Bioconversion of norethisterone, a progesterone receptor agonist into estrogen receptor agonists in osteoblastic cells

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Abstract

A number of clinical studies have demonstrated that norethisterone (NET), a potent synthetic progestin, restores postmenopausal bone loss, although its mode of action on bone cells is not fully understood, while the effect of naturally occurring progesterone in bone has remained controversial. A recent report claims that the potent effects of NET on osteoblastic cell proliferation and differentiation, mimicking the action of estrogens, are mediated by non-phenolic NET derivatives. To determine whether osteoblasts possess the enzymes required to bioconvert a progesterone receptor (PR) agonist into A-ring reduced metabolites with affinity to bind estrogen receptor (ER), we studied the in vitro metabolism of [3H]-labeled NET in cultured neonatal rat osteoblasts and the interaction of its metabolic conversion products with cytosolic –osteoblast ER, employing a competition analysis. Results indicated that NET was extensively bioconverted (36.4%) to 5α-reduced metabolites, including 5α-dihydro NET, 3α,5α-tetrahydro NET (3α,5α-NET) and 3β,5α-tetrahydro NET (3β,5α-NET), demonstrating the activities of 5α-steroid reductase and two enzymes of the aldo-keto reductases family. Expression of Srd5a1 in neonatal osteoblast was well demonstrated, whereas Srd5a2 expression was not detected. The most striking finding was that 3β,5α-NET and 3α,5α-NET were efficient competitors of [3H]-estradiol for osteoblast ER binding sites, exhibiting affinities similar to that of estradiol. The results support the concept that the interplay of 5α-steroid reductase and aldo-keto reductases in osteoblastic cells, acting as an intracrine modulator system is capable to bioconvert a PR agonist into ER agonists, offering an explanation of the molecular mechanisms NET uses to enhance osteoblastic cell activities.

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Introduction

A number of clinical trials have demonstrated that administration of norethisterone (NET), a synthetic 19-nor progestin, to postmenopausal women prevents bone mineral loss and reduces bone resorption (Abdalla et al. 1985, Horowitz et al. 1993) and also prevents bone loss in young women treated with LH-releasing agonists (Riis et al. 1990). The mechanisms of estrogen-like bone actions of NET are not fully understood, particularly since this steroid molecule neither interacts with estrogen receptors (ER; Chávez et al. 1985) nor undergoes enzyme-mediated aromatization (Gual et al. 1962). Interestingly, a study conducted in postmenopausal women and castrated patients with complete androgen resistance, strongly suggested that the antigonadotropic effect of NET is mediated through ER (Pérez-Palacios et al. 1981).

Recently, evidence has been presented indicating that two reduced derivatives of NET, 3β,5α-tetrahydro-NET (3β,5α-NET) and 3α,5α-tetrahydro-NET (3α,5α-NET), induced significant effects on rat osteoblast proliferation, differentiation, and mineralization processes, mimicking the effects of estradiol (E2). The osteoblast proliferation induced by NET reduced derivatives was suppressed by the addition of ICI 182780, a potent steroidal antiestrogen, indicating that this effect is mediated by ER. The study also provides evidence that high concentrations of unmodified NET exhibited estrogenic potency in osteoblastic cells, though these effects were suppressed by finasteride, a selective steroid 5α-reductase inhibitor (Enríquez et al. 2007).

Androgen metabolizing enzymes have been demonstrated in bone cells; thus, several reports have documented the activities and gene expression of 5α-reductase 1 and 2 in human osteoblastic cells Shimodaira et al. (1996), Issa et al. (2002) and Vittek et al. (1974), using testosterone as a substrate, demonstrated the activity of 3α-hydroxysteroid dehydrogenase (3α-HSD) in rat mandibular bone. Previous studies from our laboratory have demonstrated that synthetic 19-nor progestins are extensively metabolized in target organs

To assess if NET is bioconverted to non-phenolic reduced metabolites with intrinsic estrogenic potency in osteoblasts, we studied the metabolism of [3H]-labeled NET in rat cultured osteoblastic cells and the interaction of NET and its 5α-reduced metabolites with the osteoblast intracellular ER. The identification of NET metabolites was established by a reverse isotope dilution technique, while their interaction with ER was assessed by competition analysis using [3H]-E2 as the radioligand. In addition, the gene expression of 5α-steroid reductase type 1 (Srd5a1) and type 2 (Srd5a2) was studied using RT-PCR.

Materials and Methods

Steroids and chemicals

[6,7-3H] NET ([3H]-NET), specific activity (sp. act.) 55 Ci/nmol was kindly provided by Schering (Berlin, Germany), [2,4,6,7,16,17-3H] estradiol ([3H]-E2), sp. act. 157 Ci/nmol was purchased from Amersham International and their radiochemical purity was established by thin-layer chromatographic behavior. Authentic NET was kindly provided by Schering Mexicana, S.A. de C.V. (Mexico City, Mexico). Synthesis of 5α-dihydronorethisterone (5α-NET), 3α,5α-NET, and 3β,5α-NET was previously reported (Chávez et al. 1985). The chemical purity of NET and its reduced derivatives was assessed by their melting points, HPLC behavior, infrared absorption, and H-nuclear magnetic resonance. The physical and spectroscopic constants of the A-ring reduced derivatives of NET have been previously described (Chávez et al. 1985). Other non-radioactive steroids were supplied by Sigma Chemical Co. Fetal bovine serum (FBS) was purchased from Hyclone Laboratories Inc., and phenol red-free DMEM from Life Technologies. Reverse transcriptase RT-PCR kit and TRIzol reagent were purchased from Invitrogen. TaqMan Universal PCR Master Mix and TaqMan probes and primers were obtained from Applied Biosystems (Foster City, CA, USA). Reagents and solvents used were of analytical grade.

Animals

Female neonatal Wistar rats used in this study were obtained from the School of Medicine, Universidad Nacional Autónoma de México (UNAM). Animals were killed by decapitation and calvariae were immediately removed. All procedures were performed in accordance with the Guidelines on the Handling and Training of Laboratory Animals, published by the Universities Federation for Animal Welfare and approved by the Research Ethics Board of the Universidad Autónoma Metropolitana (UAM).

Isolation and culture of rat osteoblastic cells

Osteoblastic calvarial cells from 1 day old rats were used throughout the study. Calvariae were carefully dissected, cleaned from connective tissue, periosteum and cartilaginous part, and sequentially digested for 60 min with 0.3% type II collagenase (Robey 1995). Cells obtained in the first treatment were discarded, while cells isolated from the subsequent three digestions were pooled, plated, and cultured overnight in DMEM supplemented with 10% FBS and 100 μM non-essential amino acids and an antibiotics–antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin-B; Gibco BRL) and 50 μg/ml ascorbic acid, at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Assessment of the phenotype of cultured rat calvarial cells was done by determining the presence of alkaline phosphatase activity and osteocalcin, according to the methods described by Kaplow (1955) and Arzate et al. (1998) respectively. The results revealed the presence of these two bone-related proteins in more than 95% of the calvarial cells, thus demonstrating the distinctive features of the osteoblast phenotype. At confluence, primary rat osteoblasts were detached with 1 mM EDTA/0.25% trypsin solution, counted and submitted to metabolism and ER competition studies.

Norethisterone metabolism

To assess the bioconversion of NET to its A-ring reduced derivatives, the metabolism of [3H]-NET in cultured osteoblastic cells was studied. Osteoblasts were plated at a density of 2×10⁶/Petri dish and incubated for 24 h in culture medium (DMEM at pH 7.4, containing 10% charcoal–dextran treated (stripped) FBS, non-essential amino acids, antibiotics–antimycotic solution) and 50 μg/ml ascorbic acid, at 37 °C in a humidified atmosphere of 5% CO₂ in air. The culture medium was removed and replaced by fresh medium containing 2 μM [3H]-NET and incubated at 37 °C, for 6, 24, and 48 h, under 5% CO₂–air atmosphere. The optimal concentration of substrate ([3H]-NET) employed in these experiments, was derived from previous studies aimed to characterize the kinetic constant (Kₘ) at equilibrium of 5α-steroid reductase in different tissues, using testosterone and gestodene, other synthetic 19-norprogestin as substrates (Lemus et al. 2001). Final incubation volume was 3 ml. Cell-free and boiled inactivated cell incubations carried out under identical experimental conditions were used as negative controls. Protein content was determined by the Bradford’s dye-binding method (1976), using BSA as standard. Additional experiments with [3H]-NET were conducted in the presence or absence of 1 mM finasteride (Merck Sharp & Domhe, Mexico City, Mexico). At the end of the incubation period, the reaction was stopped by the addition of ethyl acetate and radiolabeled steroids were extracted (4×) using three volumes of water saturated ethyl acetate. The organic extracts were partitioned between petroleum ether and 10%
aqueous methanol and 5 µg each of the following steroid carriers were added to the methanolic extracts: NET, 5α-NET, 3α,5α-NET, and 3β,5α-NET. The identification of NET metabolites was established by a reverse-isotope dilution technique, including identical behavior of the steroid carriers in two different thin layer chromatographic systems (chloroform–acetone 9:1 and benzene–ethyl acetate 2:1). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer model 1900 TR, using toluene containing 4 g/l PPO and 100 mg/l dimethyl POPOP as the counting solution. Counting efficiency for [3H] was 65% and quenching was corrected in all samples by external standardization. Non-radioactive steroid carriers were detected on chromatograms using the p-anisaldehyde-sulphuric/acetic acids reagent. The formation rates of the metabolic conversion products of NET are expressed as pmol/mg of protein.

Isolation of total RNA and real-time PCR
The expression of Srd5a1 and Srd5a2 in osteoblastic cells was detected by RT-PCR. Total RNA from the cells (6 × 10⁶) was extracted using TRIzol reagent and an aliquot (3 µg) from each sample was subjected to reverse transcription using a superscript first-strand cDNA synthesis kit, according to the manufacturer’s protocol. Samples were subjected to quantitative amplification using the TaqMan probe and primers sets of Srd5a1 (Rn00567064_m1) and Srd5a2 (Rn00575595_m1). PCR amplification was carried out in triplicate for each sample and performed in a total volume of 10 µl containing 75 ng of cDNA, 900 nM of each primer, 6 µl of TaqMan Universal PCR Master Mix. All PCRs were done including one initial step of DNA polymerase activation for 10 min at 95 °C and then 40 cycles (15 s at 95 °C and 1 min at 60 °C). Gene expression was normalized with the expression of the housekeeping β-actin gene (mRNA enzyme/mRNA β-actin). mRNA rat anterior ventral prostate was used as a positive control and RNAs without RT were used as a negative control. Results are given as relative mRNA expression.

Estrogen receptor binding studies
Cytosol preparation Osteoblastic cells were plated in culture medium at a density of 5 × 10⁶ cells/flask and incubated for 2 weeks at 37 °C, in a 5% CO₂-air atmosphere, with medium replacement every other day. At the end of the incubation period, the culture medium was removed, replaced by 5% stripped FBS medium and incubated for an additional 3 h. Cells were detached using 1 mM EDTA/0.25% trypsin solution, harvested and centrifuged at 286 g for 5 min. The cellular pellet was resuspended in TEDLM buffer (20 mM Tris–HCl, pH 7.4 at 4 °C, 1.5 mM EDTA, 0.25 mM dithiothreitol, 10 µg/ml leupeptin, and 10 mM sodium molybdate), the number of cells determined (1 × 10⁶) and submitted to sonication. Cytosolic fraction was obtained by centrifugation at 160 000 g for 1 h at 4 °C in an SW 50.1 rotor (Beckman Instruments, Palo Alto, CA, USA) and protein content was determined.

Binding assays Equilibrium parameters of the reaction between [3H]-E₂ and cytosol limited-capacity binding sites were studied by incubation of cytosol aliquots (400 µg protein/ml) for 18 h at 4 °C, with 0.25 nM [3H]-E₂ and increasing concentrations (0.5–5.0 nM) of non-radioactive E₂, Buffer and free E₂ were separated by the addition of 800 µl of a dextran-coated charcoal suspension (250 mg Norit-A and 25 mg dextran T-70) in TEDLM buffer and incubated for 10 min at 4 °C with continuous shaking. Following centrifugation at 800 g at 4 °C, for 10 min, the radioactive content of the supernatant was determined in 200 µl aliquots, using Packard Insta-Gel PlusTM as the counting solution. The equilibrium Kᵣ and the number of binding sites (NBS) were determined by the method of Scatchard (1949).

Competition studies Stereospecificity of the binding of NET, 5α-NET, 3α,5α-NET, and 3β,5α-NET to ER, was assessed by competition analysis (Lemus et al. 2000), using dexamethasone as control. Cytosol aliquots (400 µg protein/ml) were incubated with 0.25 nM [3H]-E₂ at 4 °C for 18 h, in the absence or presence of increasing concentrations (0.25, 0.50, 0.75, 1.0 nM) of radioinert E₂ and NET and its 5α-reduced derivatives. The relative binding affinities (RBA) of steroid competitors to cytosol ER, were evaluated by their capability to displace bound [3H]-E₂ from the ER binding sites. The results are expressed as the inhibition constants (Kᵢ) and RBA of steroid competitors, as described by Cheng & Prusoff (1973) and Reel et al. (1979) respectively.

Figure 1 Formation of 5α-reduced norethisterone (NET) metabolites in cultured neonatal rat osteoblastic cells. Incubations were carried out using 2 µM [3H]-NET at different time periods. An early bioconversion of NET to 5α-dihydro NET (5α-NET) with a subsequent decline and a concomitant increase on the formation of 3α,5α-tetrahydro NET (3α,5α-NET) and 3β,5α-tetrahydro NET (3β,5α-NET) was noticed. At 48-h incubation, 36–4% of the [3H]-substrate was bioconverted to A-ring reduced NET metabolites. Each point represents the mean ± S.D. in three experiments in triplicate.
Results

Norethisterone metabolism in osteoblastic cells

After partition of the osteoblastic cells organic extracts, 96% of the incubated radioactive material was recovered in the methanolic fraction. When aliquots of methanolic extracts from \[^{\text{3}}\text{H}\]-NET incubations were submitted to thin-layer chromatography, four radioactive zones were detected: Zone 1 ($R_F = 0.61$), had a chromatographic behavior identical to that of the $5\alpha$-NET carrier. Zone 2 ($R_F = 0.46$) was identified as unchanged $[^{\text{3}}\text{H}]$-NET, while Zone 3 ($R_F = 0.43$), had a chromatographic behavior identical to that of the $3\alpha,5\alpha$-NET carrier and Zone 4 ($R_F = 0.38$) corresponded to $3\beta,5\alpha$-NET.

The results show that NET was extensively metabolized in rat osteoblasts to three A-ring reduced derivatives, $5\alpha$-NET, $3\alpha,5\alpha$-NET, and $3\beta,5\alpha$-NET, demonstrating the activities of $5\alpha$-steroid reductase, $3\alpha$-HSD and $3\beta$-HSD. Representative results of the formation rates of NET metabolites, as a function of time, are shown in Fig. 1. After 24 h, 27.5% of the incubated $[^{\text{3}}\text{H}]$-NET was bioconverted to its reduced metabolites ($63.5 \pm 1.9$ pmol/mg protein), while at 48 h the conversion rate increased to 36.4% ($72.6 \pm 2.2$ pmol/mg protein). The major metabolic conversion products were $3\alpha,5\alpha$-NET and $3\beta,5\alpha$-NET, presumably through a prior conversion of NET to $5\alpha$-NET. Indeed, the maximal accumulation of $5\alpha$-NET occurred at 6 h, with a subsequent decline and a concomitant increase on the formation of NET tetrahydro metabolites. Osteoblastic cells were incubated with $2 \mu$M $[^{\text{3}}\text{H}]$-NET in the absence or presence of 1 mM finasteride, a $5\alpha$-steroid reductase inhibitor, at 37 °C for 48 h. As can be seen in Fig. 2, the presence of finasteride resulted in a complete abolishment of bioconversion of NET to $5\alpha$-dihydro NET, which precluded the formation of $3\beta,5\alpha$-NET and $3\alpha,5\alpha$-NET. Data represents the mean ± S.D. of three experiments in triplicate.

Figure 2 Effect of a $5\alpha$-steroid reductase inhibitor on the metabolism of NET in cultured rat osteoblastic cells. Incubations of osteoblasts with $2 \mu$M $[^{\text{3}}\text{H}]$-NET were carried out at 37 °C, pH 7.4 for 48 h, in the absence or presence of 1 mM finasteride. The presence of the $5\alpha$-steroid reductase inhibitor resulted in a complete abolishment of bioconversion of NET to $5\alpha$-dihydro NET, which precluded the formation of $3\beta,5\alpha$-NET and $3\alpha,5\alpha$-NET. Data represents the mean ± S.D. of three experiments in triplicate.

Figure 3 5α-steroid reductase type 1 ($Srd5a1$) and type 2 ($Srd5a2$) mRNA expression levels in rat osteoblastic cells. Experiments were performed using real-time PCR. Total RNA was obtained from cultured neonatal rat osteoblasts and from rat anterior ventral prostate, used as experimental control. The expression of $Srd5a1$ was detectable in osteoblast at a similar level to that observed in control anterior ventral prostate (A). Gene expression of $Srd5a2$ in osteoblastic cells was not detectable (B), whereas gene expression of 5α-steroid reductase type 2 in control rat ventral prostate was clearly detected.
Steroid 5α-reductase genes expression

A number of experiments were performed to investigate the gene expression of type 1 and type 2 steroid 5α-reductase in neonatal rat osteoblastic cells using RT-PCR. The Srd5a1 expression (Fig. 3A) was detected in osteoblastic cells, as well as in the anterior ventral prostate from rat adult males used as positive control. The expression level of Srd5a1 in the osteoblastic cells was similar to that of control anterior ventral prostate. Expression of Srd5a2 in osteoblastic cells was not detected, while Srd5a2 expression in anterior ventral prostate was clearly noticed, as it was expected. Results are shown in Fig. 3B.

Estrogen receptor binding studies

Saturation curve and Scatchard plot of [3H]-E2 binding to cytosol preparations from rat osteoblastic cells are shown in Fig. 4. The $K_d$ was $1.19 \times 10^{-9}$ M and the number of ER binding sites was $0.39 \times 10^{-9}$ M. In addition to the high-affinity ER sites, a higher capacity specific binding site (type II) was noticed in osteoblasts at E2 concentrations above 3 nM (Fig. 4 inset).

Discussion

The results of this study clearly demonstrated that neonatal rat osteoblastic cells, efficiently biotransform the potent synthetic progestin NET to A-ring tetrahydro reduced metabolites, which possess the capability to bind with high affinity to osteoblasts ER. Indeed, rat osteoblasts incubated with radiolabeled NET exhibited a large formation of 3α,5α-NET, and 3β,5α-NET, indicating a great activity of 5α-steroid reductase and two enzymes of the aldo-keto reductases family, 3α-HSD and 3β-HSD. The results of the metabolic study suggest that the formation of the tetrahydro reduced NET metabolites is preceded by the enzyme mediated 5α-reduction of NET. Indeed, the early and high formation of 5α-NET (6 h) as an obligatory intermediary, was followed by a decline and a concomitant significant increase of 3α,5α-NET and 3β,5α-NET formation (Fig. 1).
The presence of finasteride in osteoblast incubations with NET induced a complete inhibition of $5\alpha$-NET formation, indicating that $5\alpha$-reduction of NET is an essential metabolic step for the formation of NET tetrahydro derivatives. Furthermore, these results demonstrated that $5\alpha$-reduction (trans A/B ring junction) is required, as a prior step, in the intracrine sequence of events, leading to the formation of NET metabolites with ER agonistic potency in osteoblastic cells. The data indicate that $5\alpha$-NET formation represents an essential metabolic step for the effects of NET on bone cells.

Furthermore, the expression of $\text{Srd5a1}$ in neonatal rat osteoblastic cells was clearly demonstrated while the expression of $\text{Srd5a2}$ was not detected. The striking finding of selective expression of $\text{Srd5a1}$ in neonatal rat osteoblastic cells is in line with the reports of van der Eerden et al. (2004), who have demonstrated gene expression of the $5\alpha$-steroid reductase type 1 in rat tibia metaphysis and Issa et al. (2002) who have demonstrated that gene expression of $5\alpha$-steroid reductase type 1 is the predominant enzyme in human osteoblast-like cells.

The data confirm and extend the results of previous studies, which have demonstrated the bioconversion of 19-nor synthetic progestins to their A-ring reduced metabolites in other sex steroid hormone sensitive tissues (Larrea et al. 1987, Lemus et al. 1992, 2001). The results are also in line with the presence of androgen metabolizing enzymes reported in bone and bone cells (Vittek et al. 1974, Shimodaira et al. 1996, Issa et al. 2002). More recently, McCarthy et al. (2007) have demonstrated in fetal rat osteoblasts, the conversion of tibolone, a synthetic $7\alpha$-methylated androgen receptor agonist, into its 3-hydroxylated metabolites, which are potent ER$\alpha$ agonists.

The most relevant finding of this study was that $3\beta,5\alpha$-NET and $3\alpha,5\alpha$-NET, the major metabolic conversion products of NET in intact neonatal rat osteoblasts, were also the most efficient competitors for ER binding sites in cytosol preparations of the same rat osteoblasts, exhibiting relative binding affinities similar, almost identical, to that of naturally occurring E$_2$, while unmodified NET and $5\alpha$-NET were ineffective competitors for the binding sites of ER, in a similar manner to that of dexamethasone used as a negative control. The results fit well with previous observations from our

Table 1 Relative binding affinities (RBA) and inhibition constant ($K_i$) of natural and synthetic steroids for rat osteoblast cytosol estrogen binding sites, as assessed by competition analysis. $[^{1}\text{H}]$-Estradiol was used as radioligand and dexamethasone as the negative control.

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<tr>
<th>Steroids</th>
<th>RBA (%)</th>
<th>$K_i$ (nM)</th>
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<tr>
<td>Estradiol</td>
<td>100</td>
<td>0.15</td>
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<tr>
<td>$3\beta,5\alpha$-NET</td>
<td>95</td>
<td>0.16</td>
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<tr>
<td>$3\alpha,5\alpha$-NET</td>
<td>90</td>
<td>0.17</td>
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<tr>
<td>$5\alpha$-NET</td>
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<td>Norethisterone</td>
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The presence of finasteride in osteoblast incubations with NET induced a complete inhibition of $5\alpha$-NET formation, indicating that $5\alpha$-reduction of NET is an essential metabolic step for the formation of NET tetrahydro derivatives. Furthermore, these results demonstrated that $5\alpha$-reduction (trans A/B ring junction) is required, as a prior step, in the intracrine sequence of events, leading to the formation of NET metabolites with ER agonistic potency in osteoblastic cells. The data indicate that $5\alpha$-NET formation represents an essential metabolic step for the effects of NET on bone cells.

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laboratory, which have provided evidence that the tetrahydro-reduced metabolites of 19-norprogestins, including NET, binds with high affinity to ERα but not to ERβ, and are also capable to transactivate estrogen-dependent genes in a co-transfected HeLa cells expression system (Larrea et al. 2001, García-Becerra et al. 2002), behaving as selective ERα modulators with ligand-receptor structural and functional responses similar to those induced with E2 (García-Becerra et al. 2006). The number of ER binding sites in the estrogen target osteoblastic cells employed in this study was relatively low (NBS: 0.39×10⁻⁸ M), an observation similar to those previously reported in human and rat osteoblasts (Eriksen et al. 1988, Komm et al. 1988). These data are significantly different from the large number of ER binding sites documented in hormone-dependent reproductive cells and tissues (Eriksson et al. 1978, Miller & Katzenellenbogen 1983, Chávez et al. 1985, Markaverich et al. 2001). Interestingly, the rat osteoblasts type II E2 binding sites found in this study have been previously characterized in human osteoblast-like cells and related with bone formation (Toesca et al. 2000).

A recent study from our group (Enríquez et al. 2007) demonstrated that 3β,5α- and 3α,5α-reduced metabolites of NET induced potent estrogen-mediated stimulatory effects on neonatal, cultured rat osteoblasts. Indeed, administration of increasing concentrations of 3β,5α- and 3α,5α-NET resulted in a significant, dose- and time-dependent response in osteoblastic cell proliferation, as determined by DNA content. In addition, NET-reduced metabolites induced a significant effect on cell differentiation as determined by alkaline phosphatase cell activity and osteocalcin and calcium osteoblasts content, as well as a significant effect on osteoblasts in mineralization, as determined by calcium deposition. Interestingly, that study showed that the effects of tetrahydro NET metabolites on osteoblasts were abolished in the presence of ICI 182 780, a potent ER antagonist.3β,5α-reduced metabolites of NET (3β,5α- and 3α,5α-NET). The complete intracrine cycle offers a plausible explanation for the effects of NET on bone formation and the beneficial effects observed in peri- and post-menopausal women in hormone replacement therapy, as depicted in Fig. 6.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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