from pure oxygen using a Sievers NOA 270B nitric oxide analyser (Sievers Instruments, Boulder, CO). Comparisons were made against standard curves generated by known concentrations of sodium nitrate. The results were normalised for cell number.

Western blotting. Proteins were extracted from 17β-E2 100 nM (24 h) treated cells and untreated osteoblasts using a ratio of 250 μl of lysis buffer (50 mM TRIS base and 50 mM NaCl, pH 7.4, with 1% w/v SDS) per 2 x 10^6 cells. Protein concentrations were determined by the Bradford dye-binding method (26). Lysates (standardised as 50 μg total protein per lane) were combined with an equal volume of loading buffer (0.5 M Tris–HCl [pH 6.8], 20% v/v glycerol 10% w/v SDS, 0.1 M β-mercaptoethanol, and 0.05% w/v bromophenol blue) and denatured by boiling for 5 min. Samples were electrophoresed through a 7.5% w/v SDS-polyacrylamide gel and transferred to a 0.45 μm nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Membranes were immersed in blocking solution containing 5% w/v milk before incubation with either (1) eNOS monoclonal antibody raised against a 20.4 kDA protein fragment corresponding to amino acids 1030–1209 of human eNOS (1:1000, Transduction Laboratories, Lexington, KT), (2) oestrogen receptor (ER) α monoclonal antibody raised against a protein fragment corresponding to amino acids 247–263 of the ERE–DNA binding domain (1:500, Alexis Biochemicals, Bingham, UK), (3) β-actin monoclonal antibody raised against a protein fragment corresponding to the N-terminal peptide of the β isoform of actin (1:5000, Sigma-Aldrich Chemical Co, Poole, UK), in TRIS-buffered saline with 0.1% w/v Tween-20 (TBS-T). After 2 h incubation at room temperature, membranes were washed in TBS-T and then incubated for 2 h with biotin-conjugated rat-ab sorbed horse anti-mouse antibody (1:1000). Immunoreactive bands were revealed using peroxidase-conjugated streptavidin (1:5000) for 1 h followed by detection using ECL chemiluminescence detection kit (Amersham, Little Chalfont, UK). Endothelial cell lysate was used as a positive control for detection of the 135 kDa band corresponding to eNOS. Lysate from mouse uterine tissue was used as a positive control for detection of 67 kDa band corresponding to ERα. As a negative control the primary antisera was omitted.

Statistical analysis. Data were presented as the mean ± SEM and represent three independent triplicate cell cultures. Differences between treatment were tested for by one-way analysis of variance with Tukey’s multiple comparisons post-test. A P < 0.05 was regarded as statistically significant.

**DISCUSSION AND RESULTS**

This study aimed to demonstrate that the ensuing effects of oestrogen on osteoblast activity are dependent on an NO-mediated mechanism. Dose-response curves of thymidine incorporation in human and rat osteoblasts to 17β-E2 (10^-3–10^-7 M) were plotted (Fig. 1A). Consistent with the findings of Mathieu and Merregaert (29), 10^-7 M 17β-E2 was found to give the maximum response. This was then used for the proceeding experiments. It was found that 10^-7 M 17β-E2 significantly increased alkaline phosphatase activity (Table 1) and bone formation rates (Table 2) in both the human and the rat osteoblasts. Importantly, the inactive stereoisomer 17α-E2 (10^-7 M) produced no significant increase in thymidine incorporation (Fig. 1A), alkaline phosphatase activity (Table 1) nor to bone nodule formation (Table 2), confirming that the effects of 17β-E2 are isomer-specific.

Treatment of rat osteoblasts with 17β-E2 (10^-7 M) (24 h) resulted in elevated NO metabolite levels (P < 0.01) in the culture media (Fig. 1B). This increase in NO metabolite levels was abolished in the presence of L-NNMMA (10^-3 M) (Fig. 1B). Incubation with L-NNMMA alone had no significant effect (Fig. 1B). Total eNOS enzyme expression in rat osteoblasts was quantified by Western blots and was found to be greater in 17β-E2 (10^-7 M) treated (24 h) osteoblasts cells than in the untreated osteoblasts (Fig. 2A). As an internal control, anti-β-actin was used and confirmed equal loading and transfer of proteins (Fig. 2B). Elevated eNOS protein expression seen in the 17β-E2 treated osteoblasts, suggest that the interactions between oestrogen and NO signalling pathways may occur via the eNOS isoform. Previously, it has been shown that shear stress (30) and pregnancy (31) can induce eNOS, and more recently 17β-E2 administration to a human osteoblast-like cell line (23) has been shown to induce eNOS. The eNOS gene has several half-palindromic oestrogen response binding sites in the promoter (32), suggesting a direct action on gene transcription.

A single dose of N^-monomethyl-L-arginine (L-NNMMA; 10^-3 M) in human and rat osteoblasts markedly blunted the 17β-E2-induced rise in thymidine in-
corporation (Fig. 1C), alkaline phosphatase activity (Table 1) and bone nodule formation (Table 2). However, L-NMMA (10^-3 M) alone had no significant effect on thymidine incorporation (Fig. 1C), alkaline phosphatase activity (Table 1), nor bone nodule formation (Table 2). The inactive stereoisomer D-NMMA (10^-3 M) did not inhibit the 17β-E2 induced increases in thymidine incorporation (Fig. 1B) nor alkaline phosphatase activity (Table 1). Additionally, D-NMMA (10^-3 M) alone had no significant effect on thymidine incorporation (Fig. 1C) nor alkaline phosphatase activity (Table 1) in human and rat osteoblasts. The iNOS inhibitor, aminoguanidine, but not NG-nitro-L-arginine methyl ester (L-NAME) nor L-NMMA, has been shown to inhibit bone formation in vivo (12). Moreover, Riancho et al. (13) found L-NMMA induced a dose-dependent inhibitory effect on the proliferation of the osteoblast-like cell lines MG63 and ROS 17/2.8. Considering these findings, Turner et al. (12) suggested that some NOS inhibitors might have effects on bone formation independent of the inhibition of NOS activity. Therefore, eNOS gene-deficient mice are a useful model, as this genetic approach circumvents the lack of isoform specificity of the current generation of NOS inhibitors and allows the study of the role of eNOS isoform on bone metabolism and bone regulation.

Dose response curves of thymidine incorporation in WT and eNOS KO osteoblasts to 17β-E2 (10^-9-10^-7 M) were plotted (Fig. 1D). Consistent with the human and rat findings, 10^-7 M 17β-E2 was found to give the maximum response in WT osteoblasts. However, 17β-E2 (10^-3-10^-7 M) did not induce an increase in thymidine incorporation in eNOS KO osteoblasts (Fig. 1D). Alkaline phosphatase activity (Table 1) and bone nodule formation (Table 2) were also significantly increased when treated with 17β-E2 (10^-7 M) in the WT osteoblasts but not in the eNOS KO osteoblasts.

Oestrogen acts by binding to and activating the two oestrogen receptors (ERs), which are referred to as ERα and ERβ. Vidal et al. (33) have demonstrated that ERα, but not ERβ, mediates the effects of oestrogen on the skeleton in the mouse. Therefore, oestrogen receptor α (ERα) protein levels in the eNOS KO osteoblasts and the wild-type osteoblasts were assessed to determine if an alteration in ERα expression could explain the lack of response to 17β-E2 in the eNOS KO osteoblasts. However, they were found to be equal in eNOS KO and WT osteoblasts (Fig 3A). β-actin levels were measured as an internal control and confirmed equal loading and transfer of protein (Fig 3B). Total alkaline phosphatase activity (Table 1) and bone nodule formation (Table 2) in untreated eNOS KO osteoblasts was

### Table 1
Calculated Alkaline Phosphatase Activity Units/mg Protein

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
<th>WT</th>
<th>eNOS KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>14.92 ± 0.96</td>
<td>15.8 ± 0.31</td>
<td>12.3 ± 1.39</td>
<td>9.94 ± 0.40</td>
</tr>
<tr>
<td>10^-7 M 17βE2</td>
<td>20.57 ± 1.16*</td>
<td>18.8 ± 0.65*</td>
<td>15.7 ± 0.68**</td>
<td>10.57 ± 1.16</td>
</tr>
<tr>
<td>10^-3 M 17βE2</td>
<td>16.32 ± 0.74</td>
<td>16.04 ± 0.5</td>
<td>11.3 ± 1.18</td>
<td>8.75 ± 0.96</td>
</tr>
<tr>
<td>10^-3 M L-NMMA</td>
<td>15.81 ± 0.84</td>
<td>14.8 ± 0.85</td>
<td>13.6 ± 0.74</td>
<td>10.83 ± 1.04</td>
</tr>
<tr>
<td>10^-3 M 17βE2 + 10^-3 M L-NMMA</td>
<td>20.13 ± 0.97*</td>
<td>18.3 ± 0.6**</td>
<td>14.6 ± 0.98**</td>
<td>11.3 ± 1.19</td>
</tr>
<tr>
<td>10^-3 M D-NMMA</td>
<td>15.64 ± 0.50</td>
<td>16.4 ± 0.13</td>
<td>11.57 ± 1.1</td>
<td>10.64 ± 0.51</td>
</tr>
<tr>
<td>10^-7 M 17αβE2</td>
<td>16.89 ± 1.39</td>
<td>15.74 ± 0.4</td>
<td>13.1 ± 0.76</td>
<td>8.81 ± 0.65</td>
</tr>
</tbody>
</table>

Note: Alkaline phosphatase activity units are defined as the amount of enzyme activity that liberates 1 nmol of p-nitrophenol per minute under the assay conditions. The results were normalised for total protein concentration. Results after 21 days in phenol red-free MEM supplemented with 10% v/v dextran-charcoal stripped FBS. Results expressed as mean of three independent triplicate cultures ± SEM. *P < 0.01; **P < 0.005.

### Table 2
Numbers of Bone Nodules Formed per Well

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>WT</th>
<th>eNOS KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>23 ± 1.10</td>
<td>26 ± 0.54</td>
<td>12 ± 1.29</td>
</tr>
<tr>
<td>10^-7 M 17βE2</td>
<td>35 ± 1.44**</td>
<td>37 ± 0.97**</td>
<td>13 ± 1.03</td>
</tr>
<tr>
<td>10^-7 M 17βE2 + 10^-3 M L-NMMA</td>
<td>25 ± 1.97</td>
<td>21 ± 0.93</td>
<td>11 ± 0.97</td>
</tr>
<tr>
<td>10^-3 M L-NMMA</td>
<td>24 ± 0.98</td>
<td>21 ± 1.3</td>
<td>14 ± 0.97</td>
</tr>
<tr>
<td>10^-7 M 17αβE2</td>
<td>26 ± 0.9</td>
<td>23 ± 1.5</td>
<td>10 ± 1.19</td>
</tr>
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</table>

Note: Cultures used to grow bone nodules were kept in 4 cm wells in modified α-MEM with nucleotides supplemented with 15% v/v FBS, 10 mM sodium β-glycerophosphate, 50 μg/ml ascorbic acid, antibiotics/antimycotics for 21 days. Results expressed as mean of three independent triplicate cultures ± SEM. **P < 0.001.
also significantly decreased compared with the untreated WT cells. The NO donor, S-nitro-N-acetylpenicillamine (SNAP; 10^{-6} M), recovered the eNOS KO osteoblasts to WT control levels in terms of cell proliferation (5.6 \times 10^4 cells in eNOS KO untreated vs 6.7 \times 10^4 cells in eNOS KO SNAP treated vs 6.9 \times 10^4 cells in WT untreated, after 7 days in culture), alkaline phosphatase activity (9.945 \pm 0.405 activity units/mg protein in eNOS KO untreated vs 12.3 \pm 0.235 activity units/mg protein in eNOS KO SNAP treated vs 12.3 \pm 1.39 activity units/mg protein in WT untreated, after 21 days in culture) and bone nodule formation (12 \pm 1.29 nodules/well in eNOS KO untreated vs 24 \pm 1.03 nodules/well in eNOS KO SNAP treated vs 26 \pm 0.54 nodules/well in WT untreated, after 21 days in culture).

Earlier studies have demonstrated that 17\beta-E_2 maintains bone mass by inhibition of bone resorption, which leads to a reduction in bone turnover. More recently, evidence from experimental studies (34–36) suggests that 17\beta-E_2 may also directly promote bone formation in vivo. Accordingly, oestrogen receptors have been shown to be expressed by osteoblasts (4). Studies in vitro have shown 17\beta-E_2 to enhance both proliferation and differentiation of cultured osteoblasts (37–39). Mancini et al. (40) demonstrated that low concentrations of NO, similar to the levels produced via eNOS, stimulates osteoblast replication and alkaline phosphatase activity.
In conclusion, our data has established that oestrogen-stimulated osteoblast proliferation and differentiation are dependent on production of NO. Our findings also suggest that the stimulatory effect of 17β-E2 on osteoblast differentiation and proliferation, and the actions of oestrogen on bone metabolism, rely on local production of NO by bone cells via the eNOS isoform. Furthermore, if our results reflect the in vivo action of 17β-E2, they suggest that the NO mediated actions of oestradiol are not confined exclusively to osteoclast inhibition.

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