

Activation of telomerase in BeWo cells by estrogen and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in co-operation with c-Myc

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Abstract. Telomerase activation, known to be stimulated by estrogen, is essential for cellular immortalization and transformation, both of which play a role in tumorigenesis. Dioxin and dioxin-like compounds have been shown to induce endometriosis and promote estrogen-dependent tumors. In this study, we show that either 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or a combination of TCDD and 17- β estradiol (E2) increase telomerase activity and the expression of the human telomerase catalytic subunit (hTERT) in human choriocarcinoma (BeWo) cells. Compared with estrogen or TCDD alone, the combination treatment did not show an additive effect. Likewise, treatment with either E2 or TCDD increased DNA synthesis and the cell population in S-phase, as detected by FACS analysis. However, following treatment with the E2 and TCDD combination, the proportion of cells in S-phase was actually lower than in cells treated with TCDD alone. These results suggest that TCDD alone mimics estrogenic action in telomerase activation and cell proliferation but, in the presence of estrogen, TCDD-induced actions were partially counteracted. E2 and TCDD also induced c-Myc, which is a transcriptional activator of hTERT in BeWo, but neither of these agents induced telomerase activity in HO15.19 *c-myc*-null cells. In contrast, only TCDD upregulated telomerase in TGR-1 cells, which are c-Myc expressing but lacking ER expression. The findings suggest that TCDD induces telomerase activity mediated through AhR signaling and/or ER-independent c-Myc signaling. The present study provides insight into the mechanism of promoter activity of TCDD in estrogen-related tumors.

Introduction

Exposure to the halogenated aromatic hydrocarbon, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), elicits a number of biological and toxicological responses which include both endocrine disruptive effects and cancer (1-3). In particular, recent animal experiments have demonstrated a number of female reproductive toxicities resulting from TCDD exposure, including induction of ovarian tumors in rats, placental hypoxia in pregnant rats and inhibition of estradiol secretion from human luteinizing granulosa cells (4-6). Most TCDD-induced toxic responses are thought to be mediated via binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor and a member of the basic helix-loop-helix/PER-ARNT (aryl hydrocarbon nuclear translocator protein)-SIM family of proteins (7). In addition, the mechanism of action of TCDD is similar to that proposed for steroid hormones (8,9). Ligand-bound AhR heterodimerizes with ARNT and this complex then binds to specific gene regulatory sequences, known as xenobiotic or dioxin responsive elements (XRE or DRE). The induction of *CYP1A1* gene expression by TCDD is considered to be an early response signal and also the most sensitive biomarker for assessment of exposure and sensitivity levels to this compound (10,11).

TCDD also disrupts endocrine-responsive pathways, including those involving steroid hormone metabolism (8,12). It has also been previously demonstrated that *CYP1A1* induction is influenced by the estrogen receptor (ER) and that a functional ER is required for this activation (13-15). In more recent studies, it was demonstrated that the presence of 17- β estradiol (E2) in ovariectomized rats could significantly influence the induction of *CYP1A1* by TCDD (16). These studies indicated that the presence of ER in the nucleus was an absolute requirement for the TCDD-mediated response of *CYP1A1*. Recently it has been demonstrated that the estrogenic action of AhR agonists, such as TCDD, may be activated through the formation of a functional transcriptional complex by the interaction of AhR/ARNT and unliganded ER, which then targets estrogen response element (ERE)-like regions (17). Other studies have previously shown differential expression of AhR and ARNT mRNA throughout the estrous cycle in rodents (18), suggesting that this expression is involved in estrogen and/or ER regulation in this system. Each of these findings indicates that both E2 and ER have significant effects upon TCDD-liganded AhR signaling, which can lead to the

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induction of tumorigenesis. Collectively, these studies also implicate both AhR and ER signaling in the cross-talk between a number of responses induced by both of these receptors. However, the molecular mechanism(s) underlying the development of malignant cells via an estrogen response, following AhR signaling, is still unknown. One of the new emerging functions of ER is the upregulation of human telomerase activity (19). Telomerase is a cellular reverse transcriptase that catalyzes the synthesis and extension of telomeric DNA repeats (20,21). In most normal human somatic cells the level of telomerase activity is extremely low or undetectable. It has been shown in cell culture that telomeres progressively shorten after each cell division until critically short lengths signal cellular senescence (22,23). However, a variety of cell lines and malignant tumors have been found to express high levels of telomerase activity (24-26). This suggested that telomerase activation may be a critical step in cell immortalization and oncogenesis. One of the key components of human telomerase is its catalytic subunit, hTERT, which is actively upregulated by c-Myc (27). hTERT is also transcriptionally activated by ER, which binds to the ERE present in the upstream hTERT promoter region and induces increased expression of the telomerase enzyme (19). This activation of telomerase by E2 may partly explain the carcinogenic properties of estrogen. There are no reports in the literature, however, that describe how TCDD impacts upon telomerase gene expression in cells regulated by estrogen. Nevertheless, a previous report has shown that TCDD did not affect telomerase activity in either normal human epidermal cells, immortalized cells or malignant keratinocytes (28). The human choriocarcinoma BeWo cell line expresses a functional ER that is important for the control of BeWo cell proliferation (29), and highly produces E2 (30). These characteristics in BeWo are useful for the study of interactions between E2 and TCDD in female reproductive organs.

In the present study, we have examined the effects of E2, TCDD, and the two compounds in combination on telomerase activity in BeWo cells and attempted to elucidate the mechanisms underlying the interaction of estrogen and TCDD and the subsequent activation of hTERT mediated through c-Myc.

Materials and methods

Cell culture and treatment. The BeWo (human choriocarcinoma) cell line was obtained from the American type culture collection (Rockville, MD) and routinely maintained in DMEM/Ham's F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units penicillin/ml and 100 µg streptomycin/ml) under standard conditions in a 37°C incubator with 5% CO₂. TGR-1 and HO15.19 rat fibroblast cells, which are c-Myc expressing and *c-myc* null (referred to here as Myc+/+ and Myc-/-, respectively), were a generous gift from Dr M. Cole (University of Princeton, NJ). These cells were maintained in DMEM F12 medium supplemented with 10% FBS. All tissue culture reagents were purchased from Invitrogen (Carlsland, CA). The cells were maintained by refreshing the medium twice a week and by passaging once. For each of the induction assays, cells were grown in phenol red-free media containing 10% charcoal-stripped serum for 48 h prior to treatment.

Stretch polymerase chain reaction (PCR) assay. For quantitative analysis of telomerase activity, stretch PCR assays were performed using the Telochaser system according to the manufacturer's instructions (Toyobo, Tokyo, Japan). This method was developed originally by Tatematsu *et al* (31). The PCR products were electrophoretically separated using a GenePhor electrophoresis system (Amersham Biosciences, Tokyo) unit and visualized using Sybr-Green I (FMC Bio-products, Rockland, ME). To ensure the efficiency of PCR amplification, 10 ng of internal control phage DNA sequence (Toyobo) was added to each PCR reaction. The relative telomerase activity was determined by measuring the band intensities of the telomerase ladders and comparing them with internal standards using a Molecular Imager and its software, Quality One (BioRad, Hercules, CA).

Real-time PCR and RT-PCR. hTERT mRNA expression was analyzed by real time PCR according to the method of Bieche *et al* (32). Briefly, the primer sequences for hTERT amplification were 5'-TGACACCTCACCTACCCAC-3' and 5'-CACTGTCTTCCGCAAGTTCAC-3', and the probe sequence was 5'-ACCCTGGTCCGAGGTGTCCCTGAG-3'. Ribosomal phosphoprotein P0 (RPLP0) was used as the internal control and the PCR primer sequences were 5'-GGC GACCTGGAAGTCCAAC-3' and 5'-CCATCAGCACCAC AGCCTTC-3', whereas the probe sequence was 5'-ATCTGC TGCATCTGCTTGGAGCCCA-3'. cDNA was synthesized from 1 µg of total RNA in the presence of 1.5 µM random hexamers and 50 units of superscript II RNase H-reverse transcriptase (Life Technologies, Carlsland, CA). For each PCR run, the master mix contained 150-nM of probe, 300-nM of each primer and 10 µl of each diluted c-DNA in a final volume of 50 µl. Thermal cycling was performed using an ABI PRISM 7000 PCR machine (Applied Biosystems, Foster City, CA) with the following program: 95°C for 10 min, 50 cycles at 95°C for 15 sec, 65°C for 1 min. Reverse transcriptase (RT)-PCR analysis of CYP1A1, AhR and ARNT was performed as previously described (13). Briefly, total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol and cDNA was synthesized from 1 µg of RNA using a Revertrace RT-PCR kit (Toyobo) with OligoDT₂₀. The efficiency of cDNA synthesis from each sample was estimated by PCR with glyceraldehyde-3-phosphate dehydrogenase-specific primers as the internal control and also performed as described previously (13).

Cell-cycle analysis. BeWo cells were starved of serum for 24 h and then stimulated with charcoal-stripped serum containing either E2, TCDD or both at the indicated concentrations. After 24h, the cells were trypsinized and washed in chilled phosphate-buffered saline (PBS), fixed in 70% ethanol, digested with RNase (0.02 µg/µl) and stained with 50 µg/ml propidium iodide. The cells were sorted and analyzed using a Becton-Dickinson FACScan (BD Biosciences, SanJose, CA) and ModFit LT software (Topsham, ME).

Western blotting. BeWo cells were treated with either E2, TCDD, or both, for 24 h. Then, to prepare cell extracts, cells were washed three times with PBS and lysed for 30 min on ice

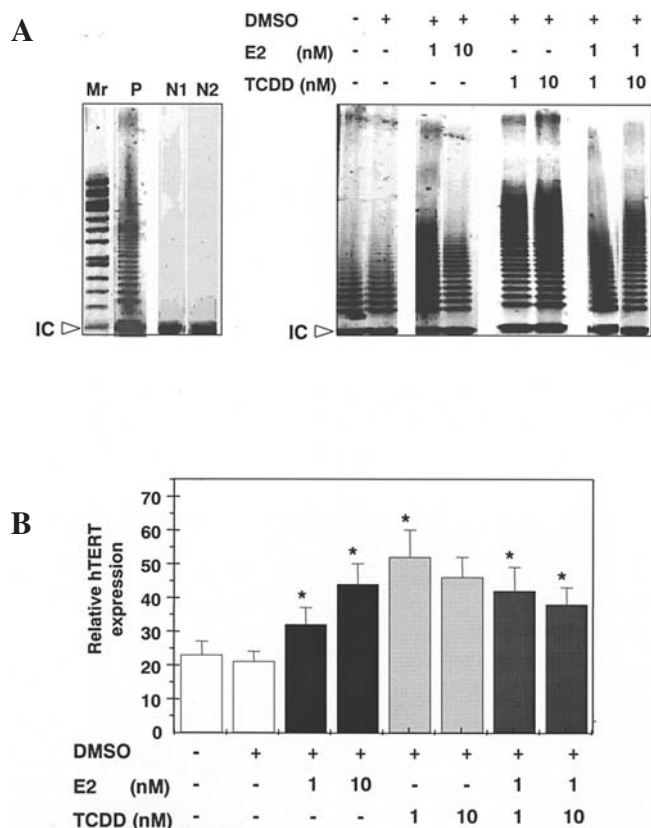


Figure 1. E2 and TCDD induce telomerase activity and hTERT expression in BeWo cells. Cells were treated for 24 h with E2 (1- and 10-nM), TCDD (1- and 10-nM) and a combination of 1-nM E2 with both 1- and 10-nM of TCDD. (A) Telomerase activity was measured by stretch PCR assay. The data shown are the results of one of three independent experiments performed in triplicate. Telomerase activity in HeLa cell extracts was used as a positive control (P), and lysis buffer only (N1) and cell extracts treated with RNase (N2) were used as negative controls. The activity of telomerase in BeWo cell extracts is shown without any treatment, with DMSO, 1- and 10-nM E2, 1- and 10-nM TCDD, 1-nM E2 with 1-nM TCDD, and 1-nM E2 with 10-nM TCDD. Mr, molecular weight markers; IC, internal control. (B) hTERT activity in BeWo cells following the indicated treatments for 24 h. hTERT induction was determined by real-time PCR. Values are the mean $\pm$ SD of three different cDNAs, each from one experiment. Triplicate experiments gave similar results.

in buffer containing 50 mM Tris-HCl (pH 7.5), 140 mM NaCl, 10% glycerol, 1% Nonidet P-40, 100 mM NaF, 200 mM Na_3VO_4 , 1 mM phenylmethanesulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin and 10 $\mu\text{g}/\text{ml}$ chymotrypsin. The lysates were centrifuged at 15000 rpm for 15 min at 4°C and the protein concentrations in supernatant were determined using a BioRad kit (BioRad). Approximately 50 μg of total cell lysate was denatured in SDS containing sample buffer (BioRad) by boiling at 100°C for 2 min. Proteins were separated on 10% SDS-PAGE and transferred to PVDF nylon membrane using a semi-dry transfer apparatus (BioRad). The membrane was blocked in 5% skimmed milk in Tris-HCl buffer, pH 7.5 for 1 h and then incubated with c-Myc antibody (C-19:sc-788; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200 for 1 h at room temperature in the same buffer. The c-Myc-specific band was detected by incubating the membrane for 1 h with anti-rabbit alkaline phosphatase-conjugated secondary antibody followed by detection with the NBT/BCIP substrate.

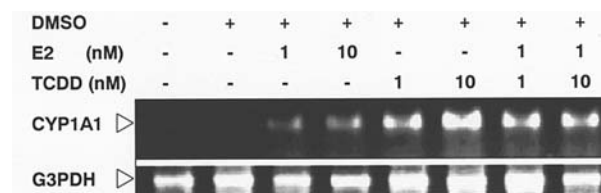


Figure 2. E2, TCDD or a combination of the two induces *CYP1A1* expression in BeWo cells. Cells were treated with E2, TCDD alone or a combination of the two for 24 h at different dose concentrations. DMSO was used as vehicle control. RT-PCR was performed using *CYP1A1*-specific primers and 10 μl of each reaction was resolved in a 1.5% agarose gel and visualized by ethidium bromide staining. G3PDH was used as the internal control.

Immunofluorescent staining of c-Myc. Cells were grown on sterile glass coverslips and then treated with E2, TCDD or both in the presence of 10% charcoal-stripped serum for 24 h. After this period, the cells were washed with chilled PBS and fixed with cold methanol for 20 min at -20°C. The cells were then washed again and further blocked with 5% normal goat serum for 1 h at room temperature, followed by another 1 h incubation with c-Myc antibody (sc-788, Santa Cruz Biotechnology) at a dilution of 1:200 in PBS with 5% goat serum. The cells were then washed three times with PBS and incubated with anti-rabbit rhodamine-conjugated secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:200 for 1 h. Finally, the cells were washed with PBS followed by incubation with 4, 6-diamino-2-phenylindole (DAPI) for 5 min and then thoroughly washed again with PBS. After this, the coverslips were placed on glass slides under glycerol and viewed under a UV-microscope (Leica, Cologne, Germany).

Results

Upregulation of telomerase activity and the mRNA expression of hTERT and *CYP1A1*. Telomerase activity in BeWo cells was determined by assaying telomere repeat lengths and telomerase catalytic subunit expression (Fig. 1). BeWo cells treated with both 1- and 10-nM of E2 for 24 h had significantly increased telomerase activity levels when compared to DMSO control-treated cells and 1-nM E2 treatment resulted in significantly higher telomerase activity than the 10-nM dosage. BeWo cells treated with TCDD at 1- and 10-nM concentrations showed a dose-dependent increase in telomerase activity. In contrast, when these cells were co-treated with 1-nM E2, they showed a marginal decrease in telomerase activity when compared to TCDD alone. Since treatment with 1-nM E2 induced the highest level of telomerase activity, this concentration was used in additional co-treatment studies with TCDD.

Among the various components that have been shown to be associated with the telomerase complex, the telomerase catalytic subunit, hTERT, has been demonstrated to be the rate-limiting determinant of telomerase activity (33). hTERT expression levels were, therefore, examined to establish whether telomerase activation by E2 and TCDD was indeed mediated through activation of the catalytic subunit. As shown in Fig. 1B, the levels of hTERT mRNA showed significant increases following E2 and TCDD treatment when compared to both the DMSO-untreated and -treated cells. Activation of

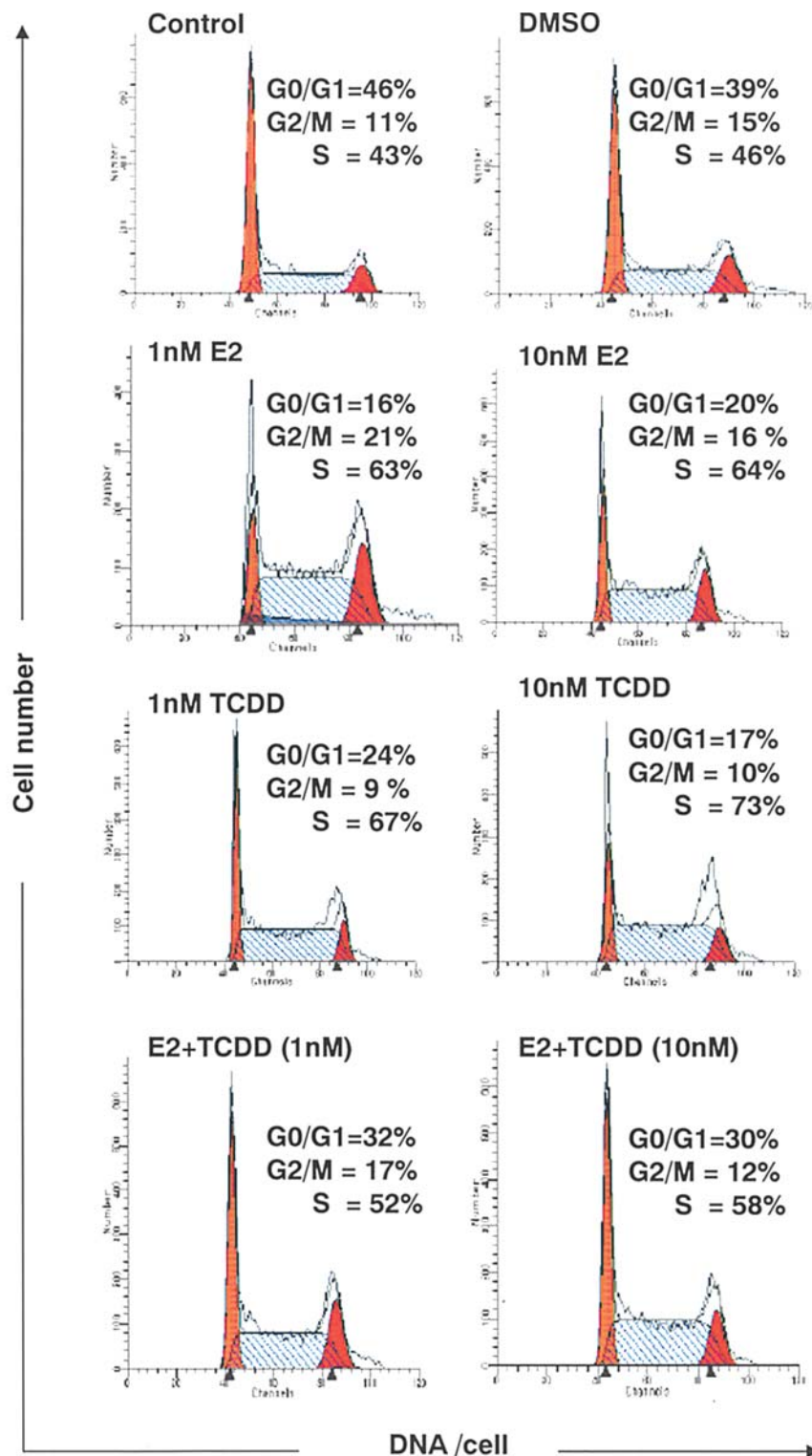


Figure 3. Representative flow cytometric data of effects of E2 and TCDD on BeWo cells. The first peak (left, red) indicates the percentage of cells in G1/G0-phase, the middle peak (blue, diagonal) indicates the percentage of cells in S-phase and the third peak (right, red) indicates the percentage of cells in G2-phase. BeWo cells were serum-starved for 24 h, treated with either E2, TCDD or both in the presence of 10% charcoal-stripped serum, and grown for 24 h prior to being harvested. The cells were then fixed with ethanol and stained with propidium iodide.

hTERT has been shown to be mediated by ER and, therefore, there was a significant increase in hTERT copy number, concomitant with increased telomerase activity, at both concentrations of E2 (1- and 10-nM). TCDD at 1- and 10-nM concentrations also significantly increased the hTERT copy number, which also effectively increased telomerase activity levels. In the presence of 1-nM E2, however, TCDD treatment

resulted in a marginal decrease in telomerase activity when compared to treatment with TCDD alone.

Induction of *CYP1A1* is a known marker for the effect of TCDD in most cells examined so far and, therefore, to confirm the action of TCDD in BeWo cells, *CYP1A1* gene expression levels were analyzed by RT-PCR (Fig. 2). TCDD treatment indeed induced *CYP1A1* in a dose-dependent manner. E2

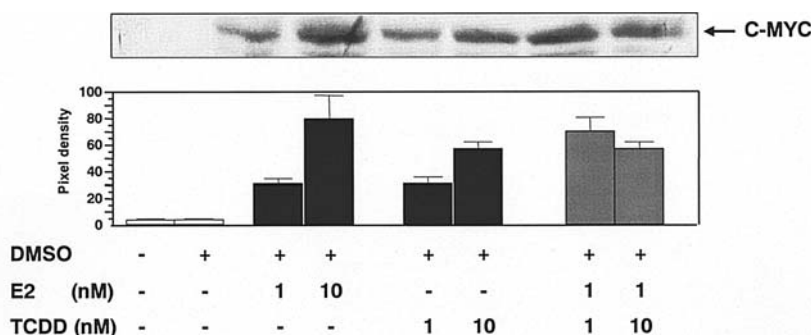


Figure 4. Induction of c-Myc by E2 and TCDD in BeWo cells. The cells were treated with agents for 24 h as described in Fig. 1 and were then harvested. Total cell lysate (50 μ g) was resolved by 12% SDS-PAGE and the proteins were transferred to PVDF membranes followed by immunoblotting analysis using c-Myc specific antibodies. This was followed by incubation with an alkaline-phosphatase conjugated secondary antibody and the specific bands were then visualized by BCIP/NBT staining. Picture data are from a representative experiment of three. Graphs are displayed as pixel density of the average of each band $\pm$ SD (n=3).

treatment alone resulted in only marginal induction of *CYP1A1*. The combination of both did not show any additive or synergic effects. These results confirm that E2 and TCDD induce *CYP1A1* in BeWo cells.

E2 and TCDD increase DNA synthesis in BeWo cells and the role of c-Myc. The cells were synchronized by serum starvation for 24 h and then treated with E2, TCDD or both in the presence of 10% charcoal-stripped serum for a further 24 h (Fig. 3). Among the cells treated with 1- and 10-nM E2 concentrations, the proportion of cells in S-phase were 63 and 64%, respectively; the corresponding percentages with the same concentrations of TCDD were 67 and 73%, respectively. The increase in the number of cells in S-phase, following E2 and TCDD treatment, suggests that the cells have a higher tendency towards proliferation, which is consistent with previous results showing the association of telomerase activity with cellular proliferation (34). However, following co-treatment with both E2 (1nM) and TCDD, the proportion of cells in S-phase was reduced to 52 and 58%, at 1- and 10-nM TCDD doses, respectively. Thus, TCDD-induced cell proliferation was decreased in the presence of E2, compared to TCDD alone.

c-Myc acts as a positive transcriptional activator of hTERT as the *hTERT* core promoter contains two c-Myc binding sites (35). E2 has been shown to activate c-Myc in MCF-7 cells, resulting in increased cellular proliferation and in the up-regulation of telomerase activity (19). To determine whether the activation of c-Myc is also involved in telomerase activation by TCDD, BeWo cells were treated for 24 h with E2 and TCDD and total cell lysates were subjected to Western blot analysis. Results from this analysis showed that there was significant induction of c-Myc by both E2 and TCDD, at concentrations of both 1- and 10-nM, in a dose-dependent manner (Fig. 4). Additionally, co-treatment of 1-nM E2 with either 1- or 10-nM TCDD induced significantly higher levels of c-Myc, compared to 1-nM E2 treatment alone but, in the presence of both E2 and TCDD, at dose concentrations of 10-nM, there was marginally less induction, suggesting that there was an antagonistic relationship between the two stimulants.

Expression of c-Myc has been closely associated with cellular proliferation and hTERT activity, which is consistent

with the induction of c-Myc by TCDD and the resulting increase in telomerase activity. To further confirm the increased levels and the localization of c-Myc expression in BeWo cells, immunostaining was performed in cells treated with either E2 and TCDD, or both (Fig. 5). In control cells, no c-Myc staining was observed, whereas clear nuclear staining of c-Myc was evident following E2 treatment at a concentration of 1-nM and also following TCDD treatment at both 1- and 10-nM concentrations. Nuclear c-Myc staining in cells treated with 1-nM TCDD was slightly defuse but was intense at 10-nM TCDD doses. The nuclear staining pattern at 1-nM E2 and 1-nM TCDD was more defuse and of lower intensity than at the co-treatment with 1-nM E2 and 10-nM TCDD. These results clearly indicate that c-Myc is induced by both E2 and TCDD and that the translocation of the c-Myc protein was not disrupted in the presence of these agents.

Telomerase activation of TCDD depends on c-Myc expression.

To confirm that the activation of telomerase by TCDD is c-Myc dependent, HO15.19 cells (Myc^{-/-}) and their parental cells, the TGR-1 cells (Myc^{+/+}) (36), were treated with TCDD for 24 h. Neither of these cell lines expressed ER, as determined by RT-PCR (data not shown), which was consistent with earlier observations (37). In the present study, it was necessary to determine the presence of AhR and ARNT in these cells since TCDD-induced cellular events are mostly mediated through the binding of AhR and the association with ARNT. Semi-quantitative RT-PCR showed that treatments with 1- or 10-nM TCDD did not increase mRNA expression levels of AhR and ARNT while the expression of AhR and ARNT was detected at steady state levels in both cell lines (Fig. 6). *CYP1A1* expression was not induced by TCDD treatment in these cells (data not shown). This lack of induction is consistent with the previous findings that the presence of functional ER is essential in TCDD-induced *CYP1A1* expression (14,18).

To investigate the telomerase activity, both rat cell lines were subsequently treated with E2 (1-nM) and TCDD (10-nM) for 24 h (Fig. 7). The results showed that telomerase activity was absent in HO15.19 cells, even following treatment with E2 and TCDD (Fig. 7, left panel). A significant increase in telomerase activity, however, was observed in TGR-1 cells

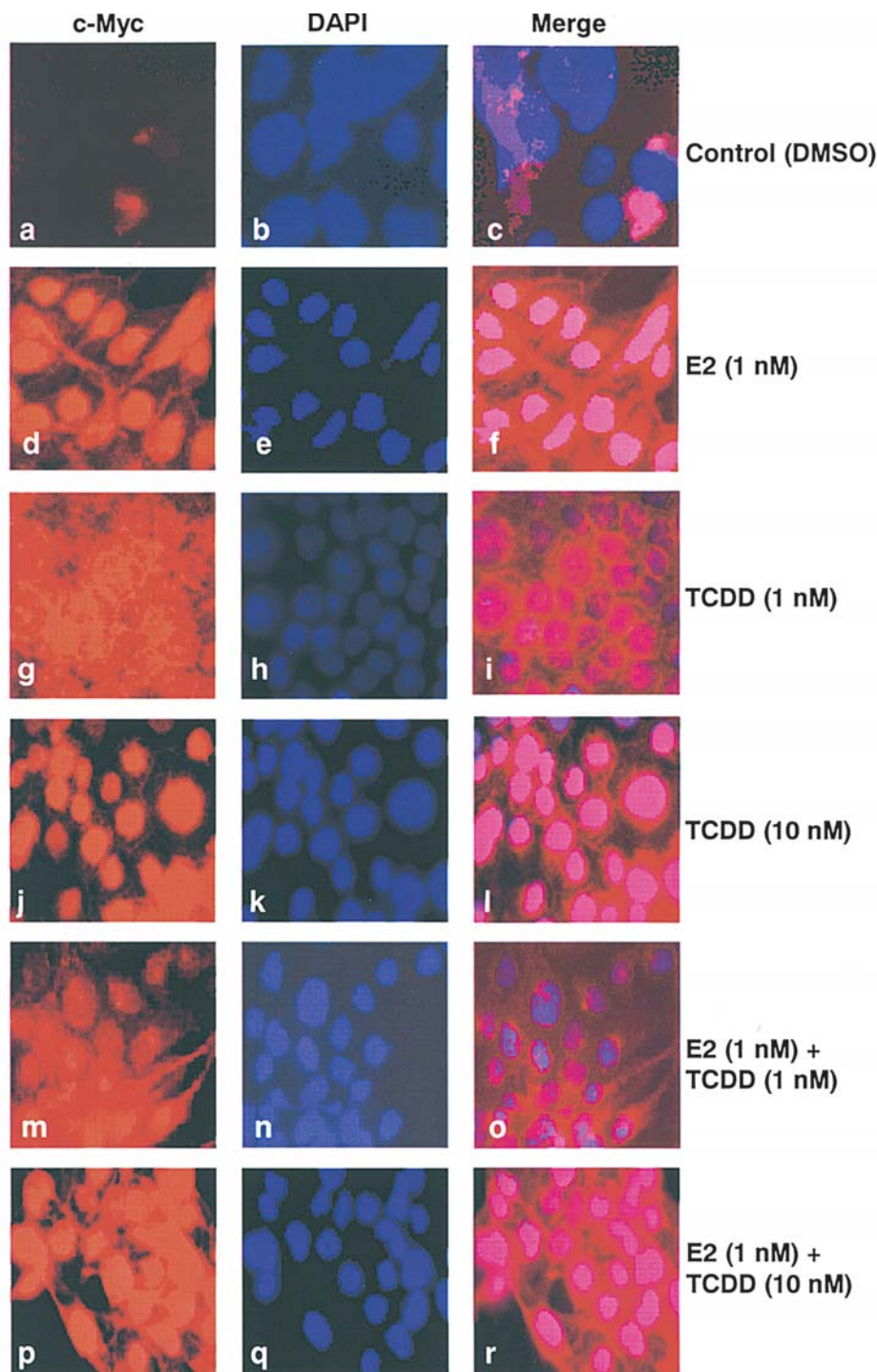


Fig. 5. Immunostaining of c-Myc in BeWo cells. Cells were grown on glass coverslips and then treated for 24 h with either E2 or TCDD alone or in combination. The cells were then washed with chilled PBS and fixed in methanol at -20°C for 12 h. This was followed by treatment with 10% normal goat serum and incubation with c-Myc-specific antibodies and, subsequently, a rhodamine-conjugated secondary antibody. The cells were washed with PBS and stained with 4, 6-diamidino-2-phenylindole (DAPI). c-Myc protein is visualized by red staining localized in the nucleus while the blue staining indicates nuclear DNA. Merged images confirm the localization of c-Myc in the nucleus.

following 10-nM TCDD treatment, but no activity was induced in these cells by E2 treatment (Fig. 7, right panel). The failure of E2 to induce telomerase in these cell lines can be accounted for by the absence of ER, which further strengthens the hypothesis that functional ER is a requirement for telomerase activation by E2. As expected, TCDD induced telomerase activity in TGR-1 cells but not HO15.19 cells, suggesting that the activation of telomerase by TCDD is directly involved in

c-Myc signaling and is different to the mechanisms of *CYP1A1* gene induction.

Discussion

The diverse effects of exposure to TCDD on various biological systems include endocrine disruption, steroid hormone metabolism, interference with cell proliferation and cancer. These

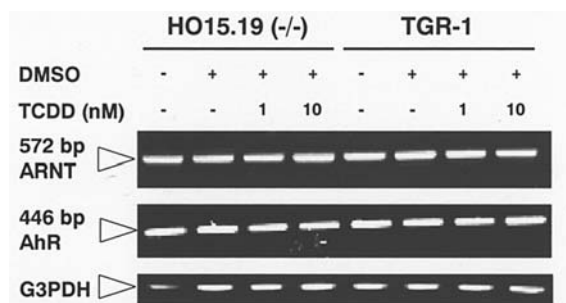


Figure 6. Expression levels of aryl hydrocarbon receptor (AhR) and aryl hydrocarbon nuclear transporter protein (ARNT). HO15.19 and TGR-1 cells were treated with either 1- or 10-nM of TCDD for 24 h prior to RNA isolation. RT-PCR was performed using primers as described in Materials and methods and PCR products were resolved in 1.5% agarose gels and stained with ethidium bromide. G3PDH was used as an internal control.

effects are mediated by the AhR-ARNT-TCDD complex, either by disruption of signaling cascades or through transcriptional activation of genes containing XRE elements located in upstream promoter regions. TCDD has been established as a potent tumor promoter and has been reviewed extensively (3). Telomerase is involved in cellular immortalization, a type of cell division leading to uncontrolled cell proliferation (25). In the present study we have attempted to address the effects of TCDD on telomerase activity, in order to further understand its role in tumorigenesis.

In BeWo cells, the increase in telomerase activity following 24 h of exposure to TCDD was dose dependent while, no such dose-dependent induction was seen under similar conditions with E2 (Fig. 1). Co-treatment of TCDD with E2 resulted in a marginal decrease in telomerase activity when compared to TCDD treatment alone, which may indicate that TCDD plays a role as a partial agonist that stimulated telomerase activity via ER under the condition of estrogen-unexposed cells and inhibited it in estrogen-treated cells. Such an antagonistic activity of TCDD towards E2 has been reported previously (37). Human telomerase is primarily controlled via its catalytic subunit, hTERT, which is a positive regulator of activity. It is possible, therefore, that TCDD increases hTERT expression levels in a dose-dependent manner, concomitant with elevated telomerase activity. E2 had been shown previously to increase hTERT activity through transcriptional activation of the hTERT promoter by the binding of ER to an ERE (19). However, co-treatment of TCDD and E2 did not result in any cumulative effects on hTERT activity that correlated with the activity of telomerase, as demonstrated in Fig. 1.

Since it has been observed that an increase in telomerase activity was associated with enhanced cell proliferation, the cell proliferation following E2 or TCDD treatment was monitored in the BeWo cells. The results showed that the percentage of the cell population in S-phase was increased by both reagents and that these increases correlated with the telomerase activity induced by the reagents. These findings strongly suggest that TCDD upregulates telomerase, with a concomitant increase in the proliferative index.

Among mitotic signals, c-Myc has been shown to be potentially involved in inducing cell division. In addition, it is well known that E2 can induce c-Myc through transcriptional activation (38) and also that c-Myc is an activator of telomerase

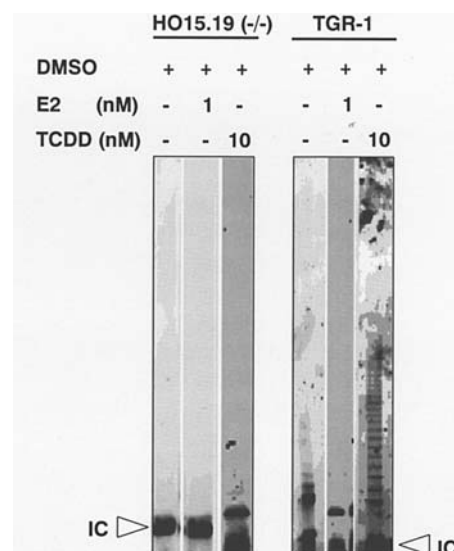


Figure 7. Activation of telomerase by E2 and TCDD in rat fibroblast HO15.19 and TGR-1 cells. Cells were treated with either E2, TCDD or a combination of the two for 24 h and telomerase activity was measured as described in Fig. 1.

(27,39). We therefore analyzed c-Myc induction by both E2 and TCDD treatment and, as shown by Western blotting, a significant increase in c-Myc protein levels was indeed induced by E2 and TCDD (Fig. 4). To confirm this induction of c-Myc protein levels, immunostaining analyses revealed intense nuclear c-Myc staining following either E2 or TCDD treatment (Fig. 5). E2 induction of c-Myc occurs via ER binding to the ERE present upstream of the promoter and this upregulation by both E2 and TCDD may well account for the significant induction of telomerase by both reagents and the increased proliferation of BeWo cells. However, since many other mitogenic signals may also be induced by both E2 and TCDD, which results in activation of telomerase, it was necessary to elucidate further the role of c-Myc in the induction of telomerase by TCDD.

HO15.19 cells (Myc^{-/-}) were used to confirm the involvement of c-Myc-induced telomerase activity via E2 and TCDD. The parental TGR-1 cells, but not the HO15.19 cells, showed induction of telomerase activity by 10-nM TCDD. In addition, HO15.19 cells also failed to show any increase in proliferation following TCDD treatment (data not shown). These findings suggest that c-Myc is involved in the activation of telomerase by TCDD. To our knowledge, this is the first report showing the induction of telomerase by TCDD and the involvement of c-Myc in its induction in BeWo cells. The activation of c-Myc by TCDD might occur directly mediated through transactivation by the AhR-TCDD complex or may occur indirectly via the upregulation of another transcription factor which in turn upregulates c-Myc. In previous studies, it has been suggested that the upstream region of the c-Myc promoter contains a consensus XRE site but functional activity has not been shown (40). The activation of c-Myc by TCDD might occur directly via transcriptional activity or indirectly through the activation of regulatory proteins that enhance transcription. We have analyzed the genome database and determined that the human c-Myc gene does indeed contain consensus XRE elements located at -59, -373, -488 and

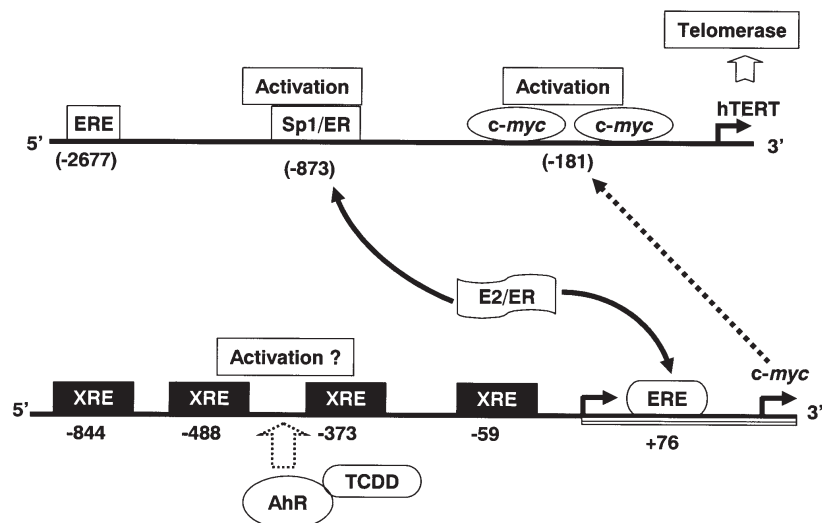


Figure 8. Schematic representation of a possible mechanism for TCDD-induced telomerase activity in BeWo cells. The sequence Accession number for the *c-Myc* gene is M16261 and the hTERT gene structure is adapted from Kyo *et al.* (19). The sequence positions are not to scale. Solid arrows indicate binding of E2/ER complexes to their respective cognate sequences in the hTERT and *c-Myc* genes. The dotted arrow indicates a possible mechanism for *c-Myc* binding to the -181 bp sequence present within the core promoter of the hTERT gene. Four putative XRE elements have been identified for TCDD/AhR/ARNT binding but have yet to be characterized. XRE, xenobiotic response element; ERE, estrogen response element.

-884 bp, but it has not yet been established whether these sites are activated by TCDD/AhR/ARNT complexes. While our present study provides strong evidence for the activation of *c-Myc* by TCDD and the subsequent induction of telomerase activity in BeWo cells, further experiments are needed to validate whether direct transcriptional activation of *c-Myc* occurs via TCDD.

A possible mechanism for the activation of telomerase by TCDD is represented in the form of a schematic diagram in Fig. 8. As described earlier, E2/ER complexes bind to EREs (-2677 bp) present upstream of the hTERT promoter sequences and activate telomerase. Also present in the hTERT promoter are two *c-Myc* binding sites within the core promoter (-181 bp) by which *c-Myc* can directly activate the transcription of hTERT and, subsequently, the telomerase enzyme. In the *c-Myc* gene regulatory region, there is a half ERE site located at 76 to 80 bp in exon 1 and this may act as an enhancer for activation of the gene by the E2/ER complex. In addition, the *c-Myc* gene also contains four XRE sites at -59, -373, -488 and -884 bp upstream of the transcription start site and these regions are the most likely binding sites for the TCDD/AhR/ARNT transcriptional complex. The results from the present study indeed suggest that TCDD might activate *c-Myc* by binding to these XRE sites. *c-Myc*, in turn, can transactivate the hTERT gene by binding to the -181-bp *c-Myc* binding site located within the core promoter region. As previously discussed, hTERT is the principal positive regulator of telomerase activity. We could not demonstrate any additive effects of E2 and TCDD upon telomerase activity, indicating that this might be due to the ERE pair present in the *c-Myc* gene region. Binding of activated ER may, therefore, suppress gene activation by TCDD/AhR/ARNT complexes bound to the more distal ERE site. In TGR-1 cells, which do not express ER, TCDD/AhR/ARNT complexes could activate telomerase, possibly through the activation of *c-Myc*, further suggesting that the E2/ER complex may hinder the induction of *c-Myc* in HO15.19 cells.

In conclusion, to our knowledge, this is the first report showing the activation of telomerase by TCDD in BeWo cells. This increase in telomerase activity by TCDD involves the induction of *c-Myc*, which is the positive regulator of hTERT, and may account for the tumor promoting activity. The present study provides insight into the disruption of telomerase activity and hTERT expression of TCDD.

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