Unoccupied Nuclear Oestradiol-Receptor Sites in Normal Human Endometrium

Carlos LÉVY,* Rodrigue MORTEL,† Bernard EYCHENNE, Paul ROBET‡
and Etienne-Emile BAULIEU

ER 125 CNRS, Physiologie et pathologie cellulaire des hormones stéroïdes, and U 33 INSERM, Métabolisme moléculaire et physio-pathologie des stéroïdes, Faculté de Médecine Paris-Sud, 78 rue du Général Leclerc, 94270 Bicêtre, France

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The existence of unoccupied nuclear oestradiol-receptor sites in normal human endometrium was investigated. Nuclei were prepared from endometrial samples obtained by curettage and exposed to [^3H]oestradiol, which became maximally bound at 0°C within 1 h. This result contrasted with the binding kinetics of oestradiol-receptor complexes, since the exchange of hormone took at least 3 h at 30°C and no displacement occurred at 0°C. Before concluding that the nuclear sites were unoccupied, the presence of endogenous low-affinity ligands was excluded, because the association rate of oestradiol was unchanged after nuclei were stripped from their putative ligands, and the displacement of oestrone bound to nuclear receptor by oestradiol was very slow at 0°C. The available sites had high affinity for oestradiol (K_D 1.3 nM) and binding-specificity characteristics of oestradiol receptors. Similar results were observed with crude and purified nuclear preparations. It was concluded that a significant proportion of nuclear oestradiol receptors in normal human endometrium is unoccupied by endogenous hormones.

Experiments in the uterus of prepubertal or castrated rats have suggested that the formation of nuclear oestradiol-receptor complexes is the result of a two-step mechanism. The hormone combines first with a soluble receptor protein in the cytoplasm. Then the transfer to the nucleus is shown by a decrease of cytosol binding sites and an increase of nuclear oestradiol-receptor complexes (Brecher *et al.*, 1967; Jensen *et al.*, 1968; Gorski *et al.*, 1968; Rochefort & Baulieu, 1968; Jensen *et al.*, 1971). Almost no oestradiol-binding sites can be extracted from nuclei in the absence of hormone. In adult intact animals, where uterine cells are exposed to endogenous oestradiol, the presence of nuclear hormone-receptor complexes is expected and was indeed demonstrated (Clark *et al.*, 1972). We have described the variations of cytoplasmic and nuclear oestradiol and progesterone receptors in endometrium of normal women throughout the menstrual cycle (Bayard *et al.*, 1975, 1978; Robel

et al., 1979). The methods used measured the sum of empty receptor sites and those occupied by endogenous hormones, and consequently the existence of available nuclear receptor sites in adult intact tissues has not been investigated.

We now report the occurrence of significant proportions of available nuclear oestradiol-receptor sites in normal human endometrium.

Materials and Methods

Preparation of endometrial nuclei

Biopsy samples were taken from women undergoing uterine curettage or hysterectomy for benign gynaecological conditions. They were obtained during the secretory phase of the menstrual cycles, with few exceptions, placed immediately in 1 ml of preservation medium and stored in liquid N_2 (Bayard *et al.*, 1978). Receptor sites are stable under these conditions.

(a) *Crude nuclei.* When necessary, the biopsies were removed, thawed, and washed twice with 10 ml of ice-cold buffer A (1 mM-MgCl₂, 1 mM-dithioerythritol, 0.01% NaN₃ and 250 mM-sucrose in 20 mM-Tris/HCl, pH 7.8). All subsequent steps were performed at 0–4°C. Eight to twelve biopsies were pooled for each experiment. They were homogenized

* On leave from Instituto de Oncologia 'Angel H. Roffo', Buenos Aires, Argentina.

† On leave from Milton S. Hershey Medical Center, Hershey, PA 17033, U.S.A.

‡ To whom reprint requests should be addressed.

in 2 vol. of buffer A and centrifuged at 800 *g* for 10 min. The pellet was resuspended in 1 ml of buffer B (same as buffer A + 3 mM-CaCl₂ + 10 mM-KCl), filtered, and washed twice with 1 ml of buffer B (Bayard *et al.*, 1978).

(b) *Purified nuclei.* They were purified by a modification of the technique of Blobel & Potter (1966). Essentially, endometrial samples were thawed, and washed as described above, except that the buffer used was buffer D (5 mM-MgCl₂, 25 mM-KCl, 1 mM-dithioerythritol, 0.01% NaN₃, 50 mM-Tris/HCl, pH 7.8) containing 0.25 M-sucrose. The tissue was homogenized at slow speed in a glass-Teflon Potter homogenizer (0.15 mm clearance) with 3 × 4 strokes alternating with three pauses of 30 s each. The homogenate was then filtered through four layers of nylon gauze (100 μm aperture). Then 2.5 ml of filtrate was transferred to a polyallomer tube fitting a SW41 Ti rotor (Spinco Beckman) and mixed with 5 ml of buffer D containing 2.3 M-sucrose. Then 2.5 ml of buffer D containing 1.9 M-sucrose was layered under this, and centrifugation was performed at 82 500 *g* for 1 h. The supernatant was carefully removed, and the nuclear pellet resuspended in 0.5 ml of buffer D containing 1 M-sucrose, followed by the addition of 1 ml of buffer D containing 0.25 M-sucrose. Usually the contents of two tubes were pooled to yield 3 ml of nuclear suspension.

In order to assess the purity of nuclei, a drop of the nuclear preparation was submitted directly to phase-contrast-microscopic examination. In addition, the nuclear pellets were treated as follows for electron-microscopic evaluation: they were fixed at 4°C in glutaraldehyde (4%, v/v) in sodium phosphate buffer (0.05 M, pH 7.4) for 1 h, washed in the same buffer (0.1 M) and fixed again for 1 h in OsO₄ (1%) in the same buffer (0.05 M). After dehydration, fragments of the pellets were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

The concentrations of nuclear DNA, protein and RNA were measured by the techniques of Burton (1956), Lowry *et al.* (1951) and Fleck & Munro (1962) respectively.

Incubation of nuclei with radioactive oestrogens

The technique used was set up and reported in detail by Bayard *et al.* (1978). The measurement of nuclear receptor sites has been recently improved by the use of a glass-fibre filter technique (C. Lévy, B. Eychenne & P. Robel, unpublished work). In brief, glass-fibre filters mounted in disposable syringes were moistened with buffer B for the crude nuclei or with buffer D containing 0.25 M-sucrose for the purified nuclei. Then 110 μl of the same buffer was added, followed by 20 μl of steroid solution and 70 μl of nuclear suspension. After

incubation with radioactive oestradiol or oestrone in the presence or absence of non-radioactive competitor at 0 or 30°C, for the time indicated, the buffer was drained. Crude nuclei were washed with 1 ml of buffer C [same as buffer B + 30% (v/v) glycerol] containing 1% Triton X-100 and 7% (v/v) ethanol, followed by 8 ml of buffer C. Purified nuclei were washed with 1 ml of buffer E (same as buffer D, containing 0.25 M-sucrose and 30% glycerol) containing 1% Triton X-100 and 7% ethanol, followed by 8 ml of buffer E. The filters were dried and transferred into scintillation vials. To each 10 ml of toluene scintillator (Packard Omnifluor in toluene, 5.5 g/litre) was added, and the radioactivity was eluted from the filter by continuous shaking at room temperature for at least 4 h. The filters were then removed and the DNA content of adhering nuclei was measured. Each determination was made in triplicate. The radioactivities (d.p.m.) in individual incubations were corrected for small variations of DNA content and expressed in d.p.m./70 μl of nuclear suspension or in pmol of radioactive hormone/mg of DNA.

Results

Binding kinetics of [³H]oestradiol at 30°C and 0°C

Crude nuclei were incubated with a saturating 20 nM concentration of [³H]oestradiol at 30°C. The association curve reached a maximum in as early as 15–60 min (Figs. 1a and 1b). However, when a large excess of non-radioactive oestradiol was added after 1 h (Fig. 1b) or 18 h (Fig. 1c), the complete displacement of [³H]oestradiol bound to saturable nuclear sites took at least 3 h (Bayard *et al.*, 1978). In contrast, [³H]oestradiol could not be displaced from its nuclear saturable binding by non-radioactive hormone when the incubation was performed at 0°C (Fig. 1d), an expected property of oestradiol-receptor complexes. These results led us to assume that the saturable binding measured within 1 h at 30°C, or after 3 h at 0°C (Fig. 1a), represented essentially available receptor sites, and that the additional binding observed between 1 and 18 h (Fig. 1b) represented the binding of [³H]oestradiol to receptor sites occupied by endogenous oestradiol. On this basis, in the experiment reported on Fig. 1(b), available receptor sites represented about 60% of total receptor sites. In addition, the results reported in Figs. 1(b) and 1(c) showed the completely reversible character of the saturable nuclear binding, thus excluding the contribution of covalent reactions.

Before concluding that human endometrial nuclei contain an oestradiol receptor with a large proportion of unoccupied binding sites, it was necessary to rule out the presence of a low-affinity endogenous ligand that could be displaced by [³H]oestradiol from receptor sites even at low temperature.

Fig. 1. Binding kinetics of $[^3\text{H}]$ oestradiol to human endometrial nuclei

(a) Samples of endometrial nuclei ($55\mu\text{g}$ of DNA/ $70\mu\text{l}$) were incubated with 20nM - $[^3\text{H}]$ oestradiol without (total binding) or with $2\mu\text{M}$ non-radioactive oestradiol (non-saturable binding). Specific binding was obtained by difference between total and non-saturable binding. $\bullet$, Incubations at 30°C ; $\circ$, incubations at 0°C . (b) Samples of endometrial nuclei ($50\mu\text{g}$ of DNA/ $70\mu\text{l}$) were incubated with 20nM - $[^3\text{H}]$ oestradiol without ($\bullet$ — $\bullet$, total binding) or with $2\mu\text{M}$ non-radioactive oestradiol ($\bullet$ — $\bullet$, non-saturable binding) at 30°C . After 1 h of preincubation, $2\mu\text{M}$ non-radioactive oestradiol was added (arrow), and residual binding was measured in the corresponding samples after the stated times ($\blacksquare$ — $\blacksquare$). (c) Samples of endometrial nuclei ($42\mu\text{g}$ of DNA/ $70\mu\text{l}$) were incubated under the same conditions as in (b), except that non-radioactive oestradiol was added after 18 h of preincubation with 20nM - $[^3\text{H}]$ oestradiol. $\bullet$ — $\bullet$, Total binding; $\bullet$ — $\bullet$, non-saturable binding; $\blacksquare$ — $\blacksquare$, residual binding. (d) Samples of endometrial nuclei ($45\mu\text{g}$ of DNA/ $70\mu\text{l}$) were incubated in the same conditions as in (b). After 2 h of preincubation at 30°C , all samples were cooled at 0°C . Some were kept as such, whereas $2\mu\text{M}$ non-radioactive oestradiol was added to the others (arrow) and residual binding was measured in the corresponding samples for the stated times. $\bullet$ — $\bullet$, Total binding; $\bullet$ — $\bullet$, non-saturable binding; $\square$ — $\square$, residual binding at 0°C .

Oestrone was chosen because it is derived from oestradiol in human endometrium (Tseng & Gurpide, 1975). A large excess of non-radioactive oestradiol was added to $[^3\text{H}]$ oestrone-labelled nuclei. It was found that at 0°C the concentration of bound $[^3\text{H}]$ oestrone was unchanged after 2 h (Geynet *et al.*, 1972) (Fig. 2a), whereas available sites were filled by oestradiol under identical conditions (Fig. 1a).

To obtain additional arguments against the presence of any low-affinity endogenous ligand, whatever its nature, endometrial nuclei were stripped of putative low-affinity ligand by dilution and heating at 30°C for 1 h, and the saturable binding was measured at 0°C after 1, 5 and 16 h (Fig. 2b). After correction for loss of binding sites, the association rates of $[^3\text{H}]$ oestradiol to stripped nuclei and

Fig. 2. Lack of evidence for a low-affinity ligand bound to nuclear receptor sites

(a) Samples of endometrial nuclei ($40\mu\text{g}$ of DNA/ $70\mu\text{l}$) were incubated with 20 nM - $[^3\text{H}]$ oestrone without (total binding, ●—●) or with $2\mu\text{M}$ non-radioactive oestradiol (non-saturable binding, ●—●), at 30°C . After 2 h of preincubation $2\mu\text{M}$ non-radioactive oestradiol was added (arrow) and binding was measured in the corresponding samples after 2 h at 0°C (□—□). Similar samples were used as controls of exchange at 30°C (■—■). (b) A suspension of endometrial nuclei ($45\mu\text{g}$ of DNA/ $70\mu\text{l}$) was prepared. One half was used to measure the specific binding of $[^3\text{H}]$ oestradiol at 30°C (●) and at 0°C (○) for the stated times. The second half was diluted 1:1 with buffer B, heated at 30°C for 1 h, centrifuged at $800g$ for 10 min, and resuspended in the initial volume of buffer B, to remove any endogenous ligand. Specific binding was then determined at 0°C (△) and corrected for the decrease of binding sites relative to untreated samples (▲).

to control nuclei were identical, suggesting again that the binding sites of the latter were not occupied by a low-affinity ligand.

Equilibrium binding of $[^3\text{H}]$ oestradiol to purified nuclei

Phase-contrast-microscopic examination of purified nuclei showed essentially clean and intact nuclei, few broken nuclei and nuclear clumps with very scattered amorphous debris (Plate 1a). Under the electron microscope (Plate 1c), the nuclear morphology appeared relatively well preserved, with conservation of nucleoli as well as condensed and uncondensed chromatin. After exposure to Triton (Plates 1b and 1d), the outer nuclear envelope disappeared. In addition, the overall nuclear preparation was cleaner, with very little amorphous extra-nuclear material. Moreover, the RNA/DNA ratio varied between preparations from 0.07 to 0.1 and the protein/DNA ratio from 1.0 to 1.8. These values agree well with those reported in the literature for pure nuclear preparations (Roodyn, 1963; Blobel & Potter, 1966).

Purified nuclei were incubated at 0 – 4°C for 18 h with $[^3\text{H}]$ oestradiol (0.1 – 20 nM), and Scatchard plots were drawn from the results after correction for the contribution of non-saturable binding (Fig. 3). A straight line was obtained with a correlation coefficient of 0.99, suggesting a single category of non-interacting binding sites. The concentration of binding sites was 342 fmol/mg of DNA and the apparent dissociation constant was 1.3 nM . The latter value is similar to those reported in the literature for the cytosol oestradiol receptor of human endometrium (0.1 – 0.3 nM at 0°C) (Krishnan *et al.*, 1973; Crocker *et al.*, 1974; Makler & Eisenfeld, 1974; Tsibris *et al.*, 1978). In a similar experiment performed at 30°C for 3 h on another pool of biopsies, again a single category of binding sites, with a K_D of 1.1 nM , was observed.

Hormone specificity of the nuclear binding

Portions of a suspension of purified nuclei containing approx. $50\mu\text{g}$ of DNA were incubated at 0°C for 18 h, and the displacement of 2 nM - $[^3\text{H}]$ oestradiol by 200 nM concentrations of several non-

EXPLANATION OF PLATE 1

Purified endometrial nuclei

Nuclei were purified by a modification of the technique of Blobel & Potter (1966). (a) and (b), Phase-contrast microscopy ($\times 500$); (c) and (d), electron microscopy ($\times 7000$). Examinations were performed before (a and c) and after (b and d) washing with buffer E containing 1% Triton X-100 and 7% ethanol as described in the Materials and Methods section.

Fig. 3 Equilibrium binding of [^3H]oestradiol to nuclei of human endometrium

Nuclei prepared from a pool of endometrial biopsies ($66\mu\text{g}$ of DNA/ $70\mu\text{l}$) were incubated in triplicate with 0.1, 0.5, 0.75, 1, 2, 5 and 20 nM - [^3H]oestradiol without or with $2\mu\text{M}$ non-radioactive oestradiol at 0°C for 18 h, and the binding was assayed by the glass-fibre-filter technique. Abbreviations: B, concentration of specifically bound oestradiol; U, concentration of unbound oestradiol.

Table 1. Hormone specificity of nuclear binding
Purified nuclei ($55\mu\text{g}$ of DNA/sample) were incubated with 2 nM - [^3H]oestradiol, without or with a 100-fold molar excess of non-radioactive hormones, at 0°C for 18 h.

Inhibitor	Bound [^3H]oestradiol (fmol/mg of DNA)
None	252
Oestradiol	91
Diethylstilboestrol	31
Dihydrotestosterone	269
Progesterone	292
Cortisol	284

radioactive steroid hormones was investigated (Table 1). Complete displacement was observed with oestradiol and diethylstilboestrol, and none with other steroids.

Discussion

The presence of available nuclear receptor sites could be related to methodological bias. For

example, the use of frozen endometrial samples was considered as bringing about dissociation of oestradiol-receptor complexes. Therefore experiments were carried out with individual fresh endometrial biopsies, taken from normal secretory or gestational endometrium, and processed within 1 h. Nuclei were prepared and incubated with a saturating concentration of oestradiol either at 0°C or at 30°C . The sites labelled at 0°C represented a variable but often large proportion of those labelled at 30°C . At both temperatures, endometrial nuclei contained a single category of high-affinity binding sites. The possibility that the proportion of unoccupied receptor sites changes with the period of the menstrual cycle has not been investigated.

It is also conceivable that the presence of available receptor sites could be due to cytoplasmic contamination, although crude nuclear preparations had been treated with buffer containing Triton X-100 and ethanol in order to remove the outer nuclear membrane and remnants of rough endoplasmic reticulum. To rule out such a possibility, purified nuclear preparations have been used, and they contained large concentrations of available receptor sites, quite comparable with those measured in crude nuclei.

Unoccupied receptor sites have been demonstrated in the nuclei of a cell line derived from a human mammary cancer (Zava *et al.*, 1977) and in samples of human mammary adenocarcinomas (Garola & McGuire, 1977; Laing *et al.*, 1977; Panko & McLeod, 1978). These authors postulated that unoccupied nuclear sites are a distinctive feature of malignant cells, and that eventually receptor could activate DNA replication without bound oestrogen (Garola & McGuire, 1977). This conclusion was reached by relating the growth-inhibitory action of an anti-oestrogen, tamoxifen, to the presence of unoccupied receptor sites in MCF-7 cells (Zava *et al.*, 1977).

We now show that normal endometrial nuclei contain very significant amounts of oestradiol receptor unoccupied by endogenous ligand(s). This is not a unique observation in normal tissues. In the liver of laying hens, oestradiol receptors are almost entirely located in the nuclear compartment, and most of them seem to be unoccupied (Mester & Baulieu, 1972). It is conceivable that the distribution of oestrogen receptors between cytosol and nuclei, in the absence of hormone, might depend on the type of cell, animal species, or physiological condition considered. Also, dissociation of oestradiol from nuclear receptor sites might occur periodically, when oestradiol secretion decreases, whereas the recycling of nuclear receptor to cytoplasm might be delayed. This does not mean that unoccupied nuclear oestradiol-receptor sites are able to trigger subsequent nuclear events.

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