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Evidence for the origin of the unoccupied oestrogen receptor in nuclei of a human breast-cancer cell line (MCF-7)

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The origin of the unoccupied nuclear oestrogen receptor (R_n) was studied. Three working hypotheses were investigated. (*a*) R_n is a dissociation product of the oestrogen occupied nuclear receptor (ER_n). (*b*) ER_n is only partially occupied, so that additional binding may occur at 0°C (the temperature at which oestradiol saturates unoccupied sites). (*c*) R_n is derived from the penetration of unoccupied cytoplasmic receptor (R_c) into the nucleus. The MCF-7 cell line was used as a model in the present investigation. The amount of unoccupied receptors was measured by saturation with 7.5 nM-[^{3}H]oestradiol at 0°C, whereas the occupied receptors were measured by exchange at 30°C. The cells at preconfluency were exposed to a medium fortified with 10 nM-[^{3}H]oestradiol for 1 h, washed and cultured up to 5 days in fresh growth medium. The distribution of oestradiol receptors was determined before exposure and during the following 5 days. After 1 h exposure only ER_n was found in the nuclear fraction. Thereafter ER_n declined continuously so that on day 5 it approached 15% of its value measured 1 h after exposure. Although after 3 days about 80% of ER_n disappeared no R_n appeared, which contradicts hypotheses (*a*) and (*b*). On day 4 R_n and R_c appeared simultaneously. The appearance of R_n and R_c was not prevented by culturing the cells in an oestrogen-free medium, supporting hypothesis (*c*). Exposure of cells to increasing concentration of [^{3}H]oestradiol (0.1–10 nM) for 1 h resulted in a parallel increase in ER_n without increasing the amount of unoccupied binding sites, which contradicts hypothesis (*b*). The present study supports the hypothesis (*c*), i.e., R_c may also penetrate the nucleus without binding to oestradiol.

R_n were demonstrated in an oestrogen-responsive human breast-cancer cell line MCF-7 (Zava *et al.*, 1977; Zava & McGuire, 1977; Geier *et al.*, 1979*a*, 1981), and in human cancerous breast tumours (Garola & McGuire, 1977*a*,*b*; Panko & McLeod, 1978; Geier *et al.*, 1979*a*,*b*). More recently it was demonstrated that R_n were present also in normal oestrogen target tissues: human endometrium (Geier *et al.*, 1980; Levy *et al.*, 1980), human myometrium (Giannopoulos *et al.*, 1980), ovariectomized and adrenalectomized pig uteri (Jungblut *et al.*, 1978) and in untreated immature rat uteri (Carlson & Gorski, 1980).

Although the current model of oestradiol action on its target cell assumed the presence of an oestradiol occupied receptor in the nucleus, the previous findings may indicate that R_n is a necessary product in the normal mechanism of oestradiol action in its target tissue. The origin and the physiological function of this R_n is as yet unknown. In the present study we tried to clarify the origin of R_n. Three working hypotheses were investigated. (*a*) R_n is a dissociation product of the ER_n. (*b*) ER_n is only partially occupied, so that additional binding may occur at 0°C (the temperature at which oestradiol saturates unoccupied sites). (*c*) R_n is derived from the penetration of R_c into the nucleus.

The MCF-7 cell line was used as a model in the present investigation. The results suggest that R_c may also penetrate the nucleus without binding to oestradiol.

Abbreviations used: R_c, unoccupied cytoplasmic oestrogen receptor; R_n, unoccupied nuclear oestrogen receptor; ER_n, occupied nuclear oestrogen receptor.

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Materials and methods

Cell culture

MCF-7 cells were generously provided by Dr. Charles M. McGrath (Michigan Cancer Foundation, Detroit, MI, U.S.A.) and grown as previously described (Geier *et al.*, 1979*a*).

In one experiment charcoal-stripped serum

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medium was used. Serum stripped of endogenous hormones was prepared by incubating the calf serum with charcoal/dextran [5% Norit A and 0.5% dextran (grade C) in 10mM-Tris/HCl buffer, pH 8.0] at 0°C for 30min. This procedure removed more than 90% of a trace amount of [^{3}H]oestradiol. The charcoal was removed by centrifugation at 5000*g* for 7min. In the present investigation cells from passage 280 were used.

The preconfluent or confluent cells in Falcon flasks were washed with cold phosphate-buffered saline [136mM-NaCl/5.3mM-KCl/8.1mM-Na_2HPO_4/14.6mM-KH_2PO_4 (pH 7.4)] and were stored for 1–5 days at −90°C until assayed.

Homogenization and fractionation

The nuclear and cytosol fractions were prepared as described previously (Geier *et al.*, 1981). Briefly, the frozen cells were thawed by adding TED buffer (10mM-Tris/HCl, pH 7.4, containing 1.5mM-EDTA and 0.5mM-dithiothreitol) and harvested by scraping the Falcon flask with a rubber policeman. The cells were homogenized and centrifuged at 800*g* for 10min to obtain the nuclear pellet. This pellet was washed twice and considered as the nuclear fraction. The cytosol was obtained by centrifugation of the low-speed supernatant at 110000*g* for 30min.

Measurement of oestrogen receptors

Oestrogen receptors were determined by a single-dose assay as described in detail previously (Geier *et al.*, 1981). Portions of nuclear or cytosol fractions were incubated wtih [^{3}H]oestradiol. Non-specific binding was determined by parallel incubation with [^{3}H]oestradiol plus a 100-fold excess of diethylstilboestrol. Unoccupied receptors were measured at 0°C after 4h of incubation. Total receptors were determined by incubation at 30°C for 3h. Occupied receptors were calculated as the difference between total and unoccupied receptors. Separation of bound and free oestradiol in cytosol was accomplished by adsorption with a charcoal/dextran mixture, and in the nuclear fraction by repeated washing with TED buffer.

Cytoplasmic progesterone-receptor assay

Progesterone receptors were assayed by using the Pichon & Milgrom (1977) technique with some modifications. Triplicate portions (200μl) of cytosol were incubated for 2h at 0°C with 10nM-[^{3}H]-R5020 (sp. radioactivity 87.0Ci/mmol; New England Nuclear Corp., Boston, MA, U.S.A.) in the presence or absence of 1000nM-R5020. After 2h TED buffer containing 60% glycerol (200μl) was added. The incubation was continued for an additional 2h. After incubation unbound radioactivity was removed by adding 50μl of charcoal/dextran mixture at 0°C for 15min and centrifugation at 5000*g* for 7min. The supernatant was collected and the radioactivity was counted.

Results

Oestrogen-receptor distribution after translocation

Cells at preconfluency were exposed for 1h to a medium fortified with 10nM-[^{3}H]oestradiol, washed and further cultured in fresh growth medium. During the following 5 days the amount of ER_n, R_n and R_c was measured. The radioactivity present in the nuclear fraction was considered as ER_n, since it was exchangeable with diethylstilboestrol at 30°C but not at 0°C (see Table 1). The additional binding of [^{3}H]oestradiol at 0°C to the nuclear fraction was considered as R_n (see Table 1). After 1h exposure only occupied receptors were found in the nucleus (Fig. 1). ER_n declined continuously thereafter so that on day 5 it approached 15% of its value measured 1h after exposure. Although after 3 days almost

Table 1. *An analysis of oestradiol-bound nuclear fraction*

Cells were treated as described in the legend to Fig. 1. The nuclear fraction was prepared and assayed by incubation with [^{3}H]oestradiol at 0°C for 4h and at 30°C for 3h. Each value represents triplicate determinations from two pooled flasks. Abbreviation used: DES, diethylstilboestrol.

		Bound [^{3}H]oestradiol (fmol/mg of DNA)			
		Incubation with 7.5nM-[^{3}H]oestradiol at 0°C		Incubation with 7.5mM-[^{3}H]oestradiol at 30°C	
Time after exposure to 10nM-[^{3}H]oestradiol (days)	Not assayed[a]	No DES	+750nM-DES	No DES	+750nM-DES
0[b]	5194	5303	5289	4988	608
1	2567	2943	2734	2922	519
2	1702	1957	1898	2097	539
3	1187	1409	1219	1410	276
4	970	2352	974	2506	286
5	927	2258	825	2355	211

[a] Bound [^{3}H]oestradiol measured in the nuclear fraction without further incubation.
[b] At 1h after exposure.

80% of ER_n disappeared no R_n appeared, opposing the hypothesis that R_n is a dissociation product of ER_n. On day 4 R_c and R_n appeared simultaneously. The possibility that R_n is a contamination product of R_c was eliminated, since similar amounts of R_n were found in nuclei purified by centrifugation through 1.8M-sucrose and treated with 0.7% Triton X-100 (results not shown). To exclude the possibility that oestrogens present in the serum participate in the R_c translocation into the nucleus, the following experiments were performed. Cells after exposure of 1h were cultured for 3 days in regular growth medium. On day 3 the medium was replaced by: (A) fresh growth medium; (B) charcoal-stripped serum medium; (C) serum-free medium. As shown in Table 2 media B and C did not prevent the appearance of R_n. The R_c and R_n values found on days 4 and 5 did not vary considerably in cells grown in the different media.

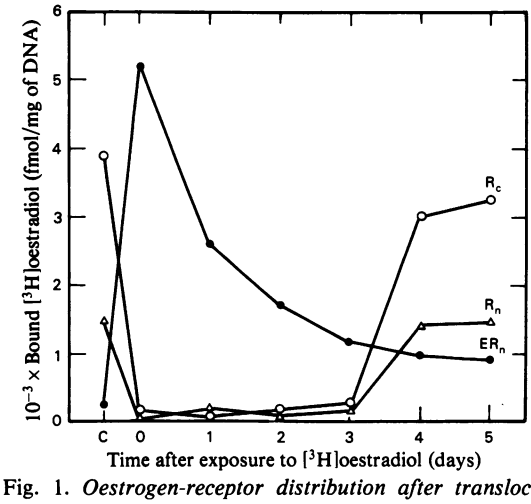

Fig. 1. *Oestrogen-receptor distribution after translocation*

Cells at preconfluency were exposed for 1h to a medium fortified with 10nM-[^{3}H]oestradiol (day 0), and the cells were further cultured in fresh growth medium up to 5 days. The radioactivity measured in the nuclear fraction was considered as occupied nuclear receptor (ER_n) (see Table 1). R_c and R_n were measured by incubation with 7.5nM-[^{3}H]oestradiol at 0°C for 4h. Abbreviation used: C, unexposed cells at preconfluency. Each point represents the mean of three determinations from two pooled flasks (experiment repeated six times).

Effect of oestrogen dose on oestrogen-receptor distribution

Cells at confluency were exposed for 1h to medium fortified with increasing concentrations of [^{3}H]oestradiol (0.1–10nM). The oestrogen-receptor distribution is shown in Fig. 2. With the increase in

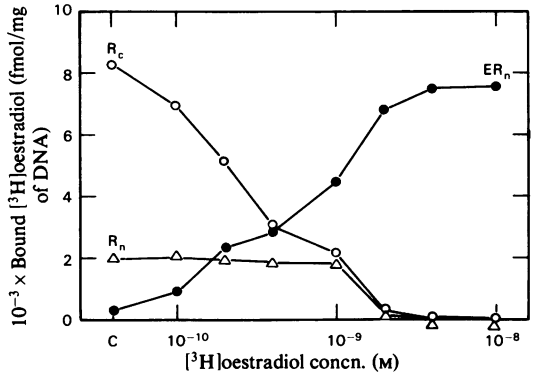

Fig. 2. *Effect of oestradiol dose on receptor distribution*

Cells at confluency were exposed for 1h to a medium fortified with increasing concentration of [^{3}H]oestradiol (0.1–10nm). ER_n, R_c and R_n were measured by incubation with 7.5nM-[^{3}H]oestradiol as described in the Materials and methods section. Abbreviation used: C, unexposed cells at confluency. Each point on the curves represents the mean of three determinations from two pooled flasks (experiment repeated three times).

Table 2. *Effect of culture medium on replenishment*

Cells at preconfluency were exposed for 1h to medium fortified with [^{3}H]oestradiol (day 0) and grown thereafter in regular medium for 3 days. On day 3 the medium was replaced by fresh growth medium (A), charcoal-stripped serum medium (B) or serum-free medium (C). R_c, R_n and ER_n were determined as described in the legend to Fig. 1. Each value represents triplicate determinations from two pooled flasks (experiments were repeated three times).

Time after exposure to 10nM-[^{3}H]oestradiol (days)	Medium	Receptor concn. (fmol/mg of DNA) R_c	R_n	ER_n
0		360	57	8756
3		304	16	2633
4	A	4728	1030	2457
	B	4526	1309	1910
	C	6114	1846	2596
5	A	4116	1043	2349
	B	4214	1001	1994
	C	5109	1860	2024

[^{3}H]oestradiol concentration R_c is depleted and a concomitant increase in ER_n occurred. Despite this increase R_n values did not change considerably up to 1 nM-[^{3}H]oestradiol, which contradicts the assumption that ER_n has additional unoccupied binding sites. At concentrations larger than 1 nM it was probable that a free penetration of oestradiol into the nucleus occurred and that oestradiol also charged the R_n.

Effect of oestrogen on progesterone-receptor induction

Cells at preconfluency were exposed for 1 h to a medium fortified with 10 nM-oestradiol, and further cultured in fresh growth medium. During the following 5 days the cytoplasmic progesterone receptor was determined in those cells and in unexposed control cells (Fig. 3). The amount of cytoplasmic progesterone receptor measured at preconfluency was about 800 fmol/mg of DNA. In the unexposed cells a 2-fold increase was observed during 5 days, whereas an 8-fold increase was observed in the oestradiol-exposed cells. These results indicate that the MCF-7 cells in our hands are hormone-responsive, and that the oestradiol-binding sites measured previously are presumably the oestrogen-receptor system that controls this induction.

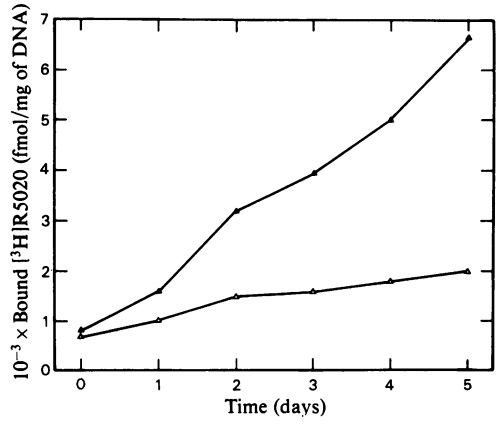

Fig. 3. *The effect of oestradiol on cytoplasmic progesterone receptor induction*

Cells at preconfluency were exposed for 1 h to a medium fortified with 10 nM-oestradiol (day 0); thereafter the cells were further cultured in fresh growth medium up to 5 days. Cytosolic progesterone receptors were determined by a single saturating dose assay with 10 nM-[^{3}H]R5020 as described in the Materials and methods section. Each point represents the mean of three determinations from two pooled flasks (experiment repeated three times). Symbols: ▲, oestradiol-exposed cells; △, unexposed control cells.

Discussion

The results of this study provided evidence that R_c may also penetrate the nucleus without binding to oestradiol. This was deduced mainly from the fact that the appearance of unoccupied receptors in the nucleus, simultaneously with the replenishment of R_c, was not prevented by culturing the cells in oestrogen-free medium (Table 2). We showed previously that R_n is not a cytoplasmic contaminant (Geier *et al.*, 1981). To exclude the possibility that R_n is a dissociation product of ER_n we followed the fate of ER_n after 1 h exposure of cells to [^{3}H]-oestradiol. Although 3 days after exposure almost 80% of ER_n disappeared, no additional oestradiol-binding sites appeared in the nuclear fraction or in the cytosol. On day 4 a substantial amount of R_n was present, and the ER_n value decreased only slightly. Since on the same day R_c appeared, it seems reasonable to assume that R_n derived from the translocation of R_c. The possibility was eliminated that ER_n is only partially occupied (i.e., containing additional unoccupied binding sites), since the exposure of cells to increasing concentrations of [^{3}H]oestradiol resulted in a continuous increase in ER_n, but did not augment the oestradiol-binding sites measured at 0°C in the nucleus. Evidence for entry of steroid cytoplasmic receptor into nuclei without the presence of the steroid was reported by Saffran & Loeser (1979). These authors showed an uptake of cytosolic progesterone receptor into isolated nuclei from guinea-pig uterus in the absence of progesterone.

The involvement of R_n in oestrogen action can only be speculated. However, an induction of cytoplasmic progesterone receptors occurred after translocation of R_c and saturation of R_n due to exposure of cells to 10 nM-oestradiol (Fig. 3). This indicates that the MCF-7 cells in our hands are hormone-responsive, and the induction of cytoplasmic progesterone receptors is presumably controlled by the oestrogen-receptor system (Horwitz & McGuire, 1978). The duration of ER_n turnover or processing and replenishment of oestradiol receptors after oestradiol treatment is different in the MCF-7 cell line as compared with the rat uterus. In the MCF-7 cell line, 80% of ER_n was lost during 3 days and the replenishment of R_c was observed on day 4 whereas in the rat uterus 70–80% of ER_n was lost during 5 h with a concomitant R_c replenishment (Anderson *et al.*, 1973; Peleg *et al.*, 1979). This discrepancy may be due to tissue specificity or to the fact that experiments in MCF-7 cells were performed *in vitro*, where only the direct effect of oestradiol was measured.

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