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Dioxin induces an estrogen-like, estrogen receptor dependent gene expression response in the murine uterus

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Running Title: Dioxin induces an estrogen-like gene expression response

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Abbreviations
TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element; EE, ethynyl estradiol; ERE, estrogen response element

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous environmental contaminant that elicits a broad range of toxicities in a tissue-, sex-, age- and species- specific manner including alterations in estrogen signaling. Many, if not all, of these effects involve changes in gene expression mediated via the activation of the aryl hydrocarbon receptor (AhR), a ligand activated transcription factor. Recent data indicate that TCDD may also elicit AhR-mediated estrogenic activity through interactions with the estrogen receptor (ER). In an effort to further characterize the estrogenic activity of TCDD, a comprehensive time course analysis of uterine gene expression was conducted using ovariectomized C57BL/6 mice. Comparison of the temporal uterine transcriptional response to TCDD with that of ethynyl estradiol (EE) revealed a large proportion of the TCDD-mediated gene expression changes were also responsive to EE. Furthermore, pretreatment of mice with the pure ER antagonist ICI 182 780 inhibited gene expression responses to both EE and TCDD, providing additional evidence that these transcriptional responses involve the ER.

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Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds are ubiquitous environmental contaminants that elicit a broad spectrum of toxic and biochemical responses in a tissue-, sex-, age- and species-specific manner (Poland and Knutson, 1982). These responses include a wasting syndrome, tumor promotion, teratogenesis, hepatotoxicity, immunotoxicity and modulation of endocrine systems, which are mediated by the aryl-hydrocarbon receptor (AhR), a member of the basic-helix-loop-helix-PAS (bHLH-PAS) family (Denison and Heath-Pagliuso, 1998; Poland and Knutson, 1982). The proposed mechanism involves ligand binding to the cytoplasmic AhR and translocation to the nucleus where it forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT), another member of the bHLH-PAS family. This heterodimer 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). Evidence suggests that the adverse effects elicited by TCDD are due to the continuous and inappropriate AhR-mediated regulation of these target genes (Denison et al., 2002). Although the mechanisms of AhR/ARNT-mediated changes in gene expression are well established, TCDD modulation of gene expression associated with the toxic and biochemical effects remains poorly understood.

Like the AhR, the estrogen receptor (ER), a member of the nuclear receptor superfamily, is a ligand activated transcription factor which mediates many of the effects of estrogens (Nilsson et al., 2001). Upon ligand binding, ERs dissociate from heat shock and chaperone proteins, homodimerize, and interact with regulatory elements near estrogen responsive genes (Klinge, 2001). Classically, ERs mediate transcriptional

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responses through binding to estrogen response elements (EREs) but also via interactions with Fos/Jun at AP-1 sites, Jun/ATF-2 at variant cyclic AMP response elements (CREs), and Sp1 at its response elements (Hall et al., 2001; Nilsson et al., 2001). The ER can also elicit cellular responses through ligand-independent, DNA-binding independent and cell-surface (non-genomic) signaling mechanisms (Hall et al., 2001).

TCDD elicits a number of AhR-dependent antiestrogenic responses in the female reproductive tract including the inhibition of estrogen induced increases in uterine wet weight, DNA synthesis, and gene expression responses (reviewed in (Safe and Wormke, 2003)). However, accumulating evidence suggests that TCDD also possesses estrogen-like activity. TCDD increases the DNA-binding activity of the ER independent of estrogen in the rat uterus (Chaffin et al., 1996) and treatment of MCF-7 cells with TCDD results in estrogen-like G_0/G_1 to S-phase transition and mitogenic effects (Abdelrahim et al., 2003). Furthermore, the ligand activated AhR/ARNT complex directly associates with the unliganded ER to form a functional complex that binds EREs and activates transcription (Ohtake et al., 2003). Independent studies also confirmed ER-AhR interactions which may account for the crosstalk between these signaling pathways (Beischlag and Perdew, 2005; Brunnberg et al., 2003). In addition, 3-methylcholanthrene (3-MC) activation of the AhR in the absence of estrogen induced estrogenic responses in the mouse uterus (Ohtake et al., 2003). Moreover, TCDD mediates the induction of estrogen dependent tumors in rats (Brown et al., 1998; Davis et al., 2000), and reportedly increases the incidence of endometriosis in laboratory animals and in women with high body burdens of TCDD (Cummings and Metcalf, 1996; Koninckx et al., 1994; Mayani et al., 1997; Rier et al., 1993).

To further characterize the apparent estrogenicity of TCDD, a comprehensive uterine time course analysis of gene expression was conducted in ovariectomized C57BL/6 mice. Temporal uterine responses to TCDD were compared to that of ethynyl estradiol (EE), an orally active estrogen, to identify similarities and differences in gene expression profiles. Moreover, mice were co-treated with the pure estrogen receptor (ER) antagonist ICI 182 780 to investigate the role of the ER in mediating the estrogen-like gene expression responses to TCDD.

Materials and Methods

Animal Husbandry

Female C57BL/6 mice, ovariectomized on PND 20 were obtained from Charles River Laboratories (Raleigh, NC) on PND 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 hr dark/light cycle (7am-7pm). Animals were provided free access to de-ionized water and Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI), and acclimatized for 3 days prior to treatment.

Animal Treatments

For the dioxin study animals (n= 5/treatment group/time point) were treated once by oral gavage with 30 μg/kg b.w. TCDD (provided by S. Safe, Texas A&M University, College Station, TX) or sesame oil (Sigma Chemical, St Louis, MO) as described previously (Boverhof et al., 2005). Animals in the estrogen study (n= 5/treatment group/time point) were treated once every 24 hrs by oral gavage on three consecutive

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days with 100 μg/kg b.w. 17α-ethynylestradiol (EE) or sesame oil vehicle (Sigma Chemical) as described previously (Kwekel et al., 2005). Mice were sacrificed by cervical dislocation 2, 4, 8, 12, 18, 24, or 72 hrs after dosing. For the co-treatment studies, animals (n= 6/treatment group) were treated orally with either TCDD (30μg/kg) or EE (100 μg/kg) with or without an i.p. injection of 10 mg/kg b.w. ICI 182 780 (Tocris Cookson Inc., Ellisville, MO) in 50 μL 1X PBS. Whole uterine weights were recorded before (wet) and after (blotted) blotting with absorbent tissue. A section of the left uterine horn was removed for histology and fixed in 10% neutral buffered formalin (NBF, Sigma, St. Louis, MO). The remaining tissue was subsequently snap-frozen in liquid nitrogen and stored at -80˚C. The doses were empirically derived to elicit robust TCDD-induced changes in gene expression or a maximal EE-induced uterotrophic response (Boverhof et al., 2005; Kwekel et al., 2005). All doses were calculated based on average weights of the animals prior to 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 μm cross-sections were mounted on glass slides and stained with hematoxylin and eosin. All embedding, mounting and staining was 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

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software (Scion Image, Scioncorp, Frederick, Maryland) 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 whole uteri using Trizol Reagent (Invitrogen, Carlsbad, CA) as per 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 The 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 by visual inspection of 1 μg on a denaturing gel.

Array 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. Briefly, 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 x 4) Chipmaker 2 pins (Telechem) at the Genomics Technology Support Facility at Michigan State University (http://www.genomics.msu.edu). Temporal changes in gene expression in mouse uteri were assessed using an independent reference design in which samples from treated animals are co-hybridized with time

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matched vehicle controls. Comparisons were performed on 3 of the 5 biological replicates (3 independent mice) with 2 independent labelings of each sample (technical replicate incorporating a dye swap) for each treatment group. For the ICI microarray experiment, gene expression changes were analyzed using a loop design with comparisons performed on 3 randomly chosen samples from the 6 biological replicates (3 independent mice) which included 2 independent labelings of each sample for each treatment group (Yang and Speed, 2002). A 3DNA Array 900 Expression Array Detection Kit (Genisphere, Hatsfield, PA) using 1 μg of total RNA was used for probe labeling in all microarray experiments, according to manufacturer's specifications. Samples were hybridized for 18–24 hrs 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 (Axon Instruments Inc., Union City, CA).

Array Data Normalization and Statistical Analysis

Data were normalized using a semi-parametric approach (Eckel et al., 2005). Model-based t-values were calculated from normalized data, comparing treated from 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 was used to filter the expression data and to define active gene lists. All arrays were subjected to quality control assessment to

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ensure assay performance and data consistency. All data are stored within dbZach (http://dbzach.fst.msu.edu), a MIAME (Minimum Information About a Microarray Experiment) supportive relational database that ensures proper data management and facilitates data analysis. Complete data sets with annotation and P1(*t*) values are available in Supplementary Tables 1 and 2. Gene expression patterns that passed the established threshold criteria were visualized using hierarchical clustering (GeneSpring 6.0, Silicon Genetics, Redwood City, CA). Comparative analysis was conducted using a multivariate correlation-based visualization application developed in-house. The program calculates correlations between the gene expression and significance values for the same genes from the EE and TCDD experiments.

Quantitative Real-Time PCR (QRTPCR) 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 each of forward and reverse gene-specific primers, designed using Primer3 (Rozen and Skaletsky, 2000), 3 mM $MgCl_2$, 1.0 mM dNTPs, 0.025 IU AmpliTaq Gold and 1x SYBR Green PCR buffer (Applied Biosystems, Foster City, CA). Gene names, accession numbers, forward and reverse primer sequences and amplicon sizes are listed in Supplementary Table 3. PCR amplification was conducted in MicroAmp Optical 96-well reaction plates (Applied Biosystems) on an Applied Biosystems PRISM 7000 Sequence Detection System 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 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 six orders of magnitude to interpolate relative template concentrations of the samples from the standard curves of log copy number versus threshold cycle (Ct). No template controls (NTC) were also included on each plate. Samples with a Ct value within 2 SD of the mean Ct values for the NTCs 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 ANOVA followed by t-test (SAS 9.1). For graphing purposes, the relative expression levels were scaled such that the expression level of the time-matched control group was equal to one.

Results

Comparison of Uterine Gene Expression Responses to TCDD and EE

The magnitude of TCDD-induced alterations in uterine gene expression was modest when compared to that of EE. In total, 345 features representing 281 unique genes were found to be differentially expressed at one or more time points in response to TCDD. A number of characteristic TCDD-inducible genes were identified including aldehyde dehydrogenase family 3a1, cytochrome P4501a1, NAD(P)H dehydrogenase, quinone 1 and TCDD-inducible poly(ADP-ribose) polymerase. In contrast, EE induced a robust transcriptional response with a total of 4,329 features, representing 3,214 unique genes, exhibiting differential expression at one or more time points.

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Many of the genes identified as differentially expressed in response to EE and TCDD were unique to each compound, however, a number of transcripts were also commonly regulated (Table 1). Comparison of the active gene lists from each study revealed that 228 of the 281 genes regulated by TCDD were also regulated by EE (Figure 1A). In order to ascertain the similarity of these 228 overlapping gene expression responses a Pearson's correlation analysis was performed on the temporal gene expression (fold-change) and significance (P1(t)) profiles. These paired data were plotted on a coordinate axis with the x-axis as the gene expression correlation and the y-axis as the significance correlation (Figure 1B). A majority of the genes fall into the upper right hand quadrant representing genes induced by TCDD and EE that exhibited highly correlated temporal gene expression and significance patterns. In total 181 of the 228 genes regulated by both TCDD and EE exhibited a gene expression correlation greater than 0.3, suggesting that the temporal expression patterns for genes induced by TCDD were similar to the expression patterns induced by EE. Visualization after gene-based hierarchical clustering reveals the similarity of the EE and TCDD gene expression profiles while also illustrating the lower magnitude of change in response to TCDD (Figure 1C). Additional clustering by treatment and time point illustrated that the 8, 12, 18 and 24 hr EE and 12 18 and 24 hr TCDD time points cluster together with the response to TCDD at 12 hrs displaying the greatest similarity to the EE groups (data not shown).

A number of uterine histological and morphological endpoints that comprise the enhanced uterotrophic assay provide complementary phenotypic information for assessing the estrogenicity of a chemical (Diel et al., 2002; Moggs et al., 2004a; Odum et

al., 1997). Previous studies have demonstrated that estrogens induce dramatic increases in uterine wet weight, luminal epithelial cell height, stromal thickness and BrdU labeling (Carthew et al., 1999; Kwekel et al., 2005; Moggs et al., 2004b). In contrast, TCDD did not induce alterations in any of these histological or morphological endpoints (data not shown). These results indicate that although TCDD induces gene expression responses similar to that of EE, these alone are not sufficient to elicit an estrogen-like physiological response in the uterus. This may be attributed to the fact that only a subset of the total number of genes activated by EE were also regulated by TCDD and these genes alone are not sufficient to mediate an estrogenic physiological response. Furthermore, the magnitude of the TCDD-mediated changes in gene expression was well below that seen for EE and may not surpass the threshold required to elicit a response.

Quantitative Real Time PCR Verification of Results

In order to independently examine the estrogen-like gene expression responses of TCDD, six genes were chosen for verification by QRTPCR. Arginine-rich, mutated in early stage tumors (Armet), asparagine synthetase (Asns), activating transcription factor 4 (Atf4), expressed in non-metastatic cells 1 (Nme1), proliferating cell nuclear antigen (Pcna) and solute carrier family 25 member 5 (Slc25a5) were specifically selected because they displayed similar responses to EE and TCDD and have been previously identified as estrogen inducible in the rodent uterus in independent studies (Fertuck et al., 2003; Kwekel et al., 2005; Moggs et al., 2004b; Watanabe et al., 2002). QRTPCR analyses confirmed the microarray results indicating these genes were induced by both EE and TCDD (Figure 2). Interestingly, the induction profiles for both the QRTPCR and microarray data reveal that the TCDD-mediated responses temporally lagged relative to

EE suggesting that these treatments exhibit different pharmacokinetic or pharmacodynamic characteristics. Alternatively, this may suggest that TCDD is mediating these responses through an indirect or secondary mechanism.

Inhibition of EE and TCDD GeneExpression Responses by ICI 182 780

Overlapping TCDD and EE gene expression responses suggest that TCDD induces an estrogen-like gene expression profile in the murine uterus. This effect has been reported to be mediated via activation of unliganded ER through direct association with activated AhR/Arnt complexes (Ohtake et al., 2003). To investigate the role of the ER in TCDD mediated induction of known estrogen responsive genes, mice were treated with the pure estrogen receptor antagonist ICI 182 780 prior to vehicle, EE or TCDD administration. Animals were sacrificed 12 hrs after treatment as this was the most active time point and exhibited the most similar EE- and TCDD-induced uterine gene expression profiles. As expected, EE induced water imbibition at 12 hrs was completely inhibited by ICI 182 780 (Figure 3A). Consistent with the earlier results, TCDD did not elicit a water imbibition response. Microarrays analyses were performed on these samples to examine the effect of ICI treatment on the gene expression responses to EE and TCDD. Of the 228 genes identified as commonly active between EE and TCDD in the temporal studies (Figure 1A), 130 were common to the 12 hour time point. Examination of these 130 genes revealed that 97 were responsive to EE and inhibited by ICI, with 91 of these genes displaying a greater than 75% inhibition. In response to TCDD treatment, 81 of these genes were regulated similarly to EE and were inhibited by ICI (Supplementary Table 4). Hierarchical clustering of the normalized intensity values from the microarrays revealed that the EE and TCDD treatment groups clustered together

while the EE/ICI and TCDD/ICI groups clustered with the vehicle control (Figure 3B), further illustrating the similarity of EE and TCDD elicited gene expression responses and their inhibition by ICI 182, 780. To verify the microarray data, QRTPCR was used to examine the same six transcripts induced by both EE and TCDD in Figure 2. Co-treatment with ICI 182 780 completely inhibited EE and TCDD gene expression responses (Figure 3C). The effect on each transcript was statistically significant with the exception of the TCDD-mediated induction of Slc25a5 which did not reach statistical significance ($p=0.10$) as the optimal time point for TCDD induction is 18 hrs. However, the expected pattern of gene induction by TCDD and the inhibition of this response by ICI 182 780 were still evident. These results indicate that the EE and TCDD induction of these transcripts is dependent on the ER.

Discussion

The present study compared TCDD and EE uterine transcriptional responses in the mouse. A subset of estrogen responsive genes was found to be responsive to TCDD indicating that TCDD elicits an estrogen-like transcriptional response in the murine uterus. In addition to the data presented here, two independent microarray reports have compared the gene expression responses of TCDD to that of estrogen. The first utilized human MCF-7 cells and compared the gene expression profiles of estrogen to a number of other estrogenic endocrine disruptors (EEDs) (Terasaka et al., 2004). Although many of the EEDs examined exhibited similar global gene expression patterns to that of estrogen, little to no correlation was observed to the responses induced by TCDD. The inability to detect the estrogenic response to TCDD in this study may have been limited by the use of a focused microarray platform that examined only a small subset of

estrogen responsive genes. Furthermore, the MCF-7 cell line may differ in cellular responses, complement of co-activators/co-repressors, receptor content or ratio of ER to AhR when compared to the *in vivo* murine uterus. A second study examined the estrogenicity of TCDD by comparing uterine gene expression responses after estrogen or TCDD treatment at a single time point (6 hrs) in ovariectomized C57BL6/J mice (Watanabe et al., 2004). However, the mice utilized in this study were ovariectomized after first estrus creating a uterine environment less responsive to estrogen (Quarmby and Korach, 1984) which may account for the smaller number of TCDD inducible genes identified. Nevertheless, the results are consistent with our research and indicate that, although the number of genes regulated by TCDD is minimal compared to estrogen, a subset of the estrogen responsive genes are also induced by TCDD.

ICI 182 780 inhibition of the estrogen-like gene expression responses of TCDD suggests that these response are ER-dependent. TCDD has been proposed to elicit estrogenic responses via direct ER binding (Watanabe et al., 2004) based on the reported estrogenic activity of PCB-77 (Nesaretnam et al., 1996), a coplanar PCB congener which binds the AhR. However, independent studies have not verified PCB-77 binding to the ER (Ramamoorthy et al., 1999), and TCDD does not bind the ER (Klinge et al., 1999). A more plausible mechanism involves the activation of unliganded ER by ligand activated AhR or a co-activator relationship between these signaling pathways. Recent studies have demonstrated that TCDD induces the AhR to interact directly with ER-alpha in the absence of estrogen (Klinge et al., 2000; Wormke et al., 2000). Moreover, TCDD increases the DNA-binding activity of the ER-independent of estrogen in the rat uterus (Chaffin et al., 1996) and treatment of MCF-7 cells with TCDD results in G_0/G_1 to S-

phase transition and estrogen like mitogenic effects (Abdelrahim et al., 2003). Studies have also shown that ER and AhR interact (Beischlag and Perdew, 2005; Brunnberg et al., 2003) and there is a discrepancy as to the co-activator or co-repressor function of ER on AhR/ARNT mediated induction of Cyp1a1 (Beischlag and Perdew, 2005; Matthews et al., 2005). Reports also indicate that the ligand activated AhR/ARNT associates with the unliganded ER to form a functional complex that binds EREs (Ohtake et al., 2003). *In vivo* studies with 3-MC corroborate that AhR activation in the absence of estrogen induces various estrogenic responses in the mouse uterus including the induction of the estrogen responsive genes Fos and Vegf, increases in uterine wet weight, and increases in BrdU positive-cells (Ohtake et al., 2003). However, in contrast to these results, we did not detect increases in uterine wet weight or increases in BrdU positive cells. These endpoints may be specific to 3-MC, or its metabolites, which have been reported to activate the ER independent of AhR (Abdelrahim et al., 2005).

Whether the ligand bound AhR is directly or indirectly activating the ER has yet to be determined. Support for direct activation can be drawn from studies indicating the interaction capabilities of these receptors (Beischlag and Perdew, 2005; Brunnberg et al., 2003; Klinge et al., 2000; Wormke et al., 2000), however, indirect mechanisms including induction of modulatory factors, activation of growth factor receptor signaling, or alterations in phosphorylation states cannot be excluded and may explain the lagging transcriptional response of TCDD when compared to EE. Moreover, TCDD activates only a subset of the estrogen responsive genes. Further investigation and comparison of the response elements associated with these genes may provide new insights into the mechanisms associated with their regulation. This subset may represent genes which

possess promoters constitutively occupied by the unliganded ER, allowing the activated AhR or induced factors to readily serve as cofactors. Alternatively, additional estrogen responsive genes may actually be regulated by TCDD through the ER but may not have met the molecular threshold for transcriptional induction or the statistical criteria for inclusion.

To date, most studies have focused on the antiestrogenic activities of TCDD in the presence of estrogen, and therefore the weak estrogenic activities of TCDD have been under reported. The modest gene expression effects (e.g., 25 - 40% of that induced by EE), and the lack of a uterotrophic response likely contributed to the preclusion of TCDD's estrogenic activity in previous studies. However, the use of immature ovariectomized mice in this study provided a more sensitive model for the detection of these responses in a physiological background devoid of estrogens. These results also illustrate the ability of microarrays can detect altered gene expression responses that, despite the absence of altered physiology, may still contribute to compromised functions or response thresholds.

The dual nature of TCDD as an antiestrogen in the presence of estrogen and estrogenic in its absence indicates that responses to TCDD may vary depending on life stage. Despite the inability to induce uterine weight alterations, TCDD may alter physiological thresholds for estrogenic responses that could affect other functions. For example, the antiestrogenic properties may be a contributing factor in compromised reproduction, breast cancer incidence, and earlier onset of menopause (Bertazzi et al., 2001; Eskenazi et al., 2005; Jongbloet et al., 2002; Safe and Wormke, 2003). In contrast, the subtle estrogen-like properties in the absence of estrogen may alter thresholds for

estrogen-mediated responses which could contribute to the earlier onset of puberty associated with TCDD exposure (Warner and Eskenazi, 2005; Warner et al., 2004; Wolff et al., 2005). This dual nature warrants further investigation and should be considered when interpreting the results of animal and epidemiological studies of TCDD.

In summary, TCDD induces an estrogen-like gene expression profile in the uteri of ovariectomized C57BL/6 mice in the absence of histopathological or morphological manifestations. Moreover, the pure estrogen antagonist ICI 182 780 inhibited the TCDD mediated induction of these responses suggesting these effects are mediated via the ER, consistent with other studies demonstrating an AhR-ER interactions. Further research is required to more fully delineate the molecular interactions that occur between the ER and AhR and their potential physiological implications.

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Footnotes

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Figure Legends

Figure 1.
Overlap of active uterine gene expression responses from temporal TCDD and EE studies. A. 3,214 and 281 unique genes were identified as differentially expressed at one or more time points in the EE and TCDD studies, respectively. Of these genes, 228 were responsive in both studies. B. Correlation analysis of temporal gene expression and significance. The majority of the genes fall into the upper right quadrant identifying genes that are highly correlated between both gene expression and significance. A number of genes also fall into the lower right quadrant which represents genes with high correlation of expression but a lower correlation of significance. These results indicate that the temporal patterns of TCDD-induced gene expression responses are similar to those elicited by EE. C. Gene-based hierarchical clustering reveals the similarity of the EE and TCDD gene expression profiles while also illustrating the lower magnitude of change in response to TCDD.

Figure 2.
Quantitative real-time PCR verification of temporal microarray results.
The same RNA used for cDNA microarray analysis was examined by QRTPCR. All fold changes were calculated relative to time matched vehicle controls. Bars (left axis) and lines (right axis) represent data obtained by QRTPCR and cDNA microarrays, respectively, while the x-axis represents the time points. Genes are indicated by official gene symbols and results are the average of 5 biological replicates. Error bars represent the SEM for the average fold change. * indicates $p<0.05$ for QRTPCR.

Figure 3.
ICI 182 780 inhibits uterine gene expression responses to TCDD.
A. Uterine wet weights for each treatment group at 12 hrs. B. Hierarchical clustering of the normalized intensity values from the microarrays illustrates that the EE and TCDD treatment groups cluster together while the EE/ICI and TCDD/ICI groups cluster with the vehicle control C. QRTPCR was used to examine gene expression responses to EE and TCDD in the absence and presence of the pure ER antagonist ICI 182 780. All fold changes were calculated relative to time matched vehicle controls. Genes are indicated by official gene symbols and results are the average of 6 biological replicates. Error bars represent the SEM for the average fold change. * indicates $p<0.05$ when compared to Vehicle control; a indicates $p<0.05$ for ICI/TCDD mice when compared to TCDD treated mice; b indicates $p<0.05$ for ICI/EE mice when compared to EE treated mice

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Table 1. Examples of unique and common gene expression responses to Ethynyl Estradiol and TCDD

Regulatory Category	GenBank Accession	Gene Name	Gene Symbol	Entrez Gene ID	EE Fold change[a]	TCDD Fold change[a]
EE specific responses						
	BG070106	lipocalin 2	Lcn2	16819	28.9	-
	AA792235	inhibin beta-B	Inhbb	16324	7.6	-
	BG089964	granzyme C	Gzmc	14940	5.9	-
	BC002005	gene rich cluster, C9 gene	Grcc9	14794	4.7	-
	W10072	insulin-like growth factor 1	Igf1	16000	4.5	-
	BG065113	branched chain aminotransferase 1, cytosolic	Bcat1	12035	4.4	-
	BG063608	eukaryotic translation initiation factor 2, subunit 2 (beta)	Eif2s2	67204	4.4	-
	W30651	eukaryotic translation initiation factor 2, subunit 2 (beta)	Eif2s2	67204	3.5	-
	U71269	CCR4-NOT transcription complex, subunit 4	Cnot4	53621	3.2	-
	AK009880	mitochondrial ribosomal protein S23	Mrps23	64656	3	-
	AA763337	signal transducer and activator of transcription 5A	Stat5a	20850	2.9	-
	BI248260	fibulin 2	Fbln2	14115	2.5	-
	BF224937	Janus kinase 1	Jak1	16451	0.24	-
TCDD specific responses						
	NM_009992	cytochrome P450, family 1, subfamily a, polypeptide 1	Cyp1a1	13076	-	9
	NM_007436	aldehyde dehydrogenase family 3, subfamily A1	Aldh3a1	11670	-	3.6
	BG067445	karyopherin (importin) alpha 6	Kpna6	16650	-	2.5
	AI315343	low density lipoprotein receptor-related protein 2	Lrp2	14725	-	2.3
	AU041966	potassium voltage-gated channel, Isk-related subfamily, gene 3	Kcne3	57442	-	2.2
	AK011746	RNA (guanine-9-) methyltransferase domain containing 3	Rg9mtd3	69934	-	1.8
	AA008629	heat shock protein 8	Hspa8	15481	-	1.6
EE and TCDD common responses						
	BE630447	arginine-rich, mutated in early stage tumors	Armet	74840	9.8	3.2
	AU051534	dynactin 2	Dctn2	69654	6.5	2.2
	AI118427	inositol polyphosphate-5-phosphatase A	Inpp5a	212111	6.4	2.9
	AA058113	expressed in non-metastatic cells 1, protein	Nme1	18102	6.2	2.6
	BG076017	asparagine synthetase	Asns	27053	5.9	2.3
	BG073595	activating transcription factor 4	Atf4	11911	5.6	1.6
	AA033138	solute carrier family 25, member 5	Slc25a5	11740	5.3	2
	BG064598	proliferating cell nuclear antigen	Pcna	18538	4.8	1.9
	BG067893	alanyl-tRNA synthetase	Aars	234734	4.6	2.2
	AI838326	serine (or cysteine) proteinase inhibitor, clade H, member 1	Serpinh1	12406	3.7	2.5
	AA117848	ornithine decarboxylase, structural 1	Odc1	18263	3.4	2.2
	BG063138	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2	Spock2	94214	0.29	0.63
	AA207607	REST corepressor 3	Rcor3	214742	0.28	0.61
	AW550374	small nuclear RNA activating complex, polypeptide 2	Snapc2	102209	0.18	0.59

[a] Fold change values represent the maximum induction or repression observed in the time course. Blank values indicate the the transcript was not differentially expressed realtive to the time matched vehicle control

FIGURE 1A

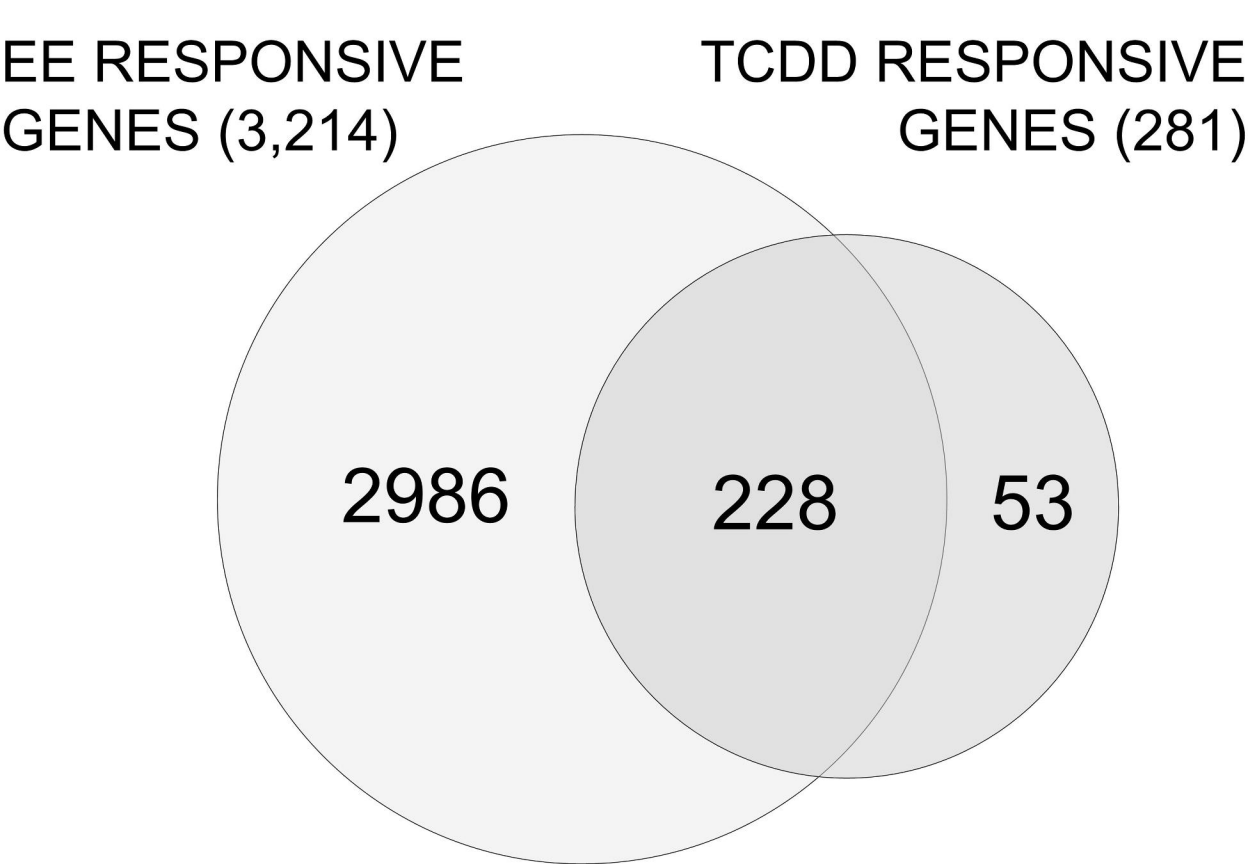

EE RESPONSIVE GENES (3,214)
TCDD RESPONSIVE GENES (281)
2986
228
53

Figure 1B

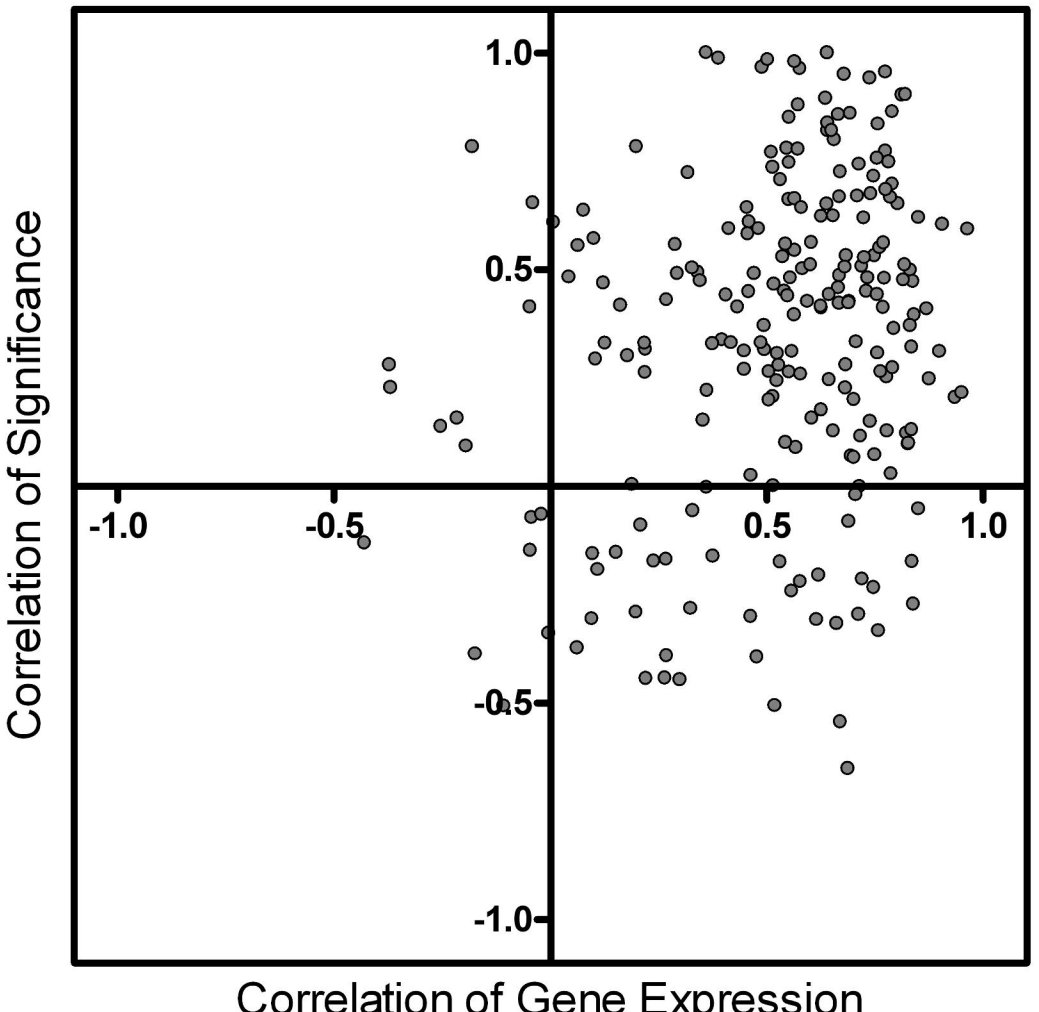

1.0
0.5
-1.0
-0.5
0.5
1.0
-0.5
-1.0
Correlation of Significance
Correlation of Gene Expression

Figure 1 C

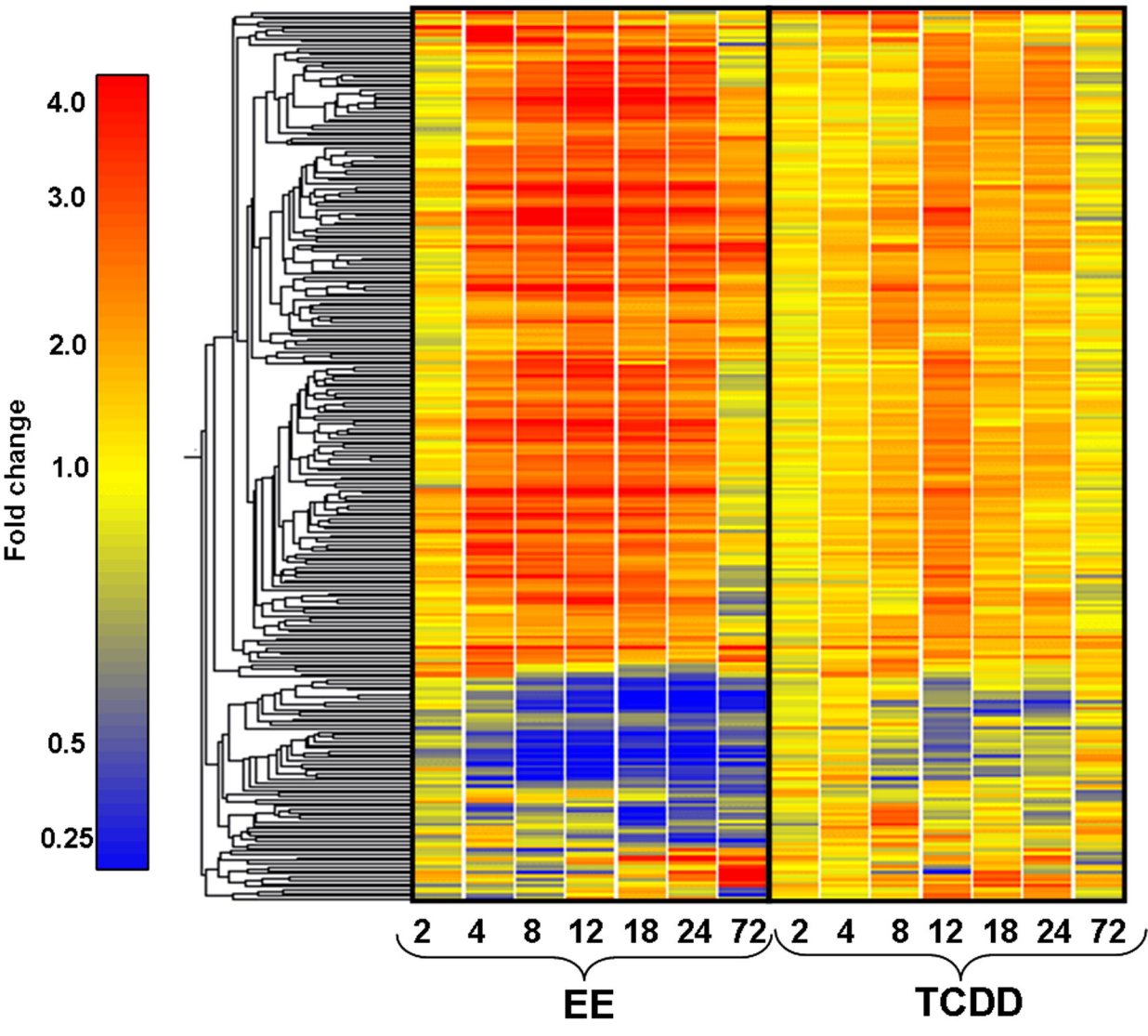

4.0
3.0
2.0
1.0
0.5
0.25
Fold change
2 4 8 12 18 24 72
2 4 8 12 18 24 72
EE
TCDD

Figure 2

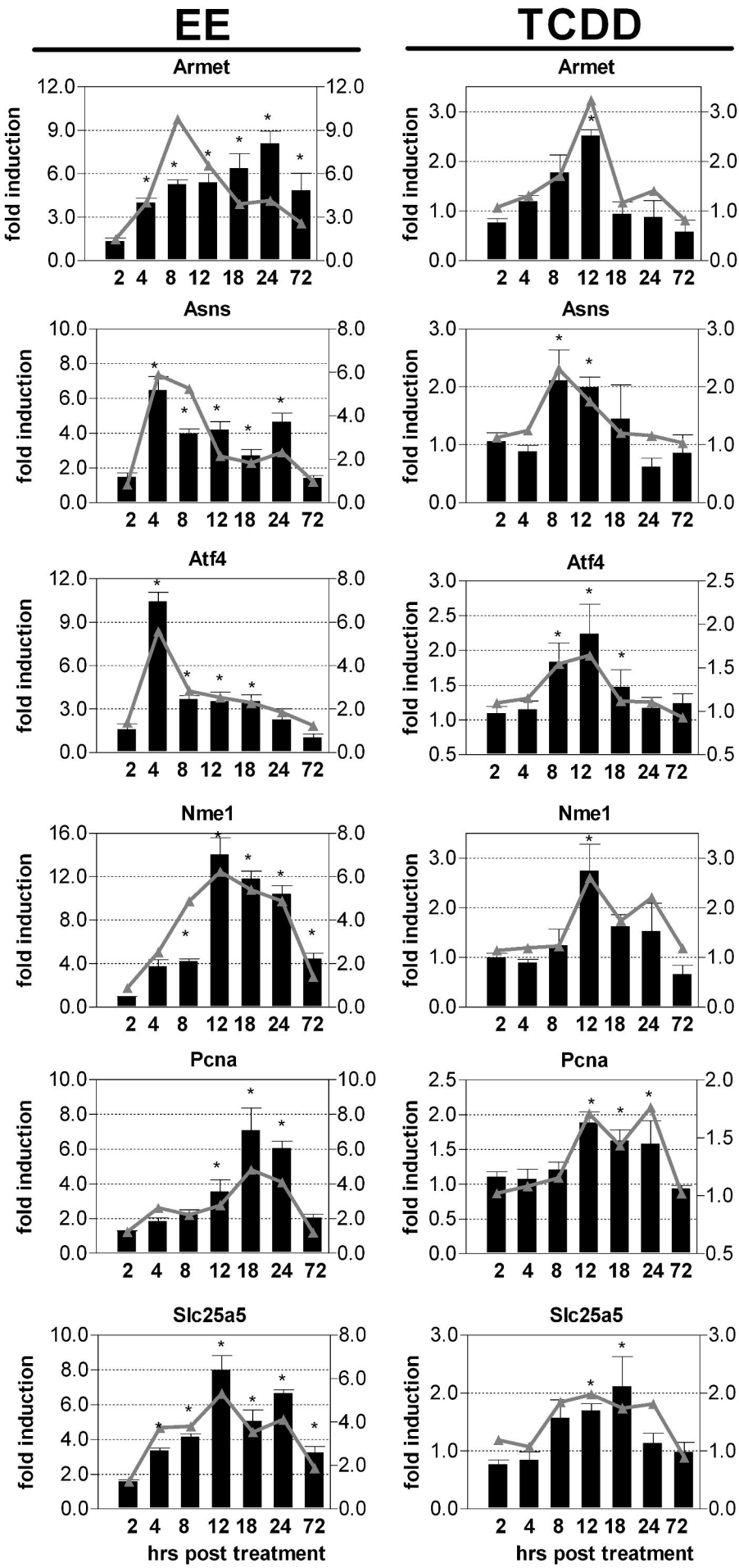

EE
TCDD
Armet
Asns
Atf4
Nme1
Pcna
Slc25a5
fold induction
2 4 8 12 18 24 72
hrs post treatment

Figure 3A

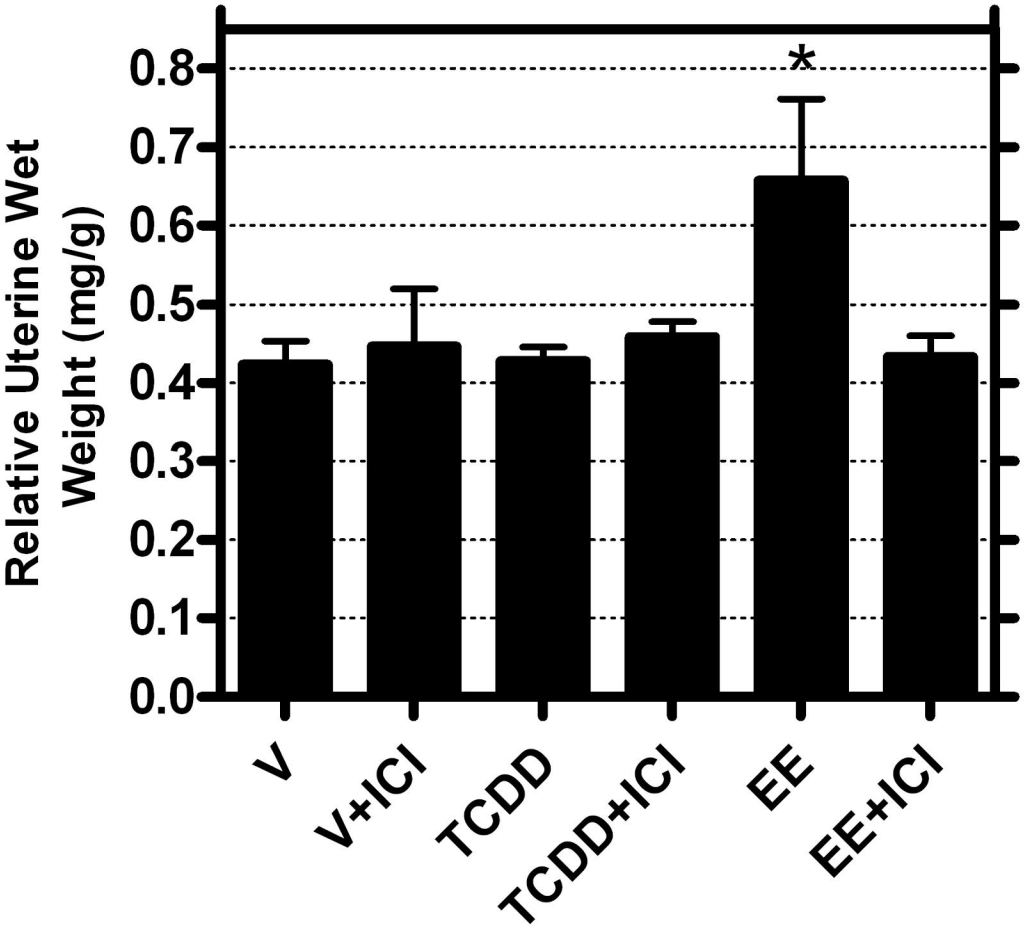

Relative Uterine Wet Weight (mg/g)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
*
V
V+ICI
TCDD
TCDD+ICI
EE
EE+ICI

Figure 3B

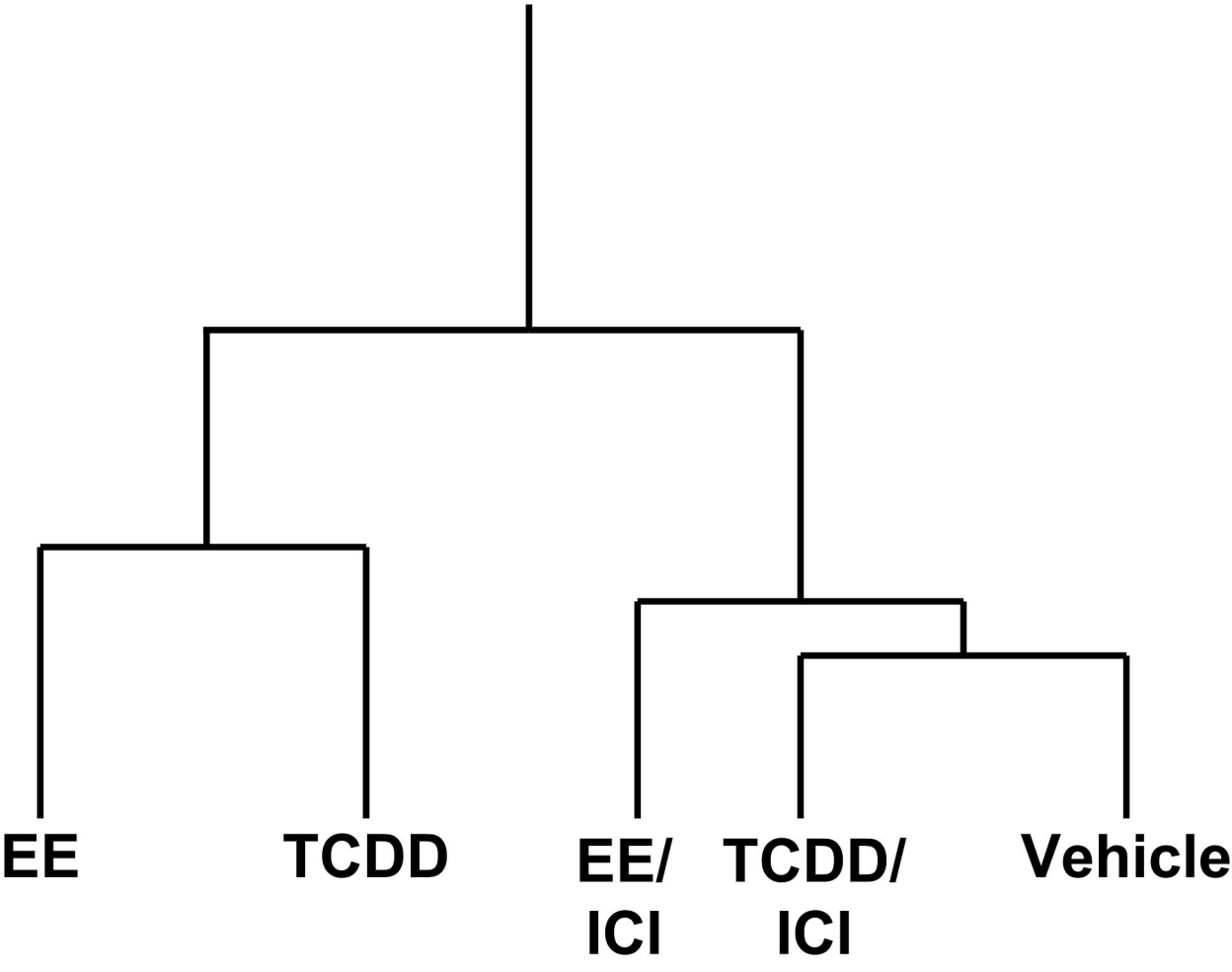
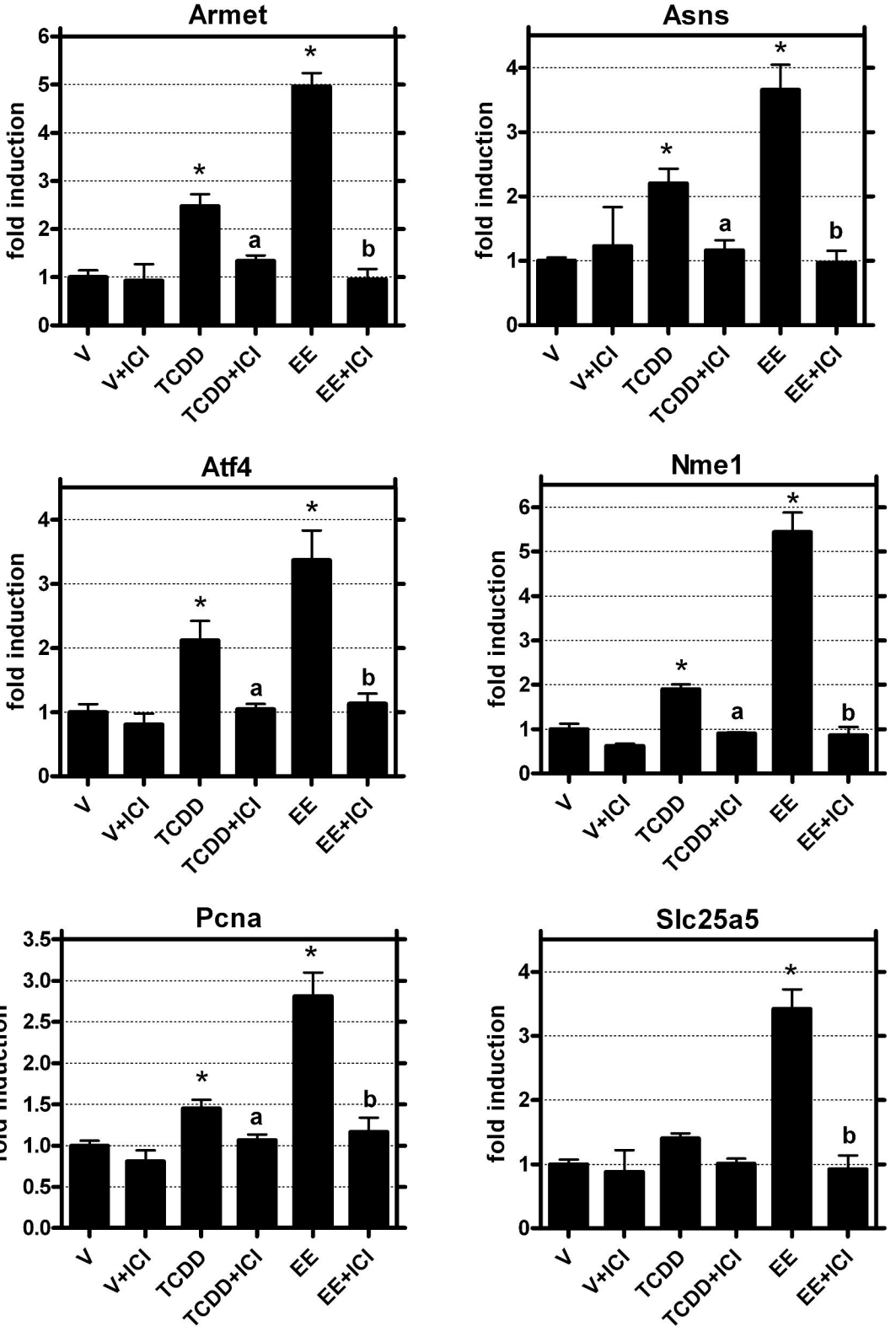


Figure 3C


Armet
Asns
Atf4
Nme1
Pcna
Slc25a5
fold induction
V
V+ICI
TCDD
TCDD+ICI
EE
EE+ICI
*
a
b