ABSTRACT

Development of resistance to hormonal therapy in breast cancer is frequently associated with a decline or loss of cellular estrogen receptors. Agents which up-regulate the receptor may reduce the incidence of hormonal resistance. Antiestrogens at concentrations ranging from 0.1 to 1 μM produced a 2- to 4-fold increase of estrogen receptors in MCF-7 and T-47D breast cancer cells. This increase, which occurred as early as 3 h and was sustained throughout the 4 days of continuous exposure to tamoxifen, was primarily due to an enhancement in receptor synthesis.

INTRODUCTION

Hormonal therapy plays a significant role in breast cancer management. Objective tumor regression can be achieved from hormonal therapy in patients whose tumors contain a significant amount of ER (1, 2). Nevertheless, hormonal resistance eventually develops when a hormonally dependent, ER-rich tumor evolves to one that is nonresponsive and ER poor (3, 4). Preventing the development of hormonal resistance may improve patient survival. With this notion, up-regulation of tumor ER may be one of the means to slow down or to reverse the process of hormonal resistance.

In most instances, the dominant factors regulating the receptors are their own ligands. The effect of estrogens on cellular ER varies depending on the developmental stage of the target organ. In immature uteri, estrogens induce ER (5); while in mature uteri and breast cancer, they down-regulate ER (5, 6), probably via a feedback mechanism to avoid excessive estrogenic function. We therefore hypothesize that tamoxifen acting as an antiestrogen may deprive the cell of this estrogenic feedback loop, thus up-regulating the ER. Furthermore, antiestrogens have been shown to induce progesterone receptors in uteri and breast cancers (7, 8), and it is conceivable that they may also induce ER synthesis.

The mechanism of action of antiestrogens is that it competes with estrogens at the ER-binding sites. Before the current knowledge that the nuclear location of ER was available, it was once thought that the ER-antiestrogen complexes remained in the nuclei for a prolonged duration, rendering the cells insensitive to estrogenic stimulation (9). Such nuclear ER-antiestrogen "retention" has been thought to be due to a reduction in receptor degradation (processing) (9-12). In this study, we attempt to investigate whether the mechanism of ER increase could be based on another reason, i.e., an increase of ER synthesis. The ER up-regulation, if proven to exist, could be a useful tool in modulating the behavior of the cancer cell and in preventing it from becoming hormonally resistant.

There are technical difficulties in studying the modulation of ER in the presence of tamoxifen, because antiestrogens interfere with the conventional ER assay in which [3H]estradiol is used to measure the amount of ER. Earlier studies on ER modulation by estrogens or antiestrogens have adopted a ligand exchange method which, however, is not completely reliable. Because of their extremely heat-labile properties, some receptor binding-activities are lost during ligand exchange at temperatures ranging from 25 to 34°C. Recent advances in anti-ER monoclonal antibodies have made it possible to accurately determine the receptor level in the presence of antiestrogens. The antigenic sites of ER are much more physically stable than the biological steroid-binding sites. In this study, we assess the effect of tamoxifen on the rate of ER synthesis in human breast cancer cells using both the anti-ER monoclonal antibodies and the density-shift technique (13).

MATERIALS AND METHODS

Chemicals. The enzyme immunoassay kits for ER were provided gratis by Abbott Laboratory (North Chicago, IL). The 4-hydroxytamoxifen and tamoxifen were kindly provided by ICI Pharmaceutics (Macclesfield, England) and ICI Americas, Inc. (Wilmington, DE), respectively. The algal amino acid mixture enriched in the dense isotopes (2H, 13C, and 15N) was purchased from Merck Sharp & Dohme (Montreal, Quebec, Canada).

Cells and Media. The MCF-7 cell line was a gift from Dr. Marc Lippman, then at the National Cancer Institute, and was maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.6 μg/ml insulin. All experiments were carried out in this regular medium except for two: (a) experiments in studying the epitope expression of ER antigen were carried out in both regular and estrogen-deprived media; the latter consisted of phenol red-free Dulbecco's modified Eagle's medium (Gibco) with 10% fetal calf serum treated with dextran-coated charcoal; and (b) experiments on the long-term tamoxifen effect were pursued in estrogen-deprived medium (Fig. 4). The MCF-7 cells used for the experiments described here were from subculture passages 36 through 55. The growth characteristics of this cell line in our laboratory have been described previously (14). The T-47D cell line was purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 8 μg/ml insulin.

Dense Amino Acid Medium. The 13C, 15N, 2H-labeled amino acid mixture (250 mg) was dissolved in 5 ml distilled water, filtered through an Amicon YM-2 membrane (Amicon Corp., Lexington, MA), and stored as 1-ml aliquots at -70°C. The dense amino acid medium was prepared according to the method of Eckert et al. (13).

ER from Whole Cell Lysate. MCF-7 cells or T-47D cells were grown in their logarithmic growth or near-confluent phase in media containing antiestrogens or vehicles (as control) for various designed intervals. Following incubation, the cells were rinsed with Hank's balanced salt solution, harvested with scrapers, and stored in liquid nitrogen. The cells were disrupted with a microultrasonics cell disrupter (Kontes Scientific, Morton Grove, IL) at a setting of 60 for 5 s in TEMG buffer containing 0.4 M KCl. Under microscopic examination no intact nucleus or cell was seen in the cell lysate. The supernatants were collected for ER-EIA and protein assays after centrifugation at 105,000 x g for

Received 2/15/89; revised 6/29/89; accepted 7/6/89.

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1 This investigation was supported by Grants PDT-323 from the American Cancer Society, CA-42439 from the NIH, and the Minnesota Medical Foundation, Inc.

2 To whom requests for reprints should be addressed.

The abbreviations used are: ER, estrogen receptors; ER-EIA, ER-enzyme immunoassay; ERIA, dense estrogen receptors; ERα, light estrogen receptors; PAF, peroxidase-antiperoxidase; TEMG buffer, 10 mM Tris-1.5 mM EDTA-12 mM monothioglycerol-10% glycerol (v/v), pH 7.4.

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35 min. DNA assay was conducted on an aliquot of the cell lysate before centrifugation.

**Sucrose Gradient Centrifugation.** The gradients were prepared in deuterium oxide containing 5–20% sucrose and 0.4 M KCl in TEMG buffer. Cell extracts (250 μl) were layered onto 3.6-ml gradients. Centrifugation was carried out at 357,000 × g for 29 h in a Beckman L5-65 ultracentrifuge with an SW60 Ti rotor. In the experiments which used 3 m urea in the gradient, the centrifugation was carried out at 308,000 × g for 40 h. All the above procedures were performed at 0–4°C. After centrifugation, four-drop fractions were collected from the bottom of the tube and assayed for ER by the ER-EIA method. Ovalbumin (3.5S) and γ-globulin (6.6S) were used as density markers.

**ER-EIA Method.** The protocol for ER-EIA was that in the product package insert (Abbott Laboratories, North Chicago, IL). Briefly, cell lysate (200 μl) or gradient fractions (180 μl) were incubated for 18 h at 2–8°C with beads coated with monoclonal anti-ER antibody D547. ER molecules were adsorbed by the beads during this incubation. Most of the proteins in the lysate were removed by washing. Afterward, a second monoclonal antibody, H-222, conjugated with PAP was added and incubated for 1 h at 37°C. Unbound PAP anti-ER was then removed by washing the beads. The amount of PAP bound to the beads through ER molecules was determined by adding an enzyme substrate solution (hydrogen peroxide and o-phenylenediamine-2HCl). The intensity of the color produced by the reaction with enzyme substrate was measured immediately within 1 h in a computerized quantum analyzer (Abbott). The DNA and protein content in the cell lysate was determined by the methods of Burton (15) and Lowry et al. (16), respectively. The amount of ER was expressed as pmol/mg DNA or fmol/mg protein. Both types of unit expression were used to assure that tamoxifen effects on protein or DNA synthesis did not artificially alter the ER values.

**Statistical Analysis.** Statistical analysis was based on Student’s t test. The confidence limits were set at the 99% level.

**RESULTS**

The effect of 1 μM tamoxifen on ER content in MCF-7 cells is shown in Table 1. In this experiment, cells were exposed to tamoxifen for 6, 24, and 48 h. There was a 3- to 4-fold increase of ER content in MCF-7 cells. Fig. 1 shows the effects of continuous exposure of cells to 1 or 0.1 μM tamoxifen for up to 5 days. There was a 325% increase of ER with 1 μM tamoxifen at 48 h, but the receptor level decreased to that of the control in 84 hr, presumably due to the cytotoxic effect from this high concentration of tamoxifen. Although there was a smaller increase in ER (220%) at the lower concentration of tamoxifen (0.1 μM), this stimulatory effect was sustained throughout the 5-day experimental period.

Fig. 2 shows the dose-dependent effect of tamoxifen and its derivative, 4-hydroxytamoxifen, on ER content. Stimulation of ER by tamoxifen or 4-hydroxytamoxifen was seen in both MCF-7 and T-47D breast cancer cells and was dose dependent. The elevation of ER by 4-hydroxytamoxifen was observed at a concentration as low as 0.1 nM and reached a maximum at 10 nM. On the other hand, the stimulatory effect of tamoxifen was not seen until the concentrations exceeded 10 nM. A concentration of 1 μM tamoxifen was required to attain the equivalent stimulatory effect of 10 nM 4-hydroxytamoxifen. Hence, 4-hydroxytamoxifen was at least 100 times more potent than tamoxifen in elevating ER. When 0.1 μM concentrations of various antiestrogens were added to MCF-7 cells, their effectiveness was of the following order: tamoxifen = keoxifene < trioxifene = LY117018 < 4-hydroxytamoxifen (Table 2).

One aspect which needs to be addressed is that the increase in ER detected by monoclonal antibody techniques might be attributed to an increase of available antigenic sites following the tamoxifen binding. This phenomenon has recently been described by Martin et al. (17) in a different buffer system. We therefore performed experiments to study the effect of antiestrogens on ER content when they were added to the cell lysate (Table 3). Tamoxifen up to 2 μM concentration or 4-hydroxytamoxifen up to 1 μM added into the cell lysate did not increase the ER measurement, while an increase of ER occurred in cells exposed to antiestrogens in the culture medium. This increase was observed in cells grown in regular media (Table 3), as well as in estrogen-deprived media (data not shown).

Increase of the ER content by antiestrogens could be attributed to either a stimulation in receptor synthesis or a decrease in ER processing. The protein synthesis inhibitor, cycloheximide, was added into the culture medium to examine its effect on receptor synthesis (Table 4). After exposure to 0.01 μM 4-hydroxytamoxifen for 3 and 6 h, the ER content of MCF-7 cells increased to 126.4 and 148.6% of the control, respectively. Cycloheximide (20 μg/ml) completely blocked the ER stimulation from 4-hydroxytamoxifen in 6 h.

Using the dense amino acid labeling technique, we measured the amount of newly synthesized ERD, and the remaining ER, in MCF-7 cells with or without exposure to 0.5 μM tamoxifen. Whole cell ER was extracted by 0.4 M KCl and layered on top of 5–20% sucrose gradients containing 0.4 M KCl-TEMG buffer in deuterium oxide. After ultracentrifugation, 4-drop fractions were collected and the ER content in each fraction was measured by the ER-EIA method. The ER was sedimented at approximately 3.8S, while the sedimentation of ERD shifted to the 5.4S region (Fig. 3). It is interesting to note that tamoxifen exposure not only increased the magnitude of the ERD peak but also caused a further shift of ERD density from 5.4S to 6.6S (Fig. 3, A and B). The shift of ERD-tamoxifen complexes to a heavier density region made it technically easier for us to identify the newly synthesized ERD. This ER density shift from tamoxifen, as described by other investigators, can be prevented...
Fig. 2. Dose dependence of estrogen receptor responses to tamoxifen (Tam) or 4-hydroxytamoxifen (4 OH-Tam) in MCF-7 (A) or T-47D cells (B). The ER-EIA assays were performed on nearly confluent cells after 24-h exposures to antiestrogens. The total cellular ER was 144.3 ± 9.5 fmol/mg protein in the MCF-7 control and 91.9 ± 5.7 fmol/mg protein in the T-47D control. Each point represents the mean ± SEM bars from triplicate samples. The curves were visually fitted. The signs (*) were designated for P < 0.01.

Table 2 Effects of different antiestrogens on ER content in MCF-7 cells

<table>
<thead>
<tr>
<th>Antiestrogens (0.1 μM)</th>
<th>ER content* (fmol/mg protein)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>93.7 ± 2.4</td>
<td>100.0 ± 2.6</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>174.0 ± 5.0</td>
<td>185.7 ± 5.3</td>
</tr>
<tr>
<td>Keoxifene</td>
<td>177.7 ± 12.1</td>
<td>190.0 ± 12.9</td>
</tr>
<tr>
<td>Trioxifene</td>
<td>236.9 ± 3.6</td>
<td>252.0 ± 3.8</td>
</tr>
<tr>
<td>LY117018</td>
<td>255.2 ± 5.4</td>
<td>272.4 ± 5.8</td>
</tr>
<tr>
<td>4-Hydroxytamoxifen</td>
<td>310.8 ± 8.7</td>
<td>331.7 ± 9.3</td>
</tr>
</tbody>
</table>

* Mean ± SEM from quadruplicate experiments. Cells were harvested 24 h after the addition of antiestrogens.

Table 3 Comparison of the estrogen receptor content in MCF-7 cells with tamoxifen or 4-hydroxytamoxifen added in culture medium (24 h before harvest) or in cell lysate (after harvest)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (μM)</th>
<th>Added to culture medium</th>
<th>Added to cell lysate</th>
<th>Conjugated ER (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>93.7 ± 2.4</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>0.5</td>
<td>146.9 ± 14.6*</td>
<td>93.7 ± 2.3*</td>
<td>100.0 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>182.1 ± 6.6*</td>
<td>103.4 ± 0.9*</td>
<td>100.0 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>193.9 ± 14.1*</td>
<td>102.6 ± 1.4*</td>
<td>100.0 ± 2.6</td>
</tr>
<tr>
<td>4-Hydroxytamoxifen</td>
<td>0.01</td>
<td>190.0 ± 8.1*</td>
<td>104.8 ± 3.0*</td>
<td>93.7 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>195.1 ± 4.9*</td>
<td>105.4 ± 10.4*</td>
<td>100.0 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>219.0 ± 5.2*</td>
<td>96.1 ± 1.0*</td>
<td>93.7 ± 2.3</td>
</tr>
</tbody>
</table>

* The ER content in control MCF-7 cells was 189 fmol/mg protein.
* Mean ± SEM. The differences from the control were significant at P < 0.01.
* No statistical significance of difference from the control.

Table 4 Effects of cyclohexamide on the estrogen receptor content of MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Estrogen receptor (% of control)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>100</td>
</tr>
<tr>
<td>OH-Tam*</td>
<td>126.4 ± 4.6*</td>
</tr>
<tr>
<td>OH-Tam + CH</td>
<td>112.3 ± 5.3*</td>
</tr>
<tr>
<td>CH</td>
<td>78.4 ± 5.1</td>
</tr>
</tbody>
</table>

* The ER content in control MCF-7 cells was 220 fmol/mg protein.
* OH-Tam + 4-hydroxytamoxifen (10-6 M); CH, cycloheximide (20 μg/ml).
* Mean ± SEM.
* Not significant.
* P < 0.01.

Discussion

In this study, we have shown that the up-regulation of ER by tamoxifen and its analogues is reproducible in two breast cancer cell lines, is dose-dependent, and is relatively long-term (at least 5 days). It was not due to an increase in receptor antigenic sites of total ER in 3 h. Following tamoxifen (0.5 μM) treatment for 3 and 6 h, there were 1.5–1.6 times as much total ER (P < 0.01). These increases were derived from the newly synthesized ERD (Table 5). The newly synthesized ERD in tamoxifen-treated MCF-7 cells was 1.97 times higher than the control at 3 h and 1.84 times higher at 6 h (P < 0.01). On the other hand, the tamoxifen did not appear to affect the ER processing judging by a rather stable ERD level at 3 and 6 h in comparison with that of the control group.

Although the experiment shown in Fig. 1 indicated that the increased cellular ER level could be maintained over the entire 5-day experimental period, it was critical to know whether the elevated ER synthesis by tamoxifen could also be maintained through this period. To study this aspect, MCF-7 cells grown in estrogen-deprived media were exposed to a relatively nontoxic concentration (20) of 0.2 μM tamoxifen for periods ranging from 3 to 96 h. The cells were then grown in dense amino acid media containing zero or 0.5 μM tamoxifen for 6 h before being harvested. The amounts of newly synthesized ERD measured by the protein density shift technique were 1.6 to 2.2 times higher in cells that were preexposed to tamoxifen for 3 to 96 h than in control cells with no exposure to tamoxifen (Fig. 4). Also, there was no difference in DNA content between control and experimental groups. Thus, cell growth was not suppressed by 0.2 μM tamoxifen, and a change in DNA content was not the cause of the observed ER increase. This study indicated that the ER increase was mainly caused by a stimulation of ER synthesis which persisted even through the 4-day tamoxifen preexposure.
caused by tamoxifen. Using hydroxytamoxifen (20 nM) in the MCF-7 culture system, other investigators also demonstrated a 2-fold increase of nuclear hydroxytamoxifen-ER complexes measured by [3H]-4-hydroxytamoxifen binding, a measurement which was unrelated to the epitope expression of ER antigen (12). These results support our findings that there is a genuine ER up-regulation from tamoxifen and its analogues. Our study further illustrated that the ER up-regulation was due to an increased rate of ER synthesis.

In our experiments, MCF-7 cells were from a range of subculture numbers (passages 36 through 55). In spite of the fact that the control ER contents in MCF-7 cells varied from 62 to 218 fmol/mg, the up-regulation of ER by antiestrogens was persistently observed. There is a trend that the lower the initial ER level, the greater is the ER up-regulation in response to tamoxifen. This phenomenon indirectly supports the concept that the ER response is a biological event rather than a physicochemical alteration of receptors. The latter, if it occurs, would provide a fixed percentage increase of antigenic sites at each given concentration of tamoxifen.

It is interesting to note that ER up-regulation (3-fold increase) was also observed in MCF-7 cells growing in an estrogen-deprived medium for 5–6 months (21). We therefore postulate that the effects of estrogen depletion and tamoxifen exposure may involve a common mechanism, i.e., a lack of an estrogen-induced feedback loop. Further elucidation on the mechanism of ER modulation by tamoxifen at the molecular level is needed.

The mechanism of the up-regulation of ER by tamoxifen may take place at the transcriptional or translational level. It is interesting to note that the antiestrogens, hydroxytamoxifen and LY117018, had no effect on ER mRNA levels (22, 23). There is a possibility that tamoxifen may regulate the ER at the translational level via a mechanism similar to the human ferritin regulation. Ferritin biosynthesis is altered without a corresponding change in the level of total ferritin mRNA (24); instead, an iron-responsive element in the 5'-untranslated portion of the ferritin mRNA mediates iron-dependent control of its translation (25). The phenomenon that rapid ER synthesis was observed within 3 h of tamoxifen exposure certainly is compatible with the hypothesis of translational regulation.

In clinical settings, successful tamoxifen therapy is frequently
associated with an ER reduction in the residual tumor (26, 27). Such ER reduction is attributed to clonal selection by eliminating an ER-rich hormonally sensitive population of tumor cells. The effects of a low-dose tamoxifen on the tumor ER content is still unknown. It is interesting to note that in athymic nude mice bearing MCF-7 cells, tamoxifen treatment for 4 months led to an increase in tumor ER (28). Such ER enhancement may be involved in perpetuating the antiestrogenic and anti-tumor effects of tamoxifen, a mechanism that may contribute to the excellent tumor regression frequently observed with tamoxifen therapy. There is also ample evidence which implies a dissociation of proliferative function from biological activity of antiestrogens (29, 30). As demonstrated in this study, tamoxifen and other antiestrogens at their optimal sublethal concentrations may alter the biological function via ER modulation without interfering with cell proliferation. In other words, they may potentially be used as “maturation” agents in preventing the tumor progression from a hormonally sensitive to resistant status.

ACKNOWLEDGMENTS

The authors thank Dr. Jonathan Li for his critical comment and discussion. The secretarial assistance from Jolene Ludvigsen and technical assistance from Steven Schulstrom are greatly appreciated.

REFERENCES


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