The effects of synthetic 19-norprogestins on osteoblastic cell function are mediated by their non-phenolic reduced metabolites

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Abstract

The key role of estrogens on osteoblastic cell function is well documented; however, the role of progesterone (P) and synthetic progestins remains controversial. While several reports indicate that P has no significant effects on bone cells, a number of clinical studies have shown that 19-norprogestins restore postmenopausal bone loss. The mechanisms by which 19-norprogestins induce estrogen-like effects on bone cells are not fully understood. To assess whether the actions of 19-norprogestins on osteoblasts are mediated by their non-phenolic metabolites, we studied the effects of norethisterone (NET), levonorgestrel (LNG), and two of their A-ring reduced derivatives upon cell proliferation and differentiation in neonatal rat osteoblasts. Osteoblast function was assessed by determining cell DNA, cell-associated osteocalcin and calcium content, alkaline phosphatase activity, and mineral deposition. P failed to induce changes on osteoblasts, while NET and LNG exerted a

number of actions. The most striking finding was that the 3 β ,5 α - and 3 α ,5 α -tetrahydro derivatives of NET and LNG induced osteoblast proliferation and differentiation with higher potency than those exerted by their parent compounds, mimicking the effects of estradiol. Interestingly, osteoblast differentiation and mineral deposition induced by NET and LNG were abolished by finasteride, a 5 α -reductases inhibitor, while the potent effect on osteoblast proliferation induced by progestin derivatives was abolished by a steroidal antiestrogen. Results demonstrate that A-ring reduced derivatives of NET and LNG exhibit intrinsic estrogen-like potency on rat osteoblasts, offering a plausible explanation for the mechanism of action of 19-norprogestins in bone restoration in postmenopausal women and providing new insights for hormone replacement therapy research.

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Introduction

Estrogens play an essential role in the maintenance of bone mass (Khastgir *et al.* 2001, Riggs *et al.* 2002). Indeed, 17 β -estradiol (E₂) enhances osteoblastic cell activity by stimulating proliferation, differentiation, and mineralization in the process of bone formation (Qu *et al.* 1998, Riggs *et al.* 2002). Even though osteoblastic cells possess the two isoforms of the estrogen-dependent nuclear progesterone receptor (PR) (Rickard *et al.* 2002), the role of progesterone (P) and synthetic progestins on bone cell function has not been completely elucidated and a number of studies have yielded controversial results. Thus, while several investigators (Scheven *et al.* 1992, Tremollieres *et al.* 1992) have

demonstrated a stimulatory effect of naturally occurring P on bone formation, Canalis & Raisz (1978) have reported that P, at relatively high doses, induces an inhibition of bone formation in cultured rat fetal calvaria. Furthermore, evidence has been presented indicating that P had very little, if any, effect on bone formation in the ovariectomized rat model (Kalu *et al.* 1991, Yamamoto *et al.* 1998). Additionally, clinical studies in postmenopausal women (Riis *et al.* 1987) have also demonstrated an absence of effect of P on bone loss.

A series of clinical trials has revealed that synthetic progestins may or may not preserve bone mass, depending on their molecular structure. Several reports have associated the long-term use of the contraceptive progestin depot medroxyprogesterone acetate, a synthetic 17-hydroxyprogesterone derivative,

with significant bone loss in women in reproductive age (Costa Paiva *et al.* 1998, Scholes *et al.* 2002). In addition, Gallagher *et al.* (1991) have demonstrated that medroxyprogesterone acetate therapy induces a decrease in bone density in postmenopausal women, when compared with estrogens. In contrast, administration of 19-norprogestins induces beneficial effects on bone turnover. Indeed, a number of clinical studies (Abdalla *et al.* 1985, Christiansen & Riis 1990, Horowitz *et al.* 1993) have shown that administration of norethisterone (NET), a synthetic 19-norprogestin, prevents bone mineral loss and reduces bone resorption in postmenopausal women, and also prevents bone loss in young women treated with luteinizing hormone-releasing hormone agonists (Riis *et al.* 1990). In addition, low-dose subdermal administration of levonorgestrel (LNG), another synthetic 19-norprogestin, also induces an increase in bone mineral density in premenopausal women (Di *et al.* 1999). Furthermore, clinical and experimental studies have demonstrated that nandrolone decanoate, a synthetic 19-nor anabolic agent, increases bone mass and inhibits bone turnover (Passeri *et al.* 1993, Li *et al.* 2000).

The mechanisms of estrogen-like bone actions of 19-norprogestins are not fully understood, particularly since these synthetic steroid molecules neither interact with estrogen receptors (ER; Chávez *et al.* 1985, Santillán *et al.* 2001) nor undergo enzyme-mediated aromatization (Gual *et al.* 1962). An early study conducted in postmenopausal women and castrated patients with complete androgen resistance strongly suggested that the antigonadotropic effect of NET is mediated through the ER, while the effect of medroxy-progesterone acetate (MPA) is mediated through the androgen receptor (Pérez-Palacios *et al.* 1981). Studies from our laboratory have demonstrated that synthetic 19-norprogestins are extensively bioconverted in target organs to A-ring reduced tetrahydro derivatives (Larrea *et al.* 1987, Lemus *et al.* 1992) which exert estrogen-like effects (Vilchis *et al.* 1986, Morali *et al.* 1990, Santillán *et al.* 2001). Furthermore, recent studies have demonstrated that the protecting neurotoxic effects of progestins are mediated by their 3 α - and 3 β -tetrahydro reduced metabolites (Ghoumari *et al.* 2003, Rhodes *et al.* 2004, Ciriza *et al.* 2006).

To assess whether the actions of synthetic 19-norprogestins on bone cell function are mediated by their non-phenolic reduced metabolites, we have studied the effects of NET, LNG, and their 3 α ,5 α - and 3 β ,5 α -reduced derivatives on cell proliferation, differentiation, and mineral deposition in cultured osteoblastic cells of neonatal rats. The effects induced by the 19-norprogestins and their tetrahydro reduced derivatives on rat osteoblasts were assessed by determining the cell DNA, cell-associated osteocalcin (OC) and calcium content, alkaline phosphatase (AP) activity, and mineral deposition. Naturally occurring P and E₂ served as the experimental controls. Parallel experiments were carried out in the presence of finasteride, a 5 α -steroid reductases inhibitor, or in the presence of ICI 182 780, a steroidal antiestrogen.

Materials and Methods

Materials

Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT, USA), phenol red-free Dulbecco's modified Eagle medium (DMEM) from Life Technologies, and Dulbecco's regular and Mg⁺⁺- and Ca⁺⁺-free PBS solutions from Invitrogen Co. Type II collagenase, E₂, P, Alizarin red-S, and salmon thymus DNA were purchased from Sigma Chemical Co. Mouse monoclonal bovine carboxylated OC antibody and peroxidase-labeled rabbit anti-mouse IgG were purchased from Zymed Laboratories, Inc. (San Francisco, CA, USA). Authentic NET (17 α -ethynyl-17 β -hydroxy-19-Nor-4-androsten-3-one) and LNG (17 α -ethynyl-18-methyl-17 β -hydroxy-19-Nor-4-androsten-3-one) were kindly provided by Schering Mexicana, SA de CV (Mexico City, Mexico) and Schering AG (Berlin, Germany) respectively. Synthesis of the 3 β ,5 α -tetrahydro derivatives of NET and LNG (3 β ,5 α -NET, (17 α -ethynyl-19-Nor-5 α -androstan-3 β ,17 β -diol); 3 β ,5 α -LNG, (17 α -ethynyl-18-methyl-19-Nor-5 α -androstan-3 β ,17 β -diol)) and their 3 α isomers (3 α ,5 α -NET, (17 α -ethynyl-19-Nor-5 α -androstan-3 α ,17 β -diol); 3 α ,5 α -LNG, (17 α -ethynyl-18-methyl-19-Nor-5 α -androstan-3 α ,17 β -diol)) was done in our laboratories and their physical and spectroscopic constants have been previously described (Chávez *et al.* 1985, Lemus *et al.* 1992). Finasteride was provided by Merck Sharp and Dhome de Mexico, SA de CV, while the antiestrogen ICI 182 780 was generously supplied by Zeneca Farma (Mexico City, Mexico). All other reagents and solvents used were of analytical grade.

Animals

Animals used in this study were female Wistar rats born in our laboratory. Rats 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 Instituto Nacional de Ciencias Médicas y Nutrición S. Zubirán.

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, and sequentially digested for 60 min with 0.3% type II collagenase. Cells obtained in the first treatment were discarded, while cells isolated from the subsequent three digestions were pooled, plated, and cultured overnight in flasks with DMEM supplemented with 10% FBS and 100 μ M nonessential amino acids and an antibiotic-antimycotic solution (100 U/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin-B; Gibco BRL) at 37 °C, in a humidified atmosphere of 5% CO₂ in air.

Figure 1 Effects of naturally occurring estradiol (E₂) and progesterone (P) on cell proliferation, differentiation, and mineralization in cultured neonatal rat osteoblasts. (A) Effect of increasing concentrations of E₂ and P on osteoblasts DNA content. Osteoblasts DNA content in the absence of steroids (vehicle alone) was 0.85 ± 0.32 μg, at all incubation time periods. (B) Effect of varying concentrations of E₂ and P on osteoblastic cells alkaline phosphatase activity (U/mg protein per min) after incubations for 15 days. (C and D) Effect of a single dose (50 nM) of E₂ and P on cell-associated osteocalcin (specific absorbance/10⁴ cells) and cell-associated calcium content (μg/mg protein), after incubations for 15 consecutive days. Results represent the mean $\pm$ s.d. of three experiments in sixuplicate each. * $P < 0.0001$ when compared with vehicle (V) control incubations. For details refer text.

Assessment of the phenotype of cultured rat calvarial cells was done by determining the presence of OC and AP activity, 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 0.25% trypsin/1 mM EDTA, counted, and submitted to different experimental studies.

Cell proliferation studies

To assess the effects of 19-norprogestins and their derivatives on bone cell proliferation, increasing concentrations (1–500 nM) of NET, LNG, 3 α ,5 α -NET, 3 β ,5 α -NET, 3 α ,5 α -LNG, and 3 β ,5 α -LNG, dissolved in propylene glycol, used as vehicle, were incubated for 4 days with cultured

osteoblasts. Identical concentrations of E₂ and P, and vehicle were used as controls. Osteoblasts were plated at 2×10^4 cells/well in culture medium (DMEM containing 5% stripped FBS treated with charcoal–dextran, nonessential amino acids, antibiotics, and antimycotic), allowing adherence for 6 h. To preclude base line cell proliferation, the medium was removed and replaced by DMEM containing 0.5% stripped FBS, 5 μ g/ml ascorbic acid, amino acids, antibiotics, and antimycotic (incubation medium), and incubated for additional 6 h. Osteoblasts were then incubated for four consecutive days in incubation medium with natural and synthetic steroids added. The medium was removed daily and replaced by fresh incubation medium containing identical steroid concentrations.

Cell proliferation was assessed by the daily determination of DNA content in both steroid-stimulated and control cultured cells on day 0 through day 4. DNA was measured using the method of Labarca & Paigen (1980), using purified salmon

Figure 2 Effect of two synthetic 19-norprogestins and their A-ring reduced derivatives on DNA cell content of cultured rat calvarial osteoblasts. Increasing concentrations of norethisterone (NET), 3 β ,5 α -NET and 3 α ,5 α -NET, levonorgestrel (LNG), 3 β ,5 α -LNG and 3 α ,5 α -LNG, were incubated with osteoblastic cells for 4 consecutive days. Cell DNA was determined at 0, 24, 48, 72, and 96 h of steroid stimulation. Values represent the mean $\pm$ s.d. of three experiments in sextuplicate each. * $P < 0.0001$ when compared with vehicle control incubations.

thymus DNA as standard, and the results were expressed as micrograms of DNA. Additional experiments using the tetrahydro derivatives of NET and LNG were carried out in the presence of ICI 182 780, using E_2 and vehicle as controls. The DNA content in steroid-stimulated and control cultured cells was assessed after 24 h of treatments.

Cell differentiation studies

To evaluate the effects of synthetic progestins and their reduced derivatives on osteoblast differentiation, two biomarkers of bone formation, AP activity and OC content, were used in cells with and without exposure to steroids. To determine osteoblast AP activity, cells were plated at 20×10^4 cells/well density in culture medium containing 50 $\mu\text{g}/\text{ml}$ ascorbic acid and increasing concentrations of NET, LNG, and their A-ring tetrahydro reduced derivatives, using E_2 , P, and vehicle as controls (day 0). Thereafter, medium was removed every other day and replaced by fresh medium with identical steroid concentrations or vehicle and incubated for 15 days. Additional experiments with NET and LNG were carried out in the presence or absence of finasteride. At the end of the steroid stimulation period, cells were harvested and lysed in a solution containing 0.1 M Tris-HCl and 0.1% Tween 20, at pH 7.5 for 2 min. Cell lysates were freeze-thawed ($2 \times$) and submitted to AP activity measurement. AP activity was determined by the method described by Lowry *et al.* (1954), using *p*-nitrophenyl phosphate as substrate. Protein concentration was determined by Bradford's (1976) dye binding method, using BSA as standard.

To determine the content of osteoblast-associated OC, cells were seeded at 4×10^4 cells/well density in culture medium, containing 50 $\mu\text{g}/\text{ml}$ ascorbic acid and 50 nM each of NET, LNG, and their tetrahydro derivatives, using E_2 , P, and vehicle as controls. Additional incubations with NET or LNG were done in the presence or absence of 75 μM finasteride. Steroid stimulation lasted 15 days, with medium removal and replacement every other day. At the end of steroid stimulation, cells were fixed to the plates using PBS containing 0.05% glutaraldehyde, at 4°C , for 30 min. Immediately after, plates were washed and dried, and the cells were incubated for 24 h with 1% BSA in 0.2 M carbonate buffer, pH 9.6, at 4°C , in the dark. The osteoblast-associated OC content in the extracellular matrix was determined by a cell-ELISA method, similar to that developed by Nibbering *et al.* (1990) for the quantification of proteins localized in the cell surface. Briefly, cells were incubated for 2 h with mouse monoclonal bovine OC antibody (1:500) in PBS-Tween 20 at 37°C . After washing the plates ($3 \times$) with 0.05% PBS-Tween 20, cells were incubated for 1 h with peroxidase-labeled rabbit anti-mouse IgG (1:1000) in 0.05% PBS-Tween 20 at 37°C . Following the removal of anti-IgG excess, cells were incubated at room temperature in the dark for 30 min with 0.4 mg/ml *o*-phenylenediamine (Sigma Chemical Co.) and 30% hydrogen peroxide in a sodium phosphate-citric acid buffer (0.2 M

Na_2HPO_4 and 0.1 M citric acid). At the end of incubation, 50 μl of 2 M H_2SO_4 were added. For the calculation of OC content in the extracellular matrix, use was made of the linear part of the curve obtained when the number of cells was plotted against the absorbance values of the peroxidase reaction product at 490 nm.

Cell-associated calcium content and mineral deposition

To assess the effect of synthetic progestins and their reduced derivatives on osteoblast mineralization, the cell-associated calcium content and mineral deposition were determined. Cells were seeded at 20×10^4 cells/well density in culture medium, added with 10 mM β -glycerophosphate, and incubated with a single dose of NET, LNG, and their $3\alpha,5\alpha$ - and $3\beta,5\alpha$ -reduced derivatives, for 15 days, using E_2 , P, and vehicle as controls. Culture medium was replaced every other day by fresh medium containing identical steroids concentration. At the end of the steroid-stimulation period, cells were submitted for determination of cell-associated calcium content and mineral deposition. Osteoblast-associated calcium content was determined after cells were washed ($3 \times$) with Ca^{++} and Mg^{++} -free PBS, decalcified overnight with 0.5 M HCl at 4°C and centrifuged (4000 g) for 5 min at 4°C . Cell-associated calcium was quantified in the supernatant using an atomic absorption spectrophotometer Perkin-Elmer Model 3110 (Norwalk, CT, USA), using pure calcium

Figure 3 Effect of ICI 182 780 (ICI), an estrogen antagonist, on the increase of cell DNA content induced in cultured rat osteoblasts by naturally occurring estradiol (E_2) and the tetrahydro reduced derivatives of norethisterone (NET) and levonorgestrel (LNG). Osteoblastic cells were incubated with 500 nM each of the following steroids: E_2 , $3\beta,5\alpha$ -NET, $3\alpha,5\alpha$ -NET, and $3\beta,5\alpha$ -LNG, in the presence or absence of 250 μM ICI, for 24 h. Results are expressed as micrograms of DNA and the values represent the mean $\pm$ s.d. of three experiments in sextuplicate each. * $P < 0.0001$ when compared with incubations in the absence of the antiestrogen.

as atomic spectroscopy standard (Perkin–Elmer Instruments, Shelton, CT, USA).

Osteoblast mineral deposition was evaluated after cells were fixed to plates with neutral formalin and incubated at room temperature for 15 min in a saturated Alizarin red-S solution in PBS, pH 4.1. Plates were rinsed (3×) in tap water, dried, and submitted for mineral deposition observation, using a Nikon stereoscopic microscope, Model SMZ 1500, and photographed with a digital Nikon E995 camera (Nikon Inc., Melville, NY, USA). An integrated density analysis in representative samples was done using a still video system Stratagene Model Eagle Eye II (La Jolla, CA, USA). Results are given as integrated density numerical values (pixels) obtained by the use of a Lab Works 4.5 software (UVP Bioimagen Systems, Upland, CA, USA). An identical set of experiments aimed to evaluate mineral deposition in cultured cells stimulated with NET and LNG was done in the presence of finasteride.

Statistical analysis

Comparisons of experimental groups with controls were done by one-way ANOVA. Statistical differences between groups were established by Student's *t*-test (SigmaStat Statistical Analysis System, Jandel Corporation, San Rafael, CA, USA). Group differences were considered significant when $P \leq 0.05$ was reached (two-tailed test; Montgomery 1991).

Results

The effects of naturally occurring sex steroid hormones on neonatal rat osteoblasts proliferation, differentiation, and mineralization are shown in Fig. 1. Addition of increasing concentrations of E_2 to cultured osteoblasts resulted in a significant, dose- and time-dependent increase ($P < 0.0001$) in cell DNA content (Fig. 1A), even at the lower dose employed (1 nM), when compared with the vehicle alone. In contrast, addition of P to osteoblast cultures, even at the higher dose employed (500 nM), completely failed to induce changes in cell DNA content. Incubations of osteoblasts with varying concentrations of E_2 resulted in a significant, dose-dependent increase ($P < 0.0001$) in AP activity, when compared with the vehicle (Fig. 1B), while P was unable to induce an increase in cell enzyme activity. Stimulation of osteoblasts with E_2 (50 nM) induced a significant increase ($P < 0.0001$) in cell-associated OC content, when compared with the vehicle alone, whereas a similar dose of P did not

induce changes in the osteoblast OC content (Fig. 1C). Addition of E_2 to cultured osteoblasts resulted in a significant increase ($P < 0.0001$) in cell-associated calcium content, when compared with the vehicle (Fig. 1D), while P completely failed to induce changes in the cell content of calcium.

The effect of NET, LNG, and their non-phenolic reduced derivatives on osteoblast proliferation is depicted in Fig. 2. Addition of NET to cultured neonatal rat osteoblasts induced a significant effect ($P < 0.0001$) on cell DNA content, noticeable only at 50 nM dose and above, particularly after long incubation periods (Fig. 2). NET at low doses (1 and 10 nM) was ineffective to induce significant changes in cell proliferation at all stimulation time periods. On the contrary, incubations of neonatal rat osteoblasts with increasing concentrations of 3 β ,5 α -NET resulted in a significant, dose-dependent increase ($P < 0.0001$) in cell DNA content, even at the lower dose employed (1 nM), at all stimulation time periods, resembling the effect of naturally occurring E_2 (Fig. 1A), though with lower potency. The 3 α ,5 α -NET derivative also induced an increase in the DNA content of cultured osteoblasts, yet with less effectiveness than its 3 β epimer. Low doses of 3 α ,5 α -NET (1 and 10 nM) were ineffective; however, at 50 nM dose and above, an increase in cell DNA was noticed at all stimulation time periods.

The synthetic progestin LNG also induced an increase in osteoblasts DNA content, though weaker than that observed with NET. The effect of LNG on osteoblast proliferation was noticed at high doses, after long-term stimulation periods (Fig. 2). The effect of the 3 β ,5 α -tetrahydro derivative of LNG in neonatal rat osteoblasts was more potent than that exhibited by LNG, but weaker than that of the corresponding 3 β ,5 α derivative of NET. Indeed, 3 β ,5 α -LNG at high doses (500 nM) was able to induce a cell DNA increase, since the first 24 h of stimulation. In contrast, 3 α ,5 α -LNG induced very little, if any, increase in osteoblasts DNA content (Fig. 2). The rank order of potency of natural and synthetic steroids to stimulate osteoblast proliferation was: estradiol > 3 β ,5 α -NET > 3 α ,5 α -NET > 3 β ,5 α -LNG > NET > LNG > 3 α ,5 α -LNG. Incubations of osteoblasts with the tetrahydro NET and LNG derivatives in the presence of ICI 182 780 resulted in a complete inhibition ($P < 0.0001$) of osteoblast proliferation induced by the synthetic progestins derivatives as depicted in Fig. 3. A similar effect of the antiestrogen on E_2 -induced cell proliferation was noticed.

The effect of increasing concentrations of NET, LNG, and their reduced derivatives on AP activity of cultured neonatal rat osteoblasts is shown in Fig. 4A and B. Addition of NET at

Figure 4 Effect of norethisterone (NET), levonorgestrel (LNG), and their tetrahydro reduced derivatives on neonatal rat osteoblast differentiation. Alkaline phosphatase activity was determined in osteoblastic cells incubated with increasing concentrations (1–500 nM) of (A) NET, 3 β ,5 α -NET and 3 α ,5 α -NET, and (B) LNG, 3 β ,5 α -LNG and 3 α ,5 α -LNG, for 15 consecutive days. Steroids were dissolved in propylene glycol, used as vehicle (V). The cell enzyme activity is expressed as U/mg protein per min. (C) The effect of 19-norprogestins and their derivatives on the cell-associated osteocalcin content (specific absorbance/10⁴ cells) was assessed after incubations with a single dose (50 nM) of each steroid for 15 consecutive days. Results represent the mean $\pm$ s.d. of three experiments in sextuplicate each. * $P < 0.0001$ compared with vehicle, ** $P < 0.0001$ compared with the corresponding parent progestin.

10 nM dose and above to cultured osteoblasts resulted in a significant increase ($P < 0.0001$) in AP activity, when compared with vehicle alone (Fig. 4A). $3\beta,5\alpha$ -NET induced a significant, dose-dependent increase ($P < 0.0001$) in osteoblast AP activity, even at the lower dose employed (1 nM), in a similar manner to that observed with E_2 (Fig. 1B), though with lower potency. Addition of $3\alpha,5\alpha$ -NET also induced a significant, dose-dependent increase in osteoblast AP activity, yet with lower potency than its 3β epimer (Fig. 4A). The synthetic progestin LNG induced a weak, though significant, effect ($P < 0.0001$) on osteoblast AP activity, noticeable only at 50 nM dose and above (Fig. 4B). $3\beta,5\alpha$ -LNG induced a significant, dose-dependent increase ($P < 0.0001$) in osteoblast AP activity as 10 nM dose is more potent than unmodified LNG, but less potent than E_2 (Fig. 1B). The $3\alpha,5\alpha$ -LNG derivative exhibited a very weak effect on osteoblasts AP activity, noticed only at higher doses (100 and 500 nM). The rank order of potency of steroids to induce enhancement in AP activity in cultured rat osteoblastic cells was: $E_2 > 3\beta,5\alpha$ -NET $> 3\alpha,5\alpha$ -NET $> 3\beta,5\alpha$ -LNG $>$ NET $>$ LNG $> 3\alpha,5\alpha$ -LNG.

The effect of NET, LNG, and their derivatives on cell-associated OC content in cultured neonatal rat osteoblasts is depicted in Fig. 4C. Stimulation of cultured osteoblasts with NET and its A-ring reduced derivatives resulted in a significant increase ($P < 0.0001$) in cell OC content when compared with the vehicle alone, resembling the effect induced with E_2 (Fig. 1C), though with a slightly lower potency. $3\beta,5\alpha$ -NET was the most effective inductor of cell-associated OC increase, exhibiting a higher potency than those of its 3α epimer and unmodified NET. Incubations of cultured osteoblasts with LNG and its A-ring reduced derivatives yielded different results. As shown in Fig. 4C, unmodified LNG had a significant effect on osteoblasts OC content, though with lower potency than NET. $3\beta,5\alpha$ -LNG induced an increase in cell OC content with higher potency than that of LNG, while $3\alpha,5\alpha$ -LNG was ineffective.

The effect of finasteride on the progestins-induced increase of AP activity in cultured osteoblasts is depicted in Fig. 5A. Experiments were designed to inhibit both 5α -steroid reductases; therefore, high doses of the inhibitor were used. Studies on human breast cancer cells (Pérez-Palacios *et al.* 2006) and cultured rat osteoblasts (unpublished) in our laboratory have shown that finasteride, at this dose level, specifically inhibits types 1 and 2 of 5α -reductases. Incubations of osteoblasts with NET or LNG, in the presence of finasteride, resulted in a complete inhibition ($P < 0.0001$) of the progestins-induced increase of cell AP activity. The results of incubations of cultured osteoblasts with NET or LNG on cell-associated OC content, in the presence or absence of finasteride, are shown in Fig. 5B. Finasteride was able to induce a complete inhibition ($P < 0.0001$) of the NET and LNG-induced increase of the osteoblast content of OC.

The effect of synthetic progestins and their derivatives on osteoblast-associated calcium content is shown in Fig. 6. Stimulation of cultured osteoblasts with NET resulted in a

significant increase ($P < 0.0001$) in cell calcium content when compared with vehicle, whereas $3\beta,5\alpha$ - and $3\alpha,5\alpha$ -NET exhibited a higher potency than that observed with unmodified NET. Stimulation of cultured osteoblasts with

Figure 5 Effect of finasteride (F), a 5α -steroid reductases inhibitor, on (A) the increase of alkaline phosphatase activity and (B) cell-associated osteocalcin content in neonatal rat osteoblasts, induced by two synthetic 19-norprogestins. Osteoblastic cells were incubated with 50 nM norethisterone (NET) or levonorgestrel (LNG), in the presence or absence of 75 μ M F, for 15 consecutive days. Results are expressed as alkaline phosphatase activity (U/mg protein per min) and cell-associated osteocalcin (specific absorbance/10⁴ cells), and the values represent the mean $\pm$ s.d. of three experiments in sextuplicate each. * $P < 0.0001$ when compared with incubations in the absence of finasteride.

Figure 6 Osteoblastic cell mineralization induced by two 19-norprogestins and their A-ring reduced derivatives, as assessed by the cell-associated calcium content. Neonatal rat osteoblasts were incubated with 50 nM norethisterone (NET) and levonorgestrel (LNG), 3β,5α-NET, 3α,5α-NET, 3β,5α-LNG or 3α,5α-LNG, for 15 consecutive days, using vehicle (V) as control. Results are expressed as µg calcium/mg protein and the values represent the mean ± s.d. of three experiments in sextuplicate each. * $P < 0.0001$ when compared with vehicle, ** $P < 0.0001$ when compared with the corresponding parent progestin.

LNG and its derivatives also induced a significant increase in cell calcium content, in a similar manner to that observed with NET and its derivatives, though with lower potency (Fig. 6).

Figure 7 shows the mineral deposition in osteoblast cultures stimulated with NET, LNG, and their tetrahydro reduced derivatives, as stained with Alizarin red-S. E₂, P, vehicle, and finasteride alone were used as controls. Naturally occurring E₂ induced a large mineral deposition (406×10^3 pixels), while P was ineffective to induce osteoblast mineralization (117×10^3 pixels). Indeed, the image of stained P-stimulated osteoblasts was similar to that observed in vehicle-treated cells (110×10^3 pixels). Stimulation of cultured osteoblasts with unmodified NET resulted in a mineral deposition (191×10^3 pixels), with lower density than that induced with E₂. Interestingly, 3β,5α-NET was more potent (289×10^3 pixels) than unmodified NET, mimicking the effect of E₂, yet with lower potency. The 3α,5α-NET derivative also induced mineral deposition (175×10^3 pixels), though with lower density than that observed after stimulation with E₂ or 3β,5α-NET. The synthetic progestin LNG and its tetrahydro reduced derivatives, particularly 3β,5α-LNG (231×10^3 pixels), were able to induce mineral deposition in cultured osteoblasts, in a similar manner to NET and its corresponding derivatives, though to a lesser extent. Addition of finasteride to incubations of cultured osteoblasts with NET or LNG precluded the progestins-induced mineral deposition as shown in Fig. 7.

Discussion

The results obtained throughout this study contribute with a plausible explanation to the understanding of the mode of action of synthetic 19-norprogestins in the bone cells. Indeed, using primary cultures of calvarial osteoblasts derived from newborn rats, we were able to show that the significant effects induced by two progestins, NET and LNG, on cell proliferation, differentiation, and mineralization processes are mediated by their A-ring reduced derivatives, mimicking the effects of E₂. The data also provide evidence that naturally occurring progesterone has no effects at all on osteoblastic cell function. The findings of a complete lack of progesterone effectiveness on osteoblasts function are in line with the observation that a female murine model, carrying a null mutation of the PR gene, has not apparent alterations in peak bone mass or bone histomorphometry, thus demonstrating that PR does not play a direct role in the processes which govern normal bone development (Bain *et al.* 1997). In contrast, NET and, to a lesser extent, LNG, at high doses (50 nM and above) after long-term incubation periods, stimulated osteoblasts proliferation in a dose-dependent fashion. The effect of both 19-norprogestins was similar to that observed with E₂, yet with significantly lower potency. This observation confirms and extends the demonstration that NET stimulates proliferation of cultured human osteosarcoma cells (Lau *et al.* 1994).

The most striking finding was that the 3β,5α-tetrahydro reduced derivatives of NET and LNG successfully stimulated osteoblasts proliferation, with higher potency than that of their parent compounds, resembling the effect of E₂. The 3α,5α-derivative of NET also induced an increase in osteoblasts proliferation, with lower potency than that of its 3β epimer, while 3α,5α-LNG was almost ineffective. These results strongly suggest that the rat osteoblasts enhancement of cell proliferation induced by the 19-norprogestins are mediated by their tetrahydro reduced derivatives and their further interaction with nuclear ER. Additional support to this concept was derived from the finding that ICI 182 780, a potent steroidal estrogen antagonist, was capable of abolishing the osteoblastic cell proliferation effect induced by both the A-ring reduced derivatives of NET and LNG, and E₂.

The two 19-norprogestins and their A-ring reduced derivatives exhibited relevant effects on rat osteoblasts differentiation, as assessed by the changes induced on cell AP activity and OC content. It must be underlined that these effects were noticed only after 15 consecutive days of stimulation, in a fashion similar to that observed with E₂. This observation correlates well with the findings of Stein *et al.* (1990) and Qu *et al.* (1998), who have demonstrated that E₂-induced expression of AP mRNA and OC mRNA in rodent osteoblast cultures are not detected before day 12 of stimulation. Interestingly, the enhancing effect on both cell biomarkers, induced by the 3β,5α-reduced derivatives of NET and LNG, was more potent than that observed with

Figure 7 Mineral deposition in osteoblastic cell cultures induced by estradiol (E_2), progesterone (P), norethisterone (NET), levonorgestrel (LNG), and their tetrahydro reduced derivatives ($3\beta,5\alpha$ -NET, $3\alpha,5\alpha$ -NET, $3\beta,5\alpha$ -LNG, and $3\alpha,5\alpha$ -LNG), as assessed by staining of osteoblasts fixed to plates, with a saturated Alizarin red-S solution. Neonatal rat osteoblastic cells were incubated with a single dose (50 nM) of each steroid, for 15 consecutive days, using vehicle (V) as control. Parallel incubations with NET or LNG were carried out in the presence of 75 μ M finasteride (F), an inhibitor of 5α -steroid reductases. The stereoscopic micrographs (magnification $\times 120$) of a representative sample show the steroid-induced mineral deposition images, as well as the effect of F on cells incubated with the corresponding unmodified synthetic progestin. The scale bar (0–200 μ m) is shown in the vehicle picture. For details refer text.

their parent compounds. The $3\alpha,5\alpha$ -derivatives of the synthetic progestins also induced a significant increase in osteoblast AP activity and OC content, though with lower potency than that of their 3β epimers. The effects of NET and LNG on osteoblast differentiation were abolished by the addition of finasteride, a steroidal inhibitor of 5α -steroid reductases, strongly suggesting that the effects of 19-norprogestins on cell differentiation were mediated by their A-ring reduced metabolic conversion products.

The results of osteoblast mineralization studies revealed that E_2 was the more potent steroid to induce an increase in cell cultures calcium content, as previously demonstrated by Qu *et al.* (1998), while P was completely ineffective. In contrast, synthetic 19-norprogestins and their derivatives exhibited a clear-cut estrogen-like mineralization effect on osteoblasts.

The most relevant finding was that the $3\beta,5\alpha$ - and $3\alpha,5\alpha$ -reduced NET derivatives stimulated an increase in cell calcium accumulation, with a potency comparable with that of E_2 . The A-ring reduced derivatives of LNG also induce cell calcium increase, yet with lower potency. The effect of E_2 , synthetic progestins, and their derivatives on mineral deposition was even more evident when Alizarin red-S was used to visualize calcium deposits in osteoblast cultures. The microstereoscopic images of stained osteoblast cultures exposed to steroids correlate with the data of cell calcium content. The most potent inducers of calcium deposition were E_2 and the $3\beta,5\alpha$ -reduced NET and LNG derivatives. The unmodified progestins and their $3\alpha,5\alpha$ -reduced derivatives also induced osteoblast mineralization, though with lower potency, while P was ineffective. Interestingly,

finasteride was able to significantly diminish the formation of calcium deposits in osteoblast cultures induced by unmodified NET and LNG, furnishing additional support to the concept that the effects of 19-norprogestins on osteoblasts differentiation are exerted through their non-phenolic reduced metabolites. Presence of androgen and progestin metabolizing enzymes has been demonstrated in bone cells; thus, the activity of 5 α -steroid reductase in human osteoblasts has been documented (Shimodaira *et al.* 1996) and Issa *et al.* (2002) have reported the expression of types 1 and 2 of the steroid 5 α -reductase genes in human osteoblast-like cells. Vittek *et al.* (1974) reported the presence of 3 α , but not 3 β , hydroxysteroid dehydrogenase in rat mandibular bone, and recent studies in our laboratory, using a reverse isotope dilution technique, have shown the presence of 3 α - and 3 β -hydroxysteroid dehydrogenases, members of the aldo-keto reductases superfamily, in cultured neonatal rat osteoblasts (unpublished results).

The overall results are consistent with the previous reports from our group, demonstrating that diverse estrogen-agonistic effects of NET and LNG are mediated by their non-phenolic A-ring reduced derivatives (Vilchis *et al.* 1986, Morali *et al.* 1990, Santillán *et al.* 2001), and are in line with the concept that 19-norprogestins are effective agents in bone remodeling, while MPA does not, as demonstrated in controlled clinical trials (Liu & Muse 2005). Furthermore, using co-transfected cell expression systems, we have shown that non-phenolic reduced metabolites of 19-norprogestins are able to transactivate estrogen-dependent genes mediated through human ER α , but not through ER β (Larrea *et al.* 2001, García-Becerra *et al.* 2002), behaving as selective ER α modulators, with ligand-receptor structural and functional responses similar to those induced with naturally occurring E₂ (García-Becerra *et al.* 2006). Additional studies on the effects of progestin reduced derivatives on breast tissue are required, particularly since an overexpression of the 5 α -steroid reductase type 1 gene has been reported in breast cancer tumors and cells (Suzuki *et al.* 2001, Wiebe & Lewis 2003, Pérez-Palacios *et al.* 2006).

The data presented herein provide new insights to understand the underlying molecular mechanisms at the genomic level involved in the estrogenic effects on osteoblastic cells induced by synthetic 19-norprogestins, which are widely used in the prevention and treatment of bone loss in postmenopausal women, and also disclose alternate avenues of approach in hormone replacement therapy research.

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