The unoccupied nuclear oestradiol receptor in the rat uterus and hypothalamus during the oestrous cycle

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The nuclear oestrogen receptor population in the rat uterus contained an unoccupied receptor component that bound oestradiol with the high affinity ($K_d \simeq 0.5$ nM) characteristic of oestrogen receptors. This unoccupied receptor was present at all phases of the oestrous cycle. Its content changed in parallel with that of the total nuclear receptor during the cycle. Oestradiol administration to the immature rat resulted in increases in the uterine content of long-term nuclear receptors (i.e., those still present 8 h after administration); these increases were due to occupied oestrogen receptors, since the content of unoccupied receptor was unchanged. Our previous experiments [White & Lim (1980) *Biochem. J.* 190, 833–837] have shown in contrast, that oestradiol administration results in an increase in the content of unoccupied nuclear receptor in the hypothalamus. However, as in the uterus, similar cyclic changes in the content of unoccupied nuclear receptor occurred in parallel with those of the total nuclear receptor population in the hypothalamus. Differences and similarities between the unoccupied nuclear receptor of the uterus and hypothalamus are briefly discussed.

It is widely accepted that the presence of oestrogen receptors in the nucleus of cells of target tissues is the result of translocation of cytosol oestrogen–receptor complexes (King & Mainwaring, 1974; Buller & O'Malley, 1976). In keeping with this view, the content of nuclear oestrogen receptors in the rat uterus increases on oestradiol stimulation and at the pro-oestrus phase of the oestrous cycle, when circulating oestrogen concentrations are maximal (Clark *et al.*, 1973). We have reported similar findings for the rat hypothalamus (White *et al.*, 1978). The nuclear receptor population has been shown to possess an unoccupied receptor component in human mammary tumours (Zava *et al.*, 1977; Panko & McLeod, 1978) as well as in normal human endometrium (Levy *et al.*, 1980). Unoccupied receptors have been reported to constitute a large proportion of the nuclear receptor population in hen liver, an oestrogenic tissue (Mester & Baulieu, 1972).

We have reported the presence of unoccupied nuclear receptors in the rat hypothalamus (White &

Lim, 1978, 1980). We now show that unoccupied nuclear receptors are present in substantial amounts in the rat uterus as well as the hypothalamus, and that changes in the content of these receptors occur during the oestrous cycle that parallel changes in the total nuclear receptor content in both tissues previously reported (White *et al.*, 1978).

Experimental

Materials

Female Wistar rats were obtained from Charles River (U.K.), Margate, Kent, U.K., or were bred in our laboratories from animals from the M.R.C. Animal Breeding Unit, Carshalton, Surrey, U.K. Where required, oestradiol (1.25 or 2.5 µg) was administered intraperitoneally in 50% (v/v) ethanol in saline (0.9% NaCl). [2,4,6,7(n)-³H]Oestradiol-17β (80–110 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation and assay of receptor in adult rats

Rats (60–65 days' old) were decapitated, and uteri and 'hypothalamic block' were removed and homogenized in 10 mM-Tris/HCl buffer (pH 7.4)/1 mM-EDTA/1 mM-dithiothreitol/10% (v/v) gly-

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cerol (TEGD buffer); cytosol and nuclear fractions were prepared as described by White *et al.* (1978).

(a) *Assay of cytosol oestrogen receptor.* Samples of cytosol were incubated with 5 nM-[³H]oestradiol for 2 h at 4°C in the presence or in the absence of excess (1 µM) of diethylstilboestrol; 0.2 ml samples were then assayed for specific high-affinity macromolecular [³H]oestradiol binding by exclusion chromatography on Sephadex LH-20 as previously described (White *et al.*, 1978).

(b) *Assay of nuclear total and 'unoccupied' receptor content.* Uterus. Crude nuclear pellets from uteri of adult rats were washed with four 25 ml volumes of ice-cold TEGD buffer; samples of a resuspension (in 10 times the original wet weight of tissue) were either (1) incubated at 4°C (unoccupied receptor) (Zava & McGuire, 1977; Levy *et al.*, 1980) or 37°C (total receptor) with [³H]oestradiol in the presence or in the absence of 1 µM-diethylstilboestrol for 1 h, left to stand for 1 h, washed with four 5 ml volumes of TEGD buffer, transferred to counting vials and their radioactivities counted in [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole]/toluene scintillant, or (2) extracted with 2 vol. of 1 M-KCl/TEGD buffer for 40 min and the supernatants incubated with 5, 13 or 30 nM-[³H]oestradiol with or without 1 µM-diethylstilboestrol for 1 h at 4°C (unoccupied receptor) or 37°C (total receptor) before measurement of specific macromolecular-bound radioactivity on Sephadex LH-20.

Hypothalamus. The crude nuclear pellets were washed, resuspended, divided into samples and incubated with [³H]oestradiol as in (b) (1) above, then washed with two 5 ml volumes of TEGD buffer and extracted with 2 vol. of 1 M-KCl/TEGD buffer for 40 min, after which the specific macromolecular-bound [³H]oestradiol was determined by using Sephadex LH-20. This procedure greatly decreased the background non-specific radioactivity, which had caused considerable problems in assays with the nuclear pellet. We found that 90% of the estimated receptor in the nuclear pellet appeared in the KCl extract, as previously reported (Roy & McEwen, 1977; White, 1978).

Preparation and assay of receptor in immature rats

A procedure identical with that for adults was followed, except that uteri from 29-day-old rats were homogenized in buffer composed of 10 mM-Tris/HCl (pH 7.4)/1 mM-CaCl₂/0.25 M-sucrose (TSC buffer) by using ten up-and-down strokes of a Teflon/glass Potter-Elvehjem homogenizer. The same buffer was used throughout. Nuclear receptor was assayed as in section (b) (1) above; the first postincubation wash contained 1% (w/v) bovine serum albumin to minimize non-specific [³H]oestradiol binding.

Results

Changes in nuclear oestrogen receptor content in the uterus during the oestrous cycle

Fig. 1(a) shows the changes in content of total and of 'unoccupied' nuclear high-affinity oestrogen receptor during the oestrous cycle. Total nuclear receptor was maximal during late dioestrus and pro-oestrus, falling 5-fold to low values during oestrus and metoestrus. This pattern is similar to that given in our previous report (White *et al.*, 1978), and corresponds closely to known changes in oestrogen secretion by the ovary during the oestrous cycle (Yoshinaga *et al.*, 1969). The 'unoccupied' nuclear receptor showed essentially the same pattern of change during the cycle.

Table 1 shows the results of Scatchard (1949) analysis of the equilibrium-binding data obtained in

Fig. 1. Total and 'unoccupied' nuclear oestrogen receptor content in rat uterus at each stage of the oestrous cycle: comparison of measurements on crude nuclei and KCl extracts of nuclei

Oestrogen receptor content, both total (▨) and 'unoccupied' (□), was measured in crude nuclear preparations (a), and KCl extracts of nuclei (b), from the pooled uteri of eight to ten rats in the same phase of the oestrous cycle. All four phases were monitored in this way. Details are given in the Experimental section.

Table 1. *Dissociation constants (K_d) for [^3H]oestradiol binding in uterine nuclei*

Equilibrium-binding studies were performed on nuclei from pooled uteri of eight to ten female rats, for each phase of the oestrous cycle, with measurement of both total receptor binding capacity and 'unoccupied receptor' binding capacity. Duplicate measurements were made at eight to ten concentrations covering the range 0.1–45 nM-[^3H]oestradiol. K_d values were calculated by Scatchard (1949) analysis of the data.

Phase	K_d (nM)	
	Total receptor	Unoccupied receptor
Pro-oestrus	1.0	0.5
Oestrus	0.4	0.6
Late dioestrus	0.8	0.3
Metoeestrus	0.3	0.4

these preparations. At each stage of the cycle the binding at 4°C showed the same high affinity for oestradiol, within experimental error, as the high-affinity component of the binding observed at 37°C, indicating that the receptor measured at 4°C is the same receptor that is measured in the 37°C assay. Similar findings were reported by Levy *et al.* (1980) for both crude and purified nuclear fractions prepared from human uterine tissue.

To eliminate the possibility of cytosolic contamination of these uterine nuclear preparations crude nuclei prepared from random cycling animals were resuspended in 2.2 M-sucrose and centrifuged at 60 000 g for 1 h at 4°C. The 'unoccupied' receptor content before and after partial purification of the nuclei was then determined; the values obtained were respectively 3.37 and 3.19 pmol/mg of DNA, showing that nuclear unoccupied receptors could not be attributed to cytosolic contamination.

The possibility that the labelling of nuclear receptor at 4°C was due to exchange was also examined. Crude nuclei were incubated for 2 h at 4°C with radioactive oestradiol (5 nM) in the presence or in the absence of diethylstilboestrol. Free steroid was removed by centrifugation and two washes with TEGD buffer. The specific radioactivity in these nuclei was then determined after periods of up to 4 h at 4°C. There was no significant change in receptor content (results not shown), indicating that dissociation of oestradiol from receptor was minimal at this temperature. It is therefore unlikely that the unoccupied sites measured in the nuclear preparations were the result of dissociation of oestradiol from the occupied sites.

Extraction of receptor from the nuclear pellet with 0.4 M-KCl-containing buffer (final concn.) and subsequent incubation with [^3H]oestradiol at 4°C (Fig.

Table 2. [^3H]Oestradiol-binding capacity and K_d in KCl extracts of uterine nuclei

Equilibrium-binding studies were performed as indicated in Table 1 on KCl extracts (see the Experimental section) of nuclei from the pooled uteri of eight female adult rats, with measurement of both total receptor binding capacity and 'unoccupied receptor' binding capacity. The K_d values were obtained by Scatchard (1949) analysis.

	Content (pmol/uterus)	Binding affinity K_d (nM)
Total receptor population	0.77	1.2
'Unoccupied' receptor population	0.56	0.5

1b) showed that KCl extracted 25–60% (average about 40%) of the total receptor under these conditions (in other experiments in this laboratory on immature, cycling and oestradiol-injected rats, these conditions have given a consistent average extraction of about 40%). Unoccupied receptors were also present in the extract. Both 'occupied' and 'unoccupied' receptor as well as total receptor followed the same pattern of change during the oestrous cycle as that observed for unextracted nuclei.

Binding of oestradiol to the KCl extracts was measured at 13 and 30 nM-[^3H]oestradiol (results not shown), as well as 5 nM-[^3H]oestradiol, confirming that the 4°C receptor was 95% saturated in 5 nM-oestradiol. In a separate experiment pooled uteri from a group of adult rats were extracted with 0.4 M-KCl-containing buffer, and the values for the binding of [^3H]oestradiol to the extract at 4 and 37°C were subjected to Scatchard analysis (Table 2): a binding component with the same high affinity as in whole nuclei was detected at both incubation temperatures.

To establish that cyclic changes in the unoccupied nuclear receptor contributed to those of the total nuclear receptor repeated measurements were made of the nuclear oestrogen receptor extracted by 0.4 M-KCl. The results in Table 3 show that cyclic changes in the content of unoccupied nuclear receptor occurred in parallel with those of the total nuclear receptor content.

Changes in uterine 'long-term' receptor after oestradiol administration

At 8 h after injection of immature female rats with 1.25 µg of oestradiol, total nuclear oestrogen receptor was about 2-fold higher than in age- and weight-matched control rats (Fig. 2). Uterine wet weight also showed a similar increase. The increase in

Table 3. *Changes in total and 'unoccupied' nuclear oestrogen receptor content in uterus during the oestrous cycle*

The cyclic changes in unoccupied and total nuclear receptor content were expressed as previously described by White *et al.* (1978). The results are obtained by relating individual measurements in each phase to the average value for the four combined phases. Values were combined from three separate experiments, each with five to eight pooled animals per phase, to give means $\pm$ s.d. Receptor content was measured in the KCl extracts of nuclear preparations (details given in the Experimental section).

Phase	Nuclear oestrogen receptor content	
	Total population	Unoccupied population
Pro-oestrus	1.64 ± 0.19	1.24 ± 0.17
Oestrus	0.52 ± 0.08	0.87 ± 0.06
Metoeustrus	0.34 ± 0.05	0.53 ± 0.05
Late dioestrus	1.49 ± 0.12	1.36 ± 0.16

Fig. 2. *Changes in total and 'unoccupied' nuclear oestrogen receptor in immature uteri, 8 h after oestradiol administration*

Oestradiol-17 β (2.5 μ g) was administered intraperitoneally to four 29-day-old female rats. These were killed after 8 h, together with four control animals of similar body weight. Nuclear oestrogen receptor content, both total and 'unoccupied', was measured in crude nuclear preparations from individual uteri. The histograms show means $\pm$ s.d. of the measurements for four individual animals. $\square$ Wet weight; other designations are as defined in Fig. 1.

receptor was accounted for by a several-fold increase in oestrogen-complexed nuclear receptor. The content of 'unoccupied' nuclear receptor (measured as specific [3 H]oestradiol binding after a 4°C incubation) at 8 h was unchanged from control values.

Fig. 3. *Changes in total and 'unoccupied' nuclear oestrogen receptor in hypothalamus during the oestrous cycle*

The results of four experiments, each with five to six pooled animals per phase, have been combined as means $\pm$ s.d. for each phase. Receptor content was measured in KCl extracts of the [3 H]oestradiol-receptor complex from crude nuclei (see the Experimental section). Figure designations are as defined in Fig. 1.

Changes in nuclear oestrogen receptor content in the hypothalamus during the oestrous cycle

The changes in total KCl-extractable nuclear oestrogen receptor during the cycle were essentially as previously reported in whole nuclei (White *et al.*, 1978), increasing during late dioestrus to a peak during pro-oestrus (Fig. 3). 'Unoccupied' receptor showed a similar pattern. The specificity of ligand binding, the affinity for oestrogen as well as the proportion of 'unoccupied' receptors present in these nuclear preparations, have been shown to be identical with those of 'unoccupied' receptors present in purified nuclei (White & Lim, 1980).

Discussion

The results reports in the present paper indicate that, in the normal adult female rat, unoccupied nuclear high-affinity oestrogen receptors are present throughout the oestrous cycle. The content of these receptors changed in parallel with that of the total oestrogen receptor in these as well as other target tissues (Thrower & Lim, 1980). This behaviour was in sharp contrast with that of unoccupied cytosol

receptors, the content of which are either unchanged, as in the uterus, or are depleted at pro-oestrus, as in the hypothalamus (White *et al.*, 1978). Our experiments on purified nuclear preparations (reported here and in White & Lim, 1980), as well as those of Carlson & Gorski (1980) on the rat uterus, and of Levy *et al.* (1980) on human endometrium, have discounted the possibility that unoccupied nuclear receptors arise from cytosolic contamination. That these receptors are complexed to oestrogens with low affinity and rapid rates of dissociation is a possibility discounted by Levy *et al.* (1980), at least in human uterine tissues.

There are, however, intriguing observations with regard to measurements of unoccupied and occupied nuclear receptor content. We have noted, that at certain phases other than pro-oestrus, the content of unoccupied receptors assayed at 4°C was in fact higher than the sum total of unoccupied plus occupied receptors assayed at 37°C (see, e.g., Fig. 3). This apparent discrepancy could be explained by a preferential and rapid destruction of unoccupied receptors during incubation at 37°C. Such a loss has in fact been observed by Carlson & Gorski (1980), who reported that incubation of unoccupied nuclear receptors at 37°C, even in the presence of 1.3 nM-oestradiol, led to their rapid destruction such that by 30 min only approx. 60% of unoccupied receptors (measured originally at 4°C) were detected. These results would imply that the measurement of total nuclear content by using assay procedures at 37°C would lead to an underestimation owing to selective loss of unoccupied sites.

Are these unoccupied nuclear receptors oestrogenically active? In the hypothalamus evidence from this laboratory has linked the unoccupied nuclear receptor population to the oestrogenic stimulus (White & Lim, 1978, 1980); the present data on the oestrous-cycle changes in hypothalamus are consistent with the possibility that once the active nuclear form of the receptor is established both occupied and unoccupied receptor may be oestrogenic.

In uterus our evidence from the oestrous-cycle study is equivocal: occupied, unoccupied and total receptor all increased during the period of oestrogenic activity, i.e. at pro-oestrus. We therefore examined the 'long-term' nuclear receptor (i.e., receptor still present 8 h later) in immature rats after oestradiol administration (Fig. 2). White & Lim (1978, 1980) have shown that, in the hypothalamus at 6 h after injection of oestradiol, occupied nuclear

receptor was barely increased, whereas unoccupied nuclear receptor had increased 3-fold. (Neonatally 'androgenized' females with defective hypothalamic responses to oestradiol did not show this increase.) A similar observation in uterus would constitute strong evidence that the unoccupied receptors in this tissue were oestrogenically active. Our results, however, did not give this pattern. The increase in uterine wet weight 8 h after oestradiol administration corresponded to an increase only in occupied nuclear receptor; unoccupied receptor was unchanged. Although this implies that the occupied receptor population alone was responsible for the response, we cannot rule out that the unoccupied receptor may be contributing to the basal degree of stimulation. The role, if any, of 'unoccupied' oestrogen receptors in the nucleus and their relevance to nuclear 'processing' of the oestrogen receptor remains unresolved.

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References

- Buller, R. E. & O'Malley, B. W. (1976) *Biochem. Pharmacol.* **25**, 1–12
- Carlson, R. A. & Gorski, J. (1980) *Endocrinology* **106**, 1776–1785
- Clark, J. H., Anderson, J. N. & Peck, E. J. (1973) *Adv. Exp. Med. Biol.* **36**, 15–59
- King, R. J. B. & Mainwaring, W. I. P. (1974) *Steroid-Cell Interactions*, p. 208, Butterworths, London
- Levy, C., Mortel, R., Eychenne, B., Robel, P. & Baulieu, E.-E. (1980) *Biochem. J.* **185**, 733–738
- Mester, J. & Baulieu, E.-E. (1972) *Biochim. Biophys. Acta* **261**, 236–244
- Panko, W. B. & McLeod, R. M. (1978) *Cancer Res.* **38**, 1948–1951
- Roy, E. J. & McEwen, B. S. (1977) *Steroids* **30**, 657–669
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Thrower, S. & Lim, L. (1980) *Biochem. J.* **190**, 691–695
- White, J. O. (1978) Ph.D. Thesis, University of London
- White, J. O. & Lim, L. (1978) *Biochem. Soc. Trans.* **6**, 1310–1312
- White, J. O. & Lim, L. (1980) *Biochem. J.* **190**, 833–837
- White, J. O., Thrower, S. & Lim, L. (1978) *Biochem. J.* **172**, 37–47
- Yoshinaga, K., Hawkins, R. A. & Stocker, J. F. (1969) *Endocrinology* **85**, 103–112
- Zava, D. T. & McGuire, W. L. (1977) *J. Biol. Chem.* **252**, 3703–3708
- Zava, D. T., Chamness, G. C., Horwitz, K. B. & McGuire, W. L. (1977) *Science* **196**, 663–664