Estrone Receptor Formation During the Processing of Estradiol-Receptor Complex in MCF-7 Cells

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ABSTRACT

Human breast cancer cells (MCF-7, maintained in long-term culture) contain separate estrogen receptors specific for either 17β-estradiol or estrone. Utilizing optimum conditions for the protamine sulfate assay, it has been possible to demonstrate both receptors in the 0.6 M KCl extract of nuclei and in the cytosol. Similarly, in the exchange assay, high-affinity low-capacity binding sites for 17β-estradiol and estrone have been found in the salt-extracted nuclear residue. Dissociation constants and binding capacities were determined for either receptor in the absence of the other [e.g., estrone receptor (E,R) in the cytosol or nuclear residue from 17β-estradiol-stimulated cells] or, when both receptors were present, a saturating amount of the other estrogen (unlabeled) was added to the assay mixture (e.g., the salt-extractable nuclear receptors). Specificity was demonstrated by the inability of estrone to compete with 17β-[2,4,6,7-3H]estradiol for the 17β-estradiol receptor (E,R) at molar excesses less than 10-fold. Likewise, there was no inhibition of [6,7-3H]estrone binding to its receptor by molar excesses of 17β-estradiol below 100-fold. Other steroid hormones were very weak competitors of [6,7-3H]estrone, even at 1000-fold molar excesses. The quantitative relationships of these two estrogen receptors were shown to fluctuate in the various cellular compartments following incubation (37°C) of MCF-7 cells with 10^-8 M 17β-estradiol. This level of 17β-estradiol elicited the translocation of all detectable cytosolic E,R to the nucleus, where, after an incubation of 1 hr, the salt-resistant 17β-estradiol disappeared and 40% of the extractable 17β-estradiol-binding capacity was lost (processed). Simultaneously, the E,R which remained in the nuclear residue appeared in the nuclear extract, and ultimately this receptor accumulated in the cytosol. The estrone-binding capacity (0.78 pmol/mg DNA) which appeared following the processing of E,R nearly equaled the loss of 17β-estradiol binding sites per cell (0.85 pmol/mg DNA). Concentrations of 17β-estradiol which elicited the greatest processing of E,R in these incubations brought about the appearance of maximum levels of E,R in MCF-7 cells.

Considering these results in the light of data previously reported from this laboratory concerning the metabolic and processing of E2R nearly equalled the loss of 17β-estradiol binding sites per cell (0.85 pmol/mg DNA). Concentrations of 17β-estradiol which elicited the greatest processing of E,R in these incubations brought about the appearance of maximum levels of E,R in MCF-7 cells.

INTRODUCTION

It is essential to the understanding of steroid effects on breast cancer cells that the total fate of physiological levels of steroid hormones in neoplastic and normal target tissues be known. For example, 17β-estradiol is destined to be metabolized by specific enzymes with which it may come into contact as well as being bound to its cytoplasmic receptor and subsequently translocated to the nucleus (1, 5, 22, 25-27). Once within the nucleus, 17β-estradiol shares an association with salt-extractable proteins and with certain salt-resistant components (4, 9, 23).

E,R complex has recently been shown to undergo processing within the nucleus of MCF-7 cells (18). Originally observed by Horwitz and McGuire (18), processing was defined as the disappearance of nuclear (as well as total cellular) E,R during the induction of progesterone receptor. The fate of the lost E,R complex has not been elucidated.

Previously reported studies with MCF-7 cells have established that within these cells 17β-estradiol is oxidized to estrone as well as being bound by its receptor followed by the translocation of the complex to the nucleus (4). It has also been demonstrated that, like uterine cells (6, 24, 29, 35-37), 17β-estradiol dehydrogenase activity exists within the nucleus of these breast cancer cells (4). Since no other fate awaits 17β-estradiol in MCF-7 cells, this system is amenable to a study of the relationship of 17β-estradiol dehydrogenase activity to the nuclear uptake and processing of E,R.

Utilizing the MCF-7 human breast cancer cell line, we have demonstrated that estrone formed from 17β-estradiol in situ is bound to a cytoplasmic macromolecule with high affinity and low capacity (4). Furthermore, estrone is formed within the nucleus from 17β-estradiol, particularly in the salt-resistant nuclear fraction. This unexpected discovery would predict the presence of estrone-specific nuclear receptors in MCF-7 cells. We have carried out studies designed to gain information regarding the presence of a nuclear estrone-binding component. Whole nuclei, the salt-extracted nuclear proteins, and the residual nuclear pellet displayed 2 separate high-affinity low-capacity binding sites specific for either 17β-estradiol or estrone (3).

The data presented herein demonstrate that formation of the E,R occurs concomitant with the nuclear processing of E,R complex in MCF-7 cells.

MATERIALS AND METHODS

Materials. [1H]Estradiol (111 Ci/mmol and [3H]estrone (48.3 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.) and purified on thin-layer chromatography before use (24). Nonradioactive hormones were obtained from Research Plus Laboratories, Inc. (Denville, N. J.).

Cell Culture and Harvesting. MCF cells of human breast tumor origin were cultured in T75 flasks utilizing 20 ml Eagle's minimal essential medium supplemented with nonessential amino acids, insulin,
and antibiotics and made 10% with respect to calf serum (33). Passages involved trypsin digestion (33) to obtain monocellular suspensions followed by the plating of 2 x 10^6 cells/flask. Cultures were confluent after 1 week and were harvested for experimentation after 2 weeks (approximately 30 x 10^6 cells/flask).

Following removal of medium, flasks were washed twice with NaCl solution (9 mg/100 ml) and once with Tris:EDTA buffer (10 mM Tris-HCl:1.5 mM EDTA (pH 7.4 at 0°)) to remove residual medium. The cells were then removed from the flask using a rubber policeman with 2 ml of Tris:EDTA buffer made 8 mM with diethiothreitol and placed in a Dounce tube.

Preparation of Cytosol, Microsomal, and Nuclear Extracts. The suspension of harvested cells from 2 flasks was homogenized in the 4 ml buffer made 0.1% with saponin utilizing 15 strokes of a tightly fitting pestle. The homogenate was centrifuged at 800 x g for 10 min (4°), and the supernatant was saved (crude cytosol). A more purified nuclear pellet was prepared by washing the crude pellet obtained with 2 ml of buffered 0.25 M sucrose solution (0.25 M sucrose:3 mM MgCl:10 mM Tris-HCl:8 mM diethiothreitol (pH 7.6 at 0°)) 3 times and centrifuging as before. The supernatants were added to the crude cytosol. The crude cytosol was centrifuged at 105,000 x g (4°) for 60 min, yielding a supernatant (cytosol) and a high-speed pellet (microsomes).

The partially purified nuclear and microsomal pellets were extracted with Tris:EDTA:KCI buffer (10 mM Tris-HCl:1.5 mM EDTA:0.6 mM KCI:1 mM thioglycolate:10% glycerol (pH 8.5 at 0°)) by triturating (with Dounce pestle) every 10 min for 1 hr. Solubilized proteins were separated from insoluble nuclear or microsomal material by centrifugation at 105,000 x g for 30 min. The concentrated nuclear or microsomal extracts were diluted with Tris:EDTA buffer to a concentration of less than 0.1 M KCI to enable precipitation by protamine sulfate (32) after subtraction of nonspecific binding.

Protamine Sulfate Assays. The method used was that of Steggles and King (34) as modified by Zava et al. (39) and Chanmness et al. (8). Fractions (500 µl) of cytosol and nuclear extracts were precipitated with 300 µl of protamine sulfate (1 mg/ml; USP injection, without phenol preservative; Eli Lilly and Co.) and centrifuged at 800 x g for 10 min.

For one-point assays, the protamine precipitates from aliquots of the cytosol or nuclear extracts were incubated with a single concentration (3.8 to 5.0 nM) of [3H]estradiol or [3H]estrone for the designated times and temperatures. Nonspecific binding was determined by a parallel reaction of less than 0.1 M KCI to enable precipitation by protamine sulfate (32) after subtraction of nonspecific binding.

Saturation analysis on cytosol, microsomal extracts, or nuclear extracts was carried out by incubating for indicated times and temperatures the protamine sulfate precipitates in duplicate with 300 µl of Tris:EDTA containing increasing concentrations of [3H]estradiol (0.2 to 4.0 nM) or [3H]estrone (0.3 to 6.0 nM) with or without 200-fold excess of respective estrogen. The protamine-precipitated proteins were then washed, extracted, and counted as described above. The binding capacities and K_d were determined using the method of Scatchard (32) after subtraction of nonspecific binding.

Nuclear Exchange Assay on 0.6 M KCl-extracted Nuclear Pellet. A modification of the exchange procedure of Anderson et al. (2) was utilized. Nuclei were purified and extracted with Tris:EDTA:KCl buffer as described above. The 0.6 M KCl-extracted pellet (105,000 x g pellet) was transferred to a Dounce homogenizer, and 2 ml of buffered 0.25 M sucrose solution were added. The pellet was homogenized into an even suspension with 15 strokes of a tightly fitting pestle. This suspension was diluted to 15 ml, 1-ml samples were taken for DNA analysis, and 500-µl samples were taken for an exchange assay. The suspension was centrifuged at 800 x g for 10 min, and the supernatant was decanted. Resulting pellets were incubated in duplicate under conditions indicated in the legend to Chart 3 with 300 µl Tris:EDTA containing increasing concentrations of [3H]estradiol (0.2 to 4.0 nM) or [3H]estrone (0.3 to 6.0 nM) with or without 200-fold excess of respective steroid. After incubation, tubes were washed 3 times with Tris:EDTA buffer and extracted with 1.5 ml ethanol, and 1 ml was counted as described before. The data were analyzed by the method of Scatchard (32).

RESULTS

Characteristics of the Protamine Assay of Various Estrogen Receptors. The 0.6 M KCl extract of nuclei from MCF-7 cells contained protamine-precipitable proteins which bind [3H]estradiol with high affinity and finite capacity. However, the rate at which this association reached equilibrium and the maximum [3H]estradiol binding observed varied with the temperature of the assay and the prior availability of 17β-estradiol to the cells (Chart 1). When cultures were grown in media containing 10% calf serum (2 to 6 x 10^{-11} M 17β-estradiol; see Footnote 4 and Ref. 28), the extractable nuclear 17β-estradiol receptor reaches equilibrium by 1 hr at 37° or 6 to 20 hr at 0°. Regardless of which temperature is used, the maximum binding is similar (approximately 0.52 pmol 17β-estradiol bound per mg DNA). Previously, this binding to (or exchange with?) the protamine-precipitated nuclear receptor at 0° has been defined as indicative of uncharged receptor (40). This does not appear to be the case for receptor extracted from substantially purified nuclei in these experiments since [3H]estrone was not capable of occupying this site during cold equilibrations (Chart 2). Even at 37°, [3H]estrone occupied (exchanged with?) only 20% of these sites (Chart 2).

After exposure of the culture to 17β-estradiol (10^{-8} M) during a 1-hr incubation at 37°, there were dramatic changes in the amount and exchange characteristics of the extractable nuclear 17β-estradiol receptor (Chart 1). A 3-fold increase (~1.5 pmol/mg DNA) was observed in the quantity of [3H]estradiol which exchanged with receptor at 37°, probably representing the translocation of cytoplasmic E2R complex to the nuclear compartment. On the other hand, little (0.2 pmol/mg DNA) [3H]estradiol was seen to exchange at 0° with nuclear receptor-bound 17β-estradiol. Presenting these cells with the optimum level of 17β-estradiol to bring about nuclear processing of the receptor complex (18) has significantly altered the exchange properties of the ligand. At the same time, 17β-estradiol stimulation of MCF-7 cells brought about the appearance of an appreciable amount of [3H]estradiol binding to (0°) or exchange (at 37°) with ligand(s) on the protamine precipitate of the 0.6 M KCl extract of nuclei (Chart 2). The 0.12 pmol/mg DNA of [3H]estradiol binding at 0° is similar to the 0.2 pmol/mg DNA of [3H]estradiol bound in the cold (Chart 1), suggesting that both Tritiated ligands were taken up by this small number of unoccupied sites in the nuclear extract, possibly from cytoplasmic contamination (13). Under the assay conditions, the observed [3H]estrone exchange at 37° would not be expected to occur with charged classical E2R complex since the affinity of estrone is considerably less than that of 17β-estradiol for this protein. Therefore, the 0.1-pmol/mg DNA [3H]estrone binding excess

Footnote 4: Radioimmunoassay of total 17β-estradiol (free plus hydrolyzed sulfates) in media containing 10% calf serum.
in the 37° exchange assay may represent specific estrone nuclear receptor.

The binding curves depicted in Charts 1 and 2 clearly demonstrate that optimum binding assays for [3H]estradiol and [3H]estrone may be carried out on the nuclear extract by equilibrating the protamine precipitate with either tritiated steroid for 1 hr at 37°. These conditions allow quantitation of nuclear estrogen receptors whether the culture has been previously exposed to 17β-estradiol or not.

Following salt extraction of the nuclear pellet, the nuclear residue has been shown to contain components with classical E2R properties (3, 9, 23). Utilizing an exchange assay for Scatchard analyses at 23° or 0°, it is possible to determine the binding properties of both tritiated estrogen ligands to these salt-resistant sites (Chart 3). Chart 3, left, depicts approximately 0.06 pmol of both [3H]estradiol and [3H]estrone exchange per mg DNA. In the salt-extracted nuclei from cells grown in regular media (10% calf serum containing 2 to 6 x 10⁻¹¹ M 17β-estradiol), [3H]estrone required elevated temperature for exchange while [3H]estradiol exchanged at both temperatures (23° and 0°). At this time, it is not possible to explain these temperature-dependent exchange variations as they pertain to receptors bound to the nuclear residue. It is, however, interesting to note that nuclei from cells grown with stimulatory levels of 17β-estradiol (10⁻⁸ M) contained only [3H]estrone exchangeable sites (23°) in the salt-extracted residue (Chart 3, middle). Furthermore, cells maintained in a hormone- and serum-free medium [chemically defined medium (16)] showed no salt-resistant estrogen-binding sites (Chart 3, right).

It is also possible to demonstrate significant cytosolic estrone binding in the absence of a detectable 17β-estradiol receptor. This is accomplished by carrying out binding studies on the protamine precipitate obtained from the 105,000 x g supernatant from cells exposed to 10⁻⁸ M 17β-estradiol for 1 hr. Assay methods which utilize an adsorbant for the free ligand [e.g., dextran-coated charcoal (1:10)] will strip the [3H]estrone from its receptor. It is also important that the protamine sulfate assay be carried out at 0° since both the E2R and E1R are destroyed if cytosol precipitates are incubated at 37°. Utilizing the described conditions, a minimum of 4 hr is required for [3H]estrone to equilibrate with its receptor, while there is no loss of binding for at least 22 hr at 0° (Chart 4). This cytosol was devoid of E2R due to its translocation to the nucleus during the previous incubation of cells with 10⁻⁸ M 17β-estradiol (Chart 4). Cytosolic E2R displays classical saturation kinetics, and the data are amenable to Scatchard analyses (Chart 5) yielding results typical of receptors with high affinity (Kd, 4.6 x 10⁻⁸ M) and limited capacity (.22 pmol/mg DNA). The [3H]estrone binding found in MCF-7 cells is not prevented by a 1000-fold excess of nonestrogenic steroid hormones in the assay (Table 1).
Specificity of 2 Estrogen Receptors for 17β-Estradiol and Estrone. The steroid receptor theory does not prohibit the binding of similar steroids to the same site. Specificity is established by the extreme molar excess of a competitor required to displace the labeled steroid ligand. It is well established that estrone will not only bind to the classical estrogen receptor but the bound estrone will activate the complex and promote nuclear translocations (21, 31). Although triggered by nonphysiological levels of estrone, the nuclear estrone complex will promote the induction of specific proteins (21). The data in Chart 6A clearly demonstrate the competition of estrone for the cytosolic and extractable nuclear E2R. Labeled 17β-estradiol was displaced from its cytosolic receptor by estrone with increasing effectiveness as the molar excess of estrone increased. However, it was not possible to displace all the bound [3H]estradiol until the assay was performed in the presence of a 1000-fold molar excess of estrone. Greater resistance to exchange with estrone was displayed by the nuclear E2R complex. In this case, there was no displacement of the ligand until the complex was exposed to a 100-fold excess of estrone (Chart 6A).

Similarly, the E2R in MCF-7 cells was very resistant to exchange with 17β-estradiol. When examined in the absence of the 17β-estradiol cytoplasmic receptor [cells previously incubated with 10⁻⁸ M 17β-estradiol (Chart 6B)], 17β-estradiol would not displace [3H]estrone on its receptor unless the unlabeled 17β-estradiol was present in the incubation at molar excesses of 100-fold or greater (Chart 6B). Similar results were obtained with salt-extractable nuclear estrone-binding component (data not shown). Clearly, at nM concentrations, [3H]estradiol does not bind to or exchange with the E2R (Chart 4).

### Table 1

<table>
<thead>
<tr>
<th>Steroids added</th>
<th>[3H]Estrone bound (pmol/mg DNA)</th>
<th>% of inhibition</th>
</tr>
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<tbody>
<tr>
<td>[3H]Estrone (5 nM)</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>+ 5 μM progesterone</td>
<td>0.38</td>
<td>42</td>
</tr>
<tr>
<td>+ 5 μM 5α-dihydrotestosterone</td>
<td>0.43</td>
<td>35</td>
</tr>
<tr>
<td>+ 5 μM cortisol</td>
<td>0.64</td>
<td>3</td>
</tr>
</tbody>
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Chart 6. Competition of 17β-estradiol (E2) and estrone (E1) for various receptors in MCF-7 cells before and after optimum processing. In A, cytosol and nuclear extract were prepared, and one-point protamine sulfate assays were performed in duplicate. Cytosol assays used 18-hr incubations at 0°, and nuclear extracts were incubated for 1 hr at 37°. Both fractions were assayed utilizing 4 nM [3H]estradiol in the presence of increasing concentrations of unlabelled estrone. O, cytosol from control cells (4 flasks) cultured only in regular media; △, nuclear extract from cells exposed to 10⁻⁸ M 17β-estradiol for 1 hr at 37° prior to harvesting (4 flasks). In B, cytosol protamine sulfate assays were performed in duplicate on preparations from control cells (B) and from cells exposed to 10⁻⁸ M 17β-estradiol for 1 hr at 37° prior to harvesting (A). These assays were carried out with 4 nM [3H]estrone being incubated in the presence of increasing concentration of unlabelled 17β-estradiol for 18 hr at 0°.
It was also possible to demonstrate the different affinities of 
[^3H]estrone for both receptors in the cytosol of cells exposed to
a minimal level of 17β-estradiol (10^(-11) M, media with 10% calf serum). A concentration of 4 x 10^(-9) M 17β-estradiol completely displaced 
[^3H]estrone from the cytosolic E2R. The remaining 
[^3H]estrone binding (to the E1R) was not totally competed out until a concentration of 4 x 10^(-6) M 17β-estradiol was reached (Chart 6B). The presence of some cytosolic E1R in control cells is also indicated by the initial slope of the unlabeled estrone competition line in Chart 6A.

**Dynamics and Cellular Distribution of 17β-Estradiol and Estrone Receptors.** Data presented above demonstrate that there are 2 estrogen-binding components in MCF-7 cells; one preferentially bound 17β-estradiol in the presence of excess estrone, and another displayed higher affinity for estrone than for 17β-estradiol. The experimental results indicated that the quantitative relationships of these individual receptor sites varied with cellular compartment and estrogen stimulation of the culture. In order to demonstrate these relationships, a quantitative analysis (Scatchard plot) of each receptor was carried out on cell fractions before and after a 1-hr exposure of the culture to 10^(-8) M 17β-estradiol (Table 2).

Cells maintained in 10% calf serum (10^(-11) M 17β-estradiol) had most of their E2R in the cytoplasm (cytosol plus microsomes, 2.04 pmol/mg DNA) with a lesser amount of salt-extractable nuclear E2R (0.44 pmol/mg DNA). These same cells also contained 0.75 pmol/mg DNA of cytosolic E1R (Table 2). E1R could not be detected elsewhere in these cells. One hour after incubating MCF-7 cells with 10^(-8) M 17β-estradiol, the previously described loss (processing) of total cellular E2R was seen [E2R decreased from 2.68 to 1.63 pmol/mg DNA (Table 2)]. During this incubation, the receptor with highest affinity for estrone had increased in the cytosolic and nuclear compartments from a total of 0.75 to 1.53 pmol/mg DNA. Therefore, incubation with 10^(-8) M 17β-estradiol had diminished total E2R by 0.85 pmol/mg DNA while increasing E1R by 0.78 pmol/mg DNA. Total cellular binding capacity for both estrogens had remained essentially unchanged during 17β-estradiol stimulation.

Examination of the time course of these alterations in recep-

<table>
<thead>
<tr>
<th>17β-Estradiol binding</th>
<th>Estrone binding</th>
</tr>
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<tbody>
<tr>
<td><strong>Capacity (pmol/mg DNA)</strong></td>
<td><strong>Kd × 10^(-8) M</strong></td>
</tr>
<tr>
<td>Cytosol</td>
<td>1.87</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.17</td>
</tr>
<tr>
<td>Nuclear</td>
<td>0.44</td>
</tr>
<tr>
<td>Total</td>
<td>2.48</td>
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</table>

**Table 2**

Scatchard analysis was carried out on each receptor utilizing the protamine sulfate assay procedure: 0° incubation for 18 hr for cytosol receptors; and 37° for 1 hr for nuclear and microsomal extracted receptors. Estrone binding was determined utilizing a range of [^3H]estrone (see "Materials and Methods") in the presence of 5 nM unlabeled 17β-estradiol, a level capable of saturating the unchanged E2R (see Chart 6B). Points, means of duplicate assays. A: total cytosolic and nuclear E2R; B: total cytosolic and nuclear E1R; C: total receptors (E2R plus E1R). B: ○, cytosolic E1R; ●, cytosolic E2R; △, nuclear E2R; ●, nuclear E2R.

The appearance of estrone-binding protein in the cytoplasm displayed a dependence on the concentration of 17β-estradiol by which the culture was stimulated. Chart 8 presents the levels of cytosolic E2R, extractable nuclear E2R, and E1R (mostly found in the cytosol; see Chart 7) which were present in MCF-7 cells incubated for 1 hr with increasing levels (10^(-11) to 10^(-8) M) of 17β-estradiol. Cytosolic E2R was seen to disappear and accumulate in the nucleus as the 17β-estradiol concentration in the incubation increased from 10^(-11) to 10^(-9) M. At the maximum 17β-estradiol-stimulatory level (10^(-9) M), the nuclear E2R reached a capacity (0.70 pmol/mg DNA) in these

[17β-Estradiol binding](#)

[3.83](#)

[0.17](#)

[0.44](#)

[3.23](#)

[0.28](#)

[5.49](#)

[1.50](#)

[0.76](#)

[1.76](#)

[1.66](#)

[1.53](#)

[3.16](#)

[1.53](#)

[3.16](#)

[1.53](#)

[3.16](#)

[1.53](#)

[3.16](#)
one-point assays which this receptor maintained throughout the incubations with elevated 17β-estradiol concentrations. The total cellular E,R rose with increasing 17β-estradiol in the incubations until the stimulatory 17β-estradiol reached 10⁻¹⁰ to 10⁻⁸ M. Thereafter, at the highest concentration of media 17β-estradiol, the E,R per mg DNA diminished, accompanied by an increase in cytosolic E,R (Chart 8). Although the binding capacity of the salt-extractable nuclear E,R after maximal processing (brought about by 10⁻⁹ to 10⁻⁶ M 17β-estradiol) was independent of the concentration of stimulatory 17β-estradiol in the incubation, the cellular E,R (mostly cytosolic) fluctuated in a manner opposite to that of the variations in cytosolic E,R and dependent on the concentration of 17β-estradiol in the media.

**DISCUSSION**

The processing (loss) of E,R has been related to the induction of progesterone receptor in MCF-7 cells (18). Experimentally, a portion of the detectable cellular E,R is seen to disappear within 1 hr of stimulation of this culture by 10⁻⁹ to 10⁻⁶ M 17β-estradiol. Since the cytosolic receptor has been translocated to the nucleus immediately following administration of 17β-estradiol, it is the diminishing level of salt-extractable nuclear E,R which initially reveals the effects of processing. Horwitz and McGuire (18) have reported that the lost receptor was not detected in the salt-resistant fraction, nor have the experiments reported herein shown additional E,R in any other cellular compartment.

Detection of the estrogen receptor, or its complex with endogenous 17β-estradiol, has heretofore relied on the specific binding of, or ligand exchange with, tritiated 17β-estradiol. Without this high-affinity adsorption of the [³H]estradiol ligand, the presence of receptor would be undetected. The observed loss of E,R could therefore be the result of any alteration to the receptor which results in a significant decrease in its affinity for the labeled 17β-estradiol in the assay. On the other hand, a circumstance could exist in which the nuclear estrogen-receptor complex was changed in a manner which no longer allowed the endogenous ligand to be exchanged with the [³H]estradiol of the detection system.

The first of these assumptions would involve the loss of total tritiated estrogen bound with high affinity in intact cells exposed to physiological levels of [³H]estradiol, while the latter proposal would possibly exhibit a consistency of protein-bound label in such experiments. An earlier publication from this laboratory (4) has shown the sum of cytosolic and nuclear tritiated estradiol not to decline during a 1-hr, 37°C incubation of MCF-7 cells containing receptor previously complexed with [³H]estradiol in the cold. It would appear then that there is no loss of bound labeled estrogen in cells exposed to physiological levels of [³H]estradiol.

These initial studies also demonstrated that the labeled ligand was composed of both 17β-estradiol and estrone. In fact, the portion of bound tritium found in estrone increased with the length of incubation of MCF-7 cells containing pulse-labeled [³H]estrone. Furthermore, the [³H]estrone increased at the expense of [³H]estradiol in the salt-resistant nuclear component while a [³H]estrone-protein complex accumulated in the cytosol in the presence of a 100-fold excess of unbound cytoplasmic [³H]estradiol. These initial experiments suggested the existence of an E,R in these cells, a fact documented in later work (3). Although unusual, our earlier reports of a specific E,R in MCF-7 human breast tumor cells have been supported by the published investigations of Kreitmann et al. showing the presence of both 17β-estradiol and estrone receptors in human uterine cytosol (19) and in monkey endometrial nuclei (20).

It has been the purpose of the studies contained herein to establish the optimum conditions for the assay of E,R and E,R in the various cellular compartments, to document the specificity of E,S,R, and to show the dynamics and cellular distribution of E,R and E,R, particularly following 17β-estradiol stimulation of MCF-7 cells.

Utilizing optimum conditions, it was possible to determine dissociation constants and binding capacities for either receptor in the absence of the other (e.g., E,S,R in the cytosol from 17β-estradiol-stimulated cells) or, in the case where both receptors were present, saturating amounts of the other estrogen (unlabeled) may be added to the assay mixture (e.g., salt-extractable nuclear receptors). These procedures have shown both E,S,R and E,R to exist in the cytosol and in the salt-extractable and the salt-resistant nuclear compartments. The binding of [³H]estradiol to the extractable nuclear E,R was not competed by estrone at molar excesses below 10-fold (Chart 6A). Below this competitive excess, there was also very little displacement of [³H]estradiol from its cytosolic receptor by estrone. At the same time, the data in Chart 6 show little or no binding of 17β-estradiol to the E,R (particularly in the cytosol) since there was no displacement of [³H]estrone by 17β-estradiol until a molar excess of 1000-fold was reached (Chart 6B). The exchange of 17β-estradiol for [³H]estrone on the classical cytosolic E,R is seen to occur in control cells which contain both receptors in their cytoplasm (half-displacement occurring at 5 x 10⁻¹⁰ M or near the K₅₀ for cytosolic E,R). Aside from the indicated specificity, 17β-estradiol binds more tightly to its cytosolic (K₅₀ = 0.3 x 10⁻⁹ M) and nuclear (K₅₀ = 0.8 x 10⁻⁹ M) receptors than estrone is bound by its receptors (cytosolic E,R, K₅₀ = 4 x 10⁻⁹ M; nuclear E,S,R, K₅₀ = 1.7 x 10⁻⁸ M).

The fact that MCF-7 cells contain separate proteins which preferentially bind either 17β-estradiol or estrone is intriguing and, since estrone is derived endogenously from 17β-estradiol,
a possible relationship between E2R and E, R is suggested. The quantitative data in Table 2 clearly show that both these receptors could be found in the cytoplasm and nucleus. Furthermore, as the 17β-estradiol exposure of this culture was increased to optimum concentrations that bring about processing, the specific E2R accumulated within the nucleus as the E, R reached its highest level in the cytosol. Most importantly, the total estrogen (17β-estradiol plus estrone) binding capacity remained virtually unaltered after a 1-hr exposure of MCF-7 cells to the processing level of 17β-estradiol. The 0.85-pmol/mg DNA value of "lost" E2R had been accompanied by a nearly equal increase in cellular E, R (0.78 pmol/mg DNA).

This quantitative similarity between the disappearance of E2R and the appearance of additional E, R in MCF-7 cells was also reflected in their temporal relationship (Chart 7). As cellular E2R was processed, the level of E, R increased. For the most part, the E, R was lost from the nuclear extract and the E, R, while present in the same extract, was shown to accumulate in the cytosol. Over the entire 5-hr period, the total detected cellular estrogen-binding capacity (17β-estradiol plus estrone) remained unchanged in these 6 timed assays which were carried out under identical saturation conditions for each ligand.

Not only did the formation of E, R occur simultaneously with the processing of E2R, but also the maximum appearance of this novel receptor depends on the exposure of MCF-7 cells to optimum levels of E2 (10−9 to 10−8 M) for the promotion of processing (Chart 8). 17β-Estradiol concentrations which were too low or too high for optimum processing of E2R resulted in decreased formation of E, R.

There are several possibilities which must be considered as possible explanations for the observed estrone binding. For example, 17β-estradiol dehydrogenase has been shown to be distributed throughout target tissues (29). Although it has been demonstrated in these investigations that there was no interconversion of 17β-estradiol and estrone during the incubation of tritiated estrogen and the protamine sulfate precipitate, it remains conceivable that a precipitated estrogen dehydrogenase might bind the estrogen. This does not appear to be the case, however, since the target tissue enzyme has a μM Km (37) indicating significantly less ligand affinity compared to that of estrone (or 17β-estradiol) found in these investigations (K0. 29. In addition, the widely different binding characteristics of the protein described herein for 17β-estradiol and estrone (10-fold 17β-estradiol required to displace estrone) are unlike those of the target tissue dehydrogenase (Km for 17β-estradiol is 20 times that of estrone). Finally, the apparent level of this dehydrogenase in target tissues is increased by progesterone (36), not by 17β-estradiol as demonstrated in these studies.

Numerous recent publications have reported the existence of a type II estrogen receptor in uterine and breast tissue (10, 14, 38). This binding has been characterized by a high K0 (30 nm) for 17β-estradiol and the lack of nuclear migration during 17β-estradiol stimulation, both properties of the E2R documented above. There are, however, several discrepancies between the properties of the type II 17β-estradiol binding and the E, R described herein. First, the cytoplasmic type II E, R concentration remains unchanged during 17β-estradiol stimulation (14). Conversely, the level of cytoplasmic E, R increases significantly following the exposure of MCF-7 cells to 17β-estradiol. Furthermore, 17β-estradiol does not compete with estrone for sites on E, R until its concentration is at least 100-fold greater. On the other hand, 17β-estradiol and estrone are equally effective in the inhibition of [3H]estradiol binding to type II sites (10). Finally, the number of binding sites on E, R does not approach that reported for type II [greater than 4 times type I in uterus, (10) and in MCF-7 cells].

The data derived from these investigations do not allow for a determination of the origins of E, R nor do they permit conclusions as to the relationship between E2R and E, R in MCF-7 cells. It is, however, pertinent to discuss receptor alterations which might account for the observations reported herein. Information presently available indicates that the D-ring of estrogens is linked to the receptor protein through a hydrogen-bonding system involving the 17-oxygen and some point on the protein which lies above Ring D (15). The 17β-hydroxy of 17β-estradiol would be expected to contribute to higher affinity if the ligand were the hydrogen donor to such bonding. However, small alterations to this site on the receptor resulting in a protein which served as the hydrogen donor above Ring D might produce a receptor with greater binding to estrone with its 17-keto group acting now as a hydrogen acceptor. A similar situation has been elucidated for the hydrogen bonding above Ring D of the estrogen-specific bovine adrenal estrogen sulfotransferase (30). More important is the contribution which the aromatic Ring A makes to the affinity of estrogens to their receptor(s). X-ray crystallographic studies have shown discrete but important differences to exist between the A ring-binding properties of estradiol and estrone (11, 12). Principally, these differences are a lower pK for the phenol group of estrone and the unique binding characteristics to exist between the A ring-binding properties of estradiol and estrone (11, 12).

REFERENCES


* Unpublished data from these laboratories.
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Estrone Receptor Formation During the Processing of Estradiol-Receptor Complex in MCF-7 Cells

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