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ESTROGEN RECEPTOR LEVELS IN ESTROGEN SENSITIVE CELLS IN CULTURE

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SUMMARY

Estradiol-17β (E_2) receptor (R) levels of *bona fide* E_2 sensitive cells were studied under a set of different cell culture conditions. E_2R concentration in $C_8$11RAP cells in culture is affected by the concentration of serum in the culture medium. Removal of serum from the medium results in a swift decrease in the E_2R concentration, reaching its lowest level after 6 h. Subculturing the cells in media containing different serum concentrations from 5 to 0.2% resulted in a sharp decrease in E_2R concentration. The dose-response relationship between serum concentration and E_2R level strongly suggests the presence in serum of (a) factor(s) responsible for the steady, high level of E_2R observed in $C_8$11RAP cells growing in culture. The swift fall of the receptor level after serum withdrawal as well as the rapid recovery after exposure to serum could be explained assuming a very high turnover of the receptor, if the serum-borne factors act by stimulating the synthesis or impeding the degradation of E_2R. Although the conditions under which these cells are grown in culture differ from those *in situ* in the animal host, it is under well defined conditions afforded by the in-culture environment that a direct evaluation of the role of putative E_2R level modifiers may be properly studied and correlated with *in vivo* data.

INTRODUCTION

The binding of Estradiol-17β (E_2) to their intracellular receptors is considered to be the first step in the sequence of events triggered by this hormone in order to elicit the putative estrogenic stimulation. In spite of this widely accepted view, few so-called estrogen (E) dependent functions in vertebrate systems have been linked in defined molecular terms to the interaction between the "activated" receptor and the genome [1]. A major drawback in this respect is the inability to consistently reproduce in cell- or organ-culture systems the trophic functions attributed to E_2 [2–4]. Nevertheless, the presence of E_2 receptors has been considered the cardinal point to define a target cell [5, 6]. Dryden and Anderson demonstrated the presence of similar receptors in cells where the E-dependent function is yet to be defined [7].

Several long-term growing mammalian clonal cell lines that carry estrogen receptors have been characterized by Sonnenschein *et al.*[4, 8, 9] and by Brooks *et al.*[10]. It has been shown that the E_2-dependent growth seen when the rat tumor cells are injected into adult hosts cannot be reproduced in culture conditions that resemble those prevalent in animals [4, 9, 11]. This represents a paradox.

In an effort to define the circumstances under which the level of E_2 receptors vary in well defined cell culture conditions we performed a series of experiments which are described below. The E_2 receptor level seems to be affected by the serum concentration added to the basic medium on which these E_2 sensitive cells grow.

MATERIALS AND METHODS

Cell lines

The clonal cell line $C_8$11RAP was established from rat pituitary tumors induced by high doses of estradiol valerate. Details of this procedure have been reported elsewhere [9].

Growth conditions of cells in culture

Briefly, cells were grown in 75 cm^2 plastic flasks; the culture medium consisted of Dulbecco modification of Eagle's medium (DME) supplemented with 10% horse serum (GIBCO, Glasgow, Scotland) (HS 10%). Other experiments were done using 10% castrated and adrenalectomized calf serum (Rockland Farms, Gilbertsville, PA, U.S.A.) (CACS).

Cells were subcultured by agitating the media-containing flasks to liberate the cells which were loosely attached to the growing surface. A similar procedure was followed to harvest cells used to estimate E_2 receptor levels.

Drugs and isotopes

Estradiol-17β (E_2) was purchased from Sigma and Co., St. Louis, MO. Tritiated estradiol ($^3HE_2$) of high specific activity (69 Ci/mole) was obtained from the Radiochemical Centre, Amersham, England. All the

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other chemicals used during this investigation were purchased from Merck & Co., Darmstadt, Germany.

E_2 binding in whole cells

Cells were detached by gentle shaking and were harvested by low speed centrifugation (800 *g* 5 min), washed twice with DME, resuspended in the same serumless media and placed in Falcon flasks. Each flask contained 6–8 × 10^6 cells. Cells were harvested at different intervals, resuspended in DME (1 × 10^6 cells/ml) and 1 ml aliquots were transferred to 35 mm diameter Falcon Plastics Petri dishes containing either saturating concentrations (10 nM as determined by saturation analysis in whole cells) of the tritiated hormone (A) or a combination of tritiated + 100 excess cold hormone (B). (A) was called total E_2 binding, (B) nonspecific binding. After 60 min incubation at 37°C in an atmosphere of 95% air/5% CO_2 and 100% relative humidity, the cells were transferred to precooled tubes. The remaining attached cells were carefully removed from the dishes by further agitation. The cell suspension was washed thrice with 10 ml ice cold phosphate buffered saline solution (140 mM NaCl, 1 mM $MgCl_2$, 2.5 mM KCl, 1 mM $CaCl_2$, 10 mM phosphate buffer, pH 7.4). The washed pellet was extracted twice with 2 ml 100% ethanol. The ethanolic extracts were transferred to glass scintillation vials. After complete evaporation, 10 ml of a scintillation cocktail (4.1 g% of 2.5 Diphenyloxazole and 0.4 g% of p-bis-(o-methylstyryl)-benzene in 1 liter of Toluene) were added. Radioactive counting was performed in a Packard Tricarb liquid scintillation spectrometer Model 3300 with a counting efficiency for tritium of 40%.

The total receptor concentration was determined by subtracting nonspecific binding from total E_2 binding, and expressed as number of sites per viable cell.

The method showed to be linear between 500,000 and 3,000,000 cells/dish.

Estradiol binding in cell extracts

$C_8$11RAP cells in culture conditions as well as those in tumors growing at the site of inoculation contain cytoplasmic and nuclear unoccupied receptors [9]. We use KCl extracted E_2 receptors with the purpose of quantitating all the E_2 receptors present in these cells regardless of their location.

Briefly, 20–30 × 10^6 cells are washed with PBS, resuspended in 0.5 ml of 10 mM Tris 500 mM KCl and 1.5 mM EDTA buffer pH 7.4. The cells were disrupted by sonication. The cell extract was obtained by centrifugation at 40,000 rev./min × 60 min in a SW 50.1 rotor (Beckman Instruments, Palo Alto, CA). 100 μl aliquots were incubated for total and specific binding for 10 h at 4°C. Bound and free hormones were separated by hydroxyl-apatite adsorption [12]. The results are expressed as sites/viable cell and femtomoles of E_2 bound per mg of KCl extractable protein.

The amount of receptor extracted through this procedure coincides with the value obtained by E_2 binding to whole live cells grown in standard medium, suggesting that most of the in culture E_2-accessible receptor could be extracted.

Protein determinations

Estimation of protein concentration was done by the microbiuret assay [13].

Cell viability

Cell viability was determined using the Trypan blue exclusion test [14].

RESULTS

Binding in whole cells

These are experiments in which E_2 binding was measured in whole cells in culture as explained in Experimental Procedures. Cells grown in horse serum (HS) 10% as cells grown in 10% castrated and adrenalectomized calf serum (CACS) supplemented medium were transferred to serumless medium (DME). Receptor levels were measured at 0, 24, 48 and 72 h. The apparent in culture binding increases within 24 h in DME and this high level was maintained unchanged throughout 72 h. Cell viability, however, showed a sharp decrease within the first 24 h of incubation in DME. It is important to point out that in assaying cell viability only the unlysed dead cells are computed. Cell health was assessed by measuring the rate of survival expressed as percent of the seeded cells recovered at the end of the experiment. This, we believe, is a more accurate way to describe the state of the cells during the experiment than cell viability measured by the dye exclusion test. In these series of experiments, cell survival was 50% after 24 h and about 10–20% at 72 h. We then decided to compare binding in culture with binding in the KCl extract of comparable treated cells, *i.e.*, kept in DME. While the level of receptors was similar with both procedures at time 0, a significant difference was recorded along the time course of this experiment. As can be seen in Table 1, dead cells spuriously bind E_2 in culture experiments in what seems to be specific binding, whereas the KCl extract which does not contain detectable proteins, of course, shows no binding. Moreover, "killing" cells by slow thawing of pellets kept in liquid nitrogen resulted in less than 10% viable cells and no detectable levels of E_2R could be extracted from them. These results suggest that only viable cells contain true receptor. To avoid these artefactual results all subsequent E_2 binding assays were performed using cell extracts instead of the in culture assays.

E_2 binding in cell extracts

As can be observed in Table 1, the amount of KCl-extractable protein per viable cell remains constant throughout the experiment. Because of it, E_2R con-

Table 1. Comparison between in culture and "*in vitro*" E_2R levels, concentration of extractable protein per cell, cell viability and percent of survival of $C_8$11RAP cells subcultured in different media

Treatment	Binding sites/ viable cell whole cells	Binding sites/ viable cell KCl extract	mol E_2 bound/ mg protein	KCl extractable protein (pg/cell)	% Viability	% Surviving cells
24 h 10% CACS	11,200 ± 2400	10,600 ± 1700	177.0 ± 31.0	100.0 ± 21	92	125.0
24 h 10% HS	10,000 ± 2000	10,400 ± 1051	155.0 ± 11.0	111.0 ± 16	89	130.0
24 h DME	21,600 ± 6600	2400 ± 460	40.0 ± 11.0	101.0 ± 25	49.0	45.0
48 h DME	19,400 ± 5400	2800 ± 539	38.0 ± 13.0	120.0 ± 20	48.0	25.0
96 h DME	222,000 22,000*	Undetectable	Undetectable	Undetectable	10.0	10.0

$C_8$11RAP cells were grown in 10% CACS and subcultured in 10% CACS and 10% HS for 24 h and in DME for 24, 48 and 96 h. The cells were harvested, washed with DME and aliquots were taken E_2 binding in whole cells, E_2 binding in the KCl extract, dye exclusion test and cell counting. E_2R levels were expressed as sites/viable cell in both assays, and in fmol of E_2 bound/mg of protein in the KCl extract. Extractable protein per cell was measured in the KCl extract. Values are means ± SD of five independent experiments.

* Binding sites/total (viable + dead) cells.

centration can be expressed as femtomoles of E_2 bound/mg of KCl extractable protein, as well as binding sites/viable cell. Both HS and CACS-grown cells showed comparable levels of receptors, whereas cells transferred to DME for 12, 24 and 48 h lost 75% of their receptor. The fall in receptor concentration seems not to be due to the stress of harvesting the cells and putting them back in culture, since cells from the same pool transferred back to HS 10% for 2, 12, 24 and 48 h maintained their basal receptor levels (Fig. 1).

Time course experiment

As shown in Fig. 2 the receptor level decreases sharply within the first two hours of incubation in DME, to reach a plateau at 6 h, where it remains throughout the next 48 h. Evaluation of the survival of cells kept in serumless media for prolonged periods of time was considered important. Cell survival for E_2R levels were estimated on cells kept for 24 h in (a) DME, (b) DME + Bovine Serum Albumin (BSA) (0.80%), (c) 1% HS and (d) 1% HS + BSA (0.80%). This experiment was done with the purpose of determining whether the change in protein concentration and hence in osmotic pressure these cells are subject to affects these two above mentioned parameters. Table 2 shows the result of such an experiment. The E_2R levels vary depending on the growth conditions these cells were in. Cells kept in DME and DME + BSA did not differ in either viability or E_2R concentration. Cells kept in 1% HS and 1% HS + BSA differed with the previous groups in both parameters but not between themselves. The addition of BSA to the media neither prevented the fall of the receptor level, nor altered the viability and survival of the cells when compared to the media without BSA. Cells in HS 1% grew exponentially and were steadily viable after one week in culture, whereas the receptor level decreased to 50% (data not shown). This experiment suggests that serum concentration rather than cell health is the factor responsible for the decrease of E_2R.

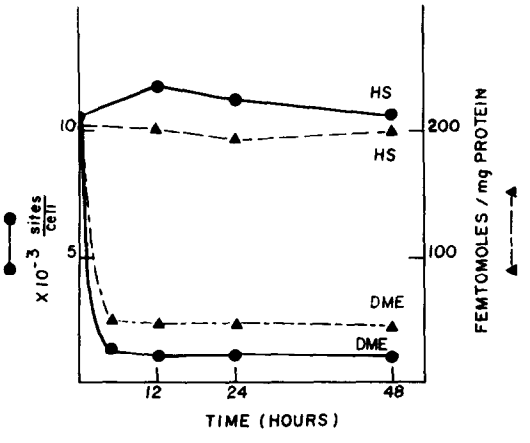

Fig. 1. Time-course experiment. E_2R levels are expressed as femtomoles/mg protein (▲---▲) and as sites/viable cell (●——●). $C_8$11RAP cells grown in 10% HS were harvested and tranferred back to 10% HS (HS) and to serumless medium (DME). E_2R levels were determined 6, 12, 24 and 48 h after subculturing.

Serum concentration-response experiment

To further explore this previous assumption a serum concentration response experiment was designed. Cells were transferred from HS 10% to

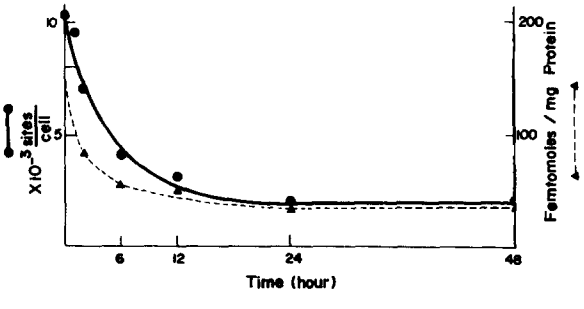

Fig. 2. Time-course of the receptor concentration after subculturing in serumless medium. Receptor levels are expressed as in Fig. 1.

Table 2. Estradiol-17β receptor levels of $C_8$11RAP cells under different experimental conditions

Treatment	Sites/cell	fmol/mg Protein	% Viability
10% HS	8635 ± 730	137.0 ± 20	82.2
DME	2800 ± 580	47.1 ± 15	83.1
DME + BSA	2887 ± 400	49.4 ± 13	77.3
1% HS	5558 ± 361	61.4 ± 21	71.1
1% HS + BSA	5217 ± 350	85.7 ± 32	86.6

$C_8$11RAP cells grown in 10% HS were subcultured in (a) 10% HS, (b) DME, (c) DME + BSA, (d) 1% HS and (e) 1% HS + BSA for 24 h. Cells were harvested, washed in PBS and extracted with 0.5 M KCl 10 mM Tris, 10 mM Tris, 1 mM EDTA pH 7.4. E_2R levels were determined in the 100,000 *g* supernatant. Values are means ± SD of 3 independent experiments.

media containing HS at 0.2, 1, 3, 5 and 10% for 24 h. An aliquot was transferred to DME. Figure 3 confirms that the level of E_2R is dependent on the concentration of horse serum supplemented to DME. The curve resembles a dose-response curve, with its maximal effect between 5–10% horse serum concentration. These results indicate that horse serum contains (a) factor(s) responsible for the high steady E_2 receptor level observed in cells growing in 10% serum.

Recovery experiment

Evidence reinforcing the idea that horse serum supplemented to DME is responsible for the maintenance of E_2R levels is presented in Fig. 4. After 24 h of incubation in DME cells transferred to HS 10% show a sharp increase in receptor level 12 h after the change in experimental condition.

DISCUSSION

The control of the level of E_2R has been the subject of several reports [15, 20]. We believe that an in animal-in culture system whereby the behavior of E_2 sensitive cells growing in animals and in culture can be compared, represents an important tool for the investigation of the elements involved in the estrogenic response.

Receptor levels in $C_8$11RAP cells were measured both in whole cells and in cell free extracts. The rationale for determining E_2 binding in whole cells is that it can be performed in conditions in which the morphological and functional integrity of the cell are preserved. This seems to be the case when cells are growing exponentially and their viability is maintained above 70%. E_2R levels obtained by this method were comparable to the ones obtained with the KCl-cell free extract, suggesting that KCl extracts nearly all the receptor molecules that are available to estrogens in culture conditions. The close correlation between results obtained in whole cells and in KCl extract is maintained as long as viability remains above 70% in the former. E_2 binding in whole cells maintained in serumless medium for prolonged intervals varied from 50 to 400% of the control grown in HS 10%. When cell viability was 10% condition in which neither proteins nor E_2R were detectable in the KCl extract, E_2 binding/cell in whole cells was 20 times higher than in cells growing in HS 10%.

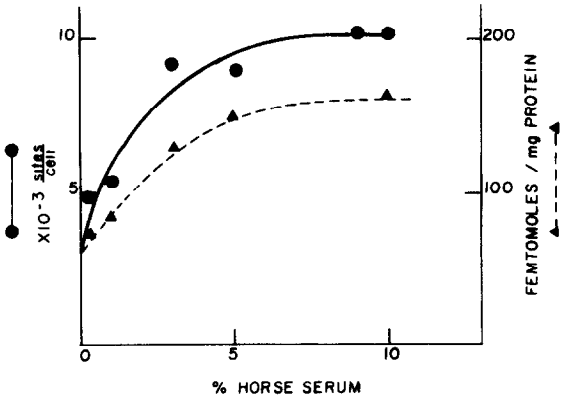

Fig. 3. Dose-response curve. $C_8$11RAP cells grown in 10% HS were transferred to medium containing 0, 0.2, 1.0, 3.0, 5.0 and 10% HS. Receptor levels were determined 24 h after subculturing.

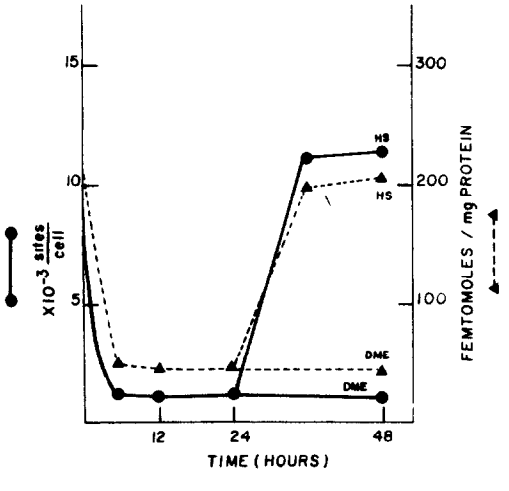

Fig. 4. Recovery of the E_2R level. $C_8$11RAP cells were maintained in serumless medium (DME) for 24 h, and then transferred to 10% HS (HS). Receptor levels were measured 12 and 24 h after adding 10% HS to DME.

Because of the wide variability in the results obtained by measuring E_2 binding in whole cells at low viability, this method was considered unreliable. We then decided to measure E_2 binding using KCl-cell free extracts.

Specific estrogen binding in rat pituitary estrogen sensitive cells in culture is affected by the concentration of serum in the medium. Removal of serum from the medium results in a swift decrease in E_2 binding, reaching its lowest level after 6 h. This low E_2 binding is maintained throughout the 48 h the experiment lasted. This phenomenon seems not to be related to the stress of harvesting, pooling and subculturing the cells, since cells from the same pool transferred back to HS 10% for comparable time intervals maintained their initial receptor level.

Subculturing cells in media containing different serum concentrations from 5 to 0.2% also resulted in a sharp decrease in E_2 binding. The dose-response relationship between serum concentration and E_2 binding strongly suggests the presence in serum of (a) factor(s) responsible for the steady, high level of E_2 binding observed in $C_8$11RAP cells growing in culture.

We should bear in mind that the serum concentrations at which we have done our experiments are hardly seen under physiological conditions in the animal. Nevertheless, if the parameter that has been considered crucial to explain the estrogenic response, be it growth or protein synthesis is to be affected at all we could state that it is under this unlikely growth conditions where the change has only been observed. The fall in E_2 binding observed at low serum concentrations does not depend on the health status of the cell population because these cells grow exponentially at serum concentrations lower than those needed to maintain maximal E_2R levels (1 and 5% respectively).

Since the results reported herein were obtained with cell extracts, the decrease of E_2R levels observed in cells maintained at low serum concentrations could be explained by postulating that cells maintained in a restricted environment would be in a catabolic situation. When these cells are disrupted, proteolytic enzymes could be released and they may degrade the E_2 receptor. A comparable suggestion has been postulated to explain the lowering of the testosterone receptor levels in the ventral prostate of castrated male rats [21]. This, however, seems not to be so in our experience since (a) $C_8$11RAP cells maintained in media supplemented with as little as 1% HS grow exponentially, that is, they apparently are not in a catabolic situation and (b) their E_2 receptor level represents 50% of the value observed in cells grown with 10% HS supplemented medium.

The swift fall in E_2 binding after serum withdrawal could be explained by assuming a very high turnover of the binding protein if the serum-borne factors act by stimulating its synthesis or impeding its degradation.

To further elaborate on the meaning of our results it would be convenient to briefly restate the working definition of E_2 receptors. This is, receptors are the intracellular E_2 binding proteins present in target cells [1]. Little is known about the homogeneity of these receptor proteins within the target cells [22]. In view of these considerations, an alternative explanation for the results obtained would be the following: as the estimated levels of E_2R represent, in fact, the binding of E_2 to protein molecules we call receptors, we cannot rule out the possibility that about 75% of the receptors present in cells transferred to DME lose their property to bind E_2. This would suggest again that the total population of E_2R represents a composite of two or more populations. In this case, the criterion to define the heterogeneity of the receptor populations would be given by the ability with which a portion of the E_2 receptors lose the property to bind with specificity to E_2.

On the other hand, the reported results raise the question of whether the diminished level of receptors measured in these cultured cells while kept on DME may represent, in fact, a dilution effect between cells that maintain their E_2R levels intact and a great majority of them (75–80%) that lose them completely. An argument against this possibility is given by the fact that cells maintained for 24 hrs in DME recover the initial E_2R concentration 12 h after being transferred to HS 10%.

The data presented suggest that the concentration of E_2R is kept at a high, steady level in these E sensitive cells. Furthermore, the lack of reproducible, consistent changes in E_2R levels in these cells by administration of E_2, prostaglandin $F_{2\alpha}$ and prolactin within a wide range of concentrations to cells growing in castrated and adrenalectomized calf serum supplemented media suggests that it takes a drastic treatment (serumless or low serum supplemented media) to consistently change the level of this parameter. The data presented is at variance from that obtained by Shafie and Brooks[20], when E_2R levels were measured in the human breast carcinoma cell line MCF7 subjected to serumless growth conditions. This may be a reflection on the different species and organs from which these cells are derived and the different serum source used (calf serum and horse serum in theirs and our case, respectively).

We have attempted to describe circumstances under which E_2 receptor levels fluctuate in cultured cells. We concluded that to do so these target cells should be kept under very restrictive conditions. These conditions are unlikely to be met in homeostatic stages prevalent in animals. Therefore, we speculate that the level of E_2 receptors may not change dramatically in target organs *in situ* despite cyclic modifications of the status of these animals related with estrogen levels.

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