Increases in levels of epoxyeicosatrienoic and dihydroxyeicosatrienoic acids (EETs and DHETs) in liver and heart in vivo by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and in hepatic EET:DHET ratios by cotreatment with TCDD and the soluble epoxide hydrolase inhibitor AUDA* 

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Running Title: CYP1A-mediated arachidonic acid metabolite detection in vivo

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Number of text pages: 28

Number of tables: 2

Number of figures: 3 (plus 2 Supplemental Figures)

Number of references: 48

Number of words in the Abstract: 248

Number of words in the Introduction: 542

Number of words in the Discussion: 1186

List of non-standard abbreviations:

AHR, aryl hydrocarbon receptor; AUDA,12-(3-adamantan-1-yl-ureido)-dodecanoic acid; CYP, cytochrome P450; EETs, eicosatrienoic acids; DHETs, dihydroxyeicosatrienoic acids; HETEs, monohydroxyeicosatetraenoic acids; MRM, multiple reaction monitoring; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.
Abstract

The environmental toxin and carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) binds and activates the transcription factor aryl hydrocarbon receptor (AHR) inducing CYP1 family cytochrome P450 enzymes. CYP1A2 and its avian ortholog CYP1A5 are highly active arachidonic acid epoxygenases. Epoxygenases metabolize arachidonic acid to four regioisomeric epoxycisatrienoic acids (EETs) and selected monohydroxyeicosatetraenoic acids (HETEs). EETs can be further metabolized by epoxide hydrolases to dihydroxyeicosatrienoic acids (DHETs). As P450-arachidonic acid metabolites affect vasoregulation, responses to ischemia, inflammation and metabolic disorders, identification of their production in vivo is needed to understand their contribution to biologic effects of TCDD and other AHR activators.

Here we report use of an acetonitrile-based extraction procedure which markedly increased the yield of arachidonic acid products by lipidomic analysis over a standard solid-phase extraction protocol. We show that TCDD increased all four EETs (5,6-, 8,9-, 11,12-, and 14,15-), their corresponding DHETs, and 18- and 20-HETE in liver in vivo and of 5,6-EET, the four DHETs and 18-HETE in heart, in a chick embryo model. As the chick embryo heart lacks arachidonic acid metabolizing activity, the latter findings suggest that arachidonic acid metabolites may travel from their site of production to a distal organ, i.e. heart. To determine if the TCDD-arachidonic acid metabolite profile could be altered pharmacologically, chick embryos were treated with TCDD and the soluble epoxide hydrolase inhibitor, AUDA (12-(3-adamantan-1-ylureido)-dodecanoic acid). Cotreatment with AUDA increased hepatic EET to DHET ratios, indicating that the in vivo profile of P450-arachidonic acid metabolites can be modified for potential therapeutic intervention.
Introduction

In addition to metabolizing xenobiotics, cytochrome P450 enzymes (CYPs) can metabolize endogenous substrates including arachidonic acid (Rifkind, 2006). CYPs exhibiting arachidonic acid epoxygenase activity include 1A2 (avian 1A5) (Gilday et al., 1996; Rifkind, 2006), 1B1 (Choudhary et al., 2004), 2J2 (Wu et al., 1996), 2B (avian 2H) (Nakai et al., 1992), 2C8, 2C9 (Daikh et al., 1994; Rifkind et al., 1995; Rifkind, 2006) and CYP2C19 (Bylund et al., 1998). Some of these epoxygenases can be transcriptionally induced. For example, CYP1A arachidonic acid epoxygenases are increased by aryl hydrocarbon receptor (AHR) activators, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene, β-naphthoflavone and tryptophan photoproducts. CYP2B (avian 2H) arachidonic acid epoxygenases are induced by phenobarbital, an activator of different nuclear receptors i.e. constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) (Nakai et al., 1992; Gilday et al., 1996; Gannon et al., 2000; Willson and Kliewer, 2002; Diani-Moore et al., 2006a; Rifkind, 2006). Imidazole based drugs (i.e. vorozole, omeprazole, benzylimidazole) increase arachidonic acid metabolism by mixed induction of CYP1 and CYP2 family P450s (Diani-Moore et al., 2006b). Significantly, the different CYP epoxygenases all produce the same arachidonic acid products: four regioisomeric epoxides, 5,6-; 8,9-; 11,12- and 14,15-epoxyeicosatetraenoic acid, and terminal hydroxyeicosatetraenoic acids (HETEs), i.e. 16-19 HETE (Capdevila et al., 1981), although the relative amounts of the products can vary for different CYPs.

Transcriptional induction of CYP1A arachidonic acid epoxygenases by AHR activation occurs in livers of birds and fish as well as mammals (Nakai et al., 1992; Rifkind et al., 1995; Schlezinger et al., 1998; Gannon et al., 2000; Diani-Moore et al., 2006a; Bui et al., 2012) albeit with species differences in the relative production of different P450 arachidonic acid metabolites.
(Capdevila et al., 1990; Lee and Riddick, 2012). \textit{In vivo} accumulation of arachidonic acid metabolites after TCDD treatment has been reported in liver of the marine fish (\textit{Stenotomus chrysops}) and of mice (Schlezinger et al., 1998; Bui et al., 2012), supporting a biologic role for P450 generated arachidonic acid metabolites.

In these studies, we have used the chick embryo near hatching, a well established model for studying TCDD toxicity and P450 mediated arachidonic acid epoxygenation (Rifkind et al., 1990), to ask whether we could improve detection of arachidonic acid epoxygenase products increased \textit{in vivo} in liver by TCDD treatment using a new extraction method. Further, as EETs and DHETs can affect cardiac function, and TCDD exerts cardiac toxicity in both the chick embryo and mammalian species (Canga et al., 1988; Canga et al., 1993; Walker and Catron, 2000), we asked whether TCDD treatment can alter the content of P450 arachidonic acid products in heart notwithstanding the lack of cardiac TCDD-induced arachidonic acid epoxygenases (Gannon et al., 2000). We also tested the hypothesis that the \textit{in vivo} profile of P450 mediated arachidonic acid products after exposure to TCDD could be modified pharmacologically.

We demonstrate here, using an improved acetonitrile-based procedure for extracting P450-arachidonic acid metabolites, that TCDD treatment increases EETs and DHETs in liver and increases 5,6-EET and DHETs in heart. We also report that treatment of chick embryos with the epoxide hydrolase inhibitor AUDA (12-(3-adamantan-1-yl-ureido)-dodecanoic acid) (Morisseau et al., 2002) alters \textit{in vivo} hepatic P450-dependent arachidonic acid metabolites, indicating that the TCDD induced arachidonic acid metabolite profile \textit{in vivo} can be modulated for potential therapeutic control.
Material and Methods

*Treatments:* TCDD (1 nmol per egg) or equal amounts of the solvent dioxane (Kanetoshi et al., 1992) were injected in ovo in 14-day old chick embryo into the fluids surrounding the embryo. Treatments were for 24 hours unless specified. For the experiments involving the soluble epoxide hydrolase inhibitor, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA, Cayman Chemical Company, Ann Arbor MI), AUDA was dissolved in DMSO (20 mg/ml) and 100 μl (2mg AUDA) were injected into the egg together with TCDD at 1 nmol per egg for 6 hours.

**Solid-phase extraction of arachidonic acid metabolites:** We initially examined a solid-phase method to extract arachidonic acid metabolites (Powell, 1982) from liver homogenates. Liver samples were homogenized in KPO₄ buffer (1:1; w/v). Ethanol (175 μL) and acetic acid (25 μl) were added to 1 ml samples of homogenates, which were then vortexed and centrifuged at 1,500 rpm for 3 min. Supernatants containing arachidonic acid metabolites were loaded on disposable extraction columns (Bakerbond spe™ Octadecyl (C₁₈), JT Baker, Phillipsburg, NJ) previously washed with 5 ml of ethanol followed by 15 ml of dH₂O. After sample loading, columns were washed 4 times with 5 ml dH₂O. Arachidonic acid metabolites were eluted with 5 ml of ethyl acetate containing 0.005% butylated hydroxytoluene (BHT). The organic (upper) phases of the eluates containing arachidonic acid metabolites, were transferred into new glass tubes. One ml of the ethyl-acetate/BHT mixture was added to the aqueous phase, passed through the column and the upper organic phase was combined with the organic phase for the first eluate. This procedure was repeated to enhance metabolite recovery. The combined organic phases were dried under N₂ gas and the pellets were stored at -80°C. The pellets were resuspended in 25 μl of 35% acetonitrile/0.1% acetic acid/65% dH₂O and used for LC-MS/MS analysis.
Acetonitrile extraction of arachidonic acid metabolites: Livers and hearts from 17-day old chick embryos were homogenized in acetonitrile (1:2; w/v) and sonicated (5 sec x 4 times). Four hundred μl of the liver homogenates or the total volumes of the heart homogenates were transferred to glass tubes. 11,12 EETd_{11} (10 ng) and 11,12 DHETd_{11} (1 ng) were added as internal standards to each tube. After adding two ml of acetonitrile, the homogenates were incubated overnight at -80°C. Then samples were sonicated (5 sec x 4 times), vortexed for 5 sec, transferred to fresh tubes and centrifuged for 10 min at 2,000 rpm. The supernatants were transferred to glass conical tubes and dried under N₂ gas. Dried pellets were resuspended in 50 μl of methanol, transferred to 1.5 ml Eppendorf tubes, and centrifuged for 5 min to remove particulates. A 5 μl aliquot of each sample was used for LC-MS/MS analysis.

LC-MS/MS analysis: LC-MS/MS analysis was performed using an Agilent 6460 Triple Quad LC/MS system (Agilent Technologies, Palo Alto, CA USA), containing an Agilent 1290 Infinity HPLC for compound separation and a JetStream ESI source for ion generation. Analysis was performed in the multiple reaction monitoring (MRM) mode. Compounds were separated on an Agilent SB-Aq C18 2.0 ×150 mm, 1.8 μm column at a flow rate of 400 μL/min. Mobile phase A was water with 0.1% formic acid, and mobile phase B, acetonitrile with 0.1% formic acid. The LC elution gradient was: 0–2 min, 30% B; 2–12 min to 65% B; 12–12.5 min, to 95% B; 12.5–14.5 min, 95% B; 14.5–15 min, to 30% B; 15–20 min, 30% B. Heated N₂ gas (12 L min⁻¹ at 400°C) was used as the sheath gas to facilitate evaporation of solvent from the ionization chamber. MRM analysis was performed in the negative ion mode. In the initial phase of these studies, compounds of interest were monitored using two different mass transitions and the one with the optimal specificity and signal was chosen. The selected parameters for MRM transitions from parent to product ions for the analysis of metabolites in the biological samples.
(Supplemental Figure 1) were as follows: From m/z 337 to m/z 207 (14,15-DHET), to 167 (11,12-DHET), to 127 (8,9-DHET), and to 145 (5,6-DHET); from m/z 319 to m/z 245 (20-HETE), to 261 (18-HETE), to 219 (14,15-EET), to 167 (11,12-EET), to 69 (8,9-EET), and to 191 (5,6-EET); from m/z 348 to m/z 167 (11,12-DHETd11); from m/z 330 to m/z 167 (11,12-EETd11). The MRM data were processed using Agilent Mass Hunter Quantitative Analysis Software. Fifteen point standard curves containing the following standards (Cayman Chemical Company, Ann Arbor, Michigan) were used for quantitation: (±)5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid; (±)8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid; (±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid; (±)14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid; (±)5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid; (±)8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid; (±)11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid; (±)14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid; (±)18-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid, 20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid. Concentrations of the standards ranged from 0.3 ng/ml to 5000 ng/ml for EETs and 0.12 ng/ml to 2000 ng/ml for DHETs and HETEs. Successive dilutions (1:2) were made in methanol. All samples and dilutions of the standards were spiked with deuterated standards: (±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic-16,16,17,17,18,18,19,19,20,20,20 acid (11,12-EETd11, 200 ng/ml) and (±)11,12-dihydroxy-5Z,11Z,14Z-eicosatrienoic-16,16,17,17,18,18,19,19,20,20,20-d11 acid (11,12-DHETd11, 20 ng/ml). Metabolite concentrations were determined by reference to the standards. Signal to noise (S/N) ratios ≥ 3 were required for a peak to be considered identifiable and ≥ 10 to be quantifiable.

**cDNA preparation and quantitative-polymerase chain reaction (RT-qPCR):** Total RNA was extracted from samples of chick embryo liver or heart using RNA STAT-60 (Tel-Test “B,” Friendswood, TX) following the manufacturer’s directions. cDNAs were prepared as follows:
1 μg of total RNA, 4 μl of qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and water (final volume of 20 μl), were mixed and incubated for 5 min at 25°C, 40 min at 42°C, 5 min at 85°C. The cDNA was diluted 1:5 with water before using it for RT-PCR. For PCR, 2 μl of cDNA, 10 μl of PerfeCTa SYBR Green FastMix (Quanta Biosciences), 1 μl of each primer (10 μM) and 6 μl of nuclease free water. Primers and annealing temperatures (in parenthesis) were as follows: for CYP1A4, 5′-tgctgcactgaaggatca-3′ and 5′-ctgggcctttattgttgc-3′ (56°C); for CYP1A5, 5′-ctctgcccttcaccatc-3′ and 5′-caggccaaagttcatcac-3′ (55°C); for CYP1B1, 5′-tggttttcctgtgaatta-3′ and 5′-tctggggtttgaccattg-3′ (55°C); for EPHX1, 5′-gatgacagcaagaaatgc-3′ and 5′-gaagtctttgagaggagaa-3′ (53°C); for EPHX2, 5′-gatgctgccttcgttttat-3′ and 5′-tcaggaaatggtagtcagtc-3′ (55°C); for CYP4V, 5′-tggttgtgcaacacccat-3′ and 5′-aactgcaatgggaaga-3′ (53°C); for CYP4B, 5′-acccacagtgaacctgg-3′ and 5′-tggaggagaaaggtcaaa-3′ (56°C); for CYP2C45, 5′-gattgaccgggtaggac-3′ and 5′-tgaagtgggtttttgtctcttc-3′ (53°C); for CYP2J2, 5′-acatcatgtgtctgcacc-3′ and 5′-tctttctgcacccaaacc-3′ (53°C); for CYP2H1, 5′-ccagtgggtatttttgttattg-3′ and 5′-gctgtgcctttggtgc-3′ (53°C); for 18s ribosomal RNA, 5′-gaccataaagatgcggct-3′ and 5′-agacagatcgtcaccacca-3′ (55°C). Denaturation and elongation temperatures were 95°C and 60°C, respectively. An Eppendorf Mastercycler epgradient machine was used for 40 cycles of amplification. The fold-changes in mRNA were calculated using the standard 2−ΔΔCt method (Livak and Schmittgen, 2001) with 18S mRNA serving as a control for normalization.

Arachidonic acid metabolism assay in vitro: Livers and hearts from chick embryos treated for 24 hour with TCDD or solvent (controls) were homogenized in three volumes of 0.1 M KPO4, pH 7.4 and stored at 80°C. Arachidonic acid metabolism was assayed as previously
described (Capdevilla et al., 1990; Rifkind et al., 1994; Gannon et al., 2000). Reaction mixtures (total volume 0.25 ml) containing 1 or 3 mg wet weight of liver or heart homogenate, 30 μM [1-14C] arachidonic acid (48 mCi/mmol) (Perkin Elmer, Torrance, CA) and 10mM MgCl2. After preincubation for 2 min at 37°C, reactions were started with a mixture of 1 mM NADPH, 10 mM isocitric acid, 0.2 U of isocitric dehydrogenase/ml, and incubated for 10 min at 37°C. Addition of 0.1 ml of acetic acid was followed by two extractions, each with 3 ml of ethyl acetate containing 0.005% butylated hydroxytoluene (BHT). The organic phases were combined, dried under N2 and resuspended in 0.11 ml of 50% acetonitrile in water containing 0.1% acetic acid. Products in 0.05 ml were resolved by reverse phase HPLC using a Vydac C18 column (Vydac, Hesperia, CA) (90 Å, 5 μm particle size, 4.6 x 250 mm), on a linear gradient from 50 to 100% acetonitrile in water containing 0.1% acetic acid, at 1 ml per min for 40 min. Radioactivity was measured using a Flo-One Beta Model S radioactivity flow monitor (Packard Instrument Company, Downers Grove, IL). Products were identified by reference to HPLC retention times of pure standards (Rifkind et al., 1994).

**Statistics:** P values for differences between group means were obtained using unpaired t tests (GraphPad Prism 5, San Diego, CA). P values <0.05 were accepted as statistically significant.
Results

Arachidonic acid metabolite production in vitro. The HPLC chromatograms in Fig. 1 demonstrate as previously reported (Gannon et al., 2000) that TCDD treatment of chick embryos in ovo enhances arachidonic acid metabolite production by homogenates of liver (Fig. 1A) but not by homogenates from heart (Fig. 1B) even at three times the protein concentration used for the liver assay. 20-HETE was the only arachidonic acid metabolite detected for liver homogenates from chick embryos treated with solvent alone (controls) while the homogenates from livers of TCDD treated chick embryos exhibited increased formation of EETs and their epoxide hydrolase products, DHETs, as well as several hydroxyeicosatetraenoic acids, HETEs. TCDD decreased 20-HETE formation. In contrast, heart homogenates from the controls or from TCDD treated chick embryo (Fig. 1B) did not generate any arachidonic acid metabolites.

Extraction of EETs, DHETs and HETEs from liver and heart; comparison of acetonitrile and solid-phase extraction. We first conducted preliminary experiments on liver samples from TCDD-treated chick embryos, using a standard solid-phase extraction protocol with C18 columns (see Materials and Methods) (Powell, 1982; Nithipatikom et al., 2003). Recoveries of most eicosanoid metabolites of interest were low or undetectable (data not shown). In order to enhance the recovery of P450 mediated arachidonic acid products, we developed an acetonitrile-based extraction procedure and compared the results with those obtained with C18 extraction. P450 arachidonic acid products were measured by LC-MS/MS. Table 1 shows that use of the solid-phase extraction method permitted measurement of 8,9- and 11,12-EETs and 8,9-, 11,12- and 14,15-DHETs but not of 5,6- or 14,15-EET or 5,6-DHET in liver after TCDD-treatment. The acetonitrile extraction procedure, in contrast, permitted measurement of all four EET regioisomers and their DHET products. Moreover, product yields were substantially
greater for acetonitrile extraction than for solid phase extraction. Mean levels of 11,12-EET<sub>d11</sub> in samples spiked with 1 ng of 11,12-EET<sub>d11</sub> were 4-fold higher for samples extracted in acetonitrile compared to C18 extraction, in agreement with recoveries shown for detectable EETs in Table 1.

To determine the extent to which EETs were recovered from liver samples after extraction in acetonitrile, we added 10 ng of 11,12-EET<sub>d11</sub> to liver samples followed by acetonitrile extraction and compared the values for peak areas for the extracted 11,12-EET<sub>d11</sub> to those for replicates of the same amount of 11,12-EET<sub>d11</sub> not added to liver samples or extracted (taken as the 100% value). The results (Supplemental Figure 2) show that acetonitrile extraction of liver samples permitted 100% recovery of the deuterated standard.

Further, to be sure that the DHETs measured in tissues had been produced in vivo and not during sample manipulation we added deuterated 11,12-EET (11,12-EET<sub>d11</sub>) to samples and measured its conversion to 11,12-DHET<sub>d11</sub>. When livers were homogenized in PBS followed by acetonitrile extraction of arachidonic acid products, high levels of 11,12-DHET<sub>d11</sub> were produced (data not shown), but 11,12-DHET<sub>d11</sub> formation was barely detectable when tissues were both homogenized and extracted in acetonitrile (e.g. mean peak areas for 11,12-DHET<sub>d11</sub> were 3200 vs. 32, respectively) indicating that homogenization in acetonitrile prevented EET breakdown during sample preparation. In addition we examined the effects of various compounds that are commonly added to tissues before homogenization for their potential to prevent alteration of biologic products during sample preparation. Neither AUDA, an inhibitor of soluble epoxide hydrolase (Morisseau et al., 2002), miconazole, an inhibitor of cytochrome P450 (Cunningham et al., 2007), nor the hydrogen peroxide scavenger, catalase, altered the results when added to acetonitrile before tissue homogenization (data not shown), providing assurance that the metabolites extracted and measured were formed in the physiological environment and not
during sample processing. Accordingly, for all subsequent studies, acetonitrile was used for tissue homogenization and arachidonic acid metabolite extraction, without other additions.

**TCDD increases arachidonic acid metabolites in liver and heart.** Using the acetonitrile method we then sought to examine the effect of TCDD on P450 mediated arachidonic acid metabolites *in vivo*, focusing on liver and heart, both of which are targets of TCDD toxicity. LC/MS results for arachidonic acid metabolite profiles of livers and hearts from 14-day old chick embryos treated for 24 hours with TCDD (1nmol/egg) or dioxane (solvent controls) are shown in Fig. 2. Low levels of 5,6-EET and 8,9-, 11,12-, and 14,15-DHETs were detected in the control livers. TCDD treatment increased the liver content of all four EETs and DHETs (Fig. 2A). TCDD produced the greatest levels for 5,6-EET and 14,15-DHET (increased from 23 ± 6 in controls to 399 ± 22 and from 31 ± 8 to 292 ± 22 ng/g liver, respectively) followed by the 11,12-DHET (from 3 ± 1 to 95 ± 9 ng/g liver). TCDD also increased *in vivo* levels of hepatic 18- and 20-HETE. Arachidonic acid metabolites were also detected in the heart although at much lower levels than in liver. 8,9-, 11,12-, 14,15- DHET and 18- and 20-HETE were detected in the control hearts (Fig. 2B), but EETs were not detected. After TCDD treatment 5,6-EET became detectable (21 ± 4 ng/g heart). A peak for 11,12-EET could be detected but not quantified (S/N <10). Levels of 8,9-, 11,12-, 14,15- DHETs and 18-HETE were also increased, particularly the 14,15-DHET (from 3.7 ± 0.8 to 42 ± 6 ng/g heart).

**Effects of TCDD on mRNA levels of enzymes that catalyze P450 dependent arachidonic acid metabolism.** We had previously shown that TCDD increased CYP1A5 in liver but not in heart (Gannon et al., 2000), and that CYP1A5 and 2H were the major arachidonic acid epoxygenases in chick embryo liver microsomes after TCDD or phenobarbital treatment respectively (Kanetoshi et al., 1992; Nakai et al., 1992). To determine whether TCDD affected
mRNA levels of P450 epoxygenases other than CYP1A5 we examined effects of TCDD on mRNA levels of chick orthologs of known mammalian P450 epoxygenases in liver and heart (Fig. 3).

mRNAs for all of the P450s tested were measurable in control liver extracts. TCDD increased hepatic mRNA levels for CYP1A4 (a positive control for TCDD activation of the AHR) and CYP1A5, but not for other CYPs with recognized arachidonic acid epoxygenase activity. Rather, TCDD diminished mRNA levels for CYP1B1 and CYP2 related epoxygenases (CYP2J2 and CYP2H1). TCDD did not affect mRNA for CYP2C45, the chicken ortholog of mammalian CYP2C19. TCDD suppressed mRNAs for CYP4V and CYP4B, avian orthologs for CYP4 enzymes, generators of 20-HETE (Kroetz and Xu, 2005; Rifkind, 2006). Both soluble and microsomal epoxide hydrolases were expressed in liver and heart. mRNA for the microsomal epoxide hydrolase (EPHX1) was not significantly affected by TCDD, while TCDD suppressed the mRNA for the soluble epoxide hydrolase (EPHX2). In heart, TCDD treatment increased only CYP1A4 but not 1A5 mRNAs and did not affect mRNAs for CYPs 2J2 or 2C45 or the epoxide hydrolases. mRNA for CYP1B1 was increased 2-fold by TCDD and was also the only transcript whose level was differently affected by TCDD in liver vs heart. mRNAs for CYP2H1, 4B or 4V were undetected in both control and TCDD treated heart extracts.

Cotreatment with TCDD and the soluble epoxide hydrolase inhibitor AUDA alters the hepatic arachidonic acid metabolite profile. In order to learn whether the P450-arachidonic acid profile elicited by TCDD could be pharmacologically modified, we examined the effect of cotreatment of chick embryos with TCDD and the epoxide hydrolase inhibitor, AUDA. A short treatment time of 6 hours was used because AUDA is rapidly metabolized (Hwang et al., 2007). Table 2 shows that cotreatment of chick embryo with TCDD and AUDA
increased levels of all four EET regioisomers in liver compared to TCDD alone (by 1.9, 7.2, 10.5 and 2.3 fold for 5,6-, 8,9-, 11,12- and 14,15-EET, respectively) and increased ratios of EETs to DHETs. These findings are consistent with reports that AUDA inhibits DHET formation (Schmelzer et al., 2005). EET levels in the heart were below the limit of detection, precluding assessment of AUDA on cardiac EET:DHET ratios.

**Discussion**

Using an acetonitrile extraction procedure for P450 dependent arachidonic acid products we report here that: (1) TCDD treatment *in ovo* increases the liver content *in vivo* of P450 epoxygenase products including all four EET regioisomers and their DHET metabolites and of 18-HETE, and in the heart, although to a lesser extent, of 5,6-EET, and 8,9- 11,12- and 14,15-DHET; (2) the soluble epoxide hydrolase inhibitor AUDA modified the arachidonic acid profile produced *in vivo* in liver by TCDD treatment, increasing EET levels and EET to DHET ratios as compared to TCDD alone; and (3) TCDD increased levels of 20-HETE, a CYP4 product, in liver and heart.

The first finding answers affirmatively a recurring question in pharmacology: Is the ability of an agent to enhance microsomal enzyme activity as assayed *in vitro* reflected in a “real world” increase in product formation *in vivo*? We show here that enhanced microsomal enzyme production of P450 arachidonic acid products by AHR activation (i.e. by TCDD) is reflected in increased levels of arachidonic acid products in liver *in vivo*, consistent with previous findings (Schlezinger et al., 1998; Bui et al., 2012) and that this occurs widely among species, including avians as well as mammals.
We expect that our increased detection of EETs and DHETs in chick embryo liver and heart compared to prior reports (Schlezinger et al., 1998; Bui et al., 2012) primarily reflects the use of acetonitrile based extraction which greatly enhanced metabolite recovery, although species differences may also be a contributing factor. Thus TCDD increased EET production less in mouse than chick embryo liver microsomes (Lee et al., 1998), and along with other AHR agonists, failed to increase arachidonic acid epoxygenation in rat liver (Lee et al., 1998; Aboutabl et al., 2009). In that context, it may be of interest to note that human CYP1A2, like avian CYP1A5, is a highly active arachidonic acid epoxygenase (Rifkind et al., 1995), suggesting that the chick embryo may be more representative than the mouse of human TCDD-induced arachidonic acid epoxygenase activity.

The small amount of EETs and DHETs detected in control liver could reflect the low levels of expression of constitutive P450 epoxygenases (Fig. 3). The evidence that among the EETs, TCDD increased the 5,6 EET most, and among the DHETs, the 5,6 DHET least and the 14,15-DHET most, is consistent with reports that soluble epoxide hydrolase has greatest substrate preference for the 14,15- EET and least for the 5,6-EET (Chacos et al., 1983; Yu et al., 2000).

Our finding that P450 arachidonic acid products also accumulate in the heart after TCDD treatment is especially noteworthy because heart homogenates from TCDD treated chick embryos lacked the ability to generate arachidonic acid epoxides, as assayed in vitro (see Fig.1). Avian TCDD-induced CYP1A5 can entirely account for increased hepatic P450 epoxygenase activity by TCDD treatment, as immunoinhibition of CYP1A5 completely abolished TCDD induced hepatic microsomal P450 arachidonic acid metabolizing activity (Kanetoshi et al., 1992), but CYP1A5 cannot account for the increase in arachidonic acid metabolites in heart as
TCDD does not induce CYP1A5 in chick embryo heart (Gannon et al., 2000) (see Fig 3). It seems unlikely that the small increase in heart of mRNA for CYP1B1 (Fig. 3), a P450 reported to have some arachidonic acid epoxygenase activity (Choudhary et al., 2004), could explain the observed increase by TCDD in cardiac arachidonic acid products in vivo (Fig. 2) since the amount of CYP1B1 in heart homogenates was insufficient to generate any P450 arachidonic acid products (as shown in Fig. 1). It seems plausible, therefore, that the P450 arachidonic acid products detected in vivo in heart after TCDD treatment reflect metabolites circulating to heart from distal sites of their production, i.e. liver or kidney, organs in which TCDD increases both CYP1A5 and arachidonic acid epoxygenase activity (Gannon et al., 2000). That suggestion is consistent with reports that arachidonic acid epoxygenase products are found in plasma (Karara et al., 1992; Jiang et al., 2013) and can be increased after TCDD treatment (Bui et al., 2012). Circulation of P450 arachidonic acid products from one organ to another would imply further that EETs have endocrine as well as previously recognized autocrine and paracrine actions (Spector, 2009; Jiang et al., 2013). DHETs in heart could reflect circulation from a different organ, or conversion of EETs in situ in the heart, by resident cardiac epoxide hydrolases (Fig. 3B).

The second major finding, the ability of cotreatment with TCDD and the well established soluble epoxide hydrolase inhibitor AUDA to increase EET:DHET ratios in liver is of interest in several respects. First, although soluble epoxide hydrolase inhibitors have been reported to alter the arachidonic acid product composition in plasma (Revermann et al., 2010; Panigrahy et al., 2013) this is the first evidence of which we are aware, for soluble epoxide hydrolase inhibition altering the endogenous P450 arachidonic acid metabolite profile in vivo in liver. The evidence that the in vivo arachidonic acid metabolite profile is susceptible to pharmacologic alteration
implies that soluble epoxide hydrolase inhibition could be used for therapeutic modification of organ arachidonic acid composition \textit{in vivo}. Notably, EETs have been shown to be beneficial by enhancing vascular flow, ameliorating inflammatory responses, cardiac reperfusion injury and metabolic disorders, while DHETs have been associated with adverse effects (i.e. hypertension, inflammation) (Roman, 2002; Imig and Hammock, 2009; Morisseau and Hammock, 2013). The therapeutic use of soluble epoxide hydrolase inhibitors has attracted increasing attention not only for cardiovascular benefits (Morisseau and Hammock, 2013) but also for treatment of hepatic metabolic disturbances (Liu et al., 2012).

The third finding reported here, an increase by TCDD treatment in the hepatic content of 20-HETE, was unexpected because 20-HETE is a prototypic product of CYP4 enzymes (Kroetz and Xu, 2005; Rifkind, 2006) which are not increased by TCDD (see Fig. 3). Interestingly, Bui et al. (2012) also reported that TCDD increased 20-HETE \textit{in vivo} in mouse liver. The finding also seems to contradict the substantial evidence that AHR activation by diverse agents, including TCDD, β-naphthoflavone, planar PCBs and imidazole drugs, suppresses hepatic 20-HETE formation by microsomes in \textit{in vitro} experiments in many species, including chick embryo, mouse, scup, rats and guinea pigs (Huang and Gibson, 1991; Nakai et al., 1992; Lee et al., 1998; Schlezinger et al., 1998; Diani-Moore et al., 2006b) (also see Fig. 1). Moreover, as shown in Fig. 3, TCDD suppresses CYP4 mRNA levels. Some possible explanations for the increase in 20-HETE levels in the setting of suppressed CYP4 mRNA include TCDD enhancement of 20-HETE release from tissues and suppression of 20-HETE metabolism by β-oxidation. The latter would also be consistent with the energy suppressing effects of the TCDD wasting syndrome. 20-HETE can have adverse effects, for example abetting hypertension and arteriolar vasoconstriction (Roman et al., 2000; Kroetz and Xu, 2005; Hama-Tomioka et al.,
Our finding that 20-HETE is detected in control hearts, although not produced by heart homogenates (see Fig. 1) (consistent with the lack of CYP4B or CYP4V mRNA expression in heart, Fig. 3B), suggests that 20-HETE, like some other P450 arachidonic acid metabolites, may derive from another organ, possibly from blood vessels, major sites of 20-HETE production (Kroetz and Xu, 2005).
Acknowledgments

We thank Dr. Kasem Nithipatikom, Medical College of Wisconsin, for suggesting the use of acetonitrile to extract arachidonic acid metabolites and Dr. Ruba Saba Deeb, Weill Cornell Medical College, for assistance in the initial phases of this project. TCDD was provided by the National Cancer Institute Chemical Carcinogen Standards Repository operated under contract by Midwest Research Institute, KS City, MO, NO2-CB-666000.

Authorship Contributions

Participated in research design, Rifkind, Diani-Moore, and Ma.

Conducted experiments, Diani-Moore and Ma.

Contributed new reagents or analytical tools, Gross

Performed data analysis, Diani-Moore, Ma, and Rifkind.

Wrote and contributed to the writing of the manuscript, Rifkind, Diani-Moore, and Ma.
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*This work was supported by National Institutes of Health [Grant RO1 ES-03606] (A.B.R), [Grants R37 HL-087062, P20 HL-113444] (S.S.G.); and a gift from the Winston Foundation (A.B.R),
**Figure Legends**

**Fig. 1.** Arachidonic acid metabolite production *in vitro* by homogenates of chick embryo liver and heart. Representative reverse phase HPLC chromatograms showing arachidonic acid metabolite production *in vitro* by 1 mg of liver homogenate (A) and 3 mg of heart homogenate (B) per reaction mixture, after treatment of chick embryos *in ovo* with TCDD (1 nmol per egg for 24 hours), or for the same amounts of homogenate from solvent controls. Arachidonic acid metabolism was assayed as described in *Material and Methods*. Peaks between 0 and 10 min are non-P450 mediated polar products. AA, arachidonic acid. Retention times (min) are shown on the abscissas.

**Fig. 2.** Arachidonic acid metabolite production *in vivo* in chick embryo liver and heart. Arachidonic acid metabolites were extracted from livers and hearts of chick embryos treated for 24 hours with TCDD (1 nmol/egg) or solvent (controls) as described in *Material and Methods*. Means ± SE for arachidonic acid metabolite levels from liver (A) and from heart (B) are plotted. n=5 individual livers or hearts for each treatment group. **, P ≤ 0.01; ***, P ≤ 0.001 and ****, P ≤ 0.0001; n.s., not significant.

**Fig. 3.** Effects of TCDD on mRNA levels of enzymes known to affect EETs, DHETs or HETEs. Mean qRT-PCR results (± SE) for total RNA extracted from livers (A) and hearts (B) from chick embryos treated for 24 hours with TCDD or solvent (controls). PCR conditions and specific primers for *CYP1A4, CYP1A5, CYP1B1, CYP2C45, CYP2J2, CYP2H1, CYP4B, CYP4V, EPHX1, EPHX2* are described in *Material and Methods*. Fold changes in mRNAs for genes in livers and hearts of TCDD treated chick embryos and controls (solvent treated) are calculated.
and compared to the values for each gene in control liver for which each mRNA is set at “1”
(n=5 individual organs per treatment group, with three replicates for each organ).
Table 1. Comparison between solid-phase (C18 column) and acetonitrile extraction for LC-MS/MS detection of AA metabolites from liver of a CE treated for 24 hours with TCDD as described in Material and Methods. Means ± SE (n=3 replicates) are shown in the table. n.d., not detected.

<table>
<thead>
<tr>
<th>Method</th>
<th>EETs</th>
<th></th>
<th>DHETS</th>
<th></th>
<th>20-HETE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5,6-</td>
<td>8,9-</td>
<td>11,12-</td>
<td>14,15-</td>
<td>5,6-</td>
</tr>
<tr>
<td>C18 column</td>
<td>n.d.</td>
<td>2 ± 0.8</td>
<td>2.4 ± 1.4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>195 ± 106</td>
<td>9 ± 3</td>
<td>10 ± 1.7</td>
<td>4.2 ± 0.5</td>
<td>5.6 ± 0.5</td>
</tr>
</tbody>
</table>

ng/g liver
Table 2. Effects of the soluble epoxide hydrolase inhibitor AUDA on EETs and DHETs in liver after treatment with TCDD or TCDD+AUDA for 6 hours. T, TCDD; n.d., not detected; n=3 individual livers for each treatment group. For EETs, p values (T+AUDA vs TCDD alone) are given in parentheses.*

<table>
<thead>
<tr>
<th>EETs (mean ng/g liver ± SE)</th>
<th>Ratio EET/DHET (mean ± SE)</th>
<th>Fold change in the ratio EET/DHET T+AUDA vs T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>T+AUDA</td>
</tr>
<tr>
<td>5,6-</td>
<td>76.6 ± 13.5</td>
<td>141.8 ± 32.2</td>
</tr>
<tr>
<td></td>
<td>(p=0.06)</td>
<td></td>
</tr>
<tr>
<td>8,9-</td>
<td>2.0 ± 0.1</td>
<td>14.4 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>(p=0.04)</td>
<td></td>
</tr>
<tr>
<td>11,12-</td>
<td>1.2 ± 0.8</td>
<td>12.6 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>(p=0.03)</td>
<td></td>
</tr>
<tr>
<td>14,15-</td>
<td>10.4 ± 0.2</td>
<td>24.1 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>(p=0.03)</td>
<td></td>
</tr>
</tbody>
</table>

* Control values for 5,6- and 14,15-EETs were 30.4±0.8 and 9.6±0.2, respectively, and for 8,9- and 11,12-EETs were not detectable.
Figure 1

A

Control - liver

TCDD - liver

B

Control - heart

TCDD - heart
Figure 2

A. Liver

- Control
- TCDD

EETs: 5,6-, 8,9-, 11,12-, 14,15-
DHETs: 5,6-, 8,9-, 11,12-, 14,15-
HETEs: 18-, 20-

B. Heart

- Control
- TCDD

EETs: 5,6-, 8,9-, 11,12-, 14,15-
DHETs: 5,6-, 8,9-, 11,12-, 14,15-
HETEs: 18-, 20-
Figure 3

A

Liver mRNAs (fold change)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>TCDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A4</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>CYP1A5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C45</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>CYP2J2</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>CYP2H1</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>CYP4B</td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>CYP4V</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>EPHX1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPHX2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Heart mRNAs (fold change)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>TCDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A4</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>CYP1A5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP1B1</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>CYP2C45</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>CYP2J2</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>CYP2H1</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>CYP4B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP4V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPHX1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPHX2</td>
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