Effect of estradiol on the progesterone receptor and on morphological ultrastructures in the fetal and newborn uterus and ovary of the rat

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Abstract. The effect provoked by estradiol after administration to pregnant rats (1 mg per day) was studied in fetal and newborn uteri and ovary. Estrogen receptors are found in the fetuses of non-treated animals. Their number (in fmol/mg DNA, ± SD) in the fetal uterus (total sites, cytosol + nuclei) was at the age of 18 days: 63 ± 15; at 20 days 101 ± 13; and in the 24-h-old newborn; 415 ± 120. The respective values in the ovary were: 105 ± 25; 520 ± 60 and 410 ± 190. Estradiol stimulated significantly the progesterone receptor in the fetal uterus at 20 days old. The progesterone receptor (in fmol/mg DNA, ± SD) which was 97 ± 17 in the non-treated animals, increased to 790 ± 90 in the E₂-primed animals. Newborns, 24-h-old, had no detectable progesterone receptor, but in the E₂-treated animals the value increased to 1210 ± 120. In the fetal ovary of non-treated animals, progesterone receptor at the age of 18 days is: 90 ± 19; at 20 days 132 ± 47, and in the newborns 260 ± 67; in the E₂-treated animals, the values are respectively 330 ± 49; 865 ± 78 and 1280 ± 307. In the fetal uteri of E₂-treated animals, histological and ultrastructural studies showed an increase in the size of the uterine horn, the height of the epithelial cells, and stromal cell differentiation. It is suggested that, as was extensively demonstrated in the fetal compartment of the guinea-pig, the machinery for estrogen responses operates also during fetal development of the rat.

The rat was the first animal species in which estrogen receptors were detected and characterized (Jensen & Jacobson 1962). In this and other studies, immature or adult ovariectomized rats were mostly used. It is also well known that progesterone receptor is a function of estrogen activity (O’Malley et al. 1971). More recently, the presence of estrogen receptor and the biological response to estrogens were extensively demonstrated in different target tissues during fetal development of the guinea pig (Pasqualini et al. 1976; Pasqualini & Nguyen 1980). To our knowledge there is limited information available at present concerning the presence in the rat of estrogen receptor and biological responses to estrogens in the fetal uterus during fetal development. One reason for this is the presence in the fetal and maternal plasma of this species of huge quantities of α-fetoprotein, a protein which binds estrogens in this animal species, with high affinity and specificity (Soloff et al. 1971; Nunez et al. 1971). It is also generally accepted that the interaction of this protein with the circulating estrogens can block the action of the hormone.

In the present study we investigated the presence of estrogen receptor in the fetal uterus of the rat, as well as the biological response to stimulation of the progesterone receptor in the fetal uterus and ovary after administration of estradiol to the maternal compartment of the pregnant rat. The effect was also studied in the uterus and ovary of newborn rats after administration of estradiol during the last days of pregnancy. The histological and ultrastructural modifications of the uterine horns of fetal and newborn rats are also included.
Materials and Methods

Biological material and treatment

Pregnant (12 days to end of gestation) and newborn (24 h old) rats of the Wistar strain were used (CESAL, Vigneul-sous-Montmedy, France). Estradiol (E2) was dissolved in a solution of 0.9% (wt/vol) NaCl-40% ethanol (vol/vol). In each study, groups of pregnant rats (50–60 animals) were injected sc (0.2–0.3 ml solution) with E2 (1 mg/kg per day). In one series of experiments (Exp. A), the animals (12 days of gestation) were treated for 6 consecutive days. Twenty-four hours after the last treatment, the animals (18 days of gestation) were anesthetized with ether and the uterus and ovaries of the fetus removed. In another series of experiments (Exp. B), pregnant rats (17 days of gestation) received the same dose of E2 for 3 consecutive days and 24 h after the last treatment, the animals (20 days of gestation) were anesthetized and the uteri and ovaries removed. Finally, (Exp. C), pregnant rats (18 days of gestation) were treated with E2 for 3 consecutive days and after birth (24 h old) the uteri and ovaries were removed in the newborn animals. Control animals received the vehicle alone. After removal, fetal and newborn uteri and ovaries were processed to determine estrogen- and progesterone-receptor contents.

In a series of experiments using 1 mg of estradiol + 50 µCi of [3H]E2 we observed that only 0.01–0.001% of the injected material was found in the fetal compartment, which corresponds to 0.1–0.01 µg of estradiol. The data indicate that, in spite of the higher quantity of E2 injected to the pregnant rats, the levels of the hormone which are reached in the fetal compartment correspond to physiological concentrations.

Radioactive steroids and other chemicals

[17α-methyl-3H]R5020 (17α,21-[17α-methyl-3H]dimethyl-19-nor-pregna-4,9-diene-3,20-dione; SA, 87 Ci/mmol), [3H]R2858 (19αβ-methoxy-19-nor-17α-pregna-1,3,5(10)-tri-en-20-yne-3,17β-diol), (6,7-3H-estradiol (E2)) (SA, 52.5 Ci/mmol), non-radioactive R5020 and R2858 were obtained from New England Nuclear Corp (NEN France, Paris, France). The purity of the radioisotopes was assessed by TLC (thin layer chromatography) in the appropriate systems. E2, diethylstilbestrol, [3,4-bis-(4-hydroxyphenyl)3-hexene], progesterone, 5α-dihydroprogesterone, cortisol and testosterone were purchased from Steraloids (Touzart et Matignon, Vitry-sur-Seine, France).

Preparation of cytoplasmic and nuclear extracts

Uteri and ovaries were homogenized (wt/vol, 1:10) in 10 mmol/l Tris-HCl, 1.5 mmol/l EDTA, and 0.5 mmol/l dithiothreitol (TED) buffer, pH 7.4, with a Teflon-glass Potter-Elvejem homogenizer. Homogenates were centrifuged at 900 × g for 10 min at 4°C. Pellets were resuspended twice in 1 ml of TED buffer and centrifuged again at 900 × g. Pooled supernatants were centrifuged at 105 000 × g for 30 min to obtain the cytosol fraction. The 900 × g pellet (containing nuclear and myofibrillar debris) was extracted with 0.6 mol/l KCl prepared in TED buffer, pH 8.5 (0.6 mol/l TKED) for 20 min at 4°C with frequent vortexing and centrifuged at 900 × g for 10 min. The procedure was done twice; the supernatants were pooled and centrifuged at 105 000 × g for 30 min to obtain the 0.6 mol/l KCl nuclear extracts.

Affinity of binding of [3H]R2858 to cytosol macromolecules

The affinity constants of [3H]R2858 binding in the cytosol fraction determined according to the method of Scatchard (1949). Aliquots (0.5 ml) of the cytosol fractions (1–1.5 mg protein/ml) were incubated in duplicate with [3H]R2858 in the concentration range of 0.5–5.0 × 10⁻⁹ mol/l. Parallel incubations were performed in the presence of 1 × 10⁻⁹ mol/l of the non-radioactive steroid. Incubations were carried out at 4°C for 16 h. Binding was measured using the dextrancoated charcoal method (Korenman 1969).

Single saturating dose assay of [3H]R2858 and [3H]R5020 binding

The measurement of specific [3H]R2858 binding in the cytoplasmic and nuclear extracts was performed by the exchange assay in protamine sulphate precipitates (Chamness et al. 1975; Zava et al. 1976) with the modifications described previously (Sumida & Pasquallini 1979). Cytosol and nuclear pellets were incubated with 1 × 10⁻⁹ mol/l [3H]R2858, with and without a 100-fold molar excess of unlabelled R2858 at 30°C for 16 h. [3H]5020-specific binding sites in the cytosol and nuclear extracts were measured after incubation with 5 × 10⁻⁹ mol/l of the radioactive steroid in the absence or presence of a 100-fold excess of the unlabelled steroid at 4°C for 16 h. Binding of radioactive steroids to macromolecules was determined after adsorption of the unbound steroid with a 0.1% (wt/vol) dextran-coated 1% (wt/vol) charcoal mixture at 4°C for 10 min.

DNA and protein assays

DNA was determined according to the method of Burton (1956), and protein was measured by the method of Lowry et al. (1951).

Measurement of radioactivity

Samples were dissolved in Ready-Solv HP (Beckman), and radioactivity was measured in a liquid scintillation spectrometer (model LS 1800, Beckman Instruments, France). Counting efficiency was 45%.

Histological and ultrastructural studies

Non-treated and estradiol-primed fetuses and newborn rats were dissected under a microscope (Zeiss). Portions were prepared for...
of the uterine horns were immediately fixed in 2.5% vol/vol glutaraldehyde (buffered with cacodylate pH 7.4 at 4°C), post-fixed in 1% vol/vol osmium tetroxide, dehydrated and embedded in Epon resin. Transverse semi-thin sections perpendicular to the long axis were obtained using a Reichert OMU 3 ultramicrotome, stained with toluidine blue and examined under a photomicroscope Ortholux Leitz. Only areas cut perpendicularly were selected for ultrathin sectioning. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an Elmiskop Siemens 101 or 102. The diameter of the uterine horns and the epithelial height of control and E₂-treated rats were measured in 0.5 µm thick sections perpendicular to the long axis. For each uterine horn, 10–20 sections were studied. Different thicknesses (100–200) of the epithelium per uterine horn were measured using a Hewlett Packard 5874A digital board and a Tektronix 4054 computer. All the results are presented as the mean ± SD. The Wilcoxon’s rank test was used to compare two distributions (control and treated).

Table 1.

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A: Aliquots (0.2–0.3 ml) of diluted fetal plasma (1:20 vol/vol) of 20-day-old fetuses were incubated with 1 × 10⁻⁸ mol/l [³H]R2858 (1) and [³H]E₂ (2) or 5 × 10⁻⁹ mol/l [³H]R5020 (3), or with the same radioactivity plus a 100-fold excess of the radioinert steroid for 16 h at 4°C. Specific binding (DPM/mg protein) was calculated from the difference in these two determinations. B: Fetal uterine cytosol (20 days old) was prepared as indicated in the Materials and Methods section and aliquots (0.2–0.3 ml) were incubated with 1 × 10⁻⁸ mol/l [³H]R-2858 (1), [³H]E₂ (2) or 5 × 10⁻⁹ mol/l [³H]R5020 (3), with or without a 100-fold excess of the unlabelled steroids for 16 h at 4°C. The unbound radioactivity was absorbed with charcoal-dextran and the total binding (DPM/mg protein) in the presence of added steroid was compared with the total binding in controls, which was assigned the value of 100%. The values represent the average of 3–4 determinations. DES: diethylstilbestrol. 5α-P: 5α-dihydroprogesterone.
Results

Competitive studies in binding of [3H]R2858, [3H]E2 and [3H]R5020 to rat fetal plasma and uterine cytosol fraction

As huge quantities of α-fetoprotein (α-FP) are present during pregnancy and in newborn (2–3 days old) rats (Gitlin & Boeman 1967), and in this species α-FP binds to estradiol with a high affinity (Kₐ = 1–2.6 × 10⁸ mol/l) (Soloff et al. 1972), the specific binding of the fetal plasma proteins was explored using the natural [3H]E₂ and the synthetic [3H]R2858 estrogens.

Table 1 shows that no specific binding for the synthetic estrogen R2858 was detected in the fetal plasma proteins, however, significant competition was found for [3H]E₂. The data suggest that the specific binding of [3H]E₂ in the fetal plasma is α-FP, as it is well known that this protein does not bind the synthetic estrogen R2858 (Raynaud 1973). On the other hand, in the cytosol fraction, specific binding was found for both [3H]E₂ and [3H]R2858. Consequently, we used [3H]R2858 to evaluate the specific estrogen binding sites in the fetal and newborn uteri and ovaries. Table 1 indicates also that in the fetal plasma, no specific binding was detected for the synthetic progestagen R5020 which was used to determine progestrone receptor levels in the uterus and ovary.

Affinity of [3H]R2858 binding sites in the fetal uterine cytosol

Fig. 1 shows the Scatchard plot of [3H]R2858 in the cytosol fraction of a pool of fetal uteri of 20 days of gestation. The calculated value gives a high affinity for the binding of this synthetic estrogen (K_d = 0.7 × 10⁻¹⁰ mol/l). To our knowledge there is no data available for the estrogen affinity in the fetal uterus of rat. The value for this K_d in the newborn rat (3–7 days old) is 0.28–1.40 × 10⁻⁹ mol/l (Medlock et al. 1981).

Fig. 2 gives the levels of specific binding sites for [3H]R2858 in the fetal uterus and ovaries (cytosol and 0.6 mol/l KCl nuclear extracts) of 18 and 20 days old rats and in 24-h-old newborns of the non-treated animals. A significant increase in
these specific binding sites is observed in both fetal tissues. In the uterus of the newborn (24 h old), the total binding sites (cytosol + nuclear extracts) are on the average 450 fmol/mg DNA. It is to be noticed that Medlock et al. (1981) in the rat uterus found the number of estrogen receptors (ER) (total: cytosol + nuclei) to be 2000 fmol/mg DNA at the age of 3 days, 3200 fmol/mg DNA at 5 days, and 5000 fmol/mg DNA at 7 days.

**Effect of estradiol on the progesterone receptor (PR) of the fetal and newborn uteri and ovaries of the rat**

The relatively high dose of E2 (1 mg/kg per day) was used in order to prevent the inactivation of the hormone by its association with the very high concentration of α-fetoprotein. Table 2 indicates total PR levels (cytosol + 0.6 mol/l KCl nuclear extracts) in both uteri and ovaries of non-treated and E2-primed animals. The fetal uterus responds from 20 days old and this response increases significantly after birth. The important response in the ovary is observed already in the 18-day-old fetus.

Comparative data of previous studies in this laboratory thus indicated that in the fetal guinea pig, the fetal uterus of this species responds to E2 from 37–42 days of fetal life (Pasqualini & Nguyen 1980). It is suggested that the presence of α-fetoprotein can block this response in early stages of rat fetal development. Concerning the dose of estradiol injected, it is also to be noted that Vannier & Raynaud (1980) observed morphological changes (hypospadias, vaginal-urethral junction) in the fetal rats whose mothers (16–20 days of gestation) were treated with 1.2 mg/rat/day of E2.

**Effect of estradiol on the uterine horn structure**

In the estradiol-treated fetus (Fig. 3B) and newborn rats (Fig. 3D) semi-thin sections show a significant increase of the lumen: (μm ± SD) control fetus 68.95 ± 10.79; E2-treated fetus 82.90 ± 8.95; control newborn 50.42 ± 8.47; E2-treated newborn 147.75 ± 39.69 (P < 0.05), and an increase in the size of the uterine horn. The estimated values of the diameter of the uterine horns (μm ± SD) are as follows: control fetus 287.03 ± 17.28; E2-treated fetus 338.28 ± 37.36; control newborn 324.80 ± 14.01; E2-treated newborn 563.04 ± 30.66 (P < 0.05).

A circular orientation of numerous stromal cells and an increase in the height of the epithelial cells are observed only in the treated newborn uteri (Fig. 3D and F) 21.06 ± 7.06 compared with the control: 14.18 ± 2.22 (P < 0.05). On the other hand, stromal cells of uterine from non-treated animals are undifferentiated (Fig. 3C and E).

Ultrastructural studies show that, in the non-treated 20-day-old fetuses (Fig. 4A), the epithelial cells are cuboidal with large nuclei. Golgi complexes, some mitochondria, ribosomes and tubules of rough endoplasmic reticulum, as well as some lipid inclusions and lysosomes, are observed in the reduced cytoplasm. The basal part of the cells presents few interdigitations (not shown). Lateral membranes of the cells show 'gap junc-
tion' and 'macula adherens' differentiation in their apical area (Fig. 4C). After treatment with estradiol (Fig. 4B and D), epithelial cells of fetal uteri are elongated with nuclei lying in their basal part. Cytoplasmic organelles are well developed ribosomes, Golgi complexes, dense mitochondria, lysosomes and, particularly, microtubules. The apical membrane with some microvilli is slightly irregular as in the control cells.

The ultrastructure of uterine epithelial cells of newborn non-treated animals (24-h-old) shows cytoplasmic characteristics similar to those observed in the 20-day-old fetuses. However, more lipidic inclusions and lysosomes are observed. After estradiol treatment of pregnant rats (18 days of gestation) (Fig. 5B), cellular modifications of the newborn uteri show a significant increase in the height of the cells, the presence of plasmic membrane, interdigitations, and numerous microvilli at the apical cell border. In addition, the apical cytoplasm contains formations of different sized granular material, apparently glycogen. These were not observed in the uterine epithelial cells of the non-treated animals (Fig. 5A).

**Discussion**

The present study has characterized estrogen receptors in the fetal uterus of the rat at the end of gestation and shows that this fetal organ responds to estrogens. With this information we extend the concept of the presence of estrogen receptors during fetal life and its biological responses to estrogens in another species, since in previous work we demonstrated that estrogen receptors and different biological responses operate during the fetal life of the guinea pig (Pasqualini et al. 1976; Pasqualini & Nguyen 1980; Pasqualini & Sumida 1986). The present information is of particular interest because in the rat during gestation, large quantities (1–3 g/l) of α-fetoprotein are present in the plasma of pregnant rats (Gitlin & Boeman 1967), and it is generally believed that α-fetoprotein, by binding specifically estrogens and with high affinity, can block the biological response of the hormone. The specific binding sites that we found in the fetal uterus are not α-fetoprotein because we determined this specific binding with the synthetic estrogen.
Fig. 3.
Fig. 5.
R-2858. As was demonstrated, R-2858 does not bind specifically α-fetoprotein (Raynaud 1973). This was confirmed by the present results (see Table 1). It is to be noticed that the biological responses, as demonstrated by an increase in the progesterone receptors, are found at the end of gestation; these responses operate also after birth in animals injected during gestation.

In order to assess statistically the significance of the $E_2$ effect on PR increase, the present data represent 3-5 experiments with 50-60 pregnant rats in each, which corresponds to 250-300 female fetuses. Different authors have given indirect evidence for estrogen receptors in the Müllerian duct of 20-day-old rat fetuses (Somjen et al. 1976). Also using autoradiographic methods, Stumpf et al. (1980) localized [3H]diethylstilbestrol in the area surrounding the Müllerian and Wolffian ducts but, to our knowledge, the present results are the first to show the stimulation of progesterone receptors in the fetal uterus of the rat.

The histological study of uteri from non-treated and treated fetuses and newborn rats confirms the biochemical results. The fetal uterus can be stimulated by $E_2$ treatment and responds by an accelerated maturation. This stimulation is revealed by an increased size of the uterine horn, the height of the epithelial cells and stromal cells differentiation. These morphological alterations are similar to those obtained in the neonatal rat by Sheehan et al. (1981).

Another attractive aspect of the present results is the stimulation of progesterone receptors in the fetal ovary. It is to be noticed that a similar finding was also observed in the fetal ovary of the guinea pig (Pasqualini & Nguyen 1980). The present information leads us to conclude that, as was extensively demonstrated in the fetal guinea pig, the machinery for the estrogen response operates also during fetal development of the rat.

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