Activation of Transcription by Estrogen Receptor α and β Is Cell Type- and Promoter-dependent*

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Tamoxifen acts as a strong estrogen antagonist in human breast but as an estrogen agonist in the uterus. The action of tamoxifen is mediated through estrogen receptors (ERα and ERβ), which bind to a variety of responsive elements, to activate transcription. To examine the role of these varied elements in the response to antiestrogens, we studied the activation of a panel of differing promoters, by these compounds, in human breast, bone, and endometrial derived cell lines. No agonistic activity was observed in breast cells, whereas all antiestrogens, particularly tamoxifen, exhibited agonistic effects in uterine cell lines. All antiestrogens studied were agonistic in co-transfections of a collagenase reporter gene and ERβ, but tamoxifen alone was agonistic with ERα in (uterine) HEC-1-A cells. The ERα mediated, agonism of tamoxifen was not observed in primary cultures of human uterine stromal cells, whereas the ERβ-mediated agonism of all selective estrogen receptor modulators was present. This suggests that the two receptors operate by distinct pathways and that the response of cells to antiestrogens is dependent on the ER subtypes expressed.

Selective estrogen receptor modulators (SERMs), such as tamoxifen, act through interaction with the estrogen receptor (ER) (1). Estrogens and SERMs bind to the ligand binding domain of the ER, allowing dimerization and binding to a palindromic estrogen response element (ERE) upstream of estrogen-sensitive genes. The bound dimer then acts to trans-activate transcription (2). SERMs act by competing with estrogen for ER binding. However, estrogenic activity is inhibited in some tissues but unaffected in others (3–5). The cloning of a second estrogen receptor (6) (ERβ) raised the possibility of two different ER homodimers together with a heterodimer of the two ERs (7, 8). The cloning of an N-terminal extended (9) and ligand binding domain insertion splice variants (10, 11) of the human ERβ suggests a wide range of possible homo- and heterodimers, each of which may possess differing ligand sensitivities. Many promoters have been identified that are estrogen-sensitive but lack an ERE. These include 1) the activation of genes possessing AP-1 elements, including collagenase (12–14), 2) the activation of expression of genes possessing Sp1 binding sites, which are often associated ERE half-sites (15–20), 3) the activation of the TGFβ3 gene through a novel sequence termed the raloxifene response element (21, 22), and more recently 4) the activation of genes possessing the antioxidant response element (23, 24). The estrogen receptor has also been found to down-regulate the interleukin-6 gene by preventing the binding of NF-κB (25–27).

The conformation of the ER allosterically varies depending on the DNA sequence bound (28) and thus will differ between response elements. Conformational changes may well affect both the ligand binding domain and its interaction with other proteins such as coactivators and corepressors (29–32). Therefore, alternative estrogen signaling pathways allow for a broad range of activities to be produced by the same compound acting through differing response elements. However, the vast majority of work on the action of SERMs has concentrated on their action through ERs and has not considered the possible roles of these alternative estrogen responsive elements.

Tamoxifen is widely used as an adjuvant therapy in the treatment of women with breast cancer. In the breast, tamoxifen acts as an estrogen antagonist, reducing or preventing the proliferation of tumor cells (33). In contrast, in the uterus, this compound acts as an estrogen agonist, resulting in cell proliferation and in the long term, a 2–5-fold increase in endometrial tumors (34, 35). In order to gain further insight into the effects of SERMs on the activation of ERα or ERβ in cells derived from breast or endometrium, a panel of reporter constructs was assembled in which transcription was driven by a range of differing promoters and response elements. These constructs were then studied, in transient transfection assays, in human breast and uterine cell lines. The activities of SERMs were found to vary dramatically both between cell type and promoter construct studied. These results suggest that the effects of SERMs, in vivo, cannot be predicted by their actions on simple elements, such as the ERE, in isolation.

MATERIALS AND METHODS

Plasmids—The reporter panel consisted of the following: 1) a consensus ERE linked to a thymine kinase promoter (pERE-TK-Luc) (36); 2) a fragment of the complement C3 gene containing three nonconsensus EREs (pC3-Pst-T1-luc) (36); 3) a small fragment of the collagenase promoter, containing an AP-1 element (pCol73-Luc) (12, 13); 4) a fragment of the TGFα promoter containing ERE and Sp1 elements in which Sp1 interaction is thought to play an important role in estrogen response pXPl-TGF1.1e-Luc (17, 37); 5) a fragment of the TGFβ3 promoter containing the putative raloxifene response element (pReg3-499-Luc) (21, 22); and 6) a fragment of the promoter of the adrenomedullin gene, which was reported to be induced by tamoxifen but not estrogen in primary uterine cell culture (p-LCF-1543-Luc) (38, 39).

The internal control plasmid was the β-galactosidase expression plasmid pCMVβ (CLONTECH). The hERα expression plasmid was pCMV5-hERα (53), and the hERβ expression plasmid was pCNX2-hERβ (40).

Cell Culture and Transfections—Human breast-derived, MCF-7 (ATCC) cells were maintained in Dulbecco’s modified Eagle’s medium/ F-12 (1:1) (Life Technologies, Inc.) supplemented with Glutamax I (Sigma) and 10% fetal calf serum (Sigma). Human osteoblast-derived, MG-63 (ATCC) cells were maintained in Eagle’s modified minimal essential medium (Sigma) supplemented as above. Human uterus-de-
rived, HEC-1-A (ATCC) cells were maintained on McCoy's 5A medium (Sigma) supplemented as detailed above. All cell lines were established to be mycoplasma-free both before and after the study was completed. Primary cell cultures were prepared as described previously (41) and maintained in Dulbecco's modified Eagle's medium/F-12 (Life Technologies) supplemented with Glutamax I (Sigma), 10% fetal calf serum (Sigma), 1× antibiotic/antifungal solution (Sigma), and 7.5% sodium bicarbonate. All media were Phenol Red-free, and all serum estrogen was stripped using dextran-coated charcoal powder (Sigma). All transfections were performed using Fugene 6 (Roche Molecular Biochemicals) according to a ratio of 1:1.5, DNA:Fugene. Cells were dosed 4 h after transfection and harvested 24 h later. Cells were lysed in reporter lysis buffer (Promega); β-galactosidase activity was determined using a β-galactosidase assay kit (Promega), and luciferase activity was determined by a luciferase kit (Promega).

**Protein Extraction and Western Blotting**—Total cell proteins were extracted as described previously (42). Protein concentrations were determined using a protein determination kit (Sigma). Proteins were separated on SDS-polyacrylamide gels using a Mini Protein II (Bio-Rad) gel kit according to the manufacturer’s instructions. Proteins were transferred to Hybond ECL membrane (Amersham Pharmacia Biotech) using a Mini-Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Blots were blocked with 10% defatted milk protein overnight at 4°C. Blots were then washed with TBS-T20 (TBS plus 0.1% Tween 20) three times for 15 min and two times for 5 min. Blots were probed with primary antibody (ERα, 1:1000 (Novacastro Laboratories NCL-ER-CF11, anti-human, mouse monoclonal to full-length human ERα); ERβ, 1:5000 (Upstate Biotechnologies, Inc., 06-629 anti-rat, rabbit polyclonal to amino acids 54–71 of rat ERβ)) in TBS-T20 containing 1% defatted milk protein for 1 h. Filters were washed as before and then incubated with secondary antibody (ERα anti-mouse (Sigma), 1:40,000; ERβ anti-rabbit (Amersham Pharmacia Biotech), 1:40,000) in TBS-T20 for 30 min. Filters were rinsed and washed three times in TBS-20 and then probed with streptavidin 3° antibody (1:2000) in TBS for 20 min before rinsing and washing three times for 5 min in TBS-T20. Detection was then performed by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**RESULTS**

SERMs Do Not Act as Agonists, in the Context of a Wide Range of Promoters, in the Breast-derived, MCF-7 Cell Line—Studies on the effects of SERMs have, generally, concentrated on signaling through the consensus ERE, and the effect of these compounds on alternative estrogen signaling pathways has yet to be fully addressed. Stimulation of a panel of six diverse estrogen-responsive reporter constructs (as described under “Materials and Methods”) by 17β-estradiol, 4-hydroxytamoxifen, raloxifene, and faslodex (IC1 182, 780) has been studied. Initially, to validate the transfection system, the activation of the ERE reporter (pERE-TK-Luc) was investigated over a wide range of doses of the above compounds in MCF-7 cells. This cell line has high endogenous levels of both ER subtypes, so these were not co-transfected in this study. As expected, 17β-estradiol was found to be strongly agonistic over a wide range of doses. A strong antagonistic dose response was also observed with all three antiestrogens examined (Fig. 1).

The trans-activation of the promoter panel was then investigated in the MCF-7 cells. The ERE construct (pERE-TK-Luc) was strongly activated, over control levels, by estradiol, while tamoxifen, raloxifene, and faslodex all exhibited strong antagonistic activity (Fig. 2a). Faslodex was a particularly strong antagonist, reducing activity to less than 1% of the control. In transfections with the complement C3 reporter (pC3-Pst-T1-Luc), estradiol again acted as an agonist, and both raloxifene and faslodex acted as strong antagonists. However, tamoxifen did not produce a response different to that of the control-treated cells (Fig. 2b). Previous studies found that in HepG2 cells, transfected with this construct, tamoxifen produced an agonistic response (36). Using the collagenase promoter (pCol73-Luc), strong estradiol agonism was observed, while all three SERMs showed no significantly different response to the control treatment (Fig. 2c).

A similar response was also observed using the TGFα promoter construct (pXP1-TGF1.1e-Luc) (Fig. 2d). These results show that, while no agonistic activity of antiestrogens was observed in any promoter context, the degree of antagonism, particularly with respect to tamoxifen, varied considerably. No significant activation of the raloxifene response element (pβ3–499-Luc) or adrenomedullin (p-LCF1543-Luc) reporters (data not shown) was observed in this cell line with any treatment. Activation of these constructs was found in all other cell lines studied; therefore, it was assumed that tissue-specific properties of the MCF-7 cell line prevented expression, rather than any inherent fault with the reporter constructs.

Effects of SERMs on the Activity of the Promoter Panel in the Osteoblast-derived Cell Line MG-63 Co-transfected with either ERα or ERβ—The osteoblast cell line MG-63 has been suggested as a model cell line for bone remodeling and is thought to express negligible levels of ERs. However, in Western blots of these cells, both ERα and ERβ expression could be detected (Fig. 3). Since we wished to compare the activity of the ERα with that of ERβ, the presence of these endogenous receptors was a potential problem. To test the ability of the endogenous ERs, expressed in this cell line, to activate the reporter genes, reporter constructs were transfected into this cell line without receptor co-transfection. No estradiol-inducible transcriptional activity could be observed in these experiments (data not shown), and it is therefore assumed that in subsequent experiments trans-activation is a result solely of the co-transfected ER.
In the MG-63 cells, all six of the reporter constructs were responsive to estradiol or antiestrogens in co-transfections with at least one of the two ERs (Fig. 4). In general, estradiol was found to be an agonist for both ERα and ERβ, although the degree of agonism did vary significantly. However, estradiol failed to enhance ERβ activity in the context of the adenomullin promoter (p-LCF-1543-Luc) and was a very weak agonist of ERα activity in the context of the ERE promoter (pERE-TK-Luc). The later result suggests that even on a simple response element such as an ERE the two ER subtypes differ markedly between the two ER subtypes. When the two receptors were co-transfected, in a 1:1 ratio, the pattern of agonism observed closely resembled that of the ERα promoter than that of ERβ.

In the HEC-1-A (uterine) cell line, co-transfections of the complement C3 reporter (pC3-Pst-T1-Luc) with ERα resulted in strong estradiol agonism. Both raloxifene and faslodex produced a strongly antagonistic effect, while tamoxifen displayed no significant activity (Fig. 5b). These results closely resemble those seen in the MG-63 cells. Co-transfections with ERβ gave very weak expression from this construct. Estradiol agonism was observed, however, and both tamoxifen and raloxifene were found to be strongly antagonistic. Faslodex produced no significant effect relative to control. When the two receptors were co-expressed, the pattern of agonism observed using this reporter construct closely resembled that seen with ERα rather than that seen with ERβ; however, the antagonism of faslodex is not as strong as observed with ERα alone.

After co-transfection with ERα and the collagenase reporter construct (pCol73-Luc), estradiol produced a weak agonistic response (Fig. 5c), and tamoxifen gave a stronger agonistic response, while the response to raloxifene or faslodex was not significantly different from control. In co-transfections with ERβ and this reporter some weak estradiol agonism was observed, but all three antiestrogens displayed significantly greater agonism than estradiol. When the two receptors were co-expressed, a pattern of agonism was observed closely resembling that seen with ERβ alone. The strong increase in the agonism of both raloxifene and faslodex in cells expressing ERβ raises the possibility of a population of cells responsive to these compounds in the uterus.

Transfections using the TGFα reporter construct (pXPl-TGF1.1e-Luc) produced results very similar to those observed in the bone-derived MG-63 cell line. In ERα co-transfections,
estradiol produced a strong agonistic activity, and tamoxifen was weakly agonistic, while both raloxifene and faslodex exhibited an antagonistic response (Fig. 5d). In ERβ co-transfections, estradiol was again agonistic, while all other treatments produced no significant effect. Co-transfection of the two receptors produced a pattern of activity resembling that of ERα; however, the estradiol agonism was weaker than that seen with ERα alone.

In the context of the TGFβ3 reporter (pβ3–499-Luc) and ERα, weak agonism was observed with both estradiol and raloxifene, while tamoxifen was strongly agonistic and faslodex gave no significant response (Fig. 5e). In cells co-transfected with ERβ, estradiol was a strong agonist and faslodex a weak agonist, while all other compounds produced no significant response. Interestingly, when the two receptors were coexpressed, a novel pattern of agonism emerged. Both estradiol and faslodex produced weak but significant agonism (p < 0.005), while both tamoxifen and raloxifene produced a strongly agonistic effect (p < 0.001).

Co-transfections using the adrenomedullin promoter (p-LCF-1543-Luc) failed to produce any tamoxifen-specific agonism. Irrespective of receptor context, estradiol produced strong agonism, while all three SERMs produced no significant response (Fig. 5f). Co-transfection of the receptors together ablated the estradiol response. The lack of tamoxifen-specific response is surprising, since this gene was originally detected as an up-regulated RNA in differential display studies of primary endometrial cell cultures treated with tamoxifen.

FIG. 4. Transfection of the reporter construct panel into the MG-63, bone derived cell line. MG-63 cells were cotransfected with either 1) pERE-TK-Luc, 2) pERβ-TGFl.1e-Luc, 3) pCol73-Luc, 4) pXP1-TGFl.1e-Luc, 5) pβ3–499-Luc, or 6) p-LCF-1543-Luc (all at 0.25 μg/well) together with the internal control plasmid pCMVβ (0.25 μg) and either an expression plasmid for hERα or hERβ (0.25 μg). Triplicate wells were then dosed with estradiol (10^{-8} M), 4-hydroxytamoxifen (10^{-6} M), raloxifene (10^{-6} M), faslodex (10^{-8} M), or Me2SO vehicle control. Cells were harvested and analyzed as described in the legend to Fig. 1. Error bars, S.E.

When the two estrogen receptor isoforms were co-transfected, the response to treatment in some cases resembled that seen with ERα alone (Fig. 5, a and c) and in some cases resembled that with ERβ alone (Fig. 5, b and d), and in some cases a novel effect was produced (Fig. 5, e and f). This suggests that not only is the coexpression of ERβ in this tissue capable of affecting the response of ERα but also that expression may give rise to a novel pattern of responses to SERMs.

We attempted to delineate the level of ERβ expression required to produce an agonistic response to raloxifene. The expression of ERβ relative to that of ERα was titrated into a set of co-transfections with the collagenase reporter construct (pCol73-Luc) (Fig. 6). In this experiment, raloxifene agonism was found to remain weak up to an approximately equimolar ratio of the two receptors. Increasing ERβ expression further produced a rapid increase in raloxifene agonism and a further increase in the agonism of tamoxifen. These results suggest that in a uterine cell, expressing both receptors, significant raloxifene agonism would only be observed if the predominantly expressed ER subtype were ERβ. Whether such cells exist in vivo is, at present, unknown.

The SERM Agonism of Promoters in Primary Cultured Uterine Cells Differ From That Observed in Immortalized Cells—One of the most striking differences observed in the above transfections was the different agonistic and antagonistic properties of antiestrogens in transfections with the ERE and the collagenase reporter construct. To investigate these differences, the responses of these two promoters were studied in...
primary uterine stromal cell cultures. After co-transfection of the ERE reporter together with ERα into these cells, strong estradiol agonism was observed (Fig. 7a). Tamoxifen had no effect, relative to control, while both raloxifene and faslodex were antagonistic. In co-transfections with ERβ, estradiol was again agonistic, while tamoxifen, raloxifene, and faslodex produced no significant effect. These results are very similar to the pattern of agonism observed in the HEC-1-A cells. However, when the collagenase reporter was co-transfected with ERα, the strong tamoxifen agonism, observed in the HEC-1-A cells, was absent (Fig. 7b). Estradiol had a strongly agonistic effect, while both faslodex and raloxifene had no significant effect, relative to control. When the primary cells were transfected with ERβ, estradiol was found to have weakly agonistic effects, while tamoxifen, raloxifene, and faslodex were all agonistic. This result is very similar to the effects of these compounds in

**FIG. 5.** Transfection of the reporter construct panel into the HEC-1-A uterus-derived cell line. HEC-1-A cells were co-transfected with either 1) pERE-TK-Luc, 2) pC3-Pst-T1-Luc, 3) pCol73-Luc, 4) pXP1-TGF1.1e-Luc, 5) pβ3–499-Luc, or 6) pLUIL-1543-Luc (all at 0.25 μg/well) together with the internal control plasmid pCMVβ (0.25 μg) and either an expression plasmid for hERα or hERβ (0.25 μg). Triplicate wells were then dosed with estradiol (10⁻⁸ M), 4-hydroxytamoxifen (10⁻⁶ M), raloxifene (10⁻⁶ M), faslodex (10⁻⁶ M), or Me₂SO vehicle control. Cells were harvested and analyzed as described in Fig. 1. Error bars, S.E.

**FIG. 6.** Titration of the two estrogen receptor subtypes into the HEC-1-A cell line co-transfected with the collagenase/AP-1 reporter construct pCol73-Luc. HEC-1-A cells were co-transfected with pCol73-Luc (0.25 μg) and pCMVβ (0.25 μg) together with varying concentrations of ERα or ERβ. These concentrations were 0.25, 0.238, 0.225, 0.1875, 0.125, 0.0625, and 0 μg of ERα and 0, 0.012, 0.025, 0.0625, 0.125, 0.1875, and 0.25 μg of ERβ for the groups 1:0, 19:1, 9:1, 7.5:1, 1:1 1:7.5, and 0:1, respectively. Cells were treated with estradiol (10⁻⁸ M), 4-hydroxytamoxifen (10⁻⁶ M), raloxifene (10⁻⁶ M), faslodex (10⁻⁶ M), or Me₂SO vehicle control in triplicate wells. Error bars, S.E. of triplicate wells from a single transfection; results are typical of several repeats. The dashed line indicates the average of the untreated control group, which did not vary significantly between transfection groups.
the HEC-1-A transfections. This suggests that the signaling via the ERα and the ERβ may be mechanistically divisible, with an ERβ pathway common to the HEC-1-A and primary cells, while the ERα pathway varies between the two cell types.

**DISCUSSION**

We have analyzed the responses of a panel of reporter constructs based on both classical EREs and "alternative estrogen-responsive elements" to treatment with estrogens and antiestrogens in a variety of cell lines. Striking differences in the response of this panel were found between the three cell lines studied. In the MCF-7 (breast-derived) cells, no detectable agonist activity of any antiestrogen was found. However, the degree of antagonism did vary between the promoters. In this cell line, no response of the TGFβ3 (pβ3–499-Luc) and adenomedullin (p-LCF-1543-Luc) promoters, to any treatment, was detected. However, these two constructs contain no inserted strong promoter or enhancer regions and so may not be sensitive enough to respond to treatment in this cell type.

The ERE (pERE-TK-Luc) and the complement C3 reporter (pC3-Pst-T1-Luc) constructs are both regulated by ERE-Sp1 interactions (50), was found to possess ERβ pathway common to the HEC-1-A cells. However, in ERα co-transfections, the tamoxifen agonism, observed in cell lines, was not found. If tamoxifen agonism via AP-1 interaction does occur through an ERα-specific pathway, these results suggest that this response is more sensitive in cell lines than in primary cultured cells. Tamoxifen may therefore only act as an agonist on uterine cells that express a "tamoxifen-sensitive AP-1 phenotype."

Co-transfections of the TGFα reporter (pXP1-TGF1.1e-Luc) produced results similar to those observed using the complement C3 construct. The expression of TGFα has been suggested to be regulated by divergent EREs (37) and several Sp1 binding sites (17, 49). Recently, the RARα promoter, also thought to be regulated by ERE-Sp1 interactions (50), was found to possess reverse pharmacology when co-transfected with ERβ, in HepG2 cells (51). We did not detect this effect with ERβ and the TGFα reporter construct in the cell lines studied here. It therefore remains unclear whether the novel response reported with the RARα promoter is unique to that gene or a more common property of ERE/Sp1-containing promoters.

The adenomedullin gene was detected as a tamoxifen-induced mRNA species in uterine cells (39). Subsequent analysis of the expression of this gene found it to be a novel, endothelial, growth factor expressed in both uterine endometrial and stromal cells. However, a construct based on this promoter (p-LCF-1543-Luc) (38), although responsive to estradiol, had no response to tamoxifen in any cell type studied, including primary uterine stromal cells (data not shown). Unlike the previous study, we co-transfected ERs into the cells; therefore, it is possible that the tamoxifen agonism is reliant on a particular pattern of ER expression. Interestingly, despite activation in
response to estradiol in experiments with either ER singly when the two receptors were co-transfected, no activation was found with any compound studied. This further suggests that the regulation of the expression of this gene is highly dependent on ER isoform expression. If this protein is a tamoxifen-induced growth factor in vivo, then it represents a further possible mechanism of tamoxifen agonism and warrants further study.

In transfections of the TGFβ3 (p63–499-Luc) construct, we were unable to detect any raloxifene agonist activity with either estrogen receptor subtype, in bone-derived MG-63 cells (Fig. 4e). This construct (52) has previously been found to be strongly induced by raloxifene, but not estradiol, in MG-63 cells when coexposed with ERα (21). The reason for this difference is unclear. In HEC-1-A cells, strong raloxifene agonism was found only after coexpression of both ER subtypes. This suggests that activity from this promoter is strongly dependent on the ER subtype expressed. The mechanism by which the ER interacts with this element is still unknown, but direct binding to the DNA seems unlikely (22). Further investigation is required to understand the ER’s interaction with this promoter and its role in the activity of raloxifene.

To study activity of the promoter panel in cells expressing both ER subtypes, both receptors were co-transfected, in equimolar ratios, into the HEC-1-A cell line. We found that some promoter constructs display a pattern of responses similar to that of ERα alone (i.e. the complement C3 promoter), some display a pattern similar to that of ERβ alone (i.e. ER and collagenase promoters), and some display novel patterns (i.e. raloxifene response element and adrenomedullin promoters). This suggests that while some promoters bind one homodimer preferentially, heterodimer binding may give rise to novel responses to antiestrogens. Therefore, cells expressing certain combinations of receptors may be particularly vulnerable to the agonistic effect of SERMs.

Our results show that antiestrogens, and in particular tamoxifen, are more agonistic in uterine than breast cells. However, the strong tamoxifen agonism, observed with some promoter constructs in uterine cell lines, is not observed in primary cultures of uterine stroma. Co-transfections of the two ER subtypes suggest that signaling occurs via a distinct pathway for each receptor and that the binding specificity of the two homodimers is promoter-specific. Further studies are required to investigate the mechanistic difference in signaling by ERα and ERβ (particularly in the context of AP-1 activation) and the observed difference in these pathways between cell lines and primary cultures.

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