Crosstalk between estrogen receptor α and the aryl hydrocarbon receptor in breast cancer cells involves unidirectional activation of proteasomes

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Abstract **2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is an environmental toxin that activates the aryl hydrocarbon receptor (AhR) and disrupts multiple endocrine signaling pathways. T47D human breast cancer cells express a functional estrogen receptor α (ERα) and AhR, and treatment of these cells with 17β-estradiol (E2) or TCDD resulted in a rapid proteasome-dependent decrease in immunoreactive ERα and AhR proteins ($>$60–80%), respectively. E2 did not affect the AhR, whereas TCDD induced proteasome-dependent degradation of both the AhR and ERα in T47D and MCF-7 human breast cancer cells, and these responses were specifically blocked by proteasome inhibitors. Thus, TCDD-induced degradation of ERα may contribute to the antiestrogenic activity of AhR agonists and this pathway may be involved in AhR-mediated disruption of other endocrine responses. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Proteasome; Estrogen receptor α;
Aryl hydrocarbon receptor; Degradation; Crosstalk;
Breast cancer cell

1. Introduction

The estrogen receptor (ER) is a ligand-activated transcription factor that is a member of the nuclear receptor superfamily [1,2], and two ER subtypes (ERα and ERβ) and multiple variants have been characterized [3–6]. Estrogens play an important physiological role in male and female reproduction and development and have also been characterized as risk factors for breast and endometrial cancer in women [7].

Drugs used for endocrine therapy of early-stage breast cancer include ER antagonists, such as tamoxifen, that inhibit ER action in ER-positive mammary tumors and breast cancer cells in culture [8]. We have been investigating aryl hydrocarbon receptor (AhR)-mediated inhibition of estrogen-induced responses in the rodent uterus and mammary gland and in breast cancer cell lines [9,10]. The ligand-activated AhR complex inhibits estrogen-induced gene expression and their derived proteins or dependent activities and also inhibits growth of estrogen-dependent mammary tumors in rodent models. Most studies with the AhR have used the environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as a prototypical ligand; we have also identified selective AhR modulators such as 6-methyl-1,3,8-trichlorodibenzofuran (MCDF)

that are relatively non-toxic but are effective inhibitors of rodent mammary tumor growth [11]. At least one mechanism of inhibitory AhR–ERα crosstalk involves direct interaction of the AhR complex with inhibitory dioxin response elements (iDREs) identified in the 5′-promoter regions of E2-responsive cathepsin D, pS2 and c-*fos* genes [12–14]. However, results of ongoing studies indicate that AhR agonists also inhibit E2-induced expression of several genes that do not have functional iDREs.

Previous studies in the rodent uterus and breast cancer cells have shown that TCDD downregulates ERα [15–20], and this response may contribute to inhibitory AhR–ERα crosstalk. Recent reports indicate that both 17β-estradiol (E2) and TCDD induce proteasome-dependent degradation of ERα and the AhR respectively [21–25], and previous studies have shown that TCDD downregulates the AhR in vitro and in vivo [26–29]. This study probes the role of proteasome activation in mediating inhibitory AhR–ERα interactions in ERα-positive MCF-7 and T47D breast cancer cell lines that also express a functional AhR [19].

2. Materials and methods

2.1. Cells, chemicals and biochemicals

T47D and MCF-7 human breast cancer cells were obtained from American Type Culture Collection (Rockville, MD). TCDD and MCDF were prepared in this laboratory, and shown to be >99% pure by gas chromatographic analysis. E2, benzo[*a*]pyrene (BaP), chloroquine, calpain inhibitor II, EST, MG132 and PSI were the highest quality available from commercial sources. Antibodies for ERα (sc-544), AhR (sc-8088), Sp1 and Arnt (sc-8076) proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The human ERα, AhR and AhR nuclear translocator (Arnt) full length cDNA probes were cloned into the pcDNA3.1 expression vector (Invitrogen) and used to translate in vitro standards for Western blots using a reticulocyte lysate method.

2.2. Cell maintenance and protein isolation

Cells were grown on monolayer cultures in Sigma MEM α modification (T47D) or DMEM Ham F12 (MCF-7) media with phenol red supplemented with 2.2 g/l sodium bicarbonate, 0.2 g/l bovine serum albumin (bovine, fraction V), 0.01 g/l apo-transferrin (human), 5% fetal bovine serum (Intergen, Purchase, NY), and antibiotic–antimycotic solution, pH 7.4. Cells for experiments were seeded into 35 mm 6 well tissue culture plates in phenol-free media (DMEM Ham F-12, Sigma) containing 2.5% charcoal-stripped fetal bovine serum. The following day, the cells were treated and harvested at designated time points as follows. Cells were washed once in ice cold phosphate-buffered saline and collected by scraping in 0.25 ml ice cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 50 mM phenylmethylsulfonyl fluoride, 50 mM sodium orthovanadate. The lysates were incubated on ice for 1 h with intermittent vortexing followed by centrifugation ($15\,000\times g$, 5 min, 4°C).

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Equal amounts of protein from each treatment group were separated by SDS–PAGE and electrophoresed to PVDF membrane using a Bio-Rad Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA); transfer buffer: 48 mM Tris, 39 mM glycine, 0.025% sodium dodecyl sulfate (SDS). Membranes were blocked for 30 min in Blotto (5% milk+TBS (10 mM Tris–HCl, pH 8.0, 150 mM NaCl), 0.05% Tween-20), probed with the polyclonal antibodies for ERα (1:1000 dilution in Blotto), AhR (1:200), or Arnt (1:200) (see Section 2.1) for 5 h, washed 2×5 min in TBS+0.05% Tween 20, and probed with secondary peroxidase-conjugated antibody (1:5000 in Blotto) for 2 h. The membranes were then washed 3×5 min in TBS+0.05% Tween-20, 1×5 min in TBS, and visualized using the ECL detection system (New England Nuclear, Boston, MA). Quantitation of Western blots was performed using a Sharp JX-330 scanner (Sharp Corp., Mahwah, NJ) and Zero-D Scanalytics software (Scanalytics Corp., Billerica, MA).

2.3. Statistical analysis

Relative ERα and AhR levels are plotted as a percentage of total densometric units observed for dimethylsulfoxide (DMSO; control)-treated cells for individual PVDF membranes. Results are presented as means ± S.E.M. for at least three separate determinations for each treatment group. All experiments were performed at least two times. Statistical differences between groups were determined by analysis of variance followed by Fisher's protected LSD test for significance ($P < 0.05$).

3. Results and discussion

The results demonstrate that after treatment of T47D and MCF-7 cells (Fig. 1a,b) with E2, TCDD or their combination, there is a rapid decrease in immunoreactive ERα protein that persists for up to 24 h. The proteasome inhibitors MG132 and PSI block both E2- (Fig. 1c) and TCDD- (Fig. 1d) mediated degradation of ERα, whereas protease inhibitors had no effect. TCDD also induces degradation of the AhR in T47D cells (Fig. 2a), and this response is blocked by proteasome inhibitors (MG132 and PS1) but not protease inhibitors (Fig. 2b). In contrast, E2 does not affect levels of AhR protein (Fig. 2a) demonstrating that proteasome-dependent inhibitory
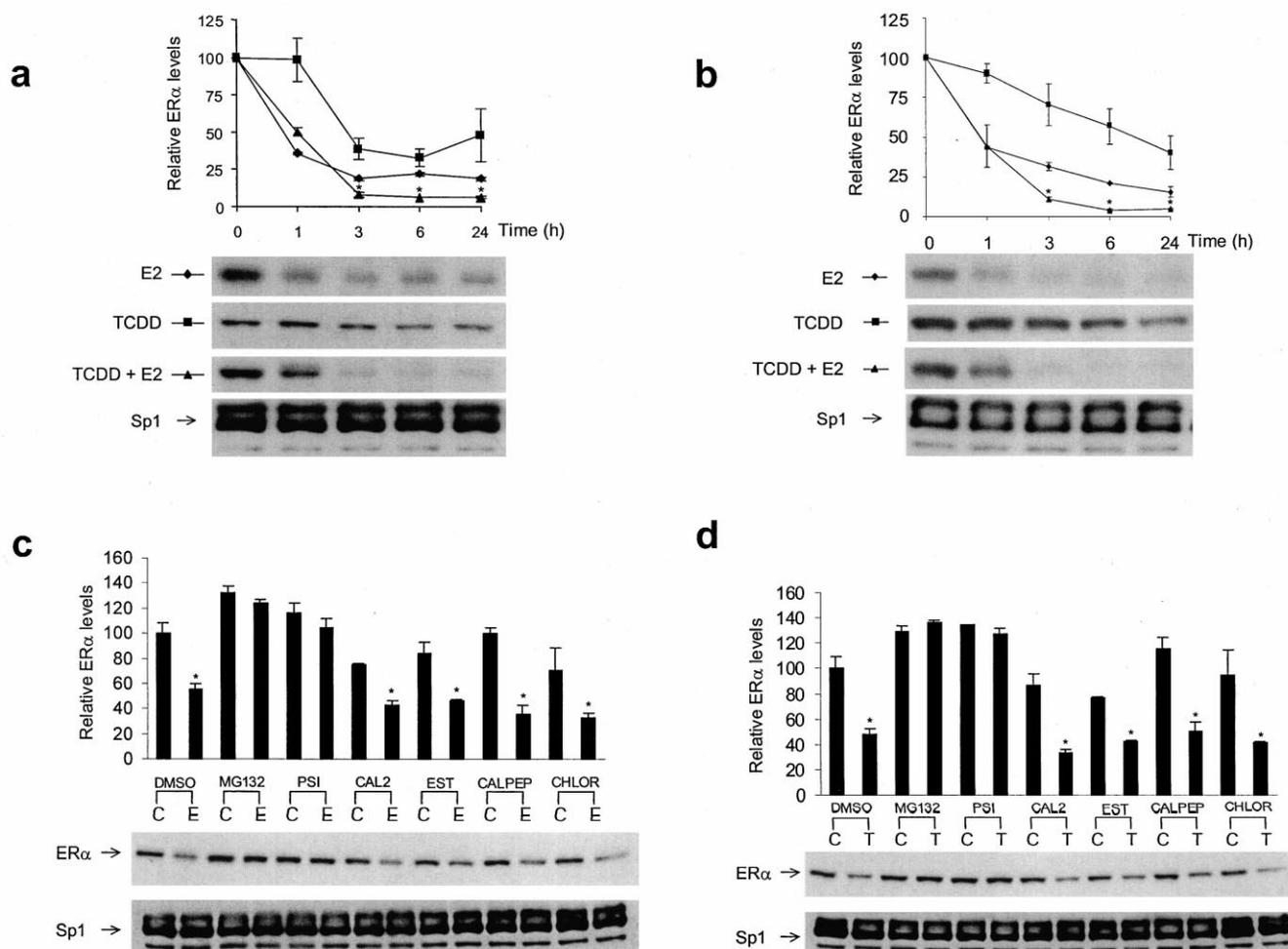

Fig. 1. Modulation of ERα protein levels through the ER and AhR pathways. T47D (a) and MCF-7 (b) cells treated with 10 nm E2, 10 nM TCDD, or 10 nM E2 plus 10 nM TCDD display a time-dependent decrease in immunoreactive ERα protein levels. 50 μg aliquots of whole cell lysates were separated by SDS–PAGE (7.5% acrylamide) and immunoblotted for ERα (see Section 2). Cotreatment with E2 plus TCDD significantly decreased ERα levels relative to E2 treatment alone by 40–80% after 3 h (a, b), 6 h (b) and 24 h (b). Pretreatment of T47D cells for 1 h with the proteasome inhibitors MG132 and PSI (10 μM) followed by a 3 h treatment of 10 nM E2 (c) or 10 nM TCDD (d) blocked degradation of ERα protein. The protease inhibitors calpeptin (CALPEP), chloroquine (CHLOR), calpain inhibitor II (CAL2), and EST (10 μM) had no effect. C = DMSO, E = E2, T = TCDD. $n = 3$, mean ± S.E.M. *$P < 0.05$. Levels of immunoreactive Sp1 protein for each treatment group (c and d) and for the E2+TCDD treatment groups (a and b) are shown as a loading control. We have previously shown that levels of this protein are unaffected by E2, TCDD or their combination (data not shown). The proteasome-dependent effects of TCDD, E2 and their combination on immunoreactive ERα in whole cell extracts were also observed in nuclear extracts as determined in gel mobility shift assays (data not shown).
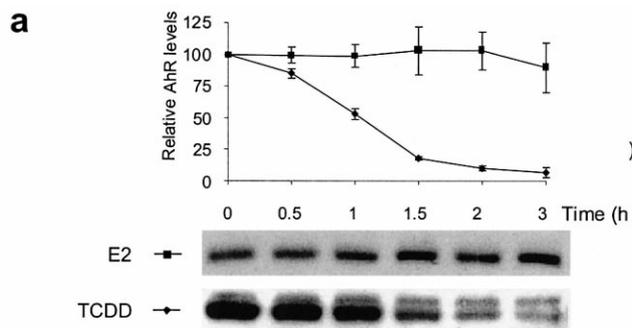
Fig. 2. E2 does not induce a reciprocal degradation of AhR protein. a: T47D cells were treated with 10 nM TCDD or 10 nM E2 for the indicated lengths of time. 50 μg aliquots of whole cell lysates were separated on SDS–PAGE (7.5% acrylamide) and immunoblotted for AhR (see Section 2). E2 did not significantly downregulate AhR protein levels. b: Degradation of AhR protein after treatment with 10 nM TCDD for 3 h was significantly inhibited (relative to TCDD/DMSO alone) by pretreatment (for 1 h) with proteasome inhibitors MG132 and PSI (10 μM). The protease inhibitors calpeptin (CALPEP), chloroquine (CHLOR), calpain inhibitor II (CAL2) and EST (10 μM) had no effect. C = DMSO, E = E2, T = TCDD. $n = 3$, mean ± S.E.M. $*P < 0.05$.
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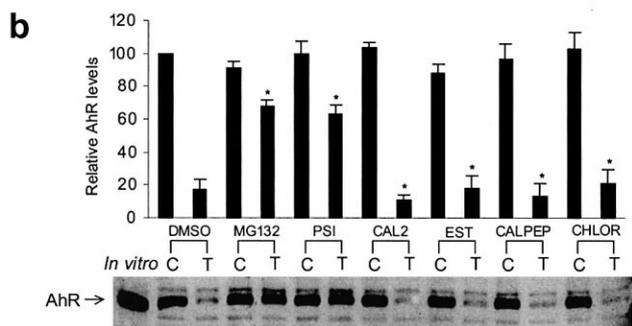
crosstalk between the two signaling pathways is unidirectional, namely AhR → ERα. Selective and timely proteasome-dependent degradation of proteins occurs at critical points during differentiation, cell cycle progression, oncogenesis, and other highly regulated cellular processes [30,31]. It has been suggested that E2-activated degradation of ER by proteasomes may be an important pathway for limiting the duration of estrogenic responses in target tissues [21], and our results (Fig. 1) demonstrate that this pathway is also induced through ligand (TCDD) activation of the AhR. The interactive proteasome-dependent effects of TCDD plus E2 are consistent with reported inhibition of E2-induced gene expression, in vitro cell proliferation and mammary tumor growth associated with AhR–ERα crosstalk [9–14].
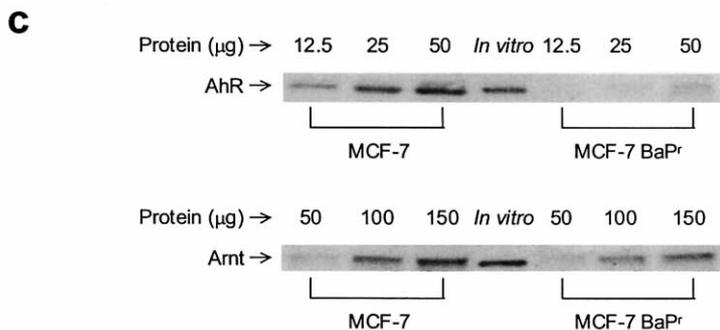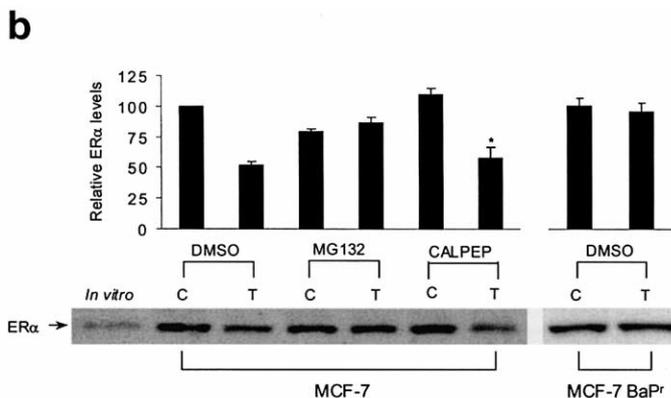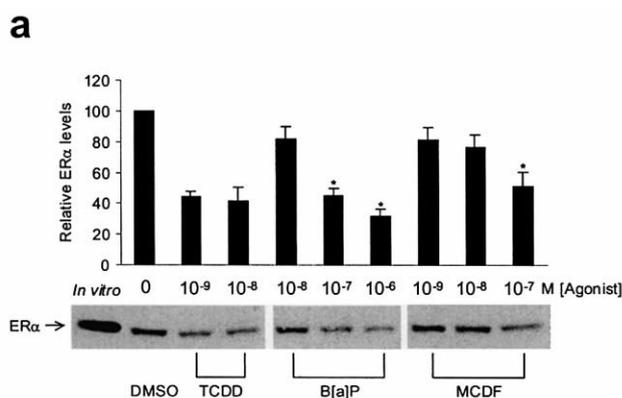

Fig. 3. Role of the AhR in ERα protein degradation. a: Treatment of MCF-7 cells with AhR agonists downregulates ERα protein levels. Cells were treated with increasing concentrations of TCDD, BaP or MCDF for 3 h, and ERα protein levels were analyzed as described in Section 2. b: Pretreatment (1 h) with the proteasome inhibitor MG132 (10 μM) followed by a 3 h treatment with 10 nM TCDD blocked ERα degradation in MCF-7 cells, whereas the protease inhibitor CALPEP had no effect. The BaP-resistant MCF-7 cell line (MCF-7 BaP^r) is AhR-non-responsive; treatment 10 nm TCDD for 3 h did not significantly alter ERα protein levels. Treatment with TCDD for longer periods did not affect ERα levels, and analysis of nuclear extracts from these cells showed increased ER DNA binding in a gel mobility shift assay [32]. c: Whole cell extracts from wild-type and BaP^r MCF-7 cells were immunoblotted for relative AhR and Arnt protein levels. MCF-7 BaP^r cells express minimal levels of immunoreactive AhR protein relative to MCF-7 wild-type cells.

The AhR interacts with structurally diverse ligands and rank order structure–binding relationships correlate with structure–activity relationships [9]. The results in Fig. 3a compare the effects of three AhR agonists, namely TCDD, BaP, and MCDF, on ERα protein levels in MCF-7 cells. Their order of potency for induction of proteasome-dependent degradation of ERα protein (TCDD > BaP$\geq$MCDF) is similar to their rank order for AhR binding and other AhR-mediated responses suggesting that ERα degradation is mediated by the AhR [9]. Moreover, the effects of TCDD on ERα degradation in wild-type MCF-7 cells are blocked by the proteasome inhibitor MG132 (Fig. 3b), whereas the protease inhibitor calpeptin did not affect TCDD action. In contrast, TCDD did not affect ERα protein in E2-responsive, BaP-resistant MCF-7 cells (Fig. 3b) that express minimal to non-detectable levels of the AhR and higher levels of Arnt protein (Fig. 3c) [32,33] confirming that the AhR is required for ligand-activated degradation of ERα in breast cancer cells. These data suggest that Ah non-responsiveness of BaP-resistant cells is associated with limiting amounts of AhR expression.

The overall mechanisms for proteasome-dependent degradation of ERα and the AhR have not been delineated and results from different reports are variable [21–25]. For example, one study showed that nuclear export was important for cytosolic degradation of the AhR [24], whereas another report suggested that nuclear localization was required for rapid AhR degradation [25].

Thus, TCDD not only activates proteasome-dependent downregulation of its own receptor, but also induces degradation of ER$_\alpha$ protein via proteasome-dependent pathways. This unique AhR-activated/proteasome-dependent degradation of two receptor proteins in breast cancer cells is highly selective since TCDD does not affect levels of several other regulatory proteins in these cells including cyclins A, E, D and H, cdk-2, cdk-4, cdk-7, p27, p21, cdc25A [34] and Sp1 protein (data not shown). These results demonstrate a novel mechanism of inhibitory AhR–ERα crosstalk in ERα-positive breast cancer cell lines. Moreover, it is possible that other cell-specific endocrine-disrupting activities of TCDD [9,10] that include downregulation of receptors for hormones and other mitogens may also be associated with selective proteasome-dependent protein degradation pathways, and these are currently being investigated.

References

[1] Evans, R.M. (1988) Science 240, 889–895.
[2] Tsai, M.J. and O'Malley, B.W. (1994) Annu. Rev. Biochem. 63, 451–486.
[3] Murphy, L.C., Dotzlaw, H., Leygue, E., Coutts, A. and Watson, P. (1998) J. Steroid Biochem. Mol. Biol. 65, 175–180.
[4] Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S. and Gustafsson, J.A. (1996) Proc. Natl. Acad. Sci. USA 93, 5925–5930.
[5] Mosselman, S., Polman, J. and Dijkema, R. (1996) FEBS Lett. 392, 49–53.
[6] Moore, J.T., McKee, D.D., Slentz-Kesler, K., Moore, L.B., Jones, S.A., Horne, E.L., Su, J.L., Kliewer, S.A., Lehmann, J.M. and Willson, T.M. (1998) Biochem. Biophys. Res. Commun. 247, 75–78.
[7] Hulka, B.S., Liu, E.T. and Lininger, R.A. (1994) Cancer 74, 1111–1124.
[8] MacGregor, J.I. and Jordan, V.C. (1998) Pharmacol. Rev. 50, 151–196.
[9] Safe, S. (1995) Pharmacol. Ther. 67, 247–281.
[10] Zacharewski, T. and Safe, S. (1998) in: Reproductive and Developmental Toxicology (Korach, K.S., Ed.), pp. 431–448, Marcel Dekker, New York.
[11] Safe, S., Qin, C. and McDougal, A. (1999) Expert Opin. Invest. Drugs 8, 1385–1396.
[12] Krishnan, V., Porter, W., Santostefano, M., Wang, X. and Safe, S. (1995) Mol. Cell. Biol. 15, 6710–6719.
[13] Gillesby, B., Santostefano, M., Porter, W., Wu, Z.F., Safe, S. and Zacharewski, T. (1997) Biochemistry 36, 6080–6089.
[14] Duan, R., Porter, W., Samudio, I., Vyhlidal, C., Kladde, M. and Safe, S. (1999) Mol. Endocrinol. 13, 1511–1521.
[15] Romkes, M. and Safe, S. (1988) Toxicol. Appl. Pharmacol. 92, 368–380.
[16] Romkes, M., Piskorska-Pliszczynska, J. and Safe, S. (1987) Toxicol. Appl. Pharmacol. 87, 306–314.
[17] Astroff, B. and Safe, S. (1988) Toxicol. Appl. Pharmacol. 95, 435–443.
[18] DeVito, M.J., Thomas, T., Martin, E., Umbreit, T.H. and Gallo, M.A. (1992) Toxicol. Appl. Pharmacol. 113, 284–292.
[19] Harris, M., Zacharewski, T. and Safe, S. (1990) Cancer Res. 50, 3579–3584.
[20] Wang, X., Porter, W., Krishnan, V., Narasimhan, T.R. and Safe, S. (1993) Mol. Cell. Endocrinol. 96, 159–166.
[21] Nawaz, Z., Lonard, D.M., Dennis, A.P., Smith, C.L. and O'Malley, B.W. (1999) Proc. Natl. Acad. Sci. USA 96, 1858–1862.
[22] El Khissiin, A. and Leclercq, G. (1999) FEBS Lett. 448, 160–166.
[23] Alarid, E.T., Bakopoulos, N. and Solodin, N. (1999) Mol. Endocrinol. 13, 1522–1534.
[24] Davarinos, N.A. and Pollenz, R.S. (1999) J. Biol. Chem. 274, 28708–28715.
[25] Roberts, B.J. and Whitelaw, M.L. (1999) J. Biol. Chem. 274, 36351–36356.
[26] Harris, M., Zacharewski, T., Astroff, B. and Safe, S. (1989) Mol. Pharmacol. 35, 729–735.
[27] Pollenz, R.S. (1996) Mol. Pharmacol. 49, 391.
[28] Pollenz, R.S., Santostefano, M., Klett, E., Richardson, V.M., Necela, B. and Birnbaum, L.S. (1998) Toxicol. Sci. 42, 117–128.
[29] Giannone, J.V., Li, W., Probst, M. and Okey, A.B. (1998) Biochem. Pharmacol. 55, 489–497.
[30] Rechsteiner, M. (1987) Annu. Rev. Cell Biol. 3, 1–30.
[31] DeMartino, G.N. and Slaughter, C.A. (1999) J. Biol. Chem. 274, 22123–22126.
[32] Moore, M., Wang, X., Lu, Y.F., Wormke, M., Craig, A., Gerlach, J.H., Burghardt, R., Barhoumi, R. and Safe, S. (1994) J. Biol. Chem. 269, 11751–11759.
[33] Caruso, J.A. and Batist, G. (1999) Biochem. Pharmacol. 57, 1253–1263.
[34] Wang, W., Smith, R. and Safe, S. (1998) Arch. Biochem. Biophys. 356, 239–248.