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PROGESTERONE TREATMENT SUPPRESSES ESTROGEN RECEPTOR IN THE SEX SKIN OF *MACACA NEMESTRINA*

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Summary—We measured tightly bound nuclear estrogen receptors (ER) in sex skin biopsies obtained from pig-tailed macaques (*Macaca nemestrina*) which were previously ovariectomized and treated with an estradiol–progesterone regimen. Incubation of fresh tissue slices with a saturating concentration of [^{3}H]estradiol (E_2) was done to determine the capacity of nuclear acceptor sites to bind activated ER with high affinity. The radiolabeled ER was extracted from nuclei with 0.5 M KCl, complexed with an anti-ER monoclonal antibody, and quantitated by analysis on sucrose gradients. Even though serum E_2 levels were unchanged, 7 and 14 days of sequential progesterone (P) treatment decreased ER amounts below those found after 7, 14 and 21–23 days of E_2 treatment. ER regulation in sex skin of this species is similar to that found in macaque reproductive tract; P suppresses ER levels even in the presence of continuous E_2. The tissue responses of sex skin to the hormone treatments correlated well with the measured fluctuations of tightly bound nuclear ER, which suggests the functional significance of this ER component.

INTRODUCTION

The sex skin of pig-tailed macaques (*Macaca nemestrina*) and some other nonhuman primates undergoes cyclic changes during the menstrual cycle. Grossly, there are alternating periods of swelling caused by estradiol (E_2) and collapse induced by sequential luteal progesterone (P). These changes have been replicated by hormone treatments of ovariectomized animals [1]. The cellular and noncellular events which cause tumescence and detumescence of sex skin have been described at both the morphological and biochemical levels [1, 2]. The pathway through which E_2 and P regulate this tissue is probably mediated by receptors for both steroid hormones. Since we know from studies on other reproductive tract tissues that the regulation of estrogen receptor (ER) quantities is an important control point, we chose to measure the ER levels in sex skin, and to relate our findings to the tissue responses. We also describe the validation in sex skin of an assay which employs a monoclonal antibody prepared against ER, [^{3}H]E_2 and sucrose gradients to measure changes in the levels of tightly bound nuclear ER. The quantity of this species of ER is intimately related to tissue function, hence its designation as functional steroid receptor [3].

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EXPERIMENTAL

Animals

Sexually mature pig-tailed macaques (*Macaca nemestrina*, $n = 8$) were ovariectomized and allowed to rest for 6 weeks. Then a 3 cm E_2 Silastic capsule (i.d., 0.132 inches; o.d., 0.183 inches) was implanted subcutaneously on the back of each animal. After 14 days of E_2 treatment, a 6 cm capsule containing P was added for an additional 14 days. The P implant was then removed and the E_2 implant left in place. A series of 28-day cycles was created in each animal by this addition and removal of P, which resulted in periodic swelling of the sex skin when E_2 acted unopposed by P, and a decline in swelling when P was added; we know from previous studies that such capsules produce serum steroid levels in the physiological range for this species [1, 2, 4]. In some cases, the E_2 treatment was extended for 21–23 days before the P was added. Wedge biopsies of sex skin (approximately 2 g each) were removed after 7 ($n = 7$), 14 ($n = 8$) or 21–23 days ($n = 5$) of E_2 treatment or after 7 ($n = 3$) or 14 days ($n = 6$) of E_2 plus P treatment. Sequential biopsies were not generally done on the same animal within 28 days. Biopsies were placed directly into Hepes buffered Hanks balanced salt solution (pH 7) and transported to the laboratory within 10 min for ER assay. All animal handling, anesthesia, analgesia and surgical procedures were carried out under the supervision of the veterinary

staff of the Oregon Regional Primate Research Center according to the stipulations of the Animal Welfare Act.

Steroid radioimmunoassay (RIA)

The serum levels of E_2 and P in blood obtained at the time of all surgical biopsies were determined by RIA as previously described [5, 6] except that different antisera for E_2 [6] and P [8] were used.

Antibodies

The monoclonal antibodies H165 and H222 were raised against purified human MCF-7 cell extranuclear ER, and were provided as a gift by Abbott Labs (North Chicago, Ill.). The nonspecific control monoclonal antibody of the same immunoglobulin subclass was affinity-purified antiantigen B, a component of Timothy grass pollen (AT) kindly provided by Dr A. Malley (Oregon Regional Primate Research Center).

Estrogen receptor assay

We have developed an ER assay for sex skin that uses an anti-ER monoclonal antibody to separate ER from nonspecific binding on sucrose gradients. A similar approach has been used by us on prostate [9] and by others on macaque liver and human breast cancer cytosols [10]. Recent evidence [11–15] suggests that tissue homogenization and cell fractionation procedures may result in a redistribution of loosely bound, unoccupied ER from the nucleus to the cytosolic fraction. We attempted to minimize this by adding [^{3}H]E_2 *in vitro* at 37°C in order to occupy all ER, activate it and thereby increase its nuclear affinity. A series of preliminary experiments, performed in order to validate the assay procedure, are described in Results. This assay has been modified somewhat from our first report on this technique [2]. The charcoal step has been eliminated and the 10,000 *g* for 1 h centrifugation step on the high salt nuclear extract has been reduced to 10,000 *g* for 10 min. The following protocol was used for the ER assay on all animals in the 5 treatment groups.

One gram pieces of sex skin biopsies were sliced into 0.5 mm sections with a Stadie-Riggs tissue slicer (Arthur H. Thomas Co., Philadelphia, Penn.). The slices were rinsed with Trowell's medium (containing 0.7 g Hepes/100 ml, pH 7 at 37°C) and then incubated for 1 h at 37°C in the same medium with 10 nM [2, 4, 6, 7, 16, 17-^{3}H]estradiol (130 Ci/mmol, New England Nuclear Corp.) in an atmosphere of 60% O_2:6% CO_2:34% N_2. After incubation the tissue slices were rinsed with TEDGM [10 mM Tris–1.5 mM EDTA–1 mM dithiothreitol (DTT)–10% (v/v) glycerol–10 mM sodium molybdate, pH 7.4] and homogenized (approximately 100 mg/ml TEDGM) in an ice bath with three 15-s bursts with a PT-10 Polytron (Brinkman Instruments) at a power setting of 7, at 1 min intervals. The homogenate was centrifuged at 2500 *g* for 10 min at 4°C yielding a crude nuclear pellet. The crude nuclear pellet was washed with TEDGM three times by resuspension (with approximately half the homogenization volume) and recentrifugation at 2500 *g* for 10 min. The final washed nuclear pellet was resuspended (2–4 mg DNA per ml) with TEDK (10 mM Tris–1.5 mM EDTA–1 mM dithiothreitol–0.5 M KCl, pH 7.4) and vortexed at 15 min intervals for 1 h at 0°C. The suspension was centrifuged at 10,000 *g* for 10 min. The pellet was assayed for DNA [16], and the resulting supernatant was divided into 500 μl aliquots. Each aliquot was added to 5 μg of monoclonal antiestrophilin antibody H222 and allowed to sit for 1 h at 0°C and then subjected to sedimentation analysis on 5–20% (w/v) sucrose gradients prepared in TEK (TEDK minus dithiothreitol). Protein solutions used as sedimentation markers (BSA 4.5*s*, IgG 6.6*s*, catalase 11.3*s*) were layered on parallel gradients. The samples were centrifuged for 18 h at 205,600 *g* in a model L5-65 Beckman ultracentrifuge using a SW-41 rotor at 4°C. Following centrifugation the gradient tubes were punctured at the bottom and 28 fractions (25 drops each) were collected. Fractions containing sedimentation markers were read at 280 nm with a model 240 Gilford spectrophotometer. Fractions containing radiolabeled samples were mixed with 5 ml of Atomlite (New England Nuclear Corp.) and the radioactivity in each was measured with a model 460-CD Packard liquid scintillation spectrophotometer. The counting efficiency was at least 40% and DPM were calculated by the instrument based on a carbon tetrachloride quench correction curve. The peak of radioactivity in the 8*s* region of the gradient is made up of [^{3}H]E_2-receptor–antibody complexes. The area of the peak is a quantitative measure of receptor and is expressed as fmols per mg of DNA.

RESULTS

Serum steroids

The serum levels of E_2 and P obtained by RIA are shown in Fig. 1. Estradiol levels remained contant and in the physiological range in all treatment groups, while the serum P levels achieved were similar to the levels found during the natural luteal phase of the menstrual cycle in this species [1, 4].

Estrogen receptor assay validations

The significant parameters involved in the assay were investigated in several preliminary experiments, each replicated at least twice. The monoclonal antiestrophilin H222 formed an 8*s* complex with the [^{3}H]E_2-radiolabeled nuclear receptor, as shown on the high salt sucrose gradient in Fig. 2. This resulted in a shift of the ER peak away from the nonspecifically bound and free [^{3}H]E_2 near the top of the gradient. When no antibody or a non-specific monoclonal antibody of the same immunoglobulin subclass (AT) was added to the radiolabeled nuclear extract,

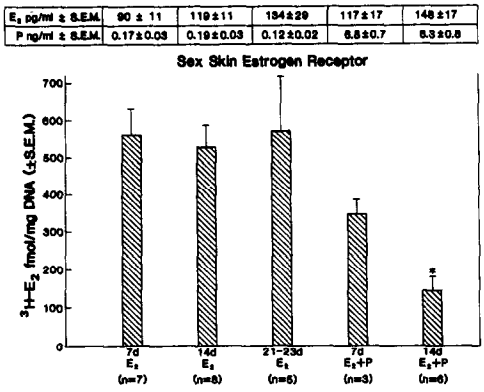

Fig. 1. The mean quantities of ER in nuclear extracts prepared from sex skin of pig-tailed macaques treated for 7, 14 or 21–23 days with E_2 or with the addition of sequential P for 7 or 14 days. The number of biopsies at each time is shown in parentheses. The mean serum levels of E_2 and P in each treatment group are presented in tabular form at the top of the figure. The asterisk indicates that the ER level in the 14 day E_2 plus P group is significantly less than in any of the groups treated with only E_2 (ANOVA, Duncan's multiple range test, $P < 0.05$).

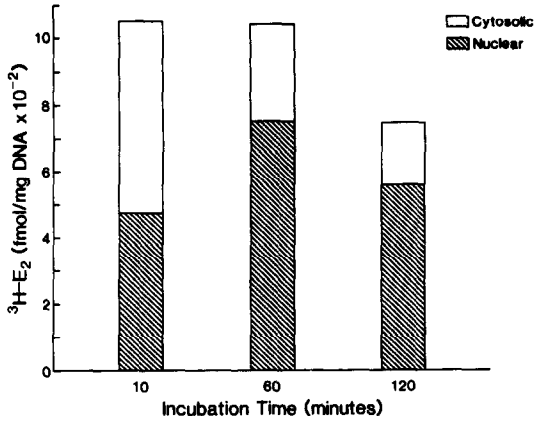

Fig. 3. The quantity of nuclear and cytosolic ER after 10, 60 and 120 min of incubation at 37°C. Slices of sex skin were incubated with 10 nM [^{3}H]E_2, homogenized and analyzed for ER as described in the legend for Fig. 2 except that cytosols were also mixed with antibody before analysis by sucrose gradient ultracentrifugation. All data are the means of two separate experiments.

only the 5*s* receptor peak was found which appears as a shoulder due to high background in this area (Fig. 2). However, when a different monoclonal antiestrophilin, H165, was mixed with the radiolabeled nuclear extract, the radioactivity was distributed in two peaks, one at 8*s* as with H222, and an additional one at 10*s*, as previously reported for this antibody-receptor complex in endometrium ([17]; Fig. 2). When a series of concentrations of [^{3}H]E_2 between 1.25 and 40 nM were used during *in vitro* incubation, there was saturation of binding at 5 nM and no difference between 5, 10, 20, 30 or 40 nM (data not shown). There was no difference in the size of the 8*s* peak when 2.5, 1.25, 0.6 or 0.3 μg of antibody H222 was used (305, 305, 301, 301 fmol/mg DNA, respectively); and 0.15 μg gave a smaller peak (284 fmol/mg DNA). 60 min of *in vitro* incubation gave a larger amount of nuclear ER than either 10 or 120 min of [^{3}H]E_2 exposure (Fig. 3). Cytosolic ER decreased from 10 to 60 min, though the quantity of total ER (nuclear plus cytosolic) was unchanged during this time (Fig. 3). By 120 min, total ER was decreased (Fig. 3). Extraction of nuclei for 1 h at 0°C with 10 mM pyridoxal 5′-phosphate (in 20 mM sodium barbitol, 5 mM DTT, pH 8.0) gave identical 8*s* peaks to when 0.5 M KCl was the extractant (data not shown). A linear relationship was shown to exist in the amount of receptor measured in a series of aliquots of nuclear extract increasing in volume from 25 to 500 μl (equivalent to from 0.1 to 4 fmol in an E_2 plus P-treated animal and from 0.7 to 24 fmol in an E_2-treated animal) of nuclear extract ($R^2 = 1.00$; Fig. 4).

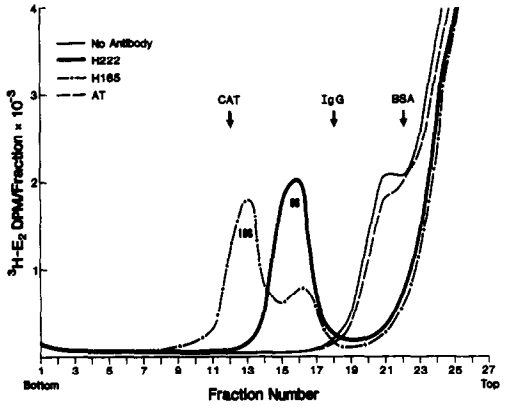

Fig. 2. Sucrose density gradient profiles of [^{3}H]E_2 binding to nuclear extracts of sex skin. Fresh tissue slices were incubated for 1 h at 37°C in the presence of 10 nM [^{3}H]E_2, homogenized at 0°C and then nuclear pellets were prepared by centrifugation. The washed nuclear pellets were extracted with 0.5 M KCl, some extracts were mixed with 5 μg each of anti-ER monoclonal antibodies (Ab) H222 or H165, or antiantigen B of Timothy grass pollen (AT), a nonspecific antibody of the same subclass (IgG_{2a}). The radiolabeled ER-Ab complexes were analyzed on 5–20% sucrose gradients prepared in TEK buffer (0.5 M KCl) for 18 h at 286,000 *g*. The positions of catalase (CAT), human immunoglobulin (IgG) and bovine serum albumin (BSA) marker proteins are indicated.

Estrogen receptor quantities in different treatment groups

There was no statistically significant difference in ER levels between the 7, 14 or 21–22 day E_2 treatment groups (Fig. 1). Fourteen days of sequential E_2 plus P treatment resulted in significantly lower E_2 receptor levels than in the 7, 14, or 21–23 day E_2-treated groups (Fig. 1; ANOVA, Duncan's multiple range test, $P < 0.05$). When the data from all groups treated with E_2 alone was pooled and compared to the data from all groups treated with E_2 plus P, there was a significant decline in the latter (Fig. 1; *t*-test, $P < 0.001$).

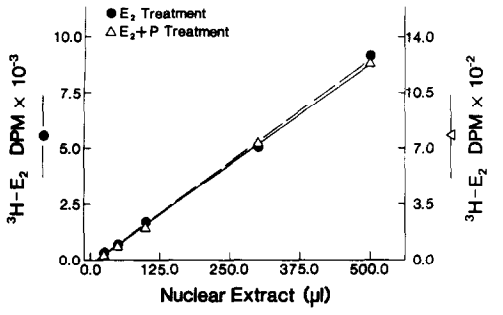

Fig. 4. Binding of [^{3}H]E_2 to receptor–antibody complexes in a series of nuclear extract dilutions. Sex skin biopsies from E_2- or E_2 plus P-treated pig-tailed macaques were analyzed on sucrose gradients and in each case a linear relationship was found between the amount of [^{3}H]E_2-receptor-antibody complex and the volume of nuclear extract ($R^2 = 1.00$). Note the 10-fold difference in the scales on the left and right ordinate axes.

DISCUSSION

Previously published studies on the ER in non-human primate sex skin have not adequately addressed the question of hormonal control by P. In our initial report on ER in sex skin [2] we used a charcoal treatment step in the assay which we have now found leads to a considerable underestimation of the quantity of ER, as has previously been reported by others [18]. Onouchi and Kato[19] showed in Japanese monkeys (*Macaca fuscata fuscata*) that estradiol benzoate injections increased nuclear ER amounts over the levels found in ovariectomized animals, however, they presented no data on cytosolic ER or on P effects. Ozasa and Gould[20] examined nuclear and cytosolic ER in sex skin obtained from chimpanzees (*Pan troglodytes*) during the menstrual cycle and found that late follicular samples had more nuclear ER (fmol/mg nuclear extract protein) than early follicular or luteal biopsies. Cytosolic ER (fmol/mg cytosol protein) did not significantly change during the cycle. Two ovariectomized chimpanzees were treated with an estrogen (mestranol) for 20 days, biopsied, then given progesterone for 1 day before another sex skin biopsy. The mean nuclear and cytosolic ER was numerically less after 1 day of P. Recent findings indicate that most or all ER is nuclear *in situ* and that cytosolic ER is an artifact of redistribution during homogenization. Thus, it is necessary to reexamine receptor regulation with new techniques which take advantage of advances in our knowledge of the mechanisms involved in hormone action. We have developed an assay that utilizes a monoclonal antibody specific for ER, in combination with a radiolabeling technique that attempts to activate all ER and measure the nuclear binding capacity. The component of activated nuclear ER tightly bound to the acceptor sites is believed to be the functionally significant form of ER.

The ER assay which we have developed is quantitative and has met the important criteria for specificity. The radiolabeling with high concentrations of steroid in fresh tissue slices at 37°C saturates unoccupied ER and, through receptor processing and exchange of endogenous E_2, achieves maximum occupancy of activated ER after 1 h. A quantitative shift of cytosolic ER to the nuclear fraction was shown between 10 and 60 min of *in vitro* radiolabeling. Between 60 and 120 min, cytosolic, nuclear and total ER were decreased, probably due to further nuclear processing and/or receptor degradation. Washing of the crude nuclear pellet with low salt buffer removes any activated ER which does not bind to nuclei with a high affinity and reduces free steroid levels. The subsequent extraction with 0.5 M KCl solubilizes the ER for antibody binding and sucrose density gradient analysis. The two monoclonal antibodies, which gave us equivalent results, are both well characterized [21]. The spatial separation of the ER–antibody complexes from excess free [^{3}H]E_2 at the top of the gradients decreases background radioactivity. The disappearance of the 5*s*[^{3}H]E_2-receptor peak as the larger complex with antibody attached shifts to 8*s* gives us confidence that nonspecific binding or [^{3}H]E_2 is not involved. We know that the antibody concentration used (5 μg) is in great excess so it is not limiting even when ER quantities vary in different preparations. The assay showed linearity even with very low ER levels.

The quantitative changes in ER which we report in this study correlate very well with the responsiveness of this tissue to the hormones and support our contention that we are measuring functionally significant ER. Our data shows that 7, 14 and 21–23 days of E_2 treatment produces identical amounts of ER, and that sequential P, even in the presence of continuous E_2, suppressed ER levels. Suppression of ER by sequential P also occurs in macaque oviduct, endometrium and cervix, although the absolute amount of ER is 2–3 times greater in reproductive tract tissues [22]. We are unable to directly compare our sex skin results with those of Ozasa and Gould[20] since they presented all of their data on a protein basis, however our ER levels during E_2 stimulation (about 550 fmol/mg DNA) are in good accord with the nuclear ER data reported by Onouchi and Kato[19] of about 275 fmol/mg DNA. The finding that ER quantities on a per unit of DNA basis are less in sex skin than in reproductive tract tissues is consistent with immunocytochemical studies which show that only the dermal fibroblasts stained for ER within the entire skin [2].

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