Influence of Cellular ERα/ERβ Ratio on the ERα-Agonist Induced Proliferation of Human T47D Breast Cancer Cells

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Received March 26, 2008; accepted July 7, 2008

Breast cancer cells show overexpression of estrogen receptor (ER) α relative to ERβ compared to normal breast tissues. This observation has lead to the hypothesis that ERβ may modulate the proliferative effect of ERα. This study investigated how variable cellular expression ratios of the ERα and ERβ modulate the effects on cell proliferation induced by ERα or ERβ agonists, respectively. Using human osteosarcoma (U2OS) ERα or ERβ reporter cells, propyl-pyrazole-triol (PPT) was shown to be a selective ERα and diarylpropionitrile (DPN) a preferential ERβ modulator. The effects of these selective estrogen receptor modulators (SERMs) and of the model compound E2 on the proliferation of T47D human breast cancer cells with tetracycline-dependent expression of ERβ (T47D-ERβ) were characterized. E2-induced cell proliferation of cells in which ERβ expression was inhibited was similar to that of the T47D wild-type cells, whereas this E2-induced cell proliferation was no longer observed when ERβ expression in the T47D-ERβ cells was increased. In the T47D-ERβ cell line, DPN also appeared to be able to suppress cell proliferation when levels of ERβ expression were high. In the T47D-ERβ cell line, PPT was unable to suppress cell proliferation at all ratios of ERα/ERβ expression, reflecting its ability to activate only ERα and not ERβ. It is concluded that effects of estrogen-like compounds on cell proliferation are dependent on the actual ERα/ERβ expression levels in these cells or tissues and the estrogen agonists to activate ERα and/or ERβ.

Key Words: estrogen receptors; SERM; breast cancer cells; T47D-ERβ; inducible; ER-U2OS-Luc.

Steroid hormones such as estrogens are needed for normal developmental, physiological, and reproductive processes in vertebrates (Harris, 2006). Many of these events are modulated by the activity of estrogen receptor (ER) α and ERβ (Pearce and Jordan, 2004; Pettersson et al., 2000). These two receptors are encoded by distinct genes and differ in their relative and absolute tissue distribution (Nilsson et al., 2001). In the absence of estrogen, ERs are sequestered within the nucleus and preserved in an inactive state by association with heat-shock proteins. Binding of estrogen or estrogen-like compounds induces a conformational change in the receptor, an event that promotes ER homo- or heterodimerization (Matthews and Gustafsson, 2003). Once the ER protein complex is bound to the DNA, it regulates the expression of estrogen-responsive genes. The ER homo- and heterodimers activate different signaling pathways and, therefore, different sets of genes (Acconcia et al., 2004; Li et al., 2004; Warner and Gustafsson, 2006).

During the last few years, an increasing number of studies have reported that xenobiotic compounds from different sources are able to mimic the natural estrogens, thus exerting comparable effects by activating gene transcription through ERα and/or ERβ (Effenberger et al., 2005; Escande et al., 2006; Gutendorf and Westendorf, 2001; Sonneveld et al., 2006; ter Veld et al., 2006; van der Woude et al., 2005). Estrogens stimulate cell proliferation in normal developing breast tissues and may prevent osteoporosis by increasing bone mineral density (Douchi et al., 2007). However, several studies also suggest that estrogens may stimulate the growth of a large proportion of ERα-positive breast cancers (Hartman et al., 2006; Lazennec, 2006; Monroe et al., 2005; Pedram et al., 2006; Weitczmann and Pacifici, 2006). It has been shown that the ratio of ERα/ERβ expression is higher in breast tumors than in normal tissues due to lower expression of ERβ (Lazennec et al., 2001) and that ERα and ERβ are antagonistic to each other; for example, ERβ appears to reduce the cell proliferation induced by ERα activation, as shown in in vitro cell transfection studies (Bardin et al., 2004; Stossi et al., 2004; Ström et al., 2004). Different breast cancer cell lines have been used for these studies, mainly MCF-7 cells, which all have a high ERα/ERβ ratio (Buterin et al., 2006; Murphy et al., 2005; Sartippour et al., 2006). It is proposed that differential responses and tissue-specific effects induced by food-born endocrine disrupters, including selective estrogen receptor modulators (SERMs), might be influenced by their relative affinity for the two ERs and the interactive effects of the estrogen-ER complex with the regulating proteins.
The overall objective of the present study was to quantitatively determine the proliferative/antiproliferative effect of two model-selective ER agonists in T47D human breast cancer cells in the presence of increasing amounts of intracellular ERβ. The model compounds studied were propyl-pyrazole-triol (PPT), a selective ERα agonist, and diarylpropionitrile (DPN), a preferential ERβ agonist. For comparison and validation of the different cellular model systems, estradiol (E2) was included in the studies as well.

The natural ligand E2 is known to stimulate both ERs, with an approximately 10-fold higher affinity for ERα than for ERβ (Kuiper et al., 1998; Quaedackers et al., 2001). DPN was reported to have a 70-fold higher relative binding affinity for ERβ than for ERα, and PPT has a reported 40-fold higher binding affinity for ERα than for ERβ (Helguero et al., 2005; Sun et al., 2003; Wang et al., 2006). In the present study, the relative isoform-specific activity of the three model compounds was characterized using the human osteosarcoma (U2OS) reporter cell lines, stably transfected with ERα or ERβ and a luciferase reporter gene with an 3×estrogen responsive element (ERE)-TATA-containing minimal promoter region (Quaedackers et al., 2001).

In subsequent experiments, the effect of the three compounds on proliferation of T47D-ERβ cells with varying ratios of ERα/ERβ expression was quantified. In wild-type T47D cells, ERα/ERβ mRNA levels were found to be present in a ratio of 9:1 (Ström et al., 2004). The T47D-ERβ cells are T47D cells stably transfected with a tetracycline-inducible ERβ which allows studying the influence of SERMs on cell proliferation in cells with varying ratio of ERα/ERβ expression, by altering expression of ERβ. Inhibition of the expression of the exogenous ERβ is expected to make the T47D-ERβ cell line function as a “pseudo”-wild-type T47D cell. Since in concurrence with the expression of ERβ, an enhanced green fluorescent protein (EGFP) from a bidirectional tetracycline-responsive promoter is coexpressed in the T47D-ERβ cells, the levels of ERβ expression can be monitored on the basis of EGFP fluorescence. To better quantify the relative levels of ERβ expression in the T47D-ERβ cells, a method to quantify the EGFP fluorescence in the cell lysate was developed in the present study.

With the newly developed method to quantify the relative ERβ expression, the effects of E2, DPN, and PPT on the T47D-ERβ cell proliferation were studied at different levels of ERβ expression to determine to what extent the estrogen-induced cell proliferation depends on the balance between the two major ER subtypes. In addition, it was investigated whether the effects observed match those that would have been predicted based on the U2OS reporter gene test results for these compounds and the hypothesis that stimulation of ERα activates and of ERβ reduces estrogen-mediated cell proliferation.

MATERIALS AND METHODS

Materials. 17β-Estradiol (E2) (> 98%) and ANTI-FLAG M2 monoclonal antibody peroxidase conjugate was purchased from Sigma (Zwijndrecht, The Netherlands). 2,3-Bis(4-hydroxyphenyl)-propionitrile (DPN) and 4,4',4''-(4-propyl-[1H]pyrazole-1,3,5-triyl) Tris-phenol (PPT) were purchased from Tocris Cookson Ltd (Bristol, UK). Dimethyl sulfoxide (DMSO) (> 99%) was obtained from Acros Organics (Pittsburgh, PA). Tetracycline, streptomycin, penicillin, and puromycin were acquired from Gibco (Paisley, Scotland). Fetal calf serum (FCS) (Australian origin, 10009), resazurin, and genetin were provided by Invitrogen Life Technologies (Paisley, Scotland). Hyclone dextran-charcoal-treated FCS (DCC-FCS, #SH30068.05) was obtained from Perbio Science NV (Etten-Leur, The Netherlands) was heat inactivated (30 min at 56°C) followed by two 45-min DCC treatment at 45°C (Horwitz and McGuire, 1978). Phosphate-buffered saline (PBS) (without Ca2+ and Mg2+) and 3% nonessential amino acids (100×, 11140-035) were purchased from Lonza (Basel, Switzerland). 2,3-Bis(4-hydroxyphenyl)-propionitrile (DPN) and 4,4''-(4-propyl-[1H]pyrazole-1,3,5-triyl) Tris-phenol (PPT) were purchased from Sigma. 

Cell lines. T47D human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA). The stably transfected T47D tetracycline-inducible cell line (T47D-ERβ) was made and provided by Ström (Ström et al., 2004). The human osteosarcoma (U2OS) cell lines stably expressing ERα or ERβ, in addition to 3×ERE-TATA-luciferase were used as described before (Quaedackers et al., 2001).

Cell culture conditions. The T47D wild-type cell line was cultured in a 1:1 mixture of Ham’s nutrient mixture F12 and Dulbecco’s modified Eagle’s medium (DMEM) (31331-035 and 31331-028), phenol red–free exposure medium (21041-025) were supplied by Gibco. Trypsin 0.25 g/100 ml in PBS was obtained from Difco (Detroit, MI). Sodium bicarbonate (NaHCO3 > 99.5%), sodium hydroxide (NaOH), ethylenedinitrotaetraacetic acid (EDTA-2H2O; Tiritplex), magnesium sulfate (MgSO4·7H2O), and 1,4-dithiothreitol (DTT) were obtained from Merck (Darmstadt, Germany). Magnesium carbonate (MgCO3·5H2O) was obtained from Aldrich (St Louis, MO). trans-1,2-Diaminocyclohexane-N,N’-N’-tetraacetic acid monohydrate (CDTA) was obtained from Prolabo (Paris, France). Hg-glycine and n-luciferin were obtained from Dufcha (Haarlem, The Netherlands). ATP and the 5-bromo-2’-deoxyuridine (BrdU) kit (colorimetric, 1164729001) were obtained from Roche Diagnostics (Mannheim, Germany). BSA Protein Assay Kit was purchased from Pierce (Bonn, Germany). Tween 20 was obtained from Merck. Sodium dodecyl sulfate (SDS) was obtained from BDH (Poole, UK). Acrylamide (30% acrylamide/bis solution 29:1), N,N,N’,N’-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), and the precision plus dual color protein marker were obtained from Bio-Rad (Veenendaal, The Netherlands). Nitrocellulose membrane was purchased from Whatman (Maidstone, The Netherlands). ERβ-specific primary (AB288/14C8) and secondary antibody (rabbit anti-mouse) were provided by Abcam (Cambridge, MA). Chemiluminescent Detection ECL Kit and photographic hyperfilm were provided by Amersham (Buckinghamshire, UK).

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proliferation in the cases where ERβ was expressed. Both the resazurin and BrdU method gave good results when compared to protein measurement assay and cell counting, and we chose to mostly apply the resazurin method as this method requires less cell handling than the BrdU method.

**Exposure conditions for T47D and T47D-ERβ cells.** Because of estrogenic activity of phenol red (Glover et al., 1988), experiments were performed in phenol red–free exposure medium supplemented with 5% DCC-FCS. Cells were seeded in 96-well plates (100 µl/well; Costar, The Netherlands, Cambridge, MA Cat. No. 3548) at densities of 103 cells/ml for proliferation and 1.8 × 104 cells/ml for fluorescence assays in the presence of different concentrations of tetracycline (0–1000 ng/ml) as indicated. The starting percentage of coverage for fluorescence experiments was higher than for proliferation experiments because wells had to be fully confluent for optimal sensitivity in the fluorescence measurements, whereas less confluent wells were needed for proliferation assays. Plates were incubated overnight at 37°C and 5% CO2. After 24 h, cells were washed with PBS to remove any trace of tetracycline and exposed to different concentrations of tetracycline and/or the test compounds as indicated.

**Cell proliferation measurements.** After 24 h of exposure, proliferation was determined by measuring BrdU incorporated into DNA following BrdU Roche’s colorimetric protocol and/or after 96 h of exposure by measuring mitochondrial activity of viable cells on the basis of chemical reduction of resazurin to resorufin as previously described (Schrems et al., 2006). Measurement of incorporated BrdU was performed in a spectrophotometer at 370-nm excitation wavelength and 492-nm emission wavelength, and resorufin was measured with a fluorometer at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Other methods for studying cell proliferation included cell counting and measurement of protein content, the latter by bichininonic acid protein assay.

**Measurement of ERβ expression–related fluorescence.** To quantify ERβ expression–related EGFP fluorescence after 24 h of exposure, medium was removed from the wells and the cells were washed with 100 µl of diluted PBS (0.5× PBS in deminwater). To each well, 110 µl of low-salt buffer, consisting of 10mM Tris-HCl, pH 7.8, containing 2mM DTT and 2mM CDTA, was added, and the cells were allowed to swell while the plates were kept on ice for 20 min. The plates were then frozen at –80°C for at least 1 h, and before analysis, they were thawed on ice and shaken briefly until reaching room temperature. Then, 100-µl aliquots of cell lysates from each well were transferred to a 96-well transparent plate with rounded bottom (Greiner, Frickenhausen, Germany) to allow fluorescence measurement in the Millipore Cytoflour 2350 fluorometer. Excitation was at 485 nm (band width of 20 nm) and emission at 530 nm (band width of 25 nm).

**Protein isolation and SDS-polyacrylamide gel electrophoresis.** For the analysis of the ERβ protein expression levels, T47D-ERβ cells were grown in growth medium with 1000 ng/ml of tetracycline in small cell culture flasks until 80–90% confluence. Cells were seeded in exposure medium with tetracycline for 24 h. Medium was removed, and cells were incubated for 24 h in new exposure medium with different tetracycline concentrations (0, 10, and 1000 ng/ml of tetracycline). Cells were collected with a scraper and suspended in lysis buffer supplemented with protease inhibitors. Lysis was done by three cycles of freezing in –80°C and thawing. Total protein content was determined using a bichininonic acid protein assay kit and a total protein amount of 20 µg/lane plus ¼ of 4X sample buffer (8% wt/vol SDS, 40% wt/vol glycerol, 0.2M Tris-HCl, pH 6.8, 0.02% bromaophenol blue, 25% vol/vol mercaptoethanol) was loaded onto the gel. Running gel (12% acrylamide) was prepared by mixing 9.9 ml of deionized water, 12 ml 30% acrylamide plus 0.8% bisacrylamide, 7.5 ml 1.5M Tris-HCl, pH 8.8, and 300 µl 10% SDS. Polymerization was started by addition of 150 µl 10% APS and 12 µl of TEMED. For the preparation of stacking gel (5% acrylamide), reagents were mixed in the following proportions: 6.66 ml water, 1.66 ml 30% acrylamide plus 0.8% bisacrylamide, 1.26 ml 1.5M Tris, pH 8.8, 100 µl 10% SDS, and 100 µl 10% APS together with 10 µl TEMED. Electrophoresis was run at 100V for approximately 2 h. After electrophoresis, gels were stained with Coomassie or used for Western blotting.

**Western blotting.** Blotting was performed at 100V for 1 h. After the transfer, unspecific binding sites on the membrane were blocked with 5% milk solution in tris-buffered saline (TBS) and 0.05% Tween 20 for 1–2 h. The membrane was washed in TBS with 0.05% Tween 20 twice for 5 min. For detection of the exogenous FLAG-ERβ, the monoclonal ANTI-FLAG M2 antibody was diluted in TBS with 0.05% Tween 20. For detection of ERβ in the control T47D cell line, ERβ mouse monoclonal antibody was used. After incubation for 1 h at room temperature with ANTI-FLAG M2 antibody, the membrane was washed with TBS with 0.05% Tween 20 six times for 5 min each time before electrochemiluminescence (ECL) treatment. Incubation with the 14C8 antibody was performed overnight at 4°C. After incubation, the membrane was washed with TBS 0.1% Tween 20 three times for 10 min. Secondary antibodies were diluted 5000 times in TBS0.1% Tween 20, and incubation was run for 45 min at room temperature. Rabbit anti-mouse antibody conjugated with peroxidase was used for ERβ. Final washing steps were done two times with TBS 0.1% Tween and one time with TBS only. Finally, the membrane was treated with peroxidase substrate (ECL kit) for protein detection. The reaction was run for 5–7 min, and bands were visualized using photographic film. As a final step, membranes were stained with Coomassie blue.

**ERβs and ERβ-specific U2OS reporter gene assay.** Cultured U2OS cells were washed with PBS, trypsinized, and seeded in 96-well plates (Greiner) at 100 µl/well at a density of 10 × 103 cells/ml (U2OS-ERα) or 7.5 × 103 cells/ml (U2OS-ERβ) in a 1:1 mixture of DMEM and Ham’s F12 medium without phenol red, buffered with 1260 mg/l NaHCO3, and supplemented with 5% DCC-FCS and 0.5% nonessential amino acids. Culture medium was refreshed after 24 h. Forty-eight hours after seeding, the cells were exposed in triplicate to E2, DPN, or PPT at the indicated concentrations (final DMSO concentration 0.2%) for 24 h at 37°C and 7.5% CO2 in a humidified atmosphere. On each plate, the cells were exposed to different concentrations of test compounds and calibration points for E2 (EC10, EC50, EC100) to be able to correct for plate to plate variations. After 24 h, the medium was removed, and cells were washed with 100 µl diluted PBS (0.5× in deminwater) per well. Cells were lysed with 30 µl of a hypotonic low-salt buffer, consisting of 10mM Tris-HCl, pH 7.8, containing 2mM DTT and 2mM CDTA. Plates were put on ice for 10 min and subsequently frozen at –80°C. Before analysis, plates were thawed on ice for 20 min and shaken briefly until reaching room temperature. Analyses were performed in a Luminoskan (RS; Labsystems, Helsinki, Finland) at room temperature as follows: background light emission of each well was measured for 2 s, then, 100 µl of flashmix was added (20mM tricine buffer, pH 7.8, supplemented with 1.07mM (MgCO3)2Mg(OH)2, 2.67mM MgSO4, 0.1mM EDTA, 2H2O, 2mM DTT, 0.47mM n-luciferin, and 5mM ATP), light emission was immediately measured for 2 s, and extinguished with 50 µl 0.2M NaOH to prevent cross-talk to the neighboring wells.

**Data analysis.** Relative light units in every well were corrected for the corresponding background signal, measured before luciferin addition. The response of the solvent control was taken as 0% induction. The maximum induction of luciferase obtained at 30pM E2 for ERα-U2OS cells and at 300pM E2 for ERβ-U2OS cells was set at 100%. The exposure concentration of the compound at which 50% of the maximum luciferase activity is reached (EC50) was determined using Slidewrite 6.10 for Windows. The estradiol equivalency factors (EEF) were calculated as EC50 estradiol/EC50 compound. The concentration of tetracycline at which 50% of the EGFP fluorescence is inhibited (IC50) was determined using Slidewrite 6.10 for Windows as well. EGFP fluorescence reflecting the level of ERβ induction was expressed relative to the fluorescence of cells exposed to the solvent control (PBS 0.2%) set at 100%. In addition, in each experiment, calibration points for E2 were included to be able to correct for plate to plate variations. The obtained data from proliferation quantified by the resazurin method was plotted after subtraction of background signal (obtained from a well containing all components except for the cells), as percent proliferation. Results from BrdU were calculated as percentage of proliferation after background subtraction. The response of cells exposed to the solvent control (DMSO 0.2%) was set at 100%.
RESULTS

Characterization of the Selected SERMs

The selectivity of PPT and DPN for ERα and ERβ was studied in the ERα-U2OS-Luc and ERβ-U2OS-Luc cells. Typical dose-response curves for the natural ligand E2 as well for the SERMs are shown in Figure 1. As previously demonstrated, E2 showed higher binding affinity for ERα than for ERβ, with EC₅₀ values of 8 and 65pM, respectively, but E2 is clearly both an ERα and ERβ agonist. The ERα-selective PPT was unable to induce any transcription of the reporter gene in the ERβ-U2OS-Luc cell line, confirming its nature as a selective ERα modulator. The EC₅₀ for the ERα-dependent response was 140pM (Fig. 1A) resulting in an EEF compared to E₂ of 0.057, and the maximal induction was 120%. As expected, DPN showed ERβ selectivity with EC₅₀ values of 2 and 59nM for ERβ and ERα, respectively. At present, a more selective ERβ agonist could not be identified, and the ERβ specificity of DPN in the U2OS cells was at least higher than that of E2 since the ratios of the EC₅₀ for ERα and the EC₅₀ for ERβ activation are, respectively, 0.12 and 29.5 for E2 and DPN. In the ERα-U2OS cells, DPN did not reach the maximal E2 induction level, but in the ERβ-U2OS, the maximal induction level of DPN was 110% of the value obtained for E2 (Fig. 1B). The EEFs for DPN were 1.3 × 10⁻⁴ in the ERα-U2OS and 0.03 in the ERβ-U2OS cells. Table 1 shows an overview of the EC₅₀, EEF, and maximum effect of PPT and DPN compared to E2 using the U2OS cell system.

Tetracycline-Dependent Expression of ERβ in the T47D-ERβ Cell Line Quantified by Measuring EGFP Fluorescence

T47D cells were stably transfected with the ERβ expression plasmid under tetracycline-responsive promoter regulation and with an EGFP gene as a coexpressed reporter also under regulation of the same tetracycline-responsive promoter. This allows qualitative/semiquantitative confirmation of ERβ expression by fluorescence microscopy. Maximal levels of fluorescence were reached after 24 h of cultivation of the cells in the absence of tetracycline. A simple method for quantitative measurements of the EGFP as sensitive reporter molecule in cell lysate of the T47D-ERβ was developed. Wells seeded with high density number of the T47D-ERβ cells were exposed to different concentrations of tetracycline (Fig. 2A). EGFP fluorescence was measured in the cell lysate. Tetracycline treatment suppressed EGFP fluorescence in T47D-ERβ cells, with concentrations above 150 ng/ml, resulting in total fluorescence suppression. Values above 2000 ng/ml of tetracycline not only completely depleted EGFP expression but also caused cytotoxicity (data not shown). The concentration of tetracycline at which 50% of the fluorescence, and thus ERβ expression, was inhibited (IC₅₀) was determined to be 9.6 ng/ml tetracycline. Since the expression of EGFP is linked to the expression of recombinant ERβ, the presence of ERβ at protein level was confirmed using Western blot (Fig. 2B). No detectable FLAG-ERβ protein was expressed in the presence of 1000 ng tetracycline/ml.

| TABLE 1 | Overview of the EC₅₀, EEF Values, and Maximum Effect of E2, PPT, and DPN Tested Using the U2OS Cell System |
|---------|---------------------------------------------------------------------------------------------------------|
|         | Maximum effect as % relative to E2 | ERβ EEF | Maximum effect as % relative to E2 |
|         | EC₅₀ maximum | ERα EEF | EC₅₀ maximum | ERβ EEF |
| E2      | 8pM | 100 | 1 | 65pM | 100 | 1 |
| PPT     | 140pM | 120 | 0.057 | 200pM | 0.03 | 2nM | 110 | 0.03 |
| DPN     | 59nM | 73 | 1.3 × 10⁻⁴ | 2nM | 110 | 0.03 |
E2-Induced Proliferation of T47D-wt Cells and of T47D-ERβ Cells with Inhibited ERβ Expression

T47D-wt cells showed a clear E2-dependent increase in cell proliferation with a maximum of 131% maximal induction of proliferation at 100pM E2 (Fig. 3). T47D-ERβ cells in which ERβ expression was completely inhibited by incubating them in the presence of 1000 ng tetracycline/ml showed an E2-dependent cell proliferation that was comparable with the response obtained in wild-type cells (Fig. 3). The T47D-ERβ-transfected cells with no ERβ expression reached the same maximum proliferation of 131% at 1nM of E2 as the wild-type cells. In both cell lines, the shape of the dose-response curves was similar and at concentrations above 1nM E2, cell proliferation decreased when increasing the concentration of E2. The dose-response curves for E2-induced proliferation obtained by measuring BrdU incorporation during the last 4 h of the 24-h period exposure were comparable to those obtained when measuring mitochondrial activity during the last 4 h of 96-h exposure using the resazurin method with both cell lines.

Expression of ERβ Inhibits E2-Induced Cell Proliferation of T47D-ERβ Cells

Mitochondrial activity of the T47D-ERβ cells treated with 1nM E2 and an increasing concentration of tetracycline, causing decreasing cellular expression levels of ERβ, showed a tetracycline-related increase in proliferation (Fig. 4). In absence of E2, the cells did not proliferate. The EC50 for tetracycline-dependent stimulation of E2-mediated cell proliferation was 41 ng tetracycline/ml. At tetracycline concentrations lower than 10 ng/ml, where ERβ expression levels were high, no E2-induced proliferation was observed.
Exposure of T47D-ERβ cells to increasing E2 concentrations in combination with 41 ng/ml (EC50) and 1000 ng/ml of tetracycline resulted in a dose-dependent cell proliferation. E2-induced proliferation was almost absent in the presence of 0 and 9.6 ng (IC50 for fluorescence) tetracycline/ml. Altogether these data demonstrate the validity of the test system and support that E2 cannot induce cell proliferation under conditions where ERβ is expressed to relatively higher levels and able to suppress ERα-mediated induction of cell proliferation.

The Proliferative Effect of Two SERMs

Figure 6 presents the results from experiments in which the proliferative effect of PPT (Fig. 6A) and DPN (Fig. 6B) was studied in the T47D-ERβ cells at the same four tetracycline concentration as used in the E2 study (Fig. 4). The straight line drawn at 164% cell proliferation represents the maximum of T47D-ERβ (ERβ absent) and T47D cell proliferation at 1nM of estradiol.

At 1000 ng/ml of tetracycline, when expression of ERβ is suppressed, PPT was able to induce cell proliferation to a level of 173%, an induction level that was slightly higher than the maximal induction of cell proliferation by E2 (164%) under these conditions. This is in spite of the fact that the affinity of ERα for PPT was lower than for E2. Although the E2-induced proliferation with full expression of ERβ was reached at 3nM of PPT (153%), no considerable reduction of proliferation compared with E2 (124%) was observed under all ERα/ERβ ratios studied due to the inability of PPT to activate ERβ.

At the same tetracycline concentration (1000 ng/ml), DPN induced similar proliferation maxima at E2 (164%) although at a higher concentration than required for maximal induction by E2. This can be due to the fact that ERα has a lower affinity for DPN than for E2. However, DPN appeared able to suppress cell proliferation when levels of ERβ expression were high. No differences in cell proliferation were observed between the two lowest tetracycline concentrations (0 and 9.6 ng tetracycline/ml) either for PPT, DPN, or E2.

E2-induced proliferation with full expression of ERβ was lower than proliferation induced with PPT and DPN. The maximum induced proliferation in the absence of tetracycline was 153% with 3nM PPT, 128% with 100nM DPN, and 124% with 1nM E2. In the presence of high levels of ERβ, E2- and DPN-induced proliferation was 40% (from 164% to 124%) lower compared to the induced proliferation in absence of ERβ.

DISCUSSION

Invasion, uncontrolled proliferation, and metastasis are the most important properties of a malignant cancer. Thus, proliferation is not the only hallmark of malignant transformation, and proliferation and invasion may under certain conditions even be contrasting events (Svensson et al., 2003). In the present study, proliferation was selected as the end point to characterize the influence of ERα/ERβ ratios and not the
invasiveness of the tumor cells since the T47D cell line in which the variable ERα/ERβ ratios can be generated is a non- or poorly invasive cell line (Adams et al., 2002).

The ratio of ERα/ERβ expression in breast tumors is higher than in normal breast tissues due to a lower expression of ERβ. This has lead to the hypothesis that low levels of ERβ may result in high proliferation rates because of the absence of ERβ-mediated modulation of the proliferative effect of ERα. This would imply that high levels of ERβ stimulation lead to decreased cell proliferation whereas high levels of ERα stimulation lead to increased cell proliferation. Therefore, the objective of the present study was to quantify the differential effect of a selective ERα and a selective ERβ agonist on cell proliferation of human breast cancer cells with varying but well-defined ratios of ERα/ERβ expression. To this end, the T47D-ERβ cell model was applied in which the levels of the ERβ receptor could be reduced by adding tetracycline. In addition to the E2-induced cell proliferation under different levels of ERβ expression, also the effect of two pseudo-estrogens reported to be specific ERα or ERβ agonists was determined.

Using human osteosarcoma (U2OS) ERα or ERβ reporter cell lines, it could be demonstrated that, compared to E2, PPT is a selective ERα modulator and DPN a preferential ERβ modulator. In the ERα- and ERβ-specific U2OS-Luc cells, E2 induced ER/ERE-mediated luciferase activity with eight times higher affinity for ERα than for ERβ. DPN was able to induce luciferase activity through both receptors with a 30 times higher potency through ERβ than ERα. PPT was found to be a fully ERα-specific inducer (EEFα = 0.057) while DPN only reasonably specifically induced ERβ (EEFβ = 1.3 × 10^{-4}, EEFβ = 0.03). This is in accordance with the results previously reported (Meyers et al., 2001). The fact that PPT was not able to activate the transcription of the reporter gene in the ERβ-U2OS-Luc is in accordance with earlier observations (Stauffer et al., 2000). The maximum induction by the partial ERα agonists DPN did not reach the maximum induction induced by E2 in the ERα-U2OS system, but DPN induced an even slightly higher maximum response than E2 in the ERβ-U2OS system.

In normal breast tissues, the ERβ to ERα ratio is high and decreases when breast tumor progresses (Lazennec et al., 2001). Earlier studies have suggested that when both receptors are expressed in the cell at the same mRNA levels, E2-induced proliferation of T47D cells is reduced compared to the E2-induced proliferation of cells in which only ERα is expressed (Ström et al., 2004). Our results in the T47D wild type cells showed that cells proliferated in the absence of high levels of ERβ and presence of the natural ligand, E2, indicating that proliferation is E2-ERα mediated. Therefore, to study the role of ERβ in cell proliferation, we used the T47D-ERβ cell line with inducible ERβ expression to directly compare the effects of ERβ levels in the same cellular background. As a validation of the cell system, it could be demonstrated that the complete inhibition of ERβ expression with 1000 ng/ml of tetracycline resulted in a “pseudo”-wild-type T47D cell with similar E2-induced proliferation responses whereas this E2-induced cell proliferation was no longer observed when ERβ expression in the T47D-ERβ cells was increased. Furthermore, given the fact that the T47D-ERβ cell line is derived from human breast cancer tissue, the expression levels of ERα and ERβ in the cells when grown in the presence of 1000 ng tetracycline/ml (no additional ERβ expression) can be expected to be physiologically relevant. Furthermore, previous data reported by Ström et al. (2004) revealed that when the cells were grown in the absence of tetracycline (full ERβ expression), the level of ERβ, as judged from mRNA expression levels, appears to be 4-fold higher than that of ERα. Given the fact that physiological levels of ERα to ERβ may vary in such a way that either one of the two receptors is dominant (Enmark et al., 1997; Mäkinen et al., 2001; Pearce and Jordan, 2004), it can be concluded that the range of ERα to ERβ ratios in the T47D-ERβ line with increasing concentrations of tetracycline reflects physiologically relevant variations in the receptor ratio.

The T47D-ERβ cell line was engineered to coexpress the EGFP in concurrence with ERβ, which allows indirect quantification of ERβ by measuring the fluorescence of EGFP. In the present study, a simple microtiter plate method was developed to be able to detect the expression levels of ERβ by measuring in the cell lysate the EGFP fluorescence. The IC_{50} for the EGFP expression after 24 h of exposure was 9.6 ng tetracycline/ml (Fig. 2). After 96 h, the EC_{50} for tetracycline-mediated stimulation of E2-induced cell proliferation was 41 ng tetracycline/ml (Fig. 4). The difference between the IC_{50} value for tetracycline-mediated suppression of EGFP and ERβ expression and the EC_{50} value for tetracycline-mediated stimulation of E2-mediated cell proliferation reflects that for 50% stimulation of E2-mediated cell proliferation, ERβ expression needs to be inhibited by more than 50%.

Our results clearly show an important role of the ERα/ERβ ratio in E2-induced cell proliferation. To better understand the interaction between ERα and ERβ, the quantification of the exact levels of expression of these receptors is crucial. Our findings also show that the ER subtype ratio determines the functional response to SERMs. Our results were consistent with the hypothesis that ERβ opposes ERα proliferative effects in response to E2. Herein, we show that the proliferative actions in the T47D-ERβ cells were mediated by the ERα, whereas ERβ played an important role in inhibiting the ERα effectiveness. It cannot yet be concluded whether the inhibition via ERβ results in a reduced transcription of genes involved in cell division or that possibly nongenomic signal transduction pathways are induced as well. It has been demonstrated that ERα/ERβ heterodimers and ERα homodimers are preferentially formed in intact cells and heterodimers bind to the ERE onto the DNA with similar affinity to that of ERα homodimers and higher affinity than that of ERβ homodimers (Cowley et al., 1997).
The ERα-selective agonist PPT was unable to induce luciferase activity through ERβ (U2OS cells) (Fig. 1B), which implies that PPT does not activate ERβ homodimer-mediated gene transcription. Moreover, it has been shown that ERα/ERβ heterodimers are only effective in coactivator interaction when both ERα and ERβ are doubly occupied with agonists (Kim et al., 2005). DPN and PPT are as effective in stimulation of cell proliferation as E2 in the absence of ERβ (Fig. 6). In the presence of ERβ, cell proliferation is decreased. DPN is more potent than PPT in inhibition of cell proliferation when both ERα and ERβ are present as in contrast to PPT DPN can activate ERβ.

In contrast to exposure to E2 and DPN, exposure to PPT in the presence of high levels of ERβ expression did not give rise to visible cell death. This corroborates a role of the activated ERβ in the induction of cell death as previously reported (Galluzzo and Marino, 2006; Nomoto et al., 2002). Therefore, it is important to explain the specific roles of the ERα and ERβ when both receptors are present and link this to the proliferation outcome.

The current results and developed method show that activation of ERβ can result in a reduction of ERα-mediated cell proliferation. In the T47D-ERβ cell line, PPT was unable to suppress cell proliferation at all ratios of ERα/ERβ expression, indicating its ability to activate only ERα. Whereas DPN appeared to be able to suppress cell proliferation when levels of ERβ expression were high since it was able to bind preferentially to ERβ. It is concluded that effects of estrogen-like compounds on cell proliferation are dependent on the actual ERα/ERβ expression levels in these cells or tissues and the potential of the estrogen agonists to activate ERα and/or ERβ.

Thus, the use of ERβ protein expression levels as a biomarker in tumor screening, in addition to protein expression levels of ERα, has the potential of more successful indication of therapeutic responses and course/outcome of the disease in ER-positive tumors. Future studies at a molecular level will be performed to further elucidate how ERβ exerts these effects.

**FUNDING**

Graduate School of Voeding, Levensmiddelentechnologie, Agrobio technologie en Gezondheid (project number 61.61.100.040).

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