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Molecular Mechanisms of the Antihormonal and Antiimplantation Effects of Norethisterone and Its A-Ring Reduced Metabolites

IVONE CASTRO,[1] MARCO ANTONIO CERBÓN,[1] ANA MARIA PASAPERA,[1] RUBEN GUTIÉRREZ-SAGAL,[1] GUSTAVO A. GARCIA,[2] CARLOS OROZCO,[3] IGNACIO CAMACHO-ARROYO,[1] RENE ANZALDUA,[1] AND GREGORIO PÉREZ-PALACIOS[1]

[1]Molecular Biology Unit in Reproductive Health, National Institute of Nutrition S. Zubirán and FES-Zaragoza, and [2]School of Chemistry, National University of Mexico, Mexico City, Mexico; and [3]Division of Science and Technology, Griffith University, Nathan, Australia

ABSTRACT Norethisterone (NET) has been used as a contragestational postcoital agent. It is biotransformed to 5α dihydro-NET (5α-NET) and 3β,5α tetrahydro-NET (3β,5α-NET) in target tissues. The participation of these metabolites in NET effects is unknown. We have examined the antiimplantation and antiprogestational effects of NET and its metabolites, in adult mated female rabbits, by assessing the number of implantation sites and the expression products of the uteroglobin (UTG) gene in the uterus, and by comparing them with those of RU-486 and estradiol. Steroids were daily administered s.c. at several doses for 7 consecutive days, starting 24 hr after coitus. To assure that fertilization occurred in all animals, the presence of early pregnancy factor was determined. The results demonstrated that high doses (5 mg/kg) of NET reduced both implantation and the expression of the UTG gene. On the other hand, lower doses (1.5 mg/kg) of 5α-NET produced an antiimplantation effect and suppressed UTG synthesis and its mRNA. These effects were similar to those of RU-486. At lower doses (1 mg/kg), both estradiol and the estrogenic metabolite 3β,5α-NET were also effective in inhibiting implantation and UTG gene expression. The overall results suggest that NET metabolites exert antiimplantation and antiprogestational effects through their interaction with progesterone and estrogen receptors, and provide an explanation for the molecular mechanisms involved in the postcoital contraceptive action of NET.

Key Words: Synthetic progestins, Uteroglobin, Pregnancy, Rabbit endometrium

INTRODUCTION

Norethisterone (NET), a synthetic 19-norprogestin, has been widely used as a potent postcoital contraceptive agent (Lei and Hu, 1981; Van Look, 1988). However, its mechanism of action has not been completely understood. It has been demonstrated that synthetic progestins and their metabolites regulate the expression of the uteroglobin (UTG) gene in rabbit endometrium (Isomaa, 1981, Jänne et al., 1983, Pérez-Palacios et al., 1992). It has been suggested that UTG plays an important role in the implantation process (Gutiérrez-Sagal et al., 1993).

We have shown that administration of NET induces a significant increase of uterine UTG and its mRNA content in prepubertal rabbits, in a manner similar to that observed with progesterone (Cerbón et al., 1990). Interestingly, it has been reported that 5α-reduction of NET precludes its progestational effects upon UTG gene expression. Indeed, when one of the NET metabolites, 5α-dihydro-NET (5α-NET), was administered simultaneously with progesterone, a clear antiprogestational effect was noticed (Cerbón et al., 1991). A further reduction of 5α-NET to the estrogenic compound 3β,5α tetrahydro-NET (3β,5α-NET) in target tissues has been reported (Larrea et al., 1987).

To elucidate the molecular mechanisms involved in the contragestational actions of NET, we have studied the antiimplantation and antiprogestational effects of NET and its metabolites 5α-NET and 3β,5α-NET in mated adult female rabbits. Fertilized rabbits treated with RU-486 and estradiol were used as control contragestational agents. Fertilization was determined by the presence of the early pregnancy factor (EPF) in serum, using a rosette inhibition test, while the recording of implantation sites and the content of uterine UTG and its mRNA were used to evaluate the antiim-

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Address reprint requests to Marco A. Cerbón, Ph.D., Instituto Nacional de la Nutrición S. Zubirán, Departamento de Biología de la Reproducción, Vasco de Quiroga No. 15, Tlalpan, México, D.F., 14000, Mexico.

plantation and antiprogestational effects of NET and its metabolites.

MATERIALS AND METHODS

Steroids

NET (17α-ethynil-17β-hydroxy-4-estren-3-one) was kindly provided by Schering Mexicana, S.A. (Mexico City). 5α-NET (17α-ethynil-17β-hydroxy-5α-estran-3-one) was synthesized by lithium ammonia reduction of NET, according to a procedure previously described (Bowers et al., 1958). The 3β,5α-NET derivative was prepared from 5α-NET by sodium borohydride reduction as previously described (Chávez et al., 1985). Chemical purity of NET and its derivatives was assessed by their melting points, high performance liquid chromatographic behavior, and H-nuclear magnetic resonance spectrometric analysis (Vilchis et al., 1986). RU-486 (17β-hydroxy-11β-[4-dimethylaminophenyl]-17α[1-propynyl]estra4,9-dien-3-one) was kindly provided by Rousell Uclaf (France). Estradiol-17β was purchased from Steraloids (Pauling, NY).

Animals and Treatments

Adult female New Zealand white rabbits (3.5–4.5 kg) were used throughout the study. Animals were housed in individual cages with food and water available ad libitum, and were mated twice on the same day with two different, experienced bucks. The day of mating was designated as day 0. Mated animals with positive serum EPF signal on day 1 (24 hr after second coitus) were included in the study. Vehicle (propylenglycol) and steroids were s.c.-administered at several doses on daily basis from day 1–7, unless otherwise specified. Blood samples were withdrawn on days 1, 3, 5, and 7, and submitted to EPF assay. One day after the last injection, the animals were deeply anesthetized with ketamine (Rhóne Mérieux, Queretaro, Mexico) and bled, the uterine horns were immediately dissected, and the number of implantation sites was recorded (Hicks et al., 1980). Uterine flushings were obtained by rinsing uterine horns with 1 ml of 0.15 M NaCl and stored at −70°C until assayed. One uterine horn was immediately frozen in a dry ice-acetone mix and stored at −70°C for total RNA extraction, while the contralateral uterine horn was fixed in a paraformaldehyde solution for histological studies.

UTG Radioimmunoassay

The UTG content of uterine flushings was determined by a specific double antibody radioimmunoassay (Mayol and Longenecker, 1974). Radioactive labeled UTG was prepared by the lactoperoxidase method (Von Schenck et al., 1976) with (^{125}I) sodium iodine (sa: 16.4 mCi/μg) purchased from Amersham International (Buckinghamshire, England). The antirecombinant UTG antibody was kindly provided by Professor M. Beato from the Institut für Molekularbiologie and Tumorforschung, Marburg, Germany. The rabbit anti-sheep serum used as second antibody was purchased from ICN Biomedicals (Costa Mesa, CA). Assay sensitivity was 0.5 ng/ml and the intra- and interassay variation coefficients were 10 and 12%, respectively. Protein content in uterine flushings was determined as previously described (Bradford, 1976).

Northern Blot Analysis

Total RNA was isolated from individual endometrial samples by the guanidine-isothiocyanate-LiCl method (Cathala et al., 1983). RNA samples were size-fractionated by electrophoresis on 1.1% agarose gels in the presence of 2.2 M formaldehyde and then transferred to Gene Screen membranes (New England Nuclear, Boston, MA). Blotted RNAs were hybridized with [α-^{32}P]-dCTP-UTG cDNA (specific activity 1–2 × 10^8 cpm/μg), labeled by the random primer method (Feinberg and Vogelstein, 1983). The UTG cDNA was kindly provided by Professor E. Milgrom from the Laboratoire des Hormones et Reproduction, Hôpital de Bicêtre, France. Hybridizations were carried out under standard conditions as previously described (Gutiérrez-Sagal et al., 1993). Membranes were then exposed to Kodak X-OMAT-AR film (Eastman Kodak, Rochester, NY) for 16 hr. After exposure, the membranes were stripped and rehybridized with [α-^{32}P]-dCTP-histone H_4, as expression control.

Early Pregnancy Factor (EPF) Activity

The determination of EPF was performed in serum samples by the rosette inhibition assay (Orozco et al., 1986; Clarke et al., 1987). In this assay a rosette inhibition titer (RIT) of 12 indicates absence of early pregnancy signal, whereas a RIT >16 demonstrates fertilization (Orozco et al., 1990).

RESULTS

Antiimplantation Effects of NET and Its Metabolites

In order to assure that mated female rabbits used in this study were fertilized, we determined the presence of EPF in serum, through the rosette inhibition assay. In mated rabbits we found a RIT value of 28 on day 1, indicating the occurrence of fertilization, whereas in control nonmated rabbits the RIT value was 12, demonstrating the absence of EPF signal. All animals with positive EPF signal were included in the study.

The administration of NET (1.0 mg/kg) had no effects on the implantation process, since all treated animals presented positive EPF activity through days 1–7, and the number of implantation sites was identical to that observed in fertilized rabbits treated with vehicle. NET was effective as an antiimplantation agent only at the high daily dose of 5.0 mg/kg, which completely inhibited both implantation and EPF signal (Table 1). It is important to mention that at this high dose, profound disrupting effects on uterus morphology were noticed (data not shown).

TABLE 1. Effects of NET and its metabolites on implantation and EPF signal

Treatments	Daily Dose* (mg/kg)	No. rabbits pregnant/mated	Implantation sites	EPF signal
Vehicle	–	6/6	9–10	+
NET	1.0	6/6	9–10	+
	5.0	0/3	0	–
5α-NET	1.0	7/9	3–4	+
	1.5	0/6	0	–
	2.5	0/6	0	–
	5.0	0/6	0	–
3β.5α-NET	1.0	0/6	0	–
17β-E_2	1.0	0/6	0	–
RU-486	1.0	0/6	0	–
	2.5	0/6	0	–

*7 consecutive days. All samples were analyzed on day 8 after mating.

When 5α-NET (1.0 mg/kg) was administered to fertilized rabbits, a slight reduction in the number of implantation sites was observed as compared with animals treated with vehicle (Table 1). Administration of 5α-NET at the daily dose of 1.5 mg/kg and above resulted in a complete inhibition of implantation as it is shown in Table 1. A close correlation between the antiimplantation effects of 5α-NET and the absence of EPF signal during different days of the treatment was observed. In contrast to NET, the administration of 5α-NET did not disrupt uterus morphology.

Both the administration of estrogenic agent 3β,5α-NET (1 mg/kg) and estradiol at the same dose induced the complete inhibition of the implantation process (Table 1), and reduction of the EPF signal. Administration of the antiprogestin RU-486 to fertilized animals at daily doses of 1.0 mg/kg and above resulted in the inhibition of implantation with the corresponding absence of EPF signal.

Effects of NET and Its Metabolites Upon Uterine UTG Content in Fertilized Female Rabbits

UTG content in uterine flushings from fertilized rabbits under different treatments was measured by RIA on day 8 after mating. Nonmated and pregnant rabbits treated with vehicle were used as negative and positive controls, respectively. UTG content in uterine flushings from adult nonmated female rabbits was very low (7.7 μg/mg protein), whereas in mated animals on day 8 of gestation a high content (163 μg/mg protein) of UTG was detected (Fig. 1).

Administration of NET to fertilized rabbits at the daily dose of 1.0 mg/kg diminished UTG content 53%, as compared with vehicle treatment. In addition, a higher dose of NET (5.0 mg/kg) reduced UTG content 90% (Fig. 1). Fertilized animals treated with 5α-NET (1 mg/kg) exhibited a significant diminution (50%) in UTG content as compared with vehicle. This effect was similar to that observed in animals treated with NET at the same dose. A daily dose of 5α-NET (1.5 mg/kg) diminished UTG content more than 85%. Furthermore, doses of 2.5 and 5.0 mg/kg reduced UTG content to levels lower than those found in nonmated adult rabbits (Fig. 1).

Fertilized animals treated with the synthetic antiprogestin RU-486 at the daily dose of 1.0 mg/kg significantly decreased UTG content (73%) in uterine flushings. Moreover, a dose of 2.5 mg/kg resulted in a further diminution (88%) of UTG (Fig. 1).

Because of the potency of antiprogestational effects of estradiol, the scheme of treatment with this steroid and 3β,5α-NET was modified. Animals were treated daily for different periods of time (3, 5, and 7 days after fertilization) with a dose of 1 mg/kg in all cases. Both estradiol and 3β,5α-NET depleted UTG content from the third day of treatment (Fig. 2).

Effects of NET Metabolites Upon Endometrial UTG mRNA Content in Fertilized Female Rabbits

Endometrial UTG mRNA content was determined by Northern blot analysis. A major band of 0.6 Kb corresponding to UTG mRNA was detected (Fig. 3) in pregnant rabbits, as previously described (Bailly et al., 1983). As indicated in Figure 3, the endometrial UTG mRNA in adult fertilized animals treated with vehicle on day 5 after mating (lane 2) was significantly more abundant than that of nonmated rabbits treated with vehicle (lane 1). Interestingly, on day 8 after mating, the endometrial UTG mRNA exhibited a slight diminution (lane 3).

Fertilized animals treated with 5α-NET (1.0 mg/kg) presented a significant diminution in UTG mRNA content on day 8 after mating (Fig. 3, lane 4), as compared with mated animals on the same day of gestation. Furthermore, UTG mRNA content in animals treated with 5α-NET was lower than in nonmated control rabbits. Daily doses of 1.5 mg/kg and above of 5α-NET completely abolished the endometrial content of UTG mRNA (Fig. 3, lanes 5–7).

The administration of RU-486 (1.0 mg/kg) significantly diminished endometrial UTG mRNA content as compared with that of pregnant rabbits (Fig. 3, lane 8). Interestingly, UTG mRNA was relatively more abun-

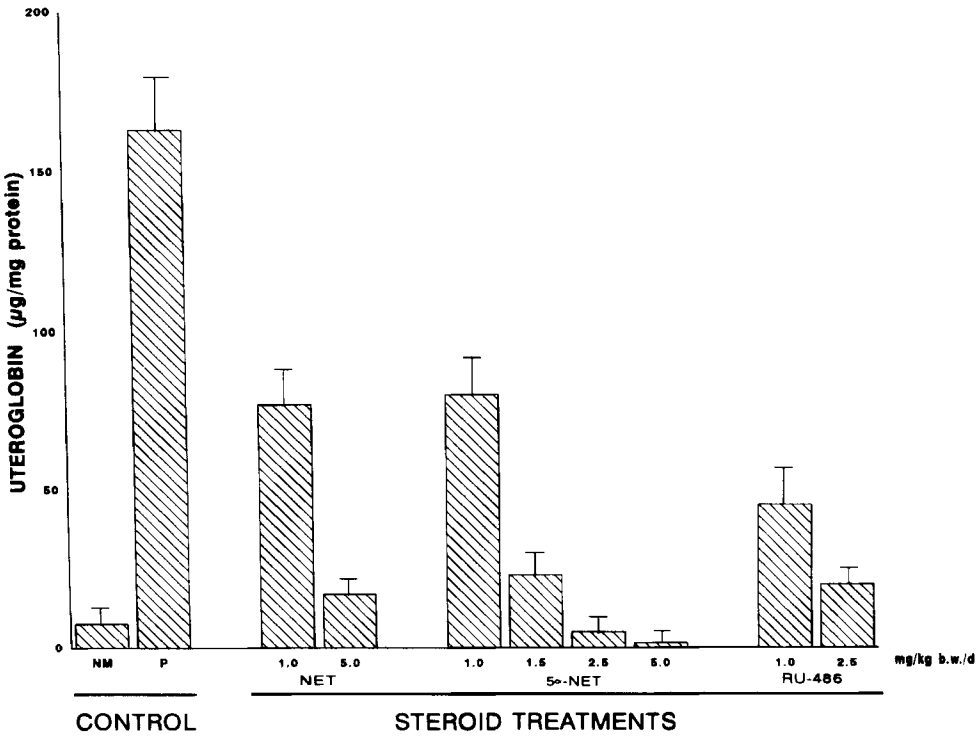

Fig. 1. Uteroglobin content in uterine flushings from fertilized rabbits treated with synthetic steroids. Animals (n = 6) were daily treated with NET, 5α-NET, and RU-486, for 7 consecutive days. Samples were analyzed on day 8 after mating by a specific double antibody radioimmunoassay. The results are expressed as µg of uteroglobin/mg protein (mean ± SD). Nonmated rabbits (NM) and pregnant rabbits (P) on the eighth day of pregnancy were used as negative and positive controls, respectively.

dant in animals receiving 2.5 mg/kg of RU-486 (lane 9) than in those treated with 1.0 mg/kg.

Pregnant rabbits treated with estradiol (1 mg/kg) for 3, 5, or 7 consecutive days showed a significant diminution in endometrial UTG mRNA content as compared with their respective controls on the same days of gestation. Indeed, no detectable UTG mRNA content was observed after estradiol treatment (Fig. 4). The daily administration of 3β,5α-NET (1 mg/kg) also significantly reduced UTG mRNA content in fertilized rabbits. In contrast with estradiol, an important diminution of UTG mRNA was noticed only after 5 days of treatment, and the suppression of UTG mRNA was observed after the seventh administration of 3β,5α-NET (Fig. 4).

All the membranes were stripped and rehybridized with H_4-histone cDNA. The lack of variation in the nonhormone-regulated H_4-histone mRNA content (Figs. 3 and 4) supported the view that the changes observed in UTG mRNA content were due to the different steroid treatments.

DISCUSSION

The present study shows the molecular effects of NET and its A-ring reduced metabolites, 5α-NET and 3β,5α-NET, as related to their antiimplantation and antiprogestational properties in pregnant rabbits. The re-

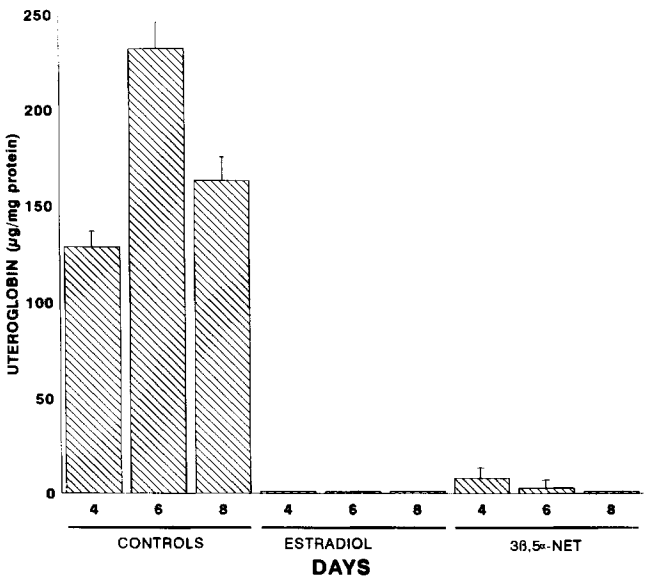

Fig. 2. Uteroglobin content in uterine flushings from fertilized rabbits treated with estradiol and 3β,5α-NET. Mated animals (n = 6) were treated daily with estradiol or 3β,5α-NET at a dose of 1 mg/kg for 3, 5, and 7 consecutive days. Uterine flushings were collected and analyzed 24 hr after the last dose on days 4, 6, and 8. The results are expressed as µg of uteroglobin/mg protein (mean ± SD). Rabbits treated with vehicle for 3, 5, and 7 days were used as controls.

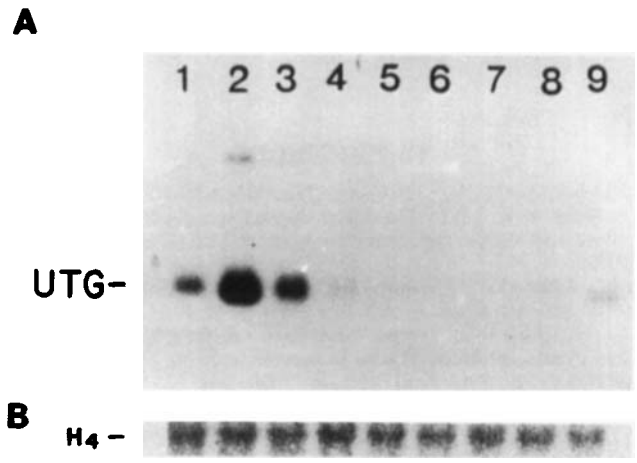

Fig. 3. Representative Northern blot of endometrial uteroglobin (UTG) mRNA from fertilized rabbits treated with synthetic steroids. Aliquots of total RNA (20 μg) from endometrium of fertilized animals treated daily with 5α-NET (1.0, 1.5, 2.5, and 5.0 mg/kg, lanes 4–7), RU-486 (1.0 and 2.5 mg/kg, lanes 8 and 9) for 7 consecutive days, and from nonmated (lane 1) and pregnant rabbits on days 5 and 8 after mating (lanes 2 and 3), were fractionated on denaturing agarose gels, transferred to Gene-Screen membranes, and hybridized with [α-^{32}P]-UTG cDNA (A) and Histone H_4 cDNA (B) probes. The membranes were exposed for 16 hr using Kodak X-OMAT-AR film.

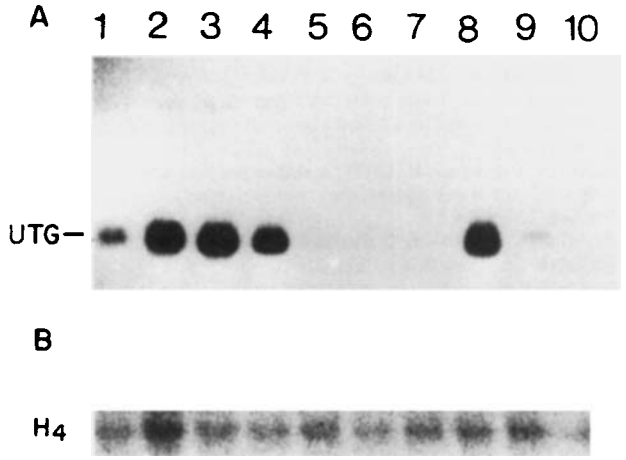

Fig. 4. Effects of estradiol and 3β,5α-NET on endometrial uteroglobin (UTG) mRNA from fertilized rabbits. Northern blot analysis of total RNA (20 μg) from endometrium of nonmated (lane 1) and pregnant animals treated daily for 3, 5, or 7 consecutive days with vehicle (lanes 2–4), estradiol (1 mg/kg, lanes 5–7), and 3β-5α-NET at the same dose (lanes 8–10), was performed as indicated in Figure 3. **A:** Hybridization with UTG cDNA. **B:** Hybridization with histone H_4 cDNA probes.

cordings of implantation sites as well as of UTG gene expression products were used as markers of the contraceptive effects of NET and its metabolites. In all cases, pregnancy was corroborated by the determination of serum EPF presence. We observed a close correlation between the antiimplantation effects of the different steroids and the reduction of EPF signal (Table 1).

The chronic treatment (7 days) of NET at a dose of 1.0 mg/kg did not reduce the number of implantation sites in fertilized rabbits. However, at a higher dose (5.0 mg/kg), NET induced a clear antiimplantation effect, since no implantation sites were found in the animals treated with this dose (Table 1). Both the chronic administration of 5α-NET and of 3β,5α-NET resulted in a more potent antiimplantation effect, since doses lower (1.5 and 1.0 mg/kg, respectively) than those of NET were able to completely inhibit the implantation process (Table 1), thus indicating that A-ring metabolites of NET were markedly more effective than their precursor molecule. Indeed, the antiimplantation effects of NET metabolites were similar to those of the antiprogestative RU-486.

In order to correlate the antiimplantation effects of NET and its metabolites with their antihormonal action, we analyzed the expression of the UTG gene, whose regulation by progesterone and estrogens is well known (Bullock and Willen, 1974; Loosfelt et al., 1981). We found that the administration of NET (5 mg/kg) reduced UTG content 90% from uterine flushings. As in the case of the inhibition of implantation, lower doses of NET metabolites were required to deplete UTG production (Figs. 1 and 2), suggesting that the biological effects of NET could be mediated by the action of its metabolites. The effects of RU-486 upon UTG synthesis were similar to those observed with 5α-NET.

The reduction of UTG content produced by NET metabolites was strongly related to a diminution in endometrial UTG mRNA content (Figs. 3 and 4). 5α-NET at doses of 1.5 mg and above completely abolished the expression of UTG mRNA. These data indicate that the inhibition of UTG gene expression by 5α-NET is exerted at the transcriptional level, although a postranscriptional effect such as the increase of mRNA degradation cannot be ruled out. Previous studies in prepubertal rabbits performed in our laboratory have also demonstrated an inhibition on progesterone-induced UTG transcription by NET metabolites (Pérez-Palacios et al., 1992). UTG mRNA content was also reduced by RU-486 treatment. The comparable effects of RU-486 and those of 5αNET observed in this study, as well as their specific binding to progesterone receptors (Reel et al., 1979; Rauch et al., 1985), suggest that these steroid receptors mediate the antihormonal effects of both compounds.

It is interesting to mention that in this study the effect of RU-486 upon UTG mRNA was greater at a low dose (1 mg/kg) than at a higher one (2.5 mg/kg). However, this pattern did not correspond to that observed in UTG content in uterine flushings, where the high dose of RU-486 induced the largest UTG diminution. The finding of a differential effect of RU-486 upon UTG mRNA and UTG protein was not unexpected since similar effects have been reported for progesterone, which exerts a dual effect upon UTG gene expression, induc-

ing UTG transcriptional and posttranscriptional actions (Loosfelt et al., 1981; Rauch et al., 1985).

The 5α-NET molecule is biotransformed at target tissues to the potent estrogenic agent 3β,5α-NET (Larrea et al., 1987). We compared its effects with those of estradiol, whose antiprogestational and antiimplantation actions at high doses are well known (Kopu et al., 1981; Gidley-Baird et al., 1986). Both 3β,5α-NET and estradiol (1 mg/kg) exert a potent antiprogestational effect by depleting UTG synthesis and its mRNA content (Figs. 2 and 4), suggesting that 3β,5α-NET could inhibit UTG gene expression through its interaction with estrogen receptors. These results confirm and extend previous data on the estrogenic properties of 3β,5α-NET in prepubertal rabbits (Cerbón et al., 1990).

Although estradiol can stimulate UTG transcription at low doses (Loosfelt et al., 1981), a negative regulation of UTG by estrogens at higher doses was found in this study. Similar high dose estradiol-induced inhibition of UTG transcription has also been reported (Kopu et al., 1981). Although the effects of estrogens upon UTG regulation appear to be dose-dependent, the molecular mechanisms involved in such regulation remain unknown. The presence of a noncanonical estrogen responsive element (ERE) located in the UTG promoter region (Slater et al., 1990) suggests that this ERE and the estrogen receptor could be implicated in the inhibition of UTG gene transcription by compounds with estrogenic activity, such as 3β,5α-NET.

Both the blockade of the progesterone receptor and the activation of estrogen receptors by 5α-NET and 3β,5α-NET, respectively, may contribute to the inhibition of UTG gene expression, and in this way produce the antiprogestational and antiimplantation effects. At this moment we cannot ascertain the contribution of both processes to the transcriptional inhibition of UTG.

In summary, our results suggest that the contragestational effects of NET involve several molecular mechanisms that include its bioconversion to 5α-NET and to 3β,5α-NET in the target tissues; the interaction of both metabolites with progesterone and estrogen receptors, respectively; and the regulation of UTG and other specific genes. The understanding of the molecular mechanisms of NET and its A-ring metabolites as contragestational agents contributes not only to clarifying the effects of synthetic steroids metabolism in implantation process, but also in the design of new postcoital contragestational agents.

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