Models of Estrogen Receptor Regulation by Estrogens and Antiestrogens in Breast Cancer Cell Lines

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ABSTRACT

The expression and stability of the estrogen receptor (ER) is the result of a complex process that is modulated by estrogens and antiestrogens. Regulation of the steady-state ER mRNA and protein levels in breast cancer cells appears to be the result of either of two distinct regulatory mechanisms. Estrogen exposure causes a rapid down-regulation of the steady-state level of ER mRNA and protein in a model I regulation, as exemplified by the MCF-7:W8 cell line. Conversely, in model II regulation, as observed in the T47D:A18 cell line, estrogen exposure causes an increase in the steady-state ER mRNA level and a maintenance of the ER protein level. In both these cell lines, the nonsteroidal antiestrogen 4-hydroxytamoxifen has little effect on the mRNA level but causes a net accumulation of the ER protein over time. In contrast, the pure antiestrogen ICI 182,780 causes a dramatic reduction of the ER protein in both the MCF-7:W8 and T47D:A18 cell lines. This loss has little effect upon the ER mRNA level in the MCF-7:W8 cells but leads to a decline in the ER mRNA in the T47D:A18 cells. The estrogen-independent MCF-7:2A cell line, which has adapted to growth in estrogen free media, expresses two forms of the ER, a wild-type M,66,000 ER and a mutant M,77,000 ER (ER77). ER77 is the product of a genomic rearrangement resulting in a tandem duplication of exons 6 and 7 (J. J. Pink et al., Nucleic Acids Res., 24: 962-969, 1996). This exon duplication has abolished ligand binding by this protein. Here we demonstrate that the loss of ligand binding has eliminated the effects of 4-OHT and ICI 182,780 on the steady-state ER77 protein level. However, in the MCF-7:2A cells, antiestrogens affect the wild-type ER protein in the same manner as observed in the MCF-7:W8 and T47D:A18 cells. Estrogen regulates the ER mRNA and wild-type ER and ER77 proteins in the MCF-7:2A cells in the same manner as observed in the MCF-7:W8 cells. Interestingly, treatment of the MCF-7:2A cells with ICI 182,780 causes a slight increase in ER mRNA, which is reflected in a net increase in the ER77 protein but a dramatic decrease in the wild-type ER. The models presented here describe the response of two human breast cancer cell lines in short-term studies. These distinct regulation pathways are predictive of the response of these cell lines to long-term estrogen deprivation. This study illustrates two alternative regulation pathways that are present in ER-positive, estrogen-dependent breast cancer cells. This variable response highlights the diversity of responses potentially present in the heterogeneous cell populations of clinically observed breast cancer.

INTRODUCTION

The treatment and prognosis of breast cancer has been improved dramatically during the past decade through the use of the nonsteroidal antiestrogen tamoxifen (1, 2). Although it has long been known that tamoxifen and its metabolites compete with estrogens for binding to the ER,3 the downstream effects of this binding are unclear. The most active metabolite of tamoxifen, which is used in all studies described here, is 4-OHT, which has a binding affinity for the ER 50-100-fold greater than that of tamoxifen (3). The continuing search for new and more effective antiestrogens lead to the synthesis of the 7α-substituted estradiol analogues such as ICI 164,384 and ICI 182,780 (4). These compounds are classified as pure antiestrogens due to their complete lack of any estrogenic activities and are currently being evaluated in clinical trials for the treatment of tamoxifen-resistant breast cancer (5). In contrast to the pure antiestrogens, the nonsteroidal antiestrogens such as tamoxifen display some estrogenic characteristics, depending upon the species and tissue type tested, and are, therefore, classified as partial agonists (6). In addition to the effects these compounds have on the function of the ER, it has been known for many years that estrogens and antiestrogens can regulate the steady-state level of the ER (7-27).

Since the isolation and characterization of the MCF-7 cell line in 1973, ER-positive breast cancer cell line have become the standard laboratory model in which to study the effects of estrogens and antiestrogens (28-31). The use of these valuable models throughout the past decade has lead to the evaluation of the effects of estrogens and antiestrogens in various cell culture systems (31-35). Previous studies have demonstrated the mechanism by which estrogens and pure antiestrogens regulate ER protein to be quite different. Both compounds cause a significant decrease in the steady-state level of the ER in specific cell lines (15, 18). Estrogens function by initially decreasing transcription of the ER and later by destabilizing the ER mRNA (15, 24). The pure antiestrogens, in contrast, have little effect on the transcription of the ER and function by directly causing a rapid degradation of the ER protein (36, 37). This degradation appears to be the result of an alteration of the nucleoplasmic shuttling of the receptor (38).

Regrettably, only a few different ER-positive cell lines are available in which to study the effects of estrogens and antiestrogens on the growth and gene expression in breast cancer cells. These represent only a few patients from which to generalize the findings. The standard cell line MCF-7 is dependent upon the presence of estrogens for maximal growth, and this growth can be competitively inhibited by antiestrogens. In virtually all published reports, E2 has been shown to lead to a rapid reduction in the steady-state levels of ER mRNA and protein in MCF-7 cells (15, 18, 39-41).

Another well-studied human breast cancer cell line, T47D, exhibits a different phenotype. The growth dependence and ER and PR expression of the various T47D clones used throughout the world reveals a remarkable diversity. The T47D cell line was originally characterized as an ER- and PR-positive, estrogen-dependent cell line (42). In the many years since its initial characterization, the cells have diverged and given rise to clones with very different growth and expression phenotypes. T47D lines have been described which range from completely estrogen independent to classically estrogen dependent (43-48). The clone that is currently used in our laboratory,
T47D:A18, requires estrogen for maximal growth, and this estrogen-stimulated growth can be inhibited by antiestrogens (32, 49). The regulation of PR expression in T47D cells, which is considered to be a marker of a functioning ER-mediated transcription pathway (50), has also been shown to be variable depending on the source of the cells. Some groups find that the PR is constitutively expressed and others, including our laboratory, find that the expression of the PR is regulated by estrogens. In T47D cells, the regulation of ER mRNA has been reported to be different than that observed in MCF-7 cells, with estrogen exposure giving rise to an increase in ER mRNA (18). For our studies, we classify the T47D:A18 cell line as ER positive and estrogen dependent.

To study the changes associated with the development of estrogen-independent growth we have developed a subclone of MCF-7 which now grows maximally in the absence of estrogens. This clonal cell line, named MCF-7:2A, was derived by growing MCF-7 cells in estrogen-free media for 8 months, followed by two rounds of limiting dilution cloning (51). In addition to being estrogen independent, these cells also express a novel M, 77,000 ER. This ER, which is referred to as ER77, is the result of a genomic rearrangement which has lead to a tandem duplication of exons 6 and 7 in the ER gene. This mutation has abrogated all ligand binding by ER77. The wild-type M, 66,000 ER is also expressed in these cells at a 4-10 fold higher level than the ER77. This cell line provides us with a unique model in which to study the regulation of two ERs with dramatic functional differences in a single cell line.

We believe that it is important to examine the differential effects of estrogens and different classes of antiestrogens on ER regulation to establish a clear understanding of this valuable therapeutic target. We have compared and contrasted the effects of compounds on three different cell lines and used two types of receptor proteins as markers of activity. This evaluation of responses to the diverse actions of these compounds in a single series of experiments gives a unique portrayal of the manner of action of estrogens and antiestrogens in human breast cancer cells. Our study reveals that distinct ER-dependent regulatory mechanisms are present in these breast cancer cells.

MATERIALS AND METHODS

Tissue Culture. MCF-7 cells were obtained from Dean Edwards (at the San Antonio Breast Cancer Group, TX; originally obtained from the Michigan Cancer Foundation Detroit, MI). T47D (42) cells were obtained from American Type Culture Collection (Rockville, MD). MCF-7:2A cells were derived from the MCF-7 line by growth in estrogen-free media and two rounds of limiting dilution cloning (51). All tissue culture components were obtained from Life Technologies, Inc. (Grand Island, NY) unless otherwise stated. MCF-7:WS8 and T47D:A18 cells were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Bioproducts for Science Inc., Indianapolis, IN), 6 ng/ml bovine insulin, 0.2 mM L-glutamine, 10 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (fully estrogenized medium). MCF-7:2A cells were routinely grown in estrogen-free medium that substitutes phenol red-free RPMI and 3% dextran-coated, charcoal-treated fetal bovine serum. Cells were passed at 1:10-1:20 dilutions once per week using 0.1% trypsin.

Growth Assays. All cells were grown in estrogen-free media for at least 2 days prior to the beginning of each experiment. Cells were seeded into each well of a 24-well plate (20,000 cells/well) in 1 ml of estrogen-free media on day 0. The following day (day 1), this media was removed, and 1 ml of media containing the appropriate compound was added. All compounds were dissolved in 100% ethanol and added to media at a 1:1000 dilution. Media was changed on day 4, and experiments were ended on day 6. DNA content was determined by the method of Labarca and Paigen (52) using a fluorocolorimeter II (SLM Aminco, Urbana, IL). 17β-Estradiol was purchased from Sigma Chemical Corp., 4-OHT was a generous gift from Zeneca Pharmaceuticals (Macclesfield, England), and ICI 182,780 was a generous gift from Dr. Alan Wakesing (Zeneca Pharmaceuticals).

Transient Transfection Assays. Cells were seeded into a 6-well plate (500,000 cells/well) in phenol red-free RPMI plus 10% 3% charcoal-stripped fetal bovine serum. The following day, medium was removed and replaced with fresh estrogen-free medium. A solution containing 1 μg of the luciferase reporter construct pVIT3-luc (51) and 0.5 μg of the β-galactosidase reporter pCMVβ (53) in 0.25 μM CaCl2 was mixed dropwise with an equal volume of 2% HBS (0.28 mM NaCl, 0.05 mM HEPES, and 1.5 mM NaPO₄, pH 7.05) by gently bubbling air through the solutions. This solution was then incubated at room temperature for 20 min to allow a DNA/CaPO₄ precipitate to form. This solution (0.4ml) was slowly added to the cells in 3.6 ml media and incubated at 37°C in a humidified incubator with 5% CO₂ for 6 h. At that time, the DNA solution was removed, and the cells were shocked with a solution of 10% glycerol in 1X HBS for 3 min. This solution was then removed, and the cells were washed 2× with 4 ml PBS. Medium with or without compounds was then added to the wells and incubated at 37°C in a humidified 5% CO₂ incubator for an additional 42 h. The media was removed, and the cells were washed once with ice-cold PBS. The cells were then scraped in extraction buffer (0.1 M KHPO₄ (pH 7.5), 1% Triton X-100, 100 μg/ml BSA, 2.5 mM phenylmethylsulfonyl fluoride, and 1 mM DTT) and pipetted vigorously to ensure complete cell lysis. Debris was then pelleted by spinning in a microfuge for 1 min, and the lysate was stored on ice until luciferase activity was assayed. Luciferase activity was assayed by mixing 50 μl of each lysate with 350 μl of reaction buffer [160 mM MgCl₂, 75 mM glycylglycine (pH 7.8), 0.5 mg/ml BSA, 19 mg/ml ATP, and 15 mM Tris-HCl, pH 7.5]. To begin each assay, 100 μl of substrate (0.4 mg/ml luciferin potassium salt in 10 mM NaPO₄, pH 6.0) was automatically injected into the lysate mixture. Each point was measured for 10 s by a Monolight 2010B luminometer (Analytical Luminescence Laboratory, San Diego, CA), and relative luciferase units were then reported. All points were corrected for transfection efficiency by dividing relative luciferase units by β-galactosidase activity. β-Galactosidase activity was measured using a β-methylumbelliferyl-borate assay (54). Briefly, an aliquot on the cell extract was mixed with 1.3 ml reaction buffer containing 0.1 μM NaPO₄, 10 mM KCl, 1 mM MgSO₄ (pH 7.0) and 2.2 X 10⁻⁵ g/ml β-methylumbelliferyl-borate. (Molecular Probes Inc., Eugene, OR). The sample was incubated at room temperature for 1 h, and 175 μl of stop buffer (15 mM EDTA and 0.3 mM glycine, pH 11.2) was added. The samples were then read in a L-S-5 fluorescence spectrophotometer (Perkin-Elmer, Foster City, CA) with excitation at 350 nm and absorption at 450 nm. All samples were corrected to a standard curve using purified β-galactosidase (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Gel Shift Assays. Nuclear extracts were prepared from cells grown in estrogen-free media for 4 days, followed by 24 h in either control estrogen-free media or media containing 1 μM ICI 182,780. Extracts were prepared by homogenization in 10 mM Tris-HCl (pH 7.5), 400 mM KCl, 10% glycerol, and 1 mM DTT. Gel shift assays were performed using the components provided in the BandShift Kit (PharMingen Biotech, Piscataway, NJ). All binding reactions contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 3 mM DTT, 0.5 mM glycerol, 0.05 mM NaPO₄, 0.1 mM ZnCl₂, 50 μg/ml poly(dIdC), and 1 ng of labeled oligonucleotide. Oligonucleotides were labeled with Klenow polymerase. Compounds were added directly to the reaction mixtures (1 μl of a 20X stock solution in 20% ethanol) and preincubated at room temperature for 15 min, at which time the radiolabeled oligonucleotide was added; then the incubation was continued for an additional 30 min. The samples were placed on ice for 15 min and then separated using nondenaturing PAGE (4%) using 1X low ionic strength buffer (7 mM Tris-HCl (pH 7.5 at 22°C), 3 mM sodium acetate, and 1 mM EDTA) with constant buffer recirculation at 4°C. Following electrophoresis, the gel was dried and exposed to X-ray film.

Western Blotting. For Western blots 1 X 10⁶ cells were seeded into a 10-cm tissue culture dish in estrogen-free media. The following day, media containing the appropriate compound was added, and cells were harvested at the indicated times. Whole-cell extracts were prepared by direct lysis of PBS washed cells in sample buffer [10% glycerol, 150 mM Tris-HCl (pH 6.8), 0.5 mM EDTA, 0.125% SDS, 1% β-mercaptoethanol, and 5 μg/ml bromophenol blue], followed by immersion in a boiling water bath for 5-10 min. Equal amounts of protein as determined in a Bio-Rad protein assay were run in a
The secondary antibody used was a horseradish peroxidase-conjugated goat antrat antibody (HyClone Laboratories, Logan, UT), and visualization was accomplished using the ECL visualization kit (Amersham Corp., Arlington Heights, IL) as per the manufacturer's directions. The membrane was then wrapped in plastic film and exposed to Kodak X-OMAT film for 15 s to 5 min and developed. Quantitation was performed using a Gel Doc 1000 (Bio-Rad, Hercules, CA).

Northern Blot Analysis. For Northern blotting, 5 × 10⁶ cells were seeded into a 100-cm² dish and allowed to attach overnight. The following day, medium containing the appropriate compound was added, and the mRNA was isolated using a procedure of direct poly(A)+ RNA purification at the indicated times (55). The mRNA was denatured by heating to 65°C for 15 min in 10 mM 4-morpholinepropane sulfoonic acid (pH 7.0), 4 mM sodium acetate, 0.5 mM EDTA, 6.5% formaldehyde, and 50% deionized formamide. The denatured mRNA was loaded onto a 1.2% agarose/formaldehyde gel and run overnight at 25 V with buffer recirculation. Transfer to Hybond N (Amersham Corp., Arlington Heights, IL) was performed using a Vacu-Gene transfer apparatus (Pharmacia Biotech, Inc., Piscataway, NJ) according to the manufacturer's directions. The membranes were then UV fixed using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) and air dried prior to prehybridization. Prehybridization was carried out at 47°C using a solution comprised of 5X SSC, 0.2% SDS, 250 μg/ml denatured salmon sperm DNA, 50% deionized formamide, and 10% sodium dextran sulfate. The DNA probes were prepared by random primer labeling using Klenow polymerase (Promega, Madison, WI). Hybridization was carried out by adding 2-4 × 10⁶ dpm/ml of the denatured probes directly to the prehybridization buffer and incubating for 12-16 h at 47°C. The membranes were then washed in 2X SSC, 0.2% SDS at room temperature for 2-3 h at room temperature with four buffer changes, followed by one wash in 0.1X SSC, 0.2% SDS at 65°C for 15 min. The membranes were then exposed to Kodak X-OMAT film in an autoradiography cassette containing double Quanta III intensifying screens at ~70°C for 24-200 h. Quantitation was performed using multiple exposures in a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). All values were corrected by reprobing the blots with β-actin following probing with the human ER cDNA; these corrected values were then compared with matched control samples, and this value is reported as fold control in Figs. 5-7.

RESULTS

Growth Effects of Estrogens and Antiestrogens. The effect of E₂ or the antiestrogens 4-OHT and ICI 182,780 on the growth of three human breast cancer cell lines was measured in a 6-day assay (Fig. 1). In the estrogen-dependent MCF-7:WS8 and T47D:A18 cell lines, growth in estrogen-free media was significantly less than that of the estrogen-independent MCF-7:2A cell line. The addition of 1 nM E₂ to the culture media caused a 5-7-fold increase in the growth of both the MCF-7:WS8 and T47D:A18 lines, as expected; however, E₂ caused an ~20% decrease in the growth of the MCF-7:2A cells. The partial agonist 4-OHT caused a slight increase in the growth of the MCF-7:WS8 and T47D:A18 cells in the absence of estrogens, but this same concentration of 4-OHT inhibited E₂-stimulated growth (data not shown). In the T47D:A18 and MCF-7:WS8 cell lines, the pure antiestrogen ICI 182,780 lead to growth rates that were indistinguishable from that in control media. In the MCF-7:2A cells, both antiestrogens inhibited growth below that in control media, with ICI 182,780 showing slightly more repression that 4-OHT.

Effects on DNA Binding. The effect of estrogen and antioestrogen on the specific DNA binding of the ERs isolated from the MCF-7:WS8 and MCF-7:2A cells was measured in a gel-shift assay (Fig. 2). Cells were grown in estrogen-free media for 4 days, after which the media was replaced with either fresh estrogen-free media or media containing 1 μM ICI 182,780. Nuclear extracts were isolated 24 h following this media change. The ability of the ER in these extracts to bind to a radiolabeled 20-bp oligonucleotide containing the consensus vitellogenin A₂ ERE was then measured in a standard gel-shift assay. The addition of estrogens or antioestrogens to the extracts from estrogen-free cells caused an increase in specific DNA binding as compared to the control groups. The binding was increased equally in all treatment groups, including the ICI 182,780 group. In contrast, the extracts isolated from cells treated for 24 h with ICI 182,780 demonstrated no measurable binding in any group. This finding is consistent with the data from the Western analyses (see below), which demonstrated the rapid degradation of the ER following ICI 182,780 treatment in intact cells. This degradation was not apparent in extracts exposed to ICI 182,780 in vitro.

ER Transcriptional Activation. The transcriptional activity of the ER in these cell lines was measured in a luciferase assay. The luciferase reporter construct pVIT3-Luc was used to measure the ability of the ER from these three cell lines to induce transcription following estradiol exposure. pVIT3-Luc contains three copies of the consensus Xenopus vitellogenin A₂ ERE upstream of a herpes simplex virus thymidine kinase minimal promoter, which controls transcription of a firefly luciferase reporter gene. This construct is exquisitely sensitive to the activity of the ER, while showing no activity in ER-negative cells (51, 56). As shown in Fig. 3, E₂ induced a dose-dependent increase in the transcription of this reporter gene in all three
Fig. 2. Gel-shift assay of nuclear extracts from MCF-7:WS8 and MCF-7:2A cells. Nuclear extracts were isolated from MCF-7:WS8 or MCF-7:2A cells following growth for 4 days in estrogen-free media followed by 24 h in control (C) or 1 μM ICI 182,780 (ICI) containing growth media. Equal amounts of each lysate were then preincubated in binding reactions containing ethanol vehicle only (Control), 1 nM E2 (Estradiol), 1 μM 4-OHT, or 1 μM ICI 182,780 as described in "Materials and Methods." Binding to a 20-mer β²P-labeled oligonucleotide containing the vitellogenin A² ERE was assessed by electrophoresis in a 4.5% nondenaturing polyacrylamide gel.

Fig. 3. Induction of luciferase in an estrogen-responsive reporter construct. MCF-7:WS8 (○), T47D:A18 (△), and MCF-7:2A (○) cells were grown for 1 day in estrogen-free media prior to transfection. Transfections were performed using standard calcium phosphate methodology and included 1 μg of the reporter pVIT3-Luc and 0.5 μg of pCMVβ as a transfection control as described in "Materials and Methods." Six h following transfection, the cells were glycerol shocked, and media containing the denoted concentration of E2 was added. Cells were harvested, and luciferase activity was determined 42 h following the addition of compound. All values are the mean of duplicate determinations, which have been corrected for β-galactosidase activity.

Regulation of Steady-State ER mRNA and Protein. The effect of E2 and two types of antiestrogens on the steady-state level of ER mRNA was assessed by quantitation of Northern blots hybridized with the human ER cDNA. Steady-state ER protein levels for all three cell lines are shown in Fig. 4 as measured in a Western blot using the monoclonal antibody H222. Both mRNA and whole-cell extracts were prepared from the cells following 1, 3, 6, 12, 24, and 48 h exposure to either 1 nM E2, 0.1 μM 4-OHT, or 0.1 μM ICI 182,780. As seen in Fig. 5, MCF-7:WS8 cells displayed a pattern of mRNA regulation consistent with previous studies (15, 18, 37). E2 caused a dramatic decrease in the steady-state level of the ER mRNA in a time-dependent manner, whereas both antiestrogens had little effect on the steady-state mRNA levels. Exposure to E2 as well as ICI 182,780 caused a dramatic reduction in the ER protein level. In contrast, the partial agonist 4-OHT caused an increase in the steady-state ER, reaching a peak at 24 h. This pattern is designated model I regulation.

Identical exposures of the T47D:A18 cell line gave rise to a distinctly different pattern of regulation (Fig. 6). Although the response of the ER mRNA and protein to 4-OHT was similar to that observed in the MCF-7:WS8 cells, the response to estradiol and ICI 182,780 was quite different. In the T47D:A18 cells, estradiol caused an initial decrease in the ER protein, as seen in the MCF-7:WS8 cells; however, this response was rapidly reversed, and by 12 h, the ER protein was present at control levels. The effect of E2 on ER mRNA was also different than that observed in the MCF-7:WS8 cells, with a rapid increase in ER mRNA levels, which remained elevated for greater than 24 h. The ER protein response to ICI 182,780 exposure in the T47D:A18 cells was exactly as in the MCF-7:WS8 cells, with a nearly total loss of ER. However, the ER mRNA response in the T47D:A18 cells was quite different from that in the MCF-7:WS8 cells. The administration of ICI 182,780 to the T47D:A18 cells lead to an ~50% drop in ER mRNA by 6 h, and the ER mRNA remained repressed through the end of the experiment. We refer to this regulation as a model II response (see "Discussion").
**Estrogen Receptor Regulation**

**MCF-7:WS8**

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**T47D:A18**

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**ER**

**ER77**

We also measured the effect of estrogens and antiestrogens on the ER in the estrogen-independent MCF-7:2A cell line (51). This cell line expresses two forms of the ER that can be easily differentiated in a Western blot. However, the mRNAs coding for these two respective receptors cannot be differentiated in a standard Northern blot. The duplication of exons 6 and 7 gives rise to an ~5% increase in size, and consequently the mRNAs coding for the two receptors cannot be resolved using this analysis. Therefore, changes in mRNA levels observed in the MCF-7:2A cell are a composite of the changes in both the wild-type and mutant ERs. Even with this limitation, the MCF-7:2A cells demonstrated a unique pattern of regulation of the two ERs (Fig. 7). Exposure to E₂ caused a repression of the ER mRNA and both the wild-type ER and the ER77. The response to the antiestrogens was unique in the MCF-7:2A cells. The partial agonist 4-OHT caused an increase in the wild-type ER protein and a decrease in the ER77. The effect of 4-OHT on the ER mRNA was also unusual, causing a decrease that was sustained for the length of the experiment. The most dramatic effect was observed in the differential effect of the pure antiestrogen ICI 182,780 on the protein level of the two ERs. The wild-type ER was rapidly decreased, as observed in the MCF-7:WS8 cells. In contrast, the ER77 protein level was increased following a short-lived decrease. The effect of ICI 182,780 on the ER mRNA was also unique, causing a rather dramatic increase in this message, which was continued throughout the length of the experiment.

**Discussion**

We have described the regulation of ER mRNA and protein by estrogens and antiestrogens in three ER-positive breast cancer cell lines. These three cell lines exhibit a diversity of phenotypes, despite the fact that all express functional ER and induce PR synthesis as well as an estrogen-responsive reporter gene expression following estrogen exposure. Using these criteria, all three cell lines would be considered to be classically estrogen responsive. This classification would predict that these cells would respond to antiestrogens in a similar manner (57). Although growth of these cell lines is inhibited by both the partial agonist tamoxifen and the pure antiestrogen ICI 182,780, the studies described here demonstrate the diversity of ER regulatory mechanisms present in these cells.

We propose that the responses observed in these cell lines can be described with two models of ER regulation. MCF-7:WS8 cells exhibit a form of regulation that we refer to as model I. In this model, the primary response to an estrogenic stimulus is a feedback inhibition of steady-state ER mRNA levels. This model of regulation is also observed in other cultured cell lines, such as the breast cancer cell line ZR-75 (58) and the ovarian carcinoma line PEO4 (59). Another result of this model of regulation is an increase in ER following estrogen deprivation. This would allow a very rapid and vigorous response to estrogen stimulation following a period of estrogen deprivation. A model I response would be consistent with rapid cell proliferation, such as that observed in the uterus, where a few rounds of cell replication are necessary for each menstrual cycle. The estrogen- and progesterone-induced down-regulation of the ER would then attenuate this growth response and prevent uncontrolled proliferation. Previous studies have been reported that show this type of ER regulation in the uterus of experimental animals (13, 20, 22).

The second model, as observed in the T47D:A18 cells, is characterized by an increase of ER mRNA synthesis in response to estrogens. This model would require prolonged periods of estrogen exposure to induce maximal ER synthesis and response. This slower and more protracted response would be compatible with a differentiation response, such as the synthesis of vitellogenin in *Xenopus* and avian liver (60, 61).
The response observed in the MCF-7:2A cells is unique and serves to reinforce our finding in the MCF-7:WS8 cells. The Mr 66,000 ER responds in a model I fashion, with decreasing expression in the presence of estradiol and ICI 182,780 and increasing expression with 4-OHT. The ER77 is a unique marker of ER synthesis that does not bind ligand and, therefore, cannot be directly regulated by estrogens or antiestrogens. However, due to expression of the wild-type ER in these cells, any effects of ER on transcription and/or mRNA stability would still be functional. Therefore, all ligand-induced changes in ER77 protein level can be presumed to be the result of the wild-type ER effects on ER77 transcription or mRNA stability. This was confirmed in studies presented here that showed that the ER77 protein is not stabilized by 4-OHT binding or degraded by ICI 182,780 binding. The fact that ER77 is down-regulated by estradiol supports the hypothesis that the mechanism of model I regulation by estradiol is through repression of ER mRNA, at either a transcriptional or post-transcriptional level. We propose that the wild-type ER present in the MCF-7:2A cells can repress expression of both the wild-type ER and the ER77. Furthermore, the increase in ER mRNA following ICI 182,780 exposure in the MCF-7:2A cells suggests that the ER77...
functions as an “unoccupied” receptor and causes a stimulation of ER transcription in a manner similar to that observed with estrogen withdrawal in model I regulation. This would argue that the complete loss of the ER following ICI 182,780 treatment is mechanistically different from estrogen withdrawal in these cells. It would also suggest that the transcription of the wild-type ER is being increased in the MCF-7:2A cells, but the increase in protein is not observed due to the rapid degradation of the ICI 182,780-occupied, wild-type ER. The concept of unoccupied ER exhibiting functions distinct from that in the complete lack of ER, as seen following ICI 182,780 exposure, is reinforced by the repression of ER mRNA in the T47D:A18 cells following ICI 182,780 treatment. If the loss of ER was no different than the presence of unoccupied ER, we would expect to see no change in the ER mRNA following ICI 182,780 exposure. In fact, ICI 182,780 causes a decrease in ER mRNA in the T47D cells.

Measurement of the DNA-binding ability of extracts isolated from MCF-7:WS8 and MCF-7:2A shows that the ICI 182,780-mediated effect on ER protein steady-state levels appears to require intact cells. Cells treated with ICI 182,780 for 24 h show a complete loss of ERE binding, whereas extracts isolated from control cells showed enhanced
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Fig. 7. Effects of E2, 4-OHT, or ICI 182,780 on steady-state ER mRNA and protein levels in MCF-7:2A cells. Following estrogen deprivation for 4 days, cells were treated with control media, 1 nM E2, 1 µM 4-OHT, or 1 µM ICI 182,780 (ICI) the next day, and protein and mRNA were harvested at the designated times as described in "Materials and Methods." A, representative Northern blot of MCF-7:WS8 poly(A)-enriched RNA probed with the human ER cDNA. To correct for loading and transfer differences the blot was stripped and reprobed with β-actin. B, quantitation of relative ER protein and RNA levels following treatment with 1 nM E2 (▲), 1 µM 4-OHT (△), or 1 µM ICI 182,780 (○) for the indicated times.

DNA binding when ICI 182,780 was added directly to the binding reactions. This also disproves previous reports that claimed that the pure antiestrogens function by inhibiting dimerization and, therefore, prevent DNA binding (62). These results suggest that the inhibition of growth and other ER-mediated responses following treatment with the pure antiestrogens is primarily the result of the rapid degradation of the ER (36–38).

The downstream effect of these two models of ER regulation is also reflected in the long-term effects of estrogen withdrawal on the MCF-7 and T47D cell lines. MCF-7 cell have for many years been shown to respond to short-term estrogen deprivation by up-regulation of the ER (33, 34). Our laboratory as well as others have isolated clones that have become estrogen independent following long-term (>8 months) estrogen deprivation. Interestingly, all estrogen-independent clones that have been characterized have continued to express high levels of the ER (33, 34, 40, 63, 64). These clones also maintained sensitivity to the growth-inhibitory effects of antiestrogens. The response of the T47D cell line to long-term estrogen deprivation was quite different than that observed in the MCF-7:WS8 cells. We isolated estrogen-independent T47D clones that no longer express
measurable amounts of ER protein, as measured by Western blotting or specific E$_2$ binding.$^5$ A number of these initially ER-negative clones failed to express ER after more than 2 months in estrogen-containing media. Further investigation of two of these clones showed that they are completely insensitive to the effects of estrogens or antiestrogens. However, two other clones, which did not express ER protein when grown in estrogen-free media, were induced to express ER by growth for 2 months in estrogen-containing media. Continued growth in estrogen-containing media caused the development of a partially estrogen-responsive phenotype. This response is compatible with a model II response in which the ER expression is very low, in this case undetectable, but the addition of estrogens leads to a slow but steady increase in the ER expression, which eventually is observed as a measurable ER-mediated response.

The scarcity of ER-positive breast cancer cell lines forced us to focus our studies of ER effects on only a few unique cell lines. Although these cell lines proved very useful in the elucidation of many aspects of ER function, understanding of the vast diversity of potential mechanisms responsible for ER activity may not be represented in the models available to us. In the studies presented here, we demonstrated that the ER can be regulated in at least two distinct manners. We investigated the regulation of the ER in response to E$_2$ and two antiestrogens with distinct modes of action in two standard ER-positive breast cancer cell lines, as well as a unique cell line that has adapted to growth in estrogen-free media. The findings presented here suggest that ER regulation in breast cancer cells can be the result of either of two distinct models. Model I regulation is characterized by an ER that is expressed at high levels in the absence of estrogen and is subsequently down-regulated following estrogen binding, primarily through repression of the steady-state level of the mRNA. Model II regulation is distinguished by a low expression of the ER in an estrogen-free environment and the subsequent induction of ER expression following estrogen binding. The fact that two distinct forms of ER regulation are observed in these studies suggests that the regulation of the ER in the vast population of clinical breast cancers may show a variety of regulation mechanisms. Elucidation of these alternative responses to estrogens and antiestrogens is necessary for a complete understanding of growth control in breast cancer and the development of the next generation of therapeutic agents in the treatment of this disease.

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Models of Estrogen Receptor Regulation by Estrogens and Antiestrogens in Breast Cancer Cell Lines

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