Unoccupied nuclear oestrogen receptors in the female rat hypothalamus

Increases on oestrogen administration

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A major proportion of the hypothalamic nuclear oestrogen receptors were available for complexing with radioactive oestradiol *in vitro* at 4°C and were apparently unoccupied. At 6 h after oestradiol administration the content of unoccupied nuclear receptors had increased 2.5-fold and represented 71% of the total nuclear receptor content. These results suggest that unoccupied receptors may be active elements in the 'long-term' receptor population of the hypothalamus. Androgenized females had lower contents of these receptors.

Sexual behaviour in mammals is regulated to a large extent by oestrogens. In the female rat these hormones act on the hypothalamus, which plays a decisive role in sexual receptivity, in the regulation of ovulation and in the release of pituitary gonadotropins (Barraclough, 1973; Pfaff *et al.*, 1974). There are specific oestrogen receptors within the hypothalamus that translocate from the cytosol to the nucleus on oestrogenic stimulation (Anderson *et al.*, 1973; White *et al.*, 1977), as has been demonstrated for other classical target organs, e.g. uterus (King & Mainwaring, 1974; Myatt *et al.*, 1978).

In the target organs, the nuclear receptor is thought to be responsible for initiating genomic and physiological activity (Brenner & West, 1975; Buller & O'Malley, 1976). In keeping with this view, we have shown an increased concentration of the nuclear receptor in the hypothalamus at the pro-oestrous phase of the reproductive cycle (White *et al.*, 1978); this is the phase when ovulation occurs in response to increased gonadotropin secretion (Barraclough, 1973). We now report that the hypothalamic nuclear receptors are comprised of two populations: one containing bound oestradiol, and the other with the binding site unoccupied, and that the content of the latter population increases on oestrogenic stimulation. The unoccupied nuclear receptors may represent the physiologically active population, since lower amounts are found in rats with defective hypothalamic functions. These results have previously been reported in a preliminary form (White & Lim, 1978).

Experimental

Adult female Wistar rats (65–70 days old) from our animal colony were used; oestradiol (2.5 µg) was administered intraperitoneally in 0.2 ml of 25% (v/v) ethanol in saline (0.9% NaCl) and animals were killed by decapitation at the indicated time periods. Hypothalamic tissue was obtained as previously described (White *et al.*, 1978) and homogenized in 4 vol. of TED buffer (10 mM-Tris/HCl, pH 7.6, 1.5 mM-EDTA, 1 mM-dithiothreitol); a crude nuclear pellet was obtained by centrifugation of the homogenate at 1400g for 10 min at 4°C followed by at least four similar re-suspension and centrifugation procedures. The initial supernatant was centrifuged at 200000g for 1–1.5 h at 4°C to prepare the cytosol fraction.

Measurement of nuclear receptors

(a) *Total receptors by exchange assay in vitro* (37°C). Nuclear samples were incubated in the presence of a saturating amount of [2,4,6,7(n)-³H]-oestradiol-17β (sp. radioactivity 90–100 Ci/mmol, final concentration 2 nM; The Radiochemical Centre, Amersham, Bucks., U.K.) with or without a 200-fold excess of diethylstilboestrol at 37°C for 1 h. Unbound radioactivity was removed by successive re-suspension and centrifugation (five to six times) of the pellet (5 ml of TED buffer, 1400g for 10 min) (White *et al.*, 1978).

(b) *Binding in vivo* ('replaceable/specific'). This method was used on nuclear samples prepared from animals that had received radioactive oestradiol. Radioactivity was either extracted directly or after

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replacement of that fraction which was bound to receptor by incubation with a 200-fold excess of diethylstilboestrol at 37°C for 1 h; radioactivity exchanged during this procedure was removed by centrifugation. This procedure gave values for the amount of [³H]oestradiol specifically and non-specifically bound to nuclear material, which were similar to that obtained when two groups of animals were injected with excess of [³H]oestradiol of either high or low specific radioactivity (8 and 0.008 Ci/mmol respectively; results not shown).

(c) *Unoccupied receptors*. A similar procedure to that used in method (a) was followed, except that the incubation temperature was 4°C/1 h. Radioactivity in the final nuclear pellets was extracted into a final volume of 10 ml of 0.4% 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/toluene and assayed in a Beckman LS.330 spectrometer at 46% efficiency. Specifically bound radioactivity was determined by difference on the basis of diethylstilboestrol competition.

(d) *Measurement of cytosol receptors*. Duplicate samples (0.2 ml) in two parallel series containing a saturating amount of [³H]oestradiol (sp. radioactivity 90–100 Ci/mmol, final concentration 5 nM) were prepared, one series with and the other without a 200-fold excess of diethylstilboestrol. After incubation Sephadex LH-20 exclusion chromatography (Ginsburg *et al.*, 1974; Thrower *et al.*, 1976) was used to fractionate macromolecular-bound radioactivity in the incubation mixtures; specific receptor binding was determined on duplicates by difference on the basis of diethylstilboestrol competition.

Results and discussion

Nuclear oestrogen receptor measurements in vivo and in vitro: presence of unoccupied receptors?

We first compared receptor measurements obtained by using the assay *in vitro* described by Anderson *et al.* (1973) with those involving determination of [³H]oestradiol already bound *in vivo* to receptor to see if they corresponded (see the Experimental section). Both measurements were made on the same nuclear fractions prepared from rats previously injected with excess (2.5 µg) of [³H]oestradiol for up to 6 h after the injection, so that translocation and retention of these oestrogen receptors could be monitored. As shown in Table 1 the administration of oestradiol results in a significant depletion of cytosol receptor within 60 min, followed by a gradual recovery to preinjection values. The corresponding values of nuclear content increased to a maximum 30–60 min after injection, and declined subsequently. The values, measured by using the two different methods, were comparable for the first 2 h. However, at later times there was a significant difference, measurements of nuclear

content made by the exchange procedure *in vitro* being in excess of those involving direct measurements of [³H]oestradiol bound *in vivo* to receptor. These results strongly suggest the presence of unoccupied receptors, the content of which at 3 and 6 h could explain differences between measurements *in vivo* and *in vitro*. Unoccupied nuclear receptors have been observed in mammary tumours (Panko & MacLeod, 1978), a human breast cancer cell line (Zava & McGuire, 1977) and more recently in normal human endometrium (Levy *et al.*, 1980). An alternative explanation would be the presence of endogenous oestrogen–receptor complexes, which would exchange *in vitro* at 37°C but would not be labelled on administration of [³H]oestradiol.

Oestrogen-stimulated increases in unoccupied nuclear receptors

We therefore performed experiments to determine whether ‘unoccupied’ receptors were present in the hypothalamus and whether these could account for the difference in the measurements shown in Table 1. Animals were injected this time with a hyperphysiological dose (2.5 µg) of unlabelled oestradiol and receptor content was determined at various times after injection. The content of ‘unoccupied’ nuclear receptors was determined by incubation of nuclear fractions at 4°C, at which temperature exchange of exogenous [³H]oestradiol with receptor-bound oestradiol is minimal, whereas exogenous [³H]oestradiol can occupy empty binding sites (Clark & Peck, 1979). Measurements of total nuclear receptor content (i.e. both ‘occupied’ and ‘unoccupied’) as well as cytosol receptor content were also made. The values for total nuclear receptor content (Table 2) matched those shown in Table 1, being maximal at 60 min after the administration and decreasing thereafter. The changes in content of cytosol receptor were also similar to that seen previously. ‘Unoccupied’ nuclear receptors were indeed found to be present. The content of these increased after oestrogen administration such that at 3 and 6 h the value of 4–5 fmol/brain was found to be approximately equivalent to the difference between the measurements of receptor content determined by the exchange procedure *in vitro* and those derived from direct estimation of receptor-bound [³H]oestradiol shown in Table 1.

When comparisons were made of the content of unoccupied receptor after periods of incubation *in vitro* from 1 to 24 h at 4°C, no significant differences were observed, showing that at 4°C negligible dissociation of oestrogen–receptor complexes occurred. A similar observation, that precharged receptors did not dissociate at 4°C, was made by Zava & McGuire (1977) in their studies on unoccupied receptors; more recently Roy & McEwen (1979) have demonstrated that there was

Table 1. *Changes in hypothalamic oestrogen receptor content on oestradiol administration*

Groups of female rats (five animals/group) were injected intraperitoneally with 2.5 µg of [³H]oestradiol-17β (sp. radioactivity 8–18 Ci/mmol). At the indicated times animals were killed and hypothalamic tissue was obtained; the tissue in each group was pooled before preparation of nuclear and cytosol fractions. The nuclear pellet was re-suspended in a final concentration corresponding to one hypothalamic block/ml of TED buffer and replicate 0.5 ml samples were used for the following measurements: (a) measurements *in vitro* by the method of Anderson *et al.* (1972); (b) nuclear receptor measured by the direct method. Radioactivity in nuclear samples, obtained from animals injected with [³H]oestradiol, was extracted either directly or after replacement of receptor-bound radioactivity with the specific competitor; (c) cytosol receptor: {cytosol from animals injected with [³H]oestradiol was incubated at 4°C for 18–24 h with a saturating and high concentration (5 nM) of [³H]oestradiol (sp. radioactivity 90–100 Ci/mmol) in the presence or absence of a 200-fold excess of diethylstilboestrol for detecting specific cytosol receptors}. All details are given in the Experimental section. Results are means ± s.d., with the numbers of determinations shown in parentheses. Statistical analysis was by Student's *t* test.

Hypothalamic oestrogen receptor content (fmol/brain)			
Nuclear content			Cytosol content
Time (h)	(a) By exchange <i>in vitro</i>	(b) By measurement of [³ H]oestradiol bound <i>in vivo</i> to receptor	
0	4.4 ± 2.3 (7)	—	62.6 ± 9.5 (10)
0.25	14.1 (2)	17.1 (2)	—
0.5	20.4 ± 2.4 (3)	21.2 (2)	16.2 (2)
1.0	19.3 ± 5.2 (7)	23.6 ± 3.7 (5)	28.1 ± 9.5 (6)**
2.0	16.7 ± 3.3 (4)	17.8 ± 3.7 (3)	27.2 (2)
3.0	10.4 ± 1.5 (6)*	5.6 ± 2.2 (4)	44.4 ± 17.6 (6)
6.0	6.4 ± 1.5 (6)*	2.1 ± 1.5 (6)	48.7 ± 14.7 (5)

* (a) significantly different from (b) ($P < 0.01$).

** Significantly different from uninjected values ($P < 0.001$).

Table 2. *Changes in content of unoccupied nuclear receptor on oestradiol administration*

Groups of female rats (65–70 days, five animals/group) were each injected intraperitoneally with 2.5 µg of unlabelled oestradiol and killed at the indicated time intervals. Tissue preparation, subcellular fractionation and determination of cytosol oestrogen receptor content were as described in Table 1. The content of total (occupied and 'unoccupied') nuclear receptor (a) and unoccupied receptor (b) was determined after incubation of samples in [³H]oestradiol at either 37 or 4°C (see the Experimental section). The unoccupied receptor contents 1 h* and 6 h** after oestradiol injection were significantly higher than that at time zero ($P < 0.05$ and $P < 0.001$ respectively; statistical analysis by Student's *t* test). Results are means ± s.d., with the numbers of determinations shown in parentheses.

Hypothalamic oestrogen receptor content (fmol/brain)				
Nuclear content			(c) Unoccupied total (%)	Cytosol content
Time (h)	(a) Total (37°C)	(b) Unoccupied (4°C)		
0	3.5 ± 1.6 (9)	2.1 ± 0.9 (8)	60	62.6 ± 9.5 (10)
1	24.8 ± 5.8 (3)	3.5 ± 0.9 (3)*	14	27.9 ± 9.2 (4)
3	12.4 ± 2.9 (5)	3.2 ± 1.8 (6)	26	57.4 ± 12.8 (4)
4.5	9.8 (2)	4.7 (2)	48	58.2 ± 8.1 (3)
6	7.2 ± 1.8 (4)	5.1 ± 1.1 (4)**	71	55.8 ± 14.9 (3)

only approx. 10% dissociation of charged hypothalamic nuclear oestrogen receptor after 16 h at 4°C, whereas at shorter time periods there was negligible dissociation. However, it is not possible at this time to discount the possibility that a ligand other than oestradiol could have dissociated from

the nuclear receptor at 4°C and allowed radioactive oestradiol to occupy the receptor *in vitro* at 4°C.

To discount the possibility that nuclear unoccupied receptors were the result of cytosol contamination, we prepared purified nuclei from rats that had received 2.5 µg of oestradiol 6 h

previously. The nuclei were purified by the method of Lovtrup-Rein & Grahn (1970) based on sedimentation of crude nuclear fractions through dense sucrose (Lovtrup-Rein & McEwen, 1966). The crude and purified nuclear fractions were substantially free of cytosol contamination, containing only 0.83 and 0.03% respectively of the lactate dehydrogenase (a cytosolic enzyme marker) activity of the homogenate. These purified nuclei indeed contained unoccupied receptors and these were present in the same proportions as in the original crude nuclear preparations. In two separate experiments, each involving eight animals, the proportion of unoccupied receptors was 60 and 54% of the total nuclear content (cf. Table 2), strongly excluding cytosol contamination of our crude nuclear preparations as being responsible for the presence of unoccupied receptors. The content of unoccupied receptors in the crude nuclear fractions was also determined before and after deliberate contamination with cytosol. No increase was noted in the content of unoccupied receptor in the deliberately contaminated nuclear preparations. The equilibrium dissociation constant of the nuclear binding measured at 4°C was determined and gave an average value of 3.5 nM (5.8 and 1.2 nM), which was higher than the K_d of <1 nM for cytosol and occupied nuclear receptor (White *et al.*, 1978). Oestradiol-17 β and diethylstilboestrol were effective competitors for this binding, whereas testosterone, progesterone and cortisol showed only limited competition.

Unoccupied receptors and defective hypothalamic functions

It is well established that a single injection of testosterone to neonatal female rats will result in adults that are sterile as a consequence of defective hypothalamic functions (Barracrough, 1967; Gorski, 1968). Anovulatory adult female rats, androgenized at 4 days after birth by a single subcutaneous injection of testosterone propionate (Barracrough, 1967), were used in groups of five when they were 65 days old. Evidence of continuous acyclicity was provided by daily microscopic examination of vaginal smears obtained from the adult. The effects of administering excess of oestradiol (2.5 μ g) to these anovulatory 'androgenized' rats were examined. In these rats gross changes in the hypothalamic receptor content are unaltered from the normal pattern of cytosol receptor depletion and replenishment, accompanied by increases and subsequent decreases in total nuclear receptor content, shown in Table 1. However, the content of 'unoccupied' nuclear receptor was lower than in the corresponding preparations of normal rats. When grouped together for all time points from 0 to 6 h with five animals per time point, the mean value for

'unoccupied' nuclear receptor (fmol/brain) in 'androgenized' rats and normal rats was, respectively, 1.91 ± 0.89 (4) and 3.72 ± 1.2 (5) [mean $\pm$ s.d. (n)], the difference between the two values being significant ($P < 0.025$).

Conclusions

It has been proposed that 'long-term' nuclear receptors (i.e. those still present 6 h after the administration of oestradiol) are responsible for mediating the physiological response of the uterus to oestrogen (Clark *et al.*, 1973; Clark & Peck, 1976). Our results, showing that in normal rats 'unoccupied' receptors constitute the majority of receptors in the hypothalamus 6 h after oestradiol administration, as well as in uninjected rats (Table 2), suggest that these receptors are active elements in the receptor population. Interestingly, in androgenized rats (Puig-Duron & MacKinnon, 1978), on oestradiol administration normal oestrogen-induced increases in serum prolactin were obtained despite a marked diminution of the lutropin response. It is possible that this diminished response in apparent production of the hypothalamic luteinizing hormone may be related to failure to produce unoccupied nuclear receptors on oestradiol stimulation (the retention of normal prolactin response may relate to retention of normal processes of oestrogen-stimulated translocation of receptor into the nucleus). The administration of luteinizing hormone to adult androgenized animals, which obviates the need for hypothalamic stimulation, will indeed restore the ability to ovulate and to conceive (Hahn & McGuire, 1978). Whether or not changes in the content of nuclear unoccupied receptors are unique to the hypothalamus, which exhibits a relatively limited response to oestradiol that does not involve tissue growth, remains to be established.

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