Specific Binding and Biological Response of Antiestrogens in the Fetal Uterus of the Guinea Pig

Alberto Gulino and Jorge Raul Pasqualini

C. N. R. S. Steroid Hormone Research Unit, Foundation for Hormone Research, 26, Boulevard Brune, 75014 Paris, France

ABSTRACT

The present data show that tamoxifen and nafoxidine compete specifically with estradiol for the estrogen receptor in the fetal uterus of the guinea pig. Furthermore, [3H]tamoxifen binds to the cytosol macromolecules of this fetal tissue at two classes of binding sites, one with a dissociation constant (Kd) of 1.8 ± 0.4 × 10^{-9} M and a concentration of specific binding sites (n) of 1800 ± 100 fmol/mg protein, which corresponds to the same specific binding site as does estradiol, and a second class of sites with a Kd of 3.9 ± 0.1 × 10^{-10} M (617 ± 77 fmol/mg protein), which does not bind estrogens. In another series of experiments, it was demonstrated that tamoxifen or nafoxidine injected into the mother (1 mg/kg/day for 3 consecutive days) stimulates fetal uterine weight with an intensity similar to that of estradiol but, in contrast to estradiol, the stimulatory effect of the progesterone receptor is limited. Also, it is observed that estradiol decreases by 80% the total number of specific estradiol binding sites (cytoplasmic plus nuclear). On the other hand, both tamoxifen and nafoxidine can translocate the estrogen receptors into the nucleus, but they have only a limited effect on the total number of specific binding sites of estradiol.

It is concluded that the fetal uterus of the guinea pig responds to the effect of estrogens and antiestrogens.

INTRODUCTION

It is well established that the estrogen response in the target tissue is a process that requires the translocation of the cytoplasmic estrogen-receptor complex into the nucleus (6, 12) and that this transfer is hormone dependent (13, 27). It was suggested that nonsteroidal antiestrogens could elicit their effect through the estrogen receptor system. Tamoxifen has been shown to be very efficient in the remission of rat mammary tumors induced by administration of 7,12-dimethylbenz(a)anthracene (14, 15) and is now often used in the treatment of human breast cancer, particularly during the post-menopausal period (20, 34). Tamoxifen can have an estrogenic action initially and an antiestrogenic response later, as has been demonstrated in the mouse vagina (7). In the fetal uterus of the guinea pig, estrogen receptors appear at an early period of gestation, increase during fetal development, and decrease after birth (22, 25). Furthermore, it has been shown that estradiol elicits a uterotrophic effect in the fetal uterus as well as an intense stimulatory effect on the progesterone receptor protein in the same fetal tissue (23, 24). Consequently, it was of interest to study, in the fetal uterus of the guinea pig, the interaction of the antiestrogens (tamoxifen and nafoxidine) with the binding of estradiol to the receptor, as well as the biological effect of these drugs in the same fetal tissue; these are reported in the present paper.

MATERIALS AND METHODS

Biological Material. Fetuses of Hartley albino guinea pigs from 54 to 64 days of gestation were used. Mothers were anesthetized with ether; the fetuses were obtained by laparotomy, decapitated, and exsanguinated; and the fetal uteri were separated and stripped of adhering fat. In treated animals, the fetal uteri were obtained after the mother had been given estradiol or nafoxidine (1 mg/kg/day s.c. for 3 consecutive days) or tamoxifen (1 or 10 mg/kg/day s.c. for 3 consecutive days), dissolved in 0.9% (w/v) NaCl:40% (v/v) ethanol; control animals received vehicle alone.

Chemicals. [6,7-3H]Estradiol [1,3,5(10)-estratriene-3,17-diol; 41 Ci/mmol] and [17α-methyl-3H]R-5020 (17α,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; 55 Ci/mmol) were obtained from New England Nuclear (Frankfurt, W. Germany) and used without further purification since the purity was found to be greater than 95% in the appropriate paper chromatographic systems. [3H]Tamoxifen [trans-1-(p-dimethylaminoethoxyphenyl)-1,2-trans-diphenylbut-1-ene; 15,8 Ci/ mmol; 97% pure] was a gift from Dr. J. Burns (Imperial Chemical Industries, England); it was stored in the dark at -20°, and its purity was checked periodically by chromatography on thin-layer plates (Merck; 0.25-mm silica gel with fluorescent indicator) using benzene:triethylamine (9:1, v/v) as solvents. Synthetic R-5020 was obtained from New England Nuclear. Tamoxifen and nafoxidine (1-2-[p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl(pyrrolidine hydrochloride) were gifts from Dr. L. Lacome, (I.C.I.-Pharma, Paris, France) and Dr. J. P. Patruraud (Upjohn Laboratories, Paris, France), respectively. The other steroids were purchased from Steroids (Touzart et Matignon, Vitry-sur-Seine, France).

Preparation of Cytosplasmic and Nuclear Extracts. Fetal uteri were homogenized in 0.01 M Tris-HCl:1.5 mM EDTA:0.5 mM dithiothreitol buffer, pH 7.4, with a Teflon:glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 900 × g for 10 min; the pellet was resuspended twice in 0.01 M Tris-HCl:1.5 mM EDTA:0.5 mM dithiothreitol buffer, pH 7.4, and recentrifuged at 900 × g for 10 min. The supernatants were pooled and centrifuged at 200,000 × g for 30 min to obtain a clear cytosol supernatant. All procedures were carried out at 4°. 0.01 M Tris-HCl:1.5 mM EDTA:0.5 mM dithiothreitol: 0.6 M KCl buffer, pH 8.5, (1 ml) was added to the nuclear: myofibrillar pellet, left for 20 min at 4°, and centrifuged at 900 × g for 10 min. This procedure was repeated, and the 2
supernatants were pooled; the remaining pellet was vortexed in 2 ml of 0.01 M Tris-HCl: 1.5 mM EDTA: 0.5 mM dithiothreitol: 0.6 M KCl buffer, pH 8.5. This suspension was added to the pooled supernatants and centrifuged at 200,000 × g for 30 min to obtain a clear nuclear extract. The pellet was suspended in 5% (w/v) trichloroacetic acid for the DNA assay.

Single-Saturating-Dose Assay of [³H]Estradiol and [³H]R-5020-specific Binding Sites in the Fetal Uterus. Specific [³H]estradiol binding in the cytoplasmic and nuclear extracts were carried out by the [³H]estradiol exchange technique in protamine sulfate precipitates (4, 35) with the modifications previously described (31). The pellets were incubated with 1 × 10⁻¹⁰ to 5 × 10⁻⁸ M [³H]estradiol, with and without a 100-fold molar excess of unlabeled estradiol, to measure nonsaturable binding. Cytosol protamine sulfate precipitate incubations were carried out overnight at 30°, and nuclear extract precipitates were incubated at 4° overnight, followed by an incubation at 37° for 3 hr. [³H]R-5020-specific binding sites in the cytosol and nuclear extracts were measured after incubation with 4 × 10⁻⁹ M concentration of the radioactive steroid, with and without a 100-fold excess of the unlabeled steroid, overnight at 4°. Binding of radioactive steroid to macromolecules was determined after adsorption of the unbound steroid with a 0.05% (w/v) dextran-coated 0.5% (w/v) charcoal mixture for 10 min at 4° (18).

Affinity of the Binding of [³H]Estradiol and [³H]Tamoxifen to Cytosol Macromolecules. The affinity of [³H]estradiol and [³H]tamoxifen binding in the cytosol was determined according to the method of Scatchard (28). Aliquots (0.5 ml) of the cytosol fractions (0.8 to 1 mg protein per ml) were incubated in duplicate with [³H]estradiol (1 × 10⁻¹⁰ to 5 × 10⁻⁸ M) including parallel incubations with the same concentration of the labeled estradiol plus an excess concentration of the nonradioactive steroid of 5 × 10⁻⁷ M. Aliquots of the cytoplasmic extract were also incubated in duplicate with [³H]tamoxifen (2 × 10⁻¹¹ M to 1.5 × 10⁻⁸ M) with and without nonradioactive tamoxifen (1.5 × 10⁻¹⁰ M). Incubations were carried out at 4° for 16 hr. Binding was measured using the dextran-coated charcoal method as described previously.

Protein and DNA Assays. Protein was measured by the method of Lowry et al. (19), and DNA was determined according to the method of Burton (1).

Measurement of Radioactivity. For aqueous samples, Ready Solv HP (Beckman) was used. Other determinations were carried out in PPO:POPOP:toluene solution. The samples were counted in a liquid scintillation spectrometer (Nuclear Chicago; Model Isocap 300). The efficiencies were, respectively, 44 and 52%.

RESULTS

Competition of Tamoxifen, Nafinoxide, and Different Steroids with [³H]Estradiol for Binding to Estrogen Receptor in the Fetal Uterine Cytosol

The displacement curves (Chart 1) show that both tamoxifen and nafinoxide compete for [³H]estradiol binding, although with a lower intensity than estradiol, and that the binding inhibition is complete at higher antiestrogen concentrations (10 × 10⁻⁶ M). No competition is observed with progesterone and dehydroepiandrosterone. Androstenediol (5-androstene-3β,17β-diol) competes at very high concentrations.
Binding and Effect of Antiestrogens in Fetal Uterus

Chart 2. Double reciprocal analysis of specific binding of [³H]estradiol to cytosol receptor in the presence of tamoxifen or nafoxidine. Aliquots of uterine cytosol (0.5 ml containing 0.8 to 1 mg protein per ml) were incubated for 16 hr at 4° with [³H]estradiol (1 x 10⁻¹⁰ to 5 x 10⁻⁸ M) and with [³H]estradiol (1 x 10⁻¹⁰ to 5 x 10⁻⁸ M) plus unlabeled estradiol (5 x 10⁻⁷ M) in the presence or absence of unlabeled tamoxifen (A) or nafoxidine (B). Specific binding of [³H]estradiol was measured using the dextran-coated charcoal method.

Chart 3. Effect of nafoxidine and some steroids on [³H]tamoxifen binding in the fetal uterine cytosol of the guinea pig. Aliquots of the cytosol fraction (0.5 ml containing 0.8 to 1 mg protein per ml) were incubated with 2 x 10⁻⁹ M [³H]tamoxifen with or without different concentrations of nafoxidine and various unlabeled steroids for 16 hr at 4°. Specific binding was determined using the dextran-coated charcoal method. The data represent the average of 2 to 4 determinations. DHT, dihydrotestosterone.

Affinities for the estrogen receptor of estradiol and tamoxifen in studies using [³H]estradiol (Chart 1). These indicated that the relative binding affinity of tamoxifen for estrogen receptor was about 10% that of estradiol itself. The stability of [³H]tamoxifen during an overnight incubation with uterine cytosol at 4° was verified by thin-layer chromatography (as described in "Materials and Methods") after extraction of tamoxifen with ethyl acetate (3 times with 1 volume). The results showed that 90 to 95% of extracted radioactivity was unchanged [³H]tamoxifen.

Affinity of [³H]Tamoxifen Binding to Fetal Uterine Cytosol

As previously described, estradiol does not completely displace [³H]tamoxifen specific binding to uterine cytosol. Consequently, it was interesting to explore the possibility of another specific binding of [³H]tamoxifen different from that of estradiol receptor. Preliminary determinations using a [³H]tamoxifen concentration range of 0.2 to 15 x 10⁻⁹ M show the presence of a single class of specific binding sites with a dissociation constant (Kd) of 1.2 ± 0.4 nM and a concentration of sites of 2400 ± 400 fmol/mg protein. However, a Scatchard analysis in a [³H]tamoxifen concentration range of 0.02 to 15 nM shows the presence of 2 classes of binding sites: site A, binding [³H]tamoxifen with a lower affinity (Kd, 1.8 ± 0.4 nM) and a concentration of 1800 ± 100 fmol/mg protein; and site B, with a higher affinity (Kd, 0.39 ± 0.01 nM) and a concentration of 617 ± 77 fmol/mg protein (Chart 4). In order to establish which of these sites is the estradiol receptor, Scatchard analysis was performed in the presence of an excess of unlabeled estradiol (2 x 10⁻⁶ M) to saturate all the estrogen receptor sites. As shown in Chart 5, a residual component binding [³H]tamoxifen with the same high affinity and concentration as that for Site B is found. Chart 5 also shows the binding of [³H]tamoxifen that can be displaced by estradiol, i.e., its binding to estrogen receptor alone. The Kd and the concentration of these binding sites are similar to those of Site A previously described. Parallel saturation binding analysis with [³H]estradiol shows a high-affinity binding and a single class of sites with a Kd of 1.29 ± 0.54 x 10⁻¹⁰ M and a concentration of binding sites of 1650 ± 200 fmol/mg protein (not shown). It is concluded that the uterine cytoplasmic [³H]tamoxifen-binding sites showing the lower affinity (Site A) correspond to the estradiol receptor.
Analysis carried out in the fetal plasma shows a saturable cytosol could derive from a plasma contamination by determin

Binding of $[^3H]$Tamoxifen to Fetal Plasma

Because $[^3H]$tamoxifen binds to a different component of uterine cytosol than does estradiol, it was necessary to exclude the possibility that the second binding component of uterine cytosol could derive from a plasma contamination by determining the binding of $[^3H]$tamoxifen in fetal plasma. A Scatchard analysis carried out in the fetal plasma shows a saturable binding of $[^3H]$tamoxifen to a single class of sites with a $K_d$ of 8.8 ± 2.0 x 10^{-9} M which is different from the binding affinities of $[^3H]$tamoxifen in the cytosol fraction (not shown). Estradiol does not compete for this $[^3H]$tamoxifen binding of fetal plasma.

Biological Effect of Tamoxifen, Nafinoxidine, and Estradiol on the Fetal Uterus of the Guinea Pig

Uterotrophic Effect. The administration of 1-mg/kg/day doses of estradiol, tamoxifen, and nafinoxidine for 3 consecutive days resulted in a similar significant ($p < 0.001$) increase in uterine wet weight (+80%) determined 24 hr after the last injection (Chart 6). It is notable that a similar uterotrophic effect is obtained with a 10-mg/kg/day dose of tamoxifen for 3 days.

Effect on Progesterone Receptors. Chart 7 shows the effect of the treatment with estradiol, tamoxifen, and nafinoxidine on cytoplasmic and nuclear progesterone receptors. As indicated, while estradiol treatment provokes a remarkable increase in the progesterone receptors, tamoxifen and nafinoxidine have only a limited, but significant, ($p < 0.001$) stimulatory effect.

Effect on the Distribution of $[^3H]$Estradiol Specific Binding Sites. In order to investigate whether the actions of both the antiestrogens (increase of uterine wet weight and progestagen specific binding sites) were mediated by their interaction with estrogen receptors, the specific binding sites of estradiol were determined both in the cytosol and in the nuclear fraction.

As indicated in Table 1, after estradiol treatment, there is a significant ($p < 0.001$) decrease of cytoplasmic and total (cytoplasmic plus nuclear) receptors, while the nuclear receptors are not significantly different from those of controls.

After tamoxifen or nafinoxidine treatment, there is a lower but significant decrease of cytoplastic and total $[^3H]$estradiol-binding sites. However, tamoxifen and nafinoxidine cause a higher content of estrogen receptors in the nuclear fraction than after estradiol treatment ($p < 0.01$ and $p < 0.05$, respectively). Furthermore, no significant differences are found in cytoplastic and nuclear estrogen receptors between 1 and 10 mg/kg/day for 3 consecutive days of tamoxifen treatment.

Chart 4. Scatchard plot of $[^3H]$tamoxifen binding in cytosol fraction of fetal guinea pig uterus. Cytosol fraction (containing 0.8 to 1 mg protein per ml) were prepared from fresh uteri of guinea pig fetuses as described in “Materials and Methods.” Aliquots (0.5 ml) were incubated with $[^3H]$tamoxifen (2 x 10^{-11} to 15 x 10^{-9} M) at 4° for 16 hr. A parallel series of tubes contained 15 x 10^{-7} M unlabeled tamoxifen to determine nonspecific binding. Binding was determined using the dextran-coated charcoal method. Specific binding of $[^3H]$tamoxifen (calculated by the difference of the binding in the 2 series of tubes) is depicted. Nonspecific binding in the different experiments was -10% of the added radioactivity. The method of Rosenthal (26) for the resolution of the 2 binding sites was used.

Chart 5. Analytical study of the 2 binding sites of $[^3H]$tamoxifen in the cytosol of fetal uterus of the guinea pig. Cytosol fraction (containing 0.8 to 1 mg protein per ml) was prepared as described in Chart 4. Aliquots (0.5 ml) were incubated with $[^3H]$tamoxifen (2 x 10^{-11} to 15 x 10^{-9} M) with or without 15 x 10^{-7} M unlabeled tamoxifen (O) $[^3H]$tamoxifen (2 x 10^{-11} to 15 x 10^{-9} M) plus unlabeled estradiol (2 x 10^{-6} M unlabeled estradiol (2) Binding was determined using the dextran-coated charcoal method.

Chart 6. Effect of estradiol, tamoxifen, or nafinoxidine on the fetal uterine wet weight of the guinea pig. Estradiol (E) or nafinoxidine (N) (1 mg/kg/day) or tamoxifen (T) (1 and 10 mg/kg/day) were administered s.c. to the mother for 3 consecutive days; control animals (C) were given injections of vehicle alone. Fetuses were separated 24 hr after the last injection, and fetal uteri were isolated, stripped of adhering fat, and weighed. Each uterine weight of treated animals was expressed as the percentage of the average weight of controls (assigned the value of 100%) for each fetal age. The 100% control was determined from the average weight on the regression curve (determined by the method of least squares) of the weight of the uterus as a function of the weight of the fetus ($y = 1.65x - 15.36$, where $y$ is weight of uterus (mg) and $x$ is weight of fetus (g); r = 0.94: n = 48). Values represent the averages of 8 to 48 uteri. Bars, S.D. The statistical comparisons ($p$) between the treated and control uteri were analyzed using Student’s t test. $p < 0.001$. 

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DISCUSSION

Previous studies using unlabeled antiestrogens (e.g., tamoxifen and nafoxidine) have shown that these compounds compete with $[^3H]$estradiol for the binding to the cytosol estrogen receptor in the target tissues of different animal species (29, 32, 33). In this report, it is shown that the 2 antiestrogens, tamoxifen and nafoxidine, also inhibit $[^3H]$estradiol binding to the uterine cytoplasmic estrogen receptor in the fetal guinea pig and that this inhibition is competitive with respect to estradiol. In agreement with previous reports (29, 32, 33), the relative binding affinity of these antiestrogens (measured by indirect methods) to the estrogen receptor in the fetal uterus is lower than that of estradiol. Studies carried out using $[^3H]$-tamoxifen also show that this compound binds to the estrogen receptor with a lower affinity than that of estradiol and that the $K_d$ found is of the same order as that found in calf uterine and human mammary tumor cytosol (2, 21).

It is interesting to note that, in the Scatchard analysis using low concentrations of $[^3H]$tamoxifen, a second class of binding sites with higher affinity ($K_d = 0.39 \pm 0.01 \text{ nM}$) was found. This second binding site does not bind estradiol and could be similar to that found in the chick oviduct cytosol (32). This second binding component is not caused by fetal plasma protein contamination because, even if $[^3H]$tamoxifen binds to plasma proteins, the binding affinity is significantly lower than that found in uterine cytosol.

It is well known that antiestrogens have estrogenic and antiestrogenic properties depending on the dose used, the type of injection, and the animal species (11, 16, 17, 33). Since the fetal uterus of guinea pig has been shown to respond to estradiol treatment, with uterine wet weight increase and induction of progesterone receptors (23, 24), we have examined the effects of both tamoxifen and nafoxidine in these fetal tissues with regard to uterotrophic effect, progesterone receptors, and distribution of estradiol receptors. Tamoxifen, nafoxidine, and estradiol show a similar uterotrophic effect in the guinea pig fetus. The 2 antiestrogens also stimulate the progesterone receptor, but much less than does estradiol. These observations of the estrogenicity of the antiestrogens can be explained by their capacity to translocate the ligand:receptor complex into the nucleus, which has been shown in in vitro studies in the fetal uterus of guinea pig (31) and by the levels of receptor present in the nucleus 24 hr after the last administered dose of antiestrogen, as shown in the present paper. However, these levels are significantly higher than in estradiol-treated animals, indicating that the nuclei of the fetal uterus of guinea pig retains the antiestrogen:receptor complex for a longer period than the estradiol:receptor complex, as has already been shown in the target tissues in extraterine life (5).

Contrary to the results found in immature rat, which show an increase of uterine cytoplasmic estrogen receptors 24 hr after estradiol administration (5), the total number of cytoplasmic estrogen receptors in the fetal uterus of guinea pig diminishes drastically after estradiol administration and remains low 24 hr after the injection of the hormone (30). Moreover, this decrease cannot be accounted for by the amount of nuclear receptor, which is the same in control uteri and in uteri 24 hr after estradiol administration; therefore, the total amount of estradiol receptors (cytoplasmic plus nuclear) is decreased. This phe-

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**Table 1**

Effect of estradiol, tamoxifen, and nafoxidine treatment on fetal uterine cytoplasmic and nuclear $[^3H]$estradiol-specific binding sites

<table>
<thead>
<tr>
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<th>Control</th>
<th>Estradiol</th>
<th>Tamoxifen</th>
<th>Nafoxidine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytosol (pmol/mg DNA)</strong></td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td><strong>Nucleus (pmol/ mg DNA)</strong></td>
<td>0.66 ± 0.27</td>
<td>0.96 ± 0.38</td>
<td>3.11 ± 0.53</td>
<td>2.71 ± 0.06</td>
</tr>
</tbody>
</table>

**Total (pmol/mg DNA)**

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</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>12.54 ± 1.7</td>
<td>&lt;0.001</td>
<td>8.57 ± 1.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Estradiol</strong></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Tamoxifen</strong></td>
<td>3.45 ± 1.13</td>
<td>&lt;0.001</td>
<td>7.34 ± 0.89</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Nafoxidine</strong></td>
<td>2.15 ± 1.0</td>
<td>&lt;0.01</td>
<td>10.20 ± 2.1</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

**Average ± S.D. of n experiments using 1 to 3 uteri in each experiment.**
nomenon has also been observed in human breast cancer (MCF-7 cells) in culture (10). Similarly, as shown in the present paper, tamoxifen and nafioxin also reduce the total amount of estradiol receptors in the fetal uterus of the guinea pig, but this decrease is limited and significantly less than after estradiol treatment, which seems to correlate with the lower induction of the progesterone receptors by antiestrogens, a phenomenon already observed in MCF-7 cells in culture (9).

Since the effect of tamoxifen on progesterone receptors has been shown to be dose dependent in MCF-7 cells (11), we have tested the effect of a 10-fold higher dose of tamoxifen. In these experiments, no significant differences were found between the administration of 1 and 10 mg of tamoxifen on estrogen receptor system. Similar observations were made recently by others (3, 6), who reported that the injection of triphenylethylene derivatives, such as clomiphene citrate, tamoxifen, or nafioxin, either during pregnancy or early neonatal period, caused uterine stimulation and reproductive tract abnormalities, some of which presumably arose because of sustained estrogenic stimulation by these compounds.

It is concluded that the guinea pig fetal uterus can be used to study the biological response to both estrogens and antiestrogens.

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