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Assessment of the oestrogenic activity of the contraceptive progestin levonorgestrel and its non-phenolic metabolites

René Santillán [a], Gregorio Pérez-Palacios [b], Martín Reyes [c], Pablo Damián-Matsumura [c], Gustavo A. García [d], Ivonne Grillasca [d], Ana E. Lemus [a,c,*]

[a] *Department of Reproductive Biology, National Institute of Medical Sciences and Nutrition S. Zubirán, Mexico City, Mexico*
[b] *School of Medicine, National Autonomous University of Mexico (UNAM) / Mexico City General Hospital, Mexico City, Mexico*
[c] *Department of Reproductive Biology, UAM-Iztapalapa, Mexico City, Mexico*
[d] *School of Chemistry, UNAM, Mexico City, Mexico*

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Abstract

Levonorgestrel (13β-ethyl-17α-ethynyl-17β-hydroxy-4-gonen-3-one), a potent contraceptive progestin stimulates growth and proliferation of cultured breast cancer cells through a receptor-mediated mechanism, even though levonorgestrel does not bind to the oestrogen receptor (ER). To assess whether the oestrogen-like effects induced by this synthetic progestin are exerted via its metabolic conversion products, we studied the binding affinity of three A-ring levonorgestrel derivatives to the ER and their capability to transactivate an oestrogen-dependent yeast system co-transfected with the human ER gene and oestrogen responsive elements fused to a β-galactosidase reporter vector. The results demonstrated that the 3β,5α reduced levonorgestrel derivative and to a lesser extent its 3α isomer interact with the oestrogen receptor, with a significantly lower relative binding affinity (2.4% and 0.4%, respectively) than that of oestradiol (100%), while levonorgestrel does not. Both levonorgestrel metabolites were able to activate, in a dose-dependent manner, the β-galactosidase reporter gene in the yeast expression system, an effect that was precluded by a steroidal antioestrogen. The oestrogenic potency of levonorgestrel metabolites was significantly lower (750-fold) than that of oestradiol. Furthermore, high doses of 3β,5α levonorgestrel (2.5 mg/day/6 days) induced an increase of oestrogen-dependent progestin receptor in the anterior pituitary of castrated rats. The overall data offer a plausible explanation for the weak oestrogenic effects induced by high, non-pharmacological doses of levonorgestrel. © 2001 Published by Elsevier Science B.V.

Keywords: Contraceptive, progestin; Levonorgestrel; Levonorgestrel metabolites; Oestrogen receptor α

1. Introduction

Levonorgestrel (13β-ethyl-17α-ethynyl-17β-hydroxy-4-gonen-3-one) is a potent, totally synthetic 19-nor progestin (Smith et al., 1963) used either alone or combined with ethynyl oestradiol in a number of contraceptive formulations, including pills (Ball et al., 1991; Rebar and Zeserson, 1991), injectables (Crabbé et al., 1983; Garza-Flores et al., 1991), medicated intrauterine devices (World Health Organization. Task Force on the Safety and Efficacy of Fertility Regulating Methods, 1990; Sivin and Stern, 1994), subdermal implants (Cravioto et al., 1997), vaginal rings (Johannisson et al., 1991; White et al., 1991; Thau and Jackanicz, 1994) and more recently emergency contraceptive pills (World Health Organization. Task Force on Post-Ovulatory Methods of Fertility Regulation, 1998; Piaggio et al., 1999).

Levonorgestrel induces hormone agonistic effects other than that of its progestational activity. Indeed, levonorgestrel specifically binds with high affinity to the mineralocorticoid (Rebar and Zeserson, 1991; Kuhl, 1996) and androgen receptors (Phillips et al., 1990; Lemus et al., 1992; Cabeza et al., 1995) and exerts the corresponding effects with a relative high potency; however, the oestrogenic actions of levonorgestrel still remain controversial. Levonorgestrel, at high concentrations, induces cell growth and proliferation in breast adenocarcinoma cells (MCF-7) (Van der Burg, 1991; Van der Burg et al., 1992; Catherino et al., 1993; Schoonen et al., 1995a) and breast ductal carcinoma cells (T47D) (Schoonen et al., 1995b), an effect

* Corresponding author. Department of Reproductive Biology, National Institute of Medical Sciences and Nutrition S. Zubirán, Vasco de Quiroga 15, Mexico City, D.F., C.P. 14000, Mexico. Tel.: +52-5573-1160; fax: +52-5655-9859.

E-mail address: anaelenalemus@aol.com (A.E. Lemus).

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that is precluded by steroidal anti-oestrogens (ICI-164,384; 4-hydroxytamoxifen) but not by antiprogestins (RU-486; ORG 31710) (Catherino et al., 1993), strongly suggesting that the oestrogen-like effects of levonorgestrel are mediated by the intracellular oestrogen receptor. These observations are in sharp contrast with a number of experimental evidences which indicate that levonorgestrel does not interact at all with the oestrogen receptor and therefore does not induce oestrogenic effects (Rebar and Zeserson, 1991; Kuhl, 1996).

Since levonorgestrel undergoes extensive peripheral metabolism (Stanczyk and Roy, 1990) and biotransformation to A-ring reduced derivatives at target organs (Lemus et al., 1992), in a similar manner to that of norethisterone (Larrea et al., 1987) and gestodene (Lemus et al., 2000), another 19-nor synthetic progestins, we felt it was of interest to investigate whether levonorgestrel may induce oestrogen agonistic effects, via its non-phenolic metabolites.

A series of studies were undertaken, including the interaction of levonorgestrel and three of its A-ring reduced derivatives with the rat uterine oestrogen receptor and the assessment of their oestrogenic potency using an oestrogen-induced transactivation yeast system co-transfected with the human oestrogen receptor gene and its cognate reporter vector, as well as the induction of oestrogen-dependent progestin receptors in the anterior pituitary of female castrated rats.

2. Materials and methods

2.1. Steroids and chemicals

[2,4,6,7-^{3}H]oestradiol ([^{3}H]oestradiol), specific activity 97 Ci/mmol and [6,7-^{3}H]ORG 2058 ([^{3}H]ORG 2058), specific activity 45 Ci/mmol were purchased from Amersham International (Buckinghamshire, England) and their radiochemical purity was established by thin-layer chromatographic behaviour and by repeated crystallizations of aliquots to constant specific activity. Radioinert natural and synthetic steroids were supplied by Sigma (St. Louis, MO, USA). The anti-oestrogen ICI-182,780 was generously supplied by Zeneca Farma (Mexico City, Mexico). All reagents and solvents used were analytical grade. Authentic levonorgestrel was kindly provided by Schering Mexicana, S.A. de C.V. (Mexico City, Mexico). 5α dihydrolevonorgestrel (5α levonorgestrel) was synthesised by lithium-ammonia reduction of levonorgestrel, while the 3α,5α tetrahydro derivative of levonorgestrel (3α,5α levonorgestrel) was prepared from reduction of 5α levonorgestrel with L-selectride under anhydrous conditions and the 3β,5α tetrahydro derivative of levonorgestrel (3β,5α levonorgestrel) was synthesised by sodium borohydride reduction of 5α levonorgestrel. Chemical purity of levonorgestrel and its derivatives was assessed by their melting points, high performance liquid chromatographic behaviour, infrared absorption, and ^{1}H-nuclear magnetic resonance. The physical and spectroscopic constants of the A-ring reduced derivatives of levonorgestrel have been previously reported (Lemus et al., 1992).

2.2. Oestradiol receptor binding studies

Immature female, intact Wistar rats (body weight 100–200 g), without oestrogen priming, were used for these studies. Animals were kept under a 14-h light/10-h darkness cycle, allowed food and water ad libitum and killed by decapitation. All procedures using animals 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 Metropolitan Autonomous University-Iztapalapa. Uteri were immediately removed, blotted and weighed; thereafter, all procedures were done at 4 °C. Uterine tissues were homogenised in chilled TEDLM buffer (20 mM Tris–HCl, pH 7.4 at 4 °C, 1.5 mM EDTA, 0.25 mM dithiotreitol, 10 μg/ml leupeptine and 10 mM sodium molibdate) in a ratio (wt/vol) 1:6, with three 10-s bursts. The homogenate was centrifuged at 180,000 × *g* for 1 h at 2 °C in an SW 50.1 rotor (Beckman Instruments, Palo Alto, CA, USA). Cytosol protein concentration was determined by the Bradford's dye binding method (Bradford, 1976) using bovine serum albumin as standard.

Stereospecificity of the binding of levonorgestrel and its derivatives to the oestrogen receptor was assessed by displacement analysis, as previously reported (Chávez et al., 1985). Uterine cytosol aliquots (4.1–5.2 mg protein/ml) were incubated with [^{3}H]oestradiol in the absence or presence of increasing concentrations (1–500 nM) of radioinert oestradiol at 4 °C for 18 h. The relative binding affinities of levonorgestrel and its derivatives to cytosol oestrogen receptors were evaluated by their capability to displace bound [^{3}H]oestradiol from the oestrogen receptor binding sites. Radioactive content in the samples was determined in a Packard Tri-Carb liquid scintillation spectrometer, Model 1900 TR (Packard, Downers Grove, IL, USA) using Insta-Gel Plus™ as counting solution. The counting efficiency for [^{3}H] was 65% and quenching was corrected in all samples by external standardisation. The relative binding affinities and the inhibition constants of levonorgestrel and its metabolites were calculated according to Reel et al. (1979) and Cheng and Prusoff (1973), respectively.

2.3. Oestrogen-induced yeast transactivation system

The oestrogen-like activity of levonorgestrel and its A-ring reduced derivatives was assessed in the yeast ex-

pression system previously reported by Lyttle et al. (1992), where a *Saccharomyces cerevisiae* hyperpermeable yeast strain RS 188 N was co-transfected with an expression vector containing the human oestrogen receptor α gene, under the control of a yeast copper metallothionein promoter and a β-galactosidase reporter vector, which expression is under the control of oestrogen responsive elements. Different concentrations of levonorgestrel, its derivatives, and/or other naturally occurring and synthetic steroids including the anti-oestrogen ICI-182,780 (Wakeling and Bowler, 1992) were added to the yeast system and incubated for 4.5 h. Yeast were harvested by centrifugation, washed with Z buffer (50 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 5 mM β-mercaptoethanol) and the cytosolic fraction was obtained by vortexing with glass beads in Z buffer followed by centrifugation at $10,000 \times g$. The supernatant was assayed for β-galactosidase activity using *o*-nitrophenyl-β-D-galactoside (4 mg/ml) as substrate, and absorbance at 420 nm was registered after exactly 10 min. Oestradiol served as the positive control, while 5α-dihydrotestosterone, progesterone, pregnenolone and testosterone were the negative controls.

2.4. Oestrogen-induced rat anterior pituitary progestin receptors

In vivo assessment of the oestrogenic potency of the 3β,5α levonorgestrel derivative was done by the induction of progestin receptors in the anterior pituitary of castrated rats, a bioassay previously described (Vilchis et al., 1986; Lemus et al., 2000). In brief, adult female Wistar rats (body weight 200–250 g) were ovariectomised under ether anaesthesia. Four weeks after ovariectomies, groups of 20 rats each were daily injected for six consecutive days with 100 μl of propyleneglycol containing 2.5 mg of 3β,5α levonorgestrel. Animals treated with oestradiol benzoate (5 μg/day/6 days) or with vehicle alone were used as experimental controls. After completion of the treatment, animals were killed by decapitation and the anterior pituitaries were immediately removed, rinsed with ice-cold TEDM buffer (20 mM Tris–HCl, pH 7.4 at 4 °C, 1.5 mM EDTA, 0.25 mM dithiotreitol and 10 mM sodium molibdate), blotted and weighed. Thereafter, all procedures were done at 4 °C. Tissues were homogenised in a glass homogeniser with a Teflon pestle with TEDM buffer supplemented with 10% glycerol (vol/vol) and 10 μg/ml leupeptine in a tissue/buffer ratio of 1:6 (wt/vol). Cytosol preparations were obtained as described for receptor binding studies. Aliquots of cytosol (300–400 μl; 10 mg protein/ml) were incubated with 3 nM [^{3}H]ORG 2058 for 4 h at 4 °C and layered on the top of linear sucrose gradients (20–35% in TEDM buffer supplemented with 10% (vol/vol) glycerol). An excess (125X) of radioinert ORG 2058 was added to an incubation set of cytosols from animals treated with 3β,5α levonorgestrel. Bovine serum albumin, on a parallel gradient, was used as an external marker. Gradients were centrifuged at $398,000 \times g$ for 2.5 h at 2 °C in a VTi 65 rotor (Beckman Instruments). Fractions of 85 μl each were collected from the bottom of the gradients and their radioactive content determined.

2.5. Statistical analysis

The results of transactivation studies in the yeast system are expressed as mean ± S.D. for each experimental group. Statistical differences were evaluated by one way analysis of variance followed by Dunnett's test and were considered to be significant when $P \leq 0.005$ (SSPS™). The relative oestrogenic potency of levonorgestrel metabolites was determined by the median effective dose (ED_{50}) calculated by a sigmoidal/logistic fit using an iterative program (Microcal, Origin™).

3. Results

3.1. Oestrogen receptor binding of levonorgestrel and its metabolites

The comparative ability of levonorgestrel and its A-ring reduced derivatives to compete with [^{3}H]oestradiol for receptor binding sites in the rat uterine cytosol, expressed as their relative binding affinities and inhibition constants are shown in Table 1. The 3β,5α tetrahydro derivative of levonorgestrel was effective as competitor for oestrogen receptor binding sites (RBA 2.4%) while the 3α,5α reduced metabolite of levonorgestrel also interacted with the oestrogen receptor, yet with lower binding affinity (RBA 0.4%). Unmodified levonorgestrel and its 5α dihydro metabolite were unable to compete for the oestrogen receptor binding sites in a similar manner to that of 5α dihydrotestosterone used as negative control (Table 1). Naturally occurring oestradiol, used as positive control, was the most potent steroid competitor for oestrogen receptors binding sites (RBA 100%).

Table 1
Relative binding affinities (RBA) and inhibition constants (K_i) of natural and synthetic steroids for cytosol oestrogen receptor, as assessed by displacement analysis
[^{3}H]oestradiol was used as radioligand.

Steroid competitors	RBA (%)[a]	K_i (nM)[a]
Levonorgestrel	–	–
5α Levonorgestrel	–	–
3α, 5α Levonorgestrel	0.4 ± 0.02	466 ± 22.10
3β, 5α Levonorgestrel	2.4 ± 0.40	84 ± 5.2
Oestradiol	100	2.1
5α Dihydrotestosterone	–	–

[a] Results represent the mean ± S.E.M. of five experiments in triplicate.

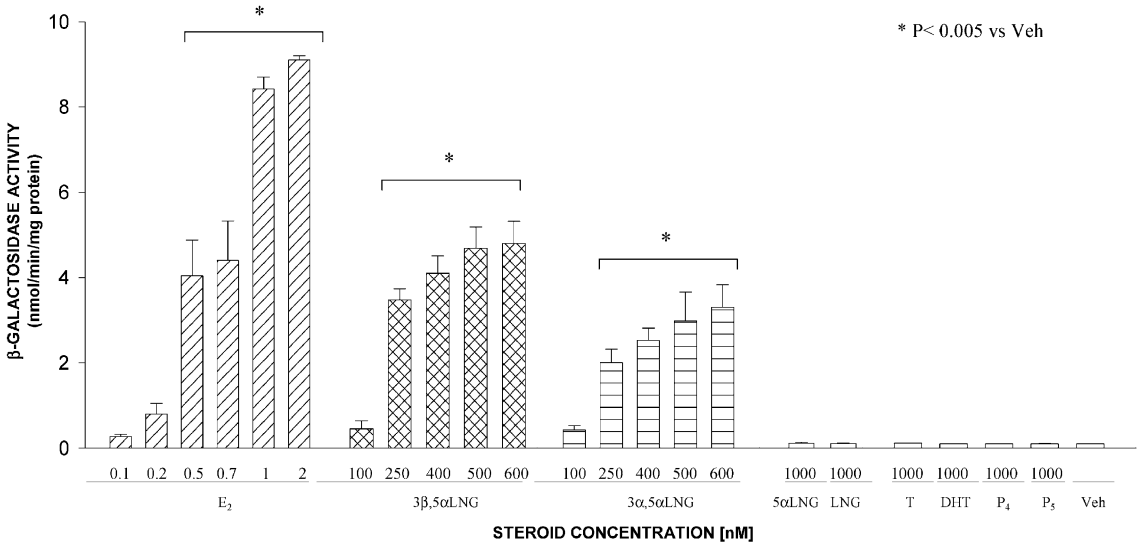

Fig. 1. Assessment of the oestrogenic potency of levonorgestrel and its A-ring reduced metabolites by their ability to transactivate oestrogen receptor-mediated β-galactosidase gene in a yeast expression system. Yeast cultures co-transfected with an expression vector containing the human oestrogen receptor α gene and oestrogen responsive elements fused to the β-galactosidase reporter vector, were grown in the presence of increasing concentrations of levonorgestrel (LNG) and its metabolites. Naturally occurring steroids were used as control. Results are expressed as β-galactosidase activity. Each point represents the mean ± S.D. of three experiments in triplicate. The results demonstrated that 3β,5α levonorgestrel (3β,5α LNG) and 3α,5α levonorgestrel (3α,5α LNG), at high concentrations, were able to transactivate the oestrogen receptor-mediated β-galactosidase in a dose-dependent manner, similar to that of naturally occurring oestradiol (E_2), used as positive control. 5α levonorgestrel (5α LNG) exhibited very little, if any, oestrogenic effect, while unchanged levonorgestrel was completely ineffective. Testosterone (T), 5α-dihydrotestosterone (DHT), progesterone (P_4), pregnenolone (P_5) and vehicle (Veh) used as negative controls were also ineffective (for details, see the text).

3.2. Activation of the oestrogen receptor-dependent β-galactosidase gene by levonorgestrel and its metabolites

The effects of different concentrations of levonorgestrel and its A-ring reduced metabolites upon the human oestrogen receptor α-mediated β-galactosidase activity in a hyperpermeable yeast expression system are shown in Fig. 1. The 3β,5α tetrahydro derivative of levonorgestrel and its 3α-isomer were able to induce the transcriptional activation of β-galactosidase reporter gene in a dose-dependent

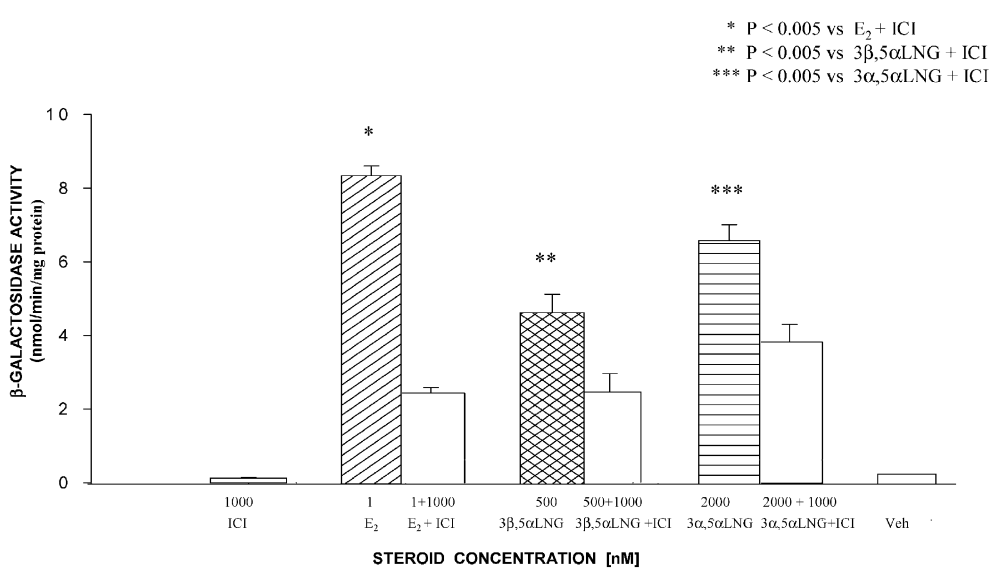

Fig. 2. Inhibition of oestradiol (E_2), 3β,5α levonorgestrel (3β,5α LNG) and 3α,5α levonorgestrel (3α,5α LNG)-induced transactivation of β-galactosidase gene by the steroidal antioestrogen ICI-182,780 in the yeast expression system. Yeast cultures were incubated with either E_2 (1 nM), 3β,5α LNG (500 nM), 3α,5α LNG (2000 nM) or vehicle (Veh) in the absence or presence of 1000 nM ICI-182,780. Results are expressed as β-galactosidase activity and each point represents the mean ± S.D. of three experiments in triplicate. The results demonstrated that the antiestrogen significantly diminished the transactivation of the yeast expression system induced by levonorgestrel metabolites.

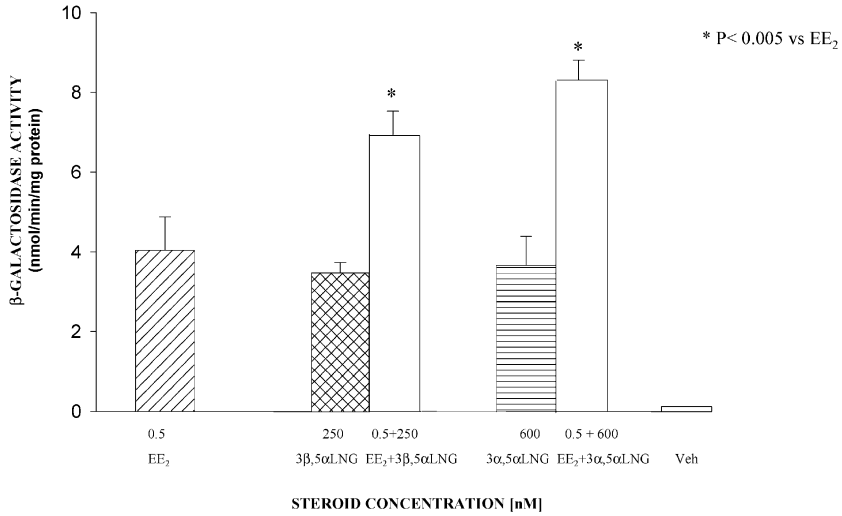

Fig. 3. Effects of ethynyl oestradiol (EE_2) on the 3β,5α levonorgestrel (3β,5α LNG) and 3α,5α levonorgestrel (3α,5α LNG)-induced β-galactosidase transactivation in the yeast expression system. Yeast cultures were incubated with either 3β,5α LNG (250 nM), 3α,5α LNG (600 nM) or vehicle (Veh) in the absence or presence of 0.5 nM EE_2. Results are expressed as β-galactosidase activity and each point represents the mean ± S.D. of three experiments in triplicate. The results demonstrated that the A-ring reduced metabolites of levonorgestrel exhibit an oestrogen additive effect rather than synergism with EE_2.

manner, an effect similar to that induced by naturally occurring oestradiol, though with a significantly lower potency. The oestrogen-like effect of 3β,5α levonorgestrel, was more potent than that of 3α,5α levonorgestrel (Fig. 1). Assessment of the median effective dose of levonorgestrel and its metabolites to induce oestrogen-like effects in the yeast expression system revealed that 3β,5α levonorgestrel (ED_{50}: 524 nM) was 750-fold less potent than oestradiol (ED_{50}: 0.70 nM), whereas 3α,5α levonorgestrel (ED_{50}: 1093) was 2.1-fold less potent than its 3β isomer. The 5α dihydro metabolite of levonorgestrel and unmodified levonorgestrel even at 1000 nM dose, were completely devoid of oestrogen-like effects (Fig. 1). Naturally occurring testosterone, 5α dihydrotestosterone, progesterone and pregnenolone used as negative controls, as well as vehicle alone, were completely ineffective (Fig. 1).

When ICI-182,780 was added to yeast cultures with either 3β,5α levonorgestrel or 3α,5α levonorgestrel, a significant diminution, though no abolition of the oestrogen receptor-mediated β-galactosidase activity was observed (Fig. 2). The antioestrogenic effect of ICI-182,780 was more potent to diminish the transactivation induced by oestradiol (70.5%) than that induced by effective doses of 3β,5α levonorgestrel (47.6%) and 3α,5α levonorgestrel (35.9%) as shown in Fig. 2. Interestingly, when lower doses of the levonorgestrel derivatives were employed, the antioestrogenic potency of ICI-182,780 was similar to that observed with oestradiol (data not shown).

To assess if the metabolites of levonorgestrel may exert oestrogen synergistic effects, co-transfected yeast cultures were incubated with 250 nM 3β,5α levonorgestrel or 600 nM 3α,5α levonorgestrel, in the absence or presence of 0.5 nM ethynyl oestradiol. As shown in Fig. 3, neither 3β,5α levonorgestrel nor its 3α isomer exhibited synergistic effects with ethynyl oestradiol. The levonorgestrel reduced metabolites exerted additive effects with ethynyl oestradiol on this expression system.

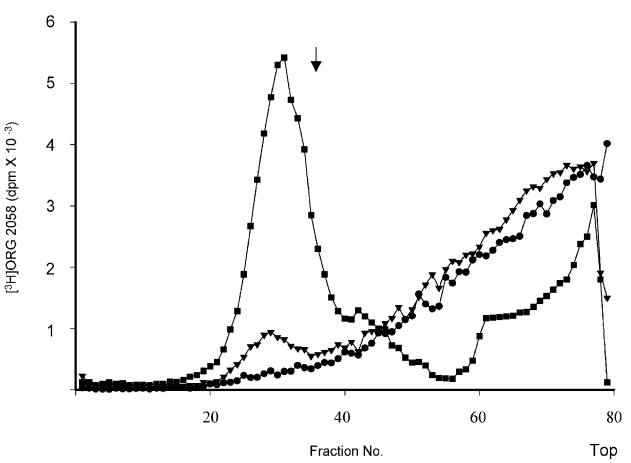

Fig. 4. Induction of oestrogen-dependent progestin receptors in the anterior pituitary of castrated female rats by the 3β,5α reduced metabolite of levonorgestrel. Animals were treated with 3β,5α levonorgestrel (2.5 mg/day/6 days), oestradiol benzoate (5.0 μg/day/6 days) or vehicle alone (propyleneglycol). Progestin receptor binding sites were assessed by a sucrose gradient labelling technique using [^{3}H]ORG 2058. Bovine serum albumin (↓) was used as an external marker. Results are expressed as the sedimentation profile of progestin receptors-[^{3}H]ORG 2058 complexes (7–9 S). Administration of oestradiol benzoate (■) fully restored the pituitary progestin receptor content in castrated animals, while the 3β,5α metabolite of levonorgestrel (▲) was able to increase oestrogen-dependent progestin receptors, only at a very high dose. Animals treated with vehicle alone (●) exhibited a complete lack of pituitary progestin receptors.

3.3. Induction of rat anterior pituitary progestin receptors by 3β,5α levonorgestrel

Castration of adult female rats resulted in a complete depletion of oestrogen-dependent progestin receptors in their anterior pituitaries, while hormone replacement with oestradiol benzoate (5 μg/day) administration was able to fully restore the pituitary content of progestin receptors, as depicted in Fig. 4. Administration of a high dose of 3β,5α levonorgestrel (2.5 mg/day) for six consecutive days to castrated animals, induced an increase of progestin receptor binding sites in the anterior pituitary. The 3β,5α levonorgestrel-induced progestin receptors sediment at 7–9 Svedgberg Units (S) in linear sucrose gradients, in an identical fashion than those induced by oestradiol benzoate, as shown in Fig. 4. Addition of an excess of the radioinert ligand ORG 2058 completely abolished the labelling of progestin receptors induced by 3β,5α levonorgestrel (data not shown). These results demonstrated a clear cut oestrogen-like effect of a neutral levonorgestrel metabolite in an in vivo animal model, though with significantly lower potency and efficacy than oestradiol benzoate.

4. Discussion

The results of this study demonstrate that the contraceptive synthetic progestin levonorgestrel exerts limited oestrogen-like effects mediated by the interaction of two of its A-ring reduced metabolites with the intracellular oestrogen receptor. Even though our laboratory reported back in 1992 that A-ring reduced derivatives of levonorgestrel exhibited very little interaction with the oestrogen receptor (Lemus et al., 1992), a number of recent studies providing evidence that high concentrations of levonorgestrel induce oestrogen-like effects (Schoonen et al., 1995a,b) prompted us to reassess the oestrogen receptor binding of levonorgestrel and its non-phenolic metabolites. The results presented herein demonstrate that the 3β,5α and 3α,5α tetrahydro metabolites of levonorgestrel specifically bind to the oestrogen receptor, though with a significantly lower affinity than oestradiol, whereas unmodified levonorgestrel does not. Interestingly, the binding affinity of levonorgestrel metabolites to the oestrogen receptor was significantly lower than that observed with the corresponding A-ring reduced metabolites of other 19-nor contraceptive progestins (Chávez et al.,1985; Lemus et al., 2000).

The studies in the yeast expression system revealed that 3β,5α levonorgestrel and to a significantly lesser extent its 3α isomer induce activation of the human oestrogen receptor α-mediated β-galactosidase reporter gene, thus indicating a clear oestrogen like-effect. The oestrogenic potency and efficacy of the levonorgestrel metabolites was significantly lower, as compared with natural occurring oestradiol in this system, an observation that correlates well with their corresponding binding affinities for the oestrogen receptor. Unmodified levonorgestrel and its 5α-dihydro derivative were devoid of oestrogenic effect, a finding which is in line with their lack of binding affinity to the oestrogen receptor.

Further evidence that the oestrogen-like effect of levonorgestrel metabolites was oestrogen receptor-mediated, was furnished by its significant diminution induced by the simultaneous addition of ICI-182,780 to the yeast cultures. The inability of the antioestrogen to completely abolish the oestradiol stimulation of β-galactosidase activity may be due to low permeability of yeast cells (Lyttle et al., 1992), since ICI-182,780 has not oestrogen agonistic effect in this system (Fig. 2).

Whether the metabolites of levonorgestrel may also exert oestrogenic actions mediated by the oestrogen receptor β (Mosselman et al., 1996; Kuiper et al., 1996, 1997), cannot be ascertained from this study and deserves additional investigation.

Since some pharmaceutical formulations combine levonorgestrel with ethynyl oestradiol, it was of interest to evaluate the synergistic effect of the levonorgestrel metabolites with ethynyl oestradiol in the yeast expression system. The results demonstrated an additive rather than a synergistic effect of levonorgestrel metabolites when they were simultaneously incubated with ethynyl oestradiol in the yeast cultures.

The oestrogen-like activity of 3β,5α levonorgestrel demonstrated in the yeast expression system was confirmed in an in vivo animal model. Indeed, this non-phenolic metabolite of levonorgestrel at a very high dose, successfully increased the anterior pituitary content of 7–9 S oestrogen-dependent progestin receptors in castrated female rats, in a similar fashion to that observed with oestradiol benzoate, though with significantly lower efficacy.

The oestrogen agonistic activity of A-ring reduced levonorgestrel metabolites, as demonstrated in this study, appears to be of a lower potency than that reported for the corresponding non-phenolic metabolites of norethisterone (Vilchis et al., 1986) and gestodene (Lemus et al., 2000), another 19-nor contraceptive progestins.

Overall, the results offer an adequate explanation for the growth and proliferation of breast carcinoma cell lines induced by high, non-pharmacological doses of levonorgestrel (Van der Burg, 1991; Van der Burg et al., 1992; Catherino et al., 1993; Schoonen et al., 1995a,b). The data underline the role of metabolism of 19-nor contraceptive progestins in the expression of their hormone-like effects.

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