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EFFECTS OF ESTROGENS ON MCF-7 CELLS: POSITIVE OR NEGATIVE REGULATION BY THE NATURE OF THE LIGAND-RECEPTOR COMPLEX

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Summary: The present study demonstrates that the nature of the binding of estrogens to the hormone-binding domain of the estrogen receptor (ER) modifies the responses of estrogen-dependent cells. We report here that 10 nM estradiol (E_2) forms noncovalent associations with the ER, increases the level of ER and Progesterone Receptors (PR) in ER^+ MCF-7 human breast cancer cells in culture following short-term or long-term exposure to E_2. In contrast, 10 nM 16-alpha-hydroxyestrone (16α-OHE_1), a physiological metabolite of E_2, in short-term cultures is equivalent to E_2, but upon long-term incubation, 16α-OHE_1 forms covalent associations with the ER, produces a marked decrease in ER and PR levels reaching values similar to, or below to that of control cells.

The MCF-7 human breast cancer cell line contains ER and is estrogen-responsive for growth and for the induction of several proteins, specially PR (1-3). The induction of ER levels has been reported to be also, estrogen-dependent although the nature of its regulation appear to be complex (4-6). The induction of PR levels is reversible: it ceases when estrogen treatment is discontinued but in response to estrogenic restimulation there is a marked increase and this is paralleled by the secretion of other proteins (2, 3, 7).

Recently we have reported that 16α-OHE_1 is able to form covalent interactions with the ER in MCF-7 cells following long-term exposure (1-4 weeks) to the metabolite (8), in contrast, to E_2 that binds to the steroid binding domain of the ER in a noncovalent reversible way in mammalian target tissues (9-10). Measurement of the ER by radiolabeled binding assays indicates that in MCF-7 cells there is a basal level of approximately 10 fmol/mg protein and there is evidence that their localization is preferentially nuclear (11-12).

The interaction of a steroid receptor with its ligand forms a transcriptionally active unit which acts after binding to the appropriate hormone responsive element on the genome (13). While the nature of the steroid hormone receptors, which are among the earliest identified transcription factors, has been extensively studied and almost all of them have been cloned and sequenced, the nature of the ligand-receptor interaction and its biological consequences are much

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Abbreviations used:

E_2, 17β-estradiol; 16α-OHE_1, 16-alpha-hydroxyestrone; ER, estrogen receptor; PR, progesterone receptor; DMEM, Dulbecco's modified essential media; DCC, dextran (0.05%) coated charcoal (0.5%).

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less well understood (14-15). The conventional view of the binding of a hormone to the steroid binding region of the ER protein is that it is of noncovalent reversible nature presumably mediated by hydrogen bonding, (9, 13). Indeed, all of the experimental evidence available for the reaction of estrogens with the ER supports this type of binding as representing the physiological norm (1, 9, 13 and references cited therein). Covalent binding to the ER has been achieved experimentally but that has involved synthetic ligands with chemically activated functions (16). The biological actions of such covalent complexes have not been characterized.

We have previously reported that a natural metabolite of E_2, 16α-OHE_1, can form a covalent bond with the lysine residues of proteins via a stabilized Schiff base and a subsequent Heyns rearrangement (17, 18). We have described the formation of such adducts with nonspecific membrane and nuclear proteins in vitro and in vivo (19-21). Most importantly, we demonstrated that this reaction occurs with the nuclear ER in a time-dependent fashion, to a very enhanced degree, presumably because of the initial conventional reversible binding of the estrogen to the receptor (8). This covalent interaction which occurs uniquely with this natural metabolite of E_2 has provided the hypothetical mechanism rationalizing the epidemiological observations that increased formation of 16α-OHE_1 is associated with an increased risk of breast cancer in the mouse and the human (22-24).

The present study was initiated to determine whether the biological properties of the covalently bound ER complex differed from those induced by a hormone bound to the receptor in the classical reversible fashion. The experimental design needed to incorporate the fact that 16α-OHE_1 can and does function initially as a classical reversible ligand for the ER and thus exhibits all of the biological effects of an estrogen agonist such as E_2 (25, 26). Upon longer exposure, however, covalent interaction does occur with 16α-OHE_1 but not with E_2. Thus, by comparing the biological responses of short-term exposure of ER positive MCF-7 cells in culture to E_2 and 16α-OHE_1 to those obtained following long-term exposure, the biological effects of the covalent and irreversible binding of 16α-OHE_1 to the ER can be determined.

MATERIAL AND METHODS

Materials: The nonradioactive steroids were obtained from Steraloids Inc. (Wilton, N.H.). Bovine insulin was purchased from Sigma Chemical Company (St. Louis, MO). All culture media were obtained from Grand Island Biological Company (Grand Island, NY). Radiolabeled compounds, [6, 7-^{3}H]E_2, sp. act. 48 Ci/mmole, [6, 7-^{3}H]R5020 (promegestone), sp. act. 87 Ci/mmole and nonradiolabeled R5020 were obtained from New England Nuclear (Boston, MA). All chemicals and reagents were of the highest available purity.

Cell Culture: MCF-7 human breast cancer cells obtained from the Michigan Cancer Foundation (Detroit, MI) were grown in plastic T-150 flasks in Dulbecco's modified Eagle's medium (DMEM) in the absence of any estrogenic stimulation by culture in phenol red-free medium containing 7.5% fetal calf serum treated twice with dextran-coated charcoal (DCC) to remove endogenous steroids and in the presence of 1 ng/ml insulin. To determine the effects of E_2 and 16α-OHE_1 on ER and PR levels, MCF-7 cells were harvested and seeded into T-75 flasks at a density of 1.5 x 10^6 cells/T-75 flask. Cells were exposed to 1 x 10^{-8} M E_2 or 1 x 10^{-8} M 16α-OHE_1 and control cells received the same volume of vehicle per flask (0.1% ethanol). Treatment of cells was extended over a period of 6 to 8 weeks. When cells reached confluence, they were harvested and replated at a density of 1:3. During treatment, culture medium was changed every 2 days.

Preparation of Nuclear Fractions: This was carried out as described by Eckert and Katzenellenbogen (28) with modifications as we have described previously (29). Procedures were done at 4° C and all buffers contained 1 mM PMSF.

Receptor Binding Activity Measurements: Before binding assays, E_2-treated cells were washed 3 times during a 2 h period and refed with fresh medium in the absence of E_2 to remove free steroid. This treatment was shown to deplete the cells of unbound E_2 prior to the exchange assay (data not shown). Purified nuclear fractions were used for radiolabeled E_2 exchange binding assays as described by Clark and Peck (9) and PR assays were done using radiolabeled R5020 as described by Horwitz and McGuire (2). Nuclear exchange assays were performed at 30° C for 30 min in the presence of 2 x 10^{-8} M [^{3}H]-E_2. In order to measure nonspecific binding, parallel assays were done in the presence of 100-fold excess nonradiolabeled E_2. Assays were completed after 30 min when tubes were placed at 4° C and bound radioactive E_2 was measured after precipitation with 5% protamine sulfate as we have described previously (8, 29). Progesterone binding activities were determined in combined nuclear and cytosol fractions using 2 x 10^{-8} M [^{3}H]-R5020 at 4° C for 4 hours. Nonspecific binding was assessed in parallel assays in the presence of 100-fold excess nonradiolabeled R5020. Samples were precipitated in the presence of 5% protamine sulfate (2). Specific binding was calculated as the difference between total and nonspecific binding per mg of DNA. DNA concentration in parallel nuclear and cytosol suspensions was measured as described by Labarca and Paigen (31).

Studies on cell proliferation: MCF-7 cells that had been grown for more than one year in DMEM in the absence of estrogenic stimulation, e.g. 7.5% fetal calf serum treated twice with DCC and in phenol red-free conditions were exposed to 10^{-8} M E_2 or 10^{-8} M 16α-OHE_1 for 1 and 4 weeks. Control cells received only the vehicle at a final concentration of 0.1% ethanol. One day after seeding at a density of 7 x 10^5 cells/25 cm^2 flask in DMEM (day 0), attached cells were harvested from 3 x T-25 flasks from each group. Their viability was assessed by exclusion of Trypan blue solution and their number was determined in duplicate samples from each flask using a Coulter cell counter (Coulter Electronics, Hialeah, FL). Viability was 95-97% of total cell number in the three experimental groups.

RESULTS AND DISCUSSION

Generally recognized defined biological responses of MCF-7 cell cultures to estrogens are the induction of the progesterone receptor (PR), changes in ER content and cell proliferation (2, 31, 32). The presence of cellular progesterone receptors (PR), as determined by radioligand binding assays, following exposure of cell cultures to E_2 and 16α-OHE_1 at different time periods are shown in Fig. 1. There is a multi-fold increase in PR with E_2 and 16α-OHE_1 with both estrogens producing similar results relative to controls for the first 150-200 h (6-8 days). Thereafter, for up to 1500 h (2 months) of exposure, when measurements were stopped, E_2 maintained high PR levels while the cell cultures grown in the presence of 16α-OHE_1 for periods exceeding 200 h (8 days) showed a precipitous drop in PR concentration to control cell values. Thus, there is a dramatic difference in the PR response to short-term and long-term 16α-OHE_1 exposure but there is no such difference in the case of E_2. This suggests that the change from a reversible to a covalent binding of 16α-OHE_1 to the ER terminates PR induction in the MCF-7 cell line.

Fig 2 shows nuclear ER levels determined by radioligand exchange binding assays. In the first hour both estrogens produced large multi-fold increases in nuclear ER content which most likely, reflect specific binding to nuclear ER and mobilization of cytosolic receptors. E_2-treated continue to exhibit increased nuclear ER for the remaining 1000 h (6 weeks) of the study which must reflect synthesis of ER, which has a relatively short half life of 4-6 h (33) and thus adds

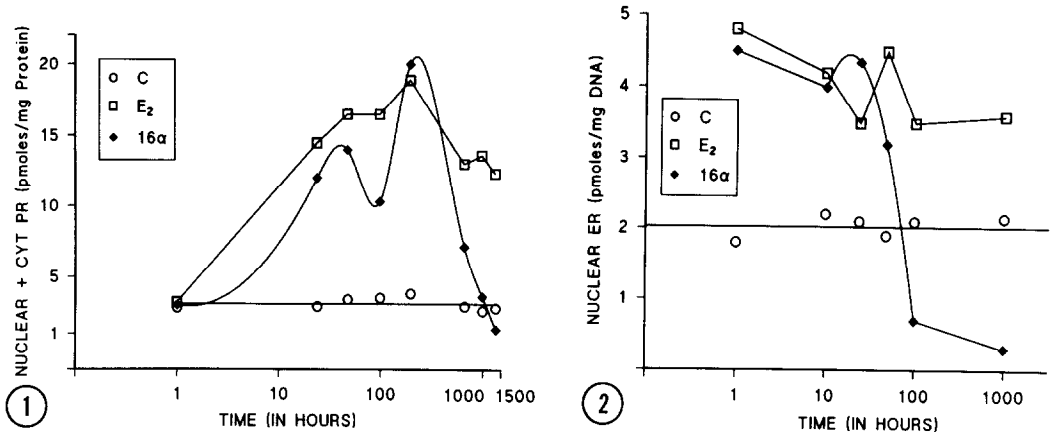

FIG. 1. The effects of E_2 and 16α-OHE_1 (10^{-8} M) on PR levels in MCF-7 cells in culture was different. The protein synthesis dependent effects of estrogens on PR was positive during the first 4-6 days (100-200 h). In a time-dependent fashion, 16α-OHE_1 failed to modulate positively PR levels that decreased markedly and remained below those of control cells, apparently exerting a negative regulation of PR levels. Because ER levels in cells treated with 16α-OHE_1 decreased earlier (see Fig 2) below baseline values, the PR effect was delayed in reference to that of ER levels, suggesting that this lag period could be related to the stability or half-life of intermediate inducers, i.e., mRNAs or trans-acting factors.

FIG. 2. Both estrogens E_2 and 16α-OHE_1 (10^{-8} M) modulate positively the regulation of nuclear ER levels during the first 24 hours of treatment in MCF-7 cells in culture. After 48 hrs, ER levels of 16α-OHE_1 -treated cells show a marked decrease that reaches values below those of control cells, as if a negative regulation, from the same estrogen was activated. ER levels in E_2-treated cells were consistently higher during the 6 weeks (1000 h) of observation.

another positive evidence to the controversial question of ER synthesis under the influence of estrogens (5, 6).

Nuclear exchangeable ER content decreases rapidly to below control levels after 50 h (2 days) of exposure to 16α-OHE_1, which may in part reflect the nonexchangeable nature of the covalently linked 16α-OHE_1. The fact that such covalently linked receptor can accumulate implies clearly that the turn over of the ER is greatly slowed or extinguished when a covalently bound 16α-OHE_1 is present. The earlier decrease in the nuclear ER, (50-100 h) than that of the PR (150-200 h) is in agreement with the temporal sequence of these events.

In contrast to the diminished or even extinguished biological activity of the ER covalently linked with 16α-OHE_1 insofar as PR and possibly ER induction is concerned, cellular proliferation was not affected by the change from classical reversible estrogen binding to the covalent type occupancy of the receptor and this lack of syncrony of protein synthesis and cell proliferation has been reported for E_2 by Katzenellenbogen et al. (27). Fig 3, A and B show the change in cell number over 7 days of culture which have been exposed for 1 week and 4 weeks respectively to E_2 and 16α-OHE_1. The rate of proliferation in the two sets is identical with the effects of E_2 and 16α-OHE_1 being essentially the same. Thus, the covalent binding of 16α-OHE_1 to the ER does not alter the proliferative effect of the hormone, which as an estrogen, activates an ER dependent function in MCF-7 cells. From this we may conclude that the covalently bound receptor is not biologically silent but that it continues to perform some of the functions of the classical reversibly bound complex, i.e. growth factors, suggesting that differential gene expression may result upon covalent binding (34).

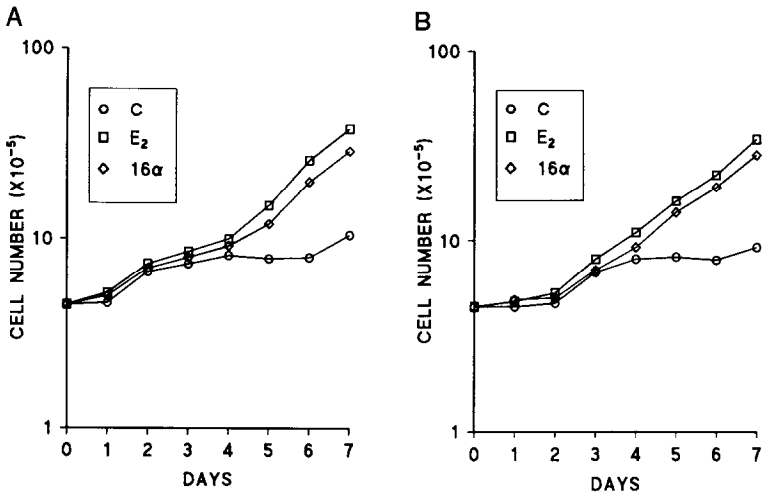

FIG. 3. **A** and **B**. Effects of 10^{-8} M E_2 and 16α-OHE_1 on cell proliferation of MCF-7 cells. **A**, cells had been incubated with 10 nM E_2 or 16α-OHE_1 for 1 week; or, **B**, for 4 weeks, then harvested and plated at a density of 7 x 10^5 cells/25 cm^2 flasks and continue under the previous treatment. Hormones and media were renewed daily. Cell number was measured daily during the 7 days of observation, after harvesting 3 flasks from each group. Values in panels **A** and **B** represent the average numbers of duplicate measurements in 3 flasks from each group.

The covalent binding of the E_2 metabolite 16α-OHE_1 probably to lysine residues of the steroid binding region of the ER is a nonenzymatic process. At all times therefore, cells exposed to 16α-OHE_1 will contain non-covalent and covalently bound 16α-OHE_1-ER complexes in a proportion that we have not determined. Since the former might turnover with a half-life of few hours, similar to that of the E_2-ER complex (33), the covalent 16α-OHE_1-ER complex reported previously by us (8) was resistant to ethanol and ether extraction and its concentration was cumulative in a time-dependent manner, characteristics that may indicate that the latter might turn over at a slower rate, a fact that we have not established. If this probability is valid, then the covalently bound complex would accumulate and eventually represent the majority of the complexes. Therefore, biological responses in cell culture will be determined by both forms of the liganded receptor with the effects of the covalent linked form increasing with time. This opposite effects were observed in the above studies. We have previously noted that in the presence of E_2 which has a much greater affinity for the ER, the covalent reaction with 16α-OHE_1 is greatly diminished or eliminated presumably because of the competitive blocking effect (8). Thus in vivo under physiological conditions such covalent interactions would be expected to be rare events which, however, would have a greater probability in the case of excess unliganded ER or higher 16α-OHE_1 presence. Once such an event occurs, the present study shows that the estrogenic action on the cell machinery is altered and this possibly may participate in cell transformation. Of necessity the present studies were conducted with transformed cells and hence the events associated to transformation could not be observed. It is hoped that studies now in progress would provide more information on the potential transforming effect of 16α-OHE_1 when bound covalently to the ER and its inherent capacity to elicit opposite interactions with regulatory elements and transcription factors.

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