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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Increases mRNA Levels for Interleukin-1β, Urokinase Plasminogen Activator, and Tumor Necrosis Factor-α in Human Uterine Endometrial Adenocarcinoma RL95-2 Cells

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This study investigated the potential role of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in uterine growth utilizing a human endometrial adenocarcinoma cell line (RL95-2). Western immunoblot analysis showed a maximal induction of cytochrome P4501A1 (CYP1A1) at 1 nM TCDD, but no change in epidermal growth factor receptor (EGFR) protein level. Northern blot analysis showed that TCDD significantly increased the steady state mRNA level of CYP1A1 and CYP1B1 which was maximal at 1 nM. TCDD significantly increased mRNA levels for interleukin-1β (IL-1β) by 6h, and for urokinase plasminogen activator (uPA) and tumor necrosis factor-α (TNF-α) by 36h. Nuclear runoff analysis showed that transcription of CYP1A1 was significantly increased by TCDD with no effect on CYP1B1, uPA or IL-1β. These results indicate that TCDD can differentially alter the expression of growth factor and cytokine gene products in uterine cells which may contribute to the promotion of uterine disease. © 1997 Academic Press

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent congener among the halogenated aryl hydrocarbons (Ahs) and arises principally as a byproduct of chemical processing, including municipal, medical waste and metal reclamation incinerators (1). TCDD exhibits a wide range of toxicities (reviewed in ref. 2) mediated through its ability to bind to an intracellular protein designated the Ah receptor which can lead to alterations in the expression of a number of gene products (3-6). TCDD is known to produce adverse immunologic effects including functional compromise of B and T lymphocytes, impaired antibody production, and decreased cytolytic activity of polymorphonuclear cells (2).

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Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; DMSO, dimethyl sulfoxide; kDa, kilodalton; kb, kilobase; IL-1β, interleukin-1β; uPA, urokinase plasminogen activator; TNF-α, tumor necrosis factor-α; EGF, epidermal growth factor.

Rier *et al* (7) have shown that rhesus monkeys exposed to TCDD in their diet manifested a dose-related increase in the severity and incidence of endometriosis. These data have been corroborated by a more recent study using a rodent endometriosis model (8). In addition, several epidemological studies provide evidence in support of a possible association between TCDD exposure and the prevalence of endometriosis in women (9-10). The etiology of endometriosis has been linked to the dissemination of endometrial cells which are able to maintain their viability and implant themselves at extra-uterine locations (11-13). Although endometriosis is treated pharmacologically as an endocrine disorder, substantial evidence indicates the presence of functional changes in both the humoral and cell-mediated immune parameters in women with endometriosis (14-17). These observations have led to the hypothesis that impaired immune surveillance and destruction of misplaced endometrial cells may underlie the development of the disease process (18).

This study presents evidence that exposure of the RL95-2 uterine endometrial adenocarcinoma cell line (19) to TCDD induced alterations in growth factor and cytokine expression that would be consistent with the reported effects of TCDD to facilitate the growth of endometrial tissue in the extra-uterine environment (7,8).

MATERIALS AND METHODS

Materials. Plasmids containing human cDNA for CYP1A1 (phP1-450-31), EGFR (pE7), TNF-α (pAW739), uPA (pHUK-8) and β-actin (no. 65128) were obtained from ATCC (Rockville, MD). The cDNAs for IL-1β and CYP1B1 were generously provided by Dr. William Greenlee (University of Massachusetts, Worchester). TCDD was obtained from Midwest Research Institute (Kansas City, MO) through the NCI Chemical Carcinogen Reference Repository. Cell culture media were purchased from GIBCO BRL/Life Technologies (Gaithersburg, MD) and Sigma Chemical Co (St. Louis, MO) with the exception of fetal bovine serum (FBS) which was obtained from Hyclone Laboratories (Logan, UT).

Cell culture. The RL95-2 cell line was obtained from ATCC and maintained in DMEM:HAM'S F-12 (1:1) supplemented with 10%

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FBS in a humidified atmosphere containing 5% CO_2 at 37°C. All media contained penicillin and streptomycin at 100 μg/ml. Stock solutions of TCDD were prepared in DMSO and added to cultures in complete medium at concentrations of 0.1 - 100 nM with final DMSO concentrations at 0.1%.

Western immunoblot analysis. Cell membrane protein (50-100 μg) was separated on 7.5% SDS-PAGE gels and transferred electrophoretically onto nitrocellulose membranes according to the methods of Laemmli (20) and Towbin *et al.* (21), respectively. Immunostaining was performed as previously described in Wang *et al.* (22). Briefly, membranes were incubated with sheep anti-human EGF receptor (Upstate Biotechnology, Inc., Lake Placid, NY) or goat anti-rat CYP1A1 (Gentest, Woburn, MA), followed by horseradish-peroxidase conjugated anti-sheep IgG for EGFR blots and anti-goat IgG for the CYP1A1 blots. Bands were visualized by incubation with 3-amino-9-ethylcarbazole and quantitated by densitometry and NIH Image software.

RNA isolation & Northern blot analysis. Total cellular RNA was isolated from cell cultures by acid guanidinum thiocyanate phenol-chloroform extraction according to Xie and Rothblum (23). For Northern blotting, 40-50 μg of total cellular RNA was denatured, fractionated on a 1.5% agarose formaldehyde gel, and transferred to nylon membranes. cDNA probes were labeled with [α-^{32}P]dCTP using a random primer labeling kit (Strategene, La Jolla, CA). Hybridization to radiolabelled probes was carried out in ExpressHyb hybridization solution (CLONTECH Laboratories, Inc., Palo Alto, CA) at 68°C for 2 - 4 h. Transcripts were then visualized by autoradiography after 3 to 7 days at −80°C. Hybridization signals were quantitated by densitometry and NIH Image software, with each message standardized against β-actin as a loading and transfer control.

Nuclear runoff assay. Nuclei were prepared by detergent lysis as described by Ausubel *et al* (24). Nuclei were resuspended in 500 μl of glycerol storage buffer (50 mM Tris, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol, pH 8.3) per 10^8 nuclei. Nuclei were stored at −80°C until ready for use. Nuclear runoff assays were performed essentially according to Merscher *et al.* (25) and Srivastava *et al* (26) with minor changes. Incorporation of [α-^{32}P]rUTP into nascent mRNA transcripts from isolated nuclei ($\approx 4 \times 10^7$ per reaction) was performed at room temperature for 30 min. After labeling, the newly transcribed RNA was isolated as described above for Northern analysis (23). Equal counts per minute ($\approx 10^6$) from DMSO control and TCDD experiments were added to 2 ml of ExpressHyb buffer and hybridized with 1 μg of appropriate cDNAs bound to nylon membranes at 65°C for 16 h. Dot blots were visualized by autoradiography for 2 - 5 days at −80°C. Spot intensities were quantitated as described for Northern blots.

Data analysis. Autoradiograms were scanned using a Microtek scanner and spot intensities compared to β-actin as an internal control. Control and treated mean RNA unit intensities from at least (n=3) experiments were compared for significance ($p<0.05$) by Student's t-test. For Western immunoblots, band intensities between treated and controls were compared for significance.

RESULTS

Effects of TCDD on epidermal growth factor receptors (EGFR) and cytochrome P4501A1 (CYP1A1). Treatment of RL95-2 cultures with 0.1 - 50 nM TCDD showed a marked induction in the expression of the 55kDa CYP1A1/CYP1A2 immunoreactive protein bands as determined by Western immunoblot analysis (Fig. 1A). The immunoreactive protein was induced in a concentration-dependent fashion, being maximal at 1 nM TCDD. In contrast, TCDD had no effect on the level of 170 kDa immunoreactive EGFR membrane protein band following exposure for up to 96h (Fig. 1A), nor was any effect observed on the binding of ^{125}I-EGF to intact cells (data not shown). Northern blot mRNA analysis further demonstrated that both CYP1A1 (3.0 kb) and CYP1B1 (5.1 kb) messages were significantly induced by TCDD in a dose dependent manner (Fig. 1B) which was maximal at 1 nM.

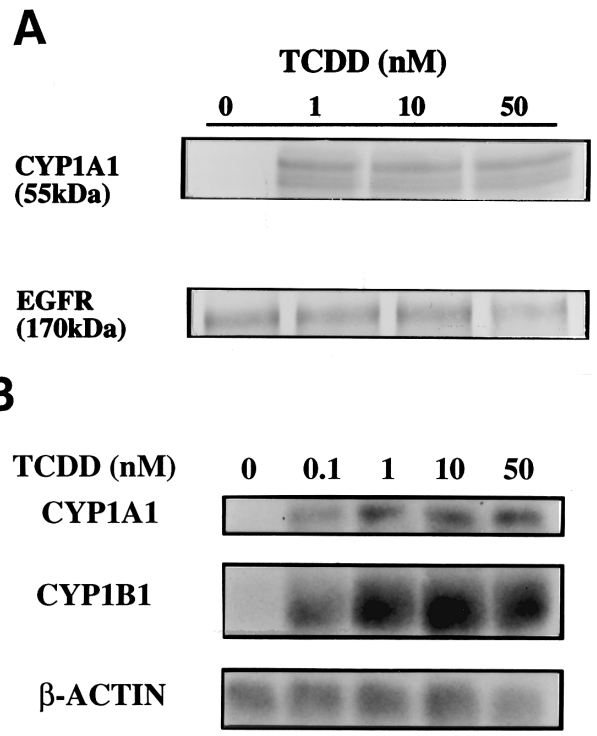

FIG. 1. Effect of TCDD on EGF receptor, CYP1A1 and CYP1B1 levels in RL95-2 cells. (A) Representative Western immunoblots after 48h TCDD exposure showing the dose-dependent induction of CYP1A1 protein. (B) Representative autoradiograms of Northern analyses of CYP1A1 and CYP1B1 induction by 0 – 50 nM TCDD exposure with β-actin as a loading and transfer control.

Effects of TCDD on interleukin-1β (IL-1β), urokinase plasminogen activator (uPA) and tumor necrosis factor-α (TNF-α) expression. TCDD exposure significantly increased the steady state level of mRNA expression for IL-1β (1.5 kb), uPA (2.8 kb), and TNF-α (1.8 kb) in both a time- and dose-dependent manner. IL-1β was significantly induced 3-fold by 6h and reached a maximal level of 7-fold that of controls by 36h (Fig. 2A, D). The mRNA levels of IL-1β remained elevated for the duration of the 48h assay period. The effect was dose-dependent with mRNA levels being significantly increased at 1nM TCDD. The level of mRNA for both uPA and TNF-α was significantly increased at 36h after 10 nM TCDD-treatment of RL95-2 cultures (Fig. 2B, C, D). Maximal induction of IL-1β, uPA and TNF-α messages occurred at 10 nM TCDD, in contrast to induction for CYP1A1 and CYP1B1 (Fig. 1B) which was maximal at

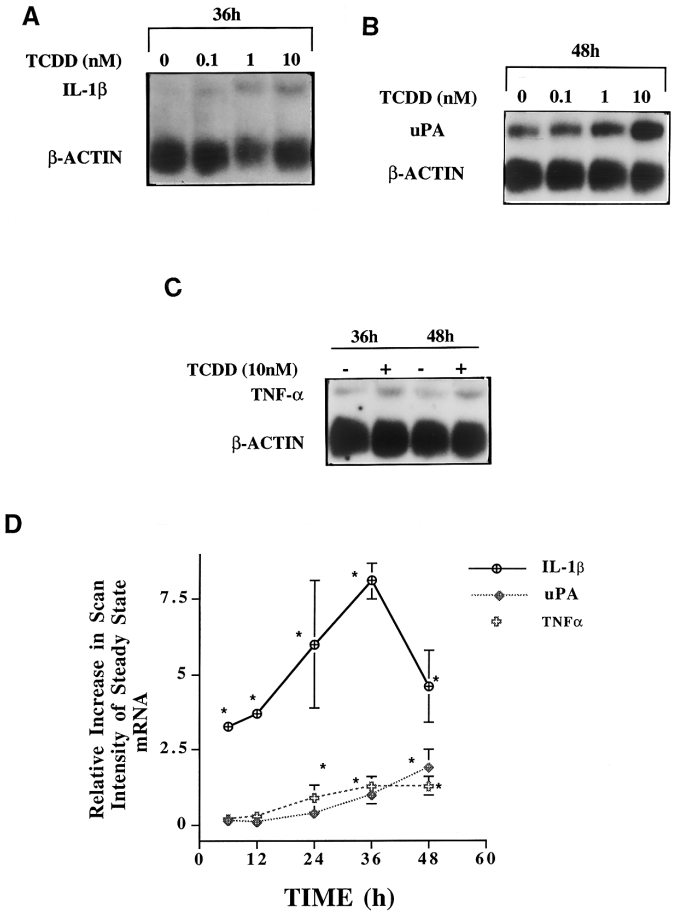

FIG. 2. Time and dose-dependent effects of TCDD on the steady state levels of mRNA for IL-1β, uPA and TNF-α in RL95-2 cells. Cells were treated with 0–10 nM TCDD as indicated. Total cellular RNA was isolated and 40–50 μg run per lane on 1.5% agarose formaldehyde gels for Northern blot analysis. (A) Representative autoradiogram of IL-1β mRNA showing the dose dependency of the response. (B) Representative autoradiogram of uPA mRNA. (C) Representative autoradiogram of TNF-α mRNA. (D) Graphical analysis of bands for IL-1β, uPA and TNF-α quantitated with NIH Image software after optical scanning. Values are the mean ± SEM for three separate experiments. Value is significantly different * p($<$0.05).

the 1nM TCDD exposure level. In addition, the CYP1A1 and IL-1β transcripts, being significantly increased at 6h, appear to be more sensitive biomarkers of early TCDD exposure in this cell line. It should be noted that TCDD did not alter all messages which were examined since Northern analysis failed to demonstrate changes in the steady state levels of mRNA for EGFR, TGFα or TGFβ1 (data not shown).

Effects of TCDD on the rate of CYP1A1, CYP1B1, uPA and IL-1β transcription in RL95-2 cells. Nuclear runoff assays were performed in order to determine whether the observed changes in uPA and IL-1β mRNA levels were the result of increased transcriptional activity. Nuclei were isolated from control and 10 nM TCDD-treated cultures of RL95-2 cells and analyzed for the level of nascent mRNA transcripts. Data in Fig. 3 show that CYP1A1 transcription is significantly increased 5-fold by TCDD, whereas no effect was observed on CYP1B1, IL-1β or uPA transcripts.

DISCUSSION

Rier *et al* (7) demonstrated that the incidence and severity of endometriotic lesions in female rhesus monkeys was associated with their dietary exposure to TCDD in a dose-related manner. Similarly, study of a surgically induced model of endometriosis in rodents showed that the administration of TCDD significantly promoted the growth of endometriotic sites (8). Recent epidemiological studies have provided further evidence linking endometriosis in women with dioxin exposure (9,10). The mechanisms by which environmental agents like TCDD might facilitate ectopic endometrial growth, however, have not been identified. In this regard, the physiological and molecular mechanisms whereby endometriotic tissue develops and persists outside the uterine cavity are not yet well understood. On the basis of observed alterations in cellular and humoral immune function in endometriosis patients, it has been hypothesized that endometriosis may be the

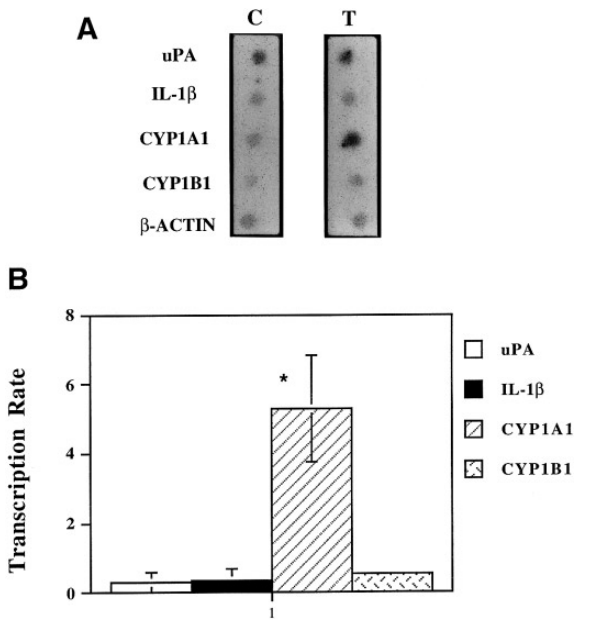

FIG. 3. Effect of TCDD on the rate of CYP1A1, CYP1B1, IL-1β and uPA transcription. RL95-2 cultures were treated with either 10 nM TCDD (T) or 0.1% DMSO (C) for 44h; nuclei were isolated and nuclear runoffs performed as described under methods. β-actin was used as a loading control and CYP1A1 as a positive control for transcriptional induction by TCDD. (A) Representative autoradiogram of a runoff analysis for transcriptional induction. (B) Results of the desitometric analysis of the band intensities after normalization to β-actin indicating relative changes in rates of transcription. Each point represents the mean of at least three experiments for 10 nM TCDD treatments. * ($p<0.05$) as compared to control.

result of a decreased immune surveillance, recognition, and destruction of misplaced endometrial tissue (18). The present study found that TCDD treatment of a human endometrial cell line produced an early and sustained increase in the cytokine IL-1β, followed by significant increases in TNF-α and the serine protease uPA. In this regard, our study is consistent with previous reports that TCDD alters the expression of CYP1A1 and CYP1B1, and growth regulatory genes involved in inflammation and differentiation, including plasminogen activator inhibitor-2 (PAI-2), IL-1β, uPA and TNF-α in human keratinocytes and in rat liver (3-5, 27, 28). Within the context of uterine biology, the observed action of TCDD to increase expression of IL-1β, TNF-α and uPA in endometrial RL95-2 cells would be consistent with a pro-inflammatory, invasive phenotype.

Endometriosis is a disease which appears to be promoted by estrogen (8). Recent data indicate that estrogen actions on the uterus may be mediated through growth factors like EGF, with subsequent increases in EGFR expression (29). The present study found no evidence that TCDD altered EGFR protein levels in RL95-2 cells. These data are corroborated by ^{125}I-EGF binding studies and Northern analysis of steady state levels of the 5.6 kb mRNA transcript for EGFR (data not shown). This is an interesting observation insofar as TCDD has been shown to down-regulate the levels of EGFR protein in reproductive tissue, as well as in other cell lines (6, 30).

Elevated numbers of activated macrophages have been detected in the peritoneal and tubal fluids of infertile women with endometriosis (31). Levels of macrophage-secreted TNF-α and IL-1β in the peritoneal fluid of women with endometriosis have also been shown to be increased above that from healthy women or those treated for the disease (16-17). Interleukin-1β and TNF-α are pro-inflammatory cytokines secreted by a variety of cell types. IL-1 has been demonstrated to stimulate collagen deposition and fibrinogen formation (32), which might account for the incidence of fibrosis and adhesions observed in advanced stages of endometriosis. TNF-α pre-treatment of mesothelial cells *in vitro* has also been shown to significantly increase the adherence of endometrial stroma, a possible step leading to endometriosis (33).

Our data further indicate that exposure of endometrial cultures to 10nM TCDD results in an increase in the level of mRNA for uPA which appears to be the result of post-transcriptional events. A similar observation has been previously reported in human keratinocytes (5). uPA activates plasminogen to plasmin, leading to additional proteolytic enzyme activation, and thus plays a major role in tissue destruction and extracellular matrix degradation (34). An increase in uPA activity could allow for an enhanced invasive profile for shed endometrium and facilitate the tissue remodeling required for extra-uterine implantation and growth (11). Previous research has demonstrated that high levels of urokinase and plasminogen are maintained in ectopic as opposed to eutopic endometrium, which has been postulated to correlate to a more invasive phenotype for the ectopic tissue (13).

In conclusion, endometrial cultures exposed to TCDD exhibited no alterations in their membrane EGFR expression. In contrast, mRNA expression for uPA, TNF-α and IL-1β were shown to be increased in a time- and dose-dependent manner. These results indicate that TCDD is able to potentially disrupt the growth factor/cytokine network via its ability to alter gene expression and, therefore, likely contribute to the development of uterine disease.

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