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STEREOSPECIFICITY OF THE INTRACELLULAR BINDING OF NORETHISTERONE AND ITS A-RING REDUCED METABOLITES[1]

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Summary—The interaction of norethisterone (NET) and four A-ring reduced metabolites of NET with cytosol receptors for progesterone (PR), androgen (AR), and estrogen (ER) was investigated. Cytosol preparations from: (a) uteri of adult estrogen-primed castrated rats, (b) ventral prostates of adult castrated rats and (c) uteri of immature rats were used as the source of PR, AR, and ER respectively. ^{3}H-Labeled ORG-2058, R-1881, and 17β-estradiol were used as the radioligands. The results of competitive studies disclosed that: (a) the most efficient competitor for PR binding sites was NET ($K_i = 1.1 \times 10^{-7}$ M) followed by 5α-dihydro NET (5α-NET), whereas the 3α,5α; 3β,5α and 3α,5β-tetrahydro NET derivatives were ineffective (b) the most efficient competitor for AR binding sites was 5α-NET ($K_i = 1 \times 10^{-8}$), immediately followed by NET, while the three tetrahydro NET derivatives were not competitors and (c) remarkable competition for ER binding sites was only exhibited by the 3β,5α-tetrahydro NET derivative ($K_i = 4.6 \times 10^{-8}$ M) and to a lesser extent by its 3α,5α-epimeric alcohol, while NET and 5α-NET were completely ineffective. These findings demonstrate the stereospecificity of the intracellular binding of NET and its reduced metabolites with cytosol steroid putative receptors, and provide biochemical support to the understanding of the variety of hormone-like effects observed after the *in vivo* administration of NET.

INTRODUCTION

It has been well established that norethisterone (17α-ethinyl-17β-hydroxy-4-estren-3-one) [NET][1], a widely used synthetic contraceptive progestin, displays a variety of hormonal effects when administered to several mammalian species [1–9]. Indeed, the estrogenic and androgenic potency of NET was early noticed in bioassays [10–13] as well as from data of clinical studies [14–16]. The interaction of NET with progesterone receptors and its resulting progestational activity has been also well documented [17–20].

More recently, we have reported [9, 21] that NET significantly suppresses serum LH levels in castrated patients with complete testicular feminization syndrome (androgen resistance). Since these patients do not have androgen receptors and are apparently deficient in progesterone receptors, this finding indicates that LH suppression by NET was due to the interaction of NET or one of its metabolites with hypothalamic-pituitary estrogen receptors. Furthermore, studies from this laboratory have demonstrated the nuclear translocation of pituitary cytosol estrogen receptors following the *in vivo* administration of NET [22] and also the abolishment of the antigonadotropic activity of this progestin in castrated rats by the simultaneous administration of a non-steroidal antiestrogen [23], thus indicating the estrogen-like effects of NET.

These findings, coupled with the observation that NET undergoes further *in vivo* metabolism [24–26], particularly enzymatic reduction at its A-ring, prompted us to study the interaction between NET and its non-phenolic metabolites with cytosol steroid receptors. The data presented herein indicate that structural modifications of the NET molecule, modulate its intracellular type of specific binding, suggesting an alternative explanation for its mode of action.

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[2]The following trivial names and abbreviations were used as follows: ORG-2058, 16α-ethyl-21-hydroxy-19-nor-4-pregnen-3,20-dione; R-5020 (Promegestone), 17α,21-dimethyl-19-nor-4,9-pregnadien-3,20-dione; R-1881 (methyltrienolone), 17α-methyl-17β-hydroxy-4,9,11-estratrien-3-one; NET, 17α-ethynyl-17β-hydroxy-4-estren-3-one; 3α,5α-NET, 17α-ethynyl-5α-estran-3α,17β-diol; 3α,5β-NET, 17α-ethynyl-5β-estran-3α,17β-diol; 3β,5α-NET, 17α-ethynyl-5α-estran-3β,17β-diol; 5α-NET, 17α-ethynyl-17β-hydroxy-5α-estran-3-one.

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EXPERIMENTAL

NET and its A-ring reduced derivatives

Authentic NET was kindly provided by Schering Mexicana S.A. 5α-NET was synthesized by lithium–ammonia reduction of NET, according to the procedure described by Bowers *et al.* [27]. The 3α,5α and 3β,5α, tetrahydro NET derivatives were prepared from 5α-NET by sodium borohydride reduction [27]. The epimeric alcohols were separated by flash chromatography [28] using the system ethylacetate–hexane (3:7, v/v). Authentic samples of the 3α,5β, tetrahydro NET derivative were generously supplied by G. D. Searle Co. (Chicago, IL). 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.

Radioactive material and chemicals

[17α-methyl-^{3}H]Methyltrienolone [R-1881] (87 Ci/mmol), [17α-methyl-^{3}H] promegestone [R-5020] (85 Ci/mmol), and [2,4,6,7-^{3}H] estradiol (107 Ci/mmol) were purchased from New England Nuclear, Co. (Boston, MA). [6,7-^{3}H] ORG-2058 (48 Ci/mmol) was obtained from Amersham International (England). Radiochemical purity of [^{3}H]steroids was greater than 98% as assessed by a reverse isotope dilution technique including paper chromatography [29] and recrystallizations of aliquots to constant specific activity. Non-radioactive steroids were purchased from Steraloids Inc. (Pauling, NY), New England Nuclear, Co., and Sigma (St Louis, MO), and their chemical purity was established prior to use.

Animals and tissues

Male and female Wistar rats used throughout the study were kept under a 14 h-light, 10 h-dark cycle and maintained on food and water *ad libitum*. Gonadectomies when required were done under light ether anesthesia. In all cases, animals were killed by decapitation. Tissues were immediately removed, blotted and weighed. Thereafter all procedures were done at 4°C.

Cytosol preparations for steroid receptor studies

Progesterone receptors. Estradiol benzoate 10 μg daily for 4 days was administered subcutaneously to female adult rats (250–300 g) starting 2 weeks after bilateral ovariectomy. Uteri were homogenized in a ratio (w/v) 1:6 in TEDAM buffer (20 mM Tris–HCl, pH 7.4 at 4°C, 1.5 mM EDTA, 0.25 mM dithiothreitol, 5000 UK/ml aprotinin, and 10 mM sodium molybdate) containing 10% glycerol (v/v) with three 10 s bursts of a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 105,000 *g* for 1 h at 2°C in a SW 50.1 rotor (Beckman Instruments, Palo Alto, CA).

Androgen receptors

Ventral prostates were obtained from adult male rats (250–300 g) castrated 48 h prior experiments. Tissue homogenization and cytosol preparation were done as described above using TEDAM buffer without glycerol.

Estrogen receptors

Immature female intact rats weighing 100–120 g without estrogen priming were used for these studies. Uterine cytosol was then prepared as described above. Glycerol was omitted from the TEDAM homogenization buffer. Cytosol protein content was determined by the Bradford's dye binding method [30] using BSA as standard.

Binding assays

Cytosol receptors analysis. Equilibrium parameters of reaction between radioligands and cytosol limited-capacity binding sites were studied by incubations of the different cytosol preparations with specific ^{3}H-ligands in the presence of increasing concentrations of the non-radioactive corresponding steroids for 14 h at 4°C. Bound and free steroids were separated by the addition of Dextran-coated charcoal suspension (250 mg Norit-A and 25 mg Dextran T-70) in 100 ml of TEDM buffer (20 mM Tris–HCl, pH 7.4 at 4°C, 1.5 mM EDTA, 0.25 mM dithiothreitol, and 10 mM sodium molibdate), and incubated for either 2 min (PR), 10 min (AR) or 5 min (ER) at 4°C with continuous shaking. Following centrifugation at 800 *g* at 4°C for 15 min, aliquots of the supernatants were assayed for radioactivity. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer model 2660, using Instagel (Packard, Downers Grove, IL) as counting solution. Quenching was corrected in all samples by external standardization. Non-specific binding was determined by the addition of an excess (100-fold) of the corresponding radioinert steroid. Specific binding was assessed by subtracting non-specific binding from total binding. Results were analyzed by the method of Scatchard [31].

Competition of NET and its metabolites for steroid receptors

The relative binding affinities (RBA) of NET, 5α-NET, 3α,5α-NET, 3β,5α-NET and 3α,5β-NET to cytosol steroid receptors were evaluated by their capability to displace bound ^{3}H-specific ligand from the corresponding cytosol receptors. The RBA and inhibition constant (K_i) of each compound were calculated according to the procedures described by Reel *et al.* [18] and Cheng and Prusoff [32] respectively. In addition, the RBA's and K_i's of natural occurring steroids were also determined.

RESULTS

Saturation curves and Scatchard plots of [^{3}H]ORG-2058, [^{3}H]R-1881, and 17β-[^{3}H]estradiol binding to cytosol preparations from estrogen-

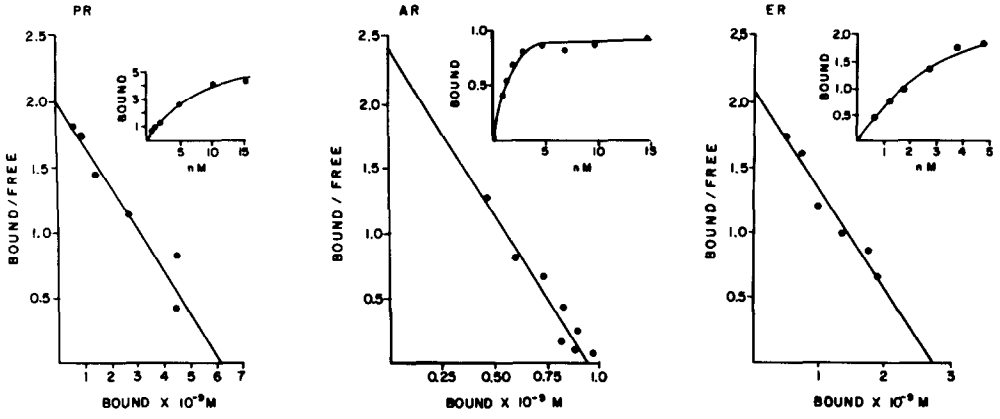

Fig. 1. Representative Scatchard plots of the *in vitro* labeling of cytosol progesterone (P), androgen (A), and estrogen (E) receptors (R). (PR) = estradiol benzoate-primed rat uterine cytosol (7.2 mg protein/ml) was incubated with [^{3}H]ORG-2058 in the presence of increasing concentrations of non-labeled ORG-2058 (ranging from 1–15 nM); apparent $K_d = 3.0 \times 10^{-9}$ M. (AR) = rat ventral prostate cytosol (6.2 mg protein/ml was incubated with [^{3}H] R-1881 in the presence of increasing concentrations of non-radioactive R-1881 (ranging from 1–15 nM); apparent $K_d = 3.0 \times 10^{-10}$ M (ER) = immature rat uterine cytosol (5.9 mg protein/ml) was incubated with 17β-[^{3}H] estradiol in the presence of increasing concentrations of non-radioactive 17β-estradiol (ranging from 1–5 nM); apparent $K_d = 1.1 \times 10^{-9}$ M. Saturation curves are also shown in the insets. Cytosol preparation and incubation conditions are detailed in the text.

treated rat uterus, rat ventral prostate, and non-treated immature rat uterus respectively, are shown in Fig. 1. The equilibrium dissociation constants (K_d) and saturation binding capacities (NBS) were as follows: PR: $K_d = 3.0 \times 10^{-9}$ M, NBS = 6.2 $\times$ 10^{-9} M; AR: $K_d = 3.9 \times 10^{-10}$ M, NBS = 0.9 $\times$ 10^{-9} M and ER: $K_d = 1.1 \times 10^{-9}$ M, NBS = 2.7 $\times$ 10^{-9} M. Similar results for K_d values were obtained when the data were analyzed by the methods of Eadie and Hofstee using the computer program developed by Zivin and Waud [33].

Competition of NET and its metabolites for progesterone receptors

Addition of increasing concentrations of radioinert ORG-2058, progesterone, NET, 5α-NET, 3α,5α-NET, 3β,5α-NET and 3α,5β-NET, induced a varying degree of displacement of [^{3}H]ORG-2058 from the uterine cytosol PR obtained from estrogen-primed castrated rats as depicted in Fig. 2. The most potent steroid competitors for PR were progesterone and NET. 5α-NET also induced a slight though significant displacement effect, whereas the three tetrahydro NET metabolites were ineffective. The RBA and K_i values for each compound are shown in Table 1. An identical competitive profile of NET and its reduced derivatives was found when [^{3}H]R-5020 was used as the radioligand (data not shown).

Competition of NET and its metabolites for androgen receptors

The effects of increasing concentrations of non-radioactive natural and synthetic steroids upon the [^{3}H]R-1881 binding to androgen cytosol receptors from rat ventral prostate are shown in Fig. 3. The order to the affinity for the androgen receptor was: 5α-DHT > R-1881 > 5α-NET > NET. The three tetrahydro NET derivatives did not exhibit any competitive potency at all. The RBA AND K_i values of each steroid are listed in Table 1.

Competition of NET and its metabolites for estrogen receptors

Competition for the 17β-[^{3}H]estradiol-labeled cytosol ER in the immature rat uterus by increasing concentrations of radioinert natural and synthetic steroids is shown in Fig. 4. The only striking competitor for the ER binding site was the 3β,5α-NET

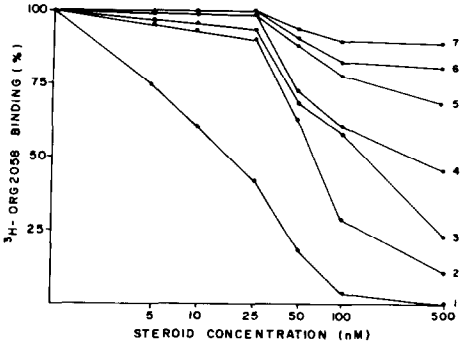

Fig. 2. Competition of non-labeled natural and synthetic steroids for uterine cytosol progesterone binding sites. Progesterone receptors were labeled *in vitro* by overnight incubations of uterine cytosol preparations from estradiol benzoate-primed castrated rats with 0.9 nM [^{3}H]ORG-2058 at 4°C (final incubation volume = 200 μl). Bound and free fractions were separated by the addition of a Dextran-coated charcoal suspension (2 min) as described in the text. Increasing concentrations of the following radioinert steroids were added to the incubations: 1 = ORG-2058, 2 = progesterone; 3 = norethisterone (NET); 4 = 5α-NET; 5 = 3β,5α-NET; 6 = 3α,5α-NET; 7 = 3α,5β-NET. The results are expressed as the percentage of [^{3}H]ORG-2058 specific binding. Each point represents the mean of three experiments in triplicate.

Table 1. Relative binding affinities (RBA) and inhibition constants (K_i) of natural and synthetic steroids for cytosol sex steroid receptors

Radioinert natural and synthetic competitors	Progesterone receptors [^{3}H-ORG-2058]		Androgen receptors [^{3}H-R-1881]		Estrogen receptors [^{3}H-17β-Estradiol]	
	RBA*	K_i† (M)	RBA	K_i (M)	RBA	K_i (M)
Norethisterone	11.2	1.1×10^{-7}	8.1	1.9×10^{-8}	<0.1	—
5α-NET	4.8	2.6×10^{-7}	14.8	1.0×10^{-8}	<0.1	—
3α,5α-NET	<0.1	—	<0.1	—	<0.1	—
3β,5α-NET	<0.1	—	<0.1	—	6.3	4.6×10^{-8}
3α,5β-NET	<0.1	—	<0.1	—	<0.1	—
Progesterone	26	5.0×10^{-8}	nd	nd	nd	nd
ORG-2058	100	1.3×10^{-8}	nd	nd	nd	nd
5α-DHT	nd‡	nd	172	8.6×10^{-10}	nd	nd
R-1881	nd	nd	100	1.5×10^{-9}	nd	nd
17β-Estradiol	nd	nd	nd	—	100	2.9×10^{-9}

*RBA were determined according to Reel *et al.* [18].
†K_i values were calculated according to Cheng and Prusoff [32].
‡Not determined.

metabolite but natural estradiol, as expected was the most potent inhibitor. The 3α,5α-NET isomer also induced a slight competitive effect, while 3α,5β-NET, 5α-NET, and particularly NET were ineffective. The RBA and K_i values for each steroid are shown in Table 1.

DISCUSSION

To assess the specific *in vitro* intracellular interactions of NET and its non-phenolic metabolites, a series of experiments were done to optimize appropriate cytosol steroid receptor assays. The results of the *in vitro* labeling of the progesterone, androgen, and estrogen receptors, disclosed identical values of the equilibrium parameters of reaction (Fig. 1) to those previously described in several target tissues by a number of investigators [34–42]. Therefore, the cytosol steroid receptor sources, radioactive ligands, and assay conditions used in these experiments, were considered to be suitable to examine the affinity of NET and its derivatives for the sex steroid hormones cytosol receptors.

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. Thus, unmodified NET was the most efficient competitor for progesterone specific binding sites, whereas the 5α-reduction of NET, significantly diminished its competitive potency for PR (see RBA and K_i values in Table 1). This finding confirms and extends the observations of Tamaya *et al.* [43] and Reel *et al.* [18] in the rabbit uterus. Further reductions at the A-ring of 5α-NET (3α or 3β) completely abolished its affinity for the PR. A

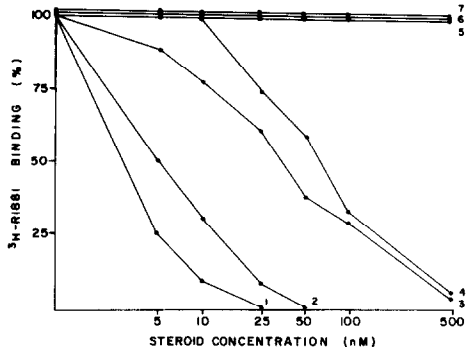

Fig. 3. Displacement effect of radioinert natural and synthetic steroids upon the specific binding of [^{3}H]R-1881 to cytosol androgen receptors. Aliquots of ventral prostate cytosol from castrated adult male rats were incubated (overnight) with 0.9 nM [^{3}H]R-1881 at 4°C (final incubation volume = 200 μl) in the absence or presence of graded concentrations of the following non-labeled steroids: 1 = 5α-dihydrotestosterone; 2 = R-1881; 3 = 5α-NET; 4 = norethisterone (NET); 5 = 3β,5α-NET; 6 = 3α,5α-NET: 7 = 3α,5β-NET. Bound and free fractions were separated by the addition of a Dextran-coated charcoal suspension (10 min). The results are expressed as the percentage of [^{3}R]1881 specific binding as described in the text. Each point represents the mean of three experiments in triplicate.

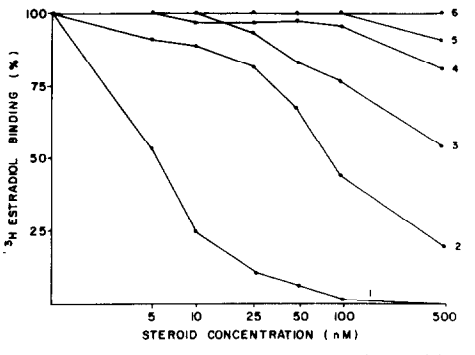

Fig. 4. Interaction of norethisterone and four of its non-phenolic metabolites with uterine cytosol estrogen receptors (ER). ER were labeled *in vitro* by overnight incubations of uterine cytosol from immature rats with 0.8 nM 17β-[^{3}H] estradiol at 4°C (final incubation volume = 200 μl), in the absence or presence of increasing concentrations of the following radioinert natural and synthetic steroids: 1 = 17β-estradiol; 2 = 3β, 5α-NET; 3 = 3α,5α-NET; 4 = 3α,5β-NET; 5 = 5α-NET; 6 = NET. Bound and free fractions were separated by the addition of a Dextran-coated charcoal suspension (5 min). The results are expressed as the percentage of 17β-[^{3}H] estradiol specific binding. Each point represents the mean of three experiments in triplicate.

similar lack of affinity of the 3α,5β-NET derivative for the progesterone binding sites was also noticed.

From these data it appears that the unmodified NET molecule specifically interacts with the PR at the target organs and that its further metabolism either diminishes or abolishes this activity. On the contrary, displacement studies with the labeled cytosol androgen receptor revealed that the structural modification of NET, particularly the 5α-reduction of the A-ring, enhanced its competitive potency. Thus 5α-NET was the most effective competitor for the ventral prostate cytosol androgen receptor ($K_i = 1.0 \times 10^{-8}$ M). NET exhibited also a significant competitive activity although to a lesser extent ($K_i = 1.9 \times 10^{-8}$ M). 5β-Reduction of NET, and 3α or 3β further reduction of 5α-NET resulted in structural impairments that precluded their interaction with the AR (Fig. 3; Table 1). The finding that 5α-A-ring reduction of the NET molecule increases its affinity for the AR is in parallel with the extensively reported observation [44, 45] that 5α-reduction of testosterone enhanced its affinity for cytosol AR. Whether 5α-NET is the active metabolite responsible for the androgenic activity of NET cannot yet be ascertained and deserves further studies.

The results of competitive studies with the estrogen cytosol receptors in the immature rat uterus, indicated that the most efficient competitor was the 3β,5α,tetrahydro derivative of NET ($K_i = 4.6 \times 10^{-8}$ M), followed by its 3α,5α-epimeric alcohol. This striking finding pointed out that non-phenolic A-ring derivatives of NET are able to interact with cytosol ER binding sites. This observation was in contrast with the finding that unmodified NET, 5α-NET, and 3α,5β-NET were unable to compete for the ER (Fig. 4, Table 1).

These data strongly suggest that non-phenolic reduced metabolites of NET (the 3β,5α-NET and/or the 3α,5α-NET) might be responsible for the well documented estrogenic potency of NET following its *in vivo* administration [6, 18, 22, 23], particularly since their *in vivo* formation to a rather large extent has been well documented [24–26].

This alternate mechanism of estrogenic action of NET seems to be relevant, since studies aimed at demonstrating the enzymatic aromatization of NET have yielded controversial results [46–47]. Recently, Barbieri *et al.* [48], have presented evidence of the aromatization *in vitro* of NET by human placental microsomes; however, the degree to which NET is converted *in vivo* to ethynl estradiol in the non-pregnant state still remains unclear. Whether the *in vitro* interactions of NET and its derivatives with intracellular molybdate and/or glycerol-stabilized receptors correlates with their expected biological activities under *in vivo* circumstances, will require further assessment.

The overall data demonstrate that structural modifications on the A-ring of norethisterone, modulate and direct its specific binding to cytosol putative steroid receptors, pointing out the importance of the *in vivo* metabolism of this synthetic contraceptive progestin. The data also provide information for the understanding of its hormone-like effects.

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