CMLS, Cell. Mol. Life Sci. 60 (2003) 567–576
1420-682X/03/030567-10

CMLS Cellular and Molecular Life Sciences

Research Article

Selective modulation of ER-β by estradiol and xenoestrogens in human breast cancer cell lines

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Received 5 November 2002; received after revision 12 December 2002; accepted 23 January 2003

Abstract. In the last decades, substances with estrogenic activity have been dispersed into the environment. Xenoestrogens act by binding to estrogen receptors, ligand-regulated transcription factors, for which two subtypes have been described, ER-α and ER-β, which are often coexpressed at variable amounts in different tissues. We investigated variations in the expression of ER-α and ER-β mRNAs following treatment with four xenoestrogens (bisphenol A, 4-tert octylphenol, 2-hydroxybiphenyl, 4-hydroxybiphenyl) and with 17β-estradiol in estrogen-sensitive (T47D) and estrogen-insensitive (BT20) breast cancer cell lines. Although to a variable extent, both estradiol and the tested xenoestrogens increased the expression of ER-β mRNA, whereas a slight effect on ER-α was observed only in T47D cells. Upregulation of ER-β expression by estradiol and xenoestrogens was observed only in the presence of detectable ER-α protein levels. These findings indicate a regulatory role for ER-β in ER-α-mediated transcription and a role for ER-β in mediating xenoestrogen toxicity.

Key words. Xenoestrogen; ER-β; ER-α; breast cancer; RT-PCR.

Rapid technological growth in the past century has led to the exposure of the population to a range of new synthetic chemicals. As a direct consequence, environment-related health issues have become a challenging problem for both the human community and for wildlife. In most cases, the effects and extension of such exposure have not been accurately documented, despite a possible association between breast cancer and substances (xenoestrogens) able to interfere with the endocrine system and mimic the activity of natural estrogens.

Bisphenol A (BPA) and related diphenylalkanes are widely employed in plastic manufacture for the synthesis of polycarbonates, polyacrylates and epoxy, phenolic or polystyrene resins, and polyacrylates and used in many products such as drug delivery systems, dental sealings and the inner coating of food cans. These biphenyls can be liberated as a consequence of incomplete polymerization or degradation due to exposure to high temperatures [1, 2].

The estrogenicity of BPA is due to its structural similarity to diethylstilbestrol, a potent synthetic estrogen characterized by high bioavailability and known to cause carcinomas and genital abnormalities following prenatal exposure [3]. The estrogenicity of BPA and related compounds has been well documented [4] by means of a bioassay (E-SCREEN) based on in vitro proliferation of the hormone-sensitive breast cancer cell line MCF-7 [5]. In fact, BPA and related compounds compete with 17β-estradiol in binding to the estrogen receptor (ER), a ligand-regulated nuclear transcription factor belonging to the superfamily of nuclear receptors [6]. At least two members of the superfamily, ER-α and ER-β, are involved in physiological responses to estradiol [7, 8]. The

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two isoforms are encoded by distinct genes located on different chromosomes but are characterized by a high degree of sequence and structural homology, especially in the DNA-binding domain. ERs regulate gene transcription by binding as homo-(ER-α/ER-α or ER-β/ER-β) or heterodimers (ER-α/ER-β) [9] to specific estrogen-responsive sequences called estrogen-responsive elements (EREs) located in the 5′ flanking region of hormone-regulated genes.

In normal and neoplastic breast cells, the two isoforms appear to be coexpressed [10] and may therefore contribute to estrogen signal transduction either independently or through the formation of heterodimers. So far, data on the specific role of the two forms are sparse, although there is strong evidence supporting a regulatory role for ER-β in ER-α-mediated transcriptional activity [11, 12]. Some estrogenic molecules exhibit differential affinity for the two forms but, more important, the balance between the agonistic and antagonistic activity of some compounds differs between ER-α and ER-β. This has been clearly demonstrated in the case of tamoxifen [13], where genetically modified cells were used and different results obtained depending on the pathway mediating transcriptional activation (AP-1 or the classical ERE), with a potential impact on the clinical responsiveness of breast cancers. A differential interaction of xenoestrogens with ER-α and ER-β would also have important implications.

Hiroi et al. [14] reported that in a cell-based transcriptional assay using a reporter gene, BPA exhibits only agonistic activity when signaling through ER-β while it behaves as a mixed estrogen agonist/antagonist when the transcription is mediated by ER-α. These preliminary findings could account for variable estrogenicity of the same compound in different tissues due to a different distribution of the isoforms. In addition, most ligands, including estradiol, are well-known to exert a regulatory effect (down- or upregulation) on their own receptors, but no data are available on a possible differential regulation of ER-α and ER-β after exposure to estrogen agonists including xenoestrogens.

In this study, we compared the effects of BPA and three related phenolic chemicals [4-tert octylphenol (4-TOP), 2-hydroxybiphenyl (2-OH-BP) and 4-hydroxybiphenyl (4-OH-BP)] with that of the endogenous ligand 17β-estradiol in regulating, at the transcriptional level, ER-α and ER-β expression in an estrogen-sensitive (T47D) and an estrogen-insensitive (BT20) breast cancer cell line.

Material and methods

Chemicals

Cell culture reagents were obtained from Sigma (St Louis, Mo.) and from Bio-Whitakker Europe (Verviers, Belgium) and [^{32}P]αdCTP from the Radiochemical Center Amersham (Amersham, UK). Kits for transcription and PCR were obtained from Roche Diagnostics (Mannheim, Germany) and Applied Biosystems (Foster City, Calif.), respectively. BPA (97% purity) was purchased from Aldrich (Steinheim, Germany); 4-TOP (>90% purity), 4-OH-BP (>98%) and 2-OH-BP (>98%) were obtained from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade.

Cell lines

Human breast cancer cell lines were kindly provided by K. Horwitz, University of Colorado at Denver (T47D) and by G. Daxenbichler, University of Innsbruck (BT20). Cells were routinely maintained in DMEM/F12 (Sigma) without phenol red and supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 4 g/l glucose.

Cell treatment experiments

Cell growth was assessed with the sulforhodamine-B (SRB) assay [15]. Briefly, cells were plated in 96-well plates in 5% FBS-containing medium at concentrations previously defined to fall into the linearity range of the assay (3000 T47D cells/well and 4000 BT20 cells/well). After 24 h for attachment, the medium was replaced with 5% charcoal-dextrane-treated FBS [16] containing xenoestrogens or 17β-estradiol at concentrations ranging from 0.001 to 50 µM, depending on the tested molecule. In cell growth experiments, the medium was changed every 3 days and after 6 days of in vitro culture; when cells were still in the exponential growth phase, experiments were stopped by removing medium from wells and fixing the cells with 10% trichloracetic acid (1 h at 4 °C). Fixed cells were washed three times with tap water and left to dry. Cells were stained for total proteins for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. After quick rinsing with 1% acetic acid, bound dye was solubilized with 10 mM Tris base (pH 10.5) and plates were read in an automatic plate reader (Elx 800; Bio-Tek Instruments, Winooski, Ve.) at 492 nm.

For measurement of progesterone receptor (PgR) fluctuations, T47D cells were plated in T150 flasks at a density of 1.5×10^6 cells/flask in 5% FBS-containing cell culture medium. After 24 h for attachment, the medium was changed to charcoal-stripped 5% FBS containing the substance to be tested. Experimental medium was changed every 3 days and cells were harvested at day 6, when they were still in the exponential growth phase. Cell pellets (approximately 2×10^7 cells) were mechanically disrupted with a dismembrator (Braun, Melsungen, Germany) and resuspended in an appropriate volume of 20 mM K_2HPO_4, 1 mM EDTA, 3 mM NaN_3, 10% (v/v) glycerol and 12 mM thioglycerol, pH 7.4. Cytosol was prepared as previously described [17] and treated with a pellet of 0.5% Norit A, 0.05% Dextran T70 to remove ex-

cess BPA and related chemicals. PgR was measured by a multipoint dextran-coated charcoal assay as described elsewhere [17] and according to EORTC standards [18], using [^{3}H]Org2058 as tracer. Results were analyzed according to Scatchard [19] and expressed as femtomoles of specifically bound ligand per milligram of cytosol protein (fmol/mgP). Total cytosol protein was determined according to Bradford [20].

For RNA extraction, cells were grown in T25 culture flasks and treated as indicated in medium containing charcoal-stripped FBS. Immediately after treatment, RNA was extracted directly from the flask with the commercially available reagent Trizol (Life Technology, Grand Island, N. Y.). The integrity and purity of spectrophotometrically quantified extracted RNA were checked by electrophoresis.

RT-PCR

One microgram of total RNA was reverse transcribed using the commercially available first-strand cDNA synthesis kit for RT-PCR (AMV; Roche Diagnostics) after incubation at 65 °C for 15 min to remove secondary RNA structures, according to the manufacturer's instructions. Amplification of cDNA was carried out using the following primers: for ER-α [10], upper (sense) 5′-CAG GGG TGA AGT GGG GTC TGC TG -3′ (corresponding to exon 4, nucleotides 1060–1083, as numbered in Green S et al. [21]) and lower (antisense) 5′-ATG CGG AAC CGA GAT GAT GTA GC -3′ (priming in exon 6, nucleotides 1520–1543), giving an amplification product of 483 bp; for ER-β [22], upper (sense) 5′-GTC CAT CGC CAG TTA ATC ACA TC -3′ (located in ER-β 130–151 according to the published sequence [8]) and lower (antisense) 5′-GCC TTA CAT CCT TCA CAC GA -3′ (located in 371–352), giving an amplification product of 242 bp spanning the A/B domain of the protein.

For the ubiquitously expressed β-actin, the following primers were used: upper (sense) 5′-ACA CTG TGC CCA TCT ACG AGG -3′ and lower (antisense) 5′-AGG GGC CGG ACT CGT CAT ACT 3′, giving an amplification product of 600 bp.

The PCR reaction contained PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 0.75 µM of each dNTP, 1.6 mM $MgCl_2$, 4 µg/µl primer, 1 unit/tube of Gold Taq DNA polymerase, 1 µl of the mixture obtained by retrotransription of 1 µg of RNA and, in the case of ER-α and ER-β only, 1 µCi [^{32}P]αdCTP (specific activity 110 TBq/mmol) in a final volume of 20 µl. The amplification conditions were as follows. ER-α: a single step of 10 min at 95 °C to activate the enzyme, followed by 20 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, elongation at 72 °C for 1 min; ER-β: a single step of 10 min at 95 °C to activate the enzyme, followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s; β-actin: a single step of 10 min at 95 °C to activate the enzyme, followed by 26 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 45 s, elongation at 72 °C for 45 s.

cDNA from the cell line MCF-7 and human testis cDNA were used as positive controls for ER-α and ER-β, respectively; negative controls included the substitution of RNA or cDNA with distilled water and were consistently negative. Each PCR amplification was performed in triplicate. Radioactive PCR products were separated on a 6% polyacrylamide gel under non-denaturating conditions. Gels were dried and the intensity of the radioactive signal was detected using a phosphorimager. Amplification of the ubiquitously present β-actin was performed in parallel and PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. The intensity of the bands was determined by densitometry and expressed as arbitrary units.

ER-α and ER-β expression levels were normalized with respect to β-actin.

Statistical analysis

Data are presented as means ± SD of three independent experiments. Differences between ER expression in control and treated cells were tested by two-tailed Student's t test for paired data. Results were considered significant at $p < 0.05$.

Results

Effect of phenolic chemicals on cell growth

Cell growth experiments were carried out at 6 days of treatment using the SRB assay. The results are shown in figure 1.

BPA and 4-TOP stimulated cell growth in a dose-dependent fashion. In particular, with BPA, an approximately twofold statistically significant ($p < 0.001$) growth stimulation was achieved with the two higher doses (i.e. 1 and 10 µM).

4-TOP was a slightly less potent growth stimulator and triggered a 1.8-fold stimulation ($p < 0.001$) with respect to the control at the highest tested dose (10 µM). In contrast, 4-OH-BP was a weaker stimulator, while the 2-OH derivative did not significantly alter cell growth over the entire range of tested concentrations.

Conversely, estradiol induced a twofold growth stimulation ($p < 0.0001$) over the entire range.

The growth of BT20 cells was not affected by estradiol or BPA and derivatives (data not shown).

Validation of RT-PCR

To define amplification conditions that allow attainment of products in the exponential phase of the reaction, total RNA, extracted from a pool of breast cancer cells,

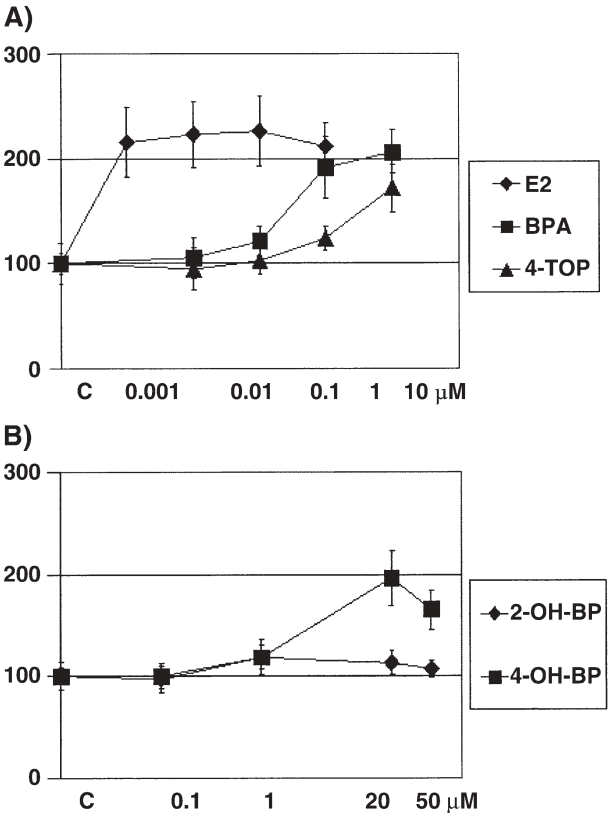

Figure 1. Cell growth variations in T47D cells as a function of treatment with xenoestrogens. Variations in the number of cells were evaluated by the SRB assay after 6 days of treatment. Results are plotted as percentages of untreated controls for 17β-estradiol, BPA and 4-TOP (*A*) and for 2-OH-BP and 4-OH-BP (*B*).

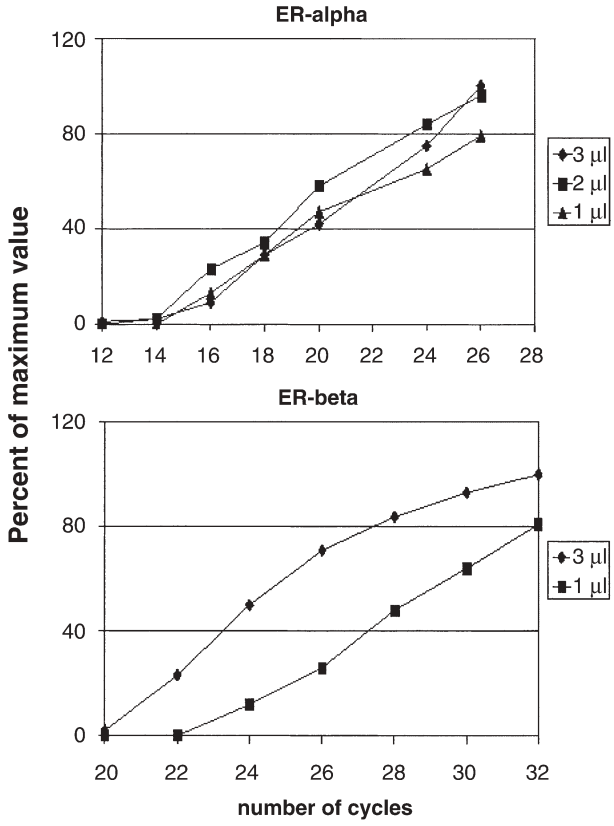

Figure 2. Densitometric quantification of ER-α and ER-β amplification products as a function of the number of PCR cycles for different aliquots of the mixture obtained by retrotranscription of 1 µg of total RNA. Data are expressed as percentages of the maximum obtained signal.

was reverse transcribed. The retrotranscription mixture obtained was used to carry out end-point amplifications at different numbers of cycles and with different amounts of template (fig. 2). Based on the reported plots, amplifications were run with 1 µl of template (corresponding to retrotranscription product obtained from 0.05 µg of RNA*)* at 20 cycles for ER-α and 28 cycles for ER-β.

Although RT-PCR is not a quantitative approach, reliable semiquantitative results can be obtained if specific experimental conditions guarantee the linearity of the assay (fig. 2), as shown in figure 3, in which a typical example of gels obtained from BPA-treated cells and the corresponding histogram of densitometric results relative to ER-β expression levels normalized for β-actin expression levels are reported. Since in a different experimental model, upregulation of β-actin in response to estradiol has been observed [23, 24], we carried out preliminary experiments to exclude regulation of β-actin by estradiol in our model. No dose- or time-dependent fluctuations of β-actin were observed upon treatment with 17β-estradiol in either T47D or BT20 cell lines (data not shown).

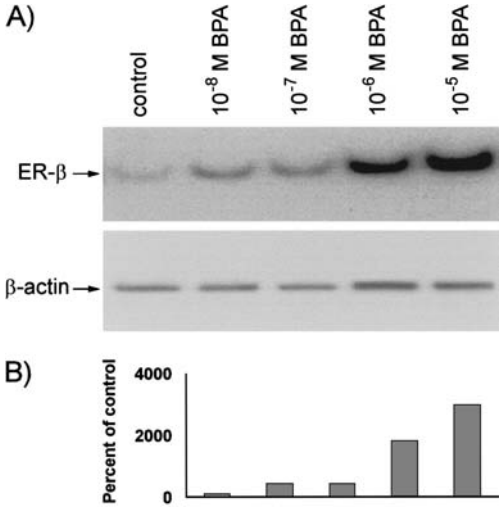

Figure 3. (*A*) Evaluation of ER-β and β-actin RNA levels in T47D cells by RT-PCR after 48 h treatment with BPA. ER-β gene products (242 bp) were separated on a 6% non-denaturating polyacrylamide gel, while β-actin gene products (600 bp) were separated on a 1.5% agarose gel. (*B*) Densitometric evaluation of ER-β expression after correction for β-actin variations: data are expressed as percentages of control.

Regulation of steady-state levels of ER-β and ER-α mRNA by BPA and its derivatives

The regulation of ER-β and ER-α levels by BPA and related phenolic compounds was investigated with two human breast cancer cell lines characterized by different receptor expression profiles. Specifically, the T47D cell line is known to be estrogen sensitive while BT20 is an estrogen-insensitive cell line.

Under steady-state conditions, in the absence of any type of stimulation, T47D cells expressed 23-fold higher levels of ER-α and 2.5-fold higher levels of ER-β than the BT20 cell line. The ER-α protein content of T47D cells was 26 fmol/mgP (determined by the ligand-binding assay) while no ligand-binding activity could be detected in the BT20 cells [25].

Modulation of ER-α and ER-β by BPA

BPA resolved in sterile-filtered DMSO (not exceeding 0.1% of the volume) was added to experimental medium at concentrations ranging from 0.01 to 10 µM, and cells were treated for 10, 24 and 48 h. The levels of ER-α and ER-β mRNA in treated and untreated cells were evaluated by RT-PCR and the extent of expression was measured by densitometry. Results are reported in figure 4. In the T47D cell line, ER-α expression transiently increased 2.5- to 3-fold after 24 h of treatment ($p < 0.01$). A similar effect was observed in the BT20 cell line, where ER-α transiently increased at 24 h of treatment to higher levels than those observed in the T47D cells: a fourfold increase at 0.1 µM BPA ($p < 0.005$) and a more than sevenfold increase at 1 µM BPA ($p < 0.01$) with respect to control.

In T47D cells, BPA induced a massive dose- and time-dependent increase in ER-β levels, which were already raised more than fourfold after 48 h of treatment at lower concentrations (0.01 µM, $p < 0.005$; 0.1 µM, $p < 0.01$) and reached a 30-fold upregulation at higher concentrations (10 µM, $p < 0.01$). By contrast, no significant effects on ER-β were observed in the BT20 cells.

Modulation of ER-α and ER-β by 4-TOP

4-TOP was administered to the cells at the same concentrations as used for BPA (fig. 5). The ER-α expression profile was not significantly changed at any time or concentration in either cell line. However, in T47D cells, 4-TOP caused upregulation of ER-β expression levels by about 3.5-fold with respect to the control at the lower concentrations (0.1 µM, $p < 0.0025$; 1 µM, $p < 0.01$), reaching a 15-fold increase at 10 µM ($p < 0.0005$) after 48 h of

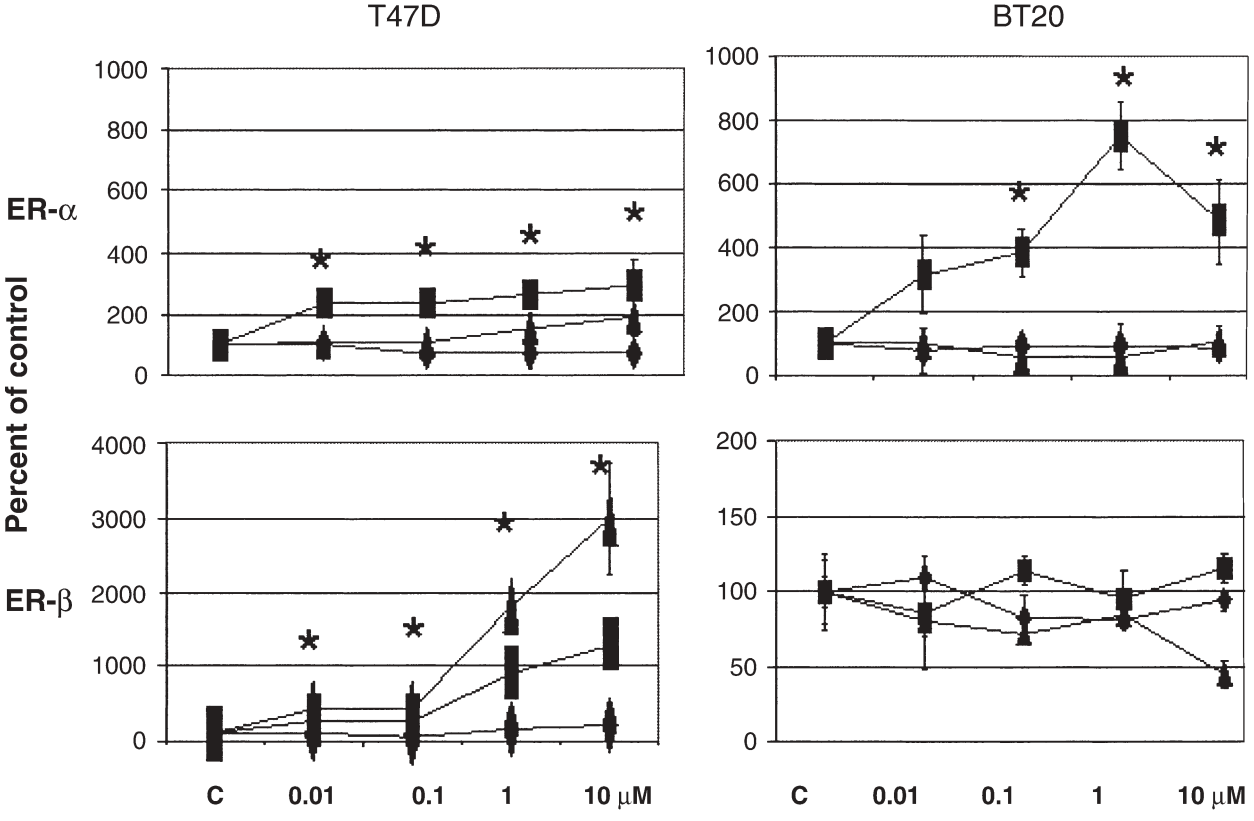

Figure 4. ER-α and ER-β expression in response to BPA treatment. ER-α and ER-β were determined by RT-PCR; specific bands were densitometrically quantified, normalized for β-actin expression levels (run in parallel) and plotted as percentages of controls by treatment doses for each treatment time: 10 h (rhombi), 24 h (squares) and 48 h (triangles). Data represent the mean of triplicate determinations ± SD. Asterisks indicate doses which significantly ($p < 0.05$) differ from the control.

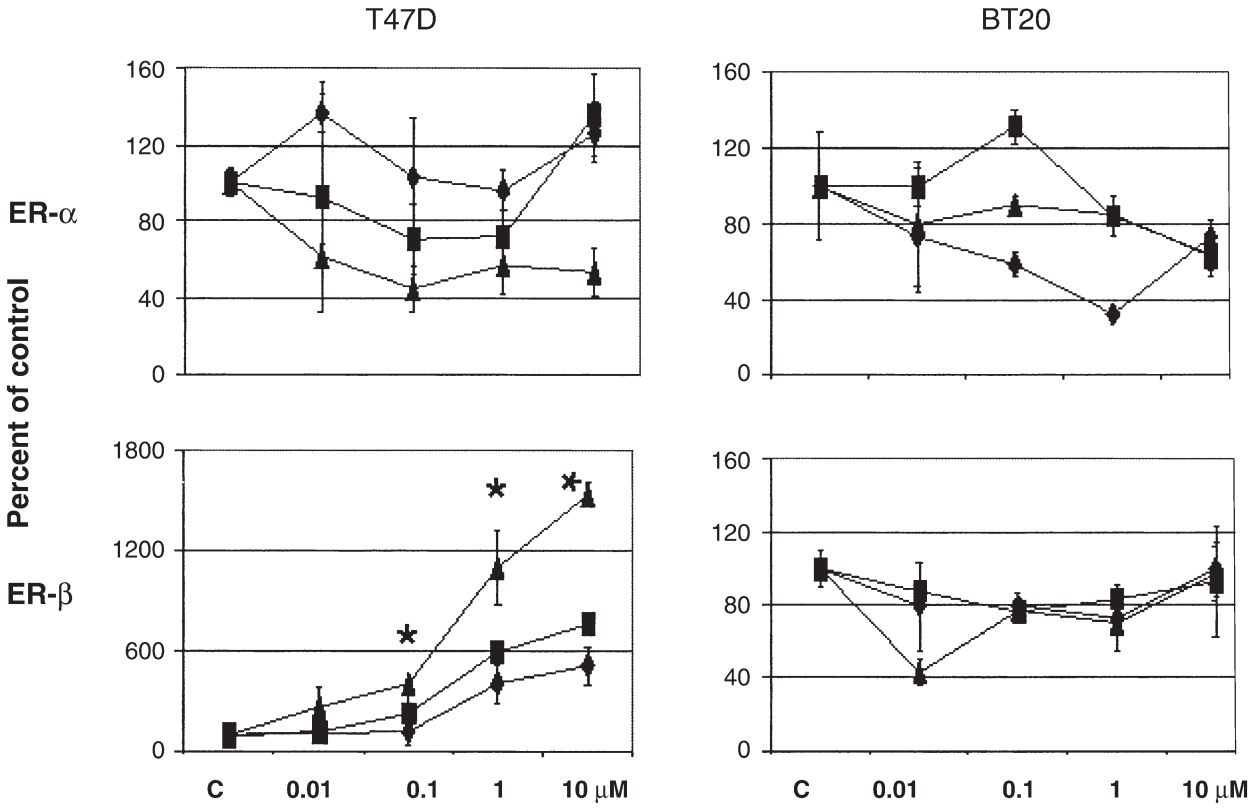

Figure 5. ER-α and ER-β expression in response to 4-TOP treatment. ER-α and ER-β were determined by RT-PCR; specific bands were densitometrically quantified, normalized for β-actin expression levels (run in parallel) and plotted as percentages of controls by treatment doses for each treatment time: 10 h (rhombi), 24 h (squares), and 48 h (triangles). Data represent the mean of triplicate determinations ± SD. Asterisks indicate doses which significantly ($p < 0.05$) differ from the control.

treatment. The induction of ER-β expression observed with 4-TOP was dose and time dependent. No significant fluctuation in ER-β expression was observed in BT20 cells.

Modulation of ER-α and ER-β by 2-OH-BP

Cells were treated with 2-OH-BP at 0.1, 1, 20 and 50 μM (fig. 6). This molecule, which proved to be a weak partial agonist in MCF-7 cells [26], did not influence the expression pattern of either ER-α or ER-β in the hormone-sensitive cell line T47D except for the 1 μM dose which caused a twofold increase in ER-β expression ($p < 0.05$). However, it surprisingly induced transient (only at 24 h) upregulation of both ER-α (about fourfold, $p < 0.01$) and ER-β (2.5-fold, $p < 0.01$) in BT20 cells.

Modulation of ER-α and ER-β by 4-OH-BP

The strong partial agonist 4-OH-BP [5] was tested over the same range of concentrations as used for 2-OH-BP (fig. 7). In T47D cells, a strong time-dependent upregulation of ER-β levels was observed at 20 μM (ninefold at 48 h; $p < 0.005$) and higher concentrations. No significant variation in receptor expression was observed in the BT20 cell line.

Modulation of ER-α and ER-β by the natural agonist 17β-estradiol

The effect of phenolic derivatives on the expression of the two receptor isoforms was compared with the effect of the natural ligand 17β-estradiol (fig. 8). ER-α was slightly upregulated in both T47D cells and BT20 cells at the longest treatment time. Similar results were observed for ER-β in the hormone-insensitive BT20 cell line. By contrast, in T47D cells, ER-β expression was markedly increased by estradiol, up to more than 25-fold at the higher concentrations given for 48 h (0.1 μM, $p < 0.05$; 1 μM, $p < 0.0025$).

Regulation of PgR steady-state levels by BPA and its derivatives compared to the natural ligand 17β-estradiol

In separate experiments, T47D cells were treated with estradiol and with BPA and its derivatives for 6 days and collected to prepare the cytosolic fraction for PgR determination by the ligand-binding assay. The results, expressed as percentages with respect to vehicle-treated control, are reported in table 1.

Estradiol at 0.001 and 0.01 μM triggered a 14- and 22-fold increase respectively, in the expression of PgR.

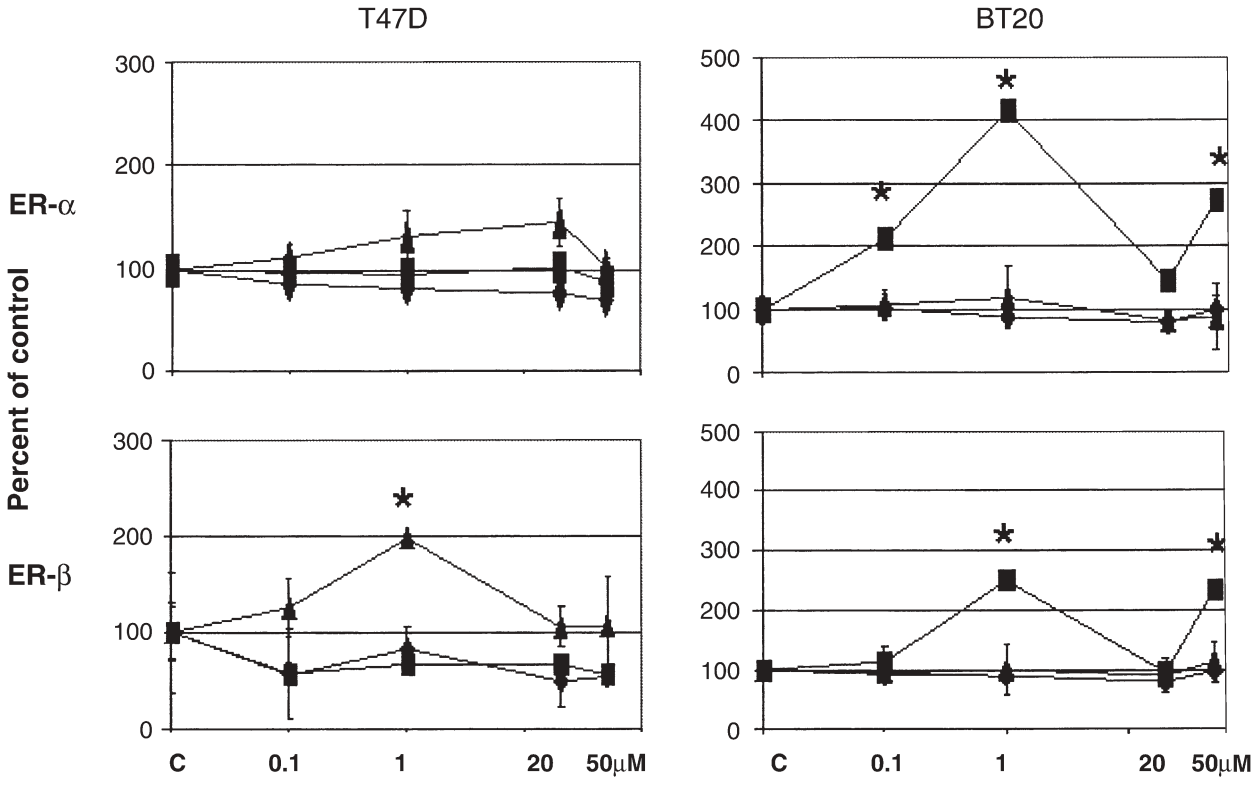

Figure 6. ER-α and ER-β expression in response to 2-OH-BP treatment. ER-α and ER-β were determined by RT-PCR; specific bands were densitometrically quantified, normalized for β-actin expression levels (run in parallel) and plotted as percentages of controls by treatment doses for each treatment time: 10 h (rhombi), 24 h (squares) and 48 h (triangles). Data represent the mean of triplicate determinations ± SD. Asterisks indicate doses which significantly ($p < 0.05$) differ from the control.

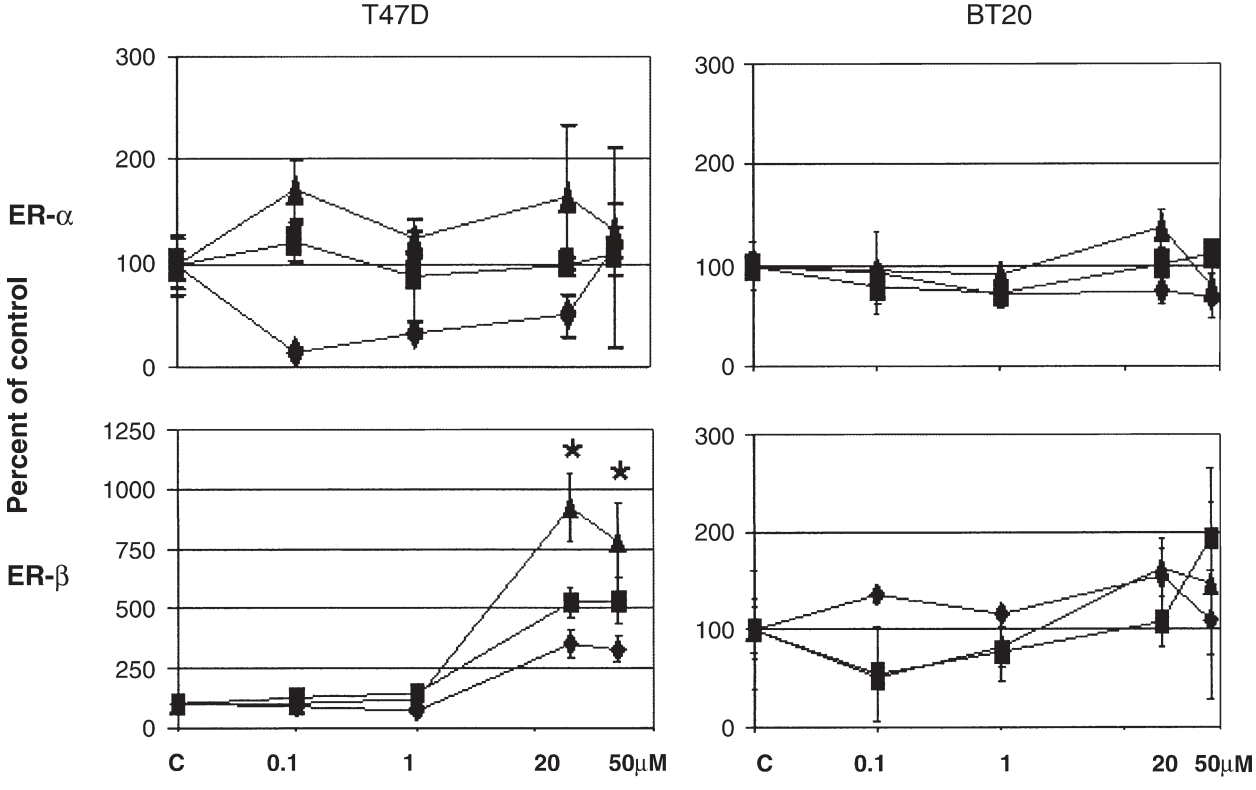

Figure 7. ER-α and ER-β expression in response to 4-OH-BP treatment. ER-α and ER-β were determined by RT-PCR; specific bands were densitometrically quantified, normalized for β-actin expression levels (run in parallel) and plotted as percentages of controls by treatment doses for each treatment time: 10 h (rhombi), 24 h (squares) and 48 h (triangles). Data represent the mean of triplicate determinations ± SD. Asterisks indicate doses which significantly ($p < 0.05$) differ from the control.

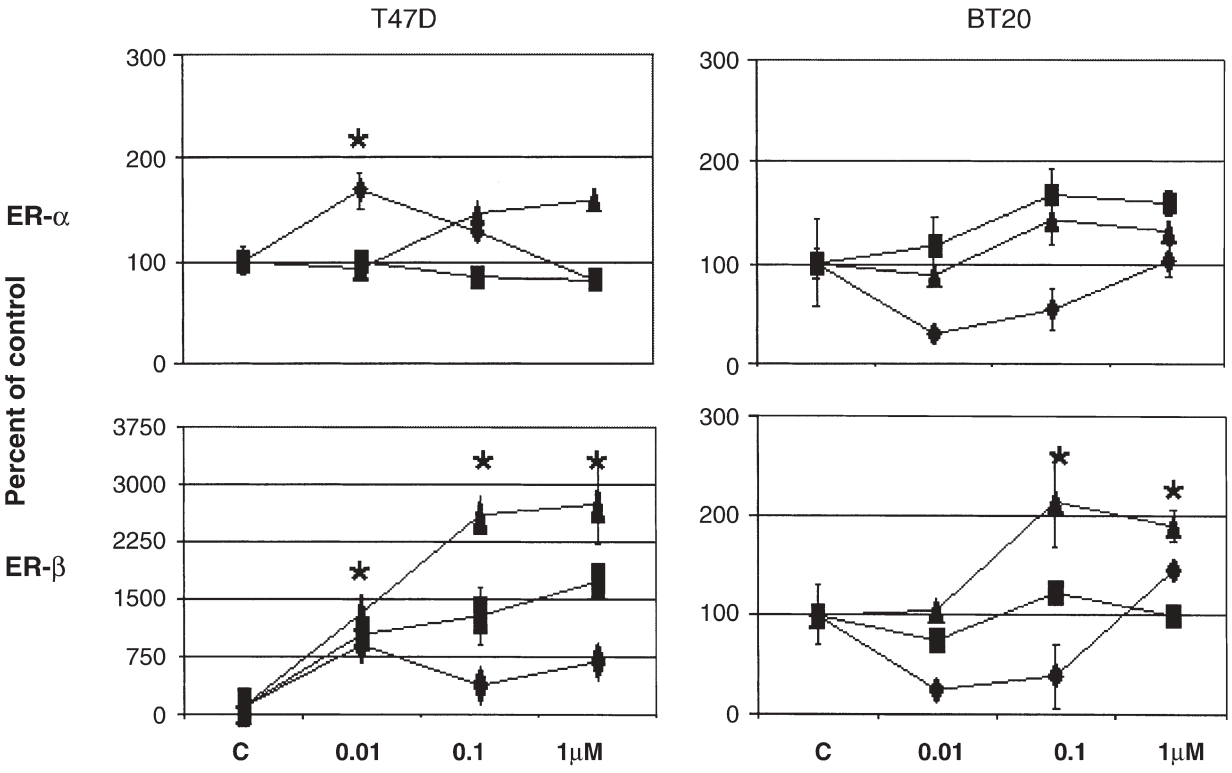

Figure 8. ER-α and ER-β expression in response to estradiol treatment. ER-α and ER-β were determined by RT-PCR; specific bands were densitometrically quantified, normalized for β-actin expression levels (run in parallel) and plotted as percentages of controls by treatment doses for each treatment time: 10 h (rhombi), 24 h (squares) and 48 h (triangles). Data represent the mean of triplicate determinations ± SD. Asterisks indicate doses which significantly ($p < 0.05$) differ from the control.

Table 1. Modulation of PgR expression in T47D cells as a percentage of the control.

Concentration (μM)	Estradiol	BPA	4-TOP	2-OH-BP	4-OH-BP
0.001	2199.0				
0.01	1391.8	120.1	188.0		
0.1		188.6	685.5	154.2	99.8
1		146.6	1947.0	112.3	124.6
10		206.6	1448.8		
20				263.5	361.9
50				351.1	382.0

Among the BPA derivatives, 4-TOP was the strongest agonist of estradiol and caused a 19.5- and 14.5-fold upregulation of PgR at concentrations of 1 and 10 μM, respectively (three orders of magnitude higher than estradiol itself).

The two derivatives 2-OH-BP and 4-OH-BP were approximately equipotent in inducing PgR, triggering an induction ranging from 2.5 to 3.8 in the 10 μM range. BPA was slightly less potent and achieved its strongest induction (twofold) at 10 μM.

These experiments were not done with the BT20 cells because they did not express detectable levels of PgR in the ligand-binding assay.

Discussion

In the present study we showed that BPA and related compounds, which are widely used for polymer synthesis and other applications and are released into the environment by various routes, mimic estradiol action. The estrogenic activity of some of these compounds has already been studied in human breast cancer cells, both in terms of proliferative activity and induction of estrogen-regulated proteins [4]. The present study, which was not planned with a toxicological perspective, was therefore performed with xenoestrogen concentrations known to cause biological effects, independently of the actual levels found in

humans. For closer insight into the action mechanism of xenoestrogens, we chose two breast cancer cell lines characterised by a different expression profile of ER-α and ER-β and by a different sensitivity to estrogens. Our results support the evidence that natural estrogen and estrogen-mimicking chemicals exert a differential regulatory effect on ER-α and ER-β. Specifically, using an RT-PCR-based approach, in the absence of validated commercially available antibodies to detect ER-β (27, 28) and because expression levels of ER-β in breast cancer cells are low [10], we observed that in T47D cells, upregulation of ER-β mRNA levels is much more dramatic than that of ER-α mRNA levels, in keeping with results obtained using estradiol by Vladusic et al. [29]. In contrast, the general view is that ER-α protein levels (evaluated by ligand-binding assay) are downregulated following extended estrogenic stimulation, although upregulation of the ER-α gene by estradiol has been observed in most tissues and experimental systems and was also recently reported for endometrial carcinoma cells, in terms of enhancement of mRNA stability [30]. Our observations on ER-β upregulation have potential clinical implications since ER-β is known to play a regulatory role in the transcriptional activity of ER-α [9, 11] and distinct effects of the ERs have been demonstrated at AP-1-containing promoters, suggesting that the balance of ER-α and ER-β may be crucial in the growth regulation of tissue [13].

Moreover, we demonstrated that the estradiol agonists BPA and 4-TOP dramatically induced ER-β mRNA in the estrogen-sensitive cell line T47D, whereas they only marginally upregulated ER-α mRNA. By contrast, no important variations in the expression of either ER were observed in the estrogen-insensitive cell line BT20. BT20 cells are considered to be hormone insensitive since estradiol fails to stimulate cell proliferation and induce estrogen-regulated proteins. In fact, in these cells, ER-α could be detected at very low levels only by the very sensitive RT-PCR approach, while ER was undetectable by the conventional radioligand assay [25]. This would indirectly suggest that the described upregulation of ER-β can only occur in the presence of detectable levels of ER-α protein.

Furthermore, our results suggest that in breast cancer cell lines, the presence of ER-β alone is not sufficient to achieve the typical estrogenic effects exerted either by estradiol itself or by less potent agonists like xenoestrogens, since growth stimulation and PgR induction could be observed only in T47D and not in BT20 cells. As a transcription factor, ER-β is generally believed to act similarly to ER-α, although with a relatively lower transcriptional potency. However, this hypothesis is based on reporter gene assays performed in cells exhibiting non-physiological expression of ER (e.g. HeLa, 293, COS) after differential transfection with ER-α or ER-β [11, 12, 14, 31]. In these cells, ER-β alone triggers activation of genes artificially under the control of an ERE, but there is no evidence that this also occurs under physiological conditions. The estrogenic potency of compounds is, in fact, a complicated matter which is strongly influenced not only by the transcription factor and its ligand but also by the specific cell and target gene promoter context. To the best of our knowledge, the ability of ER-β to regulate typical responses under estrogen control has not yet been reported. Therefore, our results challenge the belief that ER-β per se is a transcriptional factor for estrogens and support the hypothesis that ER-β regulates the transcriptional activity of ER-α.

Overall, the estrogenic upregulation of ER-β is only partially paralleled by induction of estrogen-regulated proteins.

In fact, when considering cell growth and ER-β mRNA and PgR modulation in the estrogen-responsive cell line, among the investigated substances, BPA ranked first in terms of effect on cell proliferation and ER-β upregulation, but exerted minimal activity in inducing PgR expression, even compared to 2-OH-BP or 4-OH-BP which, conversely, showed a, respectively, negligible or weaker effect on proliferation and a minimal or weaker ER-β upregulation. Conversely, 4-TOP appeared to be a potent PgR inducer and a significant upregulator of ER-β, although slightly less potent than BPA.

Our data further support the observation by Hall and McDonnel [11] and Pettersson et al. [12] that in estrogen-sensitive (i.e. ER-α-containing) tissues, ER-β is a regulator of ER-α transcriptional activity, since it can be induced by estrogen and its agonists and therefore acts as a negative feedback loop. In addition, our data suggest that the negative feedback loop mediated by the rise in ER-β could be active not only in the presence of physiologically high estradiol levels (e.g. in puberty or during pregnancy), but may also be induced by exposure to weak estrogens persistently present in the environment. Therefore, alteration of ER-β expression levels and the ensuing implications for ER-α transcriptional activity could represent an additional mechanism of action for xenoestrogens beside the classical direct interaction with ERs and with the synthesis and metabolism of hormones.

1 Brotons J. A., Olea-Serrano M. F., Villalobos M., Pedraza V. and Olea N. (1995) Xenoestrogens released from lacquer coatings in food cans. Environ. Health Perspect. **103:** 608–612
2 Olea N., Pulgar R., Pérez P., Olea-Serrano F., Rivas A., Novillo-Fertrell A. et al. (1996) Estrogenicity of resin-based composites and sealants used in dentistry. Environ. Health Perspect. **104:** 298–305
3 Bornstein J., Adam E., Adler-Storthz K. and Kaufman R. H. (1988) Development of cervical and vaginal squamous cell neoplasia as a late consequence of in utero exposure to diethylstilbestrol. Obstet. Gynecol. Surv. **43:** 15–21
4 Perez P., Pulgar R., Olea-Serrano F., Villalobos M., Rivas Metzler M. et al. (1998) The estrogenicity of bisphenol A-related diphenylalkanes with various substituents at the central

carbon and hydroxy groups. Environ. Health Perspect. **106:** 167–174
5 Soto A. M., Sonnenschein C., Chung K. L., Fernandez M. F., Olea N. and Olea-Serrano F. (1995) The E-SCREEN as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ. Health Perspect. **103(suppl 7):** 113–122
6 Evans R. M. (1988) The steroid and thyroid receptor superfamily. Science **240:** 889–895
7 Kuiper G. G., Enmark E., Pelto-Huikko M., Nilsson S. and Gustafsson J.-A. (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA **93:** 5925–5930
8 Mosselman S., Polman J. and Dijkema R. (1996) ER-β: identification and characterization of a novel human estrogen receptor. FEBS Lett. **392:** 49–53
9 Pettersson K., Grandien K., Kuiper G. G. J. M. and Gustafsson J.-A. (1997) Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. Mol. Endocrinol. **11:** 1486–1496
10 Dotzlaw H., Leygue E., Watson P. H. and Murphy L. C. (1996) Expression of estrogen receptor β in human breast tumors. J. Clin. Endocrinol. Metab. **82:** 2371–2374
11 Hall J. M. and McDonnell D. P. (1999). The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology **140:** 5566–5578
12 Pettersson K., Delaunay F. and Gustafsson J.-A. (2000) Estrogen receptor β acts as a dominant regulator of estrogen signaling. Oncogene **19:** 4970–4978
13 Paech K., Webb P., Kuiper G. G. J. M., Nilsson S., Gustafsson A., Kushner P. J. et al. (1997) Differential ligand activation of estrogen receptors ER-α and ER-β at AP1 sites. Science **277:** 1508–1512
14 Hiroi H., Tsutsumi O., Momoeda M., Takai Y., Osuga Y. and Taketani Y. (1999) Differential interactions of bisphenol A and 17β-estradiol with estrogen receptor α (ERα) and ERβ. Endocr. J. **46:** 773–778
15 Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D. et al (1990) New colorimetric cytotoxicity assay for anticancer-drug screening J. Natl. Cancer Inst. **82:** 1107–1112
16 Soto A. M. and Sonnenschein C. (1985) The role of estrogens on the proliferation of human breast tumor cells (MCF-7). J. Steroid Biochem. **23:** 87–94
17 Cappelletti V., Brivio M., Miodini P., Granata G., Coradini D. and Di Fronzo G. (1988) Simultaneous estimation of epidermal growth factor receptors and steroid receptors in a series of 136 resectable primary breast tumors. Tumor Biol. **9:** 200–211
18 EORTC Breast Cancer Co-operative Group (1980) Revision of the standards for the assessment of hormone receptors in human breast cancer. Eur. J. Cancer **16:** 1513–1515
19 Scatchard G. (1949) The attraction of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. **51:** 660–672
20 Bradford M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein dye binding. Anal. Biochem. **72:** 56–59
21 Green S., Walter P., Kumar V., Krust A., Bornet J. M., Argos P. et al. (1986) Human estrogen receptor cDNA: sequence expression and homology to v-erb-A. Nature **320:** 134–139
22 Dotzlaw H., Leygue E., Watson P. H. and Murphy L. C. (1999) Estrogen receptor-β messenger RNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins. Cancer Res. **59:** 529–532
23 Hsu C. Y. and Frankel F. R.(1987) Effect of estrogen on the expression of mRNAs of different actin isoforms in immature rat uterus: cloning of alpha-smooth muscle actin message. J. Biol. Chem. **262**: 9594–9600
24 Cicatiello L., Ambrosino C., Coletta B., Scalona M., Sica V., Breasciani F. et al. (1992) Transcriptional activation of jun and actin genes by estrogen during mitogenic stimulation of rat uterine cells. J. Steroid. Biochem. Mol. Biol. **41**: 523–528
25 Ruedl C., Cappelletti V., Coradini D., Granata G. and Di Fronzo G. (1990) Influence of culture conditions on the estrogenic cell growth stimulation of human breast cancer cells. J. Steroid Biochem. **37**: 195–200
26 Körner W., Bolz U., Süßmuth W., Hiller G., Schuller W., Hanf V. et al. (2000) Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere **40:** 1131–1142
27 Skliris G. P., Parkes A. T., Limer J. L., Burdall S. E., Carder P. J. and Speirs V. (2002) Evaluation of seven oestrogen receptor ß antibodies for immunohistochemistry, Western blotting, and flow cytometry in human breast tissue. J. Pathol. **197:** 155–162
28 Speirs V., Carder P. J. and Lansdown M. R. J. (2002) Oestrogen receptor beta: how should we measure this? Br. J. Cancer **87:** 687–690
29 Vladusic E. A., Hornby A. E., Guerra-Vladusic K. F., Lakins J. and Lupu R. (2000) Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. Oncol. Rep. **7:** 157–167
30 Robertson J. A., Zhang Y., Lindahl L. S and Ing N. H. (2000) Estradiol up-regulates estrogen receptor messenger ribonucleic acid in endometrial carcinoma (Ishikawa) cells by stabilizing the message. J. Mol. Endocrinol. **29:** 125–135
31 Pennie W. D., Aldridge T. C. and Brooks A. N. (1998) Differential activation by xenoestrogens of ER-α and ER-β when linked to different response elements. J. Endocrinol. **158:** R11–R14