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FURTHER STUDIES ON THE ANTIGONADOTROPIC MECHANISM OF ACTION OF NORETHISTERONE

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Summary—To examine the molecular mechanisms involved in the antigonadotropic effects of norethisterone (NET) and two of its A-ring reduced metabolites the 5α-norethisterone (5α-NET) and the 3β,5α-norethisterone (3β,5α-NET) at the neuroendocrine level, a series of experiments were undertaken in adult castrated rats. Animals were primed either with 0.2 mg of tamoxifen (Tam) for 4 consecutive days or 1.0 mg of cyproterone acetate (CPA) for 7 days followed by a single subcutaneous injection of 0.5 mg of NET, 5α-NET or 3β,5α-NET. Four hours later, they were sacrificed and blood obtained for the measurement of immunoreactive serum LH and FSH. The results indicated that antiestrogen (Tam) pretreatment precluded the inhibitory effects of NET and the 3β,5α-NET but not those of the 5α-NET derivative. Pretreatment with CPA did not modified the antigonadotropic action of the 3β,5α-NET metabolite but it markedly reduced the inhibitory action of the 5α-NET, thus indicating that in the experimental model used, the antigonadotropic effects of NET, are in part the result of its metabolic conversion to its A-ring reduced metabolites. While the 5α-NET displayed an androgenic effect, the 3β,5α-NET exhibited estrogen-like effect at the neuroendocrine level.

INTRODUCTION

Norethisterone NET, (17α-ethynyl-19-nortestosterone), a 19-nortestosterone derivative was first synthesized in 1951 [1]; Since then this synthetic progestin has been widely used as a contraceptive agent either alone or in combination with estrogens [2–5]. It has been recognized that NET is further metabolized to various dihydro and tetrahydro A-ring reduced metabolites which have been isolated from serum and urine of women under contraceptive therapy [6–8]. Previous experiments have shown that NET displays a variety of hormonal effects when administered to several mammalian species [9–12]. The progestogenic, estrogenic and androgenic effects of NET, have been previously reported in bioassays as well as in clinical studies [13–16]. Administration of NET as an enanthate derivative exhibits a potent gonadotropin inhibitory activity in non-estrogen-sensitized postmenopausal women, an effect which is not seen with progesterone alone, suggesting that its mode of action might differ from that of progesterone [17]. Indeed several studies from our laboratories have suggested an estrogenic mode of action of NET upon the hypothalamic–pituitary unit of ovariectomized rats [18, 19]. Moreover, further studies have indicated that biotransformation of NET to other metabolites such as the A-ring reduced dihydro derivatives can specifically interact with either estrogen or androgen receptors [20]. This interaction has been shown to be followed by specific biological responses, that with estrogen receptors there is the induction of progesterone highly specific binding sites at the pituitary level. Further support for this concept was derived from recent observation showing that some tetrahydro-NET derivatives are indeed more potent in terms of inhibitory effect on serum gonadotropins levels than NET itself [21, 22].

In the present study further observations on the mechanism of action of NET are performed by the analysis of the effects of the administration of NET and two of its A-ring reduced derivatives (5α-NET and 3β,5α-NET) upon the hypothalamic–pituitary unit of adult ovariectomized rats in the presence of tamoxifen (Tam) and cyproterone acetate (CPA).

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EXPERIMENTAL

Chemicals

Norethisterone (NET), (17α-ethinyl-17β-hydroxy-4-estren-3-one) was kindly provided by Schering Mexicana, Mexico. The NET derivatives were synthesized as previously described [24] with minor modifications. The 3β,5α-NET was prepared by sodium borohydride reduction [25]. The chemical purity of NET and its metabolites was >98% as assessed by melting points, high-performance liquid chromatography and H-nuclear magnetic resonance spectrometric analysis. Tamoxifen (Tam), (trans-(1-*p*-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene), was obtained from Imperial Chemical Industries, Macclesfield, Cheshire, Cyproterone acetate (17-acetoxy-6-chloro-1α,2α-methylene-pregna-4,6-diene-3,20-dione), was a gift from Schering Mexicana, Mexico. All other chemicals employed were analytical grade and purchased from Sigma Chemical Co., St Louis, Mo.

Animals

Adult male and female Wistar rats weighing 200–250 g were gonadectomized under light ether anesthesia 3 weeks prior to the experiments. Animals were kept under a 14 h-light, 10 h-dark day cycle and maintained on food and water *ad libitum*.

Antiestrogen administration

Tamoxifen (Tam), was administered s.c. at a daily dose of 0.2 mg for 4 consecutive days [18]. On the fourth day, the animals ($n = 6$) were treated with either the vehicle, NET or its metabolites 5α-NET and 3β,5α-NET at a single dose of 0.5 mg. Four hours after, blood samples were obtained by decapitation and serum aliquots were stored at −20°C for hormonal analysis.

Antiandrogen administration

The steroidal progestin with antiandrogen properties cyproterone acetate (CPA) was subcutaneously injected at various doses to establish the antiandrogen optimal doses. One milligram per day for 7 days was subsequently chosen for the study in male and female castrated rats.

The serum LH and FSH levels were measured before and after priming with tamoxifen or cyproterone acetate as well as after 4 h of steroid administration. This schedule was based on the the time required for NET to induce a maximum gonadotropin inhibition [18].

Hormone assays

Serum gonadotropins were measured by specific radioimmunoassays (RIA) using the double antibody technique following the protocols kindly supplied by the NIADDK Rat Pituitary Hormone Distribution Program, Baltimore, Md. The results were expressed as ng/ml (mean ± SD) according to the rLH-RP-1 and rFSH-RP1 used as standards. The intra- and inter-assay coefficients of variation for LH were 6.3 and 8.9%, respectively and for FSH 4.0 and 9.5% respectively. Direct comparison between the 2 groups was performed using the Student's *t*-test for unpaired samples.

RESULTS

Administration of NET to adult castrated male rats decreased the serum LH and FSH. This inhibitory effect upon serum gonadotropins was also observed to a greater extent with both NET metabolites indicating their greater antigonadotropic potency (Table 1).

Table 1. Effect of a single injection of 0.5 mg of NET and its reduced metabolites 5α-NET and 3β,5α-NET upon serum LH in castrated male rats. Results are expressed as the mean ± SD. The percentage of LH inhibition from the control post treatment is shown in parentheses

	Control post-treatment	NET	5α-NET	3β,5α-NET
Non primed (vehicle)	391 ± 24 (0) $n = 6$	199 ± 22* (49) $n = 6$	129 ± 10† (67) $n = 6$	157 ± 20* (60) $n = 6$
Cyproterone acetate (1 mg/day/7 days)	481 ± 25 (0) $n = 16$	259 ± 53* (39) $n = 5$	424 ± 57 (12) $n = 5$	216 ± 14† (55) $n = 5$
Tamoxifen (200 μg/day/4 days)	478 ± 30 (0) $n = 12$	— —	213 ± 19† (55) $n = 5$	351 ± 37 (27) $n = 6$

*$P < 0.01$ when compared to control post treatment group.
†$P < 0.001$ when compared to control post treatment group.

When the serum LH and FSH levels were compared before and after tamoxifen administration, a slight non significant rise was observed. As shown in Fig. 1, administration of a single dose (0.5 mg) of 5α-NET to Tam primed rats resulted in a significant ($P < 0.001$) decrease in serum LH and FSH levels. However, neither NET nor 3β,5α-NET was effective as gonadotropin inhibitor in TAM primed castrated female rats. Since the administration of 1 mg for 7 days of cyproterone acetate did not induce any significant change in the circulating pituitary gonadotropins level this dose was chosen for further experiments whereas administration of 2 mg of CPA inhibited the serum LH levels when compared to pretreatment values (Fig. 2). Figure 3 shows the results of LH and FSH in serum when NET or its A-ring

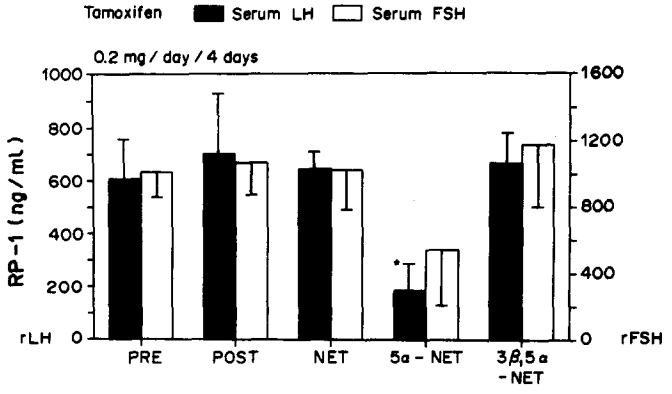

Fig. 1. Effect of NET, 5α-NET and 3β,5α-NET upon serum gonadotropins in tamoxifen primed female castrated rats. *$P < 0.001$ when 5α-NET was compared with control post tamoxifen group. The results are expressed as mean ± SD.

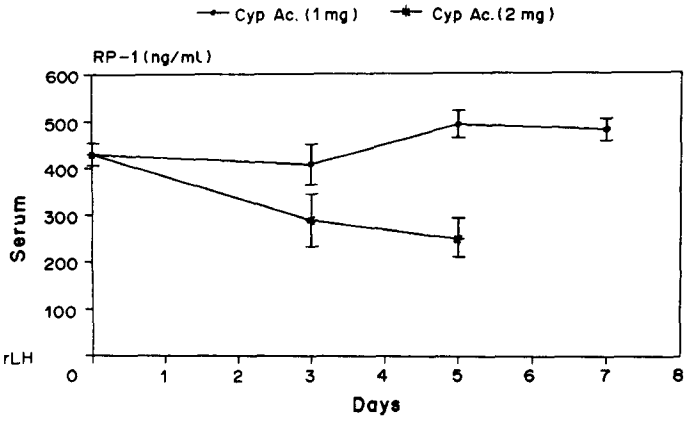

Fig. 2. Effect of cyproterone acetate upon serum LH in castrated female rats. Results are mean ± SD.

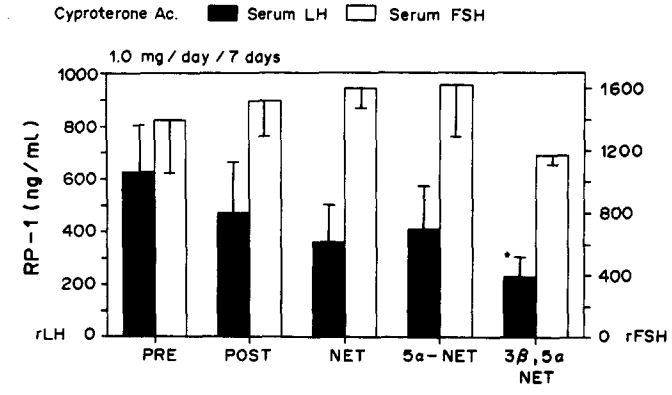

Fig. 3. Serum gonadotropin inhibition of NET, 5α-NET and 3β,5α-NET in cyproterone acetate primed animals. *$P < 0.001$ when compared with control post cyproterone acetate group.

reduced derivatives were administered to CPA-treated rats. As depicted, only the 3β,5α-NET was able to decrease significantly ($P < 0.001$) the serum concentration of LH, whereas FSH serum concentrations did not exhibit major changes though there was a slight decrease after 3β,5α-NET administration, thus suggesting the estrogenic potency of 3β,5α-NET. There was a lack of FSH inhibition after NET or 5α-NET.

DISCUSSION

The results obtained in this study confirm and extend previous observations on the mechanism of gonadotropin inhibition of NET and provides further evidence on the biological activity of two of its metabolites, the 5α-NET and the 3β,5α-NET.

Previous studies from this group have suggested that the estrogenic effects of NET are probably due to the formation of non-phenolic A-ring reduced metabolites which can interact with cytosolic receptors other than the progesterone receptors [17–19]. The demonstration that 5α-NET can interact *in vitro* with the androgen intracellular receptors and that preferentially but not exclusively the 3β,5α-NET binds the estrogen receptors [20], coupled with the data obtained on the induction of estrogen-dependent progesterone receptors of the rat anterior pituitary [21], and the enhanced *in vivo* antigonadotropic effect of NET reduced metabolites prompted us to undertake this study in antiestrogen and antiandrogen primed rats. Like the natural androgens, it is assumed that NET undergoes extensive *in vivo* metabolism in the target tissues. The 5α-reduction, NET could be further metabolized to the tetrahydroderivatives such as 3α,5α-NET and 3β,5α-NET by the 3α-hydroxysteroid dehydrogenase or 3β-hydroxysteroid dehydrogenase [26]. While a large extent of 3α-diols could be oxidized by a reversible reaction of the 3α-hydroxysteroid dehydrogenase, the formation of 3β-diols led to the rapid formation of more polar steroids indicating that further reduction of 3β-diols occur rather than oxidation [27]. These observations are important for interpreting our data since interconversion between metabolites seems to occur it is unlikely that 3β,5α-NET could be oxidized to 5α-NET. However, in the case of 5α-NET it is possible that the effects observed in the present study are not only those of 5α-NET but also the result of further reduction.

The finding that Tam pretreatment impaired the gonadotropin inhibition of NET and 3β,5α-NET suggests that its effect is mediated via the estrogen receptor. The antigonadotropic effect of 5α-NET was maintained in the presence of Tam thus indicating that 5α-NET asserts its action through interaction with a different cytosolic receptor. This suggestion is supported by the previous results indicating high affinity of 5α-NET derivative for the intracellular androgen receptors [20]. In order to further confirm the androgen-like effect of 5α-NET, CPA pretreated animals were treated with NET or its metabolites. The observations from these experiments indicated that under antiandrogen treatment, 3β,5α-NET maintained its antigonadotropic activity whereas the inhibitory effects of 5α-NET was significantly reduced.

The overall data suggests that the antigonadotropic effects of NET are the result of its A-ring reduction, leading to the formation of 5α-NET and 3β,5α-NET which exhibit androgenic and estrogenic actions respectively at the neuroendocrine level. The significance of this results could explain the wide spectrum of hormonal effects observed after the *in vivo* administration of NET and can be useful in the design of more potent and specific steroidal contraceptives.

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