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ROLE OF 17β-HYDROXYSTEROID DEHYDROGENASE IN THE MODULATION OF NUCLEAR ESTRADIOL RECEPTOR BINDING BY PROGESTERONE IN THE RAT ANTERIOR PITUITARY GLAND AND THE UTERUS

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Summary—Progesterone has been shown to decrease occupied pituitary and uterine nuclear estradiol receptor (E_2R) binding in mature and immature estrogen-primed rats. Progesterone has also been shown to stimulate pituitary but not uterine 17β-hydroxysteroid dehydrogenase (17β-HSD) in the rat. The conversion of estradiol to its less active metabolite estrone by 17β-HSD and activation of phosphatase are among mechanisms considered to be involved in the reduction of E_2R. To determine if 17β-HSD stimulation was a mechanism by which progesterone induced nuclear E_2R decrease, the synthetic estrogen ethinylestradiol, which is not oxidized by 17β-HSD, was used instead of estradiol to prime adult ovariectomized rats. When ethinylestradiol-primed rats received 0.8, 2.0 or 4.0 mg/kg body wt of progesterone 2 h before sacrifice, the total and occupied nuclear E_2R accumulation in the anterior pituitary by a subsequent ethinylestradiol injection 1 h later did not show any decrease. This response was different from that observed previously in estradiol-primed animals in which progesterone showed a multiphasic decrease of occupied form of nuclear E_2R. However, in the uterus of ethinylestradiol-primed rats, a partial decrease of total and occupied nuclear E_2R accumulation was observed in the presence of the three doses of progesterone used. The decrease of uterine nuclear E_2R with the three progesterone doses was different from the dose-dependent effect of progesterone observed in the uterus of estradiol-primed rats. Affinity constants of the interaction between [^{3}H]estradiol and the nuclear E_2R were similar among groups treated with ethinylestradiol, estradiol and progesterone. These results demonstrate the involvement of 17β-HSD in the reduction of anterior pituitary gland E_2R by progesterone in the estradiol-treated animals. Furthermore, the mechanism of decrease of E_2R by progesterone in the uterus appears to be different from the pituitary gland.

INTRODUCTION

In the estrogen-primed rat, progesterone has been shown to stimulate or inhibit gonadotropin secretion depending upon the time of administration of progesterone [1] and the dose of progesterone used [2–4]. Estrogens are necessary for the induction of progesterone receptors in the hypothalamus and anterior pituitary gland [5–7], both of which are sites for the control of gonadotropin secretion. The effects of progesterone in these tissues include rapid release of hypothalamic LH-RH [8, 9], suppression of hypothalamic LH-RH degrading activity [10] and stimulation of anterior pituitary gland 17β-hydroxysteroid dehydrogenase (17β-HSD) activity [11, 12).

It is currently recognized that the regulation of receptor levels in a tissue is one of the primary modes of intracellular control of hormonal activity [13, 14]. We have reported earlier that progesterone can decrease nuclear E_2R binding in the anterior pituitary gland of immature estrogen-primed ovariectomized rats [6, 7] and mature ovariectomized estrogen-primed rats [15]. Such a decrease is also brought about by the progesterone metabolite, 5α-dihydroprogesterone [16]. Progesterone-induced decrease of nuclear E_2R has also been observed in the hamster [17, 20] and rat uterus [15, 21] and in the rat oviduct [22]. The decrease in nuclear E_2R in a variety of tissues appears to be involved in the modulation of estrogenic activity. During the period of such a decrease estrogen-induced pituitary progesterone receptor synthesis [7] and prolactin release [23, 24] were attenuated in the immature rat. Estrogen-induced egg transport was also inhibited in rat oviduct during progesterone-induced E_2R decrease [22].

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It has been proposed that progesterone could decrease nuclear E_2R through a decrease of intracellular availability of estradiol [25]. Gurpide and Marks showed an inverse correlation between binding of estradiol to its own receptor in human endometrium and endometrial 17β-hydroxysteroid dehydrogenase activity [26]. This enzyme catalyzed the reversible oxidation of estrogen to estrone. Estrone has a lower affinity for the estrogen receptor and a higher disassociation constant, and thus is not retained in the nucleus long enough for sustained biological action [27].

The purpose of this study was to determine if 17β-HSD activation by progesterone was involved in its ability to decrease E_2R in anterior pituitary and uterus of the rat. This question was approached by using the synthetic estrogen, ethinylestradiol instead of estradiol for priming mature ovariectomized rats before the administration of progesterone. Ethinylestradiol was chosen because it cannot be oxidized to estrone by 17β-HSD due to the presence of a 17α-ethinyl group.

EXPERIMENTAL

Animals

Mature Sprague–Dawley female rats obtained from Holtzman (Madison, Wis.) were kept in an air-conditioned, light-controlled room (lights on from 0700 to 1900 h). Water and rat chow (Wayne) were given *ad libitum*. Bilateral ovariectomy was performed under ether anesthesia at 65 days of age and all animals were sacrificed by decapitation 14 days later, at 79 days of age. The first experiment was designed to study the accumulation of nuclear E_2R in the presence of estradiol or ethinylestradiol. Groups of 10 animals received 4 daily injections of either 2 μg/rat estradiol or 2, 2.5 and 3 μg/rat of ethinylestradiol, starting on day 11 after overiectomy. Exogenous steroids were administered in 0.3 ml of 20% ethanol:saline, i.p. The last injection was given 1 h before sacrifice. In subsequent experiments, we studied the modulation of nuclear E_2R in ethinylestradiol-primed animals by progesterone. Four groups of 10 rats each received either 3 μg/rat of ethinylestradiol i.p. in 0.3 ml of 20% ethanol:saline or vehicle, following the same protocol described above and detailed in Fig. 1. On the morning of sacrifice, 1 h before the last ethinylestradiol injection, ethinylestradiol-treated groups received either vehicle or 0.8, 2.0 or 4.0 mg/kg body wt of progesterone i.p. in 0.3 ml of 50% ethanol:saline. One vehicle-treated group also received 0.8 mg/kg body wt of progesterone i.p. All animals were sacrificed 1 h after the last ethinylestradiol administration. The experiments using the different doses of progesterone were repeated three times so that the results could be analyzed statistically. The average weight of the animals used for the injection was 270 $\pm$ 20 g.

Chemicals

The buffer used for homogenization consisted of 120 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl), 1.5 mM disodium ethylenediamine tetracetate (Na_2 EDTA), 1 mM dithiothreitol (DTT), 10% v/v glycerol, TEDG buffer. The buffer for nuclei consisted of: 10 mM Tris–HCl, 0.3 M sucrose, 3.8 mM magnesium chloride ($MgCl_2$); TSM buffer. All buffers were adjusted to pH 7.4 at 22°C. [2,4,6,7-^{3}H]estradiol-17β, 95.4 Ci/mmol, from New England Nuclear Corp., was purified by successive descending paper chromatograph before use [28]. Estradiol and diethylstilbestrol (DES) were obtained from Sigma Chemical Co., and progesterone from Steraloids Inc. Scintillation mixture was made with Permablend II (Packard), 5 g/l in toluene.

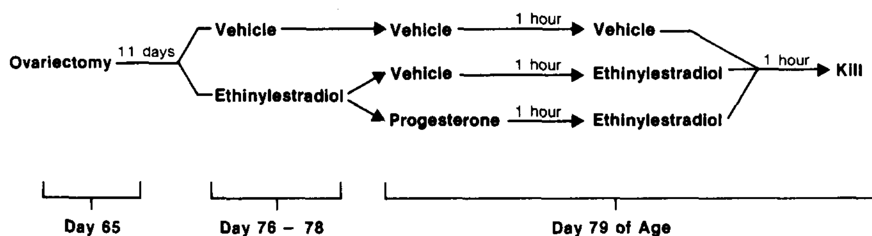

Fig. 1. Experimental protocol: adult female rats were ovariectomized at 65 days of age and 11 days later they received ethinylestradiol (3 μg/rat/day) or vehicle (20% ethanol:saline) i.p. for 3 days. On day 79 of age (day 14 post-ovariectomy), they received either progesterone (0.8, 2.0 or 4.0 mg/kg body wt) or vehicle (50% ethanol:saline) i.p. One hour later they received a final injection of ethinylestradiol (3 μg/rat) and they were sacrificed after 1 h by decapitation.

Preparation of tissue

Anterior pituitary and uteri were removed and placed immediately in ice-cold TEDG buffer. Anterior pituitaries were washed 5 times with TEDG buffer and homogenized manually by 10 strokes of a Teflon/glass homogenizer with 1 ml TEDG buffer per pituitary. Uteri were homogenized in a Polytron PT 10 in 2 ml TEDG buffer per uterus at setting No. 5 for 15 s. The procedure was repeated 3 times with 15 s cooling on ice between each burst. Crude pituitary and uterine nuclear pellets were separated by low speed centrifugation (800 *g*, 10 min) and then washed 4 times in TSM buffer. Finally, they were resuspended and diluted in TSM buffer to a final volume of 1.5 ml/pituitary and 1 ml/uterus and rehomogenized manually with a Dounce homogenizer (pestle B, 10 strokes) before use as a source of binding activity. All procedures were done at 4°C.

Binding assays

Aliquots of 250 μl of nuclear suspension were incubated with six concentrations of [^{3}H]estradiol (0.2 to 3.5 nM) in a final volume of 500 μl. Simultaneous incubations were done with [^{3}H]estradiol plus a 100-fold molar excess of DES, in order to measure nonspecific binding. The incubations were carried out at 30°C for 1 h for total nuclear E_2R and 4°C for 18 h for unoccupied nuclear E_2R. These conditions for measurement of unfilled binding sites, as well as exchange conditions for the estimation of total receptor, were previously established [29]. The difference between total receptors and unoccupied receptors was considered to be the occupied receptors.

The incubations were stopped by dilution with 0.5 ml of TSM per tube, followed by removal of unbound steroid by four successive washings with 0.5 ml of TSM buffer. Extraction of the bound steroids was then performed using 1 ml ethanol per tube, at 30°C for 30 min. After centrifugation at 800 *g* for 10 min, the supernatant were poured into vials with 10 ml of scintillation cocktail.

Analytical determinations

Radioactivity in nuclear samples was measured using a Beckman LS-9000 spectrometer (Beckman Instruments, Palo Alto, Calif.) with conversion to dpm by the H-gate method. Counting was done at a level permitting less than 2% error. Efficiency for tritium was 56%. Results were normalized for DNA,

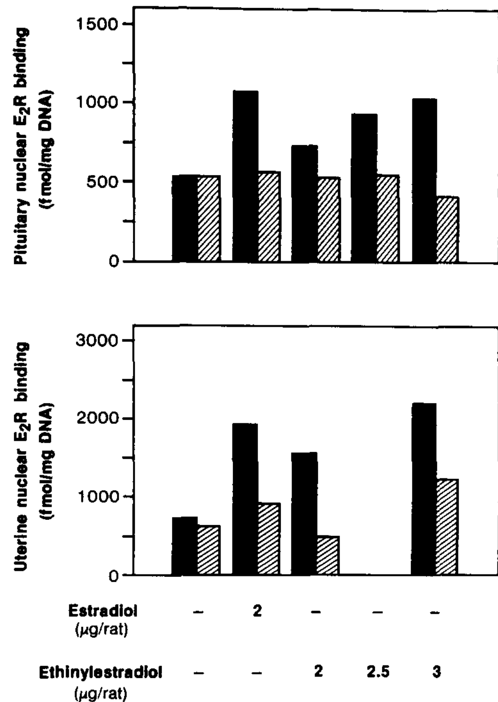

Fig. 2. Accumulation of total (■) and unoccupied (▨) nuclear E_2R induced by 17β-estradiol and ethinylestradiol in anterior pituitary (top panel) and uterus (bottom panel) of adult ovariectomized rats. After 11 days of ovariectomy, animals received four daily injections i.p. of either vehicle (20% ethanol:saline), 17β-estradiol (2 μg/rat) or ethinylestradiol (2, 2.5 or 3 μg/rat). The animals were sacrificed 1 h after the last injection. Nuclear E_2R was measured in anterior pituitary and uterus with incubations at 37°C × 1 h (total E_2R) and 4°C × 18 h (unoccupied E_2R). Bars represent B_{max} values obtained from a single experiment using Scatchard analysis of a 6-point saturation binding curve. The uterine group receiving 2.5 μg ethinylestradiol was lost during determination.

determined by the method described by Burton [30]. Binding data were analyzed according to the method of Scatchard [31]. The Duncan's multiple range test was used to determine significant difference at the $P < 0.05$ level.

RESULTS

In order to study the interactions between progesterone and nuclear E_2R in anterior pituitary gland and uterus in ethinylestradiol-primed rats, it was first necessary to establish the dose of ethinylestradiol to be used. For this purpose ovariectomized mature rats were administered either 2 μg estradiol or various doses of ethinylestradiol and the nuclear E_2R accumulation was measured in the anterior pituitary gland and uterus 1 h after the last injection. Figure 2 shows the levels of nuclear E_2R accumulation under different estrogen treatments in anterior pituitary gland and uterus measured under exchange (total E_2R) and

Table 1. Effects of 17β-estradiol, ethinylestradiol and progesterone on the equilibrium association constant of the interaction between [^{3}H]estradiol and nuclear E_2R

Priming	Progesterone	K_a range ($\times 10^9 M^{-1}$) Pituitary	Uterus
Vehicle	Vehicle	2.6–6.0	2.1–6.0
Estradiol (2 μg/rat)	Vehicle	4.8–6.6	3.4–6.1
Estradiol (2 μg/rat)	0.8 mg/kg body wt	1.5–4.5	2.6–4.3
Ethinylestradiol (3 μg/rat)	Vehicle	2.6–3.2	1.4–6.6
Ethinylestradiol (3 μg/rat)	0.8 mg/kg body wt	4.3–7.5	2.6–5.3

Mature, ovariectomized rats were sacrificed at 79 days of age (day 14 postovariectomy) after receiving one of the following treatments: (a) vehicle alone for 4 days; (b) estradiol (2 μg/rat/day) or ethinylestradiol (3 μg/rat/day) for 4 days, the last injection given 1 h before sacrifice; (c) progesterone (0.8 mg/kg body wt) administered 1 h before either the last estradiol or ethinylestradiol injection. Equilibrium association constant (K_a) for pituitary and uterine nuclear E_2R were determined from the slope of Scatchard binding analyses of 6-point saturation binding curves measured at 37°C × 1 h (total E_2R) or 4°C × 18 h (unoccupied E_2R).

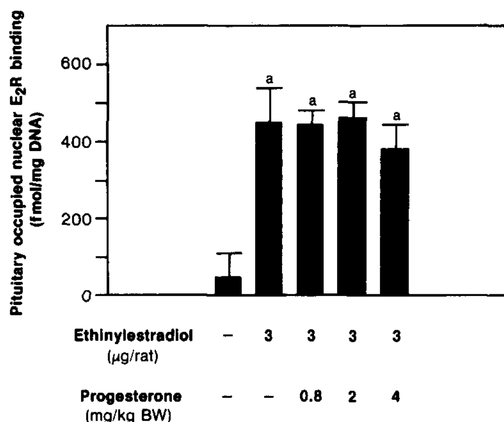

Fig. 4. Pituitary occupied nuclear E_2R response to acute exposure to progesterone in ethinylestradiol-primed rats. Experimental design and treatment protocol according to Fig. 1. Occupied nuclear E_2R were calculated as the difference between total and unoccupied nuclear E_2R. $^aP < 0.05$ when compared to vehicle-treated controls.

non-exchange (unoccupied E_2R) conditions. Control animals showed a low level of nuclear E_2R remaining after 2 weeks of ovariectomy in both tissues. The measurable E_2R appeared to be of the unoccupied E_2R form.

In ovariectomized rats, treatment with 2 μg estradiol resulted in a total nuclear E_2R accumulation of 1076 fmol/mg DNA in the anterior pituitary gland. When ovariectomized rats were treated with ethinylestradiol, the 2, 2.5 and 3 μg doses of the steroid resulted in total nuclear E_2R accumulation of 735, 940 and 1046 fmol/mg DNA respectively in the anterior pituitary gland. Since the accumulation of nuclear E_2R after priming with 2 μg of estradiol and 3 μg of ethinylestradiol was equivalent, the 3 μg dose of ethinylestradiol was used for subsequent studies. This dose of ethinylestradiol also appeared to be suitable for the uterus, the nuclear accumulation of the receptors being 1932 fmol/mg DNA with 2 μg dose of estradiol and 2212 fmol/mg DNA with a 3 μg dose of ethinylestradiol (Fig. 2). The equilibrium association constant of [^{3}H]estradiol binding to nuclear E_2R in estradiol-primed and ethinylestradiol-primed rats was also similar in the anterior pituitary gland and the uterus (Table 1).

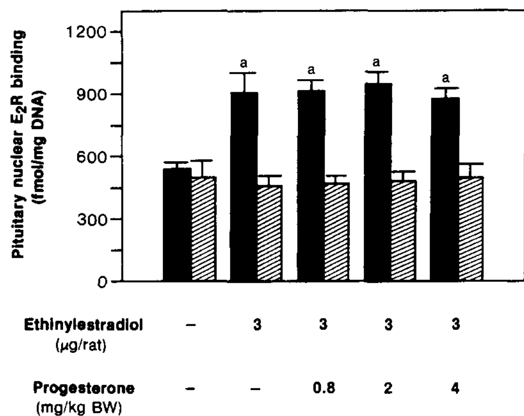

Fig. 3. Pituitary nuclear E_2R response to acute exposure to progesterone in ethinylestradiol-primed rats. Starting day 11 post-ovariectomy, groups of 10 adult ovariectomized rats were primed with 3 μg/kg body wt/day i.p. of ethinylestradiol for 3 days. On the morning of day 14 post-ovariectomy they received either progesterone (0.8, 2.0 or 4.0 mg/kg body wt, i.p.) or vehicle 2 h before decapitation and 1 h before the last ethinyl-estradiol administration (3 μg/rat i.p.). Total (■) and unoccupied (▨) E_2R was measured at 37°C × 1 h and 4°C × 18 h respectively. Bars represent average ± SE of B_{max} values obtained from 3 different experiments, using Scatchard analyses of 6-point saturation binding curves in each determination. $^aP < 0.05$ when compared to vehicle-treated control.

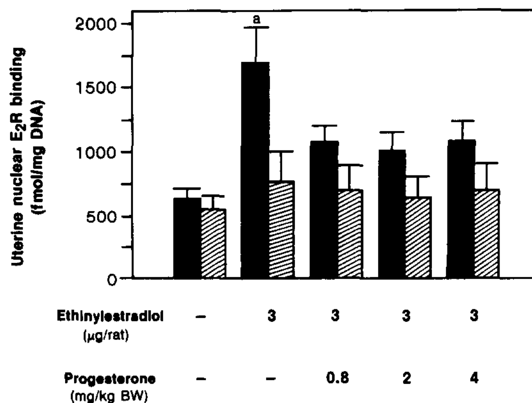

Fig. 5. Uterine total (■) and unoccupied (▨) nuclear E_2R response to acute exposure to progesterone in ethinylestradiol-primed rats. Experimental design and treatments were carried out according to Fig. 3. $^aP < 0.05$ when compared to vehicle-treated control.

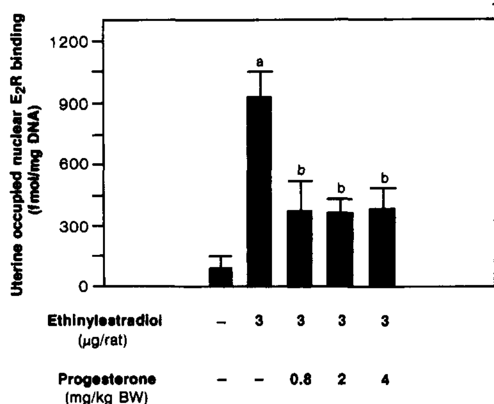

Fig. 6. Uterine occupied nuclear E_2R response to acute exposure to progesterone in ethinylestradiol-primed rats. Experimental design and treatment according to Fig. 4. [a]$P < 0.05$ when compared to vehicle-treated control. [b]$P < 0.05$ when compared to ethinylestradiol alone group.

To determine the acute effects of progesterone on nuclear E_2R accumulation in anterior pituitary gland and uterus in ethinylestradiol-primed ovariectomized rats, the 0.8, 2.0 and 4.0 mg/kg body wt doses of progesterone were administered i.p. 2 h before sacrifice (1 h before the last ethinylestradiol injection) using the protocol shown in Fig. 1. The doses of progesterone were chosen based on our previous study [15]. Treatment with 3 μg/rat of ethinylestradiol resulted in a significant rise in total nuclear E_2R of the anterior pituitary gland (540 ± 32 vs 907 ± 91 fmol/mg DNA) without changes in the unoccupied nuclear E_2R (493 ± 87 vs 459 ± 48 fmol/mg DNA; Fig. 3). The administration of the 0.8, 2.0 and 4.0 mg/kg body wt doses of progesterone did not bring about any changes in the total (910 ± 53; 942 ± 61 and 871 ± 51 fmol/mg DNA with 0.8, 2.0 and 4.0 mg/kg body wt of progesterone, respectively) and unoccupied (467 ± 39, 480 ± 44 and 492 ± 70 fmol/mg DNA with 0.8, 2.0 and 4.0 mg/kg body wt of progesterone, respectively) nuclear E_2R accumulation of the anterior pituitary gland when compared to the ethinylestradiol controls (Fig. 3). As will be evident from Fig. 4, the increase in the total nuclear E_2R accumulation in the anterior pituitary gland after ethinylestradiol treatment was almost completely due to an increase in occupied E_2R. This molecular form of E_2R, which was not found in ovariectomized rats, constituted 45% of the total nuclear estrogen binding in ethinylestradiol-treated group. Treatment with progesterone did not modify the occupied nuclear E_2R levels of the anterior pituitary gland at any of the doses used.

In the uteri of ovariectomized rats, we also found that E_2R persisted in the nuclear fraction after 2 weeks of estrogen withdrawal (Fig. 5). These receptors were the unoccupied form of the E_2R. Priming with ethinylestradiol brought about a significant increase in total nuclear E_2R (1693 ± 275 vs 636 ± 79 fmol/mg DNA in ovariectomized controls; Fig. 5). Treatment with ethinylestradiol increased nuclear E_2R binding primarily due to an increase in the occupied form of the nuclear E_2R representing 55% of total uterine nuclear estrogen binding. The three doses of progesterone used partially reduced the total nuclear accumulation of E_2R in the uterus ($P < 0.05$). However the reduction in the levels of E_2R did not vary with the dose of progesterone used (Fig. 5). No differences were found in the unoccupied E_2R of the uterus in ethinylestradiol-treated rats with or without progesterone. Figure 6 shows that occupied nuclear E_2R were reduced to a similar extent by all doses of progesterone.

DISCUSSION

Results from our laboratory and by others has shown that progesterone can bring about a reduction in E_2R binding in the anterior pituitary gland [6, 7, 15, 32]. Progesterone has also been shown to enhance the magnitude of the estrogen-induced gonadotropin surge and ovulation in intact cycling rats and pregnant mare's serum gonadotropin-treated immature female rats [33]. The decrease in anterior pituitary gland E_2R binding induced by progesterone appeared to have biological relevance because such a decrease correlated well with a decrease in estrogen action such as progesterone receptor synthesis [7] and induction of prolactin release [23, 24]. The mechanism by which progesterone decreases E_2R binding in the pituitary gland thus becomes of considerable interest.

In the human endometrium, Gurpide and coworkers [25–27] have proposed that progesterone could decrease nuclear E_2R through a decrease of intracellular availability of estradiol due to its conversion to estrone by the action of 17β-HSD. Estrone has a lower affinity for the estrogen receptor and a higher disassociation constant and is retained in the nucleus for a shorter time than estradiol [27]. A decrease of nuclear E_2R by progesterone has been reported in the rat [15, 21], and hamster [17, 20] uterus and the rat oviduct, [22]. In the hamster uterus a reduction of estrogen receptors by

progesterone was not found to be due to the action of 17β-HSD [34] but possibly due to activation of a phosphatase by progesterone [20]. Progesterone did not bring about the activation of uterine 17β-HSD in rat [11, 35], mouse [36] and hamster uteri [34]. On the other hand, increased activity of anterior pituitary gland 17β-HSD has been reported in the monkey [12] and estrogen-primed rat [11]. In addition to the differences in the activation of 17β-HSD in the rat anterior pituitary gland and the rat uterus, Fuentes *et al.* reported that progesterone [15] and 5α-dihydroprogesterone [16] reduced occupied nuclear E_2R in rat anterior pituitary gland in a multiphasic manner. Doses of 0.8 and 4.0 mg/kg body wt of progesterone were more effective in reducing the occupied nuclear E_2R than the 2.0 mg/kg body wt dose [15]. Doses of 0.2 and 2.0 mg/kg body wt of 5α-dihydroprogesterone were also more effective than the 0.8 mg/kg body wt dose in reducing E_2R [16]. The reduction of nuclear E_2R by different doses of progesterone correlated very well with its ability to decrease estrogen-induced prolactin release [24]. On the other hand, in the uterus both progesterone and 5α-dihydroprogesterone reduced nuclear E_2R in a dose-dependent manner. This led Fuentes *et al.* [15, 16] to postulate that the mechanisms involved in the reduction of rat anterior pituitary gland and rat uterine E_2R were different.

In this study ovariectomized rats were primed with the synthetic estrogen, ethinylestradiol instead of estradiol to determine if the activation of 17β-HSD was the mechanism of progesterone-induced reduction of E_2R in rat anterior pituitary gland and uterus. Three doses of progesterone 0.8, 2.0 and 4.0 mg/kg body wt were used in this study. These doses were based on multiphasic effects of progesterone on E_2R in the anterior pituitary gland and dose-dependent effects on rat uterine E_2R described in our previous study [15]. The results clearly show that whereas progesterone reduced the E_2R in the anterior pituitary gland of ovariectomized rats primed with estradiol, it was unable to do so if ethinylestradiol, a synthetic estrogen that could not be oxidized by 17β-HSD, was used. Taken together with the earlier reported stimulation of 17β-HSD by progesterone [11] these results appear to indicate that 17β-HSD stimulation by progesterone in the anterior pituitary gland is the main mechanism by which progesterone reduces E_2R. Such a mechanism has been shown to be involved with progesterone action in the human endometrium and breast cells in culture [37].

In contrast to the results observed with the anterior pituitary, progesterone was still able to reduce uterine E_2R in spite of ethinylestradiol priming. Since progesterone does not stimulate uterine 17β-HSD in the rat [11, 35] and ethinylestradiol cannot be oxidized by 17β-HSD, a mechanism different from that found in the anterior pituitary appears to be involved. Stimulation of phosphatase activity by progesterone has been suggested as a mechanism of E_2R decrease in the hamster uterus [2]. Such a mechanism may be at play in the rat uterus as well. However, in the absence of a dose-dependent effect of progesterone in ethinylestradiol-treated rats, a minor role of 17β-HSD cannot be ruled out. Several forms of 17β-HSD have been found in subcellular fractions of human placenta [38, 39] with different affinities for different substrates including progesterone and its metabolites [40–42]. Detailed studies of this type have not been done in rat tissues. In conclusion, these results further confirm our earlier observations that the mechanisms involved in the modulation of estrogen receptors by progesterone are different in the rat anterior pituitary gland and the rat uterus.

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