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THE METABOLISM OF 19-NOR CONTRACEPTIVE PROGESTINS MODULATES THEIR BIOLOGICAL ACTIVITY AT THE NEUROENDOCRINE LEVEL

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Summary—In this communication, a series of studies from our laboratory dealing with the mechanism of action of 17α-ethinyl derivatives of 19-nor testosterone are reviewed. The administration of norethisterone (NET) to long-term castrated female rats induces the nuclear translocation of pituitary estradiol receptors and is followed by some estrogenic-like effects at the hypothalamic–pituitary unit. It is established that an A-ring reduced metabolite of NET, the 3β,5α-tetrahydro NET derivative, is responsible for the observed *in vivo* estrogenic effects of the parent compound. 3β,5α-NET binds to the estrogen receptor and is efficient in inducing the pituitary estrogen-dependent progesterone receptor and in increasing the uterine weight in long-term castrated rats. Furthermore, administration of 3β,5α-NET and the 5α-reduced metabolite of NET (5α-NET) are able to inhibit the release of gonadotropins in the castrated animal to a greater extent than NET. Moreover, pretreatment with tamoxifen, an estrogen binding site competitor, results in a significant diminution of the antigonadotropic potency of 3β,5α-NET but not of the 5α-NET, which is only inhibited by the administration of cyproterone acetate. These findings underline the importance of the metabolic rate of NET for the expression of its biological effects at the hypothalamic–pituitary unit.

INTRODUCTION

Synthetic 19-nor testosterone derivatives have been widely used as contraceptive agents in a number of pharmaceutical formulations. Although it has been documented that the main mode of contraceptive activity of these progestins is exerted through the abolishment of the mid-cycle surge of gonadotropins which results in an impairment of ovulation [1], very little is known in terms of their mechanism of action at the cellular level, particularly in the neuroendocrine structures.

The interesting observation that norethisterone (17α-ethinyl-17β-hydroxy-4-estren-3-one) (NET) but not natural progesterone significantly suppresses serum LH levels in non-estrogen sensitized castrated female rats [2, 3] and postmenopausal women [4, 5], suggested that this synthetic progestin could be recognized by intracellular steroid binding sites different from the estrogen-dependent progesterone receptors. Further support for this suggestion is furnished by early reports [4, 6–8] which indicated that NET displays a variety of hormonal effects following its administration to several mammalian species. These findings coupled with the observation that 19-nor progestins undergoes further *in vivo* metabolism, particularly enzymatic reduction of its A-ring [9–11], prompted us to elucidate the mechanism by which these steroids exert their effects at the neuroendocrine level, and especially in their ability to inhibit gonadotropins in the long-term castrated rats.

The present report summarizes the results of a variety of studies from our laboratory aimed at assessing the modulating effect that structural modifications of NET molecule might have on its intracellular interactions with specific receptor binding sites and therefore on the expression of its biological activity.

NUCLEAR TRANSLOCATION OF PITUITARY CYTOSOL RECEPTORS BY THE *IN VIVO* ADMINISTRATION OF NORETHISTERONE

To determine the steroid specificity of nuclear receptor binding sites accumulated in the anterior pituitary following the *in vivo* administration of NET, a series of experiments were performed in female castrated rats. The determination of nuclear receptor–steroid complexes was performed by the nuclear exchange assay described by Anderson *et al.*[12] with minor modifications. Briefly: after NET administration (at various doses), animals were sacrificed by decapitation and anterior pituitaries and uterus were immediately excised and placed in cold TESH buffer (0.01 M Tris, pH 7.4, 0.0015 M EDTA, and 0.001 M dithiothreitol). Tissues were homogenized in TESH buffer and nuclear fractions obtained. Washed nuclei were suspended and aliquots were dispensed in assay tubes containing various concentrations of [^{3}H]labeled NET, 17β-estradiol (E2), promegestone (R-5020) and methyl-

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trienolone (R-1881). Incubations were then performed for various periods of time followed by centrifugation at 800 *g* for 10 min. [^{3}H]Steroids were extracted from washed nuclear pellets and the radioactive content determined. Non-specific binding was established by the addition of a 100 M excess of the corresponding unlabeled steroid. The cytoplasmic content of E2 receptors was determined by the exchange assay described by Katzenellenbogen *et al.*[13].

The results demonstrated that only [^{3}H]E2, among all the radioligands employed, was able to specifically exchange with nuclear pituitary receptors. Since the rate of exchangeable [^{3}H]E2 was linearly related from 2 to 100% of the original sample ($r = 0.999$), it was established that the receptor content was strictly proportional to the amount of nuclear extract. Results from dose–response exchange experiments indicated that maximal response in terms of nuclear E2 receptor accumulation was elicited at 1 mg dose of NET. Accordingly, this dose was used thereafter in all subsequent experiments. Time course exchange studies revealed that a significant depletion of pituitary cytosol receptors occurs between 2 and 4 h after NET administration, an event that temporally coincides with an important increase in the nuclear E2-receptor content (Fig. 1). Interestingly, the steroid specificity and the equilibrium parameters of reaction of these binding sites closely resemble those of the classical estradiol receptor [14]. Indeed, the addition of an excess of diethylstilbestrol (DES) and E2 inhibited the binding of [^{3}H]E2 to pituitary nuclear extracts, whereas NET was a weak *in vitro* competitor. As it was expected, R-5020 and R-1881 and medroxyprogesterone acetate had very little, if any, effect upon the binding of [^{3}H]E2 to its nuclear receptor (Fig. 2). Saturation analysis of receptor binding sites disclosed that the pituitary nuclear receptors accumulated following NET administration exhibits a high affinity with an apparent K_d and a number of binding sites (expressed per mg of DNA) within the range reported by others for the nuclear E2 receptors [12, 15, 16].

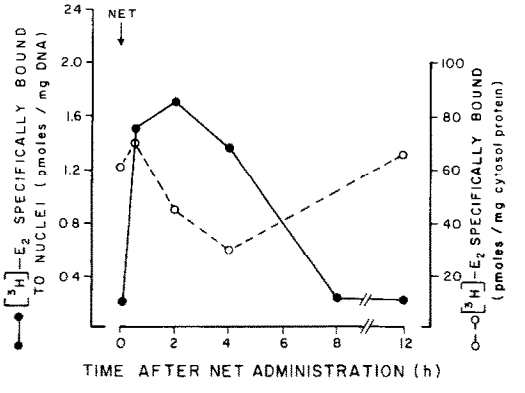

Fig. 1. Time course of depletion of cytoplasmic pituitary estrogen receptors. After NET treatment the pituitary glands of long-term castrated female rats were obtained at different time intervals and immediately assayed for nuclear (●) and cytosol (○) estradiol receptors. Each point represents the mean of 3 determinations. Larrea *et al.*[2].

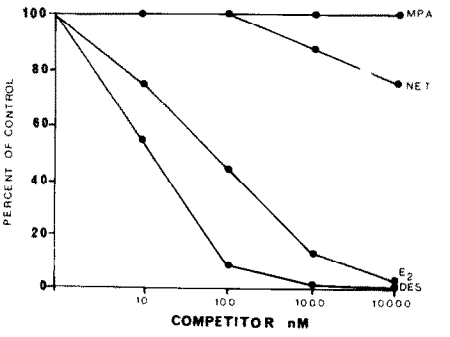

Fig. 2. Competition curves of non-labeled natural and synthetic compound for nuclear pituitary receptors. After 4 h of NET administration to long-term castrated female rats, pituitary nuclei were obtained and labeled *in vitro* with [^{3}H]E2 in the presence or absence of increasing concentrations of non-labeled competitors. Results are expressed as the percentage of [^{3}H]E2 specific binding after incubation. Each point represents the mean of 3 experiments.

ESTROGEN-LIKE EFFECTS OF NORETHISTERONE ON THE HYPOTHALAMIC-PITUITARY UNIT

To ascertain whether the NET-induced nuclear translocation of E2 cytosol receptors in the ovariectomized female rat is mediating the expression of some of its neuroendocrine actions, we considered it was of interest to study the effects of this synthetic progestin upon a variety of events taking place at the hypothalamic–pituitary unit. Plasma and pituitary LH was measured by double antibody radioimmunoassay using material and protocols kindly supplied by the National and Pituitary Program (Baltimore, MD). The pituitary LH content was determined in 1 : 1000 and 1 : 2000 dilution of aliquots from a pituitary homogenate.

Administration of a single dose (1 mg) of NET to long-term castrated animals resulted in a significant inhibition of the already elevated levels of immunoreactive LH. Time course experiments revealed that maximum LH suppression was noticed 4 h after the s.c. injection of NET, an effect which occurred concomitantly with the maximum nuclear steroid receptor retention by the anterior pituitary gland. Evidence that NET-induced LH suppression was not mediated via progesterone receptors was provided by the observation that administration of natural progesterone to castrated female rats did not modify the circulating levels of LH. Furthermore, neither the hypothalami nor the pituitaries of the animals employed in this study contained cytosol progesterone receptors, as was demonstrated by the lack of [^{3}H]R-5020 specific binding. The results at this point strongly suggested that NET was acting at the central nervous system as an estrogen rather than as a progestin at least in our

experimental model. Further studies conducted in tamoxifen-primed castrated animals showed that administration of this non-steroid antiestrogen prevented the antigonadotropic activity of NET (Fig. 3); thus indicating that this progestin was exerting its effects upon the gonadotropin dynamics through an estrogen-like mechanism of action.

To further explore the effects of NET on pituitary gonadotropins, we decide to analyze the pituitary content of LH, the hypothalamic content of GnRH, and the number of GnRH pituitary binding sites in castrated animals treated with either NET, E2 or progesterone. Additional impetus for the conduct of this study was provided by the proposal that GnRH-secreting neurons are the main target cells for the feedback actions of sex steroid hormone [17]. Endogenous GnRH was measured by a specific radioimmunoassay using GnRH antisera raised in male rhesus monkeys as previously described [18]. The assay of GnRH pituitary receptors was done according to the method described by Clayton *et al.*[19] using a lactoperoxidase-iodinated GnRH agonist analog [D-Ser(tBU)^6]des-Gly^{10}-GnRH-N-ethylamide (GnRH-A) as the radioligand. The results obtained confirmed and extended previous observations which indicate that ovariectomy is followed by an increase on pituitary LH content with a concomitant diminution of GnRH in the hypothalamus. Administration of NET and E2 but not progesterone resulted in a marked diminution of the pituitary LH content with the simultaneous increase of hypothalamic GnRH reaching values similar to those of intact rats. The effects of NET upon pituitary gonadotropin responsiveness was then studied by the exogenous GnRH administration to ovariectomized animals treated with either NET or vehicle alone. The results demonstrated that NET administration 4 h prior to GnRH stimulus induced a significant inhibition of the pituitary LH response. This finding, together with the observation that steroid-mediated gonadotropin control might be also exerted at the gonadotrop level by modifying its response to endogenous GnRH stimulus [20], prompted us to examine the effect of NET upon pituitary GnRH receptors of long-term castrated rats. The study included animals treated with E2 and progesterone for comparative purposes. Binding data analysis revealed that pituitary from animals treated with NET and E2 but not with progesterone had a lower concentration of GnRH receptors (66 fmol/gland) as compared with those of non-steroid treated castrated rats (92 fmol/gland). This finding fits well with the observation of the NET-induced diminution of pituitary gonadotropin responsiveness and strongly suggested that the antigonadotropic activity of NET in the castrated animal was due in part to an alteration in the number of GnRH pituitary binding sites exerted through an estrogenic mechanism of action.

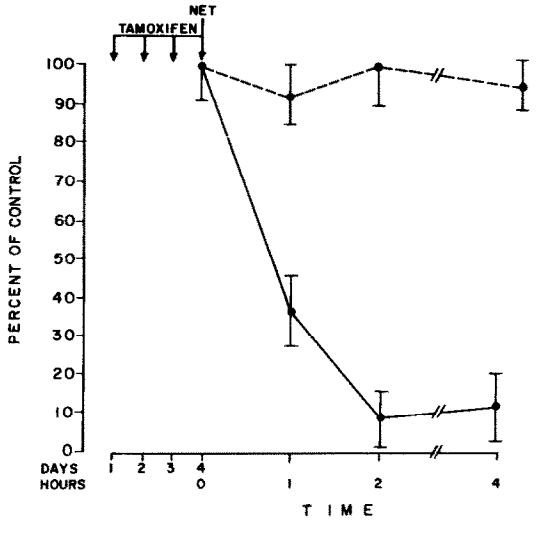

Fig. 3. Effects of tamoxifen pretreatment on the antigonadotropic activity of NET. A group of 12 rats were ovariectomized: 6 rats received 200 μg of the estrogen binding site competitor every day for 4 consecutive days (dotted line), the other 6 rats received 0.9% NaCl. All rats were so injected starting at the day 4 with 1 mg of NET. LH was determined by specific radioimmunoassay in serum samples drawn at different periods of time. Each point represents the mean ±SD of 6 rats per triplicate. Larrea *et al.*[3].

THE METABOLIC FATE OF NORETHISTERONE

Most of the results obtained from the above described studies clearly indicated that the neuroendocrine effects of NET, in the castrated female rat, were mediated via E2 receptors. However, several *in vitro* studies from this [21] and other [22–24] laboratories have failed to demonstrate the specific interaction of NET with E2 receptors in estrogen-sensitive tissues. These paradoxical findings are relevant particularly since the *in vivo* aromatization of this progestin has remained as a controversial and unsolved issue [25, 26]. In this regard, recent evidence indicated that NET may undergo aromatization *in vitro* in the presence of a purified preparation of human placenta [27]. However, the *in vivo* formation of ethinyl-E2 from NET in the non-pregnant model still remains unclear.

Since several A-ring reduced derivatives of NET have been identified in women using this progestin as a contraceptive agent, we proposed as a working hypothesis that some of the hormone-like effects of NET might be mediated by its enzyme-formed neutral non-aromatizable metabolites. A-ring reduced NET metabolites were synthesized through the kindness of Dr G. A. García (School of Chemistry, National University of Mexico). Authentic NET was provided by Shering Mexicana, S. A. 5α-Dihydro NET (5α-NET) was synthesized by lithium–ammonia reduction of NET according to the procedure described by Bowers *et al.*[28]. The 3β,5α- and 3α,5α-tetrahydro NET derivatives (3β,5α-NET

and 3α,5α-NET) were prepared from 5α-NET by sodium borohydride reduction [28]. Separation of the epimeric alcohols was done by flash chromatography [29] using the system ethyl acetate–hexane (3 : 7, v/v). Chemical purity of NET and its derivatives was assessed by their melting points, GLC and HPLC behaviour, and by HNMR spectrometric analysis. Authentic 3α,5β-NET was generously supplied by G. D. Searle Co. (Chicago, IL).

To determine if NET was biotransformed to various A-ring reduced derivatives at the target organ level, a series of experiments were carried out by incubating *in vitro* [^{3}H]NET with minced preparations and/or homogenates of rat pituitary, hypothalamus and ventral prostate. Radiochemical pure 5α-NET, 3α,5α-NET and 3β,5α-NET were isolated as metabolic conversion products, thus indicating that these tissues have the capability to modify the molecular structure of NET (data to be published elsewhere).

STEREOSPECIFICITY OF THE INTRACELLULAR BINDING OF NORETHISTERONE AND ITS REDUCED METABOLITES TO PUTATIVE STEROID RECEPTORS

To assess the specific *in vitro* intracellular interactions of NET and its four non-phenolic derivatives to progesterone (PR), androgen (AR), and estrogen (E2R) receptors, displacement analysis using [^{3}H]labeled ORG-2058, R-1881, and E2 as the radioligands were performed. The results obtained demonstrated that the competitive potency of NET and its derivatives for cytosol receptor binding sites was different not only for each hormone specific receptor but also for each particular compound. The most efficient competitor for PR was NET ($K_i = 1.1 \times 10^{-7}$ M) followed by 5α-NET, whereas the three tetrahydro-NET derivatives were completely ineffective. The most efficient competitor for AR binding sites was 5α-NET ($K_i = 1.0 \times 10^{-8}$ M) immediately followed by NET, while the tetrahydro derivatives were not competitors at all. Finally, the most striking finding was that competition for the E2R binding sites was only exhibited by 3β,5α-NET ($K_i = 4.6 \times 10^{-8}$ M) and to a lesser extent by its 3α,5α-epimeric alcohol (Fig. 4).

These data indicated that structural modifications of the NET molecule modulate its intracellular type of specific binding to putative steroid receptors. The observation that a NET derivative interacts with a given cytosol receptor does not necessarily reflect the full expression of the corresponding hormonal activity. This is of particular importance since it has been well recognized that formation of a hormone receptor complex might result in either agonistic, antagonistic or synergistic biological effects. Nevertheless, the overall data offered at least an alternate explanation for the mode of NET action.

ESTROGEN EFFECTS OF NON-AROMATIZABLE METABOLITES OF NORETHISTERONE AT THE NEUROENDOCRINE LEVEL

To assess whether the interaction of A-ring reduced derivatives of NET with E2 receptors can effectively initiate estrogen-dependent cellular responses, we conducted a series of experiments to investigate the effects of 3β,5α-NET and 3α,5α-NET on the induction of estrogen-dependent progesterone receptors of the rat anterior pituitary. The induction of pituitary PR was studied following the s.c. administration of NET derivatives for 6 consecutive days. Animals treated with estradiol benzoate (E2B) or with vehicle alone were used as the experimental controls. Pituitary PR were labeled *in*

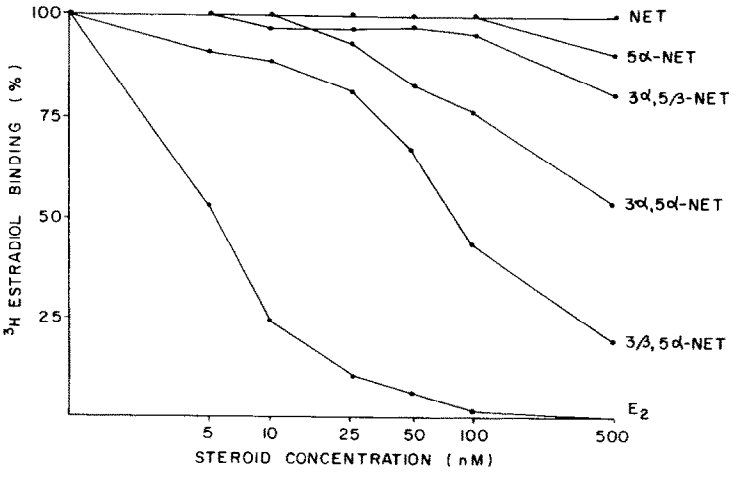

Fig. 4. Displacement curves of radioinert natural and synthetic steroids with uterine cytosol estrogen receptors (ER). Cytosol prepared from an uterine homogenate from immature rats was labeled with [^{3}H]E2 at 4°C in the absence or presence of increasing concentrations of the corresponding unlabeled steroids. The results are expressed as the percentage of [^{3}H]E2 specific binding after incubation. Each point represents the mean of 3 determinations. Chavez *et al.*[21].

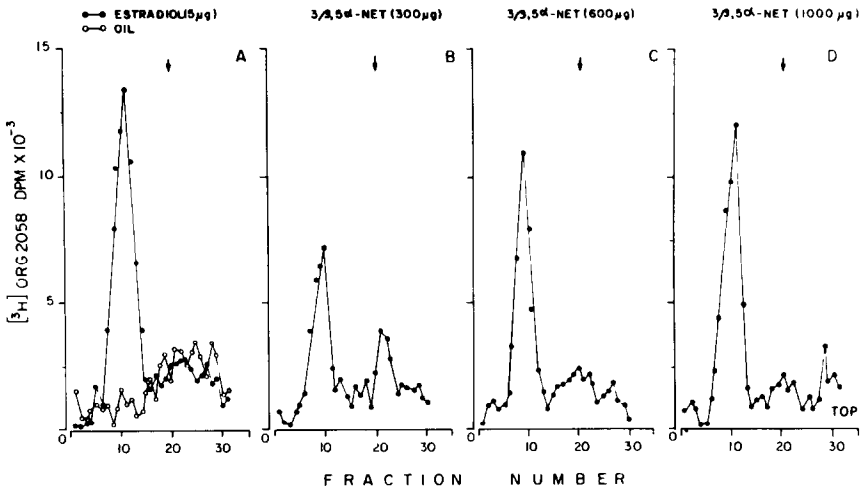

Fig. 5. Dose–response sucrose gradient centrifugation patterns of progesterone receptors (PR) in rat anterior pituitary induced by the systematic administration of 3β,5α-NET. 3β,5α-NET was daily injected to long-term castrated female rats at the dose of 300 μg (B); 600 μg (C) and 1000 μg (D) for 6 consecutive days. Control rats (A) received either oil (○) or E2B (5 μg/day 6 days) (●). Pituitary cytosols were obtained and immediately layered on the top of 5–20% linear sucrose gradients. After centrifugation at 300,000 **g** for 18 h at 2°C, aliquots from the gradient were incubated with 1.8 nM of [^{3}H]ORG-2058 for 4 h at 4°C. The arrow indicates the sedimentation pattern of BSA used as an internal marker. Vilchis *et al.*[30].

vitro after sucrose gradient centrifugation using [^{3}H]-ORG-2058 as the radioligand [30]. The PR binding specificity was determined by the use of an excess of radioinert steroids. The results of these studies demonstrated that administration of 3β,5α-NET induced specific 8–9 S pituitary cytosol progesterone receptors ($K_d = 1.0 \times 10^{-9}$ M) in a dose-dependent manner (Fig. 5). Similar results were observed after the administration of 3α,5α-NET although to a lesser extent. Binding parameters and specificity of the NET derivatives-induced PR appear indistinguishable from those induced by the administration of E2B ($K_d = 0.5 \times 10^{-9}$ M). These findings demonstrated the induction of a typical estrogen-mediated biochemical event, which was achieved by the administration of two A-ring reduced derivatives of NET. In addition to these results, both NET metabolites induced a significant increase on the uterine weight at the expense of both endometrium and myometrium. These data are in line with recent studies of Thieulant *et al.*[31], who have shown the nuclear translocation of E2R in the rat anterior pituitary by 3β,5α-androstanediol, as well as the induction of PR following the administration of this A-ring reduced metabolite of testosterone. Similar effects of 3β,5α-androstanediol in calf and rat uterus have been reported by Garcia and Rochefort [32].

ANTIGONADOTROPIC POTENCY OF A-RING REDUCED METABOLITES OF NORETHISTERONE

To examine if A-ring reduction of NET could modify its antigonadotropic potency, comparative studies using NET; 5α-NET, 3β,5α-NET; and 3α,5α-NET in castrated adult rats were recently undertaken [33]. Assessment of the antigonadotropic activity of these compounds was done by measuring the serum and pituitary content of LH and FSH following their chronic s.c. administration to animals depleted of progesterone receptors. The results demonstrated that 5α-NET and 3β,5α-NET exhibited a significantly greater gonadotropic inhibiting activity as compared with that of their parent compound (Fig. 6). The pretreatment with tamoxifen resulted in a significant diminution of the antigonadotropic potency of 3β,5α-NET, confirming that the biological activity of this derivative of NET was mediated via estrogen receptors. Interesting, however, was the finding that tamoxifen was unable to suppress the LH inhibitor activity of 5α-NET, strongly suggesting that the dihydro-NET derivative might exert its effect through androgen receptors. The pituitary content of LH following the chronic administration of 5α-NET at all doses employed remained unchanged, indicating that its LH inhibitory effect was exerted upon the pituitary release rather than on hypothalamic-regulated LH pituitary synthesis, an observation on line with the suggestion of Martini [34] that the effect of 5α-NET could be exerted on the anterior pituitary gland. The overall results underline the importance of the metabolic fate of NET for the expression of its ability to suppress or inhibit the synthesis and/or release of pituitary gonadotropins.

CONCLUDING REMARKS

To obtain an insight into the mechanisms of action of synthetic 19-nor progestins at the neuroendocrine

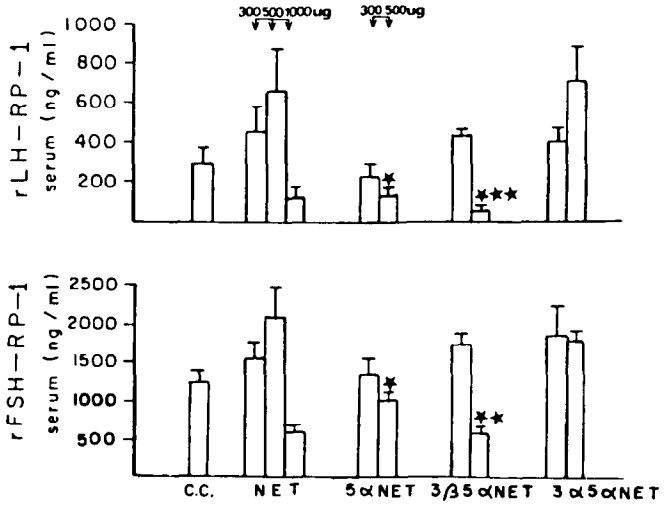

Fig. 6. Serum FSH and LH levels in chronically castrated rats (CC) treated with NET or its A-ring reduced metabolites. Serum gonadotropins were measured in serum aliquots by specific radioimmunoassays. Results were expressed as the mean ±SEM of 7 animals. $*P<0.05$; $**P<0.01$; $***P<0.005$ vs NET (500 μg) group. Garza-Flores *et al.*[33].

level, a number of studies were conducted in our laboratory over the last few years. Norethisterone, a 17α-ethinyl derivative of 19-nor testosterone was used as the model molecule not only because of its wide use but also because it represents the active compound of other 19-nor contraceptive progestins such as norethinodrel, lynestrenol and ethynodiol diacetate. The substantive outcome of these studies included the finding that enzyme-mediated hydrogenation of the double bound, by formation of the 5α-NET derivative and its further 3α or 3β reduction, determined the specific interaction of the resulting metabolites with putative progesterone, androgen or estrogen receptors and therefore their expected effects in the hypothalamic–pituitary unit. The overall data underline the important role that the metabolic fate of this synthetic progestin plays on the expression of its mode of action at the neuroendocrine level.

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