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***IN VIVO* EXAMINATION OF THE INHIBITORY EFFECTS OF 2, 3, 7, 8-TETRACHLORODIBENZO-*P*-DIOXIN ON ESTROGEN-MEDIATED GENE EXPRESSION RESPONSES**

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ABSTRACT

IN VIVO EXAMINATION OF THE INHIBITORY EFFECTS OF 2, 3, 7, 8-TETRACHLORODIBENZO-*P*-DIOXIN ON ESTROGEN-MEDIATED GENE EXPRESSION RESPONSES

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Environmental contaminants that act as estrogenic endocrine disruptors (EEDs) are of concern due to their potential to cause reproductive and developmental abnormalities as well as an increased incidence in hormone dependent cancers. Many of these effects are elicited through changes in gene expression mediated by the estrogen receptor (ER), a ligand-activated transcription factor. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is an EED which induces a wide array of toxic endpoints including lethality, hepatotoxicity and antiestrogenic effects which are mediated via changes in gene expression through activation of the aryl hydrocarbon receptor (AhR). Although the mechanisms by which the ER and AhR modulate gene expression are well established, how these changes result in the subsequent tissue-specific physiological and toxicological responses remains poorly understood. Furthermore, unlike most antiestrogenic compounds, the effects of TCDD are not mediated through binding to the ER but are hypothesized to involve ER/AhR crosstalk which involves the ability of the activated AhR to modulate ER signaling. A number of different crosstalk mechanisms have been proposed, one of which involves the inhibition of estrogen-mediated gene expression responses. However, only a limited number of inhibited responses have been identified *in vitro* which are unlikely to wholly account for the *in vivo* antiestrogenic effects. The

objective of this research was to develop a more comprehensive understanding of ethynyl estradiol- (EE) and TCDD-mediated gene expression responses *in vivo* through cDNA microarray analyses of hepatic and uterine tissues. Subsequently, cotreatment studies were performed to identify estrogen-mediated gene expression responses which are inhibited by TCDD to gain insights into this antiestrogenic mechanism.

Treatment of mice with EE or TCDD resulted in complex temporal- and dose-dependent hepatic gene expression responses which were consistent with known physiological and toxicological responses. These data further established the liver as an estrogen-responsive tissue and comparisons to uterine gene expression responses identified common, differential and tissue-specific effects and implicated the uterus as a more EE-responsive tissue. Hepatic gene expression responses to TCDD were related to physiological, histological and clinical responses and provided new insights into the potential mechanisms of TCDD-mediated hepatotoxicity. In the uterus, TCDD mediated an estrogen-like, ER-dependent gene expression response. Cotreatment studies revealed TCDD was able to inhibit EE-mediated physiological responses in the uterus which were associated with gene-specific inhibitory effects. Furthermore, these responses were independent of the estrogen-like gene expression responses to TCDD. Interestingly, neither the estrogen-like gene expression responses nor the inhibitory effects on estrogen-mediated gene expression responses were detected in hepatic tissues illustrating the diversity and tissue specificity of the responses to TCDD. These data indicate the dual nature of TCDD as a compound with both estrogenic and antiestrogenic potential which may explain its sex-, tissue- and age-specific toxicities and have expanded our overall knowledge of ER- and AhR-regulated gene expression responses and their cross-talk.

To my Wife and Family

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LIST OF ABBREVIATIONS

3MC	3-methylcholanthrene
ADME	adsorption, distribution, metabolism, and excretion
AhR	aryl hydrocarbon receptor
ALT	alanine aminotransferase
ANOVA	analysis of variance
AP-1	activating protein 1
ARNT	aryl hydrocarbon receptor nuclear translocator
ATF	activating transcription factor
bHLH	basic helix-loop-helix
BrdU	5-bromo-2-deoxyuridine
BUN	blood urea nitrogen
CHOL	cholesterol
CREA	creatinine
Cyp1a1	cytochrome P450, family 1, subfamily a, polypeptide 1
dNTP	deoxynucleotide triphosphate
DRE	dioxin response element
dUTP	deoxyuridine triphosphate
EBI	European Bioinformatics Institute
ECM	extracellular matrix
ED	endocrine disruptor
ED50	Effective Dose causing a 50% maximal response
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EDTA	Task Force on Endocrine Disruptor Testing and Assessment
EE	ethynyl estradiol
EED	estrogenic endocrine disruptor
EGF	epidermal growth factor
ER	estrogen receptor
ERE	estrogen response element
FDA	Food and Drug Administration
FFA	free fatty acids
GLU	glucose
GSH	glutathione
GST	glutathione S-transferase
HAH	halogenated aromatic hydrocarbon
HRGC	high resolution gas chromatograph
HRMS	high resolution mass spectrometer
HSP	heat shock protein

i.p.	intraperitoneal
IGF	insulin-like growth factor
LE	luminal epithelium
LEC	luminal epithelial cell
LECH	luminal epithelial cell height
MBT	model-based t-statistic
MGED	Microarray Gene Expression Data Society
MIAME	Minimum Information About a Microarray Experiment
MIAPE	Minimum Information About a Proteomics Experiment
NCBI	National Center for Biotechnology Information
NCE	new chemical entity
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NTC	no template control
OECD	Organization for Economic Co-operation and Development
PAS	PER–ARNT–SIM
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
ppt	parts per trillion
QRTPCR	quantitative real-time polymerase chain reaction
SMRS	Standard Metabolic Reporting Structures working group
Sp1	Sp1 transcription factor
TBIL	total bilirubin
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TG	triglycerides
TMVC	time-matched vehicle control
TSS	transcriptional start site
USEPA	United States Environmental Protection Agency

CHAPTER 1

REVIEW OF THE LITERATURE: ENDOCRINE DISRUPTION AND TCDD AS AN ESTROGENIC ENDOCRINE DISRUPTOR[1]

INTRODUCTION

Reports in the scientific literature and in the media have raised concerns about persistent environmental contaminants that have the potential to produce adverse effects in wildlife and human populations by interfering with the endocrine system [1-3]. These effects include reproductive and developmental abnormalities, increases in hormone dependent cancers and the overall decline in wildlife populations due to compromised reproductive fitness. These chemicals are referred to as endocrine disruptors (EDs) and act by mimicking or antagonizing endogenous hormones which are responsible for maintaining homeostasis, controlling normal development, and facilitating reproduction [1]. An endocrine disruptor can be more specifically defined as an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function [4].

The endocrine system participates in virtually all important functions of an organism including sexual differentiation, development of secondary sex characteristics, sexual maturation, and reproduction, in addition to facilitating growth, metabolism, digestion and cardiovascular functions. Due to the broad role of the endocrine system in organism homeostasis, there are increasing concerns regarding inappropriate exposure to EDs which has lead to the establishment of international efforts to develop screening and

[1] This dissertation contains color figures. All supplementary data can be found at: http://www.bch.msu.edu/~zacharet/

testing strategies to identify EDs and assess their potential adverse effects on human and wildlife health. These efforts include the United States Environmental Protection Agency's (USEPA) Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC [5]) and the Task Force on Endocrine Disruptor Testing and Assessment (EDTA) established by the Organization for Economic Co-operation and Development (OECD [6]). These groups are responsible for assessing the ability of pesticides, industrial chemicals and environmental contaminants to modulate or interfere with hormone systems in humans and wildlife. One class of EDs that has received considerable attention and research from these groups are the estrogenic endocrine disruptors (EEDs) which are compounds that inappropriately modulate estrogen signaling.

ESTROGEN AND ESTROGEN SIGNALING

The endogenous form of estrogen is the steroid hormone 17β-estradiol which is a key regulator of growth, differentiation and function in a wide array of target tissues including the male and female reproductive tracts, mammary gland, liver, prostate and the skeletal and cardiovascular systems [7]. Estrogens evoke their responses via binding to the estrogen receptor (ER), which is a ligand-activated transcription factor and a member of the nuclear receptor superfamily [8]. Two ER subtypes have been identified, ERα and ERβ, which are encoded by unique genes and exhibit distinct tissue expression patterns. ERα is more highly and widely expressed and can be found in the mammary gland, uterus, vagina, liver and kidney, while ERβ is predominantly expressed in the ovary, prostate, bladder, testis, spleen, hypothalamus and thymus [9, 10]. In the classical mechanism of ER signaling, the unliganded receptor is sequestered in the nucleus in a

multiprotein inhibitory complex with chaperones such as heat shock proteins (HSP) 90 and 70 (Figure 1) [11]. Ligand binding induces a conformational change in the ER which results in the dissociation of the chaperone proteins and the formation of ER homodimers which bind to specific DNA sequences known as estrogen response elements (EREs). The DNA bound homodimer then modulates gene transcription either directly through interactions with the basal transcriptional machinery or indirectly through interaction with cofactor proteins. The overall net effect on transcription of the targeted gene can be positive or negative depending on the cell and promoter context [12]. In addition to this classical mechanism, evidence for alternate ER signaling pathways has emerged and it is now accepted that ERs can regulate gene expression by a number of distinct mechanisms [13]. For example, the ER also mediates changes in transcription through ERE-independent mechanisms which involve interactions with Fos/Jun at AP-1 sites, Jun/ATF-2 at variant cyclic AMP response elements (CREs) and Sp1 at GC rich promoter regions [13]. In addition, growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF) can result in ligand-independent activation of the ER through alterations in the receptor's phophorylation state. Furthermore, emerging evidence suggests rapid non-genomic signaling events are mediated through putative membrane-bound ERs that activate kinase signaling pathways, a hypothesis supported by the recent identification of a G-protein coupled transmembrane ER [14, 15]. Regardless of the signaling mechanism, the ER is responsible for mediating the diverse physiological effects of estrogen which has made it a popular therapeutic target as well as a susceptible target for modulation by pharmaceuticals and environmental contaminants.

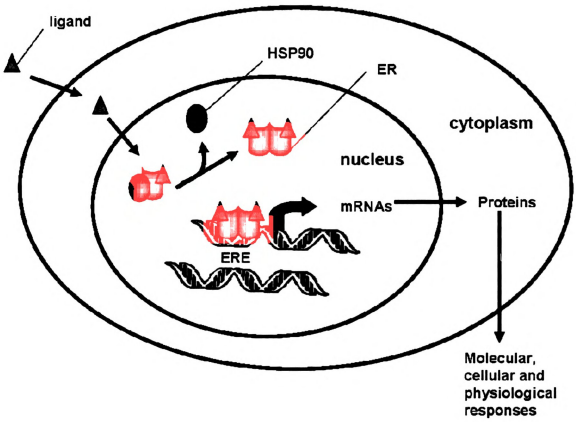


Figure 1. Classical Mechanism of Estrogen Signaling
In the absence of ligand the estrogen receptor (ER) is sequestered in the nucleus in a multiprotein inhibitory complex with proteins such as heat shock protein 90 (HSP90). Binding of ligand induces a conformational change in the ER which results in the dissociation of the inhibitory proteins and the formation of ER homodimers which bind to specific DNA sequences known as estrogen response elements (EREs). The DNA bound receptors then modulate gene transcription either directly through interactions with the basal transcriptional machinery or indirectly through interaction with cofactor proteins. The subsequent translation of the mRNAs yields proteins which are responsible for mediating the molecular, cellular and physiological effects of estrogen.

ESTROGENIC ENDOCRINE DISRUPTORS

Since as early as the 1930s, studies have shown that exogenous compounds are able to elicit and disrupt estrogenic responses *in vivo* [16, 17]. Compounds with these properties are now referred to as EEDs and encompass a wide variety of sources including industrial chemicals, pesticides, pharmaceuticals as well as natural components of plants (phytoestrogens) and fungi (mycoestrogens) [18]. Exposure to these compounds during sensitive periods of growth and development or prolonged chronic exposure is thought to be responsible for decreases in fertility and increases in the incidence of reproductive abnormalities and cancers in humans and wildlife[1-3]. To date, over 500 compounds with estrogenic potential have been identified but there are inadequate data assess their potential risks to human or wildlife health [19]. Many of these compounds are able to disrupt estrogen signaling because they possess a chemical structure similar to that of 17β-estradiol which allows them to bind the ER, albeit with affinities typically much less than that of estrogen[18]. As a result, many EEDs mediate agonistic or antagonistic estrogenic responses through the ER signaling mechanism [20-22]. However, EEDs may also elicit effects through non-ER mediated mechanisms including decreased steroid synthesis, increased estrogen metabolism or inhibition of estrogen mediated transcriptional responses [19, 22]. Chemicals that are thought to elicit endocrine disruptive effects via ER-independent mechanisms include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related halogenated aromatic hydrocarbons (HAHs).

TCDD AND THE ARYL HYDROCARBON RECEPTOR

TCDD and related compounds, which include polychlorinated -dibenzo-*p*-dioxins, -biphenyls and -dibenzofurans, represent a diverse group of widespread,

persistent and bioaccumulative environmental contaminants [23, 24]. They are formed as inadvertent by-products during the synthesis or use of chlorinated phenols, during thermal processes such as incineration and metal-processing and in the bleaching of paper pulp with free chlorine [25]. The relative amounts of these congeners produced during these processes depend on the production or incineration process and vary widely. TCDD is considered to be the most toxic HAH and has been used as a model compound to study their toxic mechanisms of action [25].

TCDD and related compounds elicit a diverse spectrum of toxic and biochemical responses in a tissue-, sex-, age- and species-specific manner [26]. These include lethality, wasting syndrome, chloracne, tumor promotion, reproductive and developmental toxicity, hepatotoxicity, immune suppression and the induction of various drug metabolizing enzymes [26]. Early research into the potential mechanisms of toxicity revealed that TCDD and 3-methylcholanthrene (3MC) both induced liver aryl-hydrocarbon hydroxylase (AHH) activity but with different potencies. Furthermore, this potency relationship was maintained across strains of mice which exhibited differential AHH induction activities [27]. Furthermore, examination of a series of halogenated aromatic compounds revealed a strong correlation between their structure-AHH induction and structure-toxicity relationships. Based on these data, Poland and co-workers hypothesized that a ligand binding protein or receptor was the initial cellular target of TCDD and subsequently, using radiolabeled-TCDD, identified the aryl-hydrocarbon receptor (AhR) in hepatic cytosol from C57BL/6 mice [28]. Additional research demonstrated that the AhR is present in multiple tissues and species and shared many characteristics with members of the nuclear hormone receptor superfamily as a ligand-

activated transcription factor [29] but is a member of the basic-helix-loop helix (bHLH) PAS family of transcription factors [30, 31].

One of the most well characterized responses to TCDD involves increases in AHH activity which is largely attributed to the induction of cytochrome P450 1a1 (Cyp1a1) transcript and protein levels. Using the human and mouse Cyp1a1 gene promoters as a model, the molecular mechanism of ligand-activated AhR-mediated transcriptional activation has been deciphered (Figure 2) [32]. In the absence of ligand, the AhR is sequestered in the cytoplasm bound to HSP90 and other chaperone proteins. Ligand binding results in a conformational change in the receptor, dissociation of chaperone proteins 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 binds specific DNA elements, termed dioxin response elements (DREs), leading to changes in gene expression [32]. Regulation of AhR-responsive genes is dependent on the promoter-, cell-, tissue- and species-context and involves interactions with a number of coactivators or corepressors of transcription [33, 34].

Many, if not all of the toxic effects of TCDD and related compounds are thought to be due to prolonged and inappropriate changes in gene expression. The obligatory involvement of the AhR/ARNT signaling pathway in mediating these effects is supported by studies demonstrating that mice with low affinity AhR alleles are less susceptible to toxicity [35] and AhR-null mice are resistant to the toxicity elicited by TCDD and related ligands [36-38]. Further support can be derived from studies utilizing mice possessing mutations in the AhR nuclear localization/DRE binding domain and mice harboring a

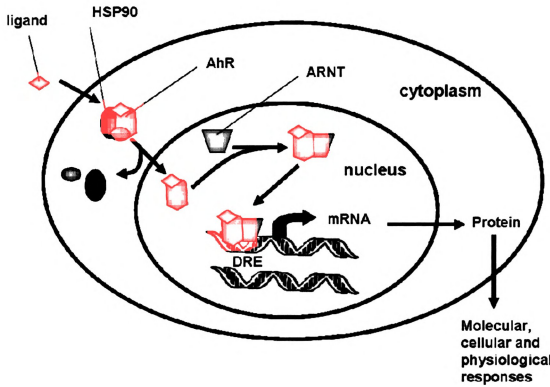


Figure 2. Aryl-Hydrocarbon Receptor Signaling Mechanism
In the absence of ligand the aryl-hydrocarbon receptor (AhR) is sequestered in the cytoplasm bound to heat shock protein 90 (HSP90) and other chaperone proteins. Ligand binding results in a conformational change in the receptor, dissociation of chaperone proteins 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 binds specific DNA elements, termed dioxin response elements (DREs), leading to changes in gene expression. The subsequent translation of the mRNAs yields proteins which are though to be responsible for mediating the toxic molecular, cellular and physiological responses to TCDD.

hypomorphic ARNT allele, which were both found to be less susceptible to TCDD-mediated toxicities [39, 40].

Although the mechanisms of AhR/ARNT-mediated changes in gene expression are fairly well established, how TCDD-elicited modulation of gene expression contributes to the observed adverse effects remains poorly understood. Well characterized AhR inducible genes are limited to various xenobiotic metabolizing enzymes including cytochrome P450s 1a1, 1a2 and 1b1, however, the significance of their induction in the observed adverse responses is questionable [41]. In addition to mediating the toxicity of TCDD, the AhR/ARNT signaling pathway also has important implications in development, differentiation and growth, as various developmental abnormalities in the liver, heart, thymus and immune system are noted in AhR null mice. These data also suggest that inappropriate activation of the AhR may interfere with normal developmental responses which may be due to modulation or crosstalk with other signaling pathways including those of the endocrine system.

TCDD AS AN ESTROGENIC ENDOCRINE DISRUPTOR

As part of its repertoire of toxic effects, TCDD is able to act as an endocrine disruptor through the modulation of thyroid-, retinoid-, androgen- and estrogen-mediated responses. With respect to estrogen, TCDD has been shown to exhibit both estrogenic and antiestrogenic consequences. The first study to identify the antiestrogenic effects was a two year chronic toxicity and oncogenicity study in rats [42]. TCDD induced sex-dependent increases in a number of tumors including hepatocellular carcinomas, stratified squamous cell carcinomas of the tongue and nasal turbinates and keratinizing squamous cell carcinomas of the lung. Interestingly, this study also noted decreases in the incidence

of several age-dependent spontaneous tumors in endocrine organs and the reproductive tract of female rats. Specifically, the incidence of both mammary and uterine tumors was decreased suggesting that TCDD inhibits the development of estrogen-dependent tumors. These results initiated a number of research projects aimed at characterizing the antiestrogenic effects of TCDD and the potential cross-talk between ER and AhR signaling pathways. This has progressed into research towards the development selective-AhR-modulators (SAhRMs) for the treatment and prevention of estrogen-dependent tumors [43].

In Vivo Antiestrogenic Responses

Rodent Studies

In vivo research into the antiestrogenic effects of TCDD has primarily utilized rat and mouse models with a focus on the mammary gland, uterus and ovary as target organs, although effects on other tissues, such as the liver [44, 45] and brain [46, 47], have also been examined. Estrogen plays an important role in the development and differentiation of these organs and each express both the ER and AhR making them sensitive targets for this cross-talk.

Uterus

Estrogen plays an important role in uterine development and induces a complex physiological response which involves induction of uterine wet weight, DNA synthesis and extensive alterations in gene expression [48-50]. TCDD impairs normal uterine development in mice [51] and dose-dependently inhibits estrogen-induced increases in uterine wet weight in both rats and mice which is accompanied by histological alterations in the uterine epithelium [51-53]. In addition, TCDD decreases uterine ER levels in both

mice and rats [45, 54, 55] and inhibits estrogen-induced expression of genes implicated in mediating uterine growth such as FBJ osteosarcoma oncogene (Fos) and epidermal growth factor receptor (Egfr) [53, 56]. Decreases in estrogen-induced epidermal growth factor and progesterone receptor binding activity have also been reported [54, 56]. More recent studies have illustrated that TCDD is able to inhibit estrogen induced uterine epithelial labeling index and induction of lactoferrin and cyclins A2, B1 and D2 in an AhR-dependent manner [57, 58].

Mammary Gland

In addition to inhibiting age-dependent mammary tumors in rats, TCDD inhibits tumor growth in carcinogen-induced mammary tumors and in athymic nude mice bearing human breast cancer cell xenografts [59-61]. Furthermore, gestational and lactational exposure of rats to TCDD impairs mammary gland development in dams and their offspring and alters mammary ER expression levels [62, 63]. However, the altered mammary development is not thought to be due to TCDD's antiestrogenic responses as the glands were still able to differentiate upon exposure to estrogen [62]. These results suggest that the antiestrogenic properties of TCDD on the mammary gland may be limited to its inhibition of tumor growth and development [64]. More recent studies have demonstrated that prenatal exposure to TCDD can actually increase the incidence of mammary cancer in rats [65], indicating that the timing of exposure may be critical to the observed outcome.

Ovary

Estrogen plays an important paracrine role in mediating ovulation, and as a result the anovulatory effects of TCDD are thought to be mediated by its antiestrogenic

properties [66]. Similar to its effects in the uterus, TCDD decreases ovarian ER mRNA levels in mice [44] and ER DNA binding activity in rats [46]. TCDD also induces decreases in ovarian weight [64] and blocks ovulation in immature rats, a response that can be alleviated by estrogen pretreatment [67]. Furthermore, TCDD delays onset of puberty and induces the early onset of reproductive senescence which may be related to alterations in ovarian function [68, 69]. Although research suggests that these effects may be mediated indirectly through interruption of the hypothalamus-pituitary-ovary axis, direct antiestrogenic effects on the ovaries cannot be dismissed [66].

Other Species

In addition to rodent species, isolated antiestrogenic effects of TCDD have been noted in fish and monkeys. In fish, TCDD inhibits estrogen-mediated increases in hepatic ER and vitellogenin, both of which play integral roles in reproduction and development [70]. In rhesus monkeys, TCDD exposure for prolonged periods caused reproductive dysfunction in combination with decreased serum estradiol and progesterone [71].

Humans

Human evidence for the antiestrogenic effects of TCDD can be drawn from reports on the decreased incidence of mammary tumors in women accidentally exposed to high levels of TCDD in Seveso, Italy [72]. Furthermore, epidemiological studies have shown that cigarette smoking may protect against the development of uterine cancer, a response which has been suggested to involve the presence of AhR active compounds in cigarette smoke condensate [73, 74].

***In Vitro* Antiestrogenic Responses**

Numerous *in vitro* studies have been conducted to explore the antiestrogenic effects of TCDD, most of which have utilized the human MCF-7 breast cancer cell line. The first reports of the antiestrogenic effects in these cells indicated that TCDD inhibited estrogen-induced tissue plasminogen activator secretion as well as multicellular foci formation in the absence of altered ER levels [75, 76]. Since these publications, TCDD has been shown to inhibit the levels or activities of numerous estrogen regulated genes and proteins in MCF-7 cells including Fos [77], heat shock protein 27 (Hsp27) [78], trefoil factor 1 (Tff1, also known as pS2) [79, 80], cathepsin D (Ctsd) [81], prolactin receptor (Prlr) [82]and progesterone receptor (Pgr) [83]. For a number of these genes, TCDD inhibited estrogen-induced reporter gene activity, suggesting direct inhibitory effects on transcription. Estrogen also induces a number of genes/proteins involved in cell cycle regulation which have been shown to mediate G1 to S phase transitions and cell proliferation [84]. TCDD is also able to inhibit these responses which is thought to be mediated via alterations in the phosphorylation status of retinoblastoma protein (Rb) and in the level and activities of estrogen-induced cyclin dependent kinases [84, 85].

The antiestrogenic effects of TCDD are not unique to MCF-7 cells. TCDD also inhibits estrogen induced cell cycle proliferation and transcriptional activation in endometrial cancer cell lines [86, 87]. In ovarian cancer cells, estrogen-mediated increases in proliferation, gene expression and protein secretion were all inhibited upon cotreatment with TCDD [88, 89]. Collectively, these results indicate that *in vitro* cell lines derived from tissues that are vulnerable to the antiestrogenic effects of TCDD retain this susceptibility in culture. However, the extension and relevance of many of these *in*

vitro responses, such as inhibition of gene expression and alterations in cyclin dependent kinase activities, have yet to be verified *in vivo*.

Mechanisms for TCDD's AhR-mediated Antiestrogenic Effects

Mechanistic studies in continuous cell lines combined with the observed *in vivo* responses have led to the development of a number of hypotheses for the mechanism by which TCDD mediates its antiestrogenic effects. These include increased estrogen metabolism, decreases in estrogen receptor levels, induction of inhibitory factors, competition for coregulators, and inhibition of estrogen-mediated gene expression responses [43]. Supporting evidence and limitations for each of these mechanisms are presented below.

Increased Estrogen Metabolism

The most well documented AhR-mediated effect of TCDD is the induction of phase I metabolizing enzymes including the cytochrome P450 isoforms 1a1, 1a2 and 1b1 [90]. These enzymes are involved in the oxidative metabolism of numerous exogenous and endogenous compounds, including estrogens [91], and as a result, it has been hypothesized that increased estrogen metabolism is responsible for the antiestrogenic effects of TCDD [75, 92]. *In vitro* studies have demonstrated that TCDD treatment increases estrogen metabolism in MCF-7 cells which coincides with the inhibition of estrogen-mediated multicellular foci formation [93-95]. However, although increased metabolism may be a contributing factor, compelling evidence indicates that this alone is not sufficient to mediate the antiestrogenic responses. For example, TCDD inhibits estrogen-induced transcriptional responses within 30-60 minutes, a response that precedes the induction of the cytochrome-P450 enzymes and estrogen metabolism [81].

Furthermore, *in vivo* studies have been unable to corroborate *in vitro* results. Pregnant rats administered TCDD exhibited decreased hepatic estrogen metabolism and no effects on serum estrogen levels [96]. Dose-response studies in mice revealed no effect on serum estrogen levels over a 21 day period after TCDD administration, despite the dramatic induction of cytochrome P450 enzymes [45]. A recent study in ovariectomized rats found that pretreatment with TCDD did not increase the clearance of estrogen when compared to vehicle controls [97]. Collectively, these results suggest that increased oxidative metabolism of estrogen cannot be solely responsible for the antiestrogenic effects of TCDD.

Decreased Estrogen Receptor Levels

The ER mediates many of the effects of estrogen; therefore down-regulation of receptor levels has been extensively examined as a potential mechanism for the antiestrogenic effects of TCDD. Results from research investigating the effects of TCDD on both ER transcript and protein levels are inconsistent and controversial. Early studies in rats revealed that TCDD reduced hepatic and uterine levels of the ER by more than 50% [54, 55]. Subsequent studies in the rat and guinea pig corroborated TCDD-mediated decreases in heptatic ER but did not detect alterations in uterine receptor levels [98]. More recent studies with immature rats reported that TCDD had no effect on the levels of hepatic or uterine ER [99]. In mice, a thirteen week repeated dose study revealed that TCDD did not alter hepatic or uterine ER levels [100]. In contrast, a shorter time course study in mice revealed that TCDD decreased hepatic and uterine ER levels by 40% within one day after administration which persisted for up to 14 days [45]. However, this down-regulation was not observed in the uteri of ovariectomized mice from the same

study, suggesting that if TCDD does down regulate uterine ER, it is likely due to indirect effects on the ovaries or the hypothalamus-pituitary-ovary axis.

Investigations into potential mechanisms involved in mediating the putative decreases in ER protein levels revealed that TCDD resulted in a 60, 50 and 30% reduction in mouse ER transcript levels in ovarian, uterine and hepatic tissues, respectively [44, 101]. In contrast, another study reported that TCDD had no effect on ER transcript levels in the rat liver or uterus [99]. Subsequent studies identified two core DREs near the transcriptional start site (TSS) of the ER which could bind AhR:ARNT complexes and were hypothesized to inhibit transcription of the ER [102]. However, studies in human MCF-7 cells and murine Hepa1c1c7 cells indicated that TCDD down regulated ER protein levels without affecting its transcript levels or rate of transcription [103]. Further *in vitro* investigations found that TCDD was able to inhibit estrogen-mediated transcriptional responses but over-expression of the ER was unable to alleviate the inhibitory effects, suggesting that effects on ER levels are not involved [104].

Recently, researchers have proposed that TCDD mediates down regulation of the ER through the AhR mediated activation of proteosomes [22, 105]. In this model, activation of the AhR by TCDD in MCF-7 cells enhances ubiquitination of the ER and its subsequent proteosome dependent degradation, an event that can be inhibited by proteosome inhibitors. This represents a novel non-genomic pathway in which ER degradation is dependent on AhR activation but independent of gene expression [43], however, this mechanism has yet to be verified in other cell types and *in vivo*.

The above results indicate that the down regulation of ER levels as a mechanism for the antiestrogenic effects of TCDD remains questionable. Inconsistencies between

these studies may be due to treatment duration, age- or species-specific effects which are known to affect the toxic outcomes to TCDD. These discrepancies indicate that additional research is required to more definitively determine the role of this mechanism.

Induction of Inhibitory Factors

As TCDD is thought to mediate its toxic effects via activation of transcription, some researchers have proposed that the antiestrogenic response is mediated via a TCDD induced inhibitory protein. Using a human ovarian cell line, Rogers and Denison studied the antiestrogenic effects of TCDD by measuring alterations in ERE luciferase reporter gene activity and transcript levels of Tff1, a known estrogen responsive gene [89]. They reported that the antiestrogenic effect of TCDD on these endpoints was not due to decreased estrogen receptor levels, increased estrogen metabolism or competition for coregulators. However, the antiestrogenic effects were blocked by treatment with cycloheximide, an inhibitor of protein synthesis, suggesting that the induction of an inhibitory factor that was responsible for TCDD's effects. However, with a limited understanding of the full spectrum of gene expression responses that are induced by TCDD, the authors were unable to speculate on the identity of the proposed inhibitory factor. These data further indicate the importance of researching the molecular targets of the AhR in an effort to identify the putative inhibitory factors.

Competition for Chaperones and Nuclear Coregulatory Proteins

The ER and AhR interact with a common chaperone and several common nuclear coactivators and corepressors of transcription. As a result, it has been proposed that altered availability or squelching of these proteins could contribute to ER/AhR crosstalk. Both the AhR and ER in their unliganded states are bound to the cellular chaperone

Hsp90 which is responsible for the proper folding and stabilization of the receptor in an inactive conformation. Therefore, the release of Hsp90 upon ligand binding to the AhR could decrease the activation potential of the ER by increasing the pool of unbound Hsp90 which could subsequently be inhibitory to ER signaling [106]. However, investigation of this hypothesis revealed that over-expression of Hsp90 was unable to block the induction of estrogen responsive genes [106]. Furthermore, it is generally accepted that cellular pools of Hsp90 are sufficiently large and availability of this protein is unlikely to be limiting [107].

The ER and AhR also interact with a number of similar coregulators of transcription including nuclear factor-1 (NF1) [108], estrogen receptor associating protein 140 (ERAP140) [33], silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) [33], receptor interacting protein 140 (RIP140) [109], and steroid receptor coactivator-1 (SRC1) [110]. These proteins contribute to alterations in transcription by acting as bridging factors between the AhR or ER and the basal transcriptional machinery or by altering chromatin structure via alterations in histone acetylation. As a result, competition for this common pool of cofactors has been proposed as a mechanism for the attenuation of ER-mediated gene expression responses by TCDD [108, 111]. Studies which over-expressed these nuclear co-activators to alleviate the suspected limiting pool revealed that this had no effect on the ER/AhR crosstalk, thereby suggesting that squelching may not play a role [89, 108]. However, these studies only examined a limited set of coactivators which may not represent the entire spectrum that are required for transcription by the ER or AhR. Furthermore, if squelching does contribute to AhR/ER crosstalk it will likely depend on the relative cell context-dependent expression

of the various coregulatory factors as well as the relative expression and activity of the receptors themselves.

Inhibition of Estrogen Mediated Gene Expression Responses

Studies using MCF-7 cells have demonstrated that TCDD is able to inhibit the induction of a number of estrogen regulated genes. Furthermore, the rapid nature of this response suggests that the inhibition may be mediated through direct interactions or interference at estrogen responsive promoters. Subsequent research revealed that the activated AhR/ARNT complex was able to bind to DREs in the promoters of these genes. Interestingly, binding alone did not influence gene expression, however it was able to block or disrupt normal ER actions to inhibit gene expression. DREs mediating these effects are referred to as inhibitory-DREs (iDREs) and have been identified in the proximal promoters of four estrogen inducible genes in MCF-7 cells, namely Ctsd, Tff1, Fos and Hsp27. In addition, the mechanism of action for each of these iDREs is gene-promoter specific.

The first iDRE was identifed in the Ctsd promoter using human MCF-7 cells [81]. TCDD was able to inhibit estrogen induced Ctsd mRNA levels, the rate of gene transcription and protein levels. Furthermore, the inhibitory effect was observed within 30 minutes after TCDD treatment. Cloning of the Ctsd promoter into a reporter construct and subsequent deletion analysis revealed estrogen mediated induction of was dependent on ERE and Sp1 binding sites in the -199 to -165 bp region upstream of the TSS. A DRE core element was identified at the -181 to -175 region, between the Sp1 and ER binding sites, which was shown to bind AhR/ARNT and block the formation of a ER/Sp1 complex [81].

Estrogen-mediated induction of Tff1 is also inhibited by TCDD through an iDRE mechanism. Estrogen mediates Tff1 induction through an ERE which is dependent on an upstream AP1 site. However, the AP1 site overlaps with an iDRE which when bound by the AhR/ARNT complex blocks appropriate assembly of AP1 and ER components at the promoter [80]. Similarly, an iDRE in the Fos promoter overlaps with a Sp1 binding site necessary for estrogen-mediated gene induction [77]. Estrogen-elicited induction of Hsp27 is dependent on a Sp1 and ERE-half site 100 bp upstream of the TSS. An iDRE is located downstream of these elements and is thought to interfere with the assembly of the basal transcriptional machinery [78].

Although these iDREs have been identified in MCF-7 cells they have yet to be characterized in other human or rodent cell lines. Furthermore, the ability of TCDD to inhibit these gene expression responses *in vivo* has not been investigated. Additional research in these models will further solidify the importance of this mechanism in mediating the antiestrogenic effects of TCDD.

ESTROGENIC ACTIVITY OF TCDD

Although much of the research surrounding the endocrine disrupting effects of TCDD have centered on its antiestrogenic effects, accumulating evidence suggests that TCDD also possesses estrogenic potential. This is supported by studies which have indicated that TCDD increases the DNA-binding activity of the ER, independent of estrogen, in the rat uterus [46] and resulted in an estrogen-like G_0/G_1 to S-phase transition and mitogenic effects in MCF-7 cells [112]. Furthermore, TCDD induces the AhR to interact directly with ERα in the absence of estrogen [105, 113]. Additional studies have also shown that ER and AhR interact [114, 115] and ligand-activated AhR/ARNT

associates with the unliganded ER to form a functional complex that binds EREs [116]. *In vivo* studies with the AhR ligand 3MC 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 [116]. However, these effects have been met with much skepticism as 3MC, or one of its metabolites, has been reported to activate the ER independent of AhR [117]. Additional evidence for the estrogenic potential can be found in studies reporting TCDD-mediated induction of estrogen dependent tumors in rats [65, 118], and increases the incidence of endometriosis in laboratory animals and in women with high body burdens of TCDD [119-122]. To date, most studies have focused on the antiestrogenic activities of TCDD in the presence of estrogen, and therefore the potential estrogenic activities of TCDD have been under-reported. Additional, more focused research is required to better characterize the estrogenic actions of TCDD and its mechanisms.

IS THE CROSSTALK BI-DIRECTIONAL?

Although much research has focused on the ability of TCDD to inhibit estrogen signaling, there has also been research to suggest that estrogen is able to influence TCDD-mediated changes in gene expression, thereby indicating that the crosstalk may be bi-directional. The first publication to suggest this bi-directional nature reported that estrogen was able to inhibit TCDD-mediated transactivation of the AhR as evidenced by decreased Cyp1a1 induction and decreased AhR binding to DRE sequences in Hepa1c1c7 and MCF-7 cell-lines [123]. However, a subsequent report was unable to repeat these results and claimed that estrogen does not affect AhR responsiveness in these

cell-lines [124]. Since the publication of these data, the influence of estrogen on AhR-mediated responses have continued to be controversial with some reports indicating its suppressive effects [125-127] while others have indicated that estrogen itself is required for maintaining or enhancing AhR responsiveness of cells in culture [128] and in the rat liver [129]. Additional reports have suggested that a functional ER is required for AhR-responsiveness in human uterine endometrial carcinoma cells, irrespective of the presence or absence of estrogen [130]. ER negative breast cancer cells do not exhibit the characteristic gene expression responses to TCDD, however, these were restored upon introduction of exogenous ER [131].

More recent studies that have attempted to shed light on the bi-directional nature of this cross-talk have explored the ability of ER to directly interact with both the AhR and ARNT [115, 116, 132]. Beischlag and Perdew [115] have shown that both the AhR and ARNT interact directly with ERα and estrogen represses TCDD-mediated Cyp1a1 induction in human MCF-7 cells. Furthermore, they illustrated that ERα is actively recruited to the Cyp1a1 promoter in response to treatment with both estrogen and TCDD and concluded that the activated ER is able to act as a transrepressor of AhR-dependent gene regulation. Concurrently, Matthews and co-workers [132] also reported that TCDD treatment resulted in the recruitment of ERα to the Cyp1a1 promoter, a response that was elevated by cotreatment with estrogen. Complementary studies in human HuH7 hepatoma cells indicated that ERα enhanced AhR-mediated transcriptional responses. Collectively, these studies indicate that ERα is recruited to the Cyp1a1 promoter but report contradictory findings regarding its co-regulator function as an enhancer or

repressor of AhR activity. This indicates that the outcome of these interactions are likely dependent on a number of factors including species, cell line and culture conditions.

Although the above data suggest that estrogen and the ER are able to influence TCDD mediated gene expression and AhR signaling, they are limited by the fact that each study has primarily focused on the response of a single gene, namely Cyp1a1. Furthermore, the majority of the data have been collected utilizing *in vitro* cell culture systems which may not accurately represent *in vivo* responses. Examination of additional AhR-regulated genes in complementary *in vitro* and *in vivo* models is necessary to more fully elucidate the potential species and tissue specificity of this putative bi-directional crosstalk.

CONCLUSIONS

A wide array of TCDD-mediated antiestrogenic responses have been observed in the rodent mammary gland, uterus and ovary and in human breast and endometrial cancer cell lines. These effects are thought to involve ER/AhR crosstalk through a number of different mechanisms including the inhibition of estrogen-mediated gene expression responses. More recent data suggest this crosstalk is bi-directional and that TCDD, in the absence of estrogen, is able to elicit estrogenic responses. These data indicate the complexity and multifaceted nature of the crosstalk which will requires additional research in order to develop a better understanding of the toxicities and risks associated with exposure to TCDD and related HAHs. Furthermore, characterization of these antiestrogenic mechanisms will increase our understanding of estrogen signaling pathways and provide valuable insights toward the development of novel therapeutic strategies for the treatment and prevention of estrogen-dependent cancers.

CHAPTER 2

RATIONALE, HYPOTHESIS AND SPECIFIC AIMS

RATIONALE

Environmental contaminants that act as estrogenic endocrine disruptors (EEDs) are of concern due to their potential to cause reproductive and developmental abnormalities as well as an increased incidence in hormone dependent cancers in both humans and wildlife. Many of these contaminants appear to utilize the classical estrogen receptor (ER) mediated mechanism to elicit their disruptive effects in either an antagonistic or agonistic manner. However, certain environmental contaminants are able to interfere with estrogen signaling through ER-independent mechanisms. One of these compounds is the persistent environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

TCDD is a ubiquitous environmental contaminant that elicits a broad spectrum of toxic and biochemical responses in a tissue-, sex-, age- and species-specific manner that include a wasting syndrome, tumor promotion, teratogenesis, hepatotoxicity and modulation of endocrine systems. Many, if not all, of these effects are due to alterations in gene expression mediated via the activation of the aryl-hydrocarbon receptor (AhR) a ligand activated transcription factor. Despite years of research the actual mechanism responsible for the estrogenic endocrine disrupting effects of this toxicant remain largely uncharacterized. One of the proposed mechanisms involves cross-talk between ER and AhR signaling at the gene expression level. The objective of this study is to further characterize this gene expression cross-talk in an *in vivo* model using a comprehensive

microarray approach. The use of a genome wide analysis approach is justified because both estrogen and TCDD primarily elicit their physiological or toxicological effects through alterations in gene expression.

Investigation of AhR/ER crosstalk is of interest due to its importance in both environmental risk assessment as well as its potential implications for the identification of novel therapeutic targets for the treatment and prevention of breast cancer. However, only a limited number of genes that are subjected to ER/AhR cross-talk have been identified and are unlikely to wholly account for the physiological antiestrogenic effects of TCDD. Furthermore, the majority of the research conducted in this area has utilized *in vitro* models; however, in order to gain a full understanding of ER/AhR cross-talk, a comprehensive *in vivo* assessment of global changes in gene expression is required. It is expected that the cross-talk that exists between these agents will be characterized at the gene expression level through the use of cDNA microarrays.

HYPOTHESIS

The *in vivo* antiestrogenic effects of TCDD are mediated through ER/AhR cross-talk which is associated with inhibition of estrogen-mediated gene expression responses in estrogen responsive tissues.

SPECIFIC AIMS

Prior to the in-depth investigation of ER/AhR cross talk, a comprehensive understanding of the global gene expression responses mediated by estrogen and TCDD alone is required. Therefore, the aims that will be used to test this hypothesis involve the

use of cDNA microarrays and a comprehensive and comparative analysis of gene expression that will:

1. Establish baseline quantitative data on the *in vivo* effects of ethynyl estradiol on global gene expression in the mouse liver.
2. Establish baseline quantitative data on the *in vivo* effects of TCDD on global gene expression in the mouse liver
3. Characterize the *in vivo* effects of ethynyl estradiol and TCDD co-treatment on global gene expression responses in the mouse liver.
4. Establish baseline quantitative data on the *in vivo* effects of TCDD on global gene expression in the murine uterus.
5. Characterize the *in vivo* effects of ethynyl estradiol and TCDD co-treatment on global gene expression responses in the mouse uterus.

CHAPTER 3

TEMPORAL AND DOSE-DEPENDENT HEPATIC GENE EXPRESSION CHANGES IN IMMATURE OVARIECTOMIZED MICE FOLLOWING EXPOSURE TO ETHYNYL ESTRADIOL[2].

ABSTRACT

Temporal- and dose-dependent changes in hepatic gene expression were examined in immature ovariectomized C57BL/6 mice gavaged with ethynyl estradiol (EE), an orally active estrogen. For temporal analysis, mice were gavaged every 24 hrs for 3 days with 100 μg/kg EE or vehicle and liver samples were collected at 2, 4, 8, 12, 24 and 72 hrs. Gene expression was monitored using custom cDNA microarrays containing 3067 genes/ESTs of which 393 exhibited a change at one or more time points. Functional gene annotation extracted from public databases associated temporal gene expression changes with growth and proliferation, cytoskeletal and extracellular matrix responses, microtubule based processes, oxidative metabolism and stress, and lipid metabolism and transport. In the dose-response study, hepatic samples were collected 24 hrs following treatment with 0, 0.1, 1, 10, 100 or 250 μg/kg EE. Thirty-nine of the 79 genes identified as differentially regulated at 24 hr in the time course study exhibited a dose response relationship with an average ED_{50} value of 47 ± 3.5 μg/kg. Comparative analysis indicated that many of the identified temporal and dose-dependent hepatic responses are similar to EE-induced uterine responses reported in the literature and in a companion study using the same animals. Results from these studies confirm that the

[2] Data contained in this chapter have been published in: Boverhof, D. R., Fertuck, K. C., Burgoon, L. D., Eckel, J. E., Gennings, C., and Zacharewski, T. R. (2004). Temporal- and dose-dependent hepatic gene expression changes in immature ovariectomized mice following exposure to ethynyl estradiol. *Carcinogenesis* **25**, 1277-91.

liver is a highly estrogen responsive tissue that exhibits a number of common responses shared with the uterus as well as distinct estrogen mediated profiles. These data will further aid in the elucidation of the mechanisms of action of estrogens in the liver as well as in other classical and non-classical estrogen responsive tissues.

INTRODUCTION

Although estrogens are among the most widely prescribed pharmacological agents [133], many aspects of their action following receptor binding remain unresolved. Published research has primarily focused on the biological effects of estrogens on classical estrogen responsive tissues such as the uterus, mammary gland and ovary [134, 135]. However, estrogens also exert profound effects on other non-classical estrogen responsive tissues including the kidney, bone, and liver [136].

Compounds with estrogenic activity, which include endogenous steroids, natural products, industrial chemicals, environmental contaminants and pharmaceutical agents [137], elicit a broad spectrum of physiologic and toxic effects in the liver [135, 138]. Many of these responses are mediated by α and β estrogen receptor (ER) isoforms. The liver predominantly expresses ERα [10], although low levels of ERβ have been reported [139]. In the classic signaling model, estrogen diffuses into cells and binds to the nuclear localized ER resulting in the dissociation of associated proteins. Homodimers of liganded complexes then act as transcription factors by binding to specific estrogen response element (ERE) sequences in the regulatory regions of target genes, evoking a wide range of transcriptional responses. In addition, rapid non-genomic responses mediated by membrane ERs, also stimulate signal transduction pathways [140].

Although the transcriptional actions of estrogen in reproductive tissues are well characterized, less is known about estrogenic responses in the liver. Recent reports utilizing genetically engineered mice that possess ERE-regulated reporter genes indicate that the liver is one of the most estrogen responsive tissues [141, 142]. While not considered a classical target tissue, accumulating evidence indicates that the liver mounts a multifaceted transcriptional and translational response that includes increased DNA synthesis and the modulation of cell growth [143, 144]. Estrogens have also been implicated in liver growth during ontogenesis and enhance liver regeneration after partial hepatectomy [145]. Moreover, they dramatically alter lipid metabolism and transport and elicit anti-atherosclerotic effects via alterations in the levels and activities of lipid metabolizing enzymes and lipoproteins [146]. Estrogens also elicit toxic responses in the liver including cholestasis, oxidative damage, mitotic abnormalities and carcinogenesis [135, 138, 147, 148].

Despite the wide array of physiological and toxic responses, the number of known estrogen mediated hepatic responses is limited [134]. In the present study, cDNA microarrays were utilized to examine the temporal and dose-dependent changes in hepatic gene expression following treatment of immature ovariectomized mice with 17α-ethynyl estradiol (EE), a pharmaceutical agent with enhanced oral bioavailability [149] that elicits a transcriptional response similar to that of 17β-estradiol [150]. Rigorous statistical approaches were used to identify treatment-induced changes in gene expression while accounting for variability between replicates. Comparisons between hepatic responses and those observed in classical estrogen responsive tissues were drawn in order

to identify common as well as tissue specific gene expression responses to further elucidate the mechanisms of action of estrogenic compounds.

MATERIALS AND METHODS

Animal treatment

Female C57BL/6 mice, ovariectomized by the vendor on postnatal day 20 and all having body weights within 10% of the average body weight, were obtained from Charles River Laboratories on postnatal day 26 (Raleigh, NC). The mice were housed in polycarbonate cages containing cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) in a 23°C HEPA-filtered environment with 30-40% humidity and a 12hr light/dark cycle (07 00hr – 19 00hr). Animals were allowed free access to deionized water and Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI), and acclimatized for four days prior to dosing. On the fourth day, animals were weighed, and 17α-ethynyl estradiol (EE) (Sigma Chemical Co., St. Louis, MO) was dissolved in sesame oil (Loriva, Ronkonkoma, NY) to achieve the desired dose based on the average weight of the animals. For the time course study, animals were treated by gavage with 0.1 ml of sesame oil for a nominal dose of 0 (vehicle control) or 100μg/kg bw of EE. Five animals were treated per dose group and time point and groups for each dose and time point were housed in separate cages. Mice were sacrificed 2, 4, 8, 12 and 24 hrs after dosing. Additional groups of 5 animals were included which were dosed for three consecutive days with either the vehicle or EE (100 μg/kg bw) and were sacrificed 24 hrs after the final dose, referred to here after as the 3x24 hr group. An untreated group of mice was also included which was sacrificed at time zero, the time at which the other animals were dosed. For the dose response study, 5 mice per group were gavaged with 0.1ml of vehicle or 0.1, 1, 10, 100 or 250 μg/kg EE and

sacrificed 24 hr after dosing. In both studies, treatment was staggered to ensure exposure times were within 5% of the desired length. Animals were sacrificed by cervical dislocation and a section of the liver was removed and stored in RNAlater (Ambion Inc., Austin TX) at -80°C until further use. All procedures were performed with the approval of the Michigan State University All-University Committee on Animal Use and Care.

RNA isolation

Liver samples (approximately 70 mg) were transferred to 1.0 ml of Trizol (Invitrogen, Carlsbad, CA) in a 2.0 ml microfuge tube and homogenized using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was isolated according to the manufacturer's protocol with an additional phenol:chloroform extraction. Isolated RNA was resuspended in RNA storage solution (Ambion Inc., Austin, TX), quantified (A_{260}) and assessed for purity by determining the A_{260}/A_{280} ratio and by visual inspection of 1.0 μg on a denaturing gel.

Experimental design

Temporal changes in gene expression were assessed using cDNA microarrays by comparing EE treated samples to time matched vehicle controls using a modified loop design (Figure 1a). One loop utilizes one of the five animals from each time/treatment group, with four independent labelings of each sample, with appropriate dye swaps, for a total of 26 arrays per loop. Three loops, and therefore three biological replicates, were conducted for a total of 78 arrays. Therefore, within a dose group (n=5) three animals were used to assess temporal changes in gene expression by cDNA microarrays.

Dose response changes in gene expression were analyzed using a common reference design in which samples from EE treated mice are co-hybridized with a common vehicle control (Figure 1b). Each design replicate uses one of the five animals from each dose

group with two independent labelings per sample, with appropriate dye swaps, for a total of 10 arrays. Four biological replicates were conducted for a total of 40 microarrays. Therefore, within a dose group (n=5) four animals were used to assess dose-dependent changes in gene expression by cDNA microarrays

Microarray analysis of differential gene expression

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 in duplicate onto epoxy coated glass slides (Quantifoil, Germany) using an Omnigrid arrayer (GeneMachines, San Carlos, CA) equipped with 16 (4x4) Chipmaker 2 pins (Telechem) at the Genomics Technology Support Facility at Michigan State University (http://www.genomics.msu.edu). Total RNA (25μg) was reverse transcribed in the presence of Cy3- or Cy5-dUTP to create fluor-labeled cDNA which was purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA). Cy3 and Cy5 samples were mixed, vacuum dried and resuspended in 32μl of hybridization buffer (40% formamide, 4xSSC, 1%SDS) with 20μg polydA and 20μg of mouse COT-1 DNA (Invitrogen, Carlsbad, CA) as competitor. This probe mixture was heated at 95°C for 3 minutes and was then hybridized on the array under a 22x40 mm coverslip (Corning Inc., Corning NY) in a light protected and humidified hybridization chamber (Corning Inc.). 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 nm (Cy5) and 535nm (Cy3) on an Affymetrix 428 Array Scanner (Santa Clara, CA). Images were analyzed for feature and background intensities using AnalyzerDG (MolecularWare, Cambridge, MA).

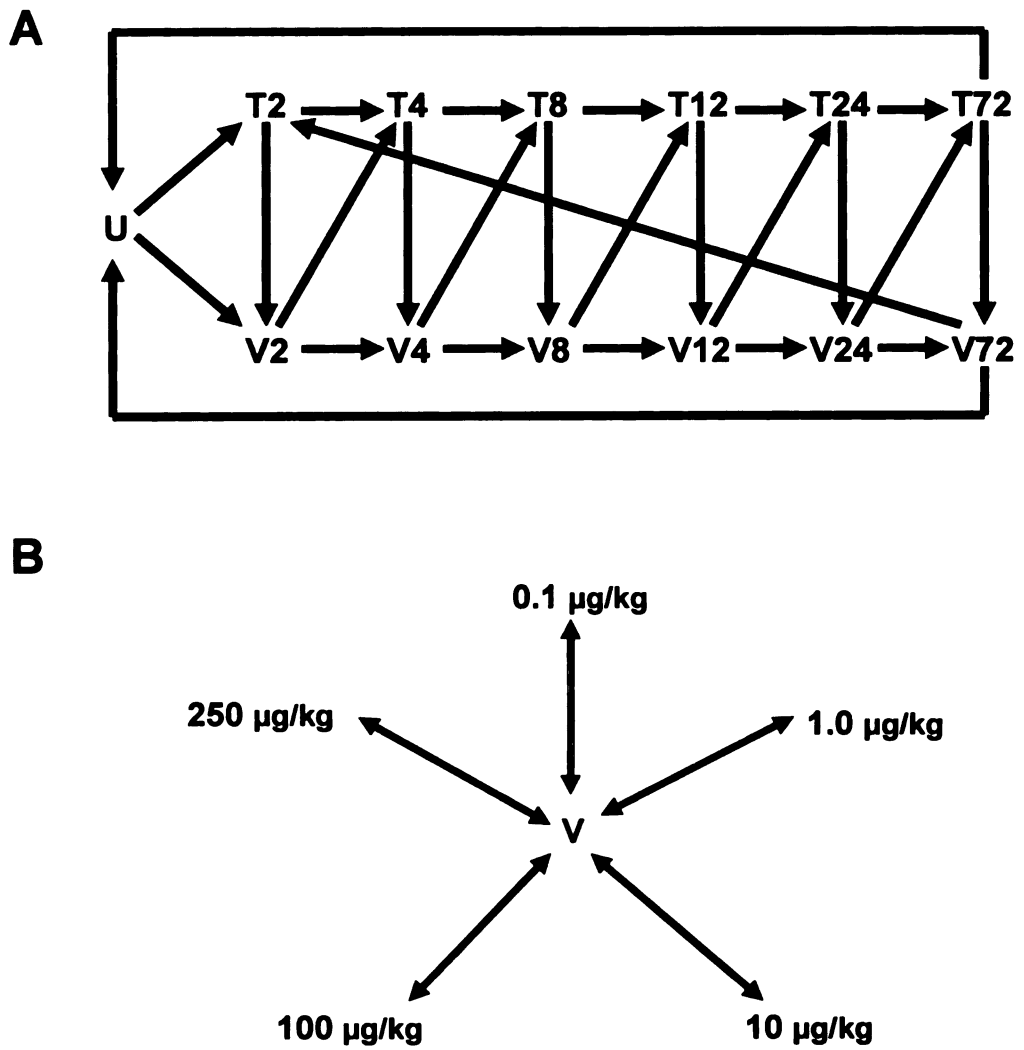


Figure 1. Microarray experimental designs for (A) temporal (B) and dose-response studies.

A. Temporal gene expression patterns were analyzed with cDNA microarrays using a modified loop design that included four independent labelings of each sample, with appropriate dye swaps, for a total of 26 arrays per loop. One loop utilizes one of the five animals from each time/treatment group. Three loops, and therefore three biological replicates, were conducted for a total of 78 arrays. Arrow bases represent labeling with Cy3 while arrow heads represent labeling with Cy5. U= untreated animal at time zero, V and T = vehicle and EE treated animals, respectively; numbers indicate time of sacrifice (hrs). B. Dose-dependent changes in gene expression were analyzed 24hr after treatment using a common reference design in which samples from EE treated mice were co-hybridized with a common vehicle control. This design uses one of the five animals from each dose group with two independent labelings per sample, with appropriate dye swaps, for a total of 10 arrays per replicate. Four biological replicates were conducted for a total of 40 microarrays. Double headed arrows indicate dye swap (each sample labeled with Cy3 and Cy5 on different arrays). V= vehicle control, doses represent animals treated with the indicated dose of EE.

Microarray Data Normalization

Data normalization and identification of treatment-related effects were performed using the general linear mixed model

$$y_{ijkbmqsr} = \mu + A_j + D_k + (AD)_{jk} + Bb + (AB)_{jb} + \varepsilon_{ijkbmqsr} \quad (1)$$

where $y_{ijkmqsr}$ denotes the is the base-2 logarithm-transformed uncorrected median signal intensity of the i^{th} gene (i = 1, .., J), j^{th} array (j = 1, .., J), k^{th} dye (k = 1,2), m^{th} treatment (m = UR,V,T), q^{th} time (q = 0,2,4,8,12,24, 72 hrs; not used in the dose response), s^{th} spot (s = 1,2) and the r^{th} design replicate (r = 1,..,R), μ is the overall mean, D_k is a fixed effect associated with the k^{th} dye, A_j is a random effect associated with the j^{th} array, $(AD)_{jk}$ is a random effect associated with the array by dye interaction, B_b is a random effect associated with the b^{th} block, and $(AB)_{jb}$ is a random effect associated with the array by block interaction (Eckel and Gennings, submitted for publication).

The residuals from the normalization model are defined as:

$$r_{ijkbmqsr} = y_{ijkbmqsr} - (\mu + A_j + D_k + (AD)_{jk} + Bb + (AB)_{jb}) \quad (2)$$

and are used as the normalized response for the gene model. Specific treatment effects on genes relative to vehicle effects were determined using the gene model:

$$r_{ijkmqsr} = \mu_i + A_{ij} + D_{ik} + H_{iq} + T(H)_{im(q)} + S_{is} + R_{ir} + (AR)_{ijr} + (AS)_{ijs} + \gamma_{ijkmqsr} \quad (3)$$

where $r_{ijkmqsr}$ is the normalized response as defined in (Equation 2), μ_i is the overall mean for the i^{th} gene, A_{ij} is a random effect associated with the j^{th} array for the i^{th} gene, D_{ik} is a fixed effect associated with the k^{th} dye for the i^{th} gene, H_{iq} is a fixed effect associated

with the q^{th} time point for the i^{th} gene, $T(H)_{im(q)}$ is a fixed effect associated with the m^{th} treatment nested within the q^{th} time point for the i^{th} gene, S_{is} is a fixed effect associated with the s^{th} spot for the i^{th} gene, R_{ir} is a fixed effect associated with the r^{th} design replicate for the i^{th} gene, $(AR)_{ijr}$ is a random effect associated with the array by design replicate interaction for the i^{th} gene, and $(AS)_{ijs}$ is a random effect associated with the array by spot interaction for the i^{th} gene. The model for the dose-response experiment is similar to the temporal model, except there is no effect for the q^{th} time point. This model provides estimates of the treatment effect, relative to the vehicle effect, which was used in the data filtering step.

Data filtering and analysis

For analysis purposes, a reduced data set was desired in order to remove those genes with highly variable responses that are potentially unrelated to the treatment. A model-based t-statistic (MBT) was used to rank gene expression changes based on absolute t-score values in order to initially prioritize treatment related effects for subsequent analysis. The MBT was calculated based on the results of the general linear mixed model for the gene-specific treatment effects (Equation 3). The MBT is defined as:

$$t = \frac{\hat{\theta}_i}{se(\hat{\theta}_i)} \tag{4}$$

where $\hat{\theta}_i = T(H)_{iT} - T(H)_{iV}$ is the contrast estimate from the gene-specific treatment model, and $se(\hat{\theta}_i)$ is the standard error of $\hat{\theta}_i$. For the time-course experiment all values are based on the i^{th} gene and the q^{th} time-point.

To focus subsequent analyses and data interpretation on the most reproducible differentially regulated genes, a stringent t-score threshold of 3.3 was used to obtain a subset of differentially regulated genes. This threshold is an absolute t-score value that accounts for changes in gene expression on both tails of the t-distribution. Gene expression changes that passed the threshold were subsequently analyzed using K-means clustering (GeneSpring 6.0, Silicon Genetics, Redwood City, CA). Dose response analysis was performed using Graph Pad Prism 4.0 (GraphPad Software, Inc. San Diego, CA)

Quantitative real-time PCR

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, Carlsbad, CA). The cDNA (1.0μl) was used as a template in a 30 ul PCR reaction containing 0.1 μM each of forward and reverse gene-specific primers designed using Primer3 [151], 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 Table I. 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 seconds and 60°C for 1 minute. 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 7 orders of magnitude to interpolate relative

Table 1. Gene names and primer sequences (5'- 3') for select transcripts verified by QRTPCR

Genbank Accession	Gene Name	Gene Symbol	Locus Link	RefSeq Number	forward primer	reverse primer	size (bp)
	actin, beta, cytoplasmic	Actb	11461	NM_007393	GCTACAGCTTCACCACCACA	TCTCCAGGGAGGAAGAGGAT	123
AA000327	actin, gamma, cytoplasmic	Actg	11465	NM_009609	CCACTCCTTCTTGCCAGTCT	AGGCAACTAACAACCGATGG	104
AA518577	apolipoprotein A-IV	Apoa4	11808	NM_007468	GGCTCTGGAAGACCTGAACA	ACCCAGCTGCTGTCTGAACT	130
W10324	apolipoprotein C-II	Apoc2	11813	NM_009695	GCAGGGCTCCCTCTTAAGTT	TTTCTCATCCATGCTGATCG	91
AA170585	Carbonic anhydrase 3	Car3	12350	NM_007606	CACACTTTGACCCATCATGC	AGCTCACAGTCATGGGCTCT	129
AI323806	cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	12575	NM_007669	TCTATCACTCCAAGCGCAGA	CACACAGAGTGAGGGCTAAGG	130
AI561913	cytochrome P450, family 17, subfamily a1	Cyp17	13074	NM_007809	ATTTACCCTTCGGAGCTGGT	AGGGCAGCTGTTTGTCATCT	
AI893885	Cytochrome P450, 2b10, phenobarbitol inducible, type	Cyp2b10	13088	NM_009998	CCCAGTGTTCCACGAGACTT	GGTGCCGACAAAGAAGAGAG	129
AA002910	FBJ osteosarcoma	Fos	14281	NM_010234	GTCCGGTTCCTTCTATGCAG	TAAGTAGTGCAGCCCGGAGT	129
AA122891	glyceraldehyde-3-phosphate dehydrogenase	Gapd	14433	NM_008084	GTGGACCTCATGGCCTACAT	TGTGAGGGAGATGCTCAGTG	125
AA710940	glutathione S-transferase, pi	Gstp2	14870	NM_013541	TTGCCGATTACAACTTGCTG	GAGCCACATAGGCAGAGAGC	96
	hypoxanthine guanine phosphoribosyl transferase	Hprt	15452	NM_013556	AAGCCTAAGATGAGCGCAAG	TTACTAGGCAGATGGCCACA	104
W10072	insulin-like growth factor 1	Igf1	16000	NM_010512	TGGATGCTCTTCAGTTCGTG	GCAACACTCATCCACAATGC	118
W83086	insulin-like growth factor binding protein 1	Igfbp1	16006	NM_008341	CACTCGGAATTCCTCATCGT	ATGTATGGGACGCAGCTTTC	115
AA867135	lipase, hepatic	Lipc	15450	NM_008280	GCTGCTGGGAACAAAAGAAG	GATCAACTCGCCGATGTCTT	118
AA009268	myelocytomatosis oncogene	Myc	17869	NM_010849	ACGAGCACAAGCTCACCTCT	TCCAGCTCCTCCTCGAGTTA	122
AA763337	signal transducer and activator of transcription 5A	Stat5a	20850	NM_011488	ACCAAGATGGCGAGTTTGAC	GGAGGTGAAGAGACCAGCAG	125
AA212445	signal transducer and activator of transcription 5B	Stat5b	20851	NM_011489	ATGCGACTGTCCCAGTAACC	CCCATGCTGCTCTACTCTCC	102
AA165755	transglutaminase 2, C	Tgm2	21817	NM_009373	TGGAGAATCCCGAAATCAAG	TCATACAGGGGATCGGAAAG	106

template concentrations of the samples from the standard curves of log copy number vs 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 the geometric mean of 3 house-keeping genes (Bactin, Gapd and Hprt) to control for differences in RNA loading, quality and cDNA synthesis. Statistical significance of induced or repressed genes was determined using the t-test. For graphing purposes, the relative expression levels were scaled such that the expression level of the time matched control group was equal to 1.

RESULTS

Experimental quality assurance

The production of accurate and precise microarray results requires repeated measures of individual samples as well as biological replication in order to minimize noise associated with the experimental method and its biological samples. The experimental designs utilized address these issues by incorporating multiple independent labelings of each sample as well as completing biological replicates for each study (Figure 1). To assess image quality, raw microarray data for each dye was monitored for 1) background signal intensity, 2) feature signal intensity, 3) feature/background signal intensity ratios, 4) the number of features with background intensities greater than the feature intensity for each array, and 5) relationships between feature and background signal intensities (Table II). Background signal intensities between time course and dose response studies were very similar despite the chance occurrence of some areas of a few

arrays having background signal intensities that approached saturation. All parameters within and across the two studies (i.e. 3 x 26 arrays for time course; 4 x 10 arrays for dose response) were highly consistent which facilitated the identification of temporal and dose response associations.

Microarray analysis of EE-induced temporal changes in hepatic gene expression

A model-based t-test identified 447 microarray features, representing 419 annotated clones and 393 unique genes, which were differentially expressed ($t > |3.33|$), relative to time matched vehicle controls, at one or more time points. The data revealed that the 2 and 3x24 hr time points were the most active based on the number of significant changes in gene expression (Figure 2). Sample data were also compared to the untreated control at time zero (time of dosing) to assess potential vehicle or circadian effects; while this was not used as a filtering criterion, it was considered during data interpretation.

K-means analysis of the 419 annotated genes indicated that five clusters accurately and concisely described the data (Figure 3). The clusters consisted of up- and down-regulated early and late responses as well as an up-regulated early/sustained group, consistent with recently published reports of temporal transcriptional responses to estrogen in the uterus and MCF-7 cells [152, 153]. In general, there was an equal distribution between up and down-regulated expression patterns although the magnitude of the response was greater for induced transcripts.

Table 2. Microarray global quality control statistics

		Time Course Study			Dose-response Study		
		Range	Mean	Standard Deviation	Range	Mean	Standard Deviation
Background Signal Intensity[a]		0 - 65535	298.3	448.8	8 - 13247	260.8	269.8
	Cy3	0 - 65535	270.3	577.8	23 - 12649	214	193.7
	Cy5	0 - 37342	326.3	319.8	8 - 13247	307.6	322
Feature Signal Intensity[b]		1 - 65535	7232.8	10415.7	13 - 65535	5512.7	8269.2
	Cy3	1 - 65535	7258.4	10573.9	29 - 65535	5519	8270.2
	Cy5	42 - 65535	7207.2	10257.5	13 - 65535	5506.3	8268.3
Signal vs Background Ratio[c]		N/A	24.5	25.2	N/A	21.1	30.6
	Cy3	N/A	26.9	18.3	N/A	25.8	42.7
	Cy5	N/A	22.1	32.1	N/A	17.9	25.7

[a] Background signal intensity is based on mean background calculated for each individual microarray.

[b]Feature signal intensity is based on mean feature signal intensity calculated for each individual microarray.

[c]The mean and standard deviation are estimates based on the global statistics.

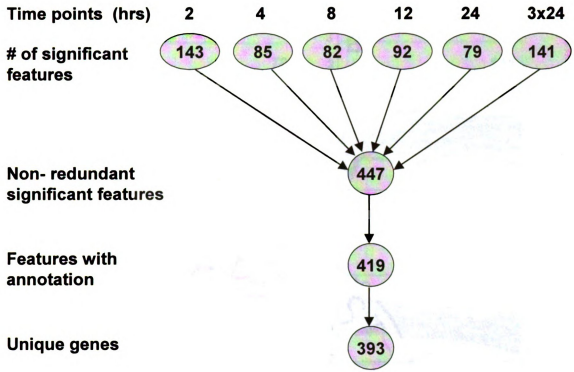


Figure 2. Microarray data analysis and filtering.
Microarray data was analyzed for significant changes in gene expression using a model-based t-statistic by comparing EE treated samples to the corresponding time matched vehicle controls. Significant changes in gene expression at each time point were combined, filtered for redundancy and ranked according to an absolute t-score value (3.3) that accounted for changes in gene expression on both tails of the t-distribution. Published literature and LocusLink identifiers were then used to associate genes with functional categories. Additional genes from the microarrays were considered for analysis provided that the gene approached the t-score threshold and was associated with a functional category based on published literature or functional annotation extracted from public databases.

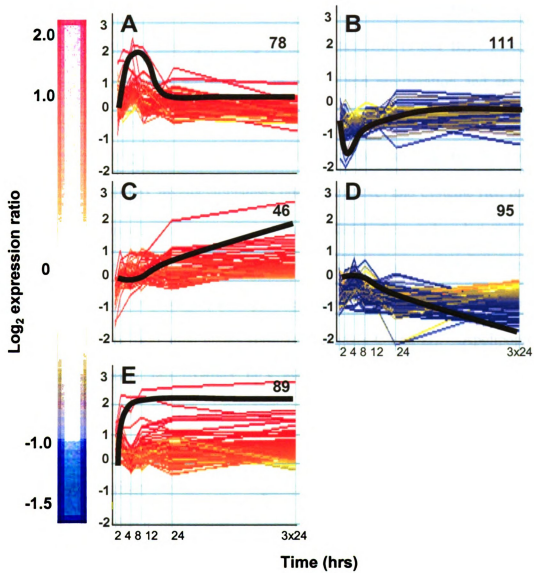


Figure 3. K-means clustering of significant temporal gene responses.
Five K-means clusters corresponding to (A) up-regulated early, (B) down-regulated early, (C) up-regulated late, (D) down-regulated late, and (E) up-regulated early/sustained responses best described the selected genes. The number of genes in each cluster is indicated. Graphs are expressed as $\log_2$ expression ratios relative to time-matched vehicle controls. A pseudogene line is drawn in bold to illustrate the representative response that defines the pattern in each cluster.

Functional annotation extracted from public databases and the literature revealed that many genes exhibited functions associated with cell cycle, growth and proliferation, cytoskeleton and extracellular matrix (ECM), microtubule based processes, oxidative stress and metabolism, and lipid transport and metabolism (Table III). Many of the immediate early responses are involved in growth and proliferation and are classical estrogen responsive genes. These genes exhibited significant changes in mRNA levels at 2 and 4 hrs (primarily clusters A and E) and included FBJ osteosarcoma oncogene (Fos), Jun-B oncogene (Junb), myelocytomatosis oncogene (Myc) and cysteine rich protein 61 (Cyr61). Genes involved in microtubule based processes were induced at early to mid-phases of the time course (cluster A) while those involved with cytoskeleton and ECM, oxidative stress and metabolism, and lipid metabolism and transport displayed induction or repression at the later time points (primarily clusters C and D). Apolipoprotein E (Apoe) and Junb were "misclassified" by K-means clustering as down regulated early and late, respectively (clusters B and D) due to a non-significant repression at these time points. Inappropriate cluster assignments occur due to the inability of K-means clustering to consider the statistical significance of a change in gene expression at multiple points within a time course.

Quantitative real-time PCR (QRTPCR) was used to verify changes in transcript levels for a selected subset of genes (Figure 4). In total, 25 of the 29 genes examined by QRTPCR exhibited a pattern of gene expression comparable to the microarray results (see supplementary data). In general, fold-change ratios of mRNA expression levels were lower for the microarrays when compared to QRTPCR. For example, microarray analysis revealed that signal transducer and activator of transcription 5A (Stat5a) was

Table 3. Functional categorization and temporal regulation of select hepatic genes identified as differentially regulated in response to EE.

Functional Category	Accession Number	Gene name	Gene symbol	Locus Link	Regulation[a]	Time points hrs[b]	Cluster[c]
Growth and proliferation							
	AA396123	E2F transcription factor 1	E2f1	13555	↑	2, 4	A
	AA002910	FBJ osteosarcoma oncogene	Fos	14281	↑	2, 4	A
	W83086	insulin-like growth factor binding protein 1	Igfbp1	16006	↑	2, 4, 8, 24	A
	AA009268	myelocytomatosis oncogene	Myc	17869	↑	2, 4, 12	A
	AA690767	insulin-like growth factor 1	Igf1	16000	↓	24, 3x24	D
	AA759387	Jun-B oncogene	Junb	16477	↑	2, 4	D
	AA241105	Jun proto-oncogene related gene d1	Jund1	16478	↑	12	D
	AA419858	cysteine rich protein 61	Cyr61	16007	↑	2, 4	E
	AA014761	E26 avian leukemia oncogene 2, 3' domain	Ets2	23872	↑	2, 4	E
	AA739325	zuotin related factor 2	Zrf2	22792	↑	4, 24	E
Cytoskeleton and Extracellular Matrix							
	AA000327	actin, gamma, cytoplasmic	Actg	11465	↑	8, 12	A
	AA097808	WD repeat domain 1	Wdr1	22388	↑	8, 12	A
	W88115	procollagen, type IV, alpha 1	Col4a1	12826	↑	8, 12	C
	NM_010663	keratin complex 1, acidic, gene 17	Krt1-17	16667	↑	24	C
	AA009138	myosin, heavy polypeptide 3	Myh3	17883	↑	24, 3x24	C
	AA230924	myosin light chain, alkali, nonmuscle	Myln	17904	↑	3x24	C
Microtuble based processes							
	AA008134	dynein, cytoplasmic, light chain 1	Dnclc1	56455	↑	2	A
	AA033406	MAD2 (mitotic arrest deficient, homolog)-like 1	Mad2l1	56150	↑	4, 8	A
	AA498356	microtubule-associated protein 1 light chain 3	Map1lc3	67443	↑	2, 4, 8, 24	A
	AA125125	microtubule-associated protein 2	Mtap2	17756	↑	2	A
	W89405	pericentrin 2	Pcnt2	18541	↑	4, 8, 12	A

Table 3. Continued

Functional Category	Accession Number	Gene name	Gene symbol	Locus Link	Regulation[a]	Time points hrs[b]	Cluster[c]
Oxidative Metabolism and Stress							
	AA162908	gamma-glutamyl transpeptidase	Ggtp	14598	↑	2 - 12	A
	AA710940	glutathione S-transferase, pi 2	Gstp2	14870	↑	24, 3x24	C
	W54349	glutathione S-transferase, alpha 4	Gsta4	14860	↓	3x24	D
	AA461810	glutathione S-transferase, mu 5	Gstm5	14866	↓	3x24	D
	AA230348	glutathione S-transferase, theta 2	Gstt2	14872	↓	3x24	D
	AA139714	glutathione reductase 1	Gsr	14782	↑	12	E
Lipid metabolism and Transport							
	AK010261	apolipoprotein E	Apoe	11816	↓	24	B
	AA518577	apolipoprotein A-IV	Apoa4	11808	↑	24, 3x24	C
	W10324	apolipoprotein C-II	Apoc2	11813	↑	24, 3x24	C
	AA521869	steroidogenic acute regulatory protein	Star	20845	↑	8, 12	C
	AA867135	lipase, hepatic	Lipc	15450	↓	24	D

[a] Arrows represent up- and down- regulated responses

[b] Time points indicated were considered to be significantly changed in the microarray analysis based on $t > |3.33|$

[c] Cluster designation corresponds to respective K-means cluster (Figure 3)

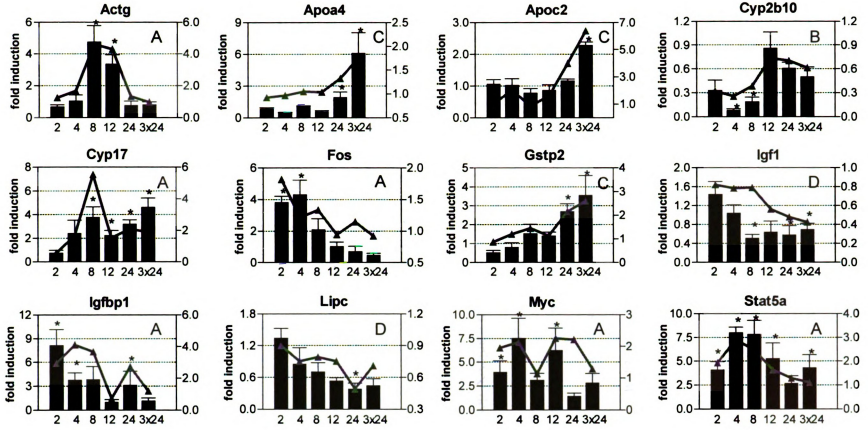


Figure 4. Verification of time-course microarray data using QRTPCR. RNA from the same sample that was used for cDNA microarray analysis was examined using QRTPCR . All fold changes were calculated relative to time matched vehicle controls. Bars (left axis) and lines (right axis) represent data obtained 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 3 biological replicates. Letters represent the cluster designation for each gene. Error bars represent the SEM for the average fold change. * = $p<0.05$ for QRTPCR.

maximally induced 3-fold at 4 hrs, while QRTPCR analysis measured an 8-fold increase. Data compression has been previously documented when comparing microarray data to other, more gene specific, measurement techniques [154].

Microarray analysis of dose-dependent changes in gene expression induced by EE

Of the 79 genes that were differentially expressed at 24 hrs in the time course study, 39 exhibited a dose-dependent pattern of expression. Cytoplasmic gamma actin (Actg), procollagen IV alpha 1 (Col4a1), and mitotic arrest deficient, homolog-like 1 (Mad2l1) also displayed dose-dependent expression; although the time course study clearly indicated that 24 hrs was not the optimal induction time for these genes (Table IV). It is likely other genes identified in the time course study, including some of those identified at the 24 hr time point, would have also displayed dose response kinetics had additional optimal exposure times been investigated. These observations demonstrate the complexity of conducting and interpreting dose-response experiments due to the fact that gene expression is not static.

Despite the data compression, gene expression patterns across doses were comparable between microarray and QRTPCR assays (Figure 5). Moreover, there was strong concordance between the time course and dose-response studies. For example, cytochrome P450 17 (Cyp17), leukemia inhibitory factor receptor (Lifr) and transglutaminase 2, C polypeptide (Tgm2) were up regulated 2.1, 5.9 and 3.8 fold, respectively, in the time course study and 2.4, 4.3 and 4.4-fold at the same dose in the dose-response study. Similarly, carbonic anhydrase 3 (Car3), insulin-like growth factor 1 (Igf1) and hepatic lipase (Lipc) were down regulated 0.63, 0.62 and 0.49-fold, respectively, in the time course study, and 0.50, 0.77 and 0.67-fold in the dose-response

Table 4. Dose response data for select genes as obtained with cDNA microarray analysis[a]

Functional Category	GenBank Accession	Gene Name	Gene Symbol	Locus Link	Dose of EE (µg/kg) 0.1	1	10	100	250	Cluster	ED_{50}[b]
Cell cycle, growth and proliferation											
	AA000468	cell division cycle 20 homolog (S. cerevisiae)	Cdc20	107995	0.98	1.20	0.95	1.35	1.78	A	>250[c]
	W83086	insulin-like growth factor binding protein 1	Igfbp1	16006	1.83	1.02	1.68	3.35	3.97	A	>250[c]
	AA009268	myelocytomatosis oncogene	Myc	17869	0.96	1.15	1.18	2.17	1.68	A	20.70 ± 7.16
	AA690767	insulin-like growth factor 1	Igf1	16000	0.85	0.96	0.90	0.77	0.79	D	46.88 ± 24.89
	AI323806	cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	12575	0.99	1.00	1.17	1.46	1.47	E	18.28 ± 1.25
	AA419858	cysteine rich protein 61	Cyr61	16007	0.67	0.94	0.94	1.30	1.63	E	166.72 ± 4.15
	AA739325	zuotin related factor 2	Zrf2	22792	1.11	1.12	1.23	1.80	1.99	E	66.68 ± 1.19
Cytoskeleton and extracellular matrix											
	AA000327	actin, gamma, cytoplasmic	Actg	11465	1.07	0.99	1.07	1.33	1.65	A	>250[c]
	AA097808	WD repeat domain 1	Wdr1	22388	1.19	1.02	0.96	1.19	1.35	A	>250[c]
	W88115	procollagen, type IV, alpha 1	Col4a1	12826	1.38	1.52	1.61	2.12	3.19	C	>250[c]
	AA009138	myosin, heavy polypeptide 3, skeletal muscle, embryonic	Myh3	17883	1.21	1.38	1.61	1.62	1.68	C	1.44 ± 1.44
	W98341	keratin complex 2, basic, gene 4	Krt2-4	16682	0.78	1.07	1.06	4.14	9.33	NS[d]	>250[c]
Microtuble based processes											
	AA008134	dynein, cytoplasmic, light chain 1	Dnclc1	56455	1.00	1.07	1.08	1.31	1.69	A	>250[c]
	AA033406	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)	Mad2l1	56150	1.14	1.07	1.13	1.20	1.84	A	>250[c]
	BG070050	microtubule-associated protein 1 light chain 3	Map1lc3	67443	1.69	1.06	1.13	2.14	1.89	A	59.70 ± 76.56
	W89405	pericentrin 2	Pcnt2	18541	0.87	0.96	1.17	1.40	1.53	A	13.46 ± 1.54

Table 4. continued

Functional Category	GenBank Accession	Gene Name	Gene Symbol	Locus Link	0.1	1	10	100	250	Cluster	ED_{50}[b]
Oxidative metabolism and stress											
	AA162908	gamma-glutamyltransferase 1	Ggt1	14598	0.95	1.03	1.17	1.81	2.61	A	>250[c]
	AA710940	glutathione S-transferase, pi 2	Gstp2	14870	0.79	0.99	0.93	1.15	1.32	C	179.47 ± 7.56
	W54349	glutathione S-transferase alpha 4	Gsta4	14860	1.14	1.12	1.00	0.56	0.56	D	28.12 ± 1.65
	BG075231	glutathione S-transferase, mu 6	Gstm6	14867	0.91	0.84	0.67	0.65	0.69	D	1.56 ± 2.05
Lipid metabolism and transport											
	AK010261	apolipoprotein E	Apoe	11816	1.04	1.12	1.04	0.89	0.82	B	86.10 ± 3.14
	AA518577	apolipoprotein A-IV	Apoa4	11808	1.18	1.06	1.37	2.21	1.88	C	18.58 ± 3.88
	W10324	apolipoprotein C-II	Apoc2	11813	0.85	1.16	1.44	1.80	1.59	C	2.16 ± 2.55
	AA521869	steroidogenic acute regulatory protein	Star	20845	1.51	1.56	1.76	2.77	2.94	C	43.75 ± 1.42
	AA867135	lipase, hepatic	Lipc	15450	1.21	0.74	0.78	0.67	0.59	D	10.72 ± 2.42
Other responses											
steroid biosynthesis	AI561913	cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17	13074	1.10	1.10	1.17	2.36	2.41	A	50.23 ± 2.39
signal transduction	AA763337	signal transducer and activator of transcription 5A	Stat5a	20850	0.95	1.12	1.09	1.87	2.74	A	>250[c]
immune signaling	AA571998	CD3 antigen, delta polypeptide	Cd3d	12500	1.17	1.08	0.68	0.40	0.34	B	7.24 ± 1.10
acute phase/ inflammatory	AA271451	C-reactive protein, petaxin related	Crp	12944	1.17	1.24	1.35	2.10	1.97	C	26.79 ± 2.67
cellular homeostasis	BC011129	carbonic anhydrase 3	Car3	12350	1.39	1.03	0.85	0.50	0.36	D	9.16 ± 2.49
signal transduction	AA207338	leukemia inhibitory factor receptor	Lifr	16880	1.74	1.82	3.72	4.25	7.12	E	>250[c]
	AA165755	transglutaminase 2, C polypeptide	Tgm2	21817	1.20	1.03	1.60	4.41	6.96	E	>250[c]

[a] all values expressed as fold change realtive to vehicle control

[b] $LogED_{50}$ ± Standard Error

[c] Estimated ED_{50} outside the range of doses investigated

[d] NS = Not significant in the time course study and therefore not clustered

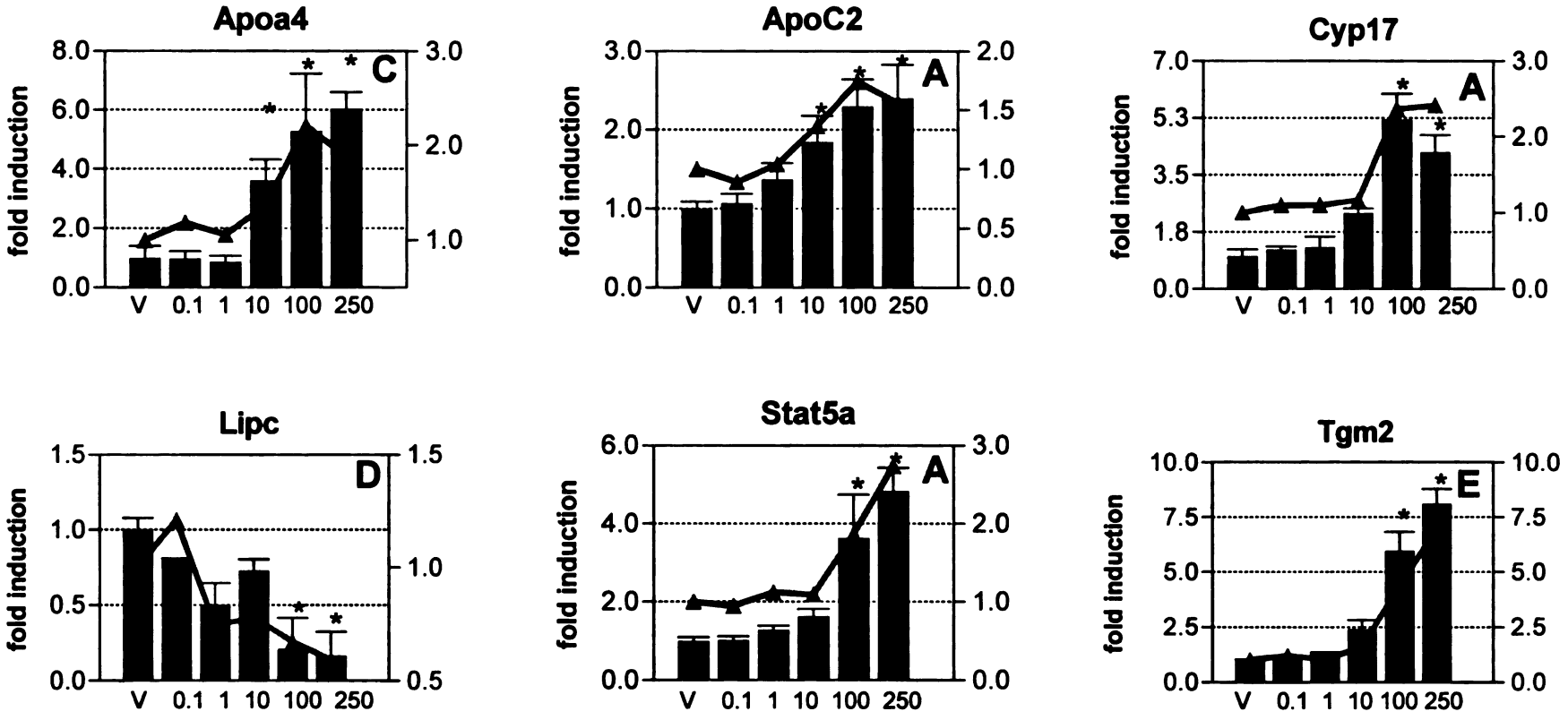


Figure 5. Verification of dose-response microarray data using QRTPCR. RNA from the same sample was used for both cDNA microarray and QRTPCR analysis. All fold changes were calculated relative to vehicle controls. Bars (left axis) and lines (right axis) represent data obtained by QRTPCR and cDNA microarrays, respectively, while the x-axis represents the dose groups. Genes are indicated by official gene symbols and results are the average of 4 biological replicates. Letters represent the cluster designation for each gene. Error bars represent the SEM for the average fold change. * = $p<0.05$ for QRTPCR.

study. These results indicate the reproducibility of the responses in independent experiments. This, combined with the observed dose-response relationships for these and other genes, provides strong evidence that these genes are either primary or downstream estrogen target genes.

ED_{50} values for dose-dependent changes in gene expression ranged from approximately 1.4 μg/kg to greater than 250 μg/kg with an average ED_{50} of 47 ± 3.5 μg/kg (Table IV). This average ED_{50} value for gene expression responses is comparable to the ED_{50} for induction of uterine weight in mice treated by gavage with EE [155, 156] indicating that the transcriptional responses observed in the liver display similar dose kinetics to known EE elicited physiological endpoints. Furthermore, most of the genes for which the ED_{50} could not be determined (ED_{50} > 250) belonged to cluster A (Table IV) which is likely due to the non-optimal sampling times for these transcripts.

Comparison of hepatic and uterine responses

In order to evaluate EE mediated hepatic and uterine gene expression responses, microarray data from these tissues, collected from the same mice, were compared. Changes in uterine gene expression elicited by EE were assessed using Affymetrix Mu11KSubA GeneChips as described [48]. Common genes represented on the Affymetrix Mu11KSubA GeneChip and the cDNA microarrays were determined using LocusLink identifiers. Of the 1318 genes common between the two platforms, 680 exhibited a change in expression in the hepatic or uterine samples at one or more time points. Ninety-three of these genes exhibited changes in gene expression in both tissues at one or more time points (Figure 6). Of the 587 genes that exhibited a gene expression

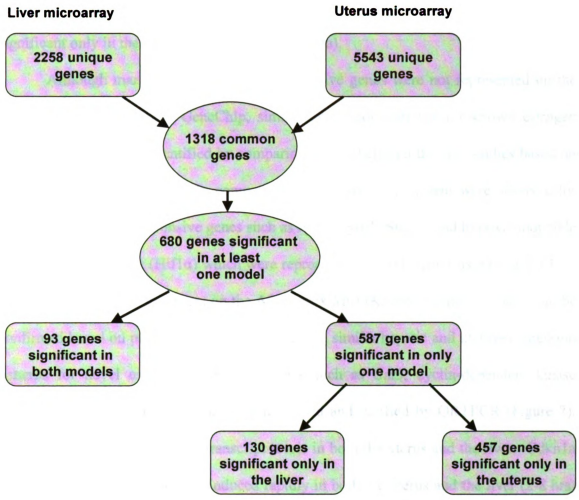


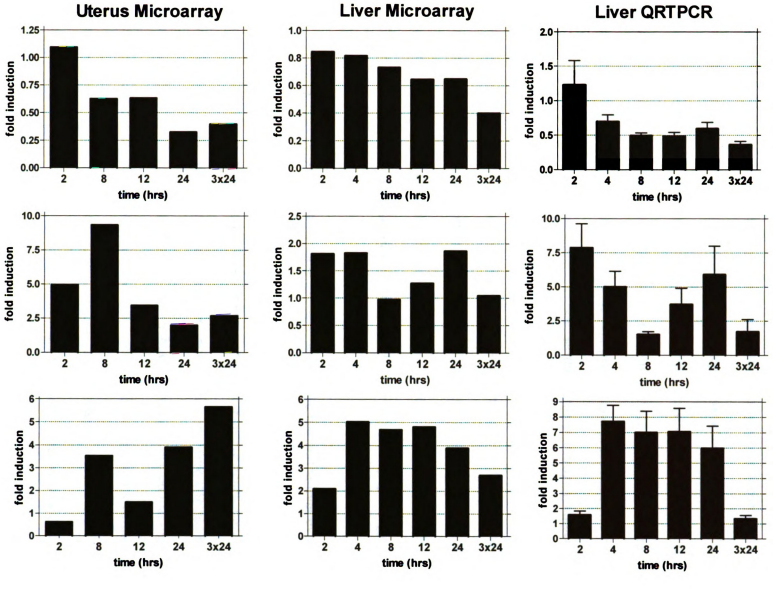
Figure 6. Comparison of hepatic and uterine gene expression responses.
Hepatic gene expression data was obtained using cDNA microarrays while uterine data was obtained using Affymetrix Mu11KSubA GeneChip arrays. Genes in common between the two platforms were identified using LocusLink numbers. Statistical cut-offs utilized were $t > \lfloor 3.33 \rfloor$ for the hepatic study and $p1z > 0.90$ for the uterine study.

change in only one tissue, 130 were significant only in the liver while 457 were significant only in the uterus (see supplemental data).

Although many classical estrogen responsive genes were not represented on the Affymetrix Mu11K SubA GeneChip, similar expression patterns for known estrogen responsive genes were identified by comparing results between the two studies based on published reports. For example, comparable gene expression patterns were observed for established estrogen responsive genes such as Actg, Cyr61, Stat5a, and hypoxia inducible factor 1, alpha subunit (Hif1α) which were represented on both arrays as well as for Fos, Jun and Myc which were absent on the Affymetrix Mu11K SubA GeneChip but could be confirmed based on published reports. In addition, similar hepatic and uterine expression patterns for novel estrogen responsive genes such as Car3, cyclin-dependent kinase inhibitor 1A (Cdkn1a) and Tgm2 were identified and verified by QRTPCR (Figure 7). Car3 displayed down regulated transcript levels in both the uterus and the liver. Cdkn1a (aka p21) transcript levels were induced rapidly in both the uterus and the liver (2-8 hrs) and again at 24 hrs in the liver samples only. Interestingly, the induction of Cdkn1a in the uterus lagged compared to hepatic expression, with maximal induction observed at 8 and 2 hrs, respectively. Tgm2 was induced at nearly all time points for both the uterus and the liver. It is important to note that differences in induction kinetics between tissues can be influenced by a number of factors including route of exposure, blood flow, tissue vascularity and lipid content [157] as well as by other tissue and cell specific factors that affect transcription and mRNA stability.

Several genes including various tRNA synthetases, ornithine decarboxylase (Odc), thymidine kinase (Tk1) and cyclin B1 and D2 (Ccnb1 and Ccnd2) were

Figure 7. Car3, Cdkn1a and Tgm2 expression in uterine and hepatic tissues.
Uterine and hepatic gene expression changes obtained using Affymetrix Mu11K SubA GeneChips, cDNA microarrays and QRTPCR were compared. All fold changes were calculated relative to vehicle controls. Car3 (A), Cdkn1a (B) and Tgm2 (C) represent genes that displayed a similar expression pattern between the two tissues. Generally, expression kinetics across the tissues are similar, although uterine responses are lagging compared to hepatic responses which may be due to differences in blood flow and vascularization, or lipid content of the tissue as well as other factors including pharmacokinetics, pharmacodynamics and factors that could alter mRNA stability. For QRTPCR results, error bars represent the SEM for the average fold change.


Uterus Microarray
Liver Microarray
Liver QRTPCR
A. Car3
B. Cdkn1a
C. Tgm2
fold induction
time (hrs)
2
4
8
12
24
3x24

upregulated only in the uterus while significant expression changes of cytochrome P-450 enzymes and glutathione transferases were specific to hepatic samples. Odc, Tk1, Ccnb1 and Ccnd2 are known to play integral roles in cell cycle progression [48] and the inability to detect changes in the liver may be due to hepatic cells actively cycling in response to normal circulating factors. In contrast, uterine cell growth and proliferation is highly inducible in the immature, ovariectomized mouse, as the tissue is estrogen starved and most cells are arrested in G_0 prior to treatment. However, following exposure to EE, these uterine cells are synchronously activated and simultaneously enter into the cell cycle resulting in a pronounced response. Conversely, changes in the expression of the cytochrome P-450s and glutathione transferases are limited to the liver which is consistent with their important roles in xenobiotic metabolism in hepatic physiology.

DISCUSSION

Although not considered a classical estrogen responsive tissue, the liver evoked a number of temporal and dose-dependent changes in hepatic gene expression in response to EE. Transcript levels of many novel and known estrogen responsive genes, previously reported to be ER regulated in classical estrogen responsive tissues, were affected by EE in the liver. Functional annotation obtained from public databases indicated that many of the changes in gene expression may contribute to growth and proliferation, cytoskeleton and extracellular matrix (ECM) reorganization, microtubule based processes, oxidative metabolism and stress, as well as lipid metabolism and transport, which will be described in detail below.

Cell proliferation and growth - Induction of the early immediate genes Fos, Junb, Myc and E26 avian leukemia oncogene 2 (Ets2) was observed at 2 and 4 hrs, consistent with

their roles in growth and proliferation and their known ER-mediated regulation in classical estrogen responsive tissues [158, 159]. Although some studies suggest that hepatic Fos and Myc are not affected by estrogens [159, 160], the induction of Fos in primary rat hepatocytes and Myc in rats by 17β-estradiol (E2) and EE, respectively[143, 161], corroborate the observations of this study. EE induction of Junb and Ets2 also extends the list of estrogen inducible hepatic protocongenes and is consistent with their estrogen-mediated regulation in other estrogen responsive tissues [162].

Cyr61, zuotin related factor 2 (Zrf2) and insulin-like growth factor binding protein 1 (Igfbp1) were also temporally and dose-dependently induced. Cyr61, a member of the ctgf/cyr1/nov (CCN) gene family which is critical for estrogen dependent DNA synthesis, MCF-7 cell proliferation and the uterotrophic response in ovariectomized rats [163, 164], was induced at 2 and 4 hrs in the liver. Zrf2 mRNA, which has not been previously reported to be estrogen regulated, was significantly induced at 2 and 24 hrs. The Zrf2 protein interacts with Id proteins and plays an important role in the promotion of cell growth, cell cycle progression and DNA synthesis [165]. Igfbp1, a member of a group of proteins that bind and modulate the signaling of insulin-like growth factors (IGFs), was significantly induced between 2 and 8 hrs and again at 24 hrs. Igfbp1 is induced rapidly after partial hepatectomy and plays an important role in liver regeneration [166]. Furthermore, estrogen is known to promote liver regeneration after partial hepatectomy [145] which may involve the induction of Igfbp1.

Immediate early induced transcription factors included Hif1a, E2F transcription factor 1 (E2f1), and signal transducer and activator of transcription 3, 5a and 5b (Stat3, Stat5a and Stat5b). Hif1a is implicated in angiogenesis, apoptosis, and energy

metabolism and is induced by estrogens in the mouse uterus [48] by an undefined mechanism [167]. E2F transcription factors are critical to G_1/S progression. E2F is inducible by estrogen in MCF-7 cells as mediated by ER and Sp1 [168]. Stat 3, 5a and 5b were all induced within 4 hrs after estrogen treatment and the induction of Stat5a and 5b is consistent with their induction in the mouse kidney [169]. These Stat gene products are phosphorylated by receptor associated kinases, which facilitates the formation of transcriptionally active homo- or heterodimers. Stat5 and Stat3 are downstream targets for non-genomic effects of estrogen that contribute to growth regulation and may be involved in carcinogenesis [170]. Stat5a has also recently been reported to be estrogen regulated in the liver [171] while there are no published reports of hepatic Stat 3 and 5b induction by estrogen.

EE-mediated down-regulated genes included basic helix-loop-helix domain class B2, transcription factor 4, and transcription factor 12 (Bhlhb2, Tcf4 and Tcf12) which act as repressors and co-repressors of transcription [172, 173]. Bhlhb2 specifically inhibits Myc mediated transcription, an important regulator of growth and proliferation [172]. Down-regulation of these repressors provides further evidence that EE creates an environment supportive of hepatic growth and proliferation which is consistent with the effects of estrogen on cultured rat hepatocytes and MCF-7 cells [153, 174, 175].

Collectively, induction of Fos, Junb, Myc, Ets2, Cyr61, Zrf2, Igfbp1, Hif1a, E2f1, and Stat 3/5a/5b, as well as the down-regulation of Bhlhb2, Tcf4 and Tcf12 are supportive of a proliferative environment [138, 161]. Products of these genes act as effectors of estrogen signaling by modulating the expression of downstream targets that support cell cycle progression and proliferation [158]. Consequently, chronic deregulated

expression of these genes may contribute to the hepatocarcinogenic effects of estrogens [158, 164, 176]. Interestingly, many of these genes have been implicated in estrogen induced endometrial and breast cancers [176, 177]. Although no increase in liver weight was observed in the present study (data not shown), previously reported hepatotrophic effects used significantly larger doses of estrogens (5mg/kg EE [161]), alternate dosing regimens and longer exposure periods. However, increases in DNA synthesis have been reported at doses as low as 0.1 μg/rat when administered subcutaneously [178].

Cytoskeleton and extracellular matrix (ECM) - Changes in gene expression that favor hepatic cell growth and proliferation were followed by alterations in the expression of many structural genes including Actg, myosin, heavy polypeptide 3 (Myh3), myosin light chain (Myln), Col4a1, and fibronectin 1 (Fn1). Moreover, these genes exhibited dose-dependent increases in expression at 24 hrs. Actg, a major structural component in eukaryotic cells with roles in cytoskeletal maintenance, intracellular motility and cytokinesis, is known to be estrogen regulated [179]. Non-muscle myosins are also involved in cytoskeletal maintenance as well as cell migration and proliferation and are regulated by estrogen in the rabbit endometrium as well as in smooth muscle cells [180, 181]. Col4a1 is known to serve an important function in estrogen induced uterine hypertrophy [182]. Fibronectin, an ECM component critical for development and wound healing, is also induced by estrogen in rat cardiac fibroblasts and in the mouse mammary gland [183, 184]. The regulation of these cytoskeletal and ECM genes in the EE-treated liver are likely a response to mitogenic changes in gene expression in preparation for cell division and growth. Their sequential response suggests that the expression of these

genes is dependent on the induction of other signaling molecules, which is supported by data indicating that Actg induction is blocked by protein synthesis inhibitors [185].

Microtubule related processes - Estrogenic compounds increase hepatic mitotic activity and the number of cells in metaphase and anaphase that exhibit spindle disturbances [135, 186]. Hepatocytes exposed to estrogens also exhibit abnormal mitosis as well as alterations in cytoplasmic microtubules and disarrangement of chromosomes [135, 187]. Consistent with these observations, microtubule-associated protein 1 light chain 3 (Map1lc3), microtubule-associated protein 2 (Map2), mitotic arrest deficient, homolog like 1 (Mad2l1), pericentrin 2 (Pcnt2), and dynein, cytoplasmic, light chain 1(Dnclc1) were induced by EE. These gene products are involved in microtubule stabilization, organization, and centrosome attachment, as well as spindle assembly checkpoint and mitotic movement [188-193]. Although not previously shown to be estrogen regulated, their induction may be part of a larger cascade of events in preparation for cellular division. Alternatively, their inappropriate induction may contribute to abnormal hepatic mitotic features as observed after estrogen exposure.

Oxidative metabolism and stress - Although the hepatocarcinogenic effects of estrogens have been attributed to stimulated growth and proliferation, cytochrome P450 (Cyp)-mediated formation of genotoxic catechol and quinone metabolites and the resulting oxidative damage to DNA, proteins and lipids, may also be a contributing factor [148, 194]. Hepatic induction of Cyp2b19 and Cyp17 as well as the pronounced down-regulation of Cyp2b10 was observed in the present study. Cyp2b19 is a relatively uncharacterized enzyme that is involved in the metabolism of arachidonic acid [195], while hepatic Cyp17 is involved in estrogen biosynthesis during development by

ensuring the conversion of circulating progestogens to estrogen [196]. The down-regulation of Cyp2b10 contradicts studies reporting induction in the liver, but is consistent with its ER mediated regulation [197]. Gamma-glutamyl transpeptidase (Ggt1) was also induced by EE and may play a pro-oxidant and hepatocarcinogenic role via the generation of reactive oxygen species [198]. Increases in Ggt1 positive foci in the rat liver after EE administration have been reported and are thought to be ER dependent [178].

The glutathione S-transferase (GST) family of enzymes facilitates the conjugation of glutathione with exogenous and endogenous compounds [199]. Genetic polymorphisms in these genes have been attributed to increased cancer susceptibility [200]. Glutathione S-transferase isoforms alpha 1 (Gsta1), alpha 4 (Gsta4), mu 5 (Gstm5), and theta 2 (Gstt2) were all repressed at 3x24 hr, with concomitant up-regulation of the pi 2 isoform (Gstp2). Many of these transcripts were also dose-dependently regulated at 24 hrs. E2 is known to block the immunoreactivity of the alpha and mu class isoforms in epithelial cells of the vas deferens in Syrian hamsters [201] while significant down-regulation of Gstt2 transcripts in the mouse uterus has been previously reported [202]. Overall, down regulation of GST enzymes would reduce conjugation and elimination of the oxidative catechol and quinone metabolites, thus increasing susceptibility to oxidative stress and genotoxicity, which may contribute to the hepatocarcinogenic effects of EE in addition to its promotion of cell growth and proliferation.

Lipid metabolism and transport - Estrogens modulate lipid metabolism and transport and elicit anti-atherosclerotic effects in mammals [146, 203] primarily through alterations

in hepatic lipase and plasma lipoprotein levels which lead to decreases in plasma VLDL and concomitant increases in HDL [204, 205]. Apolipoprotein C-II (Apoc2), which plays an important role in plasma lipid clearance and when deficient results in hypertriglyceridemia [206], was temporally and dose-dependently induced, consistent with its ER-mediated induction in primate liver and human HepG2 cells [204, 205]. Temporal and dose-dependent induction of apolipoprotein A-IV (Apoa4) transcript was also detected. Apoa4 is involved in cholesterol transport and increasing HDL plasma levels and exerts atherosclerotic protective effects [207]. Both Apoc2 and Apoa4 are cofactors that modulate key enzyme activities involved in lipoprotein metabolism including lecithin:cholesterol acyltransferase and lipoprotein lipase, which clear plasma chylomicrons and decrease plasma VLDL levels [206, 208].

Increased plasma Apoe levels are also associated with decreased risk for atherosclerosis [206]. Translationally mediated increases in plasma Apoe have been reported in male mice treated with estrogen for 5 consecutive days at 3 mg/kg [146]. However, in this study Apoe transcripts were repressed at 24 and 3x24 hrs suggesting that transcriptional regulation may also exist. The directional discrepancy may be due to differences in dose, time of sacrifice, sex and route of exposure. Lipc mRNA levels also exhibited temporal and dose dependent repression, in agreement with published studies using HepG2 cells [209]. Lipc is an important enzyme in lipoprotein metabolism and there is an inverse correlation between its activity and plasma levels of HDL cholesterol [210]. Collectively, these alterations in lipid transport and metabolism mRNAs illustrate the complex role that estrogen plays in the regulation of transcripts that modulate plasma lipid profiles, and provide supporting evidence for estrogen mediated decreases in plasma

VLDL levels with concomitant increases in HDL levels that are associated with a reduced risk for atherosclerosis.

Dose response analysis

Conducting dose response experiments allows for further interpretation of the sensitivity of the responses to the chemical agent. A majority of the 39 dose responsive genes identified in this study exhibited ED_{50} values comparable (10 to 100 μg/kg) to the uterotrophic ED_{50} values reported for EE in the literature [155, 156] (Table IV). However, a number of transcripts had ED_{50} values less than 10 μg/kg indicating that changes in gene expression can occur at doses that may not elicit a physiological effect. Therefore, these responses could serve as molecular markers for exposure that are more sensitive than the physiological response. Several genes also had ED_{50} values greater than 250 μg/kg which may represent weak estrogenic responses or an effect due to an inappropriate sampling time for these responses. Alternatively, these genes may represent ER-independent responses to supraphamacological doses that may contribute to EE toxicity.

Comparison of uterine and hepatic responses

Intuitively, differences in gene expression in response to EE are expected between tissues. By harvesting multiple tissues from the same animal, this study provided an opportunity to comparatively assess EE elicited shared and tissue-specific gene expression responses. Despite differences in data analysis (i.e. empirical Bayes analysis of two replicates in uterine study vs. model t-statistic of three replicates in hepatic study), and the array platform (i.e. Affymetrix Mu11KSubA GeneChips for uterine study vs. cDNA/EST microarrays for hepatic study) used in the two studies, 93 of

the 1318 genes in common, were significantly affected in both tissues. For example, Car3 was down-regulated in both tissues whereas Car1 was up-regulated, indicating a common isoform-specific regulation. Carbonic anhydrases catalyze the reversible hydration of CO_2 which serves functions in cellular homeostasis related to energy metabolism and the maintenance of pH, both of which are likely to be important in tissue responses to estrogen[211]. Although typically associated with cell cycle arrest; Cdkn1a (aka p21) induction in both tissues in response to estrogen stimulation suggests a more complex role in the cell cycle[212] as does its immediate early induction at both the mRNA and protein level [152]. These data suggest that Cdkn1a may play a regulatory role in S-phase progression during cell growth and proliferation. Likewise, Tgm2 was also induced in both tissues and is likely involved in maintaining structural integrity during tissue growth [213]. Other genes that displayed similar expression profiles included Stat5a, Hif1a, Actg, and Cyr61 suggesting that these gene products may serve common functions and have common mechanisms of regulation. Although these genes shared similar expression patterns, there were noticeable variations in expression kinetics that may be due to differences in blood flow (i.e. first passed through liver), tissue vascularity and lipid content [157] and other tissue and cell specific factors that may affect transcription and mRNA stability.

Although there is a high degree of similarity in the responses observed in the liver to those reported for reproductive tissues, there are also examples of genes that displayed opposite regulation. For example, Igf1, a well characterized estrogen inducible gene in the uterus [152, 214], that is involved in proliferation, was down-regulated in the liver. Conversely, Igfbp1 is known to be down regulated in the uterus [214] but was induced

over 8-fold in the liver. The products of these genes play important roles in estrogen mediated proliferation in the uterus. Their opposite regulation in the liver suggests that they may be involved in liver specific responses such as mediating the mito-inhibitory effect that is observed after a more prolonged exposure of the liver to estrogen [215]. Cyp17 exhibited a dose-dependent induction in the liver while dose-dependent repression has been reported in the uterus and ovary [216]. This differential regulation of these likely involves tissue specific co-activator/co-repressor expression or may be a function of chromatin structure and promoter accessibility [217]. Their tissue specific regulation and the manner in which their functions contribute to the unique physiology of these tissues, requires further investigation.

General conclusions

Results from these studies clearly establish the liver as an estrogen responsive tissue. Temporal and dose response studies not only extended the number and classes of estrogen regulated genes but also further elucidated potential mechanisms associated with known hepatic physiologic responses to estrogen. Furthermore, as a result of comparative studies, common estrogen elicited expression profiles across tissues as well as potential tissue-specific biomarkers of exposure were identified that may support drug development as well as the assessment of the endocrine disrupting activities of xenobiotics and natural products. However, additional studies using complementary technologies are needed to establish causal relationships between changes in gene expression and physiological outcomes. Furthermore, other estrogen responsive tissues will need to be examined in order to more clearly define the shared and tissue-specific effects of estrogens.

CHAPTER 4

TEMPORAL AND DOSE-DEPENDENT HEPATIC GENE EXPRESSION PATTERNS IN MICE PROVIDE NEW INSIGHTS INTO TCDD-MEDIATED HEPATOTOXICITY[3]

ABSTRACT

In an effort to further characterize the mechanisms of TCDD-mediated toxicity, comprehensive temporal and dose response microarray analyses were performed on hepatic tissue from immature ovariectomized C57BL/6 mice treated with TCDD. For temporal analysis, mice were gavaged with 30 μg/kg of TCDD or vehicle and sacrificed after 2, 4, 8, 12, 18, 24, 72 or 168 hours. Dose response mice were gavaged with 0, 0.001, 0.01, 0.1, 1, 10, 100 or 300 μg/kg of TCDD and sacrificed after 24 hours. Hepatic gene expression profiles were monitored using custom cDNA microarrays containing 13,362 cDNA clones. Gene expression analysis identified 443 and 315 features which exhibited a significant change at one or more doses or time points, respectively, as determined using an empirical Bayes approach. Functional gene annotation extracted from public databases associated gene expression changes with physiological processes such as oxidative stress and metabolism, differentiation, apoptosis, gluconeogenesis, and fatty acid uptake and metabolism. Complementary histopathology (H&E and Oil Red O stains), clinical chemistry (i.e. ALT, TG, FFA, cholesterol) and high resolution gas chromatography/mass spectrometry assessment of hepatic TCDD levels were also performed in order to phenotypically anchor changes in gene expression to physiological

[3] Data contained in this chapter have been published in: Boverhof, D. R., Burgoon, L. D., Tashiro, C., Chittim, B., Harkema, J. R., Jump, D. B., and Zacharewski, T. R. (2005). Temporal and dose-dependent hepatic gene expression patterns in mice provide new insights into TCDD-Mediated hepatotoxicity. *Toxicol Sci* **85**, 1048-63.

endpoints. Collectively, the data support a proposed mechanism for TCDD mediated hepatotoxicity, including fatty liver which involves mobilization of peripheral fat and inappropriate increases in hepatic uptake of fatty acids.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds are legacy environmental contaminants that cause human health effects at controversial environmental levels. This class of chemicals elicits a broad spectrum of toxic and biochemical responses in a tissue-, sex-, age- and species-specific manner that include a wasting syndrome, tumor promotion, teratogenesis, hepatotoxicity, modulation of endocrine systems, immunotoxicity and enzyme induction [26]. TCDD exposure in human populations has also been linked to increases in various cancers including hepatocellular carcinoma [218]. Many, if not all, of these effects are due to alterations in gene expression mediated via the activation of the aryl-hydrocarbon receptor (AhR), a member of the basic-helix-loop-helix-PAS (bHLH-PAS) family [23, 26]. Ligand binding to the cytoplasmic AhR complex triggers the dissociation of interacting proteins and results in the subsequent translocation of the ligand-bound AhR to the nucleus where it heterodimerizes 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 that ultimately result in the observed toxic and biochemical responses [32].

The obligatory involvement of the AhR/ARNT signaling pathway in mediating the toxic and biochemical responses to TCDD is supported by studies demonstrating that mice with low affinity AhR alleles are less susceptible to the effects of TCDD [35], and AhR-null mice are resistant to the prototypical toxicities of TCDD and related compounds [36-38]. More recent studies have shown that mice possessing mutations in the AhR nuclear localization/DRE binding domain and mice harboring a hypomorphic ARNT allele, fail to exhibit the classical TCDD toxicities [39, 40]. Furthermore, the AhR/ARNT signaling pathway plays an important role in development, differentiation and growth, as AhR null mice experience various liver, heart, thymus and immune system abnormalities. Developmental effects are most notable in the liver as AhR null mice exhibit reduced liver weight, transient microvesicular fatty metamorphosis, prolonged extramedullary hematopoiesis, and portal hypercellularity with thickening and fibrosis [219]. Moreover, mice expressing a constitutively active AhR exhibit increased heptocarcinogenesis which has further implicated AhR activation in tumor promotion [220].

Although the mechanisms of AhR/ARNT-mediated changes in gene expression are fairly well established, TCDD elicited modulation of gene expression and pathways associated with toxicity remains poorly understood. Well characterized AhR inducible genes are limited to various xenobiotic metabolizing enzymes including cytochrome P450s 1a1, 1a2 and 1b1. However, a significant role for cytochrome P450 induction alone in the observed adverse responses is questionable [41]. Global gene expression technologies provide a comprehensive strategy whereby critical AhR-regulated target

genes can be identified and used to elucidate target pathways involved in the etiology of TCDD and related compound toxicity.

Sustained activation of the AhR and its target genes has been hypothesized as a prerequisite for toxicity that typically requires days or weeks to develop. Alternatively, activation of the AhR may initiate a cascade of secondary and tertiary gene expression changes leading to the compromised physiological state. Hepatotoxicity is a classical endpoint of TCDD exposure characterized by hepatomegaly accompanied by hepatocyte hypertrophy, fat accumulation, immune infiltration, necrosis and alterations in liver enzymes [221] which likely contribute to tumor promotion and hepatocarcinogenesis. In order to identify gene expression changes causal to hepatotoxicity and carcinogenesis and to further characterize the spectrum of AhR/ARNT responsive transcripts, temporal and dose-dependent effects of TCDD on hepatic gene expression were examined in the context of complementary histological and clinical chemistry endpoints. This integrative approach has provided a powerful strategy to comprehensively assess the *in vivo* effects of TCDD.

MATERIALS AND METHODS

Animal Handling

Female C57BL/6 mice, ovariectomized by the vendor on postnatal day (PND) 20 and all having body weights (BW) within 10% of the average BW, were obtained from Charles River Laboratories (Raleigh, NC) on PND day 26. This animal model is utilized in our lab for the research of estrogenic endocrine disruptors and was employed in the present study for consistency and to facilitate future research goals examining the estrogenic endocrine disrupting effects of TCDD. The mice were housed in polycarbonate cages

containing cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensberg, NY) in a 23°C HEPA-filtered environment with 30-40% humidity and a 12hr light/dark cycle (07 00hr – 19 00hr). Animals were allowed free access to deionized water and Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI), and acclimatized for four days prior to dosing. On the fourth day, animals were weighed, and a stock solution of TCDD (provided by S. Safe, Texas A&M University, College Station, TX) was diluted in sesame oil (Sigma, St. Louis, MO) to achieve the desired dose based on the average weight. All procedures were performed with the approval of the Michigan State University All-University Committee on Animal Use and Care.

Time Course and Dose Response Studies

For the time course study, mice were treated by gavage with 0.1 ml of sesame oil for a nominal dose of 0 (vehicle control) or 30μg/kg bw of TCDD. Eight animals were treated per dose group and time point and groups for each dose and time point were housed in separate cages. Mice were sacrificed 2, 4, 8, 12, 18, 24, 72 or 168 hours after dosing. An untreated group of mice was also included which was sacrificed at time zero, the time at which the other animals were dosed. For the dose response study, 5 mice per group were gavaged with 0.1ml of vehicle or 0.001, 0.01, 0.1, 1, 10, 100 or 300μg/kg TCDD and sacrificed 24 hr after dosing. In both studies, treatment was staggered to ensure exposure duration was within 5% of the desired length. Animals were sacrificed by cervical dislocation and tissue samples were removed, weighed, flash frozen in liquid nitrogen and stored at -80°C until further use. For the dose response study, the right lobe of the liver was fixed in 10% neutral buffered formalin (Sigma), for histological analysis.

Clinical Chemistry and Histological Analyses

Mice were gavaged with 0 (vehicle control) or 30μg/kg bw of TCDD and were sacrificed 2, 4, 8, 12, 18, 24, 72 or 168 hours after dosing. At sacrifice, mice were anesthetized with 0.1ml of a 5% solution of sodium pentobarbital and blood was collected by cardiac puncture and placed in Vacutainer® SST gel and clot activator tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were allowed to clot and serum was separated by spinning at 1500×g for 10 minutes after which they were stored at -80°C until analysis. As sample was limiting, only select clinical chemistry endpoints were monitored which included blood urea nitrogen (BUN), creatinine (CREA), free fatty acids (FFA), glucose (GLU), total bilirubin (TBIL), alanine aminotransferase (ALT), cholesterol (CHOL), and triglycerides (TRI). Endpoints were monitored by standard clinical chemistry assays using an Olympus AU640 Automated Chemistry Analyzer, (Olympus America Inc., Melville, NY) at the Clinical Pathology Laboratory at MSU (http://cvm.msu.edu/clinpath/new.htm).

Tissues were harvested and fixed in 10% neutral buffered formalin (NBF, Sigma). Sectioned tissues were processed sequentially in ethanol, xylene and paraffin using a Thermo Electron Excelsior (Waltham, MA). Tissues were then embedded in paraffin using a Miles Tissue Tek II embedding center after which paraffin blocks were sectioned at 5 microns with a rotary microtome. Sections were placed on glass microscope slides, dried and stained with the standard hematoxylin and eosin stain. All histological processing was performed at the histology laboratory (http://www.lahms.msu.edu). Histological evaluations were preformed by a veterinary pathologist. For Oil Red O staining, liver cryosections were fixed in NBF, stained with Oil Red O solution, washed and counterstained with hematoxylin.

Quantification of TCDD in liver samples

Liver samples were processed in parallel with lab blanks and a reference or background sample at Wellington Laboratories Inc. (Guelph, ON, Canada). Samples were weighed, spiked with $^{13}C_{12}$ TCDD surrogate, digested with sulfuric acid and then extracted. Extracts were cleaned, concentrated and spiked with an injection standard. Analysis was performed on a high resolution gas chromatograph/high resolution mass spectrometer (HRGC/HRMS) using a Hewlett Packard 5890 Series II GC interfaced to a VG 70SE HRMS. The HRMS was operated in the EI/SIR mode at 10,000 resolution. A 60m DB5 column (J&W Scientific, Folsom, CA) with an internal diameter of 0.25mm and film thickness of 0.25μm was utilized. Injection volumes were 2ul and a splitless injection was utilized.

RNA isolation

Frozen liver samples (approximately 70mg) were transferred to 1.0ml of Trizol (Invitrogen, Carlsbad, CA) and homogenized using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was isolated according to the manufacturer's protocol with an additional phenol:chloroform extraction. Isolated RNA was resuspended in RNA storage solution (Ambion Inc., Austin, TX), quantified (A_{260}) and assessed for purity by determining the A_{260}/A_{280} ratio and by visual inspection of 1.0μg on a denaturing gel.

Microarray Experimental design

Changes in gene expression were assessed using customized cDNA microarrays containing 13,362 features representing 7,952 unique genes (Unigene build #144, Supplementary Table 1). For temporal analysis, TCDD treated samples were compared to time matched vehicle controls using an independent reference design. In this design, a

treated animal is compared to a time matched vehicle control with two-independent labelings per sample (dye swap) for a total of 16 arrays per replicate (8 time points × 2 arrays/time point comparison). Four replicates of this design were performed, each using different animals, for a total of four biological replicates and 64 arrays.

Dose response changes in gene expression were analyzed using a common reference design in which samples from TCDD treated mice are co-hybridized with a common vehicle control. Each design replicate uses one of the five animals from each dose group with two independent labelings per sample (dye swap) for a total of 14 arrays (7 doses × 2 arrays/dose comparison). Four replicates of this design were performed, each using different animals, for a total of four biological replicates and 56 microarrays.

Microarray analysis of differential gene expression

Detailed protocols for microarray preparation, labeling of the cDNA probe, sample hybridization and 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 (4×12) Chipmaker 2 pins (Telechem) at the Genomics Technology Support Facility (http://www.genomics.msu.edu). Total RNA (30μg) was reverse transcribed in the presence of Cy3- or Cy5-dUTP to create fluor-labeled cDNA which was purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA). Cy3 and Cy5 samples were mixed, vacuum dried and resuspended in 48μl of hybridization buffer (40% formamide, 4xSSC, 1%SDS) with 20μg polydA and 20μg of mouse COT-1 DNA (Invitrogen, Carlsbad, CA) as competitor. This probe mixture was heated at 95°C for 3 min and hybridized on the

array under a 22×40 mm lifterslip (Erie Scientific Company, Portsmouth, NH) in a light protected and humidified hybridization chamber (Corning Inc., Corning, NY) for 18-24 hours in a 42°C water bath. Slides were then washed, dried by centrifugation and scanned at 635nm (Cy5) and 532nm (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, Union City, CA).

Microarray Data Normalization and Analysis

Data were normalized using a semiparametric approach [222]. Model-based t-values were calculated from normalized data, comparing treated and vehicle responses per time-point or dose group. Empirical Bayes analysis was used to calculate posterior probabilities (P1(t)-value) of activity on a per gene and time-point or dose-group basis using the model-based t-value [223]. A stringent P1(t) cutoff of 1.0 was used to obtain a subset of differentially regulated genes to initially focus analysis and data interpretation on the most reproducible differentially regulated genes,. Gene expression changes that passed the threshold were subsequently analyzed using hierarchical and K-means clustering (GeneSpring 6.0, Silicon Genetics, Redwood City, CA). Dose-response analysis was performed using Graph Pad Prism 4.0 (GraphPad Software, San Diego, CA). The P1(t) value is a Bayesian posterior probability that is different from the p-value in that it can be used to provide an initial ranking of genes, based on their expression, in order to prioritize those transcripts for further investigation relative to biologic/toxic relevance. It is only a guide to rank the probability of identifying the most active genes, and is not equivalent to a p-value. Therefore, it is not intended to be used for hypothesis testing. Posterior probabilities generated by Bayesian analyses are better suited for

microarray data when compared to parametric analyses since no assumptions are required regarding the distribution of the gene expression data which is typically not normally distributed. Consequently, gene expression changes that approach the initial P1(t) cut-off will also be considered provided supporting published evidence indicates its relevance in the emerging pathway. These genes would also be candidates for verification by QRTPCR

Quantitative real-time PCR

For each sample, 1.0μg of total RNA was reverse transcribed by SuperScript II using an anchored oligo-dT primer as described by the manufacturer (Invitrogen, Carlsbad, CA). The cDNA (1.0μl) was used as a template in a 30μl PCR reaction containing 0.1μM of forward and reverse gene-specific primers designed using Primer3 [151], 3 mM $MgCl_2$, 1.0mM dNTPs, 0.025IU AmpliTaq Gold, and 1× 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 2. 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 7 orders of magnitude to interpolate relative template concentrations of the samples from the standard curves of log copy number vs. 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 the geometric mean of 3 house-keeping genes (β-actin, Gapd and Hprt) to control for differences in RNA loading, quality and cDNA synthesis. For graphing purposes, the relative expression levels were scaled such that the expression level of the time matched control group was equal to 1.

Statistical Analysis

Statistical analysis was performed using SAS 8.02 (SAS Institute, Cary, NC). Data were analyzed using analysis of variance followed by Dunnett's or Tukey's post hoc tests. Differences between treatment groups were considered significant when $p<0.05$.

RESULTS

Organ and Body Weights

TCDD treatment resulted in a significant ($p<0.05$) dose-dependent increase in relative liver weights at 100 and 300 μg/kg (Table 1). In the time course study, relative liver weights were significantly ($p<0.05$) increased at 24, 72 and 168 hours with maximal increases observed at 168 hours (Table 2). No additional significant treatment related alterations in organ weights were noted in either study. Although wasting syndrome is a hallmark of TCDD toxicity, no effects on body weight or body weight gain were noted at any of the doses or time points in either study when compared to time matched controls. These results are consistent with a recent study that reported increases in liver weights but no alterations in body weights after a single oral dose of TCDD at concentrations up to 200 μg/kg [224].

Table 1. Terminal body, whole liver and relative liver weights for mice treated with vehicle or increasing doses of TCDD and sacrificed after 24 hours.

Dose[a]	Treatment	Body Weight (g)	Liver Weight (g)	RLW[b]
0	Vehicle	14.52 ± 1.27	0.864 ± 0.112	0.059 ± 0.003
0.001	TCDD	15.88 ± 1.37	0.972 ± 0.246	0.061 ± 0.004
0.01	TCDD	15.24 ± 1.71	0.921 ± 0.092	0.061 ± 0.010
0.1	TCDD	14.94 ± 1.24	0.891 ± 0.036	0.060 ± 0.005
1	TCDD	15.30 ± 1.02	0.967 ± 0.122	0.063 ± 0.005
10	TCDD	14.72 ± 1.13	0.962 ± 0.111	0.065 ± 0.004
100	TCDD	15.46 ± 0.48	1.042 ± 0.076	0.067 ± 0.003 *
300	TCDD	15.14 ± 0.91	1.002 ± 0.109	0.067 ± 0.004 *

[a] All doses in µg/kg
[b] RLW = relative liver weight
* $p<0.05$

Table 2. Terminal body, whole liver and relative liver weights for mice treated with vehicle or 30 µg/kg of TCDD.

Sacrifice Time (hrs)	Treatment	Body Weight (g)	Liver Weight (g)	RLW[a]
2	Vehicle	12.58 ± 1.61	0.753 ± 0.127	0.059 ± 0.004
	TCDD	12.23 ± 1.31	0.722 ± 0.136	0.059 ± 0.005
4	Vehicle	12.25 ± 1.49	0.718 ± 0.115	0.058 ± 0.003
	TCDD	12.16 ± 1.48	0.652 ± 0.0.86	0.054 ± 0.004
8	Vehicle	12.31 ± 1.65	0.618 ± 0.093	0.050 ± 0.004
	TCDD	12.50 ± 1.70	0.655 ± 0.109	0.052 ± 0.003
12	Vehicle	12.64 ± 1.26	0.687 ± 0.084	0.054 ± 0.004
	TCDD	12.97 ± 0.79	0.741 ± 0.060	0.057 ± 0.006
18	Vehicle	13.86 ± 1.56	0.844 ± 0.109	0.060 ± 0.005
	TCDD	13.33 ± 1.67	0.830 ± 0.165	0.062 ± 0.006
24	Vehicle	13.11 ± 1.63	0.816 ± 0.100	0.062 ± 0.003
	TCDD	12.54 ± 1.43	0.879 ± 0.112	0.070 ± 0.003 *
72	Vehicle	14.44 ± 1.58	0.858 ± 0.127	0.058 ± 0.009
	TCDD	14.00 ± 1.97	1.061 ± 0.209	0.075 ± 0.007 *
168	Vehicle	15.89 ± 1.51	0.9735 ± 0.139	0.060 ± 0.004
	TCDD	15.53 ± 1.84	1.262 ± 0.212	0.081 ± 0.006 *

[a] RLW = relative liver weight
* $p<0.05$

Hepatic TCDD Concentrations

Hepatic TCDD levels were determined in dose-response and time course studies in order to relate tissue concentrations to molecular responses. TCDD levels were significantly ($p<0.05$) increased in a dose dependent manner at doses as low as 0.1 μg/kg (Figure 1A). In the time course study, significant ($p<0.05$) increases in hepatic TCDD content were noted at all time points with a gradual increase between 2 and 72 hours followed by a 60% decrease at 168 hours (Figure 1B). Hepatic TCDD levels in this study are comparable to other reports using similar exposure regimens. For example, acute administration of 1 μg/kg to rats, resulted in 7,000 ppt TCDD in the liver after 24 hours [225], comparable to 5,100 ppt in this study. Similarly, 54,000 ppt TCDD was detected in the mouse liver at 7 days following acute administration of 10 μg/kg[226], while we report 125,000 ppt TCDD 7 days after a 30 μg/kg dose. Moreover, a recent 13 week subchronic National Toxicology Program study reported that mice dosed with 0.1 ug/kg/day 5days/week achieved hepatic TCDD levels of 18,300 ppt [227], a value within the range of this study. The accurate determination of hepatic TCDD levels is essential in order to elucidate correlations between gene expression and physiological effects across studies and to comparatively assess hepatic accumulation and clearance of TCDD between species as there are significant species differences in the half-life of TCDD.

Histological Endpoints

In the dose-response study, the principal TCDD related alteration was a minimal to moderate cytoplasmic vacuolization of hepatocytes primarily in the periportal and midzonal regions of the liver. This effect was absent or minimal in mice from the 0.001-

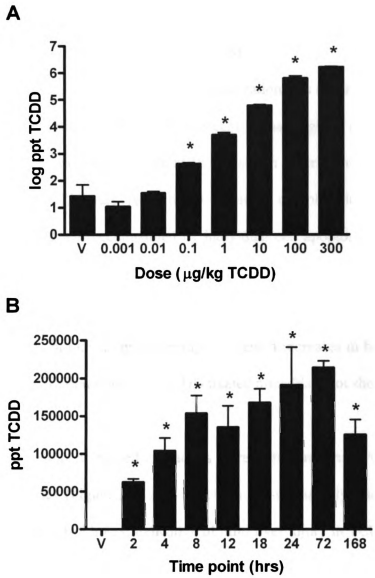


Figure 1.
TCDD concentrations in hepatic tissue of mice from both dose response (A) and temporal (B) studies determined using HRGC/HRMS. Dose response concentrations are displayed on a log scale to allow for visualization of tissue concentrations at all doses. All results are displayed as the mean ± standard error of at least 3 independent samples.
ppt= parts per trillion (equivalent to pg/g), * $p<0.05$

0.1μg/kg groups, whereas in mice from the 1-300μg/kg dose groups, mild to moderate cytoplasmic vacuolization was consistently observed.

In the time course study, cytoplasmic vacuolization was observed in the periportal and midzonal regions with extension into the centriacinar regions at later time points. Minimal vacuolization was first observed at 18 hours with severity progressing from mild to moderate at 24 and 72 hours, respectively. Marked cytoplasmic vacuolization was noted at 168 hours and was accompanied by individual cell apoptosis and immune cell accumulation (Figure 2, A and B). Oil Red O staining confirmed that the dose- and time-dependent vacuolization was due to lipid accumulation (Figure 2, C and D). Analysis of liver lipid extracts by thin layer chromatography revealed increases in both triglycerides, free fatty acids and cholesterol esters in TCDD treated mice (data not shown).

Clinical Chemistry

Significant treatment related alterations were noted in serum ALT, cholesterol, FFAs, and triglycerides (Figure 3). ALT levels increased steadily after 24 hours to a maximum of 2.6-fold at 168 hours, indicative of mild liver injury in TCDD-treated mice. Serum cholesterol was significantly ($p<0.05$) decreased by 33 and 28% at 72 and 168 hours, respectively. Serum FFAs were increased 33, 16 and 28% at 24, 72 and 168 hours, respectively. Triglyceride levels were also elevated by 24, 15 and 40% in TCDD treated mice at 24, 72 and 168 hours, respectively. No significant treatment related effects were noted on serum BUN, CREA, GLU or TBIL.

Microarray Data Filtering

Empirical Bayes analysis of the dose response data identified 443 microarray features, representing 374 annotated clones and 349 unique genes, which were

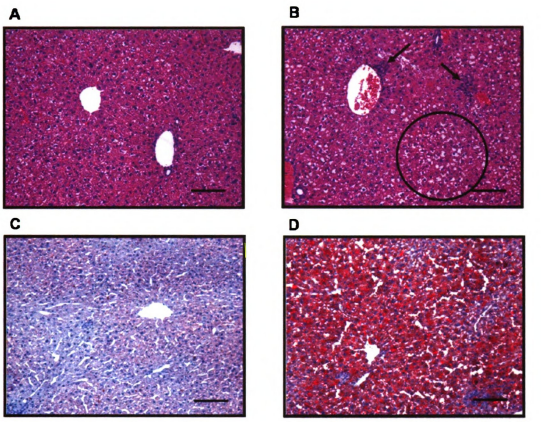


Figure 2.
Liver histology from control and TCDD treated mice at the 168 hour time point. A and B are H&E stains from a control and TCDD treated mouse, respectively. Arrows indicate immune cell accumulation while the circle highlights an area of extensive vacuolization. C and D are Oil Red O stains from a control and TCDD treated mouse, respectively. Red staining areas denote fat accumulation. Bars = 10 μm

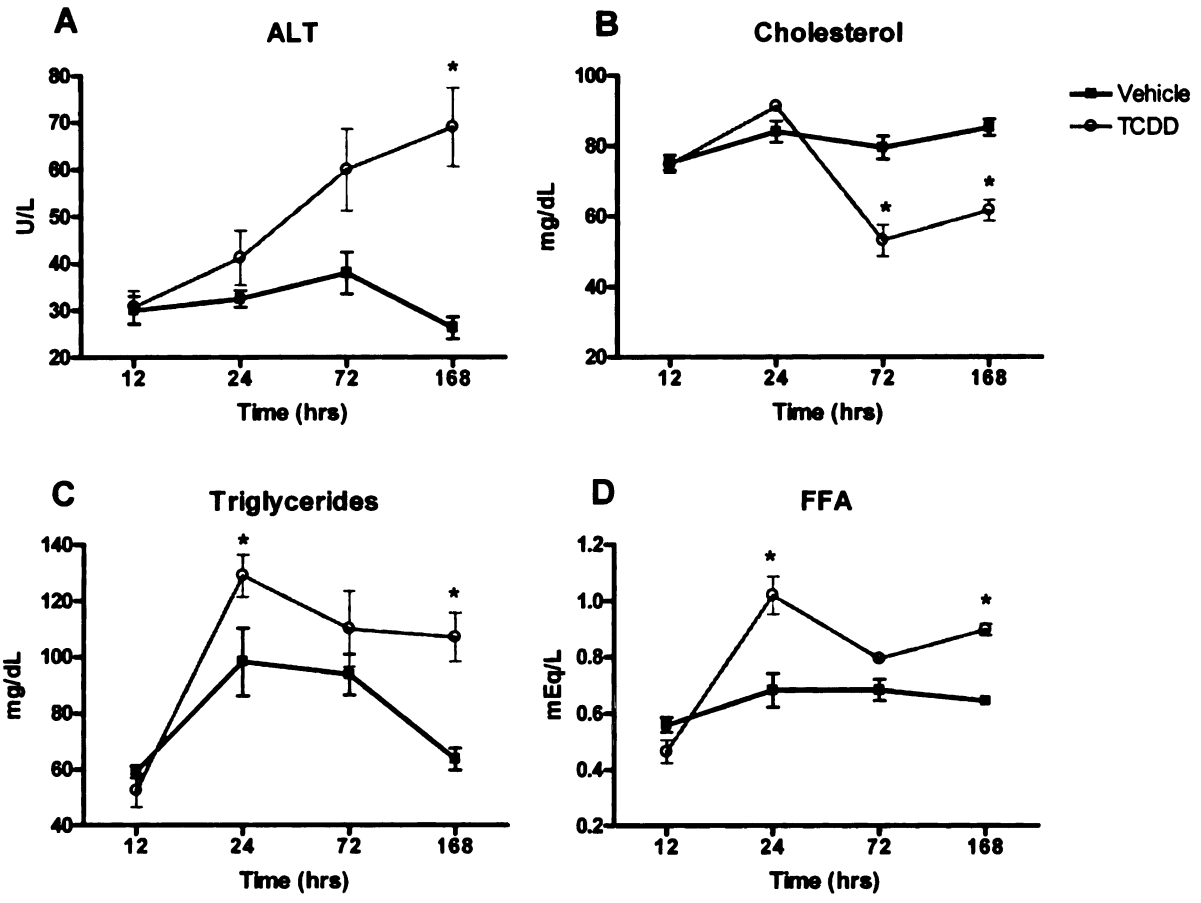


Figure 3.
Serum clinical chemistry values of significantly affected endpoints after treatment with vehicle (squares) or 30 μg/kg TCDD (circles) for 12, 24, 72 or 168 hours. Triglycerides (A), cholesterol (B), alanine aminotransferse (ALT; C), and free fatty acids (FFA; D). There were no significant treatment effects on serum BUN, CREA, GLU or TBIL. Results represent the mean ± standard error of at least 3 independent samples. * $p<0.05$

differentially expressed (P1(t) = 1.0) relative to vehicle controls, at one or more doses. A dose dependent increase in the number of active genes was observed which began to plateau at 100 and 300μg/kg TCDD (Figure 4A). Based on these results, a dose of 30μg/kg was chosen for temporal studies as it represents the approximate ED_{50} for overall gene responses and would avoid overt toxicity and lethality in a longer term study. Analysis of the time course data identified 315 microarray features, representing 269 annotated clones corresponding to 255 unique genes, which were differentially expressed (P1(t) = 1.0), relative to the time-matched vehicle controls at one or more time points. The 2 hour time point displayed the fewest number of active genes followed by a large increase at 4 hours which was largely stable through 18 hours and followed by an additional increase between 24 and 168 hours. This temporal pattern indicates that a majority of the gene expression responses are preceding the observed histological alterations. In addition, the later increases in active features coincide with the appearance and severity of hepatotoxicity indicating that these responses may be a result of the emerging toxicity. The gene lists obtained from these initial stringent filtering criteria were used for data clustering, organization and the identification of functional pathways affected by TCDD. Complete data sets for both dose response and time course experiments are available in Supplementary Tables 3 and 4, respectively.

Of the 98 active genes identified by these criteria in the time course study at 24 hours, 83 also exhibited active dose-dependent responses. Although the dose utilized in the time course study (30μg/kg) was not included in the dose-response study, comparison of responses from flanking doses (10 and 100μg/kg) indicated that there was a good agreement in the magnitude of the responses between these studies. For example, Notch

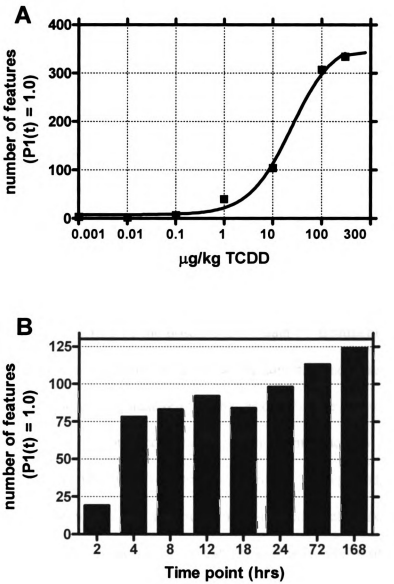


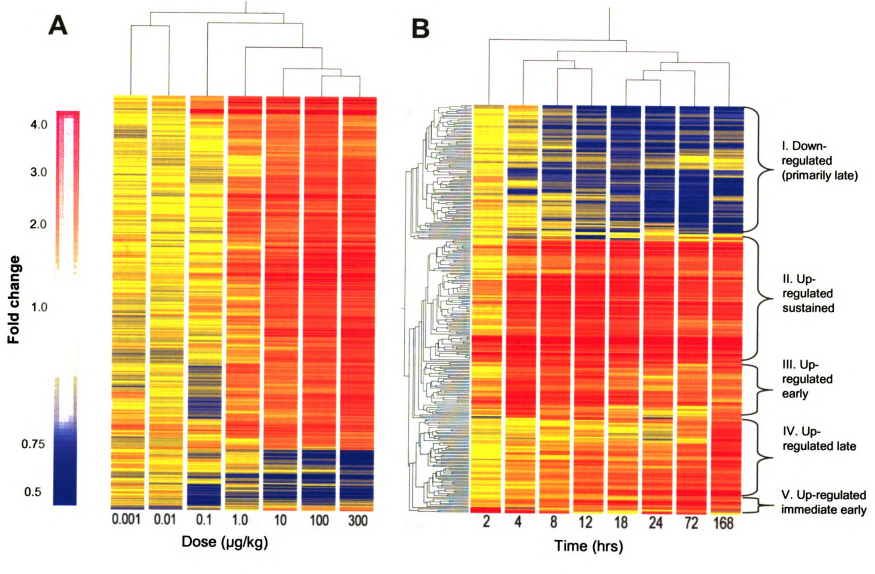
Figure 4.
Number of features exhibiting significant changes in gene expression for dose response (A) and temporal (B) microarray studies (P1(t) = 1.0). The dose response study revealed a dose-dependent increase in the number of active features which maximized at 100 and 300 µg/kg. The temporal study displayed a large increase at 4 hours followed by a consistent increase in the number of active genes with time.

gene homolog 1 (Notch1), NAD(P)H dehydrogenase, quinone 1 (Nqo1) and peroxisomal acyl-CoA thioesterase 2A (Pte2a) were induced 3.1, 3.1 and 3.0 fold, respectively, in the time course study and 3.4, 3.3 and 2.6 fold at 100μg/kg in the dose response study. Similarly, carbonic anhydrase 3 (Car3), glutamate oxaloacetate transaminase 1 (Got1) and torsin family 3, member A (Tor3a) exhibited -2.7, -2.3 and -2.1 fold repression in the time course study and -2.3, -2.4 and -2.5 fold repression at 100μg/kg in the dose response study, respectively. These results indicate the reproducibility of the gene expression responses in independent microarray experiments.

Data Clustering

Hierarchical clustering of the dose response data by treatment revealed a strong concord between gene expression responses and the administered dose of TCDD (Figure 5A). The two low dose groups clustered together as did the five highest doses. The high dose cluster also branched out sequentially by dose with the top dose of 300μg/kg exhibiting the greatest difference from low dose groups, as expected. Three K-means clusters, one down-regulated cluster and two up-regulated clusters, most accurately represented the dose response data (data not shown). The existence of two up-regulated clusters for the dose response data indicates that TCDD elicits gene specific dose dependent responses. For example, genes such as Cyp1a2, phosphoenolpyruvate carboxykinase 1 (Pck1), and Got1 displayed similar ED_{50} values to that of Cyp1a1 (0.37 – 0.95 μg/kg). ED_{50} values for Notch1 and Nqo1 induction were an order of magnitude greater (2.17 – 8.8 μg/kg), while tumor necrosis factor, alpha-induced protein 2 (Tnfaip2) and Cyp1b1 were two orders of magnitude greater (28 – 39.5 μg/kg) (Figure 6). These results may be due to gene specific thresholds, differential temporal regulation, or

Figure 5.
Hierarchical clustering of active gene lists for dose response (A) and temporal (B) microarray studies. Similar temporal clusters were identified empirically using K-means clustering (C) and were classified into down regulated (primarily late; I), up-regulated sustained (across time course; II), up-regulated early (4-12 hours; III), up-regulated late (24-168 hours; IV) or up-regulated immediate early (2 hours; V).


A
B
4.0
3.0
2.0
1.0
0.75
0.5
Fold change
0.001
0.01
0.1
1.0
10
100
300
Dose (µg/kg)
2
4
8
12
18
24
72
168
Time (hrs)
I. Down-regulated (primarily late)
II. Up-regulated sustained
III. Up-regulated early
IV. Up-regulated late
V. Up-regulated immediate early

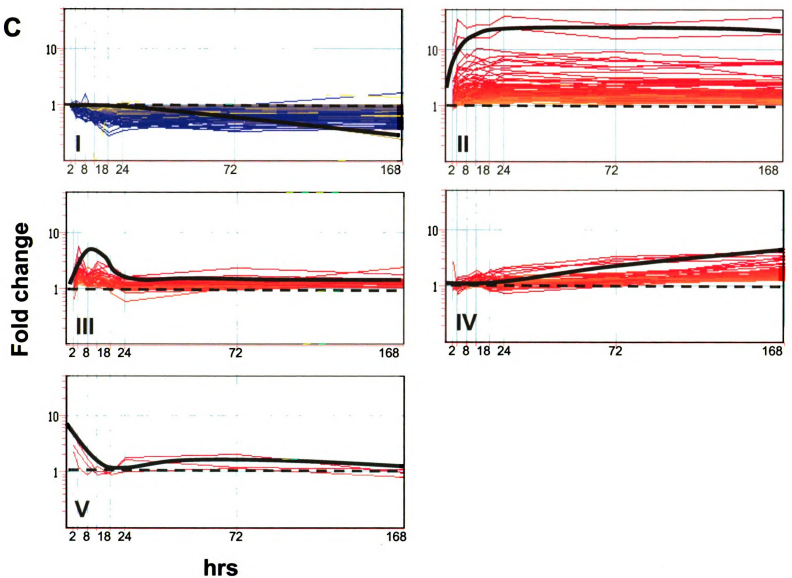
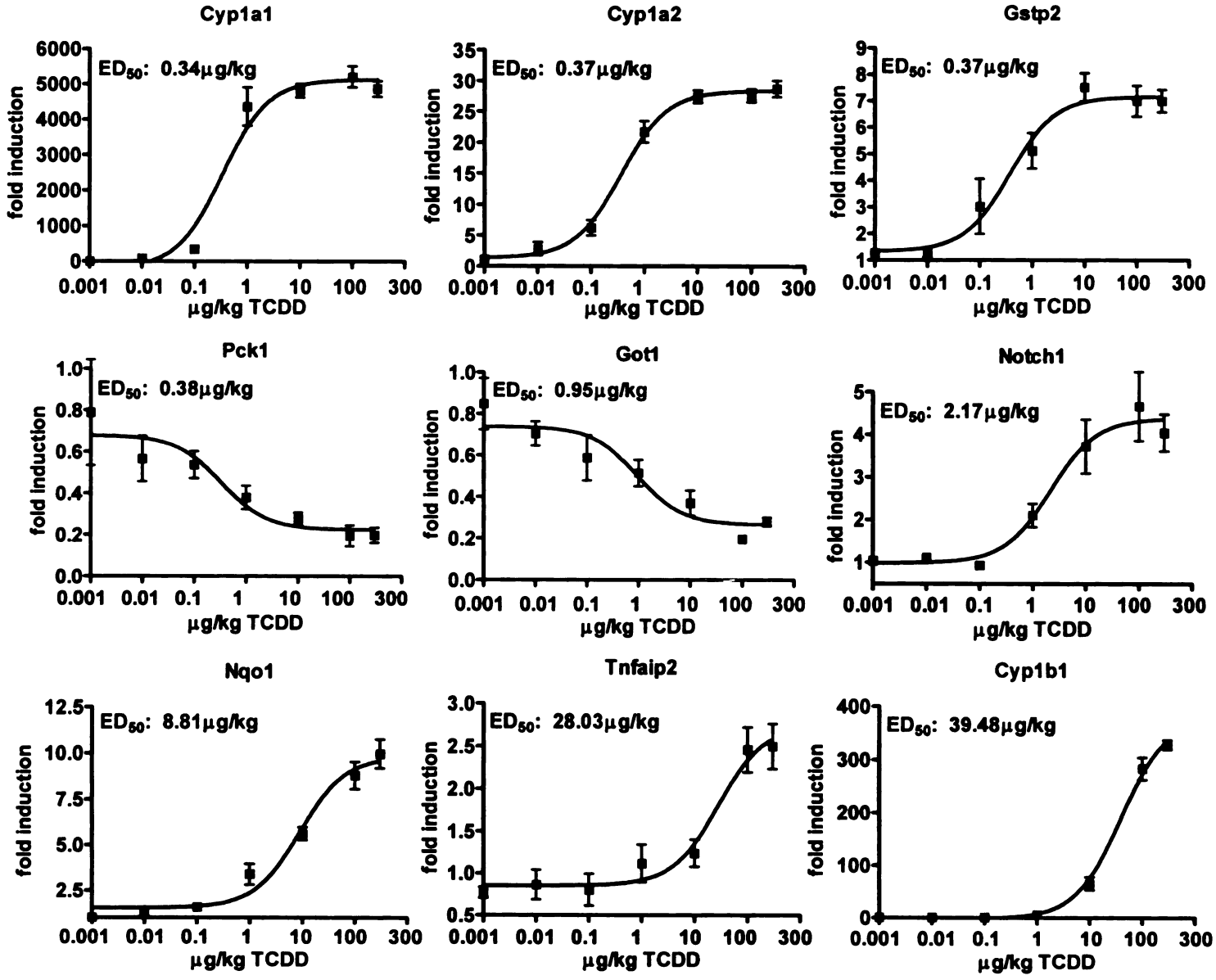

C
Fold change
10
1
I
II
III
IV
V
2 8 18 24
72
168
hrs

Figure 6.
QRTPCR verification of select dose-dependent gene expression responses. The same RNA used for cDNA microarray analysis was examined by QRTPCR. The y-axis represents the fold change calculated relative to time matched vehicle controls, while the x-axis represents the dose groups. Data points represent the fold change ± standard error of at least 4 independent samples. Dose response curves and ED_{50} values for QRTPCR data were generated in Graph Pad 4.0 using non-linear regression dose-response analysis. Genes are indicated by official gene symbols.


Cyp1a1
ED50: 0.34µg/kg
fold induction
µg/kg TCDD
Cyp1a2
ED50: 0.37µg/kg
fold induction
µg/kg TCDD
Gstp2
ED50: 0.37µg/kg
fold induction
µg/kg TCDD
Pck1
ED50: 0.38µg/kg
fold induction
µg/kg TCDD
Got1
ED50: 0.95µg/kg
fold induction
µg/kg TCDD
Notch1
ED50: 2.17µg/kg
fold induction
µg/kg TCDD
Nqo1
ED50: 8.81µg/kg
fold induction
µg/kg TCDD
Tnfaip2
ED50: 28.03µg/kg
fold induction
µg/kg TCDD
Cyp1b1
ED50: 39.48µg/kg
fold induction
µg/kg TCDD

differing basal expression levels which would affect the dose at which transcriptional regulation may be initiated or detected.

Hierarchical clustering of temporal data by experimental time points identified three primary branches. The two hour time point clustered alone, while the remaining time points clustered into early (4, 8 and 12 hour) and late (18, 24, 72 and 168 hour) responsive groups (Figure 5B). Both hierarchical and K-means clustering revealed distinctive gene expression patterns across time including down regulated (primarily late; I), up-regulated sustained (across time course; II), up-regulated early (4-12 hours; III), up-regulated late (24-168 hours; IV) or up-regulated immediate early (2 hours; V) (Figure 5 B and C). These patterns reflect the complex transcriptional hepatic to TCDD which involves induction and repression as well as early and late responses.

Functional Categorization of Microarray Data

Functional annotation extracted from public databases revealed that many of the transcriptional responses were associated with metabolizing enzymes, development and differentiation, fatty acid uptake and metabolism, gluconeogenesis, immune signaling and apoptosis (Table 3). Metabolizing enzymes included oxidoreductases, monooxygenases and xenobiotic metabolizing enzymes such as the well characterized TCDD inducible genes, Cyp1a1 and Nqo1. Novel responsive oxidoreductase and xenobiotic metabolizing genes included abhydrolase domain containing 6 (Abhd6), carbonyl reductase 3 (Cbr3), dehydrogenase/reductase (SDR family) member 3 (Dhrs3), epoxide hydrolase 1 (Ephx1) and UDP-glucose dehydrogenase (Ugdh). Glutathione S-transferases alpha2, alpha4 and pi2 (Gsta2, a4 and p2) as well as glutamate-cysteine ligase (Gclc) and glutathione synthetase (Gss) were also regulated by TCDD, which is consistent with the induction of

both phase I and II metabolizing enzymes by TCDD, commonly referred to as the AhR gene battery.

Genes involved in development and differentiation were also induced or repressed in response to TCDD treatment including Notch1, tumor necrosis factor, alpha-induced protein 2 (Tnfaip2), hairy and enhancer of split 6 (Hes6), growth arrest and DNA-damage-inducible 45 beta (Gadd45b) and growth arrest specific 1 (Gas1), all of which have not been previously reported to be regulated by TCDD. AhR-mediated dysregulation of these genes may play a role in mediating the effects of TCDD on cellular differentiation or in AhR signaling which has been implicated in the differentiation and development of various organ systems. TCDD also repressed transcripts encoding enzymes involved in gluconeogenesis including Pck1, which has been previously reported [228]. In addition, Got1 and glycerol phosphate dehydrogenase 2 (Gpd2), two additional enzymes involved in the gluconeogenic pathway, were also down regulated. These results suggest that TCDD may affect multiple steps in gluconeogenesis, although clinical chemistry did not detect any alterations in circulating glucose.

Effects on fatty acid uptake and metabolism, immune signaling and apoptosis are consistent with the observed hepatic histological findings. H&E and Oil Red O staining revealed marked fatty vacuolization of hepatocytes at 24 hours with maximal effects at 168 hours. Numerous genes involved in fatty acid transport including fatty acid binding protein 4 and 5 (Fabp4 and 5), CD36 antigen (Cd36), solute carrier family 27, member 2 (Slc27a2) and lipoprotein lipase (Lpl), were significantly induced and may mediate the fatty accumulation. Induced apoptotic genes included receptor (TNFRSF)-interacting

Table 3. Functional categorization and temporal regulation of select hepatic genes identified as differentially regulated in response to TCDD

Functional Category	Accession	Locus Link	Gene Name	Gene Symbol	Regulation	Fold change[a]	Time Points (hrs)[b]	Cluster[c]	DRE[d]
Metabolizing Enzymes	W34507	66082	abhydrolase domain containing 6	Abhd6	▲	3	4 - 168	II	Yes
	AA710940	14870	glutathione S-transferase, pi 2	Gstp2	▲	2.5	8,12,24,168	II	Yes
	AA067191	22235	UDP-glucose dehydrogenase	Ugdh	▲	3	2 - 168	II	Yes
	AA472074	22436	xanthine dehydrogenase	Xdh	▲	3	4 - 168	II	Yes
	W29265	14858	glutathione S-transferase, alpha 2 (Yc2)	Gsta2	▲	7	12 - 168	II	No
	BG062989	14629	glutamate-cysteine ligase, catalytic subunit	Gclc	▲	2	4,8,12,24,72,168	II	No
	BG072453	13849	epoxide hydrolase 1, microsomal	Ephx1	▲	2	4 - 72	II	Yes
	NM_009992	13076	cytochrome P450, family 1, subfamily a1	Cyp1a1	▲	40	2 - 168	II	Yes
	BE623489	18104	NAD(P)H dehydrogenase, quinone 1	Nqo1	▲	5	4 - 168	II	Yes
	AK003232	109857	carbonyl reductase 3	Cbr3	▲	2.5	8,12,18,72	II	Yes
	BE457542	20148	dehydrogenase/reductase (SDR family) 3	Dhrs3	▲	2	4 - 168	II	Yes
	W54349	14860	glutathione S-transferase, alpha 4	Gsta4	▲	2	12 - 168	IV	Yes
Development/ Differentiation	W84211	14451	growth arrest specific 1	Gas1	▼	-2	4,8,18,24,72,168	I	Yes
	W98998	18128	Notch gene homolog 1 (Drosophila)	Notch1	▲	3.5	2 - 168	II	Yes
	AA009268	17869	myelocytomatosis oncogene	Myc	▲	4	4,12,18,24,168	III	Yes
	BG065761	21928	tumor necrosis factor, alpha-induced protein 2	Tnfaip2	▲	5.5	2 - 168	III	Yes
	BE951829	55927	hairy and enhancer of split 6 (Drosophila)	Hes6	▲	2	168	IV	Yes
	BB025141	14673	guanine nucleotide binding protein, alpha 12	Gna12	▲	1.5	12,24,72,168	IV	Yes
	NM_008655	17873	growth arrest and DNA-damage-inducible 45b	Gadd45b	▲	5	2,4,8,24,72	V	Yes
Fatty acid uptake/ metabolism	AA032375	11806	apolipoprotein A-I	Apoa1	▼	-2.5	18 - 168	I	Yes
	BG063838	14104	fatty acid synthase	Fasn	▼	-2	72, 168	I	No
	BG066626	64898	lipin 2	Lpin2	▲	3	4, 12, 24, 72	II	Yes
	XM_130363	14725	low density lipoprotein receptor-related protein 2	Lrp2	▲	3	4 - 168	II	Yes
	AJ223958	26458	solute carrier family 27a2- fatty acid transporter	Slc27a2	▲	2	8 - 168	II	No
	BC002008	16592	fatty acid binding protein 5, epidermal	Fabp5	▲	4	8,12,18,72,168	III	Yes
	AA458178	12491	CD36 antigen	Cd36	▲	3.5	18 - 168	IV	No
	BG063416	16956	lipoprotein lipase	Lpl	▲	3.5	18,72,168	IV	Yes

Table 3. Continued

Functional Category	Accession	Locus Link	Gene Name	Gene Symbol	Regulation	Fold change[a]	Time Points (hrs)[b]	Cluster[c]	DRE[d]
Gluconeogenesis	W41175	14571	glycerol phosphate dehydrogenase 2	Gpd2	▼	-2.5	4 - 168	I	Yes
	AA014441	103988	glucokinase	Gck	▼	-2.5	12, 24	I	Yes
	BG064771	26384	glucosamine-6-phosphate deaminase 1	Gnpda1	▼	-1.5	4,18,72,168	I	No
	BG066689	14718	glutamate oxaloacetate transaminase 1	Got1	▼	-2.5	8 - 168	I	Yes
	NM_011044	18534	phosphoenolpyruvate carboxykinase 1	Pck1	▼	-2.5	18, 24	I	Yes
Apoptosis	AI506719	215114	huntingtin interacting protein 1	Hip1	▲	2	4 - 168	II	Yes
	AA269857	19766	receptor (TNFRSF)-interacting serine-threonine kinase 1	Ripk1	▲	4.5	12 - 168	IV	No
	AA711151	12125	BCL2-like 11 (apoptosis facilitator)	Bcl2l11	▲	1.5	12 - 168	IV	No
	BG076240	12368	caspase 6	Casp6	▲	2	24 - 168	IV	Yes
Immune Response	AA273296	12505	CD44 antigen	Cd44	▲	2	168	IV	No
	AA419750	16149	Ia-associated invariant chain	Ii	▲	3	168	IV	No
	AA145865	110454	lymphocyte antigen 6 complex, locus A	Ly6a	▲	2.5	72, 168	IV	No
	AA175329	14961	histocompatibility 2, class II antigen A, beta 1	H2-Ab1	▲	3	168	IV	No
	BG063553	14969	histocompatibility 2, class II antigen E beta	H2-Eb1	▲	3.5	168	IV	No

[a] Induction or repression as determined by microarray analysis

[b] Significant at P1(t) > 0.99

[c] Clusters designations as in Figure 5

[d] DRE identified in -1500 to + 1500 of TSS

serine-threonine kinase 1 (Ripk1), caspase 6 (Casp6), BCL2-like 11 (Bcl2l11) and huntingtin interacting protein 1 (Hip1), which is also consistent with the histopathologic identification of hepatocyte apoptosis at 168 hours. In general, these gene expression responses preceded or paralleled the observed histopathology for each functional category. In contrast, the induction of immune signaling genes was largely confined to168 hour coincident with the histology. Consequently, these gene expression changes are likely due to the infiltration of immune cells as opposed to changes in hepatocyte gene expression. Moreover, all functional categories included genes in each of the five identified clusters of up-regulated immediate early, early, late and sustained as well as down regulated responses. The exceptions were the TCDD elicited changes in gluconeogenesis and immune signaling which were primarily represented by down regulated and up-regulated late clusters, respectively.

Verification of Microarray Responses

QRTPCR was used to verify changes in transcript levels for a selected subset of active genes representing different responses and functional categories in Table 3 (Figure 7). In total 24 genes were verified by QRTPCR, all of which displayed temporal expression patterns comparable with the microarray data (See supplementary Table 2 for complete list of genes). For genes such as Myc, Tnfaip2, Fabp5, and Cd36, there was also good agreement in the magnitude of the fold-change when comparing microarray and QRTPCR data. However, microarray data compression was evident for Cyp1a1 and Gstp2 due to the smaller dynamic fluorescence intensity range (0-65 535) of the microarrays which resulted in signal saturation for these genes and compression of the true induction. Cross hybridization of homologous probes to a given target sequence on

Figure 7.
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 4 biological replicates. Numbers indicate respective clusters as illustrated in Figure 5 B and C. Error bars represent the SEM for the average fold change. * = $p<0.05$ for QRTPCR.

Got1
Pck1
Cyp1a1
Gstp2
Notch1
Nqo1
Ugdh
Fabp5
Tnfaip2
Cd36
Lpl
Gadd45b
fold induction
hrs
2 4 8 12 18 24 72 168

the microarray may also be a contributing factor especially when in comparison to other, more gene specific, measurement techniques [154].

Identification of Putative DREs in Responsive Genes

Genomic sequence (-1500 to +1500 relative to the TSS) for genes represented in Table 3 were also examined for the presence of putative DREs which were identified by computational scanning [229]. Tentative functionality was assessed by comparing matrix similarity scores calculated using a position weight matrix (PWM) developed using sequences from *bonafide* functional DREs. Of the 42 TCDD regulated genes listed in Table 2, 28 contained putative DREs within this region (Table 3 and supplementary Table 5). Bach, Cbr3, Dhrs3, Gadd45b, Myc, and Ugdh all possessed high scoring DREs within this region and displayed an early induction response to TCDD treatment, strongly suggesting that they are primary AhR responsive genes.

DISCUSSION

The present study examined the hepatotoxicity elicited by a single oral dose of TCDD using histological, clinical and global gene expression approaches. TCDD induced temporal- and dose-dependent increases in relative liver weight due to fatty vacuolization and altered serum FFA, TRIG, and CHOL. At later time points, there also was evidence of immune infiltration and apoptosis. Gene expression responses exhibited temporal- and dose-dependent patterns consistent with these endpoints thereby providing mechanistic information regarding the etiology of TCDD induced fatty liver and hepatotoxicity. Functional annotation of the responses associated changes in gene expression with metabolizing enzymes, development and differentiation, fatty acid uptake and

metabolism, gluconeogenesis, apoptosis and immune signaling which are consistent with known responses to TCDD as well as the histological and clinical results from this study. The following sections integrate these results with published data to further elucidate AhR-mediated gene expression effects involved in the hepatotoxic effects of TCDD.

TCDD Induction of Metabolizing Enzymes and Oxidative Stress

Genes encoding products associated with oxidoreductase, monooxygenase and xenobiotic metabolism activities were induced by TCDD, some of which have been previously characterized as members of the "AhR gene battery", including Cyp1a1, 1a2 and 1b1 as well as Nqo1 and Ugt1a6 [230]. Although their induction serves an important role in detoxification, their activity also contributes to the formation of reactive oxygen species (ROS) which can lead to cellular oxidative stress, lipid peroxidation and DNA fragmentation [231, 232]. TCDD is a particularly potent mediator of ROS formation due to its pronounced induction of P450 enzymes such as Cyp1a1 [231], while Cyp1a2 is considered only a minor contributor [233]. However, Cyp1a1 null mice still exhibit the hallmarks of TCDD toxicity suggesting the involvement of additional members of the AhR gene battery in mediating these adverse effects [234].

Further examination identified previously uncharacterized TCDD-induced transcripts encoding enzymes with oxidoreductase activity. As with classic members of the AhR gene battery, xanthine dehydrogenase (Xdh), Ugdh, Dhrs3, and Cbr3 were up-regulated early (within 4 hours) and dose-dependently and were likely significant contributors to TCDD mediated oxidative stress. For example, Xdh is a known major producer of ROS in ischemic-reperfusion injury due to its ability to catalyze the reduction of molecular oxygen leading to the formation of superoxide anions and hydrogen

peroxide [235]. Xdh transcript induction also complements reports of sustained induction of hepatic Xdh enzyme activity following TCDD treatment [236]. Moreover, comparative computational scanning using a PWM has identified high scoring putative DREs in the proximal promoter sequences of each of these enzymes.

Induction of ROS generating enzymes was accompanied by increases in glutathione transferases (GSTs - Gsta2, a4 and p2), epoxide hydrolase (Ephx1) and Ugdh, which prevent cellular damage by oxidative stress. GSTs catalyze the conjugation of reduced glutathione (GSH) to electrophiles and products of oxidative stress, thereby facilitating their elimination. Although GSH protects against oxidative stress, production of ROS by TCDD depletes cellular GSH levels leaving cells susceptible to oxidative damage. Consistent with this, both GSH synthesis enzymes, glutamate-cysteine ligase, which catalyzes the first and rate limiting step, and glutathione synthetase, which catalyzes the second step, were induced by TCDD. Ugdh catalyzes the formation of UDP-glucuronic acid (UDPGA) from UDP-glucose. In subsequent phase II glucuronidation reactions, UDPGA is conjugated to reactive xenobiotics to facilitate their elimination. Interestingly, these conjugation reactions are catalyzed by Ugt1a6 and 1a7, both members of the AhR gene battery, indicating that TCDD induces multiple levels of this phase II metabolism pathway. The induction of these phase II enzymes may play an important protective role in response to TCDD elicited oxidative stress.

TCDD-Induced Fatty Acid Uptake and Metabolism

The integration of histopathology and clinical chemistry with microarray data provides compelling evidence that TCDD-mediated increases in liver weight can be attributed to fatty accumulation involving the disruption of hepatic lipid uptake and

metabolism. Cellular uptake of lipids from chylomicrons and VLDL occurs via hydrolysis by lipoprotein lipase (Lpl) and hepatic lipase which enables FFAs to accumulate via membrane associated transporters such as fatty acid binding proteins (Fabps), fatty acid translocase, and fatty acid transport proteins [237]. Lpl mRNA was up-regulated within 18 hours and achieved maximum induction by 168 hours, which would increase FFA availability for hepatic uptake. In addition, TCDD induction of Fabp4 and 5, Slc27a2 and Cd36 would facilitate increased hepatic fatty acid uptake and a resultant fatty liver. Cell models with increased or reduced expression of Fabps exhibit increased and decreased fatty acid uptake, respectively [238]. Fatty acid transporter 2 (Slc27a2), which was up-regulated at 4 hours and remained elevated through to 168 hours, facilitates long chain fatty acid transport across the plasma membrane and accounts for high affinity and specific FA transport in hepatocytes [239]. Fatty acid translocase (Cd36), up-regulated in a pattern similar to that of Lpl, is a key enzyme involved in the uptake of FA and oxidized LDL across the plasma membrane. Null mutations of Cd36 result in reduced FA uptake while over-expression increases FA uptake and metabolism [240, 241]. Lipoprotein receptor-related protein-2 (Lrp2) was also up-regulated for the duration of the time course and may account for the increased hepatic cholesterol ester content and reduced serum levels. Lipin2 was up-regulated throughout the time course and belongs to a family of genes whose deficiency prevents normal lipid accumulation and the induction of key lipogenic enzymes in adipocytes [242, 243]. Furthermore, mice deficient in Lipin exhibit dramatically reduced Lpl activity [244]. Consequently, TCDD induction of Lipin2 may be linked to the subsequent up-regulation of Lpl. Collectively, induction of these key genes supports an

environment for increased lipid uptake into the liver. Furthermore, their dose dependent and temporal expression profiles precede or parallel the observed histological increases in hepatic fat accumulation, strongly suggesting that their induction is involved in mediating this response.

Fatty acids also act as signaling molecules that regulate gene expression. Fatty acid synthase (Fasn), involved in *de novo* lipogenesis, and Apoa1 were both down regulated-late following TCDD treatment, consistent with their previously reported decreases in transcript and activity levels after increases in hepatic FA accumulation [245]. Similarly, the oxidative phosphorylation uncoupling gene, uncoupling protein-2 (Ucp2), was induced late, in agreement with its regulation by FAs [246]. These results suggest that a subset of the late changes in gene expression may be secondary to the increased FA content of the liver, and not direct AhR-mediated responses.

Inhibition of Gluconeogenic Enzymes

TCDD-induced lethality involves feed refusal, body weight loss and exhaustion of energy stores, collectively referred to as a wasting syndrome [247]. However, feed refusal alone does not sufficiently account for the wasting effect as pair fed animals still exhibit this response [248]. TCDD exposure also inhibits gluconeogenesis by repressing key gluconeogenic enzymes, which in combination with feed refusal, is thought to result in TCDD-induced wasting syndrome lethality [247]. Pck1, pyruvate carboxylase (Pcx) and to a lesser extent G6pc (glucose-6-phosphatase) are known to be repressed by TCDD. In this study, repression of Pck1, Gpd2 and Got1 was also detected. Gpd2 catalyzes the irreversible conversion of glycerol phosphate to dihydroxyacetone phosphate (DHAP) required for the formation of fructose-1-6-bisphosphate. Got1 is involved in the malate-

aspartate shuttle and the conversion of aspartate to oxaloacetate, which provides substrate for use by Pck1 in gluconeogenesis. The multiple gluconeogenic enzymes down regulated further implicate this pathway as a target in the etiology of TCDD-induced wasting syndrome.

In adipose tissue, TCDD inhibits lipid synthesis, decreases uptake of FFA due to reduced Lpl activity and increases the mobilization of fat [249]. Gluconeogenic enzymes also serve roles in glyceroneogenesis, a process that plays an integral but opposite role in fatty acid cycling and triglyceride turnover in hepatic and adipose tissues. In adipose tissue, inhibition of glyceroneogeneis induces FFA release due to decreased triglyceride storage. Increased FFA output combined with the reduced uptake due to down regulation of adipose Lpl, is a likely contributor to the increased serum FFA levels observed in the present study. Furthermore, inhibition of hepatic glyceroneogenesis reduces triglyceride output thereby contributing to the TCDD elicited fatty liver. Collectively, FFA mobilization from adipose tissue and the decreased triglyceride export from the liver, combined with the increased expression of genes for fatty acid uptake, would facilitate the loss of body fat (i.e. wasting) and its accumulation in the liver.

Immune cell accumulation

Histological analysis revealed the presence of immune cell accumulation, primarily in the centrilobular regions, coincident with the up-regulation of numerous immune signaling genes at 168 hours. These genes included a number of cluster of differentiation and lymphocyte antigens (Cd and Ly antigens) as well as major histocompatabilty complex (MHC) molecules. Cd and Ly antigens are surface molecules on hemopoietic cells which are important in a number of immune signaling functions

including rolling and migration and T-cell activation [250, 251]. H2-Ab1 and H2-Eb1 belong to the MHC class II and are involved in antigen presentation and processing [252]. Changes in immune gene expression are likely a secondary AhR-independent response to hepatic damage mediated by ROS or fatty accumulation as induction was confined to the 168 hour time point when immune cell infiltration was detected by histology, consistent the absence of DREs in the promoters of these genes. These results further illustrate the importance of complementary histology to facilitate the interpretation of changes in gene expression in complex tissue analysis.

Apoptosis

Induction of a number of genes involved in the initiation of apoptosis was also detected by microarray analysis including Ripl k, Casp6 and Hipl. Ripl k and Hipl are both able to activate apoptotic pathways [253, 254] while Casp6 induction lowers the threshold for apoptotic signals[255]. The collective induction of these and other genes is supportive of a cellular environment conducive to apoptosis, in agreement with histological evidence of late apoptotic events. Although induction of these apoptotic genes may be a response secondary to oxidative stress and toxicity, it is also possible that these are primary response genes involved in TCDD-mediated apoptosis or alterations in differentiation.

TCDD Regulates Genes Involved in Development and Differentiation

TCDD treatment also resulted in the induction of a number of genes involved in development and differentiation including Tnfaip2 and Notch1. Although these genes may not be involved in mediating the hepatotoxicity observed in this study, they may play an important role in normal AhR signaling during hepatic development as AhR null

mice are known to exhibit reduced liver size and altered hepatic vasculature [219, 256, 257]. Both genes have previously been implicated in tissue development and exhibit specific patterns of expression in the developing liver [258] [259, 260]. Activation of Notch receptors induce the hairy and enhancer of split (Hes) family of genes, whose expression mediates many aspects of Notch signaling [261]. Consistent with this regulation, induction of Hes6 at the 72 and 168 hour time points was also observed. The hepatic expression patterns of Tnfaip2 and Notch1 occur during embryonic days 12 – 18 [258, 260] which coincides with the period of AhR activation required for normal liver development [262]. Furthermore, treatment of AhR hypomorphs, which exhibit a 90% reduction in AhR levels and display altered hepatic development, with TCDD on embryonic days 12 – 18 is able to restore normal liver development, presumably due to the potent activation of low levels of AhR [262]. Therefore, these genes provide putative candidates for mediating the hepatic developmental role of the AhR.

Summary

The present study represents the first comprehensive *in vivo* examination of the acute transcriptional response of the liver to TCDD. Alterations in gene expression were directly related to physiological outcomes demonstrating the importance of phenotypic anchoring when interpreting microarray data. Integration of gene expression, histological and clinical chemistry endpoints facilitated the development of a response network that further elucidates potential mechanisms involved in TCDD mediated hepatotoxicity (Figure 8). The comprehensive time course analysis also allowed for the identification of gene expression responses that precede and may mediate subsequent physiological/toxicological responses. Early and sustained induction of ROS-generating

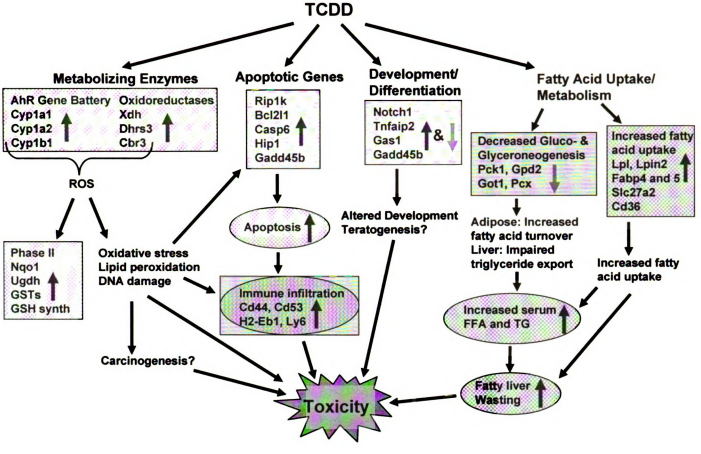


Figure 8.
Integration of gene expression, histology and clinical chemistry endpoints and their potential involvement in the etiology of TCDD mediated hepatotoxicity. Responses in boxes represent gene expression observations; circles represent clinical or histological observations; arrows in the boxes or circles indicate direction of indicated response.

oxidoreductase enzymes likely contribute to later liver damage, as indicated by mild increases in ALT levels, and the subsequent accumulation of immune cells. Changes in gene expression and histopathology also indicated the occurrence of apoptosis which may be due to direct transcriptional responses or may be a secondary response to oxidative stress. Dysregulation of gene expression responses involved in fatty acid uptake and metabolism concomitant with serum TRIG and FFA increases and inhibition of glyceroneogenesis, suggests a putative mechanism for mediating the subsequent fatty liver response. Additional studies are required to more fully delineate these responses and determine if other hepatotoxicants use common pathways to elicit comparable steatotic effects. Furthermore, examination of additional target tissues and animal models will reveal whether these responses are tissue- and/or species-specific which will aid in development of accurate models of toxicity for TCDD and related compounds as well as human risk assessments.

CHAPTER 5

EXAMINATION OF THE ABILITY OF 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN TO INHIBIT ETHYNYL ESTRADIOL-MEDIATED INCREASES IN UTERINE WEIGHT INDUCTION IN MICE

ABSTRACT

The objective of the present study was to examine the ability of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to inhibit ethynyl estradiol- (EE) mediated increases in uterine weight using the uterotrophic assay dosing regimen. Mice were dosed with increasing doses of EE, alone or in combination with TCDD, and wet and blotted uterine weights were measured. The results indicate that TCDD was able to significantly and dose-dependently inhibit EE-mediated increases in uterine weight. To examine the inhibitory effects of TCDD on EE-mediated gene expression responses a preliminary microarray experiment was conducted on hepatic and uterine tissues. These analyses revealed that TCDD did not modulate EE-mediated changes in hepatic gene expression. In contrast, a number of the EE-mediated uterine gene expression responses were inhibited by TCDD. These results indicate the tissue-specific inhibitory responses of TCDD on EE-mediated gene expression responses and suggest the uterus may serve as a useful *in vivo* model to study the gene expression crosstalk that exists between TCDD and estrogen.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous environmental contaminant that elicits a broad spectrum of toxic and biochemical responses in a tissue-, sex-, age- and species-specific manner that include a wasting syndrome, tumor promotion, teratogenesis, hepatotoxicity, modulation of endocrine systems, immunotoxicity and enzyme induction [26]. Many, if not all, of these effects are due to alterations in gene expression mediated via the activation of the aryl hydrocarbon receptor (AhR), a member of the basic-helix-loop-helix-PAS (bHLH-PAS) family [23, 26]. Ligand binding to the cytoplasmic AhR complex triggers the dissociation of interacting proteins and results in the subsequent translocation of the ligand-bound AhR to the nucleus where it heterodimerizes 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 that ultimately result in the observed toxic and biochemical responses [32].

As part of its repertoire of toxic effects, TCDD elicits a number of antiestrogenic responses in the female reproductive tract including the inhibition of estrogen induced increases in uterine wet weight, DNA synthesis, and gene expression responses [51-53, 57]. These effects are not mediated through TCDD binding to the estrogen receptor (ER) [263]; rather, they are thought to involve crosstalk between the ER and AhR signaling pathways. Furthermore, the obligatory role of the AhR in mediating these responses has been revealed in studies examining these antiestrogenic effects in AhR-knockout mice [58]. Despite this evidence, the antigestrogenic effects of TCDD on uterine weight and

gene expression responses have been inconsistent in the literature [99, 264]. These inconsistencies are likely due to the species-, sex-, age-, tissue-, and species- specific effects of TCDD. Therefore, prior to an in-depth investigation into the inhibitory effects of TCDD on EE-mediated hepatic gene expression responses, a preliminary evaluation of the responsiveness of the animal and tissue model is warranted. The present study was conducted to examine and optimize the antiestrogenic effects of TCDD on the inhibition of EE-mediated increases in uterine weight in the uterotrophic assay using immature ovariectomized mice. Subsequently, the inhibitory effects of TCDD on EE-mediated gene expression responses were examined in both hepatic and uterine tissue.

MATERIALS AND METHODS

Animal Treatments

Immature 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 4 days prior to treatment.

Animals (n = 4/treatment group/time point) were treated by oral gavage at time zero with sesame oil vehicle (Sigma Chemical, St Louis, MO), 17α-ethynylestradiol (EE, Sigma Chemical), TCDD (provided by S. Safe, Texas A&M University, College Station, TX) or a combination of EE and TCDD. These doses were followed by two additional

oral administrations of vehicle, in the vehicle and TCDD groups, or EE, in the EE and EE/TCDD groups, at 24 and 48 hrs, as per the uterotrophic assay (Figure 1). Only a single dose of TCDD was given to be consistent with previous studies examining TCDD-mediated gene expression and antiestrogenic effects and due to its negligible metabolism [265-267]. For the first study, doses of 0, 3, 10, 30 and 60μg/kg EE were used alone or in combination with 10 or 100μg/kg TCDD. In the second study doses of 0, 1, 3, and 10μg/kg EE were used alone or in combination with doses of 1, 3, 10 or 30μg/kg TCDD. Mice were sacrificed by cervical dislocation 24hrs after the final dose. Whole uterine weights were recorded before (wet) and after (blotted) blotting with absorbent tissue. Uterine and hepatic tissues were snap-frozen in liquid nitrogen and stored at -80°C. 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.

RNA Isolation

Total RNA was isolated from whole uteri and liver samples (~100 mg from left liver lobe) 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

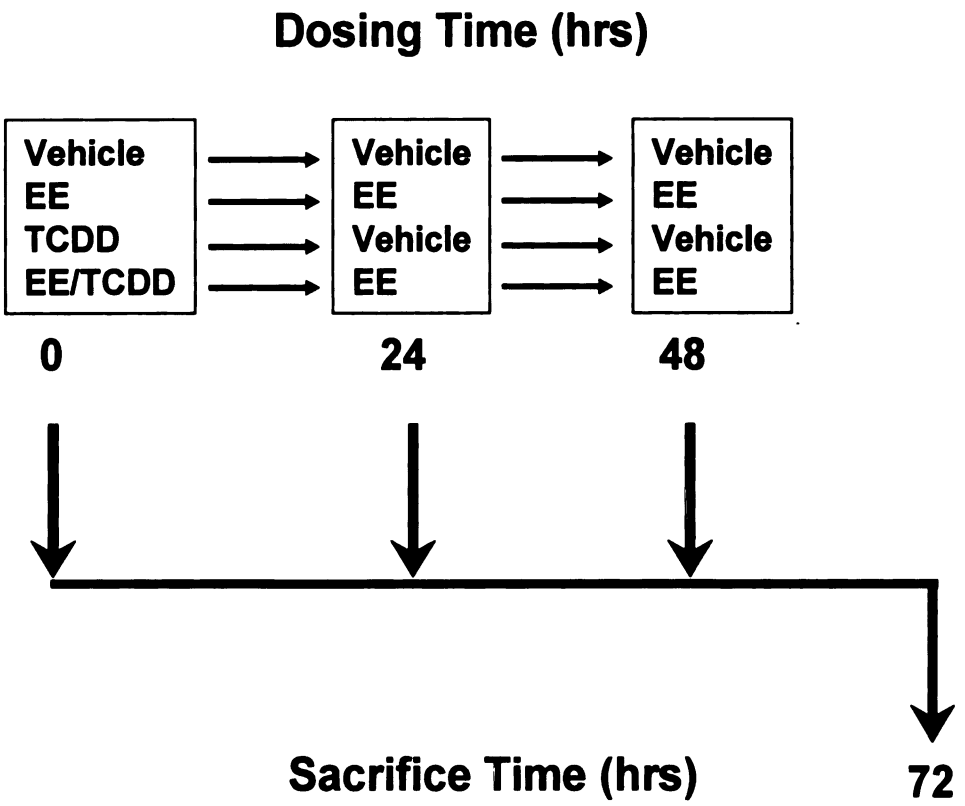


Figure 1.
Experimental design for the EE/TCDD cotreatment uterotrophic assay.
Mice were dosed with vehicle, EE, TCDD or a mixture of EE and TCDD at time zero followed by doses of vehicle (vehicle and TCDD groups) or EE (EE and EE/TCDD groups) at 24 and 48 hours as per the uterotrophic assay. Mice were sacrificed 72 hours after the initial dose at which time uterine and hepatic tissue samples were harvested.

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). For hepatic samples, total RNA (30μg) was reverse transcribed in the presence of Cy3- or Cy5-dUTP to create fluor-labeled cDNA which was purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA). Cy3 and Cy5 samples were mixed, vacuum dried and resuspended in 48μl of hybridization buffer (40% formamide, 4xSSC, 1%SDS) with 20μg polydA and 20μg of mouse COT-1 DNA (Invitrogen, Carlsbad, CA) as competitor. For uterine samples a 3DNA Array 900 Expression Array Detection Kit (Genisphere, Hatsfield, PA) using 1.0μg of total RNA was used for probe labeling in all microarray experiments, according to manufacturer's specifications. For both hepatic and uterine arrays, 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 [268]. Model-based t-values were calculated from normalized data, comparing treated and vehicle responses on a per time-point basis. 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 [223]. 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. All arrays were subjected to quality control assessment to ensure assay performance and data consistency. All data are stored within dbZach (http://dbzach.fst.msu.edu), a MIAME supportive relational database that ensures proper data management and facilitates data analysis. Gene expression patterns that passed the established threshold criteria of ± 1.5 fold induction or repression and a statistical P1(t) > 0.9999 were visualized using hierarchical clustering (GeneSpring 6.0, Silicon Genetics, Redwood City, CA).

Quantitative Real-Time PCR (QRT-PCR) Analysis

For each sample, 1.0μg of total RNA was reverse transcribed by SuperScript II using an anchored oligo-dT primer as described by the manufacturer (Invitrogen). The resultant cDNA (1.0μl) was used as the template in a 30μl PCR reaction containing 0.1μM each of forward and reverse gene-specific primers, designed using Primer3 [151], 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 Table 1. 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

Table 1. Gene names and primer sequences (5'- 3') for select transcripts verified by QRTPCR

Gene Name	Gene Symbol	Locus Link	Ref Seq Accession	forward primer	reverse primer	product size (bp)
arginase 1	Arg1	11846	NM_007482	TCACCTGAGCTTTGATGTCG	CTGAAAGGAGCCCTGTCTTG	134
aquaporin 1	Aqp1	11826	NM_007472	CCGAGACTTAGGTGGCTCAG	TGATACCGCAGCCAGTGTAG	92
complement component 3	C3	12266	NM_009778	GAAAAGCCCAACACCAGCTA	CTGTGAATGCCCCAAGTTCT	121
lipocalin 2	Lcn2	16819	NM_008491	CTGAATGGGTGGTGAGTGTG	GCTCTCTGGCAACAGGAAAG	101
lactotransferrin	Ltf	17002	NM_008522	GAAGGCAGGAAATGTTGCAT	CATCAAGGCACAAAAGCTCA	122
ribosomal protein L7	Rpl7	19989	NM_011291	AGCCCAAAGGTTCGTAAGGT	CATGCAATGTATGGCTCCAC	122

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 [269]. 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 AND DISCUSSION

Inhibition of EE-mediated increases in uterine weight by TCDD

The present dose-range finding studies were conducted to identify the concentrations of EE and TCDD that would allow for the optimal detection of the antiestrogenic effects on EE-mediated physiological and gene expression responses. For these experiments an uterotrophic dosing regimen was utilized and the induction of uterine weight, a well established estrogenic endpoint, was monitored [270]. This model is currently being validated by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) and the Organization for Economic Co-operation and Development (OECD) for the detection of estrogenic endocrine disruptors [271]. For this

assay, the detection of antiestrogenic activity involves monitoring effects on uterine weight in the presence of a potent reference estrogen such as EE. In the present study, mice were dosed with EE or a mixture of EE and TCDD at time zero followed by doses of EE alone at 24 and 48 hrs and animal sacrifice at the 72 hr time point (3 daily doses of EE).

The first dose-range finding study was conducted to determine the ability of TCDD to inhibit uterine weight induction as well as to identify an appropriate dose of EE for more extensive temporal cotreatment studies. Mice were gavaged with 0, 3, 10, 30, or 60 μg/kg EE combined with a 10 or 100 μg/kg dose of TCDD. The results indicate that TCDD was able to significantly inhibit EE-mediated increases in uterine wet and blotted weights by 35-40% at doses of 3 and 10μg/kg EE (Figure 2).

The second dose range finding study was conducted to identify the appropriate dose of TCDD for the temporal cotreatment studies. Mice in this experiment were gavaged with 0, 1, 3, or 10μg/kg EE combined with a 0, 1, 3, 10 or 30 μg/kg dose of TCDD. The results revealed that TCDD significantly and dose dependently inhibited EE-mediated increases in uterine wet and blotted weights by 35-40% at EE doses of 3 and 10 μg/kg and TCDD doses of 10 and 30 μg/kg (Figure 3).

Collectively, these results indicate that TCDD is able to consistently elicit inhibitory effects on uterine weight induction in the female ovariectomized mouse model using the uterotrophic assay dosing regimen. Furthermore, the observed responses are consistent with the magnitude of the antiestrogenic responses observed in previously published reports [265] and are within the range of other anitestrogenic compounds. Based on these data, the doses chosen for a more in-depth temporal examination of the

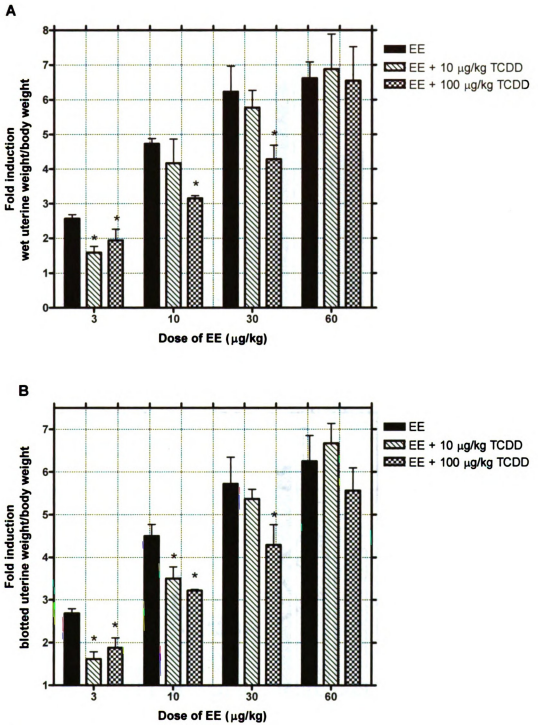


Figure 2.
Inhibitory effects of TCDD on EE-induced wet and blotted uterine weights.
Administration of EE at doses of 3, 10, 30 and 60µg/kg resulted in a dose dependent increase in both wet (A) and blotted (B) uterine weights. Cotreatment with TCDD was able to inhibit EE-mediated increases in uterine wet and blotted weights by 35-40% at EE doses of 3 and 10µg/kg. Data are represented as fold induction relative to vehicle treated controls. * = $p<0.05$ when compared to dose matched EE controls.

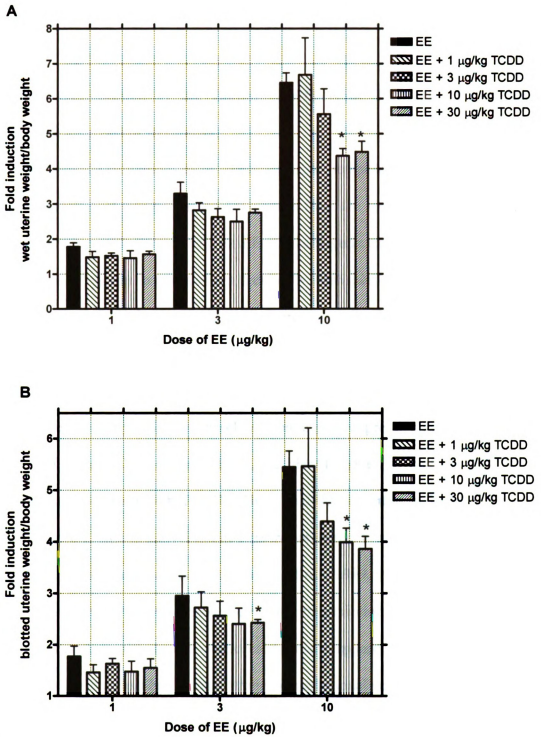


Figure 3.
Dose-dependent inhibitory effects of TCDD on EE-induced wet and blotted uterine weights. Cotreatment studies indicate that TCDD dose-dependently inhibited EE-mediated uterine wet (A)and blotted (B) weight induction by 35-40% at EE doses of 3 and 10μg/kg. Data are represented as fold induction relative to vehicle treated controls. * = $p<0.05$ when compared to dose matched EE controls

inhibitory effects of TCDD on EE-mediated gene expression responses are 10μg/kg EE and 30μg/kg TCDD.

Microarray Examination of the Inhibitory Effects of TCDD on EE-mediated Hepatic Gene Expression Responses

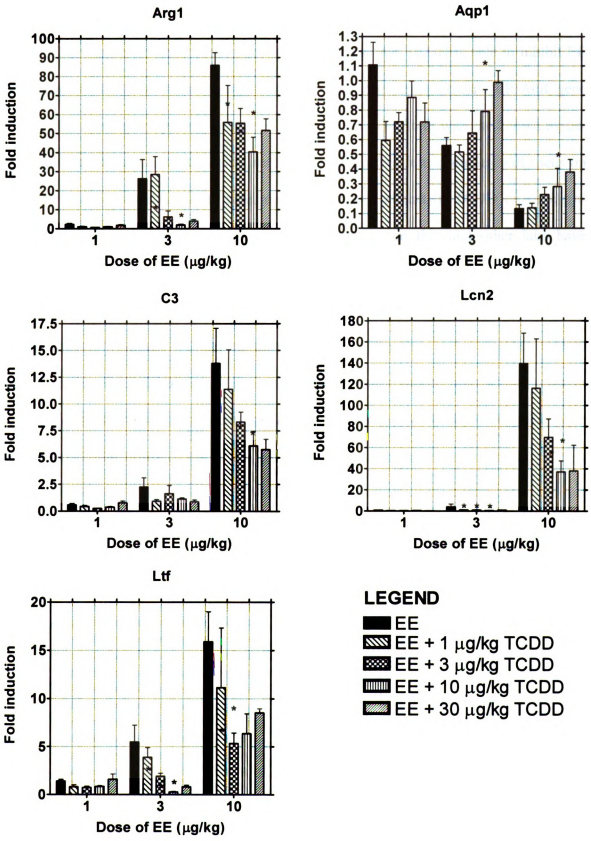
Prior to the initiation of a comprehensive temporal investigation, a pilot microarray experiment was performed on samples from the second dose-range study to assess the inhibitory effect of TCDD on EE-mediated gene expression responses. The liver was initially chosen to complement previously published reports that examined the effects of EE and TCDD on hepatic gene expression. In the present study, labeled hepatic cDNA from mice dosed with 10μg/kg EE were co-hybridized with that from mice dosed with 10μg/kg EE and 30μg/kg TCDD. The observed gene expression responses were subsequently compared to the baseline hepatic gene expression profiles previously obtained for EE and TCDD alone [266, 272] to identify genes regulated by EE that are modulated by cotreatment with TCDD. These analyses identified only 6 EE-regulated genes which were significantly inhibited upon cotreatment with TCDD treatment by greater than ± 1.5 fold and a statistical P1(t) cut-off of 0.9999, indicating TCDD does not exhibit extensive inhibitory effects on the hepatic transcriptional response to EE. This may be attributed to the overall weak estrogenic response induced in this tissue. Furthermore, the liver exhibits a very strong transcriptional response to TCDD which may confound the identification of estrogen regulated genes that are influenced by cotreatment with TCDD. The cross-talk may also be dependent on the relative expression levels of the ER and AhR which differ dramatically in the liver when compared to tissues in which the antiestrogenic effects of TCDD have been observed.

However, the absence of inhibitory gene expression responses are consistent with previously published data which have not reported any antiestrogenic physiological responses in the liver. Alternatively, these results may be indicative of the absence of ER/AhR cross-talk *in vivo*.

Microarray Examination of the Inhibitory Effects of TCDD on EE-mediated Uterine Gene Expression Responses

Historically, physiological antiestrogenic responses to TCDD have been observed in the mammary gland, ovary and uterus [42, 51-53, 66]. Therefore, a microarray study was performed on uterine tissue from the same animals used in the above hepatic study to further investigate gene expression crosstalk in a more estrogen responsive tissue. Microarray results from this study were compared to uterine gene expression profiles obtained for EE and TCDD alone [49] to identify genes induced by EE that are influenced by cotreatment with TCDD. In contrast to the results obtained in the liver, gene expression analyses identified a number of estrogen regulated genes that were negatively influenced by TCDD in the uterus. In total, 76 genes were identified which were regulated by EE and significantly inhibited by TCDD cotreatment by greater than ± 1.5 fold and a statistical P1(t) cut-off of 0.9999. This list included many genes previously reported to be regulated by estrogen in the uterus including arginase 1 (Arg1), aquaporin 1 (Aqp1), complement component 3 (C3), lipocalin 2 (Lcn2) and lactotransferrin (Ltf). Quantitative real-time PCR (QRTPCR) was performed for these five genes on all uterine RNA samples obtained from the second dose range finding study which verified the microarray data and indicated that TCDD is able to dose dependently inhibit EE-mediated transcriptional responses of these genes (Figure 4).

Figure 4.
Quantitative real-time PCR verification of pilot microarray results examining the inhibitory effects of TCDD on EE regulated transcripts.
The same RNA used for cDNA microarray analysis was examined by QRTPCR. All fold changes were calculated relative to vehicle treated controls. Genes are indicated by official gene symbols and results are the average of 4 biological replicates. Error bars represent the SEM for the average fold change. * = $p<0.05$ when compared to dose matched EE controls


Arg1
Aqp1
C3
Lcn2
Ltf
Fold induction
Dose of EE (μg/kg)
1
3
10
LEGEND
EE
EE + 1 μg/kg TCDD
EE + 3 μg/kg TCDD
EE + 10 μg/kg TCDD
EE + 30 μg/kg TCDD

Conclusions

The results of these initial pilot studies indicate that TCDD is able to inhibit EE mediated increases in uterine weight. Furthermore, the uterus exhibits ER/AhR gene expression crosstalk responses while the liver does not, consistent with the known tissue-specific antiestrogenic physiological responses. Therefore, future studies will focus on the uterus as a model tissue to research the inhibitory effects of TCDD on estrogen-mediated gene expression responses. Previous studies in this and other labs have extensively characterized the transcriptional response of the uterus to EE which will serve as the baseline quantitative data [48-50]. However, no studies to date have examined the transcriptional response of the uterus to TCDD. Therefore, prior to the in-depth investigation of EE/TCDD crosstalk in the uterus, studies should first be conducted to establish baseline quantitative data on the *in vivo* effects of TCDD on global gene expression in the mouse uterus.

CHAPTER 6

DIOXIN INDUCES AN ESTROGEN RECEPTOR DEPENDENT, ESTROGEN-LIKE GENE EXPRESSION RESPONSE IN THE MURINE UTERUS[4]

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.

[4] This chapter has been submitted for publication in: Boverhof, D. R., Kwekel, J. C., Humes, D. G., Burgoon, L. D., and Zacharewski, T. R. Dioxin induces an estrogen receptor dependent, estrogen-like gene expression response in the murine uterus. *manuscript in submission.*

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 [26]. 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 [23, 26]. 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 [32]. Evidence suggests that the adverse effects elicited by TCDD are due to the continuous and inappropriate AhR-mediated regulation of these target genes [273]. 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 [274]. Upon ligand binding, ERs dissociate from heat shock and chaperone proteins, homodimerize, and interact with regulatory elements near estrogen responsive genes [11]. Classically, ERs mediate transcriptional 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 [13, 274]. The ER can also elicit cellular responses through ligand-independent, DNA-binding independent and cell-surface (non-genomic) signaling mechanisms [13].

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 [43]). 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 [46] and treatment of MCF-7 cells with TCDD results in estrogen-like G_0/G_1 to S-phase transition and mitogenic effects [112]. Furthermore, the ligand activated AhR/ARNT complex directly associates with the unliganded ER to form a functional complex that binds EREs and activate transcription [116]. Independent studies also confirmed ER-AhR interactions which may account for the crosstalk between these signaling pathways [114, 115]. In addition, 3-methylcholanthrene (3-MC) activation of the AhR in the absence of estrogen induced estrogenic responses in the mouse uterus [116]. Moreover, TCDD mediates the induction of estrogen dependent tumors in rats [65, 118], and reportedly increases the incidence of endometriosis in laboratory animals and in women with high body burdens of TCDD [119-122].

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 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 [266]. Animals in the estrogen study were treated once every 24 hrs by oral gavage on three consecutive days with 100 μg/kg b.w. 17α-ethynylestradiol (EE) or sesame oil vehicle (Sigma Chemical) as described previously [49]. Mice were sacrificed by cervical dislocation 2, 4, 8, 12, 18, 24, or 72 hrs after dosing. For the co-treatment studies, animals 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 [49, 266]. 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 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 matched vehicle controls. Comparisons were performed on 3 biological replicates each with 2 independent labelings of each sample (incorporating a dye swap) for each treatment group. 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 [268]. 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 [223]. 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 ensure assay performance and data consistency. All data are stored within dbZach (http://dbzach.fst.msu.edu), a MIAME 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 [151], 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 [269]. 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 (Aldh3a1), cytochrome P4501a1 (Cyp1a1), NAD(P)H dehydrogenase, quinone 1 (Nqo1) and TCDD-inducible poly(ADP-ribose) polymerase (Tiparp). 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.

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

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

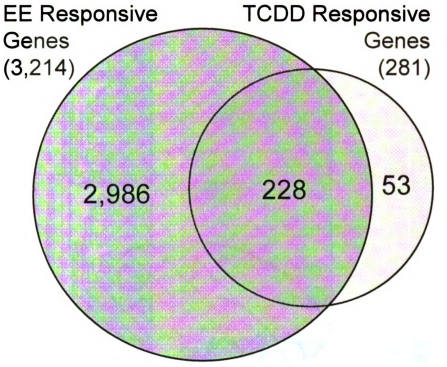
Regulatory Category	GenBank Accession	Gene Name	Gene Symbol	Locus Link	EE Fold change[a]	TCDD Fold change[a]
EE specific responses						
	BG070106	lipocalin 2	Lcn2	16819	28.9	-
	AA792235	inhibin beta-B	Inhbb	16324	7.6	-
	BG089964	granzyme C	Gzmc	14940	5.9	-
	BC002005	gene rich cluster, C9 gene	Grcc9	14794	4.7	-
	W10072	insulin-like growth factor 1	Igf1	16000	4.5	-
	BG065113	branched chain aminotransferase 1	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, gene 3	Kcne3	57442	-	2.2
	AK011746	RNA 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	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, H 1	Serpinh1	12406	3.7	2.5
	AA117848	ornithine decarboxylase, structural 1	Odc1	18263	3.4	2.2
	BG148607	protein phosphatase 2, regulatory subunit B, epsilon	Ppp2r5e	26932	0.18	0.38
	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

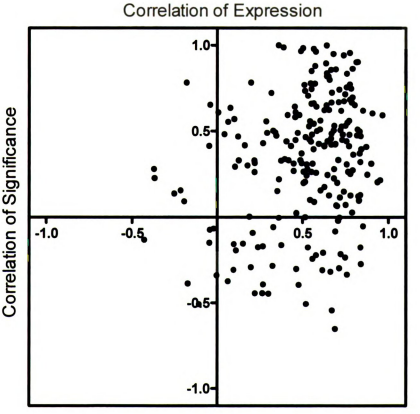
genes regulated by both TCDD and EE exhibited a gene expression correlation greater than 0.3, suggesting that the temporal expression patterns for genes induced by TCDD were similar to the expression patterns induced by EE. Visualization after gene-based hierarchical clustering reveals the similarity 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 [155, 270, 275]. Previous studies have demonstrated that estrogens induce dramatic increases in uterine wet weight, luminal epithelial cell height, stromal thickness and BrdU labeling [49, 50, 276]. 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.

A


EE Responsive Genes (3,214)
TCDD Responsive Genes (281)
2,986
228
53

B


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

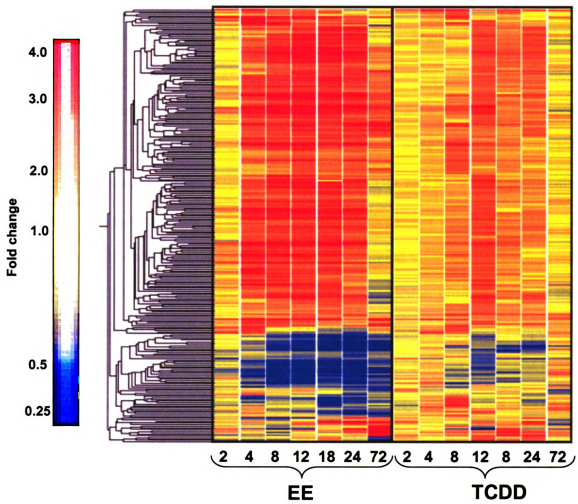


Figure 1.
Overlap of active uterine gene expression responses from temporal TCDD and EE studies. A. 3,214 and 281 unique genes were identified as differentially expressed at one or more time points in the EE and TCDD studies, respectively. Of these genes, 228 were responsive in both studies. B. Correlation 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.

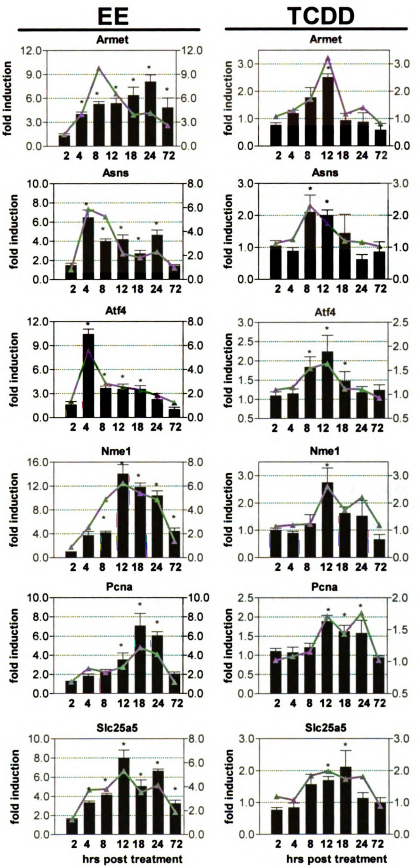
Quantitative Real Time PCR Verification of Results

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 [48-50, 202]. 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 [116]. To investigate the role of the ER in TCDD mediated induction of known estrogen responsive genes, mice were co-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

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.


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

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. 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 3B). The effect on each transcript was statistically significant with the exception of the TCDD-mediated induction of Slc25a5 which did not reach statistical significance 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) [277]. 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

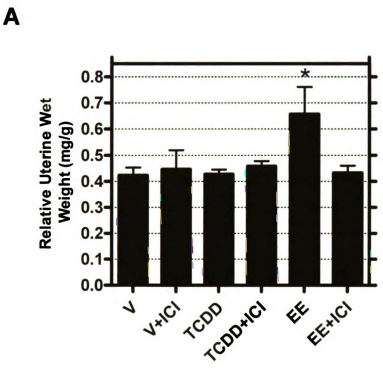
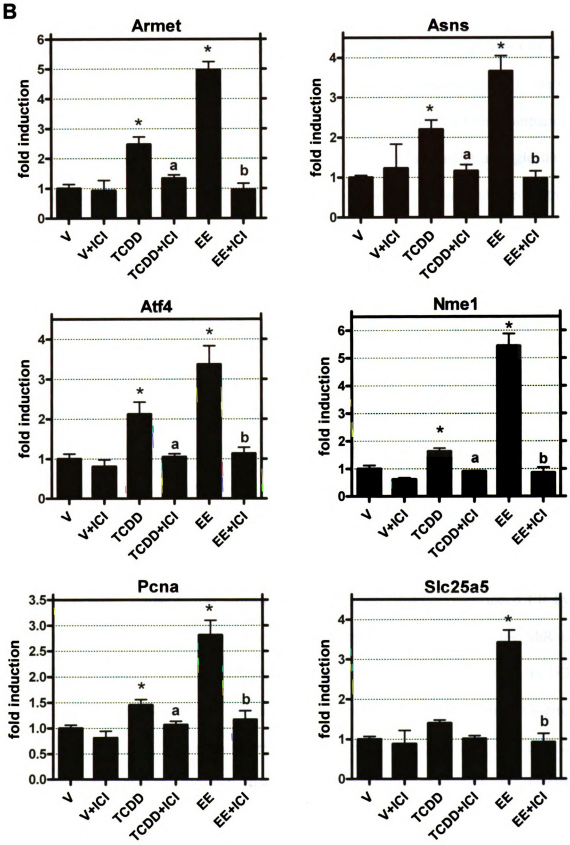


Figure 3.
ICI 182 780 inhibits uterine gene expression responses to TCDD.
A. Uterine wet weights for each treatment group at 12 hrs. B. QRTPCR was used to examine gene expression responses to EE and TCDD in the absence and presence of the pure ER antagonist ICI 182 780. All fold changes were calculated 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


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

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 [278]. However, the mice utilized in this study were ovariectomized after first estrus creating a uterine environment less responsive to estrogen [279] 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 [278] based on the reported estrogenic activity of PCB-77 [280], a coplanar PCB congener which binds the AhR. However, independent studies have not verified PCB-77 binding to the ER [281], and TCDD does not bind the ER [263]. A more plausible mechanism involves the activation of unliganded ER by ligand activated AhR. Recent studies have demonstrated that TCDD induces the AhR to interact directly with ER-alpha in the absence of estrogen [105, 113]. Moreover, TCDD increases the DNA-binding activity of the ER-independent of estrogen in the rat uterus [46] and treatment of MCF-7 cells with TCDD results in G_0/G_1 to S-phase transition and estrogen like mitogenic effects [112]. Studies have also shown that ER and AhR interact [114, 115] and ligand activated AhR/ARNT associates with the unliganded ER to form a

functional complex that binds EREs [116]. *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 [116]. 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 [117].

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 [105, 113-115], 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 [43, 72, 282, 283]. 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 [284-286]. 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.

CHAPTER 7

INHIBITION OF ESTROGEN-MEDIATED GENE EXPRESSION RESPONSES BY DIOXIN IN THE UTERI OF C57BL/6 MICE

ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) possesses antiestrogenic properties and has previously been shown to inhibit estrogen-induced uterine cellular growth and proliferation. These effects are not mediated through binding to the estrogen receptor (ER) but are thought to involve ER/ aryl hydrocarbon receptor (AhR) crosstalk, for which a number of different mechanisms have been proposed. One mechanism involves the inhibition of estrogen-mediated gene expression, however, only a limited number of inhibited responses have been identified which are unlikely to wholly account for the antiestrogenic effects. Furthermore, most studies have utilized *in vitro* systems which may not accurately reflect *in vivo* responses. Therefore, the inhibitory effects of TCDD on ethynyl estradiol (EE) mediated gene expression responses were investigated in the murine uterus using a microarray approach combined with phenotypic anchoring to physiological and histological endpoints. A 2 × 2 factorial microarray design was utilized to facilitate the identification of gene expression responses to EE and TCDD alone as well as their interactive effects. Of the 2,753 genes regulated by EE in the uterus, only 133 were significantly modulated upon cotreatment with TCDD, indicating a gene-specific inhibitory response. Functional annotation of these genes was associated with cell proliferation, water and ion transport, and maintenance of cellular structure and

integrity. These responses were correlated with the observed histological alterations and may collectively contribute to the antiestrogenic effects of TCDD on the uterus.

INTRODUCTION

Estrogens are essential regulators of growth, development and reproductive function in both males and females and have been implicated in the etiology of breast and endometrial cancers [7]. Many of the effects of estrogens are mediated through the estrogen receptor (ER) which is a ligand-activated transcription factor and a member of the nuclear receptor superfamily [274]. In the classical 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)[11]. However, the activated ER can also mediate effects via interactions with Fos/Jun at AP-1 sites, Sp1 at GC rich promoter regions [13, 274], and through ligand-independent, DNA binding-independent and cell-surface (nongenomic) signaling mechanisms [13]. 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-PAS (bHLH-PAS) family of transcription factors. The AhR is responsible for mediating many, if not all, of the diverse toxic and biochemical responses to TCDD and related compounds. These responses 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 [23, 26]. The proposed mechanism for AhR signaling involves ligand binding to the cytoplasmic receptor 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 [32]. Although the mechanisms of AhR/ARNT-mediated changes in gene expression are well established, how the modulation of gene expression results in the subsequent physiological and toxicological effects remains poorly understood.

As part of its repertoire of toxic effects, TCDD elicits a number of antiestrogenic responses 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 [51-53, 57]. Chronic administration decreased the incidence of both mammary and uterine tumors in female rats suggesting that TCDD inhibits the development of estrogen-dependent tumors [42]. These effects are not mediated through TCDD binding to the ER [263]; rather, they are thought to involve cross-talk between the ER and AhR signaling pathways. Studies in continuous cell lines combined with the physiological responses observed *in vivo* have led to the development of a number of proposed mechanisms for ER-AhR cross-talk including increased estrogen metabolism, decreased estrogen receptor levels, induction of inhibitory factors, competition for cofactors, and direct inhibition of gene expression responses through interactions at estrogen responsive promoters (reviewed in [43]).

Inhibitory ER-AhR crosstalk at the gene expression level has been investigated *in vitro* and a small number of estrogen responsive genes which are inhibited by TCDD have been identified [77, 78, 80, 81]. However, the full spectrum of modulated gene expression responses and their relationship to *in vivo* antiestrogenic physiological endpoints has yet to be characterized. Therefore, to investigate the inhibitory effects of TCDD on estrogen mediated gene expression *in vivo*, temporal gene expression responses to EE and TCDD, both alone and in combination, were monitored in the uteri of C57BL/6 mice using a microarray approach. 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 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 4 days prior to treatment.

Animals (n= 5/treatment group/time point) were orally gavaged at time zero 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 and TCDD followed by additional doses of vehicle (vehicle and TCDD groups) or EE (EE and EE/TCDD groups) at 24 and 48 hrs as per the uterotrophic assay (Figure 1). Doses of 10 and 30μg/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. Mice were sacrificed by cervical dislocation 4, 12, 24 or 72 hrs 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 histology and fixed in 10% neutral buffered formalin (NBF, 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 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 software (Scion Image, Scioncorp, Frederick, Maryland) and standard morphometric techniques. The length of basal lamina underlying the luminal epithelium (LE) and

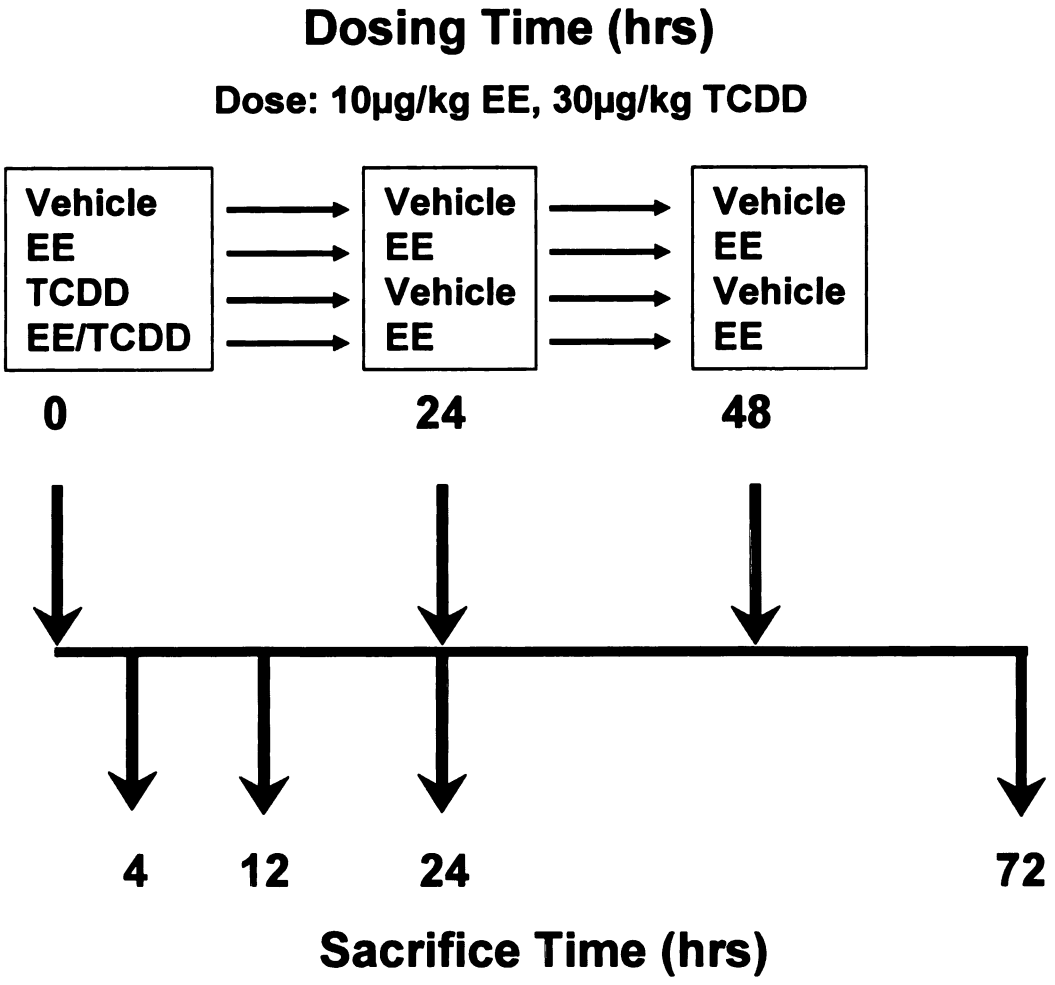


Figure 1.
Experimental design for EE/TCDD cotreatment time course study.
An *in vivo* time course study was performed in which immature ovariectomized C57BL/6 mice were orally administered vehicle (sesame oil), 10µg/kg ethynyl estradiol (EE), 30µg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or a mixture of EE and TCDD at time zero followed by doses of vehicle (vehicle and TCDD groups) or EE (EE and EE/TCDD groups) at 24 and 48 hrs as per the uterotrophic assay. Mice were sacrificed 4, 12, 24 or72 hrs after the initial dose at which time uterine tissues were harvested.

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) 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 visual inspection of 1μg 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). Changes in uterine gene expression were assessed using a 2 × 2 factorial design (Figure 2) [287]. In this design, arrow bases represent samples labeled with Cy3 and arrow heads represent samples labeled with Cy5. Within each replicate a sample is labeled and hybridized on three independent arrays for a total of 6 arrays/replicate/time point. Three biological

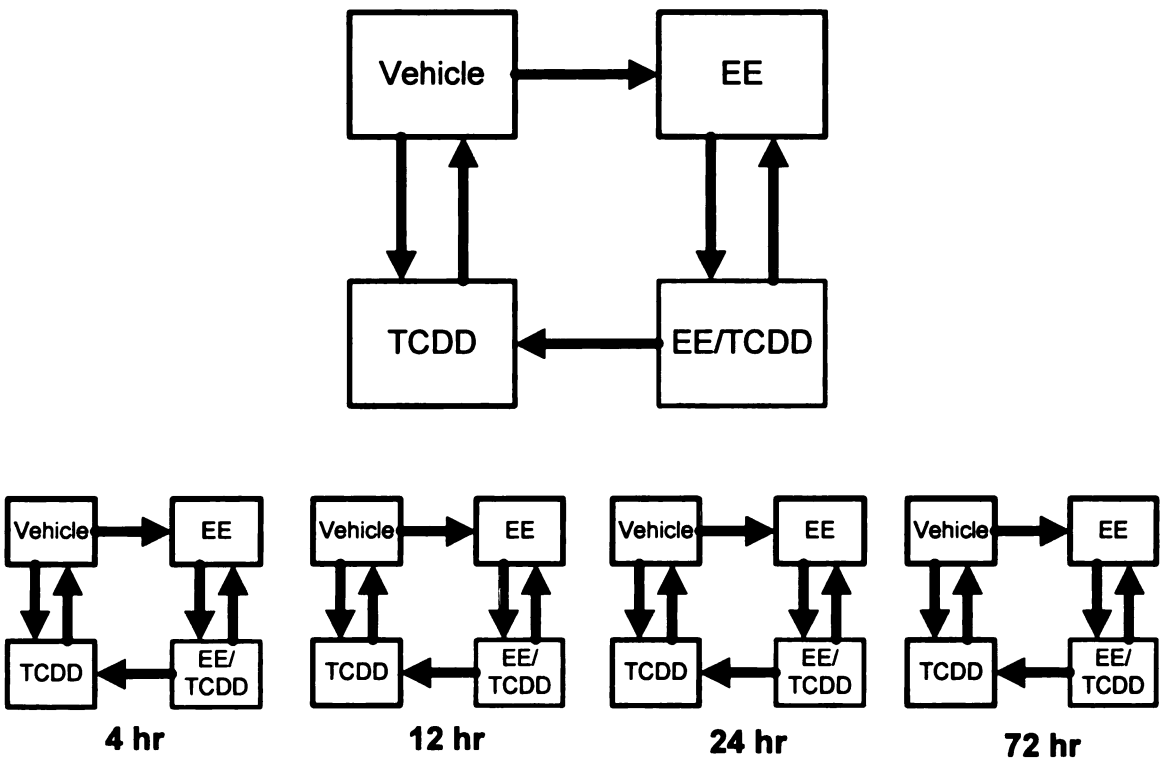


Figure 2.
2 × 2 Factorial design utilized for the microarray experiments
A 2×2 factorial design was used to investigate the effects of EE and TCDD alone while also facilitating testing of the interactive effects between EE and TCDD. Each arrow represents a microarray with the arrow bases representing Cy3 labeled samples and arrow heads 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 were completed for a total of 72 microarrays.

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 1.0μg 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 [268]. 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 [223]. 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[288]. Data are stored within dbZach (http://dbzach.fst.msu.edu), a MIAME 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 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 (QRTPCR) Analysis

For each sample, 1.0μg of total RNA was reverse transcribed by SuperScript II using an anchored oligo-dT primer as described by the manufacturer (Invitrogen). The resultant cDNA (1.0μl) 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 [151], 3 mM $MgCl_2$, 1.0mM 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 2. 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 [269]. Statistical significance of differentially expressed genes was determined

using two-way ANOVA followed by a Tukey's post hoc test (SAS 9.1). For graphing purposes, the relative expression levels were scaled such that the expression level of the TMVC was equal to one.

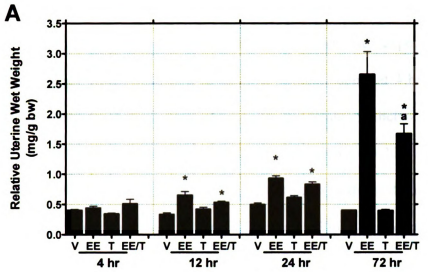
RESULTS

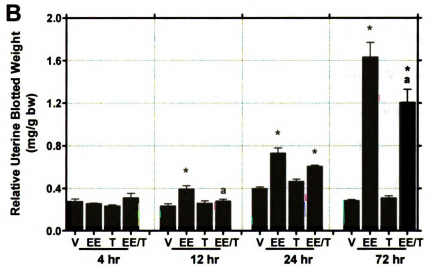
Uterine and Hepatic Weights

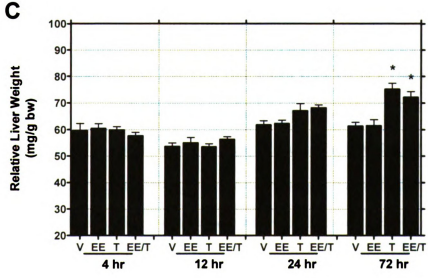
Increases in uterine weight due to water imbibition, hypertrophy and hyperplasia are well-characterized responses to estrogenic compounds and serve as the basis of the uterotrophic assay [270]. As expected, EE induced a significant increase in uterine wet and blotted weights at 12, 24 and 72 hrs after treatment, relative to the TMVC, while treatment with TCDD alone had no effect (Figure 3A and B). Cotreatment with TCDD was able to significantly inhibit this response relative to that of EE alone. TCDD inhibited EE-mediated increases in uterine wet weight by 37, 23 and 45% ($p<0.05$) 12, 24 and 72 hrs time points, respectively (Figure 3A). Similar effects were noted on blotted uterine weights with an inhibition of 71 ($p<0.05$), 38 and 30% ($p<0.05$) at 12, 24 and 72 hrs, respectively (Figure 3B). These results illustrate the antiestrogenic effects of TCDD on the inhibition of EE-mediated induction of uterine weight in the standard uterotrophic assay.

Increases in relative liver weights are a well-known TCDD-mediated response. TCDD alone and TCDD in combination with EE resulted in a significant increase in relative liver weights at 72 hrs (Figure 3C). Cotreatment of EE with TCDD did not enhance or inhibit the response when compared to TCDD alone. EE did not have any effect on liver weights and no effects on body weight or body weight gain were noted in any of the treatment groups.

Figure 3.
EE, TCDD and EE/TCDD effects on temporal uterine and hepatic weights. EE induced the expected increases in uterine wet (A) and blotted (B) weights at 12, 24 and 72 hrs while TCDD (T) had no effect. Cotreatment with TCDD was able to inhibit EE-mediated increases in both wet and blotted uterine weights. TCDD alone and TCDD in combination with EE resulted in a significant increase in relative liver weights at 72 hrs. Cotreatment with EE did not modulate the TCDD-mediated increases in relative liver weights. * = $p<0.05$ when compared to time-matched vehicle controls (V). a = $p<0.05$ when compared to time matched EE-treated animals.


A
Relative Uterine Wet Weight (mg/g bw)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
V EE T EE/T
4 hr
12 hr
24 hr
72 hr
*
a


B
Relative Uterine Blotted Weight (mg/g bw)
2.0
1.6
1.2
0.8
0.4
0.0
V EE T EE/T
4 hr
12 hr
24 hr
72 hr
*
a


C
Relative Liver Weight (mg/g bw)
100
90
80
70
60
50
40
30
20
V EE T EE/T
4 hr
12 hr
24 hr
72 hr
*

Histopathology and Morphometry

Treatment of mice with EE resulted in the expected complex uterine histopathology which consisted of minimal stromal edema at 4 hrs which progressed to moderate severity by 12 hrs. At 24 hrs moderate epithelial cell hypertrophy and hyperplasia with moderate stromal edema was observed which progressed to marked epithelial and stromal hypertrophy and hyperplasia with mild stromal edema at 72 hrs (Figure 4). Cotreatment of EE and TCDD resulted in the same histology observed with EE treatment alone with the exception of reduced stromal edema at 12, 24 and 72 hrs, subnuclear vacuolization in epithelial cells at 24 hrs, and reduced stromal hypertrophy and hyperplasia with marked luminal epithelial cell (LEC) apoptosis at 72 hrs (Figure 4). The effects observed on the LEC layer are consistent with previous reports of TCDD in the murine uterus [51]. These alterations in uterine histology may contribute to the associated decreases in uterine weight observed with TCDD cotreatment.

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

Increased luminal epithelial cell height (LECH) is a classic marker of estrogen exposure and has been used to assess the estrogenicity of a number of structurally diverse ligands [289-291]. EE induced significant increases in LECH at 24 and 72 hrs, consistent with previous reports [49], however, cotreatment with TCDD did not inhibit this response. The inability to detect antiestrogenic effects on LECH may indicate that

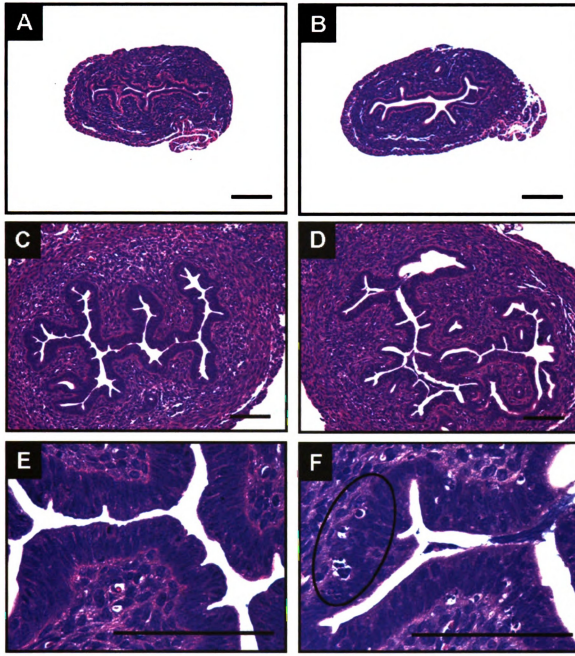


Figure 4

Comparison of uterine histology at 72 hrs between vehicle, TCDD, EE, and EE/TCDD treatment groups.

Relative to the time matched vehicle control (A), TCDD did not induce any alterations in uterine histology (B). EE induced marked epithelial and stromal hypertrophy and hyperplasia with mild stromal edema at 72 hrs (C and E). Cotreatment of EE and TCDD resulted in the same histology observed with EE treatment alone at 72 hrs with the exceptions of reduced stromal edema, decreased stromal hypertrophy and hyperplasia and marked luminal epithelial cell (LEC) apoptosis (circled) (D and F). Bars = 10 microns

TCDD does not influence this response. Alternatively, it may be attributed to 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 which facilitates investigations into the responses to EE and TCDD alone as well as their interactive effects [287]. Following normalization and statistical analyses, a conservative statistical P1(t) cutoff of 0.9999 combined with a differential expression of ±1.5 fold relative to TMVCs was used to filter the gene expression data and to define active gene lists. Gene expression responses to EE alone displayed the expected complex transcriptional profile as previously reported [48, 49] with a total of 3,746 features, representing 2,753 unique genes, identified as differentially expressed at one or more time points. Uterine gene expression responses to TCDD were modest compared to EE. In response to TCDD, 793 features representing 628 unique genes were found to be differentially expressed. EE/TCDD cotreatment resulted in an overall gene expression response similar to that of EE alone, with at 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 to EE, TCDD and EE/TCDD, hierarchical clustering was performed on features which 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 the EE/TCDD group was essentially indistinguishable from that of EE alone (Figure 5A). In addition, the TCDD group displayed similarities to that of EE, consistent with

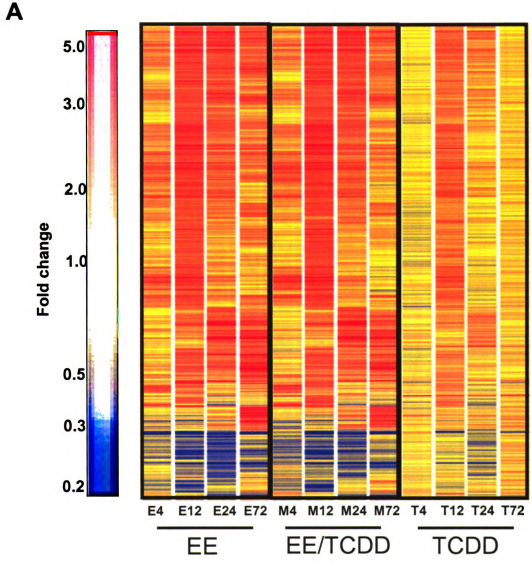
previous microarray studies illustrating the estrogen-like gene expression profile of TCDD [292]. Clustering by treatment and time point further revealed the temporal similarity of the EE and EE/TCDD treatment groups as each of their gene expression time points clustered with one another (Figure 5B). Furthermore, the 12 hr gene expression responses to TCDD, EE and EE/TCDD clustered together, further demonstrating the estrogen-like response to TCDD at this time point.

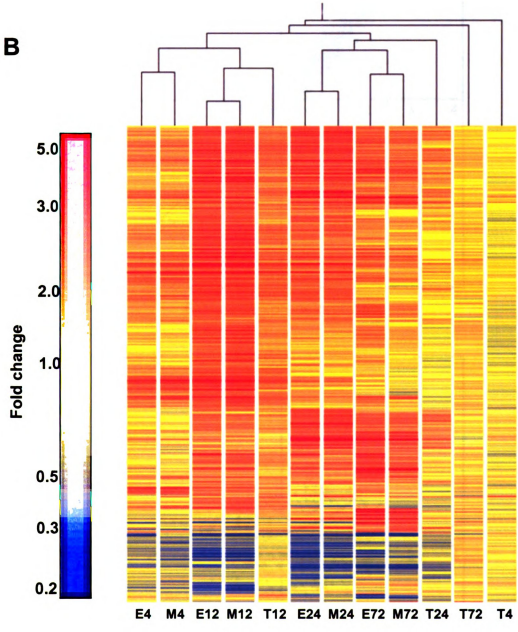
Although these clustering approaches are useful to illustrate the similarity of gene expression patterns across treatments, they do not provide a measure of the variation between the magnitudes of the response, an important consideration when examining TCDD inhibition of EE-mediated gene expression. Scatter plots of the $\log_2$ expression ratios of EE versus EE/TCDD revealed that most responses were of the same magnitude, relative to the TMVCs. Figure 5C illustrates a plot comparing the 12 hr EE and EE/TCDD group which displays a correlation value of 0.95. This graph also indicates that a small subset of genes is more highly expressed in the EE treated group, suggesting inhibition upon cotreatment with TCDD. A similar comparison between EE and TCDD groups at 12 hrs revealed a low correlation of 0.064, indicating differences in the magnitudes of the response despite similar expression patterns (Figure 5D).

Genes Differentially Regulated Between EE and EE/TCDD Groups

To characterize the inhibitory effects of TCDD on EE-mediated responses, genes were identified which were differentially expressed by EE/TCDD cotreatment when compared to EE treatment alone. To be considered in this category two criteria had to be met, namely, differential expression by EE treatment relative to TMVCs and differential expression in EE/TCDD cotreatment relative to EE. This approach identified genes

Figure 5.
Comparison of global gene expression responses between EE, TCDD and EE/TCDD.
A. Comparison of temporal gene expression profiles indicates the similarity of the EE and EE/TCDD patterns while the responses to TCDD appear minimal by comparison with the exception of the 12 hr time point. B. Hierarchical clustering of the treatment/time categories further illustrates the temporal similarity between the EE and EE/TCDD groups as each treatment/time point clustered together. The 12 hr TCDD samples clustered with the 12 hr EE and EE/TCDD samples indicating the estrogen-like pattern at this time point. The 24 hr TCDD sample exhibited modest similarity to the 24 and 72 hr EE and EE/TCDD groups while the 4 and 72 hr TCDD groups clustered separately. C. Scatter plots of the $\log_2$ expression ratios of EE versus EE/TCDD revealed that the majority of the responses were of the same magnitude relative to the time matched vehicle controls (TMVCs) with a correlation of 0.95. This graph also reveals a small subset of genes which 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 hrs revealed a low correlation of 0.064, indicating high variation between the magnitudes of the response despite similar expression patterns. Comparisons were performed on features which 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).


A
5.0
3.0
2.0
1.0
0.5
0.3
0.2
Fold change
E4 E12 E24 E72 M4 M12 M24 M72 T4 T12 T24 T72
EE
EE/TCDD
TCDD


B
5.0
3.0
2.0
1.0
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0.3
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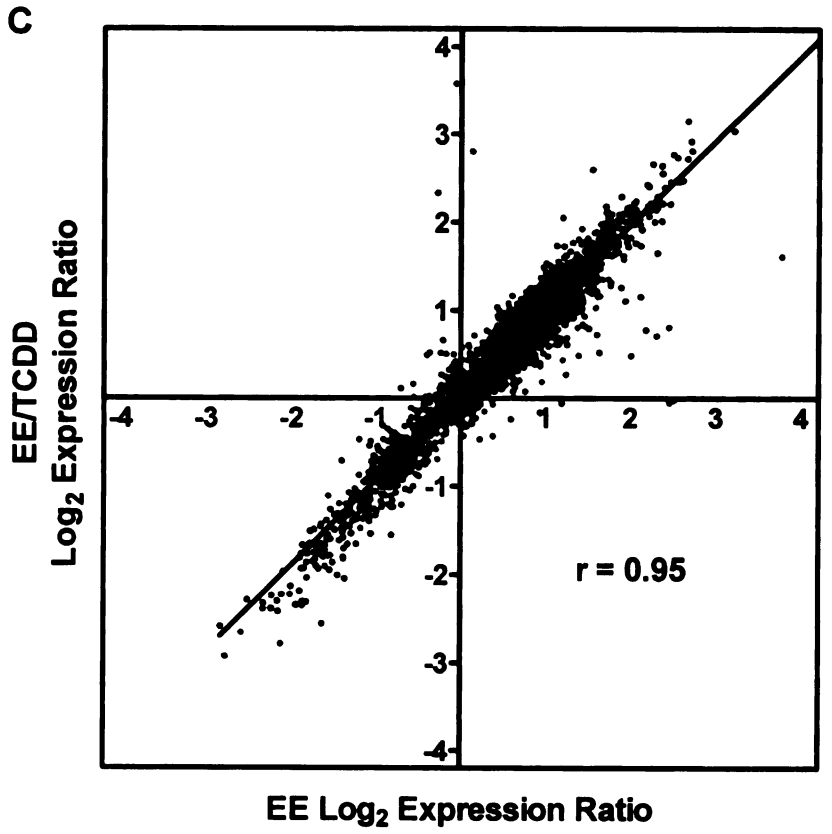
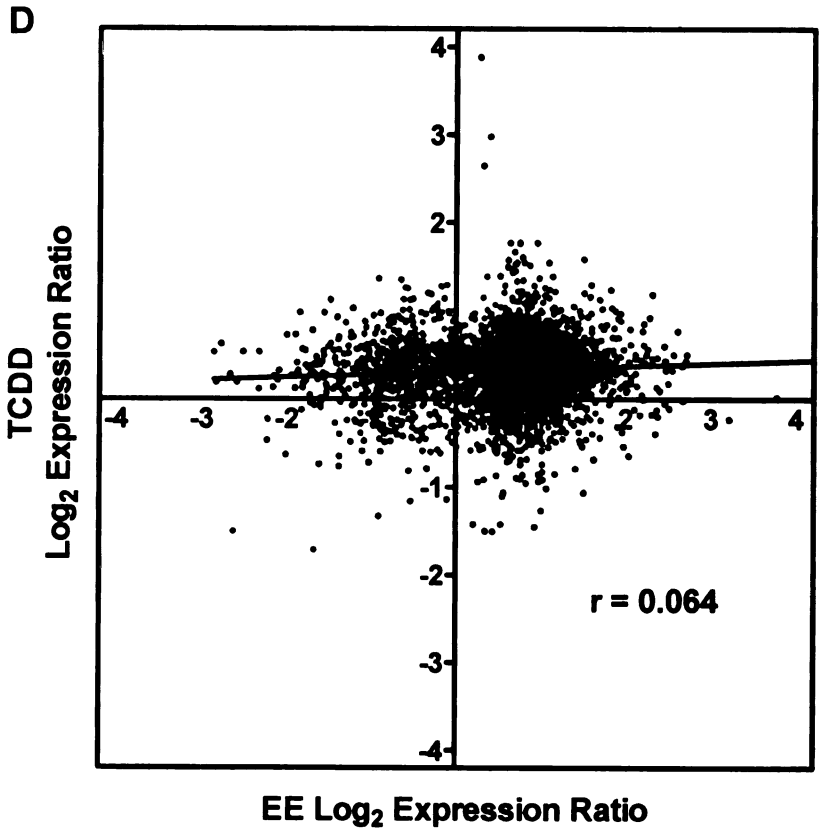
Fold change
E4
M4
E12
M12
T12
E24
M24
E72
M72
T24
T72
T4


C
EE/TCDD
Log2 Expression Ratio
4
3
2
1
-4
-3
-2
-1
1
2
3
4
-1
-2
-3
-4
r = 0.95
EE Log2 Expression Ratio


D
TCDD
Log2 Expression Ratio
4
3
2
-4
-3
-2
2
3
4
-1
-2
-3
-4
r = 0.064
EE Log2 Expression Ratio

which were regulated by EE and modulated upon cotreatment with TCDD. This identified 163 features representing 133 EE-regulated genes which were modulated by TCDD cotreatment at one or more time points. These data indicate that TCDD inhibition of EE-mediated responses is selective and most genes are unaffected by cotreatment consistent with the data in Figure 5C. On a per time point basis, 9, 23, 32 and 130 features representing 5, 21, 28, and 106 genes were differentially expressed between EE and EE/TCDD groups at 4, 12, 24 and 72 hrs, respectively. This indicates a time-dependent increase in the inhibitory effects of TCDD on EE-mediated gene expression responses suggesting 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 and were not EE-regulated responses but were differentially expressed due to the presence of TCDD. This included 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 as the induction of inhibitory factors is a previously proposed mechanism [89].

Functional Categorization of Microarray Data

Functional annotation of gene expression responses was performed using data extracted from public databases and published literature. Classification of active genes in the EE and EE/TCDD groups identified previously reported functional categories including transcription factors, mRNA and protein synthesis, cell cycle regulation, cellular proliferation, energetics and structural constituents [48-50]. Functional

annotation of genes whose EE-mediated regulation was inhibited upon cotreatment with TCDD were associated with the regulation of cell proliferation and growth, water/ion 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[84-87] and uterine tissue [57]. 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. Inhibition of genes encoding structural molecules included 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). Collectively, the inhibition of these EE-mediated responses may contribute to the antiestrogenic effects of TCDD on uterine histology, growth and LEC integrity

Verification of Microarray Results

QRTPCR was used to verify changes in transcript levels for a selected subset of the EE-inducible genes inhibited by TCDD (Figure 6). These analyses indicated a good agreement between the microarray and QRTPCR results although compression in the magnitude of the response was observed for the microarray data, a previously reported phenomenon when comparing microarray analysis to other methods [154]. QRTPCR

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 (hr)[a]				% Inhibition of EE Response by TCDD Time point (hr)[b]			
				4	12	24	72	4	12	24	72
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

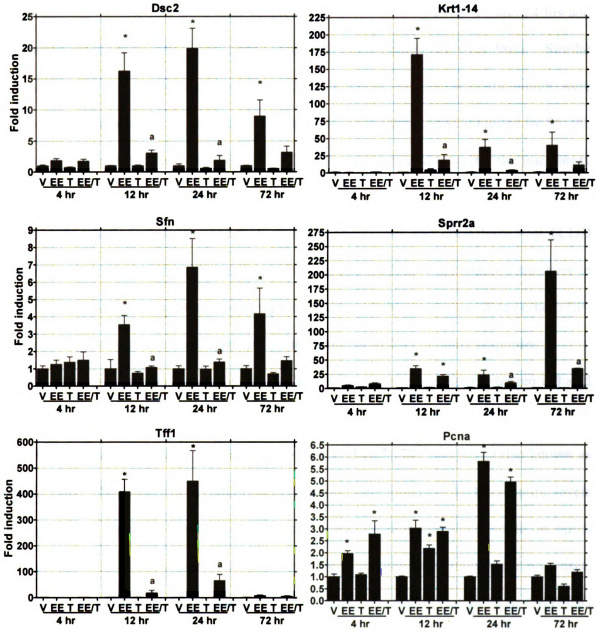


Figure 6.

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 5 biological replicates. Error bars represent the SEM for the average fold change.

* = $p<0.05$ for treatment groups relative to time matched vehicle controls

a = $p<0.05$ for EE/TCDD when compared to time matched EE controls

revealed Tff1 transcripts were induced greater than 400-fold by EE at 12 and 24 hrs and cotreatment with TCDD significantly inhibited this response by over 90%. Similar confirmatory responses were noted for Dsc2, Krt1-14, Sprr2a, and Sfn which were maximally induced 20, 171, 206 and 6.8 –fold by EE treatment and inhibited by 95, 90, 83 and 93 %, respectively, by TCDD. QRTPCR was also used to verify the microarray data for proliferating cell nuclear antigen (Pcna) and solute carrier family 25, member 5 (Slc25a5) which represent genes that were induced by EE and not affected by TCDD cotreatment. The results indicate that both Pcna and Slc25a5 were induced similarly in the EE and EE/TCDD groups, revealing the accuracy of the microarray data on both EE-mediated gene expression changes and the influence of TCDD on these responses.

Previous research using human MCF-7 cells characterized four estrogen induced genes which were inhibited upon cotreatment with TCDD through an inhibitory DRE (iDRE) mechanism, including Fos[77], Ctsd [81], Hsp27 [78], and Tff1 (also known as pS2) [80]. The dramatic TCDD-mediated inhibition of uterine Tff1 transcript levels suggests the cross-species (human to mouse) and cross-model (*in vivo* to *in vitro*) 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, however, TCDD cotreatment did not inhibit their induction (data not shown), suggesting the inhibitory effects on these genes may be specific to *in vitro* models or human MCF-7 cells.

DISCUSSION

The present study was conducted to develop a further understanding of the inhibitory effects of TCDD on estrogen mediated gene expression responses *in vivo*. The model utilized was the standard uterotrophic assay which is currently being validated by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) and the Organization for Economic Co-operation and Development (OECD) for the detection of estrogenic endocrine disruptors [271]. In this assay, the detection of antiestrogenic activity involves monitoring effects on uterine weight in the presence of a potent reference estrogen such as EE. TCDD significantly inhibited EE-mediated increases in uterine weight and disrupted the LEC layer, consistent with previous reports [51, 52, 265]. Comparison of EE and EE/TCDD gene expression responses revealed that the majority of EE-mediated changes were unaffected by cotreatment. However, a small subset of EE-responsive genes was inhibited upon cotreatment with TCDD suggesting a gene-specific inhibitory response. Functional annotation revealed the targeted inhibition of genes involved in cell proliferation, growth and differentiation, water and ion transport, and maintenance of cellular structure and integrity. Furthermore, these responses were consistent with the histological alterations observed with TCDD cotreatment suggesting a role for their inhibition in mediating the antiestrogenic effects.

Inhibition of Cellular Growth and Proliferation Responses

Estrogen induction of uterine weight involves a coordinated proliferative response which is mediated through a well orchestrated series of changes in gene expression [48-50]. Cotreatment with TCDD disrupted several EE-induced genes with important roles in the regulation of cell cycle, 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 hrs. Bcat regulates G1 to S phase transition and cells with reduced expression exhibit faster growth rates, a shorter G1 stage and an increased frequency of mutations [293]. Sfn serves as a component of the G2 checkpoint and is a positive mediator of growth-factor-induced cell cycle progression [294, 295]. TCDD also inhibited the EE-mediated induction of Serpinb5 at 12, 24 and 72 hrs. This gene is plays an essential role in development, as exhibited by embryonic lethality in knockout mice [296], while decreased expression results in reduced cellular proliferation and adhesion [296]. Sestrin 1 (Sesn1) was induced by EE between 12 and 72 hrs and TCDD attenuated this response at 24 and 72 hrs. Sesn1 induction is associated with positive regulation of cell growth and protection from apoptosis [297, 298]. Additional EE-induced genes implicated in cellular growth, proliferation and development included retinol binding protein 2[299], Tnfsf8 [300] and Vezf1 [301] which were also inhibited by cotreatment between 12 and 72 hrs. Although not regulated by EE, Ing1 was significantly induced by TCDD between 12 and 72 hrs, and has been associated with cell cycle arrest at G1 and can also lead to apoptosis [302]. The inhibition of genes involved in regulating cell cycle progression is consistent with a previous study, however, we did not detect inhibition of estrogen-induced cyclin transcripts which may be attributed to different experimental treatments and time points[57]. Overall, the inappropriate 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 [303, 304].

Tffs mediate these protective effects by blocking p53-dependent and independent pathways of apoptosis and promoting growth and regeneration by allowing cells to break attachments with the basement membrane to replace epithelial defects without cell death [305]. Tff peptides have also been shown to have anti-inflammatory actions and are a component of protective epithelial mucous layers [305, 306]. Therefore, the greater than 90% inhibition of EE-induced Tff1 by TCDD may play an important role in the in the increased degeneration and apoptosis observed in the LECs of the uterus.

Tff1 is a also a prognostic marker in human breast cancer and is an estrogen responsive gene in breast cancer cells and the human endometrium [307-309]. Furthermore, the inhibitory effect of TCDD on estrogen induction of Tff1 has been characterized in human MCF-7 breast cancer cells [79]. Inhibition is dependent on an iDRE which interferes with AP-1 and ERE mediated transcriptional activation [80]. Tff1 induction and inhibition in 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 which may play a role in the observed inhibition.

Water/Ion Transport Responses

Histopathological examination of uteri from EE treated mice revealed stromal edema which was reduced upon cotreatment with TCDD. Microarray analysis identified a number of EE-regulated genes involved in water and ion transport which were inhibited upon cotreatment with TCDD including the EE-mediated down-regulation of Aqp1 and

up-regulation of Aqp3. Isoform-specific regulation of aquaporins is thought to play an integral role in mediating the water imbibition response of the uterus to estrogens [310, 311]. In addition, a number of EE- regulated transcripts involved in sodium and chloride transport were inhibited by TCDD including Slc4a2, Slc38a3 and Fxyd4 [312, 313]. The combined inhibition of these EE-mediated gene expression responses may be a contributing factor to the reduction of uterine wet weight observed upon cotreatment with TCDD.

Inhibition of Structural Constituents

The uterus must undergo extensive changes in cytoarchitecture to accommodate the dramatic proliferation and growth response to estrogen. To facilitate this, numerous structural, adhesion and extracellular matrix genes were dramatically induced by EE including a number of keratins, actins, procollagens, tubulins, desmocollins, and small proline-rich proteins. Furthermore, genes in these categories have previously been reported as estrogen-inducible uterine responses in independent microarray studies in mice, rats and humans [50, 152, 202, 314-316]. TCDD cotreatment inhibited a number of these EE-mediated responses which likely contributed to its antiestrogenic effects on uterine growth as well as increases in apoptosis.

In the present 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 in epithelial cells which consists of an extensive array of filamentous networks and their disruption results in cell fragility and lysis [317, 318]. Keratins 18 and 19 have previously been identified as estrogen inducible transcripts whose induction is blocked upon cotreatment with TCDD in MCF-7 cells [319]. Their inhibition in the

murine uterus suggests that this may represent a conserved response between rodents and humans. Furthermore, 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 [320].

Desmocollin 2 (Dsc2) is primarily expressed in epithelial cells and is an important component of desmosomes which play an integral role in cell adhesion by forming links with the intermediate filament network [321]. Dsc2 was induced by EE treatment at 12, 24 and 72 hrs and cotreatment with TCDD was able to inhibit this response at each time point. TCDD also inhibited Perp induction, which promotes the stable assembly of desmosomal complexes [322]. EE induction of small proline rich protein 2a (Sprr2a) at 12, 24 and 72 hrs was also inhibited by cotreatment with TCDD. The Sprr2 family consists of 11 genes (Sprr2a-2k) which are important structural components of epithelial cells due to their ability to form extensive cross-links [323, 324]. Furthermore, many of the Sprr2 genes are up-regulated in the luminal epithelial cells of the uterus in response to estrogen where they are thought to serve important roles in the cytoarchitectural changes [324]. TCDD also inhibited the EE-mediated induction of many additional structural molecules including Marco, procollagen 6a2, troponin T1, and tubulin beta 6. Collectively, the TCDD-mediated inhibition of these structural constituents may compromise the ability of uterine cells to accommodate the rapid proliferation and growth induced by EE resulting in altered histology, increased apoptosis and overall decreased uterine growth.

TCDD as estrogenic and antiestrogenic

Previous reports have indicated that TCDD elicits an estrogen-like, ER-dependent gene expression profile in the uterus and results from this study further support these data [116, 278, 292]. Regulation of a similar battery of genes by EE and TCDD suggested these responses may represent sensitive targets for inhibition upon cotreatment. However, EE-mediated responses for these genes were largely unaffected by TCDD cotreatment including well-known estrogen inducible 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 crosstalk [43, 78, 79]. These data suggest that TCDD elicits its estrogenic gene expression responses independent of those associated with its antiestrogenic effects.

Decreased ER levels [55] and increased estrogen metabolism [95, 325] have previously been proposed as potential mechanisms for the antiestrogenic effects of TCDD. However, a number of reports have indicated that these are unlikely to wholly account for the antiestrogenic effects as TCDD did not increase estrogen metabolism *in vivo* [45, 97] and uterine ER levels were unaffected [45, 99, 100]. In the present study, TCDD inhibited a select subset of EE responses suggesting increased metabolism and decreased ER levels are not the primary mechanisms as these would be expected to affect gene expression responses on a more global scale. The gene-specific inhibitory effects may involve an iDRE mechanism or induction/inhibition of an upstream regulatory protein. These effects are likely to be tissue and species-specific and additional research will be required to more definitively assign the antiestrogenic mechanisms.

Conclusions

The present study has identified *in vivo* gene expression responses to EE that are inhibited upon cotreatment with TCDD. Only a small subset of EE regulated transcripts were inhibited, indicating the gene-specific nature of this response. TCDD-mediated inhibition of estrogen-induced Tff1 transcripts is consistent with previous *in vitro* studies in human MCF-7 cells, indicating the potential conservation of this response between different models and species. Functional categories represented by the inhibited genes were related to the observed histological and physiological antiestrogenic responses and represent potential mediators of TCDD's anti-uterotrophic/antiestrogenic response. Moreover, the estrogenic and antiestrogenic gene expression effects of TCDD are independent, further highlighting the need for additional research to more fully delineate the dual nature of TCDD as an estrogenic and antiestrogenic compound.

CHAPTER 8

TOXICOGENOMICS IN RISK ASSESSMENT: APPLICATIONS AND NEEDS[5]

ABSTRACT

Since its inception, there have been high expectations for the science of toxicogenomics to decrease the uncertainties associated with the risk assessment process by providing valuable insights into toxic mechanisms of action. However, the application of these data into risk assessment practices is still in the early stages of development and proof of principle experiments have yet to emerge. The following discusses some potential applications as well as impediments that warrant a concerted investigation from all stakeholders in order to facilitate the acceptance and subsequent incorporation of toxicogenomics into regulatory decision making.

[5] This chapter has been published in: Boverhof, D. R., and Zacharewski, T. R. (2005). Toxicogenomics in Risk Assessment: Applications and Needs. *Toxicol Sci.*

INTRODUCTION

Genomic technologies are rapidly evolving as powerful tools for discovery- and hypothesis-driven research, a fact evidenced by the exponential increase in the number of publications involving microarrays, proteomics and metabolomics [326, 327]. Toxicogenomics, the integration of *omic* technologies, bioinformatics and toxicology, has seen significant investment in the pharmaceutical industry for both predictive and mechanism based toxicology in an effort to identify candidate drugs more quickly and economically [328-330]. Despite significant progress in its development and implementation, deciphering meaningful and useful biological information from toxicogenomic data remains challenging for toxicologists, risk assessors and risk managers. In general, toxicogenomic studies have been limited to a qualitative description of alterations in transcript, protein and metabolite levels with little correlation to toxicity or contributions towards the elucidation of mechanisms of toxicity. Despite this, reviews and commentaries continue to pledge that toxicogenomics will support the development of high-throughput assays and computational models and revolutionize mechanistically-based quantitative risk assessment, thereby improving predictions of environmental and human health safety [331-335]. Although laudable goals, significant challenges impede the incorporation of toxicogenomic data into risk assessment practices [328, 333, 336].

To date, drug discovery and development has been the driving force behind toxicogenomics in an effort to identify and prioritize new chemical entities (NCEs) with a greater likelihood of success in clinical trials. The high cost associated with the development of a single drug, which ranges from $500-900 million with a 12-15 year

commitment [337], has prompted efforts to improve the preclinical evaluation of NCEs to reduce failures in clinical trails due to unfavorable adsorption, distribution, metabolism, and excretion (ADME) characteristics as well as unacceptable toxicity [338]. Historically, only 1 in 5,000-10,000 screened chemicals successfully reaches the market, with 30-50% of drug candidates failing due to toxicity, and only 30% of marketed drugs producing sufficient revenue to recover research and development investments. These factors significantly contribute to the time and cost of drug development [339-341] and therefore, even incremental improvements in the success rate will have favorable impacts for all stakeholders [328]. This, combined with the increasing pressure for cheaper and safer drugs, has the pharmaceutical sector re-organizing their screening and preclinical development strategies. Many are examining toxicogenomic approaches in order to develop and incorporate high-throughput toxicology screening earlier in the drug development pipeline.

Regulatory agencies such as the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) also recognize the potential of toxicogenomics and encourage the use and submission of complementary toxicogenomic data in an effort to establish guidelines, and eventually protocols, for its inclusion in submitted applications and incorporation into regulatory decision-making [342, 343]. At this time, the FDA and EPA, along with European and Asian regulatory bodies, are carefully monitoring developments as the field continues to mature and a workable consensus is reached among the various stakeholders.

Fundamental differences in drug versus environmental safety/risk assessment may be a factor contributing to the predominant use of toxicogenomics in the pharmaceutical

sector. For example, some level of toxicity may be acceptable provided it can be monitored and managed, and the new drug provides clear health benefits relative to available treatments. Moreover, pharmaceutical companies will likely utilize toxicogenomics data to "screen out" candidates with unacceptable levels of toxicity or to demonstrate that toxicity exhibited in rodents, dogs or non-human primates is irrelevant to humans. Economic influences could also play a role in the predominance of toxicogenomics in pharmaceutical research as investigative toxicology may be supported to a greater extent in this industry. In contrast, chemical and agrochemical sectors have been less receptive to the implementation of toxicogenomics due to its questionable benefits in supporting risk assessment. Furthermore, there are significant concerns regarding its potential naive and premature use in hazard identification, possibly leading to unfounded product de-selection [344]. The demonstration of any effects elicited by commerce chemicals is considered by some advocacy groups to be an adverse, involuntary and therefore, unacceptable risk. Companies are concerned that unsubstantiated toxicogenomic data could be inappropriately extrapolated to toxicity which could evoke actions such as the Precautionary Principle [344, 345]. The inability to place all toxicogenomic data into biological context may therefore increase the uncertainty of the exposure-to-outcome linkage associated with commerce chemicals and environmental contaminants which could "screen in" more chemicals requiring further investigation in the absence of any toxicity. Nevertheless, the use of toxicogenomic data in environmental risk assessment must continue to be explored in parallel with drug safety assessments in an objective manner to determine its potential role and further define its limitations (Table 1).

Table1. Applications and Needs for Toxicogenomics in Risk Assessment Practices

Applications	Needs
Prioritization of chemical lists	Establishment of a comprehensive knowledgebase
Deciphering mechanisms of action	Effective and user-friendly databases
Identifying biomarkers of exposure	Conserved genetic, protein and metabolite
Identifying biomarkers of toxicity	Consistent analysis approaches
Cross-species extrapolations	QA/QC standards
Identifying species sensitivities	Examples of application to risk assessments
Analysis of mixtures toxicity	Education of stakeholders
	Tools for integration of disparate data sets Cross-species (human, rat, mouse), cross-platform (oligo, cDNA), cross technology (microarray, proteomic, metabolomic)

APPLICATIONS OF TOXICOGENOMICS

One of the most promised applications involves the screening and prioritization of commerce chemicals and drug candidates that warrant further development and testing. This consists of comparing their toxicogenomic profiles to databases containing profiles of known toxicants and identifying biomarkers of exposure and toxicity that can be used in high-throughput screening programs. These applications are analogous to the development of diagnostic signatures and classification protocols for disease states which can identify more effective treatment regimens for selected populations and can also be used to monitor drug efficacy during clinical trails [346, 347]. Toxicogenomic-based biomarkers will likely comprise an agglomeration of responses that allow for further stratification of the population to identify sensitive groups which could then be treated more effectively while minimizing the risk of unacceptable toxicities. Ideally, these biomarkers will be mechanistically-based and causally associated with the adverse effect, which is expected to further minimize uncertainties in the source-to-outcome continuum and extrapolations between species (rodent to human) and across models (*in vitro* to *in vivo*). Classifications based on mechanisms of action will identify biomarkers with greater predictive accuracy that could be used for exposure assessments in humans and extended to include wildlife species. Moreover, they will provide evaluations of the appropriateness of cross-species extrapolations by assessing the degree of conservation of mechanisms of toxicity, which would facilitate the implementation of mechanistically-based chemical-specific uncertainty factors that account for both within and across species variability. Furthermore, toxicogenomics provides strategies for the comprehensive assessment of mixtures since all possible chemical, gene, protein,

metabolite, and network interactions that may be important in eliciting mixture-specific toxicities can be considered. However, these applications including the identification of biomarkers will require broad acceptance and comprehensive validation procedures such as those proposed by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the European Centre for the Validation of Alternative Methods (ECVAM)[348, 349].

Overall, expectations that toxicogenomics will facilitate the development of safer drugs and commerce chemicals are justified. Initial reports have demonstrated that chemicals and drugs can be classified based on their gene and metabolite profiles [350-359] but these approaches are not yet ready to be utilized as stand alone tools. Consequently, it is likely that expression profiles and agglomerative biomarkers will initially be used to: (i) rank and prioritize the potential toxicity of NCEs in the early stages of development and therefore would not be included as a regulatory reporting requirement (e.g. investigational new drug application), and (ii) demonstrate that toxicities observed in traditional models (i.e., rodent, dog, non-human primate) are not relevant to humans since the mechanisms of action are not conserved across species.

Both EPA and FDA are encouraging the use of toxicogenomics and have described its applicability in regulatory decision-making. EPA's interim policy states that toxicogenomic data may be considered but these data alone are insufficient as a basis for decisions and therefore, will be used on a case-by-case basis [360, 361]. However, the recent establishment of a Computational Toxicology Program to build systems biology capacity within the agency signals its intent to use more computational

approaches in the future to prioritize data requirements and reduce uncertainties in the source-to-outcome continuum used in quantitative risk assessments [362].

Concurrently, the FDA recognizes that toxicity and human safety testing has not kept pace with the emerging technologies, and drug development has become more challenging, inefficient and costly [363]. Although traditional toxicology testing has a proven track record of safety, the approaches are laborious, time-consuming, and have failed to predict specific human toxicity [328, 364]. Consequently, the FDA is encouraging the incorporation of new tools, such as toxicogenomics and computational toxicology, to improve the critical path to the development of new therapeutics. They are also requesting the voluntary submission of complementary toxicogenomic data in order to facilitate training and to establish guidelines, which will eventually lead to policies regarding its submission and use in regulatory decision making [330, 342, 343].

IMPEDIMENTS AND NEEDS OF TOXICOGENOMICS

There are a number of technical, interpretation and implementation issues that impede the use of genomic, proteomic and metabolomic approaches in biomedical research, regulatory decision-making and quantitative risk assessment. These include the lack of uniform study designs, multiplicity of normalization and analysis strategies [365], questionable reproducibility of microarray data across platforms [366-369], the semi-quantitative nature of proteomics [370, 371], limited availability of metabolite annotation to support metabolomics [372], absence of data quality control measures and standards [326, 373], and lack of effective data sharing and reporting standards. Fortunately, several organizations (MGED [374], MIAPE [375], and SMRS [376] (Table 2)) are

Table 2. Toxicogenomic Standards and their Organizations

Standard	Organization	URL
MIAME- Minimum Information About a Microarray Experiment[1]	MGED Society- Microarray Gene Expression Data Society	http://www.mged.org
MIAPE- Minimum Information About a Proteomics Experiment [2]	HUPO PSI - Human Proteome Organization Proteomics Standards Initiative	http://psidev.sourceforge.net/gps/index.html
Minimum requirements for designing and recording the results of a metabolic study[3]	SMRS- Standard Metabolic Reporting Structures working group	http://www.smrsgroup.org

References: [1](Brazma et al. 2001), [2](Orchard et al. 2003), [3](Lindon et al. 2005))

addressing a number of these issues by developing guidelines and standards for the user community. Many journals are now requiring omic data to be uploaded into public database repositories that adhere to these standards as a prerequisite for publication in an effort to ensure unhindered public access to the primary data [377, 378]. However, the availability of published toxicogenomic data, and more specifically microarray and proteomic data, will only be of value if issues regarding cross-platform comparisons and the lack of uniform data quality control measures are resolved.

One of the most challenging aspects of implementing toxicogenomics in risk assessment involves establishing the appropriate supportive infrastructure to facilitate the effective management, integration, interpretation and sharing of toxicogenomic data. An effective, flexible and comprehensive knowledgebase is required that is populated with phenotypically anchored toxicogenomic data complemented with ADME, histopathology, clinical chemistry and toxicity data. Currently, several public and commercial toxicogenomic database efforts have been initiated (Table 3) utilizing the Minimum Information About a Microarray Experiment (MIAME) standards [374] as a guide. Although commercial databases are highly promoted, there is a lack of peer reviewed publications critically assessing their utility, although these are now starting to emerge [379, 380]. Future publications from independent laboratories will further demonstrate their utility of and facilitate increased acceptance of toxicogenomics in the scientific community. In contrast to the commercial databases, public database efforts are still in development.

Regardless of their origin, it is imperative that these databases are able to effectively communicate and share deposited data. Strategies to facilitate electronic data

Table 3. Toxicogenomic Supportive Databases

Database	Availability	URLs
Array Track	non-commercial	http://www.fda.gov/nctr/science/centers /toxicoinformatics/ArrayTrack/
ArrayExpress	non-commercial	http://www.ebi.ac.uk/arrayexpress/
Chemical Effects in Biological Systems (CEBS)	non-commercial	http://cebs.niehs.nih.gov/
CIBEX	non-commercial	http://cibex.nig.ac.jp/index.jsp
Comparative Toxicogenomics Database (CTD)	non-commercial	http://ctd.mdibl.org/
dbZach	non-commercial	http://dbzach.fst.msu.edu/
EDGE	non-commercial	http://edge.oncology.wisc.edu/
Gene Expression Omnibus (GEO)	non-commercial	http://www.ncbi.nlm.nih.gov/geo/
PharmGKB	non-commercial	http://www.pharmgkb.org/
SYMATLAS	non-commercial	http://symatlas.gnf.org/SymAtlas/
Toxicogenomics Project in Japan	non-commercial	http://wwwtgp.nibio.go.jp/index-e.html
Tox-MIAMExpress	non-commercial	http://www.ebi.ac.uk/tox-miamexpress/
Gene Logic	commercial	http://www.genelogic.com/
Iconix Pharmaceuticals	commercial	http://www.iconixpharm.com/

exchange between databases such as Microarray Gene Expression-Markup Language (MAGE-ML) [381] and Systems Biology Markup Language (SBML) [382] are being developed and will facilitate effective electronic data exchange between compliant repositories. Ideally, these databases will provide access to the large, disparate and robust toxicogenomic data sets required to develop the necessary computational algorithms and models needed to support quantitative risk assessment.

Although databases provide effective data management solutions, the ability to integrate toxicogenomic data across chemical and biological space to develop mechanistic pathways and networks remains limited. With few exceptions, most toxicogenomic studies to date provide a qualitative description of changes with minimal reporting regarding the implications to physiological outcomes and limited contributions towards further elucidating mechanisms of toxicity [383]. Similarly, reproducibility problems, quantification issues, and limited throughput compromise the utility of proteomics [370, 371]. The lack of comprehensive peptide and metabolite reference databases also hinders the ability to elucidate mechanisms of toxicity associated with changes in protein and metabolite profiles [371, 372]. Nevertheless, these technologies have demonstrated their utility in classification and diagnostics, but significant contributions toward deciphering mechanisms of toxicity and aiding in risk assessment have yet to materialize. This is not surprising since most studies lack the required replication and appropriate bioinformatic and statistical support and fail to phenotypically anchor the data to adverse outcomes. Moreover, it is not clear what toxicogenomic data is required and how it would be used in the current regulatory paradigms. Ideally, disparate gene, protein and metabolite data would be integrated with phenotypic toxicity data and

other traditional toxicology endpoints in order to identify mechanistically-based agglomerative biomarkers and elucidate mechanistic networks that could be used to develop predictive quantitative models. These data could then be used to determine points of departure, establish thresholds of toxicity, and predict exposure levels to a contaminant or complex mixture required to elicit a particular biomarker or adverse response [384-387].

Comparative toxicogenomics has the potential to identify conserved responses between humans and animal research models that are associated with toxicity which can be used to develop predictive toxicity tools. In addition, these approaches are likely to provide empirical evidence supporting the transfer of functional annotation from known human and mouse genes to unknown genes or ESTs in the rat or ecologically-relevant species, based on sequence similarity and comparable expression patterns. To date, very few studies exploit comparative approaches to transfer functional annotation between orthologous genes based on comparable gene expression patterns and conserved protein interactions in addition to the traditional use of sequence homology [388-390]. However, platform differences, inaccurate annotation across species and microarrays, the lack of tools to facilitate comparative analysis, one-to-many relationships between genes and probes (e.g., one gene in rat has two or more orthologs in humans), incomplete or poorly annotated genomes, discrepancies between databases which define orthologous relationships (NCBI vs EBI), and the limited availability of functional annotation complicate effective cross-species comparisons all confound comparative analyses. Current gene ontologies are also imprecise, incomplete and inconsistent across species which compromises the accurate interpretation of toxicogenomic data relative to a

phenotypic endpoint. For example, a large proportion of the current gene annotations for human, mouse and rat are inferred exclusively by electronic associations (Table 4) which includes low quality associations prone to changes and errors [391, 392]. Therefore consistent approaches to annotation curation are required to ensure the accurate interpretation of the data [393]. In addition, despite more complete and accurate annotation for the human and mouse genomes, the rat continues to be the traditional rodent model of choice for toxicology studies (Table 4). More comprehensive human and mouse annotation provides the information necessary for a more thorough interpretation of the data, and facilitates a more complete elucidation of pathways and networks involved in mediating toxicity. The availability of murine knock-out models also allows for more in-depth and definitive mechanistic studies. Consequently, from a toxicogenomic perspective, the mouse is a more powerful mechanistic model that is under utilized in toxicology.

The interpretation of toxicogenomics data will continue to be a difficult task and more effective tools to facilitate their integration and interpretation are required. Currently a number of tools exist to aide in the interpretation of genomic, proteomic and metabolomic data independently, however, tools that integrate these disparate data are required. Typically toxicity is a persistent and easily identified endpoint, however, toxicogenomic responses are dynamic and subject to reversible temporal changes, that can be displaced in time relative to toxicity. Therefore, capturing predictive profiles will be time sensitive and temporal toxicogenomic data will need to be collected and phenotypically anchored to well established endpoints of toxicity [394]. Comparison of transcriptomic, proteomic and metabolomic data will require sampling at multiple time

Table 4. Number of Human, Mouse and Rat gene products that have been annotated to gene ontologies[a]

Contributor[b]	Gene Ontology Category						Total gene products associated	Total references included as evidence
	Biological Process		Molecular Function		Cellular Component			
	All codes	non-IEA[c] codes	All codes	non-IEA codes	All codes	non-IEA codes		
GO annotations @ EBI- Human	20940	8371	24213	7837	18206	7366	26939	14722
GO annotations @ EBI- Mouse	20158	4588	24438	3812	19321	6817	28044	5128
GO annotations @ EBI- Rat	18936	1091	26002	892	15242	649	27902	1769
Mouse Genome Informatics (MGI)	12788	8565	13987	8967	13117	9952	16299	5274
Rat Genome Database (RGD)	10394	3614	11016	4169	9973	2683	12409	4041

[a]Data adapted from Gene Ontology Consortium (http://www.geneontology.org/GO.current.annotations.shtml)- Data extracted 07-21-2005

[b]There are various organizations that contribute to GO annotations, those specific to human, mouse and rat are listed.

[c]IEA codes are those Inferred by Electronic Association, meaning no human involvement in the association of the annotation. This is the only evidence code that does not require human judgment during curation and thus represents the lowest quality evidenc

points as the relationships that exist between these measures will also exhibit temporal displacement. Relating early toxicogenomic changes to distant effects is further complicated when only a subpopulation of the treatment group experiences the toxic effect as in the case of carcinogenesis, reproductive toxicity and teratogenicity. The added challenge is to accurately determine whether acute or short term toxicogenomic responses are predictive of sub-chronic or chronic toxicity outcomes. In addition, dose response studies are required to differentiate adaptive versus toxic responses and to establish toxicogenomic thresholds that need to be exceeded prior to the initiation of the cascade of molecular responses leading to an adverse effect. Each of these applications will require the development of powerful bioinformatics tools that can integrate disparate data across time, dose and technologies to develop comprehensive toxic response profiles.

Historically, the data used in risk assessment has largely been descriptive and agencies often differ in the choice of the critical toxic effect that is utilized when conducting risk assessments. The application of toxicogenomics has the potential to reduce the occurrence of such discrepancies by aiding in the identification of mechanisms of action which will lead to increase confidence and consistency in risk assessment practices. Reductionist approaches have been successful in providing insights into mechanisms of toxicity by examining individual cellular components, their families and functions. Despite this success, clear adverse effects can rarely be attributed to an individual event. Instead, most toxic responses likely involve complex interactions between genes, proteins and metabolites. The emergence of toxicogenomics provides the opportunity to simultaneously interrogate the broad molecular status of an organism,

tissue or cell experiencing toxicity within its gene, protein and metabolite domains (Figure 1). Studies in simpler organisms such as yeast, fly and worm demonstrate that individual responses are not independent, but form a network of interacting networks [395-398]. Similar approaches have also been used to examine toxicologically relevant models [399-401]. The challenge that remains is to comprehensively integrate the disparate chemical, biological, toxicological and toxicogenomic data in order to elucidate the mechanisms and networks involved in toxicity and to develop quantitative models capable of accurately predicting thresholds. Complex network theory has been used to investigate technological and social networks and similar principles have also been shown to govern complex biological networks [387], and are also likely to regulate toxicity. Therefore, the most significant challenge will be the application of comparable network approaches that integrate disparate toxicity data in order to reduce uncertainties and to support mechanistically-based quantitative risk assessment [361]. This will require multidisciplinary collaborative efforts, as well as significant retraining of toxicologists, modelers, risk assessors and risk managers consistent with the recommendations made by the Biomedical Information Science and Technology Initiative (BISTI) to integrate information and quantitative sciences into biomedical research [402]. Traditional toxicologist must understand the potential value and applications of toxicogenomics so it can be effectively tested and implemented alongside traditional research practices. In addition, individuals providing bioinformatic service and support need to understand the basic principles of toxicology in order to facilitate the development of effective and user-compliant toxicogenomic-based interpretation and storage tools.

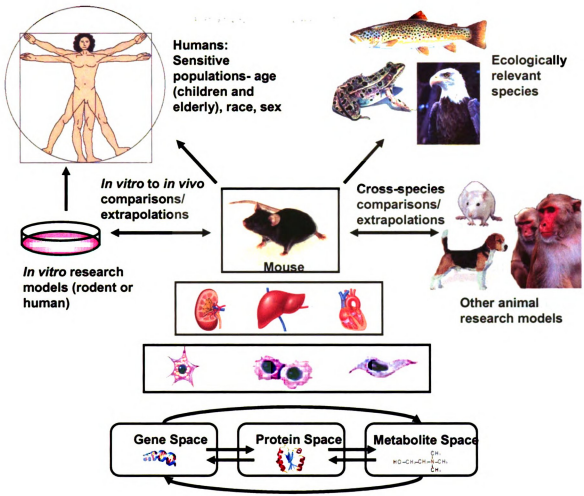


Figure 1.
Paradigm for Toxicogenomics in Risk Assessment.
Toxicogenomics, the combination of genomics, proteomics and metabonomics, allows for the examination of toxicant induced alterations in biochemical networks which involves perturbations in gene, protein and metabolite space. These data can be phenotypically anchored to toxicity observed at the cellular and/or tissue level in a model organism to provide insights into the toxic mechanism of action. Comparison of these data across *in vivo* and *in vitro* research models allows for the identification of conserved responses which can be used to identify predictive biomarkers of exposure or toxicity and reduce the uncertainties in understanding the risk posed to humans and environmentally relevant species.

CONCLUSIONS

The suggestion that toxicogenomic data such as changes in gene expression, protein levels or metabolite levels may be used in risk assessment creates considerable unease with some stakeholders [403]. Conversely, others are actively pursuing toxicogenomic approaches to identify putative high-throughput biomarkers to rank and prioritize lead candidates that warrant further development [337, 404]. The anxiety on one hand and enthusiasm on the other has created a discord within the risk assessment community on how to proceed with toxicogenomics. The concerns are justifiable due to the potential naive and premature use of the data which could have dire consequences for all stakeholders, including the general public. Nevertheless, there is general agreement that toxicogenomics will play an increasingly larger role in regulatory decision-making. Given the opportunity, effective and productive communication and collaboration will be a critical factor in establishing protocols for the interpretation and incorporation of toxicogenomics into quantitative risk assessment which will iteratively evolve in the presence of existing strategies as all stakeholders gain further experience with these emerging technologies.

CHAPTER 9

CONCLUSIONS AND FUTURE RESEARCH

The preceding studies have characterized the gene expression responses mediated by EE and TCDD in murine hepatic and uterine tissues. Each compound elicited a complex temporal and tissue-specific gene expression response which could be related to physiological and toxicological outcomes of exposure. The data also revealed that TCDD, when administered in the absence of estrogen, is able to elicit estrogen-like gene expression responses in the uterus. Furthermore, upon co-administration, TCDD is able to inhibit EE-mediated physiological responses in the uterus which are associated with gene-specific inhibitory effects. These data indicate the dual nature of TCDD as a compound with both estrogenic and antiestrogenic potential which may explain its sex, tissue and age-specific toxicities. Interestingly, neither the estrogen-like gene expression responses nor the inhibitory effects on estrogen-mediated gene expression responses were detected in hepatic tissues further illustrating the diversity and tissue specificity of the responses to TCDD.

These data have expanded our knowledge on the diversity of gene expression responses mediated by EE and TCDD alone. Furthermore, *in vivo* evidence has been acquired to support the gene-specific inhibitory effects of TCDD on estrogen-mediated gene expression responses as a potential mechanism for its antiestrogenicity. However, the generated data have also opened new research avenues which should be explored to develop an increased understanding of the hepatotoxic, estrogenic and antiestrogenic effects of TCDD. These anticipated research areas and suggested approaches are outlined below.

Hepatic gene expression responses to TCDD

TCDD mediates toxicity in a species-specific manner, and therefore developing a more complete understanding of the conserved and divergent gene expression responses between species will be integral to understanding its toxic mechanisms of action. As a result, studies examining the hepatotoxicity of TCDD as well as its estrogenic and antiestrogenic responses should make use of cross-species comparative approaches. Data presented in Chapter 4 provide baseline quantitative data on the *in vivo* gene expression responses of murine hepatic tissue to TCDD which can be used in future comparative studies. Similar experiments have already been completed using Sprague Dawley rats and comparison of these responses to those observed in mice can be used to examine the species-specific physiological and gene expression responses to TCDD. Previously published reports indicate that rats are more sensitive to TCDD-mediated toxicity and do not exhibit the same spectrum of toxicological endpoints when compared to mice. However, unlike strain-specific responses, this cannot be explained by differences in receptor affinity or differences in the AhR transactivation domain. Comparative microarray analyses are expected to identify species-specific gene expression responses that are responsible for the increased sensitivity and differential *in vivo* toxicity observed in rats.

Toxicogenomics approaches have been expected to help reduce, refine and replace the use of animal models in toxicology by using gene expression profiling with cells in culture to extrapolate to responses in the whole organism. Despite these expectations, proof of principle studies have yet to emerge. Therefore, to determine the utility of *in vitro* models for predicting *in vivo* responses, a comparison of TCDD-mediated hepatic gene expression responses obtained in mice and rats should be made to those obtained in murine Hepa1c1c7

and H4IIE rat hepatoma cell lines. Subsequently, these responses can be compared to those obtained in the human HepG2 hepatoma cell line. These cross-model (*in vitro* to *in vivo*) and cross-species (mouse, rat, human) comparisons will help to assess the ability of in *vitro* toxicogenomic approaches to contribute to risk assessment practices.

Estrogen-like gene expression responses to TCDD

Studies completed to date have shown that TCDD induces an estrogen-like gene expression profile in the murine uterus, an outcome which is dependent on the availability of the ER. However, these studies have not investigated the involvement of the AhR in mediating this response which should be examined *in vivo* through the use of AhR knock-out mice. It is expected that the uterine responses will depend entirely on the presence of the AhR as shown recently for hepatic gene expression responses to TCDD [405]. Subsequently, chromatin immuno-precipitation assays should be performed to examine the presence of both the AhR and ER at the promoter regions of these genes.

Studies in this lab have also completed time course experiments with Sprague Dawley rats treated with TCDD and uterine samples from these animals should be examined for the ability of TCDD to elicit an estrogen-like gene expression profile. Subsequently, these responses can be compared to that seen in the mouse to assess the species conservation of this response. Similar cross-species comparative studies have been completed for the uterine response to EE in mice and rats which revealed an overwhelming similarity in gene expression responses. The results from these comparisons were taken into consideration when choosing transcripts for QRTPCR analysis between murine uterine responses to EE and TCDD such that an initial inspection of the rat could focus on the induction of Armet, Asns,

Atf4, Nme1, Pcna and Slc25a5. Results from these studies will indicate the conserved nature of this response which will provide further evidence for the estrogenic activity of TCDD.

Inhibitory effects of TCDD on EE-mediated gene expression responses

The preceding data have identified a small subset of EE-responsive genes which are inhibited upon cotreatment with TCDD. The majority of estrogen regulated genes are unaffected by co-treatment suggesting that down regulation of ER or EE levels are unlikely to be responsible for mediating this effect. However, in order to more definitively dismiss the involvement of these previously proposed mechanisms, uterine ER levels and serum EE levels should be monitored. ER antibodies are readily available and an EE ELISA kit has been identified which will facilitate these analyses.

The uterine response to EE involves a complex interaction between multiple cells and tissue types including the myometrial, stromal and epithelial compartments. However, global profiling of transcript levels with cDNA microarrays does not allow for discrimination between responses which may be specific to a given compartment. Therefore, an approach that allows for detection in a defined tissue location will enable a more informed assignment of the functional implications of the dysregulated responses. This could be accomplished using multiple techniques including laser capture microdisseciton, *in situ* hybridizations or immunohistochemsitry.

Although rodent models provide valuable data on the physiological implications of toxicant exposure, they are not amenable to high-throughput or mechanistic studies. Therefore, suitable rat or murine cell lines should be identified to facilitate a more detailed investigation to the observed *in vivo* gene expression responses. One potential cell line is the HC11 murine mammary epithelial cell line which expresses both ER and AhR [406]. The

characterization of ER/AhR cross-talk in this cell line will facilitate cross-species comparisons with human MCF-7 breast cancer cells which have been used extensively to characterize AhR/ER cross-talk. In addition, a number of human endometrial cancer cell lines are available which express both AhR and ER, have been characterized as estrogen and TCDD responsive and susceptible to inhibitory crosstalk[86]. These *in vitro* models will serve as valuable tools to test new and emerging hypothesis regarding ER and AhR signaling.

Previous data have identified iDREs in the promoters of the human genes CTSD, TFF1, FOS and HSP27. In human MCF-7 cells, TFF1 induction by estrogen involves an ERE and an AP-1 site and TCDD mediates its inhibitory effects through an iDRE which overlaps with the AP-1 site. In the present studies we have identified the TCDD mediated inhibition of EE-induced Tff1 in the murine uterus. Examination of the mouse promoter region identified ERE and AP-1 sites which may be responsible for mediating transcript induction in response to EE as seen with the human promoter. However, unlike the human promoter, an iDRE did not overlap with the AP1 site, although putative iDREs were identified further upstream at -1920 and -2637 relative to the TSS. Future research should be directed at cloning this promoter to determine the estrogen dependent elements as well as those responsible for meditating the inhibitory response to TCDD. Similar approaches should also be applied to the remaining subset of TCDD inhibited EE-mediated gene expression responses. The identification of the functional response elements in the promoter regions of these genes should be initiated using bioinformatics approaches combined with cross-species gene expression data when available. Subsequently promoter occupancy by the ER or AhR at the identified elements can be examined both *in vitro* and *in vivo* using chromatin immunoprecipitation assays. These *in vivo* experiments will more definitively

characterize the physiological relevance of the gene expression crosstalk between ER and AhR which can then be further explored for its therapeutic potential.

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