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Differential Expression of Estrogen Receptor Alpha (ERα) Protein in MCF-7 Breast Cancer Cells Chronically Exposed to TCDD

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Abstract Estrogens play a key role in the development and evolution of breast cancer tumors. Estrogen receptor alpha (ERα) mediates many of the biological activities of estrogens, and its expression is associated with low invasiveness and good prognosis. Recent epidemiological reports suggest that long-term exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is implicated in the increased incidence of breast cancer in exposed women. TCDD interferes with the expression of some ERα-dependent genes and inhibits estradiol (E2)- dependent growth of breast cancer cells in vitro. However, E2-dependent xenographs of MCF-7 human breast cancer cells resumed growth after a 2-week exposure to TCDD. The mechanisms involved in the resumption of cell growth are not completely understood. In this study, we show that short term-exposure (16 days) to 1 nM TCDD results in the suppression of ERα protein expression, while chronic exposure for more than 1 year (LTDX cells) results in the partial re-expression of the receptor. Immunocytochemistry studies showed that re-expression of ERα in LTDX cells occurred in some of the cells. Analysis by Western immunoblots indicated that four out of five LTDX clones expressed ERα at levels comparable to those in unexposed MCF-7 cells. Removal of TCDD treatment for 16 days restored the expression of ERα in the ERα-negative clonal cells. These results suggest that MCF-7 cells chronically exposed to TCDD contain at least two cell subpopulations that may respond differently to the ERα-mediated effects of TCDD. J. Cell. Biochem. 103: 636–647, 2008.

Key words: estrogen receptor alpha; chronic exposure; TCDD; MCF-7; breast cancer

Breast cancer is the most common form of cancer in women. Each year, approximately 40,000 women die of breast cancer in the United States alone. Although some cancer cases are associated with one or more of the known risk factors, the majority of causes remains unknown. Some recent epidemiological reports suggest that long-term exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is implicated in the increased incidence of breast cancer in women exposed to the chemical [Revich et al., 2001; Warner et al., 2002]. After the 1976 accidental TCDD exposure in Seveso, Italy, a study was conducted to examine a possible association between TCDD exposure and breast cancer incidence. According to Warner et al. [2002], the study failed to find any such link, because it covered only the first decade after the accident and the time elapsed was insufficient for TCDD's effects to manifest themselves. However, a recent study of a cohort of 981 women that were residents at Seveso at time of the accident, found that a 10-fold increase in dioxin levels in serum was associated with a 2.1-fold increase in the risk for breast cancer. Despite the small number of cases, the increase was statistically significant. That study also showed that for the seven women for whom ER status was reported, six were positive and nine out of the 15 women diagnosed (60%) were pre-menopausal at the time of diagnosis [Warner et al., 2002]. Such a high percentage distribution is unusual,

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since breast cancer is associated with postmenopausal women.

TCDD is a ubiquitous, persistent organic contaminant that is stored in mammalian adipose tissue; its half-life in humans has been calculated to be between 7 and 9 years [Geyer et al., 2002]. Most of the reported effects of TCDD are exerted through the ligand-activated aryl hydrocarbon receptor (AhR). TCDD has been classified as an antiestrogen, because it inhibits the estrogen-dependent preconfluent and postconfluent in vitro growth of breast cancer cells [Fernandez and Safe, 1992; Oenga et al., 2004], as well as the transcription and expression of some estrogen receptor (ER)-dependent genes [Gierthy et al., 1987, 1991; Biegel and Safe, 1990; Nodland et al., 1997]. However, the growth-inhibitory effect of TCDD appears to be transient given that estradiol (E2)-dependent xenographs of MCF-7 human breast cancer cells resumed growth after 2 weeks of TCDD exposure [Gierthy et al., 1993]. The mechanisms involved in the resumption of cell growth after an initial arrest of E2-dependent growth are not completely understood.

Breast lesions are classified into benign, or pre-malignant, and malignant. Benign lesions, such as atypical ductal hyperplasia (ADH), atypical lobular hyperplasia (ALH), ductal carcinoma in situ (DCIS), and lobular carcinoma in situ (LCIS) appear to have the potential for malignancy. The cells of these lesions possess some malignant properties, such as relative loss of growth control, but they lack the ability to invade and metastasize, and in this sense, are pre-malignant. Most premalignant breast lesions express high levels of estrogen receptor alpha (ERα) [Allred et al., 2001]. It is generally accepted that ERα and ER beta (ERβ) mediate the biological activities of estrogens [Chan, 2002]. Activated receptors, together with some co-activators and co-repressors, regulate the expression of other genes that in turn regulate growth and differentiation in breast epithelium [Pike et al., 1993]. Because ERα-positive lesions are more likely to respond to anti-hormonal therapy than ERα-negative lesions, the expression of ERα has been associated with low invasiveness and good prognosis. In contrast, ERα-negative tumors have been related to higher invasiveness and poor prognosis [Crowe et al., 1986]. Therefore, expression of ERα receptor at the time of diagnosis has a major influence on the treatment and the course of the disease.

In this study, we explored the apparent inconsistency between the observed effects of TCDD on cell growth *in vitro* and the in vivo effects. We hypothesized that chronic exposure to TCDD induces alterations in the cells that are responsible for the observed loss of TCDD's antiestrogenic effects, and that one of these alterations is the modulation of ERα. Because ERα is involved in MCF-7 cell growth, and because TCDD interferes with some of the ERα-mediated processes, we investigated the in vitro effects of chronic exposure to TCDD, on ERα. We compared the levels of ERα protein expression in MCF-7 cells that have been exposed for more than 1 year to TCDD (LTDX cells) or TCDD plus E2 (LTDXE2 cells) to the levels of ERα in MCF-7 cells exposed to TCDD or TCDD plus E2 for a short-term period (16 days). We used MCF-7 cells as in vitro model system for an ERα-positive ($ER\alpha^+$) pre-malignant lesion because these cells also express the aryl hydrocarbon receptor (AhR) that has been shown to mediate most of the effects of TCDD, such as the induction of estrogen metabolizing enzymes [Spink et al., 1990]. Our results indicate that MCF-7 cultures undergoing chronic exposure to TCDD maintain reduced levels of ERα protein expression, resulting from the re-expression of ERα protein by a fraction of the cell population. This finding suggests the presence of at least two subpopulations of cells that differentially express ERα in the presence of TCDD.

The relevance of this study design to public health derives from the long half-life of TCDD in humans that results in a chronic cellular exposure condition, even when the duration of the actual environmental exposure was limited. The studies presented here provide an in vitro model of this condition for the examination of the paradoxical consequences of short- and long-term TCDD exposure.

MATERIALS AND METHODS

Cell Lines

The MCF-7 cells used here were derived from clone 33, isolated in 1991 [Gierthy et al., 1991] from the original strain of the human mammary adenocarcinoma cell line MCF-7, obtained from Dr. Alberto C. Baldi, Institute for Experimental Biology in Medicine, Buenos Aires, Argentina.

Stock cultures were maintained in 75 cm^2-plastic tissue culture flasks (Costar), using DC5 medium, prepared with Dulbecco's modified Eagle's medium with penicillin (100 units/ml) and streptomycin (100 μg/ml), 10 ng/ml insulin, L-glutamine (2 mM), and non-essential amino acids, supplemented with 5% defined iron-supplemented bovine calf serum (Cat. No. SH 30072.03; Hyclone™, Logan, UT). Calf serum was used because it contains a lower concentration of conjugated estrogens and growth factors than does fetal bovine serum [Spink et al., 2003]; thus, it more accurately models the conditions prevailing in the plasma of a postmenopausal woman. The complete DC5 medium was filter-sterilized using 500-mL 0.2-μm pore size plastic Nalgene (Rochester, NY). LTDX cells were obtained by culturing MCF-7 cells for about 1 year in the presence of 1 nM TCDD. LTDXE2 were obtained by culturing MCF-7 cells for 1 year in the presence of 1 nM TCDD plus 1 nM E2. Cells were passed weekly or at confluence. Single-cell derived clones were obtained through seeding of cells from low-density single-cell suspension in 96-well plates. LTDX and LTDXE2 cultures were maintained in the presence of 1 nM TCDD or 1 nM TCDD plus 1 nM E2, respectively for 1 month. Cultures showing only one colony per well were selected, trypsinized, and transferred to 25 cm^2 plastic cultured flasks.

Fluorescence Microscopy

Cells were seeded into eight-well chamber slides at a concentration of 10^5 cells per 0.4 ml DC5 per cm^2. Twenty-four hours later, the medium was replaced with fresh DC5 or DC5 containing 1 nM TCDD, or 1 nM E2, or 1 nM TCDD plus 1 nM E2 in DMSO as solvent. After 16 days, the cultures were fixed with formalin for 10 min and delipidized with a solution of acetone and ethanol (1:1) for 15 min. After removal of the ethanol–acetone solution, a solution of 10% fetal bovine serum in phosphate buffered saline (PBS) was used for rinsing off solvent and for blocking. Cells were labeled overnight with anti-ERα rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a concentration of 1:250 in PBS at 4°C. On the next day, slides were washed three times for 3 min each time with PBS and were then stained with a secondary antibody, Alexa Fluor® 488 goat–anti-rabbit IgG (green) (Molecular Probes) at a concentration of 1:250 in PBS for 1 h. Slides were washed three times for 3 min each. Photographs of representative fields were taken using a Nikon 50i fluorescence/phase microscope.

Western Immunoblots

Cells were cultured in six-well plates and treated with the indicated concentrations of TCDD and E2 for 16 days. Cells were washed twice with PBS and protein was extracted with 300 μl/well sample buffer–SDS reducing buffer. The protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) by comparison with a standard curve obtained with bovine serum albumin. An equal amount of total protein was resolved using the NOVEX NuPAGE Bis-Tris electrophoresis system with the Bis-Tris–HCl buffered polyacrylamide gel as described by the manufacturer, and electroblotted to nitrocellulose. Blotted membranes were blocked using 5% non-fat dried milk overnight at 4°C and were then hybridized to anti-ERα primary rabbit-polyclonal antibodies (Santa Cruz Biotechnology, Inc.) in a 1/200 dilution for 1 h at room temperature. Membranes were washed three times in Tris-buffered saline Tween-20 (TBST) for 10 min per wash. Horseradish peroxidase-conjugated anti-rabbit (Pierce) was used as the secondary antibody in a 1/7,500 dilution and was incubated for 1 h at room temperature. Membranes were washed again three times in TBST for 10 min per wash. The antigens were detected using the SuperSignal Enhanced Chemiluminescence System (Pierce). Magic Mark™ Western Standard (Invitrogen, Carlsbad, CA) was used to estimate the molecular masses of the bands. A non-specific band of approximately 85-kDa mass was used as loading control. Band density was measured by Kodak Digital Science 1D™ software.

Ethoxyresorufin-*O*-Deethylase (EROD) Activity Assay

The EROD assay was performed directly in whole cell cultures of MCF-7, LTDX, and LTDXE2 cells based on the method described by Kennedy et al. (1993). Approximately 5,000 cells/well were seeded in a 96-well plate. Twenty-four hours after plating, EROD activity was induced by treating cell cultures with 1 nM TCDD, 1 nM TCDD plus 1 nM E2 or DMSO (0.1%) and allowed to incubate at 37°C for an additional 72-h period. Eight replicates were prepared for each treatment. The cultures were

rinsed twice with 250 μl PBS per well. Then, 100 μl/well of DC5 medium containing 4 μM ethoxyresorufin and 10 μM dicumarol were added, followed by incubation at 37°C. Fluorescence measurements were obtained every 15 min, up to 90 min, with a Fusion™ Universal Microplate Analyzer (Packard Instrument Company, Meriden, CT) using 535-nm excitation and 590-nm emission filters. Ethoxyresorufin activity was calculated using a standard curve for resorufin prepared in medium and ethanol. Resorufin production was linear for 60 min. For protein correction, three wells per treatment were washed twice with PBS before lysis with 0.2% sodium dodecyl sulfate. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce), with bovine serum albumin used as a standard.

Statistical Analysis

Microsoft Excel™ Software was used for all analyses. Comparisons were considered significant at $P < 0.05$.

RESULTS

Short-Term (16 days) Exposure of MCF-7 Cell Cultures to E2, TCDD, or TCDD Plus E2 Completely Inhibited ERα Protein Expression

In order to compare the effects of short-term (16 days) and long-term exposures (>1 year), we exposed MCF-7, LTDX, and LTDXE2 cells for 16 days to the media indicated in Figure 1. As shown in this figure, MCF-7 cells expressed ERα in the absence of treatment (lane 1). Short-term treatment with 1 nM E2 inhibited expression of the ERα (lane 4), a finding consistent with previous reports [Gierthy et al., 1996]. Similarly, short-term treatment with TCDD or TCDD plus E2 abolished the protein expression of ERα (lanes 7 and 10). These results indicate that the effects of TCDD and E2 on ERα protein expression in the short-term (16 days) are similar, since both suppress the expression of this receptor.

Chronic Exposure of MCF-7 Cell Cultures to TCDD (LTDX cells) Reduced ERα Protein Expression

As shown in Figure 1, chronic exposure of MCF-7 cell cultures to TCDD for 16 days expressed approximately 34% of the ERα protein level of unexposed MCF-7 cells (lanes 1 and 8). In contrast, chronic co-exposure of MCF-7 cell cultures to TCDD plus E2 (LTDXE2 cells) completely inhibited ERα protein expression (lanes 1 and 12). Together these results indicate that with time there is a partial restoration of the ERα protein expression, which had been suppressed following the short-term exposure to TCDD (lanes 7 and 8) and that this partial re-expression of ERα is inhibited by E2.

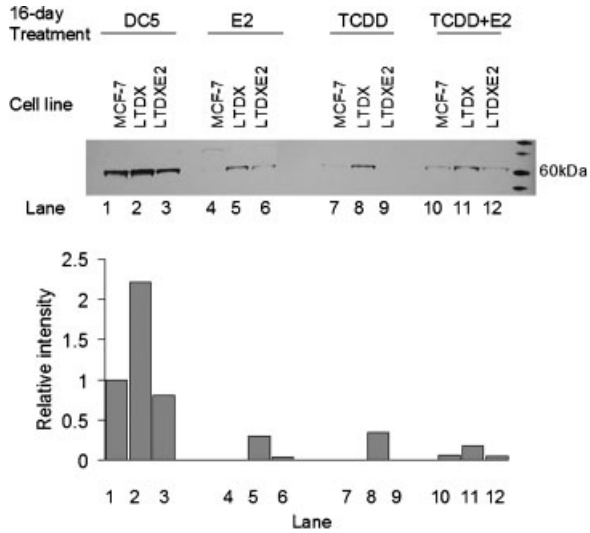

Fig. 1. Levels of ERα protein expression in MCF-7 cells chronically exposed to TCDD. MCF-7 cells cultured for more than one year in 1 nM TCDD (LTDX cells) and 1 nM TCDD plus 1 nM E2 (LTDXE2 cells) were treated for 16 days in DC5 (**lanes 1–3**), 1 nM E2 (**lanes 4–6**), 1 nM TCDD (**lanes 7–9**) and 1 nM TCDD plus 1 nM E2 (**lanes 10–12**). Cell lysates were analyzed by Western immunoblots as described in Materials and Methods.

Treatment Removal for 16 Days Enhanced ERα Protein Level in LTDX Cells But Not in LTDXE2 Cells

In order to determine whether the observed inhibitory effect of TCDD on ERα in LTDX cells is reversible, we removed TCDD or TCDD and E2, and cultured LTDX cells and LTDXE2 cells in DC5 for 16 days. ERα protein expression was restored after removal of the TCDD treatment. In fact, ERα protein expression in LTDX cells was more than twofold higher than that in the untreated control MCF-7 cells (Fig. 1, lanes 1 and 2). In contrast, treatment removal from LTDXE2 cells only restored about 80% of the ERα of protein level in control MCF-7 cells (lanes 1 and 3). These results suggest that the repressive effect of chronic TCDD treatment on ERα protein expression is reversible in the short term.

Chronic Exposure to TCDD Restored ERα Protein Immunolabeling in a Portion of LTDX Cells

Since chronic exposure to TCDD down-regulated ERα protein expression in LTDX cell cultures by about 70% (Fig. 1, lanes 8 and 1), we wondered whether TCDD was exerting its down-regulating effects on all cells or on a portion of the cell population. Fluorescence microscopic examination indicated that confluent MCF-7 cells express ERα and that treatment with 1 nM E2 for 16 days inhibits this expression (Fig. 2, panels B,D). Similarly, short-term exposure to TCDD suppressed ERα protein expression in MCF-7 cells as compared to unexposed controls (Fig. 2, panels B,F). Immunolabeled ERα protein was observed in some LTDX cells as compared to MCF-7 cells exposed to TCDD for 16 days (panels F,H). In contrast, relatively fewer cells expressed ERα in LTDXE2 cells (panel J). These results suggest that chronic exposure to TCDD restores the expression of ERα in a subpopulation of LTDX cells and the presence of E2 diminishes this partial restoration.

E2 or TCDD Alone or TCDD Plus E2 Reduced, But Did Not Completely Inhibit ERα Expression in LTDX Cells, Suggesting the Existence of a Resistant Subpopulation

Because LTDX cells showed a partially restored ERα expression compared to unexposed MCF-7 cells, we investigated whether LTDX cells were responsive to the ERα-down-regulating effects of E2. While the level of expression of ERα receptor in MCF-7 cells was abolished by short-term treatment with 1 nM E2 (Fig. 1, lanes 1 and 4), consistent with previous reports about the effects of E2 on ERα in these cells [Gierthy et al., 1996], the level of ERα expression in LTDX cells after short-term treatment with E2 was about 29% as compared to unexposed MCF-7 cells (lanes 1 and 5). This level of ERα protein expression was similar to

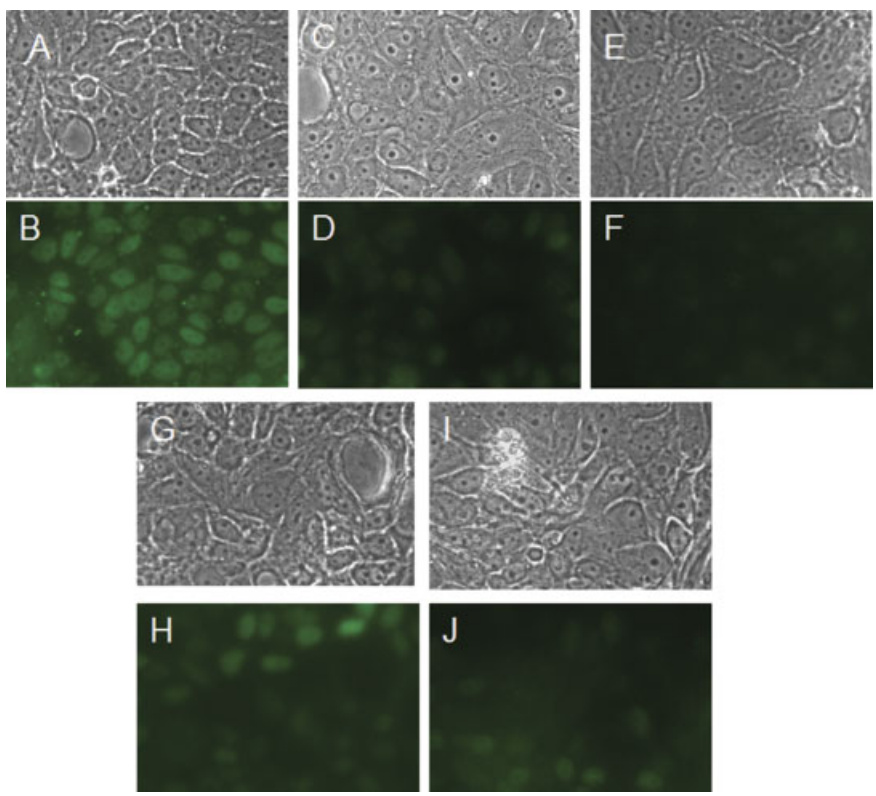

Fig. 2. Chronic exposure to TCDD partially restored ERα protein expression in LTDX cells. MCF-7 cells that have been chronically exposed to TCDD for more than 1 year (LTDX cells), partially restored ERα protein expression (**panels G**,**H**) as compared to untreated MCF-7 cells (**A**,**B**) or MC-7 cells exposed for 16 days to TCDD (**E**,**F**). E2 down-regulated expression of ERα in MCF-7 cells (**C**,**D**) and LTDXE2 cells (**I**,**J**). Fluorescence microscopic analysis was performed as described in Materials and Methods. Magnification 400×. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that maintained by LTDX cells (34%) in the presence of TCDD alone (lanes 1 and 8). These results suggest those MCF-7 cells chronically exposed to TCDD are not only partially resistant to the ERα down-regulating effects of TCDD, but that about the same proportion of cells is also partially resistant to the ERα down-regulating effects of E2.

E2, TCDD, or TCDD Plus E2 are Involved in the Inhibition of Expression of ERα in LTDXE2 Cells

Because 1 nM E2 represses ERα protein expression in MCF-7 cells (Fig. 1, lanes 1 and 4), one might expect a restored expression of ERα after E2 is removed from LTDXE2 cells. However, the expression of ERα was not restored after the removal of E2 alone (Fig. 1, lanes 9 and 12). Similarly, weak receptor expression level (about 5%) was retained when LTDXE2 cells were cultured for 16 days in the presence of E2 alone (lanes 12 vs. 6). ERα protein expression was restored to a level about 80% that of the control MCF-7 cells only when both TCDD and E2 were removed from the medium (Fig. 1, lanes 12 and 3). This result suggests that TCDD and E2 are both involved in the repressed expression of ERα, and that this expression is not restored after removal of either TCDD or E2 alone in the short term (16 days).

Subpopulations of LTDX Differentially Express ERα, in an All-Or-Nothing Manner

Based on the response to E2, it has been previously determined that the MCF-7 cell line is composed of heterogeneous cell subpopulations [Gierthy et al., 1991]. Since results in Figure 2 showed differential expression of ERα, we conducted additional exposure experiments in clones derived from LTDX and LTDXE2 cells, to determine whether the decrease in ERα protein was due to a reduced expression in all of the cells or, alternatively, due to a total suppression of the receptor in some of the cells. The clonal subpopulations were cultured for 16 days in the presence of 1 nM TCDD or 1 nM TCDD plus 1 nM E2. The results in Figure 3 show that four of the five LTDX clones analyzed expressed ERα at levels comparable to those in the control MCF-7 cells. The fact that one of the clones, X2B failed to express detectable levels of the immunoreactive ERα constitutes evidence of heterogeneity among LTDX cells. The lack of expression of ERα in clone X2B, suggests either

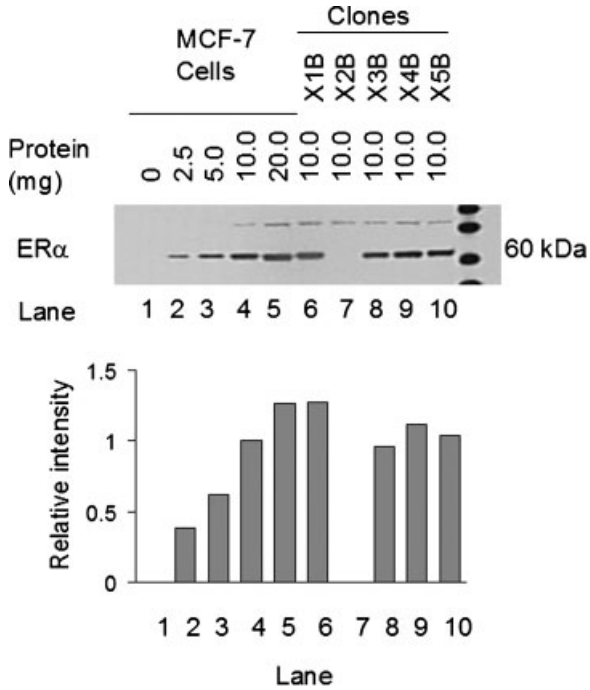

Fig. 3. Chronic exposure to 1 nM TCDD maintained the expression of ERα in four of the five clones derived from LTDX cultures. Dose–response levels of ERα protein in unexposed control MCF-7 cells are shown for comparison. Immunoreactivity of the ERα was analyzed by Western blots as described in Materials and Methods.

that chronic exposure to TCDD differentially down-regulates the expression of ERα, or else that the ERα regulation differentially "recovers" from the short-term inhibition. Complete inhibition of ERα by TCDD was observed in MCF-7 cells treated for a 16-day short-term period (Fig. 1, lanes 1 and 7) but not in cells chronically exposed to TCDD (lanes 1 and 8). The result of ERα suppression in clone X2B suggests that the LTDX cell line, like the parental MCF-7 cell line, is not homogeneous, and that the expression or suppression of ERα in LTDX cells is the result of the presence of at least two cell subpopulations that respond differently to the ERα down-regulating effects of TCDD.

We were unable to detect any differences in the expression of ERα among the five MCF-7-derived clones tested. All of the clones expressed ERα protein at levels similar to those in the parental MCF-7 cell population (results not shown). This finding is consistent with a previous a report showing that all of eight MCF-7-derived sub-lines expressed ER [Butler et al., 1986].

Short-Term (16 days) TCDD Withdrawal Or Co-Exposure to TCDD Plus E2 Enhanced ERα Protein Expression in LTDX Clonally Derived X2B Cells

In order to determine whether the lack of expression of ERα in the LTDX-derived clone X2B cells was reversible in the short-term, we cultured cells for two weeks in 1 nM TCDD in the presence or absence of 1 nM E2. Consistent with our previous results in Figure 3, the results in Figure 4 indicate that the ERα protein was expressed in the parental MCF-7 cell line, but not in the X2B clonal cell line that had been maintained in TCDD (Fig. 4, lanes 1 and 4). However, when the X2B clonal cells were cultured for 16 days in the absence of TCDD, the expression of the ERα was restored (lanes 1 and 2). Removal of treatment for 16 days increased ERα protein expression in X2B cells to a level that was 1.4 times the level of the control MCF-7 cells. Unlike the pattern in LTDX cells, X2B cells showed expression levels of ERα 83% higher than levels in control MCF-7 cells, after short-term culture in the presence of both TCDD and E2 (Fig. 4, lanes 1 and 5). These

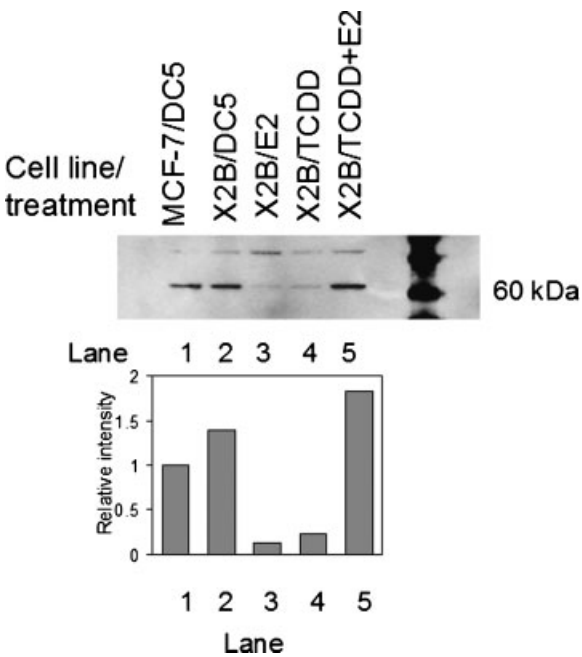

Fig. 4. ERα protein expression in X2B cells was down regulated in the presence of TCDD or E2 (lanes 2, 3 and 4), but not in the presence or absence of both of them (lane 5). LTDX-derived clonal X2B cells were cultured in the presence or absence of 1 nM TCDD, or 1 nM E2, or 1nM TCDD plus 1 nM E2 for 16 days. Immunoreactivity of the ERα was analyzed by Western blots as described in Materials and Methods.

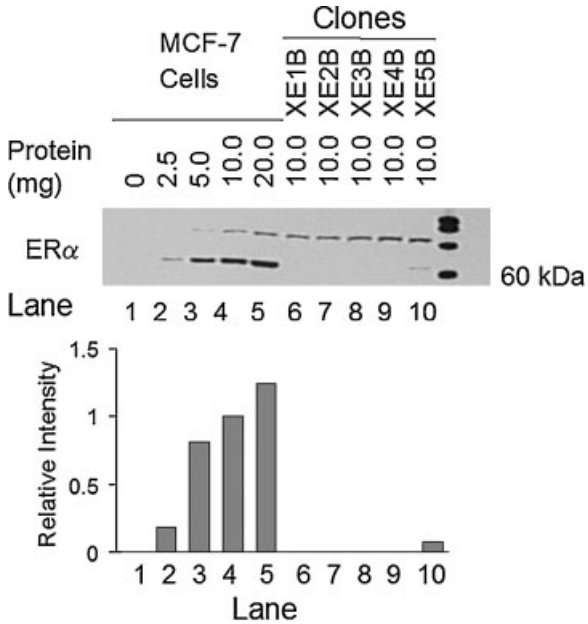

Fig. 5. Chronic co-exposure to 1 nM TCDD and E2 inhibited the expression of ERα in clones derived from LTDXE2 cells. Dose–response levels of ERα protein in unexposed control MCF-7 cells are shown for comparison. Immunoreactivity of the ERα was analyzed by Western blots as described in Materials and Methods.

results suggest that in the X2B subpopulation of LTDX cells, either TCDD or E2 is required to maintain the inhibition on ERα protein expression, and that in the presence of TCDD, X2B cells are insensitive to the ERα-down-regulating effects of E2.

Subpopulations of LTDXE2 Cells Exhibited Homogenously Inhibited Expression of ERα

Clones derived from LTDXE2 cells were tested for their expression of ERα. Results in Figure 5 indicate that all of the five clones analyzed, XE1B-XE5B, showed an almost complete inhibition of ERα protein expression. These results are consistent with our previous results (Fig. 1) indicating that LTDXE2 co-treatment with TCDD and E2 for 16 days resulted in a major inhibition of immunoreactive ERα protein expression.

MCF-7 Cells Chronically Exposed to TCDD or TCDD Plus E2 Exhibited Higher Levels of EROD Activity as Compared to Untreated MCF-7 Controls

The EROD assay provides a measure of the activity of CYP1A1/1A2 enzymes (Navas and Segner, 2000), which are involved in E2 metabolism. Incubation with E2 for 72 h had no

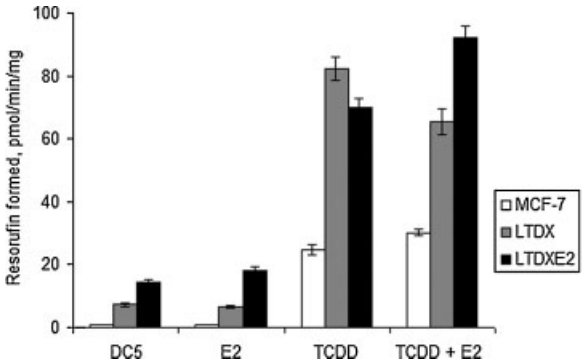

Fig. 6. LTDX and LTDXE2 cells exhibited higher EROD activity than MCF-7 cells. Cells were incubated for 72 h in DC5, 1 nM E2, 1 nM TCDD, or 1 nM TCDD plus 1 nM E2. The EROD assay was performed as described in Materials and Methods. Results are typical of three different determinations. Values represent the mean of eight replicates ± SEM.

effect on the constitutive low EROD activity in MCF-7 cells (Fig. 6). As expected, a 72-h treatment of MCF-7 cells with TCDD or TCDD plus E2 resulted in 44- and 59-fold higher EROD activities, respectively. Since LTDX cells have been cultured in the presence of 1 nM TCDD for more than 1 year, 72-h induction with TCDD was a continuation of the accustomed conditions for the cells. Under these conditions, EROD activity was 3.3 times higher in LTDX cells than in MCF-7 cells in which EROD activity has been induced for 72 h. Similarly, LTDXE2 cells had been chronically exposed to TCDD plus E2, showed a 3.7-fold higher EROD activity than did MCF-7 cells co-exposed to TCDD and E2 for 72 h. These results suggest that chronic exposure to TCDD is involved in maintaining the high levels of E2 metabolizing CYP1A1/1A2 enzymes in LTDX and LTDXE2 cells.

In summary, our current results indicate that chronic exposure of MCF-7 cells to TCDD (LTDX cells) results in reduced but sustained levels of ERα expression. The presence of both ERα-positive and ERα-negative cells in LTDX cultures suggests the existence of at least two subpopulations of cells that respond differently to the ERα-down-regulating effects of TCDD. Our results also indicate that chronic co-exposure of MCF-7 cells to TCDD and E2 maintains complete inhibition of ERα protein expression; the failure to reverse this effect by the short-term removal (16 days) of either TCDD or E2 suggests that both agents participate in this inhibition of ERα protein expression.

DISCUSSION

Estrogens play a key role in the development and evolution of breast lesions and expression of ERα is critical for proliferation of both normal and pre-malignant breast cells [Pike et al., 1993]. AhR agonists such as TCDD have been shown to modulate the expression of ERα in breast cancer cells. A 50% reduction in the levels of ERα mRNA expression in MCF-7 cells has been previously reported in cells exposed for 24 h to 10 nM of TCDD [Wormke et al., 2000]. Previous studies from this laboratory also found minor, although significant, reductions in the levels of nuclear ERα immunoreactive protein after 72 h of exposure to TCDD [Gierthy et al., 1996a]. Although these experiments provide insight into the immediate effects of TCDD, they have limited value in any efforts to extrapolate the effects of these persistent agonists of the AhR to the long-term exposures that arise from the long half-life of TCDD in humans.

In this study, we investigated the effects of chronic exposure to 1 nM TCDD on the expression of ERα; MCF-7 cells were used as an in vitro model of an E2-dependent breast cancer. Here we report a complete inhibition of the ERα receptor expression after short-term exposure (16 days) to 1 nM TCDD and the restoration of ERα protein expression to approximately 30% of the levels found in untreated MCF-7 cells after 1 year chronic exposure to 1 nM TCDD (LTDX cells). This suppressive effect of the chronic TCDD treatment was reversed after 16 days of treatment removal.

While the reduction in ERα protein expression in LTDX cells could be the result of the TCDD-induced degradation of ERα that has been previously reported [Wormke et al., 2003], the cause of the partial restoration of ERα protein expression in LTDX cells is still unknown. We propose that this partially restored expression of ERα is the result of the development under selective pressure, of a TCDD-resistant cell subpopulation with subsequent clonal expansion favored by the low-estrogen conditions in which LTDX cells have been maintained.

The hypothesis of selection and clonal expansion implies that at least two cell subpopulations were present in the MCF-7 cell culture. In this study, the first evidence of the presence of at least two cell subpopulations in LTDX cultures

was the heterogeneity in the immunolocalized ERα; further lines of evidence were the isolation of clones that differentially expressed ERα and the two-fold higher expression of ERα in LTDX cells than in control MCF-7 cells, after treatment removal. Together these results suggested that expression of ERα in parental MCF-7 cells maintained in DC5 was partially inhibited or repressed. Further support for this idea came from the fact that four of the clones analyzed were resistant to the ERα-down-regulating effects of TCDD (Fig. 3). The results presented here are consistent with previous findings from our laboratory suggesting the presence of various cell subpopulations in MCF-7 cells that respond differently to E2 [Gierthy et al., 1991]. Studies from other laboratories have characterized subpopulations of MCF-7 cells in terms of their ability to regenerate subpopulations differing in growth rate and expression of marker proteins such as ERα and *ras* p21 [Resnicoff et al., 1987; Podhajcer et al., 1988]. Therefore, it is likely that in the population of MCF-7 cells used in the study described here, there are at least two cell subpopulations that differentially respond to the inhibitory effects of TCDD and that this TCDD-resistant cell subpopulation is responsible for the 30% recovery of ERα protein expression that is observed in LTDX cells.

We propose that the clonal expansion of TCDD-resistant cells is favored by the low levels of estrogens in the medium. The medium in which the cells are cultured (DC5) contains only 5% bovine calf serum, which itself is inherently low in estrogens as compared to the fetal bovine serum that is used in routine MCF-7 cell culture. In addition, low estrogen conditions could also have resulted from increased estrogen metabolism. TCDD activates estrogen metabolizing enzymes in MCF-7 cells to a level that essentially depletes E2 from the medium [Spink et al., 1990]. In this study we used the EROD assay, which indirectly provides a measure of the activity of some of the main estrogen-metabolizing enzymes. Our results from this assay indicated that EROD activity was about 3.3 times higher in LTDX cells than in MCF-7 cells after 72 h induction with TCDD. Therefore, it is likely that in LTDX cells, the TCDD-chronically induced estrogen metabolism contributed to the depletion of the already low levels of estrogen in the calf serum-supplemented medium, creating conditions that allowed the re-expression of ERα in the TCDD-resistant subpopulation.

The cellular mosaicism of $ER\alpha^+$ and $ER\alpha^-$ observed in this study with MCF-7 cells could be representative of the conditions in the human breast. In the mammary ducts, about 60% of breast cells are $ER\alpha^+$ and this percentage varies during the menstrual cycle, being higher during the luteal phase than during the follicular phase [Stute et al., 2004]. Since breast cancer tumors are heterogeneous in their expression of ERα [Russo et al., 1999], and since proliferation has been observed primarily in $ER\alpha^-$ cells [Russo et al., 2006], the concept of clonal selection has already been proposed to explain the etiological distinction between $ER\alpha^+$ and $ER\alpha^-$ breast cancers [Kurbel, 2005].

In summary, chronic exposure to TCDD results in decreased expression of ERα. We propose that the later development of an $ER\alpha^+$ subpopulation is the result of clonal expansion of TCDD-resistant cells favored by low-estrogen conditions. This condition models the situation for post-menopausal women. In the next section, the situation for pre-menopausal women will be discussed.

Effects of Co-Exposure to TCDD and E2 on ERα

It has been shown that both E2 and TCDD individually can reduce the levels of ERα protein by multiple mechanisms. E2 down-regulates ERα gene expression [Borras et al., 1994] and modulates the concentration of the receptor [Nawaz et al., 1999; Wijayaratne and McDonnell, 2001; Laios et al., 2005]. TCDD also modulates the concentration of ERα by degradation through the proteosome pathway [Wormke et al., 2000]. It is possible that some, if not all, of these mechanisms that have been shown to occur in the short-term are involved in the selective pressure that, in the long-term, results in the sustained down-regulation of ERα protein expression. However, while E2-dependent down-regulation of the ERα is a cyclic process in pre-menopausal women, TCDD-dependent down regulation of the receptor is not. It has been estimated that TCDD's half-life in the human body is from 7 to 9 years [Geyer et al., 2002]. Therefore, in TCDD-exposed pre-menopausal women, a significant proportion of $ER\alpha^+$ cells could become $ER\alpha^-$ and therefore unresponsive to the ERα-mediated effects of E2 for as long as TCDD is present in the breast

microenvironment in a concentration sufficient to maintain such down-regulation. In this way, TCDD could continuously interfere with the cyclic breast remodeling that in pre-menopausal women occurs as a result of E2-induced cell growth, migration, differentiation, and apoptosis. As proliferation occurs primarily in $ER\alpha^-$ breast epithelial cells [Russo et al., 1999], $ER\alpha^-$ cells have the advantage that their growth does not depend on cyclic increases of E2, and an increase in the rate of proliferation of $ER\alpha^-$ cells could also increase the likelihood that these cells will acquire mutations. This concept supports the proposed hypothesis of the etiological distinction between $ER\alpha^+$ and $ER\alpha^-$ tumors.

The factors that render a cell or a subpopulation of MCF-7 cells susceptible to the ERα-down-regulating effects of TCDD or E2 are still unknown. Previous reports indicated that estrogens regulate AhR responsiveness [Spink et al., 2003]. In the present work, we show that chronic exposure to TCDD, regulates ERα protein expression and possibly its responsiveness to TCDD or other AhR agonists.

In addition to the sustained down-regulation of ERα, the maintenance of E2 metabolism, with consequent elevated levels of E2 metabolites could have profound biological implications for the initiation and progression of breast cancer. Specifically, TCDD-induced CYP1A1 and CYP1B1 increase E2 metabolism [Spink et al., 2003]. The carcinogenic metabolite 4-catechol estrogen (4-CE) that is oxidized to catechol estrogen-3,4-quinone (CE-3,4-Q), which in turn reacts with DNA to form depurinating adducts may lead to oncogenic mutations (Li and Li, 1987). Increased formation of 4-CE as a result of elevated hydroxylation of E2 at C-4 and C16α positions occurs in human breast cancer patients, and also in women at risk of developing this disease [Malins et al., 1993]. Moreover, the 4-CE is 4-fold more abundant in women with breast carcinoma than in the women without breast cancer [Rogan et al., 2003]. In this study, we show that prolonged exposure to TCDD, or to TCDD plus E2 results in increased EROD activity that is indicative of induced expression of CYP1A1/A2 enzymes involved in estrogen metabolism. In the present work, we determined that EROD activity in LTDXE2 cells was about 3.7-fold higher than the activity in MCF-7 cells after 72 h-induction with TCDD and E2. Therefore, since the concentration of E2 in the medium that we used is expected to be low, we do not believe that the down-regulation of ERα in LTDXE2 cells be the result of E2 exclusively; rather such down-regulation results from the interaction between TCDD and E2. Since E2 down-regulates ERα protein expression, it may do so in that part of the population that is resistant to the ERα down-regulating effects of TCDD.

It has recently been proposed that a subpopulation derived from the mammary epithelial cell population represents stem/progenitor mammary cells with self-renewal properties, that due to their longevity, also represent a major target for mutations leading to cancer-stem cells (Kalirai and Clarke, 2006). Although there is still a controversy about the ERα status of that proposed subpopulation, some reports suggest that $ER\alpha^+$ cells represent a stem-progenitor mammary epithelial cell population that is able to proliferate and to generate $ER\alpha^+$ and $ER\alpha^-$ cells that transiently amplify populations in the presence of estrogen [Dontu et al., 2004]. Moreover, MCF-S cells, a subpopulation derived from MCF-7 cells, have been shown to express higher levels of the putative stem cell markers and had higher tumor-initiating capability than did their parental MCF-7 cells [Ponti et al., 2005]. Therefore, it is likely that the $ER\alpha^+$ MCF-7 cells used in our experiment represent a self-renewal cell population that under the ERα-modulating conditions imposed by the chronic exposure to TCDD generates $ER\alpha^+$ and $ER\alpha^-$ cell subpopulations.

Finally, we show that chronic exposure to TCDD results in the sustained induction of enzymes involved in estrogen metabolism and the likely generation of genotoxic E2 metabolites. We propose that the chronic and artificial down-regulation of ERα by TCDD, together with the resulting sustained up-regulated estrogen metabolism can have profound effects for the diagnosis and therapeutic treatment of breast cancer arising in women exposed to TCDD or other AhR agonists.

Although more studies are needed to determine the public health significance of these findings and the molecular mechanisms underlying them, the in vitro results shown here provide evidence that chronic exposure to TCDD can cause alterations resulting in the progression of $ER\alpha^+$ pre-malignant lesions to $ER\alpha^-$ malignant tumors in vivo by interfering

with the estrogen-modulated physiology in breast tissue.

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