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Inhibition of Estrogen-Mediated Uterine Gene Expression Responses by Dioxin[S]

Darrell R. Boverhof, Lyle D. Burgoon, Kurt J. Williams, and Timothy R. Zacharewski

Departments of Biochemistry & Molecular Biology (D.R.B., L.D.B., T.R.Z.) and Pathobiology & Diagnostic Investigation (K.J.W.), Center for Integrative Toxicology (D.R.B., L.D.B., T.R.Z.), National Food Safety & Toxicology Center, Michigan State University, East Lansing, Michigan

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) exhibits antiestrogenic properties, including the inhibition of estrogen-induced uterine growth and proliferation. The inhibition of estrogen-mediated gene expression through ER/AhR cross-talk has been proposed as a plausible mechanism; however, only a limited number of inhibited responses have been investigated that are unlikely to fully account for the antiuterotrophic effects of TCDD. Therefore, the effects of TCDD on ethynyl estradiol (EE)-mediated uterine gene expression were investigated using cDNA microarrays with complementary physiological and histological phenotypic anchoring. Mice were gavaged with vehicle, 3 daily doses of 10 μg/kg EE, a single dose of 30 μg/kg TCDD, or a combination of EE plus TCDD and sacrificed after 4, 12, 24, and 72 h. TCDD cotreatment inhibited EE-induced uterine wet weight by 37, 23, and 45% at 12, 24, and 72 h, respectively. TCDD cotreatment also reduced EE-mediated stromal edema, hypertrophy, and hyperplasia and induced marked luminal epithelial cell apoptosis. A 2 $\times$ 2 factorial microarray design was used to identify EE- and TCDD-specific differential gene expression responses as well as their interactive effects. Only 133 of the 2753 EE-mediated differentially expressed genes were significantly modulated by TCDD cotreatment, indicating a gene-specific inhibitory response. The EE-mediated induction of many genes, including trefoil factor 1 and keratin 14, were inhibited by greater than 90% by TCDD. Functional annotation of inhibited responses was associated with cell proliferation, water and ion transport, and maintenance of cellular structure and integrity. These inhibited responses correlate with the observed histological alterations and may contribute to the antiuterotrophic effects of TCDD.

Estrogens regulate growth, development, and reproductive function in men and women and have been implicated in the etiology of breast and endometrial cancers (Hewitt et al., 2005). Many estrogen effects are mediated through the estrogen receptor (ER), a ligand-activated transcription factor and member of the nuclear receptor superfamily (Nilsson et al., 2001). In the traditional mechanism, ligand binding to the ER results in dissociation from heat shock and chaperone proteins, homodimerization, and interaction with regulatory elements near estrogen-responsive genes known as estrogen response elements (EREs) (Klinge, 2001). However, the activated ER can also mediate effects via interactions with Fos/Jun at AP-1 sites, via Sp1 at GC-rich promoter regions (Hall et al., 2001; Nilsson et al., 2001), and through ligand-independent, DNA binding-independent, and cell-surface (nongenomic) signaling mechanisms (Hall et al., 2001). These ER-mediated alterations in gene expression and signaling pathways are responsible for the subsequent molecular and physiological responses to estrogens.

Like the ER, the aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor but is a member of the basic helix-loop-helix-PER/ARNT/SIM (periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded) family of transcription factors. The AhR is responsible for mediating many, if not all, of the toxic and biochemical responses to TCDD and related compounds. These include a wasting syndrome, tumor promotion, teratogenesis, hepatotoxicity, immunotoxicity, and modulation of endocrine systems, which are mediated in a tissue-, sex-, age-, and species-specific manner (Poland and Knutson, 1982; Denison and Heath-Pagliuso, 1998). The proposed mechanism involves ligand

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ABBREVIATIONS: ER, estrogen receptor; ERE, estrogen response element; AP-1, activator protein-1; AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element; EE, 17α-ethynylestradiol; LE, luminal epithelium; LEC, luminal epithelial cell; PCR, polymerase chain reaction; TMVC, time-matched vehicle controls; QRTPCR, quantitative real-time PCR; LECH, luminal epithelial cell height; PCNA, proliferating cell nuclear antigen.

binding to the cytoplasmic AhR and translocation to the nucleus, where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT), another member of the basic helix-loop-helix-PER/ARNT/SIM family. This complex then binds specific DNA elements, termed dioxin response elements (DREs), in the regulatory regions of target genes, leading to changes in gene expression (Hankinson, 1995). Although the mechanisms of AhR/ARNT-mediated changes in gene expression are well established, how changes in gene expression results in the subsequent physiological and toxicological effects remains poorly understood.

As part of its repertoire of effects, TCDD exhibits antiestrogenic activity in the rodent female reproductive tract, including the inhibition of estrogen-induced increases in cellular growth and proliferation, uterine wet weight, DNA synthesis, and gene expression responses (Gallo et al., 1986; Umbreit et al., 1988; Astroff et al., 1991; Buchanan et al., 2002). Long-term administration decreased the incidence of both mammary and uterine tumors in female rats, suggesting that TCDD inhibits the development of estrogen-dependent tumors (Kociba et al., 1978). These effects are not mediated through TCDD binding to the ER (Klinge et al., 1999), but seem to involve AhR-ER cross-talk. Results from in vitro and in vivo studies have led to a number of proposed antiestrogenic mechanisms for TCDD, including increased estrogen metabolism, decreased estrogen receptor levels, induction of inhibitory factors, competition for cofactors, and the inhibition of estrogen-induced gene expression through interactions at estrogen responsive promoters (reviewed by Safe and Wormke, 2003).

Only a limited number of estrogen responsive genes that are inhibited by TCDD have been identified to support the ER-AhR gene expression cross-talk mechanism (Krishnan et al., 1995; Gillesby et al., 1997; Duan et al., 1999; Porter et al., 2001). However, a comprehensive assessment of inhibitory gene expression responses and their relationship to in vivo antiestrogenic endpoints has not been investigated. Therefore, to further examine the inhibitory effects of TCDD on estrogen-mediated uterine gene expression, temporal gene expression responses to EE, TCDD, and EE plus TCDD were investigated. Results indicate that the inhibitory effect of TCDD on EE-induced uterotrophy is associated with the selective inhibition of EE-mediated gene expression responses.

Materials and Methods

Animal Treatments. Female C57BL/6 mice, ovariectomized on postnatal day 20, were obtained from Charles River Laboratories (Raleigh, NC) on postnatal day 25. Animals were housed in polycarbonate cages containing cellulose fiber chip bedding (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) and maintained at 40–60% humidity and 23°C on a 12-h dark/light cycle (7 AM–7 PM). Animals were provided free access to deionized water and rodent food (22/5 Rodent Diet 8640; Harlan Teklad, Madison, WI) and acclimatized for 4 days before treatment.

Animals (n = 5/treatment group/time point) were orally gavaged at time 0 with sesame oil vehicle (Sigma Chemical, St. Louis, MO), TCDD (provided by S. Safe, Texas A&M University, College Station, TX), 17α-ethynylestradiol (EE; Sigma Chemical) or a combination of EE plus TCDD followed by additional doses of vehicle (vehicle and TCDD groups) or EE (EE and EE + TCDD groups) at 24 and 48 h as per the uterotrophic assay (Fig. 1). Doses of 10 and 30 μg/kg EE and TCDD, respectively, were empirically determined to elicit an optimal inhibitory effect on the EE-mediated induction of uterine weight in cotreatment studies (data not shown). Mice were sacrificed by cervical dislocation 4, 12, 24, or 72 h after dosing. Uterine weights were recorded before (wet) and after (blotted) blotting with absorbent tissue. A section of the left uterine horn was removed for histologic examination and fixed in 10% neutral buffered formalin (Sigma). The remaining tissue was subsequently snap-frozen in liquid nitrogen and stored at −80°C. All doses were calculated based on average weights of the animals before dosing. All procedures were performed with the approval of the Michigan State University All-University Committee on Animal Use and Care.

Histological Processing and Assessment. Fixed uteri were embedded in paraffin according to standard histological techniques. Five-micrometer cross-sections were mounted on glass slides and stained with hematoxylin and eosin. Embedding, mounting and staining were performed at the Histology/Immunohistochemistry Laboratory, Michigan State University (http://humanpathology.msu.edu/histology/index.html). Histological slides were evaluated according to standardized National Toxicology Program (NTP) pathology codes. Morphometric analyses were performed for each sample using image analysis software (Scion Image; Scioncorp, Frederick, MD) and standard morphometric techniques. The length of basal lamina underlying the luminal epithelium (LE) and corresponding area of the luminal epithelial cells (LECs) was quantified for multiple representative sectors of each section to calculate LEC height.

RNA Isolation. Total RNA was isolated from uteri using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Uteri were removed from −80°C storage and immediately homogenized in 1 ml TRIzol reagent using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was resuspended in RNA Storage Solution (Ambion, Austin, TX). RNA concentrations were calculated by spectrophotometric methods (A_{260}) and purity assessed by the A_{260}:A_{280} ratio and visual inspection of 1 μg on a denaturing gel.

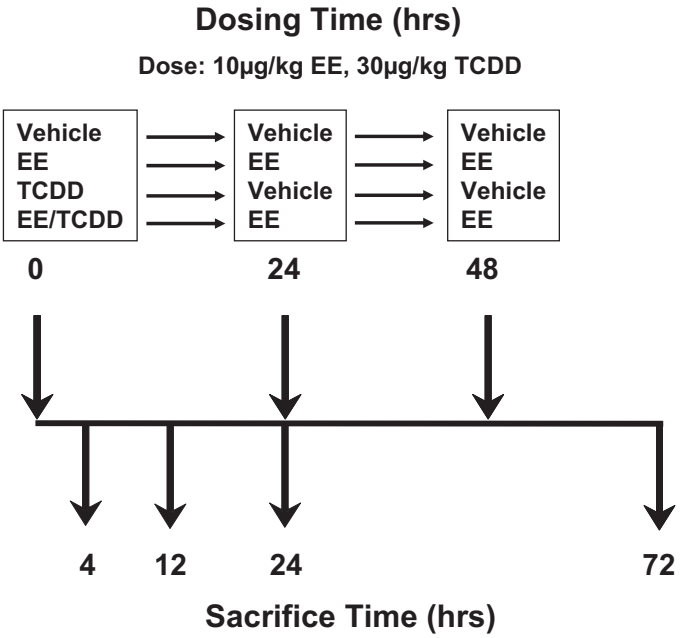

Fig. 1. Experimental design for EE + TCDD cotreatment time course study. An in vivo time course study was performed in which immature ovariectomized C57BL/6 mice were orally administered vehicle (sesame oil), 10 μg/kg ethynyl estradiol (EE), 30 μg/kg 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), or a mixture of EE and TCDD at time 0 followed by doses of vehicle (vehicle and TCDD groups) or EE (EE and EE + TCDD groups) at 24 and 48 h as per the uterotrophic assay. Mice were sacrificed 4, 12, 24, or 72 h after the initial dose, at which time uterine tissues were harvested.

Microarray Experimental Design and Protocols. Spotted mouse cDNA microarrays were prepared in-house and consist of 13,361 features, representing 7,948 unique genes (Unigene Build 144). Detailed protocols for microarray construction, labeling of the cDNA probe, sample hybridization, and slide washing can be found at http://dbzach.fst.msu.edu/interfaces/microarray.html. In brief, PCR-amplified DNA was robotically arrayed onto epoxy-coated glass slides (Schott-Nexterion, Duryea, PA) using an Omnigrid arrayer (GeneMachines, San Carlos, CA) equipped with 48 (12 × 4) Chipmaker 2 pins (Telechem) at the Genomics Technology Support Facility at Michigan State University (http://www.genomics.msu.edu). Changes in uterine gene expression were assessed using a 2 × 2 factorial design (Fig. 2) (Yang and Speed, 2002). In this design, arrow bases represent samples labeled with Cy3 and arrowheads represent samples labeled with Cy5. Within each replicate, a sample is labeled and hybridized on three independent arrays for a total of six arrays/replicate/time point. Three biological replicates were completed at each time point for a total of 72 microarrays. A 3DNA Array 900 Expression Array Detection Kit (Genisphere, Hatsfield, PA) using 1.0 μg of total RNA was used for probe labeling in all microarray experiments, according to manufacturer's specifications. Samples were hybridized for 18 to 24 h at 42°C in a water bath. Slides were then washed, dried by centrifugation, and scanned at 635 (Cy5) and 532 nm (Cy3) on an Affymetrix 428 Array Scanner (Santa Clara, CA). Images were analyzed for feature and background intensities using GenePix Pro 5.0 (Molecular Devices, Sunnyvale, CA).

Array Data Normalization and Statistical Analysis. Data were normalized using a semiparametric approach (Eckel et al., 2005). Model-based t values were calculated from normalized data, comparing treated and vehicle responses per time-point. Empirical Bayes analysis was used to calculate posterior probabilities of activity [P1(t)-value] on a per gene and time-point basis using the model-based t-value (Eckel et al., 2004). Gene lists were filtered for activity based on the P1(t)-value which indicates a greater likelihood of activity as the value approaches 1.0. A conservative P1(t) cutoff of 0.9999 combined with a differential expression of ± 1.5-fold relative to time-matched vehicle controls (TMVC) was used to filter the expression data and to define active gene lists. All arrays were subjected to quality control assessment to ensure assay performance and data consistency (Burgoon et al., 2005). Data are stored within dbZach (http://dbzach.fst.msu.edu), a MIAME supportive relational database that ensures proper data management and facilitates data analysis (Burgoon et al., 2006). Complete data sets with annotation and P1(t) values are available in Supplementary Table 1. Gene expression patterns that passed the established threshold criteria were visualized using hierarchical clustering (GeneSpring 6.0; Silicon Genetics, Redwood City, CA).

Quantitative Real-Time PCR Analysis. For each sample, 1.0 μg of total RNA was reverse-transcribed by SuperScript II using an anchored oligo-dT primer as described by the manufacturer (Invitrogen). The resultant cDNA (1.0 μl) was used as the template in a 30-μl PCR reaction containing 0.1 μM forward and reverse gene-specific primers, designed using Primer3 (Rozen and Skaletsky, 2000), 3 mM $MgCl_2$, 1.0 mM dNTPs, 0.025 IU of AmpliTaq Gold, and 1× SYBR Green PCR buffer (Molecular Devices). Gene names, accession numbers, forward and reverse primer sequences, and amplicon sizes are listed in Supplementary Table 2. PCR amplification was conducted in MicroAmp Optical 96-well reaction plates (Molecular Devices) on an PRISM 7000 Sequence Detection System (Molecular Devices) using the following conditions: initial denaturation and enzyme activation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation protocol was performed to assess the

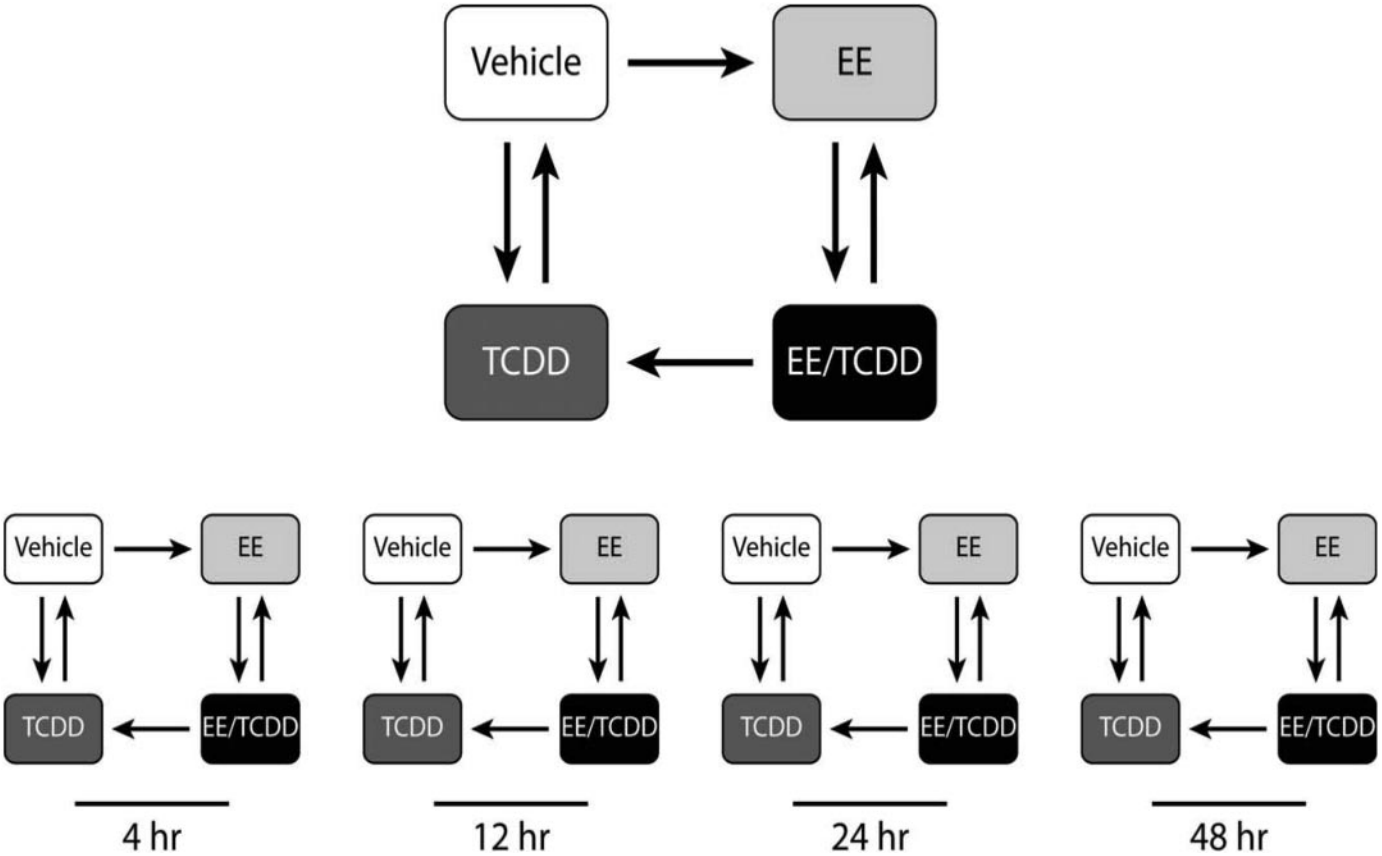

Fig. 2. 2 × 2 Factorial design used for the microarray experiments. A 2 × 2 factorial design was used to investigate the effects of EE and TCDD alone while also testing for interactive effects between EE and TCDD. Each arrow represents a microarray; arrow bases represent Cy3-labeled samples, and arrowheads represent Cy5-labeled samples. This design was applied at each of the four time points, with each biological replicate consisting of 6 arrays (6 arrows). Three biological replicates (18 arrays per time point) were completed for a total of 72 microarrays.

TABLE 1
Functional categorization of EE-regulated genes inhibited by TCDD cotreatment as determined by microarray analysis

Gene Name	Gene Abbrev	Entrez Gene ID	GenBank Accession	EE-Mediated Response Time Point[a]				Inhibition of EE Response by TCDD Time Point[b]			
				4 h	12 h	24 h	72 h	4 h	12 h	24 h	72 h
									%		
Growth and Proliferation											
Branched chain aminotransferase 1	Bcat1	12035	AA003372	**3.41**	**3.71**	**2.36**	**5.80**	0.84	48.77	65.33	**60.43**
Serine proteinase inhibitor B 5	Serpinb5	20724	BF021354	0.90	**4.02**	**3.56**	**2.31**		**86.92**	**89.73**	**93.07**
Sestrin 1	Sesn1	140742	AA154829	1.14	**1.80**	**1.55**	**4.74**		8.73	48.08	**56.18**
Stratifin	Sfn	55948	AA009229	1.06	**5.47**	**4.89**	**3.31**		**83.36**	**89.46**	75.12
Trefoil factor 1	Tff1	21784	NM_009362	0.91	**5.70**	**12.08**	**1.82**		**100.00**	**92.31**	19.62
Tumor necrosis factor (ligand) superfamily, member 8	Tnfsf8	21949	NM_134131	0.76	**3.11**	**3.29**	**2.68**		**79.65**	**85.95**	72.57
Vascular endothelial zinc finger	Vezf1	22344	NM_016686	0.81	**1.41**	**1.33**	**5.36**				**64.87**
Water/Ion Transport											
Aquaporin 3	Aqp3	11828	AI788487	0.76	**2.29**	**2.66**	**2.85**		**100.00**	65.28	60.15
FXYD domain-containing ion transport regulator 4	Fxyd4	108017	BG072055	1.02	**0.61**	**0.55**	**1.60**		**100.00**	**84.73**	
Solute carrier family 38, member 3	Slc38a3	76257	NM_023805	1.15	1.07	**0.60**	0.95			**83.55**	
Solute carrier family 40, member 1	Slc40a1	53945	BG074144	0.71	1.32	1.44	**6.19**				**62.32**
Solute carrier family 4, member 2	Slc4a2	20535	AA048952	0.92	**1.95**	1.43	**1.59**		**85.49**		88.74
Structural Function											
Desmocollin 2	Dsc2	13506	BG063370	1.08	**3.83**	**4.01**	**2.55**		**59.44**	**73.53**	70.63
Keratin complex 1, acidic, gene 14	Krt1–14	16664	NM_016958	0.86	**13.63**	**13.94**	**9.35**		**83.73**	**91.96**	**90.38**
Keratin complex 1, acidic, gene 19	Krt1–19	16669	BG064706	0.87	**2.06**	**1.53**	**3.28**		52.18	**100.00**	**66.04**
Keratin complex 2, basic, gene 4	Krt2–4	16682	W98341	0.71	**4.95**	**4.66**	**3.25**		**83.93**	52.56	0.00
Keratin complex 2, basic, gene 7	Krt2–7	110310	AA014127	1.04	**4.97**	**2.52**	**4.00**		46.12	**78.99**	**64.88**
Macrophage receptor with collagenous structure	Marco	17167	NM_010766	1.01	**3.05**	**4.93**	**3.11**		**68.35**	**80.67**	**89.22**
TP53 apoptosis effector	Perp	494479	NM_022032	0.41	**4.54**	**4.13**	**2.75**		**79.92**	73.48	54.14
Small proline-rich protein 2A	Sprr2a	20755	AI596101	1.11	**4.85**	**8.89**	**18.75**		22.02	59.23	71.55
Troponin T1, skeletal, slow	Tnnt1	21955	AA637201	1.06	**1.92**	**3.37**	1.12		**100.00**	**98.58**	0.00

[a] Values in bold indicate active genes based on statistical criteria of ±1.5-fold induction and P1(t) ≥ 0.9999.
[b] Only values statistically significant for EE are indicated. Values in bold indicate significantly inhibited responses based on statistical criteria of ±1.5 fold induction and P1(t) ≥ 0.9999.

specificity of the primers and the uniformity of the PCR-generated products. Each plate contained duplicate standards of purified PCR products of known template concentration covering 6 orders of magnitude to interpolate relative template concentrations of the samples from the standard curves of log copy number versus threshold cycle (C_t). No template controls were also included on each plate. Samples with a C_t value within 2 SD of the mean C_t values for the No template controls were considered below the limits of detection. The copy number of each unknown sample for each gene was standardized to Rpl7 to control for differences in RNA loading, quality, and cDNA synthesis (Couse et al., 1995). Statistical significance of differentially expressed genes was determined using two-way analysis of variance followed by a Tukey's post hoc test (SAS 9.1; SAS Institute, Cary, NC). For graphing purposes, the relative expression levels were scaled such that the expression level of the time-matched vehicle control was equal to 1.

Results

Uterine and Hepatic Weights. Increases in uterine weight as a result of water imbibition, hypertrophy, and hyperplasia are well characterized responses to estrogenic compounds and serve as the basis of the uterotrophic assay (Diel et al., 2002). EE induced an expected increase in uterine wet and blotted weights at 12, 24, and 72 h relative to the TMVC, whereas TCDD elicited no effect (Fig. 3, A and B). Cotreatment of mice with EE and TCDD significantly ($p < 0.05$) inhibited EE-mediated induction of uterine wet weight by 37, 23, and 45% at 12, 24, and 72 h, respectively (Fig. 3A). Blotted uterine weights were also inhibited at levels of 71, 38, and 30% at 12, 24, and 72 h, respectively (Fig. 3B). These results confirm previous reports of the antiestrogenic effects of TCDD on the inhibition of EE-mediated induction of uterine weight in the standard uterotrophic assay (Gallo et al., 1986; Umbreit et al., 1988; Astroff et al., 1991)

Histopathology and Morphometry. Treatment of mice with EE resulted in the expected complex uterine histologic examination results, consisting of minimal stromal edema at 4 h that progressed to moderate severity by 12 h. At 24 h, moderate epithelial cell hypertrophy and hyperplasia with moderate stromal edema were observed that progressed to marked epithelial and stromal hypertrophy and hyperplasia with mild stromal edema at 72 h (Fig. 4). Cotreatment of EE and TCDD resulted in comparable histological effects compared with EE treatment alone with the exception of reduced stromal edema at 12, 24, and 72 h, subnuclear vacuolization in epithelial cells at 24 h, and reduced stromal hypertrophy and hyperplasia with marked luminal epithelial cell (LEC) apoptosis at 72 h (Fig. 4). The LEC layer effects are consistent with previous reports of TCDD in the murine uterus (Gallo et al., 1986). These alterations in uterine histologic results may contribute to the associated decreases in uterine weight observed with TCDD cotreatment.

Treatment with TCDD alone exhibited negligible effects compared with vehicle controls. Inconsistent responses of minimal stromal edema at 12 h, minimal stromal hypertrophy at 24 h and minimal subnuclear vacuolization at 12 and 24 h were noted. No significant differences were noted between TCDD and vehicle samples at 4 and 72 h.

Increased luminal epithelial cell height (LECH) is a well recognized marker of estrogen exposure and has been used to assess the estrogenicity of a number of structurally diverse ligands (O'Connor et al., 1996; Nakagawa and Tayama, 2001). EE-induced LECH at 24 and 72 h, as reported previously (Kwekel et al., 2005). Cotreatment with TCDD did not inhibit this response. The inability to detect antiestrogenic effects on LECH may indicate that TCDD does not influence this response. Alternatively, it may be attributed technical difficulties in the measurement of such a change because of the complex pseudostratified nature of the proliferating LE cells combined with the histopathological alterations induced by TCDD on this cell layer.

Microarray Data Filtering and Clustering. Microarray analyses were preformed using a 2 × 2 factorial design that allowed for the identification of differentially expressed genes after EE and TCDD treatment alone as well as the interactive effects of EE + TCDD compared with the single treatment groups (Yang and Speed, 2002). A conservative statistical P1(t) cutoff of 0.9999 combined with a differential expression of ±1.5 fold relative to TMVCs was used to identify lists of differentially active genes. Gene expression responses to EE alone displayed the expected complex transcriptional profile as reported previously (Fertuck et al., 2003; Kwekel et al., 2005) with a total of 3,746 features, representing 2,753 unique genes, identified as differentially

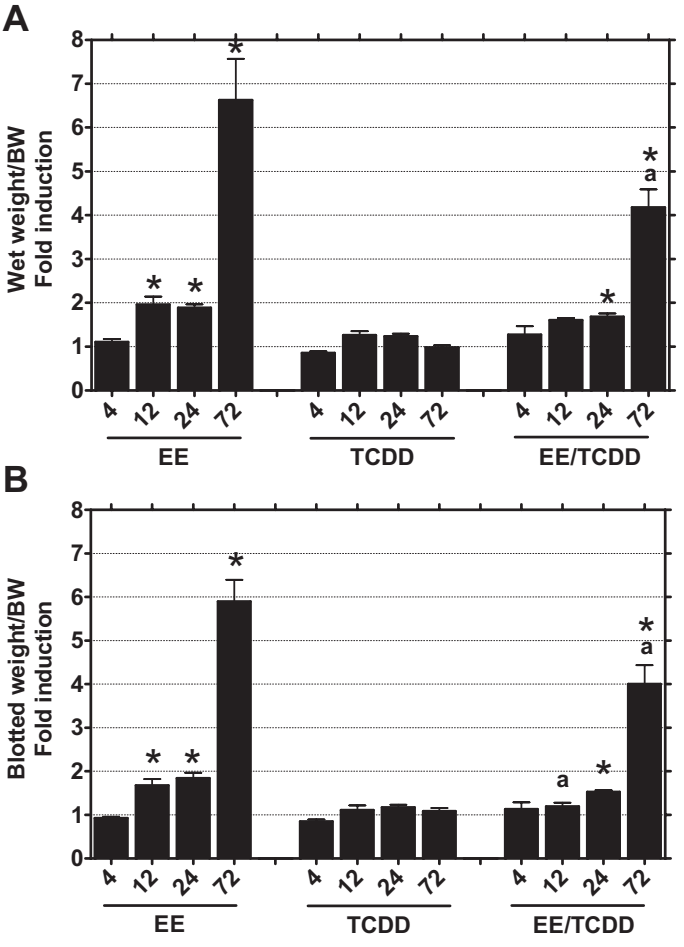

Fig. 3. EE, TCDD, and EE + TCDD effects on temporal uterine weights. EE induced the expected increases in uterine wet (A) and blotted (B) weights at 12, 24, and 72 h, whereas TCDD (T) had no effect. Cotreatment with TCDD inhibited EE-mediated increases in wet (72 h) and blotted (12 and 72 h) uterine weights. Data are expressed as -fold change in uterine weight (normalized to body weight) for each treatment relative to the TMVC group. *, $p < 0.05$ compared with TMVC. a, $p < 0.05$ compared with time matched EE-treated animals. BW, body weight

expressed at one or more time points. The number and magnitude of uterine gene expression responses elicited by TCDD were modest compared with EE. Seven hundred ninety-three features representing 628 unique genes were found to be differentially expressed in response to TCDD. EE + TCDD cotreatment resulted in an overall gene expression response similar to that of EE alone, with a total of 3,631 features representing 2,647 unique genes identified as differentially expressed at one or more time points.

To compare the global gene expression responses of EE, TCDD, and EE + TCDD, hierarchical clustering was performed on features that were differentially expressed in any of the treatment groups at any time point relative to the TMVCs. Visualization of the global responses for each treatment group revealed that the temporal expression pattern of EE + TCDD was essentially indistinguishable from EE alone (Fig. 5A). In addition, TCDD displayed similarities to EE, consistent with our previous studies describing the estrogen-like gene expression profile of TCDD (Boverhof et al., 2006). Clustering by treatment and time point further revealed the temporal similarity of the EE and EE + TCDD treatment groups as each of their gene expression time points clustered with one another (Fig. 5B). The 12-h gene expression responses to TCDD, EE, and EE + TCDD also clustered together, further demonstrating the estrogen-like response to TCDD at this time point.

Although these clustering approaches illustrate the similarity of gene expression patterns across treatments, they do not adequately demonstrate variations in the magnitudes of the responses between treatments, an important consideration when examining the inhibition of EE-mediated gene expression by TCDD. Scatter plots of the $\log_2$ expression ratios of EE versus EE + TCDD revealed that most responses were of the same magnitude (Fig. 6A). However, a small subset of genes was more highly expressed in the EE-treated group, suggesting inhibition upon cotreatment

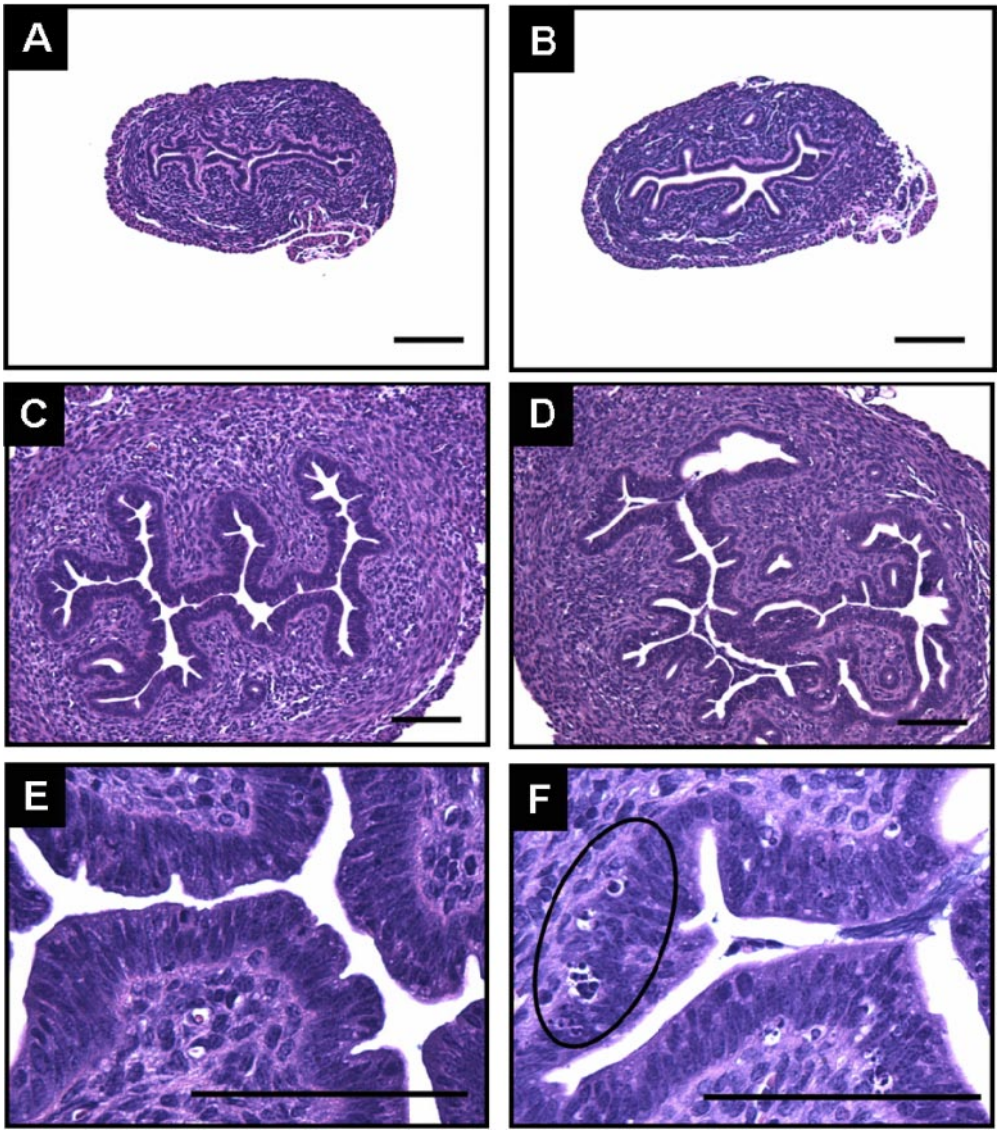

Fig. 4. Comparison of results of uterine histologic examination at 72 h after vehicle, TCDD, EE, or EE + TCDD treatment. Relative to the time matched vehicle control (A), TCDD did not induce any histologic alterations in uteri (B). EE induced marked epithelial and stromal hypertrophy and hyperplasia, with mild stromal edema at 72 h (C and E). Cotreatment of EE plus TCDD exhibited results of uterine histologic examination comparable with those produced by EE treatment alone at 72 h with the exceptions of reduced stromal edema, decreased stromal hypertrophy and hyperplasia, and marked LEC apoptosis (circled) (D and F). Bars, 10 μm

with TCDD. Similar comparisons between EE and TCDD groups revealed low correlations, indicating differences in the magnitudes of the response despite similar expression patterns (Fig. 6B).

Genes Differentially Regulated by EE and EE + TCDD Treatments. Genes identified as differentially expressed by EE + TCDD cotreatment compared with EE treatment alone were identified and investigated further. To be considered in this category, two successive criteria were required to be met. First, these genes needed to exhibit differential expression after EE treatment relative to TMVCs; second, these genes needed to exhibit differential gene expression after EE + TCDD cotreatment relative to EE. This approach identified genes regulated by EE that were subsequently modulated upon cotreatment with TCDD and identified 163 features representing 133 EE-regulated genes at one or more time points. In many cases, EE-mediated gene expression responses were inhibited by more than 80% upon cotreatment with TCDD. The data also indicate that only a select number of EE-mediated gene expression responses experience inhibition after cotreatment with TCDD, most EE responsive genes being unaffected by the cotreatment. On a per-time-point basis, 9, 23, 32, and 130 features representing 5, 21, 28, and 106 genes were inhibited by TCDD cotreatment at 4, 12, 24, and 72 h, respectively. This indicates a time-dependent increase in the inhibitory effects of TCDD on EE-mediated gene expression responses, suggesting that direct early primary responses may subsequently mediate more extensive secondary and tertiary indirect inhibitory responses.

A small number of gene expression responses were differentially expressed between the EE + TCDD and EE groups but were not EE-regulated responses; rather, they were differentially expressed due to TCDD alone. This included the well characterized induction of Cyp1a1 as well as the induction of inhibitor of growth 1 (Ing1), karyopherin alpha 6 (Kpna6), and replication protein A2 (Rpa2). The induction of these genes cannot be dismissed as a contributing factor to the antiestrogenic effects of TCDD because the induction of inhibitory factors is a previously proposed mechanism (Rogers and Denison, 2002).

Functional Categorization of Microarray Data. Functional annotation of gene expression responses was performed using data extracted from public databases and published literature. The functions of EE differentially expressed genes have been associated with transcription factors, mRNA and protein synthesis, cell cycle regulation, cellular proliferation, energetics and structural constituents (Fertuck et al., 2003; Moggs et al., 2004; Kwekel et al., 2005). Functional annotation of EE-mediated gene expression responses that were inhibited upon cotreatment with TCDD were associated with the regulation of cell proliferation and growth, water/ion

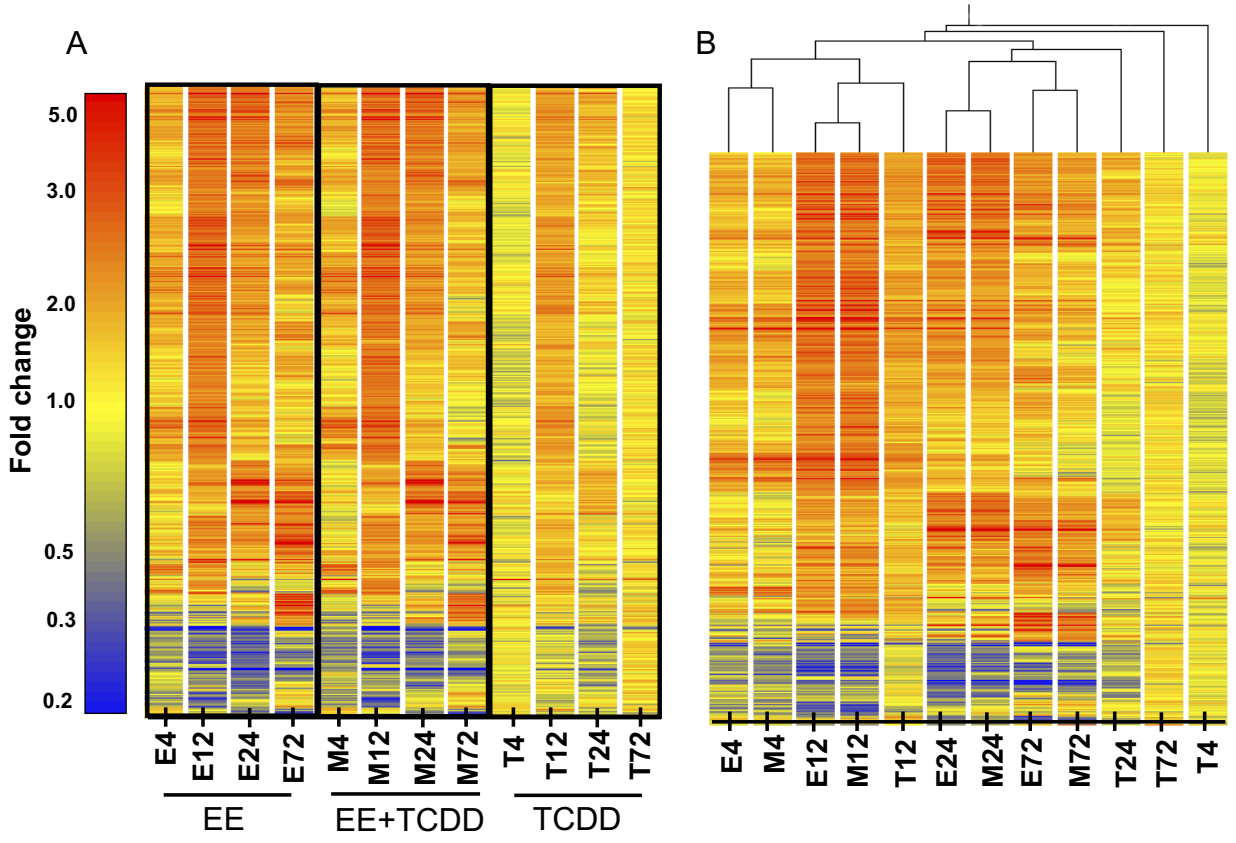

Fig. 5. Comparison of global gene expression responses to EE, TCDD, and EE + TCDD. A, comparison of temporal gene expression profiles indicates the similarity of EE and EE + TCDD patterns, whereas TCDD were minimal by comparison except for the 12-h time point. B, hierarchical clustering further illustrates the temporal similarity between EE and EE + TCDD groups, in that each treatment/time point clustered together. The 12-h TCDD samples clustered with the 12-h EE and EE + TCDD samples, indicating estrogen-like expression. The 24-h TCDD sample exhibited modest similarity to the 24- and 72-h EE and EE + TCDD groups, whereas the 4- and 72-h TCDD groups clustered separately. Comparisons were performed on features that were differentially expressed in any of the treatment groups at any time point relative to time-matched vehicle controls (E = EE, T = TCDD, and M = EE + TCDD mixture).

transport, and the maintenance of cellular structural architecture (Table 1). Cellular growth and proliferation genes included branched chain aminotransferase 1 (Bcat), serine proteinase inhibitor B5 (Serpinb5), sestrin 1 (Sesn1), stratifin (Sfn), and trefoil factor 1 (Tff1). Inhibition of this functional category is consistent with previous reports of decreased cellular growth responses in breast and endometrial cancer cell lines (Wang et al., 1998; Castro-Rivera et al., 1999; Puga et al., 2000; Wormke et al., 2000) and uterine tissue (Buchanan et al., 2002). TCDD-inhibited water and ion transport genes included aquaporins 1 and 3, (Aqp1 and 3) solute carriers 4a2, 38a3, and 40a1 (Slc4a2, 38a3 and 40a1), and FXYD ion transport regulator 4 (Fxyd4). Inhibition of these responses may contribute to TCDD-mediated decreases in stromal edema and uterine wet weight. Desmocollin 2 (Dsc2), keratins 4, 7, 14 and 19 (Krt2–4, Krt2–7, Krt1–14 and Krt1–19), macrophage receptor with collagenous structure (Marco), TP53 apoptosis effector (Perp), and small proline-rich protein 2A (Sprr2a) represent structural genes inhibited by TCDD. Collectively, the inhibition of these EE-mediated responses may contribute to the antiestrogenic effects of TCDD on uterine histology, growth, and LEC integrity.

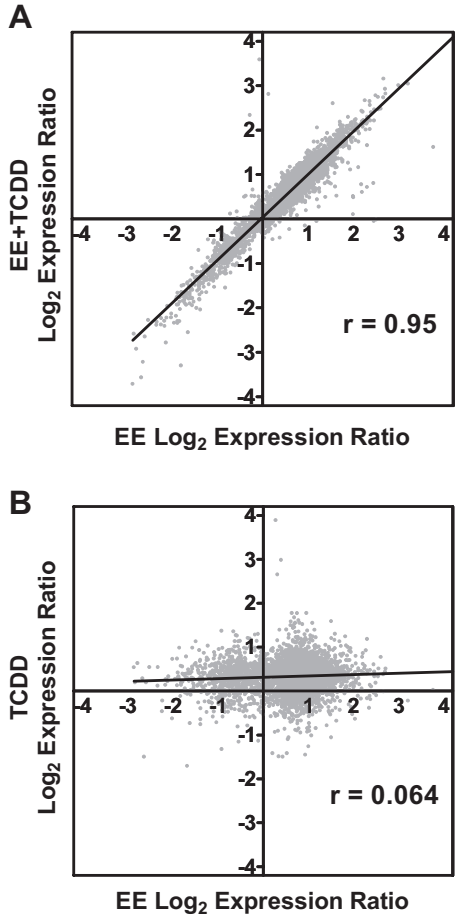

Fig. 6. Scatter plot comparisons of gene expression magnitudes between treatment groups. A, scatter plots of the $\log_2$ expression ratios of EE versus EE + TCDD at the 12-h time point revealed that the majority of the responses were of the same magnitude relative to the TMVCs with a correlation of 0.95. This graph also reveals a small subset of genes that were more highly expressed in the EE-treated group, suggesting inhibition upon cotreatment with TCDD. D, a similar comparison between EE and TCDD groups at 12 h revealed a low correlation of 0.064, indicating high variation between the magnitudes of the response despite similar expression patterns. Similar responses were noted at the other time points (data not shown).

Verification of Microarray Results. QRTPCR was used to verify changes in transcript levels for a selected subset of EE-inducible genes inhibited by TCDD (Fig. 7). There was good agreement between the microarray and QRTPCR results, although compression of the gene expression response was observed in the microarray data, which has been previously reported comparing microarray analysis to other methods (Yuen et al., 2002). QRTPCR revealed that Tff1 transcripts were induced more than 400-fold by EE at 12 and 24 h, whereas cotreatment with TCDD inhibited this response by more than 90%. Similar confirmatory responses were noted for Dsc2, Krt1–14, Sprr2a, and Sfn, which were maximally induced 20-, 171-, 206-, and 6.-fold by EE treatment and inhibited 95, 90, 83, and 93%, respectively, by TCDD. QRTPCR was also used to verify the EE induction of proliferating cell nuclear antigen (PCNA) and solute carrier family 25, member 5 (Slc25a5), because microarray analysis indicated that these genes were not affected by TCDD cotreatment. Induction levels of PCNA and Slc25a5 by EE and EE + TCDD were comparable, verifying that TCDD inhibited select EE induced responses.

Four estrogen-induced genes, including Fos (Duan et al., 1999), Ctsd (Krishnan et al., 1995), Hsp27 (Porter et al., 2001), and Tff1 (also known as pS2) (Gillesby et al., 1997), have been reported to be inhibited after TCDD cotreatment in human MCF-7 cells. The dramatic inhibition of EE-induced uterine Tff1 transcript levels by TCDD suggests cross-species (human to mouse), cross-model (in vivo to in vitro), and cross-tissue (breast to uterus) conservation of this response. To further investigate the conservation of these responses, the effect of TCDD on the remaining three transcripts was also investigated by QRTPCR. EE significantly induced Fos, Ctsd, and Hsp27 transcript levels in the mouse uterus; however, TCDD cotreatment did not inhibit the induction of these genes (data not shown), suggesting that the inhibitory effects of TCDD on these genes may be model specific.

Discussion

The present study was conducted to further elucidate the antiuterotrophic effects of TCDD. TCDD significantly inhibited EE induced uterine weight and altered the integrity of the LEC layer, consistent with previous reports (Gallo et al., 1986; Astroff and Safe, 1988; Umbreit et al., 1988). Comparison of EE and EE + TCDD gene expression responses revealed that the majority of EE-mediated changes were unaffected by cotreatment. However, a subset of EE-responsive genes was inhibited upon cotreatment with TCDD, suggesting a gene-specific inhibitory response. The inhibited genes are involved in cell proliferation, growth and differentiation, water and ion transport, and maintenance of cellular structure and integrity and were consistent with the observed histological alterations.

Inhibition of Cellular Growth and Proliferation Responses. Estrogen induction of uterine weight involves a coordinated proliferative response that is mediated through a well orchestrated series of changes in gene expression (Fertuck et al., 2003; Moggs et al., 2004; Kwekel et al., 2005). Cotreatment with TCDD disrupted several EE-induced

genes with important functions in cell cycle regulation, growth, and proliferation. For example, EE-mediated induction of Bcat and Sfn, important regulators of cell cycle progression, was inhibited by TCDD between 12 and 72 h. Bcat regulates G_1-to-S phase transition, and cells with reduced expression exhibit faster growth rates, a shorter G_1 stage, and an increased frequency of mutations (Schuldiner et al., 1996). Sfn serves as a G_2 checkpoint component as a positive mediator of growth-factor-induced cell cycle progression (Hermeking et al., 1997; Zhang et al., 2004). TCDD also inhibited EE induction of Serpinb5 at 12, 24, and 72 h, which plays an essential role in development, as exhibited by embryonic lethality in knockout mice (Gao et al., 2004), whereas decreased expression results in reduced cellular proliferation and adhesion (Gao et al., 2004). Sesn1 is a positive regulator of cell growth and protects against apoptosis (Velasco-Miguel et al., 1999; Budanov et al., 2004) and was also inhibited by TCDD cotreatment. Additional EE-induced genes implicated in cellular growth, proliferation, and development included retinol binding protein 2, Tnfsf8, and Vezf1, which were also

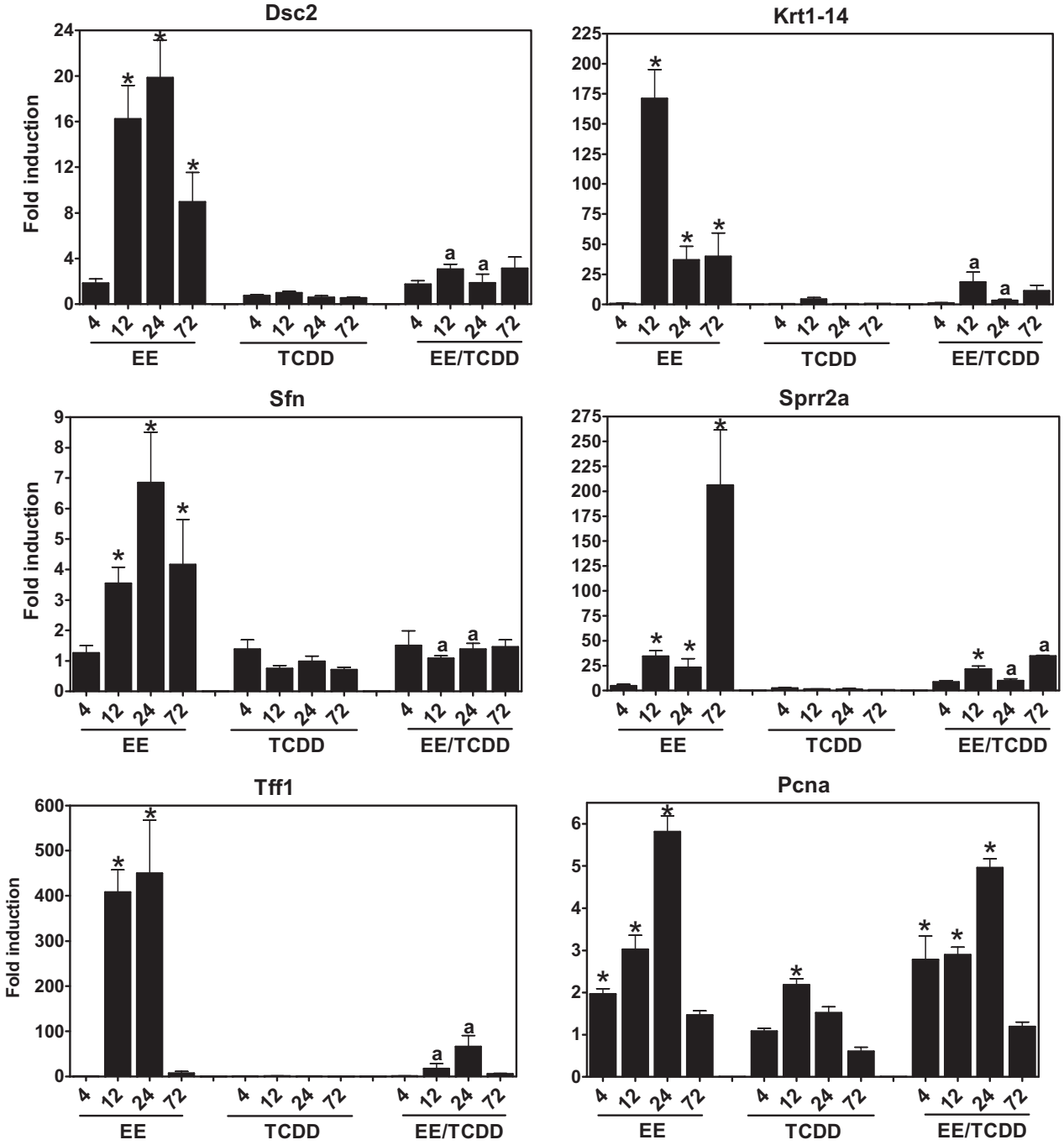

Fig. 7. Quantitative real-time PCR verification of the selective inhibition of EE-induced gene expression responses by TCDD. TCDD cotreatment inhibited the EE-mediated induction of Dsc2, Krt1–14, Sfn, Sprr2a, and Tff1 but did not affect the induction of PCNA. The same RNA used for cDNA microarray analysis was examined by QRTPCR. All -fold changes were calculated relative to time-matched vehicle controls. Genes are indicated by official gene symbols and results are the average of five biological replicates. Error bars represent the S.E.M. for the average -fold change. $*$, $p < 0.05$ for treatment groups relative to time-matched vehicle controls. a, $p < 0.05$ for EE + TCDD compared with time matched EE controls

inhibited upon cotreatment between 12 and 72 h. The inhibition of genes involved in regulating cell cycle progression is consistent with that reported in a previous study; however, the inhibition of estrogen-induced cyclin transcripts was not detected, which may be attributed to different experimental treatments and time points (Buchanan et al., 2002). Overall, the alteration of these responses may be a contributing factor to the observed reduction in stromal cell hypertrophy and hyperplasia as well as the marked LEC apoptosis.

One of the most dramatic TCDD-inhibited responses was that of Tff1, which plays a fundamental role in epithelial maintenance, protection, and regeneration (Lefebvre et al., 1996; Playford et al., 1996). Tffs block p53-dependent and independent pathways of apoptosis and promote growth and regeneration by allowing cells to break attachments with the basement membrane to replace epithelial defects without cell death (Hoffmann et al., 2001). Tff peptides also have anti-inflammatory actions and protect the epithelial mucous layers (Hoffmann et al., 2001; Vieten et al., 2005). Therefore, TCDD's inhibition of EE-induced Tff1 by more than 90% may play an important role in the increased LEC degeneration and apoptosis.

Tff1 is also a prognostic marker in human breast cancer and is an estrogen-responsive gene in breast cancer cells and the human endometrium (Gillesby and Zacharewski, 1999; Punyadeera et al., 2005). The inhibitory effect of TCDD on estrogen induction of Tff1 has been characterized in human MCF-7 breast cancer cells and depends on an inhibitory DRE that interferes with AP-1- and ERE-mediated transcriptional activation (Gillesby et al., 1997). Tff1 induction and inhibition by TCDD in human MCF-7 breast cancer cells and the mouse uterus indicates that this mechanism may be conserved across sensitive species and tissues. Examination of the mouse promoter region for Tff1 identified a variant ERE at −475 as well as an AP-1 site at −998 relative to the transcriptional start site. Although a DRE does not overlap with the AP-1 site, two putative DREs are located further upstream at −1920 and −2637 and may play a role in mediating the inhibition.

Water/Ion Transport Responses. EE induced stromal edema, which was significantly inhibited by TCDD. Several EE-regulated genes involved in water and ion transport were inhibited by TCDD, including the EE-mediated down-regulation of Aqp1 and up-regulation of Aqp3. Isoform-specific regulation of aquaporins plays an integral role in mediating the water imbibition in the uterus (Richard et al., 2003). EE-regulated transcripts involved in sodium and chloride transport were also inhibited by TCDD, including Slc4a2, Slc38a3, and Fxyd4 (Garty et al., 2003; Quentin et al., 2004). Collectively, the inhibition of these EE-mediated gene expression responses may have contributed to reductions in uterine wet weight after cotreatment with TCDD.

Inhibition of Structural Constituents. The uterus undergoes extensive cytoarchitectural changes to accommodate the dramatic proliferation and growth response to estrogen, which involves numerous structural, adhesion, and extracellular matrix genes, including a number of keratins, actins, procollagens, tubulins, desmocollins, and small proline-rich proteins in mice, rats, and humans (Watanabe et al., 2002; Hewitt et al., 2003; Watanabe et al., 2003; Moggs et al., 2004). TCDD cotreatment inhibited a number of these EE-mediated responses, which probably contributed to its anti-uterotrophic effects as well as increases in apoptosis.

In this study, the EE induction of keratins 4, 7, 14, and 19 was significantly inhibited by TCDD cotreatment. Keratins are involved in the formation of the cytoskeleton, which consists of an extensive array of filamentous networks. Their disruption results in epithelial cell fragility and lysis (Sørensen et al., 2003; Wong et al., 2005). Keratins 18 and 19 are estrogen-inducible transcripts, the induction of which is blocked by TCDD in MCF-7 cells (Chen et al., 2001). Inhibition of uterine keratin 18 and 19 suggests that this may represent a conserved response between rodents and humans. Moreover, the inhibition of multiple keratin genes suggests that TCDD may disrupt signaling at a common regulatory region as the basic (Krt2–2 through -8) and acidic (Krt1–9 through -19) keratin genes are encoded in a tandem array on chromosomes 15 and 11, respectively (Chu and Weiss, 2002).

Desmocollin 2 (Dsc2), a component of desmosomes, is expressed primarily in epithelial cells, serves an integral role in cell adhesion by forming links with the intermediate filament network (Marsden et al., 1997), and was inhibited by TCDD. TCDD also inhibited Perp induction, which promotes desmosomal complex assembly (Ihrie et al., 2005). EE induction of small proline rich protein 2a (Sprr2a) was also inhibited by cotreatment with TCDD. The Sprr2 family consists of 11 genes (Sprr2a-2k), which are important structural components of epithelial cells because of their ability to form extensive cross-links (Hong et al., 2004). Several Sprr2 genes are up-regulated in the luminal epithelial cells of the uterus in response to estrogen, where they are important for cytoarchitectural changes (Hong et al., 2004). TCDD also inhibited the induction of other structural molecules including Marco, procollagen 6a2, troponin T1, and tubulin β6. Together, the inhibition of these structural constituents could compromise the rapid proliferation and growth induced by EE, resulting in altered results on histologic examination, increased apoptosis, and overall decreased uterine growth.

TCDD as Estrogen and Antiestrogen. Reports have indicated that TCDD elicits an estrogen-like, ER-dependent gene expression profile in the uterus (Ohtake et al., 2003; Watanabe et al., 2004; Boverhof et al., 2006). The regulation of similar genes by EE and TCDD suggests that these responses may represent targets for inhibition. However, these genes were largely unaffected by TCDD, including well characterized estrogen-responsive genes, such as PCNA, Slc25a5, cell division cycle 2 homolog A (Cdc2a), and ornithine decarboxylase (Odc). Instead, many of the inhibited responses were unaffected by TCDD treatment alone, consistent with previous reports of estrogen/TCDD gene expression cross-talk (Zacharewski et al., 1994; Porter et al., 2001; Safe and Wormke, 2003). These data suggest that the anti-uterotrophic effects of TCDD are independent of its weak estrogenic activity.

Decreased ER levels (Romkes et al., 1987) and increased estrogen metabolism (Spink et al., 1994) have also been proposed as mechanisms for the antiestrogenic effects of TCDD. However, several reports indicate that these mechanisms do not account for the antiestrogenic effects of TCDD. TCDD does not increase estrogen metabolism in vivo (DeVito et al., 1992; Petroff and Mizinga, 2003), and uterine ER levels were unaffected by TCDD (DeVito et al., 1994; White et al., 1995).

In the present study, TCDD inhibited only a select subset of EE responses, further indicating that increased metabolism and decreased ER levels are not the primary mechanisms, because these would be expected to elicit a more global effect on gene expression. The gene-specific inhibitory effects may involve an inhibitory DRE mechanism, alteration in the activity or availability of gene-specific cofactors, or induction/inhibition of an upstream regulatory protein that are likely to be tissue and species-specific.

Summary. The present study has identified a small subset of EE- induced uterine gene expression responses that are inhibited by TCDD. Moreover, the repressed functional categories can be related to the observed histological and physiological responses and therefore represent potential mediators of TCDD's anti-uterotrophic effect. Select responses, including Tff1, are also in agreement with in vitro studies, indicating the potential conservation of these responses among different models and species. Furthermore, the results indicate that the estrogenic and antiestrogenic gene expression effects of TCDD are independent. The importance of these genes in the uterotrophic response as targets for the antiestrogenicity of TCDD can be addressed through comparative studies with rats and mice and through receptor- and gene-specific null mice. Additional research is also required to fully delineate the mechanism of the gene-specific inhibitory response and should employ molecular approaches including receptor interaction (fluorescence resonance energy transfer), chromatin immunoprecipitation, promoter dissection/reporter gene assays and siRNA approaches in appropriate in vitro models (e.g., uterine based cell lines, primary cells or cocultured primary systems) that closely simulate in vivo conditions. These approaches will more comprehensively elucidate the dual nature of TCDD as an estrogenic and antiestrogenic compound.

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Address correspondence to: Dr. Timothy R. Zacharewski, Michigan State University, Department of Biochemistry and Molecular Biology, 224 Biochemistry Building, Wilson Road, East Lansing, MI, USA 48824-1319. E-mail: tzachare@msu.edu