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Flavin-containing monooxygenase-3: induction by 3-methylcholanthrene and complex regulation by xenobiotic chemicals in hepatoma cells and mouse liver¹

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Abstract

Flavin-containing monooxygenases often are thought not to be inducible but we recently demonstrated aryl hydrocarbon receptor (AHR)-dependent induction of FMO mRNAs in mouse liver by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Celius *et al.*, *Drug Metab Dispos* 36:2499, 2008). We now evaluated FMO induction by other AHR ligands and xenobiotic chemicals *in vivo* and in mouse Hepa1c1c7 hepatoma cells (Hepa-1). In mouse liver, 3-methylcholanthrene (3MC) induced FMO3 mRNA 8-fold. In Hepa-1 cells, 3MC and benzo[*a*]pyrene (BaP) induced FMO3 mRNA >30-fold. Induction by 3MC and BaP was AHR-dependent but, surprisingly, the potent AHR agonist, TCDD, did not induce FMO3 mRNA in Hepa-1 cells nor did chromatin immunoprecipitation assays detect recruitment of AHR or ARNT to *Fmo3* regulatory elements after exposure to 3MC in liver or in Hepa-1 cells. However, in Hepa-1, 3MC and BaP (but not TCDD) caused recruitment of p53 protein to a p53 response element in the 5'-flanking region of the *Fmo3* gene. We tested the possibility that FMO3 induction in Hepa-1 cells might be mediated by Nrf2/antioxidant response pathways but agents known to activate Nrf2 or to induce oxidative stress did not affect FMO3 mRNA levels. The protein synthesis inhibitor, cycloheximide (which causes “superinduction” of CYP1A1 mRNA in TCDD-treated cells) by itself caused dramatic upregulation (>300-fold) of FMO3 mRNA in Hepa-1 suggesting that cycloheximide prevents synthesis of a labile protein that suppresses FMO3 expression. Although FMO3 mRNA is highly induced by 3MC or TCDD in mouse liver and in Hepa-1 cells, FMO protein levels and FMO catalytic function showed only modest elevation.

¹**Abbreviations:** AHR, aryl hydrocarbon receptor; AHRE, AH response element; ARE, anti-oxidant response element; ARNT, aryl hydrocarbon receptor nuclear translocator; BaP, benzo[*a*]pyrene; BHA, butylated hydroxyanisole; BNF, β-naphthoflavone; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; DMSO, dimethylsulfoxide; ETA, ethionamide; FMO, flavin-containing monooxygenase; 3-MC, 3-methylcholanthrene; Nrf2, NF-E2-related factor 2; PCB126, 3,3',4,4',5-pentachlorobiphenyl; SS, sulindac sulfide; *t*-BHQ, *tert*-butylhydroquinone; SOX, sulindac sulfoxide; SUL, sulphoraphane; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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Potential conflicts of interest Allan Okey has received compensation as a member of the Dioxin Scientific Advisory Board of The Dow Chemical Company within the last three years. All other authors declare no potential conflicts of interest.

Keywords

flavin-containing monooxygenase; 3-methylcholanthrene; aryl hydrocarbon receptor; induction; hepatoma cell

Introduction

Flavin-containing monooxygenases (FMOs) metabolize a broad range of substrates that contain nitrogen or sulfur atoms, including therapeutic agents, environmental compounds and endogenous compounds derived from the diet such as trimethylamine (reviewed in: (Krueger and Williams, 2005; Cashman and Zhang, 2006)). For example, sequential FMO-dependent S-oxygenations of thiocarbamides/thioureas produce the mono-oxygenated sulfenic and di-oxygenated sulfinic acid metabolites. Both reactive metabolites have been isolated and confirmed for a number of thioureas in timecourse studies (e.g. (Poulsen, 1979; Francois *et al.*, 2009)). Sometimes, only one of the oxygenated metabolites has been observed, presumably due to differences in the reaction rates that either favor accumulation of the mono- or di-oxygenated metabolites, or absence of detection favored by further fast non-FMO dependent reactions. In addition, sulfenic acids are known to bind glutathione and initiate futile redox cycling in the cell producing oxidative stress and toxicity in liver and other target organs (Krieter *et al.*, 1984; Ruse and Waring, 1991; Smith and Crespi, 2002; Henderson *et al.*, 2008).

Regulation of FMO expression by endogenous factors, including hormones is complex (Klick and Hines, 2007; Hines *et al.*, 2008; Klick *et al.*, 2008). FMOs traditionally have been viewed as not being inducible by xenobiotic chemicals (Krueger and Williams, 2005; Cashman and Zhang, 2006; Hines, 2006). However, we recently found that the mRNAs for FMO1, FMO2 and FMO3 are highly induced in livers of male mice treated with the potent aryl hydrocarbon receptor (AHR) agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Tijet *et al.*, 2006; Celius *et al.*, 2008). Induction of FMO2 and FMO3 mRNAs by TCDD occurs in mice that have wildtype AHR but not in *Ahr*-null mice; this confirms that the AHR is essential for FMO upregulation by dioxin-like chemicals *in vivo* (Tijet *et al.*, 2006). Furthermore, we demonstrated recruitment of AHR and AHR nuclear translocator (ARNT) to regulatory regions in the mouse *Fmo3* gene indicating that induction by TCDD is an AHR-dependent process (Celius *et al.*, 2008). Two other AHR agonists also have been reported to induce FMO mRNAs in rodent livers *in vivo*: FMO3 in mouse liver by β -naphthoflavone (BNF) (Patel *et al.*, 2007) or benzo[*a*]pyrene (BaP) (Yauk *et al.*, 2010) and FMO1 in rat liver by 3-methylcholanthrene (3MC) (Chung *et al.*, 1997).

The Hepa-1 cell model has been exceptionally useful for defining the mechanism of cytochrome P4501A1 (CYP1A1) induction by AHR agonists (Okey *et al.*, 1980; Denison and Whitlock, 1995; Hankinson, 1995). Thus, in the current research, to facilitate mechanistic studies of FMO regulation, we employed the Hepa-1 cell model to investigate induction of FMOs by various chemical agents. By using Hepa-1 variant cell lines deficient in AHR or ARNT, our experiments indicate that the AHR and its dimerization partner, ARNT, are required for induction of FMO3 mRNA by 3MC and BaP in Hepa-1 cells but that additional mechanisms must also be involved in upregulation of FMO3 by these carcinogenic polycyclic aromatic hydrocarbons.

Materials and Methods

Chemicals and reagents

3MC, BaP, butylated hydroxyanisole (BHA), sulforaphane (SUL), menadione, *tert*-butylhydroquinone (*t*-BHQ), MDL28170, chloroquine, MG132, sulindac sulfide (SS) and sulindac sulfoxide (SOX) as well as antibodies to IgG used in the ChIP assay were obtained from Sigma-Aldrich (St. Louis, MO). Ethionamide sulfoxide (ETASO) was a kind gift from Dr. Paul R. Ortiz de Montellano (University of California, San Francisco, CA). Mouse anti-AHR antibody (SA-210) was from BIOMOL Research Laboratory (Plymouth Meeting, PA); human anti-p53 antibody (FL-393) and human anti-ARNT were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The Hepa-1c1c7 (Hepa-1) mouse hepatoma cell line, the AHR-deficient cell line c35 (B16GBi1c3), and the ARNT-deficient cell line c4 (B13NBii1) were obtained from the American Type Culture Collection (Manassas, VA). TCDD was from Wellington Laboratories (Guelph, ON, Canada); BNF and PCB126 from Accustandard (New Haven, CT); Power SYBR Green PCR Master Mix, TaqMan Universal PCR Master Mix, and 96-well real-time PCR plates were from Applied Biosystems (Foster City, CA). Reagents for cell culture were provided by Invitrogen (Carlsbad, CA). All other reagents were of the highest quality available from commercial sources.

In vivo experimental design

Adult (8-week old) male C57BL/6J mice from Charles River Laboratories (Montreal, QC, Canada) were housed under a 12-h light/12-h dark cycle and given food and water *ad libitum*. Mice were injected intraperitoneally with 3MC (80 mg/kg body weight) or corn oil vehicle. Livers were harvested 6 h after treatment and were snap-frozen in liquid nitrogen, then stored at -80°C for later use in chromatin immunoprecipitation (ChIP) assays, RNA isolation and real-time PCR analysis, proteomic analysis and measurements of catalytic activity.

Cell culture experimental design

All hepatoma cell lines were cultured in nucleoside-free α -minimal essential medium containing 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) and 1% penicillin/streptomycin (PEST). Cells were maintained at 37°C in a 5% CO_2 /air incubator with 90% humidity. Medium was changed every second day and cells were sub-cultivated once a week. For FMO induction experiments cells were seeded in 6-well plates (200,000 cells/well) and cultured for 24 h prior to treatment. Cells were exposed to the known AHR agonists: TCDD (10 nM), 3MC (1 μM), BaP (1 μM), BNF (1 μM), or PCB 126 (1 μM) for 6, 12, or 24 h. We also exposed cells to several compounds known to activate Nrf2 or oxidative stress pathways: BHA (50 μM), SUL (20 μM), menadione (10 μM), and *t*-BHQ (30 μM). Potential superinduction of FMO3 and CYP1A1 was studied by exposing Hepa-1 cells to 10 $\mu\text{g/ml}$ cycloheximide (CHX) for 1 h prior to exposure of the cells to 1 μM 3MC for 5 h or 23 h.

Real-time reverse transcription quantitative polymerase chain reaction (qPCR)

Probes and primers for mouse *Fmo1* (NM_010231), *Fmo2* (NM_018881), *Fmo3* (NM_008030), *Fmo4* (NM_) and *Fmo5* (NM_010232) were designed as reported in Celius et al., (Celius et al., 2008). Total RNA from Hepa-1 cells was isolated using RNeasy mini kits (Qiagen, Mississauga, ON) according to the manufacturer's instructions. Real-time RT-PCR was performed using 1 μg of total RNA and a random hexamer primer in a standard procedure. Two μl of RT-reaction was used for each real-time qPCR reaction. Each reaction (25 μl) contained optimized probe and primer concentrations as well as the TaqMan

universal mastermix. Expression levels of target mRNAs were normalized to the levels of 18S rRNA and analyzed using the comparative C_T ($\Delta\Delta C_T$) method.

Chromatin Immunoprecipitation (ChIP) assay

ChIP assays were performed according to Matthews et al. (Matthews *et al.*, 2005) except that 100–120 mg of snap-frozen liver was homogenized and cross-linked in 1% formaldehyde and the DNA was isolated using a PCR purification kit (BioBasic) and eluted in 50 μ l of elution buffer provided in the kit. When Hepa-1 cells were used for preparation of chromatin for ChIP assays, cells (1.8×10^6) were plated in 10-cm dishes and cultured for 24 h prior to exposure to 1 μ M 3MC or 1 μ M BaP for 1, 6, 15 or 24 h. Cell pellets were cross-linked in 1% formaldehyde and the *in vitro* ChIP assay followed the same protocol as for *in vivo* ChIP assays. ChIP DNA (1 μ l) was amplified by PCR with primers 5'-AAGCCAAGCCAGAAAATCAA3' and 5'-TCGAGGAACAGAGTGCAATG3' for the *Fmo3* AH response element (AHRE); 5'-GAGGATGGAGCAGGCTTACG-3' and 5'-GGGCTACAAAGGGTGATGCTT-3' for the mouse *Cyp1a1* AHRE; 5'-ATGGAAGGGTCTTGACACCA3' and 5'-CAAATTCTGCCCCATCTGTT3' for the mouse *Fmo3* p53RE; and 5'-CCCGAAACCCAGGATTTTAT-3' and 5'-GGTCTGTCCCTGACCAACT-3' for the p53 response element in the *Cdk1a* gene (also known as p21). For real-time PCR, Power SYBR green PCR master mix was used to amplify the DNA fragments.

Protease inhibitor exposure and cell pellet preparation

Hepa-1 cells were plated (1.8×10^6) in a 10-cm dish and pre-exposed to protease inhibitors (5 μ M MG132; 25 μ M MDL28170 or 50 μ M chloroquine) for 1 h followed by 23 h exposure to DMSO or 3MC. Cells were scraped and washed once with PBS and pellets were resuspended in buffer (10 mM phosphate buffer, pH 7.4, 20% glycerol, 1.0 mM EDTA, 0.2 mM PMSF) and frozen at -80°C . Cells were subsequently lysed after adding 1 μ l/ml CalBiochem protease inhibitor cocktail III, by alternately freezing the cell suspension in liquid nitrogen and thawing on ice, three times. The homogenate was further degraded by passage through a 28-gauge needle 5 to 10 times. Protein concentration was measured using Pierce's Coomassie Plus assay.

Assays for FMO catalytic activity

Mouse liver microsomes (100 μ g for ethionamide (ETA); 200 μ g for SS) were incubated for 15 min at 37°C with 200 μ M substrate and 1 mM NADPH in 0.1 M Tricine buffer at pH 8.5. Reactions were stopped by the addition of 75 μ l CH_3CN (ETA) or 2 ml ethyl acetate (SS) and chilling on ice. For ETA, samples were transferred to microcentrifuge tubes and centrifuged for 30 min at $15,000 \times g$ to remove protein and the supernatant analyzed by reverse phase HPLC (Henderson *et al.*, 2008) for presence of ETA S-oxide (ETASO). We assessed activity toward SS as described previously (Koukouritaki *et al.*, 2007): samples were vortexed for 1 min and the ethyl acetate layer removed to a new tube. The extraction was repeated and the samples taken to dryness under vacuum in a Speed Vac. After resuspension in 50:50 $\text{CH}_3\text{CN}:\text{H}_2\text{O}$, samples were analyzed by reverse phase HPLC for presence of sulindac sulfoxide (SOX). Statistical analysis was performed with GraphPad Prism 5 software unpaired two-tailed t-test. * = significant at $p \leq 0.05$.

AQUA mass spectrometry for mouse FMO3 protein quantification

AQUA (absolute quantification) mass spectrometry quantifies proteins by tandem mass spectrometry and MRM (multiple reaction monitoring) (Gerber *et al.*, 2003). AQUA employs synthetic peptide standards identical to unique tryptic peptides except that standards incorporate stable isotopes. A manuscript providing a detailed description of

AQUA for quantification of mouse FMOs 1–3 and 5 will be forthcoming (MCH, SKK and DEW; in preparation); a brief description is included here.

An FMO3 standard candidate DSFPGL*NR (mouse Fmo3 amino acids 158–165) containing dual ^{13}C - and ^{15}N -labeled leucine (L^*) was synthesized (Sigma, St. Louis, MO) after QTOF experiments confirmed that trypsinized microsomes from both Sf9 cells expressing mouse FMO3 and mouse liver contained the corresponding unlabeled peptide. Protein samples (25 $\mu\text{g}/\text{well}$) were separated on NuPAGE 3–8% Tris-Acetate gels (Invitrogen). Subsequently, FMO bands were excised, spiked with labeled peptide, and in-gel trypsinization performed (Shevchenko *et al.*, 2006) with sequencing-grade trypsin (Promega, Madison, WI).

Peptides were separated by UPLC on a Waters Symmetry C_{18} (180 $\mu\text{m} \times 20 \text{ mm}$) trapping column, and eluted onto a Waters nano Acquity UPLC column (1.7 μm , BEH130 C_{18} , 100 $\mu\text{m} \times 100 \text{ mm}$) connected to a nano Acquity UPLC (Waters Corporation, Milford, MA.). Solvents were 0.1% formic acid (A) and ACN/0.1% formic acid (B), (Burdick & Jackson) at a flow rate of 0.47 $\mu\text{L}/\text{min}$ with a gradient from 5–35% B. Mass spectrometry was performed using an Applied Biosystems 4000 Qtrap (Applied Biosystems/MDS Analytical Technologies, Concord, ON, Canada) using the nanospray ion source. Data was acquired in MRM mode with a 50 mSec dwell time for each transition. Precursor and product ions were 453.2 (precursor), 703.4, and 556.3 for the native protein and 456.7 (precursor), 710.4 and 563.3 for the standard.

Values obtained for area under the curve of the first product ions of sample and standard were used to calculate protein content (Analyst Software, Applied Biosystems). The second product ion provided additional confirmation of identity. The standard curve range was 50 to 1000 fmol.

Results and Discussion

FMO gene expression is regulated by multiple agents including hormones, developmental factors and nutritional status (Krueger and Williams, 2005; Hines, 2006). Although FMOs generally have been considered not to be inducible by xenobiotic chemicals, recent studies indicate that several AHR agonists – including TCDD, 3MC and BNF – can upregulate FMO3 mRNA (Chung *et al.*, 1997; Tijet *et al.*, 2006; Patel *et al.*, 2007; Celius *et al.*, 2008; Novick *et al.*, 2009). Induction of FMO3 mRNA by TCDD in mouse liver *in vivo* proceeds essentially via the same well-characterized AHR-dependent mechanism that regulates CYP1A1 induction (Celius *et al.*, 2008). However, the mechanisms responsible for FMO3 induction by non-halogenated xenobiotics have not been explored in detail. The goals of our current study were to extend the range of compounds tested for FMO3 induction and to identify induction mechanisms, primarily using the Hepa-1 cell model.

3MC induces FMO2 and FMO3 mRNAs in mouse liver *in vivo* but does not recruit the AH receptor to AH response elements in the Fmo3 gene

3MC is a well-known inducer of the classic AHR-regulated gene, *Cyp1a1* in a wide variety of rodent tissues *in vivo* (Okey, 2007). Thus, as a prelude to studies in the Hepa-1 cell culture model, we tested the non-halogenated AHR agonist 3MC to determine if it could induce FMO mRNA in mouse liver *in vivo*. Treatment of adult male C57BL/6 mice with 80 mg/kg of 3MC for 6 h significantly increased mRNA levels both for FMO3 (8-fold) and for FMO2 (2.5-fold) (Fig. 1). 3MC had no effect on mRNA levels for FMO1, FMO4 or FMO5 in mouse liver (data not shown).

Previously we found, by ChIP assays, that *in vivo* treatment with the potent AHR agonist, TCDD, causes significant recruitment of AHR and ARNT proteins to an AH response

element (AHRE) in an upstream regulatory region of the mouse *Fmo3* gene (Celius *et al.*, 2008). Although 3MC treatment induced FMO3 mRNA 8-fold in mouse liver (Fig. 1), we did not detect any recruitment of AHR or ARNT to AHREs in the *Fmo3* gene in livers of 3MC-treated mice (data not shown). The *Cyp1a1* gene served as a positive control in the ChIP experiments. There was strong recruitment of AHR and ARNT to an AHRE regulatory region in the *Cyp1a1* gene in mice treated with 3MC (Fig. 2), confirming that the 3MC treatment regimen we employed activates the AHR/ARNT pathway. Thus the lack of recruitment of AHR and ARNT to AHREs in the *Fmo3* gene is not due to a general inability of 3MC to activate AHR pathways *in vivo* but reflects a selective failure of recruitment to the *Fmo3* gene.

The carcinogenic PAHs 3MC and BaP induce FMO3 mRNA in Hepa-1 cells

Given that a non-halogenated AHR agonist, 3MC, induced FMO3 mRNA in mouse liver *in vivo*, we tested additional well-characterized AHR agonists, including both non-halogenated and halogenated compounds in the Hepa-1 cell culture model. The non-halogenated polycyclic aromatic hydrocarbons, 3MC and BaP, both caused significant induction of FMO3 mRNA in Hepa-1 cells after a 24 h exposure (Fig. 3). In addition to FMO2 and FMO3, we examined FMO1, FMO4 and FMO5 but none of these mRNAs was induced by 3MC or BaP in Hepa-1 cells (data not shown).

TCDD induces FMO3 mRNA in mouse liver *in vivo* but not in Hepa-1 cells

Although we previously found the prototypical AHR agonist, TCDD, to be a very efficacious inducer of multiple species of FMO mRNA in mouse liver *in vivo* (Celius *et al.*, 2008), surprisingly TCDD did not induce FMO3 mRNA in Hepa-1 cells (Fig. 3). The coplanar dioxin-like PCB126 and BNF, both of which are known AHR agonists for induction of CYP1A1 (Denison and Nagy, 2003) and Figure 4), also failed to induce FMO3 mRNA. FMO2 mRNA was not induced in Hepa-1 cells (data not shown) by any of the compounds illustrated in Figure 3, including 3MC and TCDD, in contrast to our previous *in vivo* study which showed TCDD to be a potent inducer of FMO1, FMO2 as well as FMO3 in mouse liver (Celius *et al.*, 2008).

FMO3 mRNA induction in Hepa-1 cells does not involve oxidative stress pathways or Nrf2 mechanisms

As described above, 3MC did not cause recruitment of AHR/ARNT to AHREs in the *Fmo3* gene in mouse liver *in vivo*; thus FMO3 induction by 3MC does not appear to adhere to the canonical AHR induction model. Therefore we evaluated alternative mechanisms that might explain induction. We initially focused on the Nrf2/ARE pathway because this system mediates induction of multiple enzymes that protect cells from xenobiotic chemicals and oxidative stress (Kohle and Bock, 2006). It also has become increasingly apparent that the AHR pathway and the Nrf2 pathway are interconnected in a way that facilitates protection by synergistically regulating a partially-overlapping set of genes involved in cell defense from xenobiotics (Kohle and Bock, 2006). In fact, Nrf2 itself is induced by TCDD, in an AHR-dependent fashion providing a further link between these two regulatory systems (Miao *et al.*, 2005; Franc *et al.*, 2008). In addition, we gave attention to the Nrf2/ARE pathway because we previously found that the 5'-flanking region of the mouse *Fmo3* gene contains multiple copies of the anti-oxidant response element (ARE) (Celius *et al.*, 2008). Moreover, 3MC is categorized as a "bifunctional inducer" (Prochaska and Talalay, 1988; Kwak and Kensler, 2006) or "mixed activator" (Kohle and Bock, 2006) meaning that it activates not only the AHR but also the Nrf2/ARE pathway. Thus we tested other bifunctional inducers as well as "monofunctional inducers" (which activate only the Nrf2/ARE pathway) to determine whether the Nrf2/ARE system might be responsible for upregulation of FMO3 mRNA.

The bifunctional inducers/mixed-activators, BHA, BNF and *t*-BHQ (Kohle and Bock, 2006) had little effect on FMO3 mRNA levels in Hepa-1 cells (Fig. 3). Sulphoraphane (SUL) activates Nrf2 (Miao *et al.*, 2004) and has been reported to also activate the AHR (Anwar-Mohamed and El-Kadi, 2009); however, SUL also failed to induce FMO3. If FMO3 induction in Hepa-1 cells occurs through combined activation of the AHR and Nrf2, then bifunctional inducers such as SUL and BNF should upregulate FMO3 mRNA but BNF was unable to accomplish this induction. *t*-BHQ is a Nrf2-activator that induces multiple “Phase II” enzymes such as glutathione-*S*-transferases, UDP-glucuronosyltransferases and NQO1 (Kohle and Bock, 2006; Kohle and Bock, 2007). Recently *t*-BHQ was reported to also act as an agonist for the AHR and to directly induce CYP1A1 in Hepa-1 cells; thus *t*-BHQ now is classed as a bifunctional inducer (Gharavi and El-Kadi, 2005; Kohle and Bock, 2006). Despite the fact that *t*-BHQ can act directly on both AHR and Nrf2 pathways, *t*-BHQ did not induce FMO3 mRNA in Hepa-1 cells in our experiments (Fig. 3). The results with BNF and *t*-BHQ indicate that combined activation of AHR and Nrf2 is not sufficient to upregulate FMO3. Nrf2 was a plausible candidate to regulate induction of FMO3 by 3MC and BaP in Hepa-1 cells but Nrf2 is ruled out by our experimental evidence.

TCDD is known to provoke oxidative stress in rodent liver *in vivo* (Hassoun *et al.*, 2002) and in Hepa-1 cells (Puga *et al.*, 2000) but TCDD did not induce FMO3 mRNA in Hepa-1 cells in our experiments (Fig. 3). We also subjected the Hepa-1 cells to oxidative stress by exposing them to menadione but this, too, failed to produce much elevation in FMO3 mRNA (Fig. 3). Thus it seems very unlikely that FMO3 induction reflects a component of the complex cellular response to oxidative stress.

Time-course of FMO3 induction

In Hepa-1 cells there was no significant increase in FMO3 mRNA levels at 6 h after exposure to any of the AHR agonists tested (Fig. 4). FMO3 mRNA was significantly increased in cells exposed to 3MC or BaP for 12 h but the maximum response was not obtained until a 24-h exposure to 3MC (Fig. 4). CYP1A1 mRNA, which served as a benchmark for induction by AHR agonists, was significantly induced within 6 h by all 5 AHR agonists tested. CYP1A1 mRNA levels continued to increase up to 24 h for all compounds tested except for BNF which produced its maximal induction at 12 h (Fig. 4).

Dose-response induction of FMO3 by 3MC

To further characterize FMO3 induction we obtained dose-response curves for induction of FMO3 mRNA by 3MC and found that 1 μ M of 3MC was required to significantly elevate mRNA levels in Hepa-1 cells after 24 h of exposure (Fig. 5), whereas CYP1A1 was induced at 3MC concentrations as low as 0.01 μ M (Fig. 5).

Cycloheximide induces FMO3 mRNA in Hepa-1 cells in the absence of any other xenobiotic chemical

CHX has been reported to suppress AHR turnover in TCDD-exposed Hepa-1 cells (Ma *et al.*, 2000) and to cause “superinduction” of CYP1A1 mRNA in these cells (Lusska *et al.*, 1992; Ma *et al.*, 2000) or in MCF10A breast cancer cells (Joiakim *et al.*, 2004). Therefore we tested whether CHX would cause superinduction of FMO3 mRNA (as it does for CYP1A1) by pre-treating Hepa-1 cells with 10 μ g/ml CHX for 1 h followed by 5 h or 23 h exposure to 1 μ M 3MC (Fig. 6). Similar to what was shown in previous studies with TCDD (Lusska *et al.*, 1992; Ma *et al.*, 2000; Joiakim *et al.*, 2004), CHX potentiated induction of CYP1A1 mRNA by 3MC, particularly after 24 h (Fig. 6; data not shown for 6 h). However, CHX did not potentiate induction of FMO3 mRNA by 3MC.

Unexpectedly, CHX, on its own, caused a very large increase in FMO3 mRNA levels (Fig. 6). Our ChIP studies did not show any recruitment of AHR or ARNT to *Fmo3* regulatory regions in cells exposed to CHX, indicating that the AHR/ARNT complex is not directly responsible for upregulation of FMO3 mRNA by CHX (Fig. 7). Previously CHX, in the absence of any exogenous AHR agonist, had been found to cause only a small increase in CYP1A1 mRNA levels (Ma, 2007). Superinduction of CYP1A1 by the combination of CHX plus an AHR agonist has been attributed to the fact that CHX treatment increases the level of AHR protein in cells by blocking AHR proteolysis (Ma and Baldwin, 2000; Ma *et al.*, 2000). The proteolytic process may require the presence of a labile protein whose synthesis is blocked by CHX (Ma, 2007). The dramatic upregulation of FMO3 mRNA that we observed in cells treated with CHX alone might occur because CHX prevents synthesis of a labile protein that acts as a repressor of *Fmo3* gene transcription or that facilitates degradation of FMO3 mRNA. We postulate a labile protein as a negative regulator of constitutive FMO3 mRNA expression because our data indicate that this protein requires constant synthesis given that, in the presence of the protein-synthesis inhibitor CHX, levels of FMO3 mRNA increased rapidly and dramatically. This rise plausibly reflects decreased synthesis of a labile protein that normally suppresses basal expression of FMO3. If the protein was stable, we would expect that basal levels of FMO3 would be unchanged after CHX treatment, since the expression of a stable protein would be not affected by CHX within the time-course of this experiment.

FMO3 induction in Hepa-1 cells requires both AHR and ARNT

We previously found that an intact AHR signaling pathway is essential to FMO3 mRNA upregulation by TCDD in mouse liver *in vivo* since induction does not occur in *Ahr*-null mice (Tijet *et al.*, 2006). The wildtype Hepa-1 cells used in experiments up to this point contain high levels of the AHR (Okey *et al.*, 1980). In order to determine whether the AHR and its dimerization partner, the ARNT protein, are essential to the observed upregulation of FMO3, we tested the ability of 3MC to induce FMO3 mRNA in variant cell lines derived from the Hepa-1 parent: an AHR-deficient cell line (c35), and the ARNT-deficient cell line (c4). 3MC was completely unable to induce FMO3 mRNA in variant cells that are deficient in either AHR or ARNT (data not shown); thus both the AHR and ARNT are required for FMO3 mRNA induction by 3MC.

In addition to the mouse wildtype Hepa-1 cell line and its AHR-deficient and ARNT-deficient derivatives, we also tested whether AHR agonists could induce FMO3 in the rat 5L hepatoma cell line and the human HepG2 hepatoma cell line. Both the 5L cell line and the HepG2 cell line contain AHR and both lines exhibit CYP1A1 induction when treated with AHR agonists such as TCDD (Reiners *et al.*, 1999) or 3MC (Li *et al.*, 1998). However, neither TCDD nor 3MC was able to induce FMO3 mRNA in 5L cells or in HepG2 cells despite the presence of an intact AHR mechanism in these cell lines (data not shown).

FMO3 induction by 3MC or BaP in Hepa-1 cells clearly requires the AHR pathway. However, induction by these non-halogenated PAHs does not seem to be mediated simply by the conventional mechanism involving binding of the ligand:AHR:ARNT complex to AH-responsive elements in the 5'-flanking region of the *Fmo3* gene. We were unable to detect any binding of AHR:ARNT to the *Fmo3* regulatory region by ChIP assays with DNA from cells treated with 3MC although the positive control (the *Cyp1a1* gene) from the same cells demonstrated a strong signal of AHR:ARNT (Fig. 7). In liver of mice treated *in vivo*, TCDD produces robust binding of AHR:ARNT to an AHRE-I motif in the 5'-flank of the *Fmo3* gene accompanied by elevation of FMO3 mRNA levels (Celius *et al.*, 2008). Induction of FMO3 by TCDD in liver *in vivo* probably proceeds by a mechanism very similar to that which regulates CYP1A1 induction but this does not appear to be the case for FMO3 induction by 3-MC *in vivo* nor by 3-MC or BaP in Hepa-1 cells in culture.

New mechanism of FMO3 mRNA induction: 3MC recruits p53 protein to a p53 response element in the *FMO3* gene

There is precedent for an alternative mode of transcriptional regulation by 3MC and BaP in Hepa-1 cells. Mathieu and co-workers (Mathieu *et al.*, 2001) found that p53 mediates induction of the multidrug resistance gene, *mdr1*, in Hepa-1 cells treated with 3MC or BaP and that 3MC and BaP require metabolic activation in order to stimulate the p53 pathway. 3MC and BaP induce their own metabolism by binding to the AHR and upregulating expression of CYP1A1 and other members of the AH gene battery (Okey and Vella, 1982; Okey *et al.*, 1984; Riddick *et al.*, 1994; Nebert *et al.*, 2004).

To test whether 3MC could recruit p53 to p53-response elements in the *Fmo3* gene we used ChIP assays with the *Cyp1a1* gene as a positive control. As expected, 3MC caused robust recruitment of AHR and ARNT proteins to regulatory regions in the *Cyp1a1* gene in Hepa-1 cells (Fig. 7, top panel). Recruitment to the *Cyp1a1* regulatory region in 3MC-treated cells was significantly higher in cells that had been pre-incubated with CHX than in cells not exposed to CHX (Fig. 7, top panel). Despite the fact that 3MC caused a substantial increase in FMO3 mRNA in Hepa-1 cells, our ChIP analyses did not detect significant recruitment of AHR or ARNT to regulatory regions in the *Fmo3* gene in either the presence or absence of CHX (Fig. 7, bottom panel). Nor did CHX, on its own, stimulate recruitment of AHR or ARNT to the regulatory regions of the *Fmo3* gene (Fig. 7, bottom panel).

However, both 3MC and BaP caused a significant increase in binding of the p53 protein to a p53-response element in the 5'-flanking region of the *Fmo3* gene in wildtype Hepa-1 cells that contain AHR and ARNT (Fig. 8a) as these PAHs did to the *Cdk1a* gene, a known p53-regulated gene (Mathieu *et al.*, 2001) used as a positive control (Fig. 8b). In ARNT-deficient cells, neither 3MC nor BaP caused recruitment of p53 to the p53 response element in the *Fmo3* gene (data not shown).

The AHR pathway is the central mechanism for FMO3 mRNA induction by TCDD in mouse liver *in vivo* as well as being the extensively-characterized central mechanism for CYP1A1 induction in Hepa-1 cells. Despite abundant AHR, Hepa-1 cells fail to exhibit induction of FMO3 mRNA by TCDD. Both 3MC and TCDD are classic agonists for the AH receptor and both induce FMO3 mRNA in mouse liver *in vivo*. However, our experiments indicate that the mechanism of FMO3 induction is different for these two agents. TCDD, a halogenated polycyclic aromatic, upregulates FMO3 transcription by direct recruitment of AHR/ARNT to AH response elements in the 5'-flanking region of the *Fmo3* gene. 3MC is unable to promote binding of AHR/ARNT to the *Fmo3* AHRE.

The aggregate data from our experiments suggest a model (Fig. 9) in which 3MC and BaP initially bind to the AHR and upregulate CYP1 enzymes. In turn the CYP1 enzymes convert 3MC or BaP into reactive metabolites that damage DNA, leading to activation of the p53 protein and its subsequent binding to a p53-response element in enhancer regions of the *Fmo3* gene. TCDD is poorly metabolized, forms few reactive metabolites (Poland and Glover, 1979), and does not induce FMO3 expression in Hepa-1 cells, again consistent with a mechanism in which the inducer must first be metabolically activated in order to upregulate FMO3. *Fmo3* regulation in Hepa-1 cells differs from that in mouse liver *in vivo*. In liver *in vivo*, TCDD induces FMO3 mRNA expression in parallel with recruitment of TCDD-bound AHR to an upstream regulatory region of the *Fmo3* gene. With 3MC as the inducer, we did not detect AHR binding to an AHRE-containing region of the *Fmo3* gene in mice *in vivo*. The predominant mechanism of *Fmo3* upregulation by TCDD in liver *in vivo* appears to be via the canonical AHR:AHRE system whereas induction of FMO3 mRNA by 3MC or BaP in Hepa-1 cells in culture requires the AHR/AHRE mechanism followed by activation of p53. There is a lag-time for elevation of FMO3 mRNA when compared with

the rapid upregulation of CYP1A1 mRNA which is mediated by direct binding of activated AHR to AHREs. The requirement for AHR/ARNT, taken together with the delayed response for FMO3 induction, is consistent with a model in which 3MC and BaP first induce CYP1A1 which then activates these PAHs to metabolites that stimulate p53 binding to its regulatory elements.

There is another very recent example of induction of drug-metabolizing enzymes by reactive metabolites. Weems & Yost (2010) found that concentrations of 3-methylindole that damage DNA also induce CYP1A1 in human bronchial epithelial cells and that CYP1A1 induction was attenuated by the inhibitor of P450 catalytic activity, 1-aminobenzotriazole. The potential role of p53 in mediating induction of CYP1A1 by 3-methylindole was not examined in the study by Weems & Yost but seems worthy of testing.

FMO enzyme activity and FMO protein levels after treatment with compounds that induce FMO3 mRNA

Catalytic activity—We previously observed (Celius *et al.*, 2008) with methimazole as substrate that the marked induction of FMO mRNA by TCDD in mouse liver was not accompanied by a comparable rise in catalytic activity. To ensure that this apparent lack of induction of catalytic activity was not peculiar to methimazole, we examined activity towards two additional FMO substrates of clinical importance, ETA and SS. ETA is a prodrug, activated by FMO-dependent S-oxygenation to a metabolite toxic to *Mycobacterium tuberculosis* (Shimizu *et al.*, 2007; Henderson *et al.*, 2008). SS is a nonsteroidal anti-inflammatory (NSAID) which is S-oxygenated by FMO to the pharmacologically inactive S-oxide (Hamman *et al.*, 2000). Genetic polymorphisms in expression of FMO3 allelic variants are responsible for the disease known as trimethylaminuria (Yeung *et al.*, 2007); these patients also exhibit altered activity towards other pharmaceuticals known to be FMO3 substrates (Shimizu *et al.*, 2007). A clinical trial of SS in chemoprevention of Familial Adenomatous Polyposis demonstrated that this FMO3 genetic polymorphism significantly impacted the chemopreventive potency of SS (Hisamuddin *et al.*, 2004).

There was a small but significant increase in S-oxygenation of ETA to the sulfenic acid in liver microsomes from TCDD-treated male but not female mice (Fig. 10). The basal activity of female liver microsomes toward ETA was higher than control or TCDD-treated males and was not further increased by TCDD. There was a slight increase in SS S-oxygenation in males, but this was not significant. No increase in S-oxygenation was observed in liver microsomes from TCDD-treated females. Based on these observations showing a lack of any marked impact on FMO activity by TCDD, it is not apparent that exposure to environmental levels of TCDD would impact drug metabolism even though there is such a large increase in FMO mRNA levels.

Protein levels—The constitutive FMO3 protein content of liver microsomes was approximately 15-fold higher in female mice than in male mice. However, despite robust induction of FMO3 mRNA, TCDD had no significant effect on FMO3 protein content in either male or female (Fig. 11). There is precedent with another drug-metabolizing enzyme for mRNA induction by 3MC and TCDD without an increase in the encoded protein. Deb and Bandiera (Deb and Bandiera, 2010) found that CYP2S1 mRNA was induced by 3MC and TCDD in several rat tissues, including liver, but that CYP2S1 protein levels were unaffected by these inducers. Multiple mechanisms can be postulated (see (Deb and Bandiera, 2009; Hrycay and Bandiera, 2009) to account for the failure of the induced mRNA to yield significant increases in protein levels or protein function. One possibility is that the induced protein is rapidly degraded by cellular proteases. However, chloroquine, an

inhibitor of autosomal/lysosomal pathways (Lee *et al.*, 2008), did not increase FMO3 protein levels over that observed in DMSO-treated control cells or cells treated with 3MC nor did MDL28170, an inhibitor of calpain-like activity (Dale and Eltom, 2006) (Supplemental Fig. 1). We also tested the proteasome inhibitor, MG132 (5 μ M), but MG132 was toxic to the Hepa-1 cells (data not shown). Our measurements of FMO3 protein levels in Hepa-1 cells must be considered pilot experiments because only a single sample was able to be analyzed for each treatment condition. Nonetheless, it seems clear that the large elevation of FMO3 mRNA in 3MC-treated mouse liver *in vivo* or in Hepa-1 cells in culture is not accompanied by any meaningful increase in FMO3 protein or catalytic activity.

Conclusion

It is clear that FMO3 regulation is exceptionally complex. The discrepancy between mRNA levels and protein levels for FMO3 exemplifies this complexity. We also have found that upregulation of FMO mRNAs by AHR agonists does not occur uniformly between sexes or across different animal species. Our previous study revealed that FMO2 and FMO3 mRNAs are highly induced by TCDD in liver of adult male mice. In adult female mice the magnitude of hepatic FMO3 induction is much more modest than in males and there is no induction of FMO2 in females (Celius *et al.*, 2008). Although strong upregulation of FMO3 mRNA by TCDD occurs in mouse liver (Celius *et al.*, 2008; Novick *et al.*, 2009), TCDD does not induce FMO3 in liver of adult rats (Celius *et al.*, 2008). Transgenic mice that express wildtype rat AHR exhibit full FMO2 and FMO3 induction by TCDD (Celius *et al.*, 2008); thus the rat AHR is competent to drive induction of these mRNAs. The species-difference in induction must reside at a regulatory level other than the AHR.

Our current study shows that FMO induction also differs between mouse liver *in vivo* and mouse hepatoma cells in culture; i.e., the prototypical AHR agonist, TCDD, is a potent inducer of mRNAs encoding FMO1, FMO2, and FMO3 in liver of male mice *in vivo* (Celius *et al.*, 2008) but TCDD is completely inactive as an FMO3 inducer in the hepatoma-derived Hepa-1 cell line that has been extensively used as a model for regulation by the AHR (Okey, 2007). The non-halogenated AHR agonists, 3MC and BaP, induce FMO3 mRNA in mouse liver and in Hepa-1 cells. However, even though both 3MC and BaP are AHR ligands, the mechanism of FMO3 induction by these PAHs is distinctly different from the conventional AHR mechanism by which TCDD acts *in vivo*.

The profound variations in response between different agonists, between rodent species and between cells in culture versus *in vivo* indicates that multiple regulatory mechanisms are involved, even when all of the xenobiotic inducers tested are confined to compounds known to be AHR agonists. Our current study has revealed a new mechanism, p53, by which FMO3 mRNA is upregulated by 3MC and BaP. Clearly much more remains to be clarified regarding the multiple mechanisms that alter FMO3 expression, particularly the reason why the highly-induced FMO3 mRNA does not yield a proportional increase in FMO3 protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Upregulation of FMO2 and FMO3 mRNA levels by 3MC in livers of adult male mice. Mice received injections of 80 mg/kg 3MC. Livers were removed after 6 h and mRNA levels were measured by real-time qPCR as described in Materials and Methods. mRNA levels are expressed relative to the level of that particular FMO mRNA in the control animals set at 1.0. Plotted bars in all figures represent mean $\pm$ S.D.; n=3 per group. ** p <0.01 compared to vehicle control.

Fig. 2.

In vivo recruitment of AHR and ARNT to the *Cyp1a1* enhancer region after 3MC exposure. Adult male mice were injected with 80 mg/kg 3MC or the corn oil vehicle (control). Livers were removed 6 h later. ChIP assays were performed as described under Materials and Methods using antibodies to AHR and ARNT as well as to IgG (negative control). Plotted bars represent mean $\pm$ S.D.; n=3 per group. ** p <0.01 compared to vehicle control.

Fig. 3.

Chemical selectivity of FMO3 mRNA induction in Hepa1c1c7 mouse hepatoma cells. Cells were cultured as described under Materials and Methods and exposed to 1 μ M 3MC, BaP, BNF or PCB126, or to 10 nM TCDD, 50 μ M BHA, 10 μ M menadione, 20 μ M SUL or *t*-BHQ for 24 h. Control cells were exposed to DMSO (solvent control) at a concentration in the medium of less than 1%. Cells were harvested and total RNA was analyzed by real-time qPCR as described in Materials and Methods. Cells were treated in duplicate and the entire experiment was performed twice. Plotted bars represent mean $\pm$ S.D. for data combined from the two experiments (n=4). * p < 0.05; ** p < 0.01 compared to solvent control.

Fig. 4.

Time-dependent induction of FMO3 and CYP1A1 mRNA. Cells were exposed to 1 μ M 3MC, BaP, BNF, PCB126, or 10 nM TCDD for 6, 12 or 24 h. Cells were harvested and mRNA levels were measured by real-time qPCR as described in Materials and Methods. Cells were treated in duplicate and the entire experiment was performed twice. Plotted bars represent mean $\pm$ S.D. for data combined from the two experiments. * p < 0.05; ** p < 0.01 compared to solvent control.

Fig. 5.

Dose-dependent induction of mRNAs for FMO3 and CYP1A1. Cells were exposed to varied concentrations of 3MC or to a single concentration of TCDD (10 nM) and harvested 24 h after exposure. Cells were treated in duplicate and the entire experiment was performed twice. Plotted bars represent mean $\pm$ S.D. for data combined from the two experiments. Plotted bars show mean $\pm$ S.D. * p < 0.05; ** p < 0.01 compared to solvent control.

Fig. 6.

Induction of FMO3mRNA by cycloheximide and superinduction of CYP1A1 mRNA by combined exposure to cycloheximide plus 3MC. Cells were pre-exposed to 10 μ g/ml of CHX for 1 h, then 1 μ M 3MC was added and cells were incubated for a further 23 hours in the continued presence of CHX and 3MC. Cells were harvested and mRNA levels were measured by real-time qPCR as described in Materials and Methods. * p <0.05; ** p <0.01 compared to solvent control.

Fig. 7.

Effect of 3MC treatment on recruitment of AHR or ARNT to *Cyp1a1* and *Fmo3* enhancer regions in Hepal1c7 cells. Cells were preincubated for 1 h with or without 10 μ g/ml cycloheximide (CHX), then incubated for 4 h with 1 μ M 3MC or the DMSO vehicle. ChIP assays were performed in triplicate as described in the Materials and Methods. Significant differences compared to matched DMSO samples are represented by an asterisk ($p < 0.01$), whereas significant difference between 3MC-treated matched samples in the presence or absence of CHX are shown by the number sign ($p < 0.01$).

Fig. 8.

Treatment with 3MC or BaP recruits p53 to a p53 response element (p53RE) in the 5'-flanking region of the *Fmo3* gene. Hepa1c1c7 cells were exposed to TCDD (10 nM), 3MC (1 μ M), BP (1 μ M), or the vehicle (DMSO) for 15h. ChIP assays were performed as described in Material and Methods using antibodies to IgG (2 μ g per reaction) and p53 (3 μ g per reaction). Primers were designed for the p53RE (-4885 to -4982) shown in the diagram. Results are reported as percentage enrichment of p53 binding to the p53RE of the total input and compared to the DMSO IgG control. Plotted bars represent mean $\pm$ S.D. for data combined from three experiments (n=3). * p < 0.05; ** p < 0.01 compared to DMSO control. Panel A is a positive control which demonstrates binding of p53 to a p53 response element (p53RE) in the *Cdkn1a* gene, a gene that is a known target for regulation by p53. Panel B illustrates recruitment of p53 to a p53RE in the 5'-flanking region of the *Fmo3* gene in Hepa-1 cells treated with either 3MC or BaP.

Fig. 9. Postulated mechanism of FMO3 mRNA induction by 3MC and BaP. (Adapted from Mathieu et al. (Mathieu *et al.*, 2001).)

Fig. 10.

Catalytic activity of mouse liver microsomes for FMO substrates after treatment with TCDD *in vivo*. Mice were treated with TCDD (30 $\mu\text{g/kg}$) and liver was harvested 24 h later. Enzyme activity was measured with ethionamide (ETA) and sulindac sulfide (SS) as substrates as described in section 2.7. Plotted bars represent mean $\pm$ S.D.; $n=3$ for control males, TCDD-treated males and TCDD-treated females; only one female control was available. * $p<0.05$ compared to vehicle control.

Fig. 11.

FMO3 protein content in mouse liver after treatment with TCDD *in vivo*. The ACQA/mass spectrometry method described in section 2.8 was used to quantitate the FMO3 protein content in microsomes from the same mice shown in Fig. 10.