Endosulfan induces CYP2B6 and CYP3A4 by activating the pregnane X receptor

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Abstract

Endosulfan is an organochlorine pesticide commonly used in agriculture. Endosulfan has effects on vertebrate xenobiotic metabolism pathways that may be mediated, in part, by its ability to activate the pregnane X receptor (PXR) and/or the constitutive androstane receptor (CAR) which can elevate expression of cytochrome P450 (CYP) enzymes. This study examined the dose-dependency and receptor specificity of CYP induction in vitro and in vivo. The HepG2 cell line was transiently transfected with CYP2B6- and CYP3A4-luciferase promoter reporter plasmids along with human PXR (hPXR) or hCAR expression vectors. In the presence of hPXR, endosulfan-alpha exposure caused significant induction of CYP2B6 (16-fold) and CYP3A4 (11-fold) promoter activities over control at 10 µM. The metabolite endosulfan sulfate also induced CYP2B6 (12-fold) and CYP3A4 (6-fold) promoter activities over control at 10 µM. In the presence of hCAR-3, endosulfan-alpha induced CYP2B6 (2-fold) promoter activity at 10 µM, but not at lower concentrations. These data indicate that endosulfan-alpha significantly activates hPXR strongly and hCAR weakly. Using western blot analysis of human hepatocytes, the lowest concentrations at which CYP2B6 and CYP3A4 protein levels were found to be significantly elevated by endosulfan-alpha were 1.0 µM and 10 µM, respectively. In mPXR-null/hPXR-transgenic mice, endosulfan-alpha exposure (2.5 mg/kg/day) caused a significant reduction of tribromoethanol-induced sleep times by approximately 50%, whereas no significant change in sleep times was observed in PXR-null mice. These data support the role of endosulfan-alpha as a strong activator of PXR and inducer of CYP2B6 and CYP3A4, which may impact metabolism of CYP2B6 or CYP3A4 substrates.

Introduction

Endosulfan is an organochlorine insecticide belonging to the cyclodiene group that is widely used in agriculture. It is sold under the trade name of Thiodan® which is a mixture of 70% endosulfan-α (endosulfan I) and 30% endosulfan-β (endosulfan II). Occupational exposure of pesticide applicators is of greatest concern, and it has been demonstrated that even with proper protective measures total endosulfan concentrations in urine can reach 1.3 × 10⁻⁸ M (Lonsway et al., 1997; Arrebola et al., 2001). In a study of the general male population of southern Spain detected endosulfan-α, endosulfan-β, or their metabolites in the serum of all individuals tested (Carreno et al., 2007). The mean and maximum serum concentrations measured were 5.2 × 10⁻⁹ M & 4.8 × 10⁻⁸ M for endosulfan-α, 3.2 × 10⁻⁹ M and 1.7 × 10⁻⁸ M for endosulfan-β, and 6.3 × 10⁻⁸ M & 3.58 × 10⁻⁷ M for total endosulfan, respectively (Carreno et al., 2007).

In laboratory animals, endosulfan has been shown to be toxic to the liver, kidney, nervous system, and reproductive organs (Gupta and Chandra, 1977; Paul et al., 1994; Hack et al., 1995; Paul et al., 1995; Sinha et al., 1997). Endosulfan exposures can modify the activity of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione S transferase (GST) causing oxidative stress (Bebe and Panemangalore, 2003). Endosulfan has also been shown to increase cytochrome P450s (CYPs) levels in vivo, in both hepatic and extra-hepatic tissues (Siddiqui et al., 1987), as well as in human hepatocytes in vitro (Lemaire et al., 2004). Endosulfan is an endocrine disruptor in rodents, and its endocrine disrupting effects in humans are a concern. In developing and adult rats, endosulfan induces testicular toxicity and damage including abnormal spermatogenesis, and decreased sperm counts and sperm motility (Rao et al., 2005). In rodents, endosulfan treatment lead to increased testosterone metabolism and clearance (Singh and Pandey, 1990; Wilson and LeBlanc, 1998).

CYP enzymes are members of a superfamily of hemoproteins that play an important role in the human metabolism of drugs and xenobiotics (Estabrook, 2003). CYP3A4 is the most abundant CYP in human liver, and it plays a major role in the metabolism of xenobiotics, including approximately 50% of drugs, as well as...
endogenous substances such as steroid hormones (Usmani et al., 2003). As previously shown in this laboratory, endosulfan-α is metabolized by CYPs, specifically by CYP2B6 and CYP3A4 (Casabar et al., 2006).

The induction of the CYP2B6 and 3A4 is mediated by the activation of nuclear receptors, such as PXR and the CAR. Induction of CYPs can lead to enhanced detoxification or greater bioactivation of xenobiotics, and it has only recently been recognized that CYP2B6 plays a significant role in the metabolism of a number of pesticides (Hodgson and Rose, 2007). Activated PXR and CAR mediate the upregulated expression of numerous genes involved in xenobiotic detoxification including phase I CYP enzymes, phase II enzymes, and transporters (Kretschmer and Baldwin, 2005; Timsit and Negishi, 2007; Tompkins and Wallace, 2007). Numerous drugs and environmental contaminants have been shown to activate PXR and/or CAR, and activation may be protective by detoxifying xenobiotics and increasing levels of detoxification enzymes such as CYP2B and CYP3A (Kretschmer and Baldwin, 2005). Endosulfan has been shown to act a PXR agonist in vitro at a single concentration (10 µM) (Coumoul et al., 2002; and a DR3 motif.

336


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Materials and methods

Chemicals and reagents. Endosulfan-α, endosulfan-β, technical-grade endosulfan (60:40 mixture of endosulfan-α and β isomers), and endosulfan sulfate were purchased from ChemService (West Chester, PA) and stock solutions were dissolved in ethanol or acetonitrile (ACN). 5α-androst-16-en-3 α-ol (androstenol) and 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPDBP) were obtained from Sigma-Aldrich (St. Louis, MO). Androstenedol was dissolved in ethanol and TCPDBP was dissolved in DMSO, with dilutions dissolved in ethanol. 6-(4-Chlorophenyl)imidazol[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (Citco) was purchased from BIONOL International, Inc. (Plymouth Meeting, PA). Rifampicin (Rif), dexamethasone (Dex), phenobarbital (PB), and all other chemicals, unless specified otherwise, were purchased from Sigma-Aldrich (St. Louis, MO). Z-DEVD-FMK is a product of Alexis Biochemicals supplied by AXXORA, LLC (San Diego, CA).

Antibodies and plasmids. Rabbit polyclonal human CYP2B6 and mouse monoclonal human CYP3A4 antibodies were purchased from BD Biosciences (Woburn, MA). Goat anti-mouse IRDye800 and goat anti-rabbit IRDye800 fluorescently labeled secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). Rabbit β-actin primary antibody was purchased from Sigma-Aldrich. The Monoclonal Anti-Rat Cytochrome P450 3A1 which has been shown to detect mouse CYP3A11 was purchased from Oxford Biomedical Research (Oxford, MI). The pSV-Beta-galactosidase and the firefly luciferase reporter plasmids pGL3 and pGL4 basic vectors were purchased from Promega Corp. (Madison, WI). The plasmids CYP3A4-luciferase and pSG5-hPXR were provided by Dr. Dr. Jean Marc Pascussi (French National Institute for Health and Medical Research (INSERM), France), and the pSG5-mCAR was kindly provided by Dr. John T. Moore (GlaxoSmithKline Research Triangle Park, NC). The human CAR-3 expression plasmid was provided by Curtis J. Omiecinski (Penn State University, University Park, PA). The pSG5 control vector was obtained from Stratagene (La Jolla, CA).

CYP2B6 promoter cloning and pGL4-PBREM-XREM-luciferase construct. The location and sequences of the proximal phenobarbital responsive enhancer module (PBREM) and distal xenobiotic responsive enhancer module (XREM) regions in the CYP2B6 promoter were previously characterized by Wang et al (2003). The pGL4.10 (Promega Corp)
Endosulfan toxicity to HepG2 and primary human hepatocytes was assessed after exposures up to 72 h were done as described by Auerbach et al. (2005), using 5 µM CITCO as a positive control. The primary human hepatocytes was assessed after exposures up to 72 h were done as described by Auerbach et al. (2005), using 5 µM CITCO as a positive control.

Endosulfan toxicity studies. Endosulfan toxicity to HepG2 and primary human hepatocytes was assessed after exposures up to 72 h using the luciferase-based Toxilight® (Lanza, Rockland, ME) and Caspase-Glo®-3/7 (Promega Corp, Madison, WI) assay systems. Luminescence produced by luciferase is proportional to adenylyl kinase release (Toxilight®) or caspase activation (Caspase-Glo®-3/7) and was measured as RLU (relative light unit) using a Fusion™Universal Microplate Analyzer (Packard Bioscience Company, Meriden, CT). These assays were performed according to the manufacturer’s protocols.

Endosulfan-treated primary human hepatocytes. Primary human hepatocytes purchased from ADMET (Research Triangle Park, NC) that had been plated (1.5 × 10⁵/well) in 6-well culture plates coated with Collagen Type I and overlaid with Matrigel™ (Hamilton et al., 2001). The hepatocytes were equilibrated in a humidified incubator at 5% CO₂/95% air at 37 °C for 48 h and cultured in William’s medium E, which was replaced every 24 h prior to treatment. The media was supplemented with penicillin G (100 U/mL), streptomycin sulfate (100 µg/mL), Dex (10⁻⁷ M), insulin (10⁻⁷ M), and 10% fetal bovine serum. Hepatocytes were treated with or without rifampicin (10 µM) or phenobarbital (100 µM) or endosulfan-α (0.1, 1, 5, 10, and 50 µM) every 24 h for up to 3 days. Control cells were treated with ACN at 0.1% solvent concentration.

Cells were then harvested from 2 wells of a 6-well plate using a cell scraper and plated in microfuge tubes for protein collection. Cells in microfuge tubes were then centrifuged at 5000 g for 3 min and the supernatant was discarded. The cells were then suspended in 75 µL chilled CYP storage buffer (0.1 M potassium phosphate buffer with 10% fetal bovine serum (FBS), 1% gluta mine, 1% penicillin-streptomycin, 1% sodium pyruvate, and 1% non-essential amino acids. Prior to transfection, HepG2 (3 × 10⁵ cells per well) were plated into six-well plates. Twenty-four hours later, cells were washed and transfected with expression plasmids pSG5 or pSG5-hPXR, the control vector pSV-Beta-galactosidase (0.1 µg per well), and reporter plasmids pCYP2B6 pGL4-XREM-PBREM-luc or pCYP3A4-luc (1.0 µg per well). Transfections were done using Transit reagent (Mirus Corp, Madison, WI). After 4–6 h, the media was changed. Twenty-four hours after transfection, the cells were treated with endosulfan-α, -β, technical-grade endosulfan or endosulfan sulfate. Cells were treated for a total of 24 h. Cell lysates were prepared and luciferase activity was measured to determine CYP2B6 and CYP3A4 promoter activity following Promega’s luciferase assay system protocol. The β-galactosidase assay system (Promega Corp.) was used to measure the amount of β-galactosidase activity, which was used as a control for transfection efficiency.

Induction of CYP2B6 promoter activity via mouse CAR and human CAR-3. The protocol used for this experiment was the same as the above, with the following exceptions. The expression plasmids pSG5-mCAR or human CAR-3 were transfected into HepG2 cells or COS1 cells, 24 h before treatment of cells. In studies using mouse CAR, transfected cells were treated with 4 µM androstenediol to block mCAR constitutive activity as described by Sueyoshi et al. (1999). Treatments included androstenediol alone, or both androstenediol and 0.25 µM TCPOBOP, or both androstenediol and 10 µM endosulfan-α for 24 h. All treatments had 0.1% solvent concentration per well. Experiments using human CAR-3 were done as described by Auerbach et al. (2005), using 5 µM CITCO as a positive control.

Endosulfan cytotoxicity studies. Endosulfan toxicity to HepG2 and primary human hepatocytes was assessed after exposures up to 72 h using the luciferase-based Toxilight® (Lanza, Rockland, ME) and Caspase-Glo®-3/7 (Promega Corp, Madison, WI) assay systems. Luminescence produced by luciferase is proportional to adenylyl kinase release (Toxilight®) or caspase activation (Caspase-Glo®-3/7) and was measured as RLU (relative light unit) using a Fusion™Universal Microplate Analyzer (Packard Bioscience Company, Meriden, CT). These assays were performed according to the manufacturer’s protocols.

Sleep studies. All animals were maintained and bred at the University of Northern Colorado as described previously (Stover et al., 2000) and in compliance with National Institutes of Health guidelines for the humane use of laboratory animals. Wild type [Hsd: NSATM (CF-1®)] mice were originally obtained from Harlan (Indianapolis, IN). MPXR-null (PXR-null) and MPXR-null/hPXR+/+(hPXR) mice were originally obtained as a generous gift from Dr. Ronald Evans and were generated on a 129 S4/SvJae X C57Bl/6J background. (The Salk Institute for Biological Studies, La Jolla, CA). Dosing solutions were prepared by dissolving endosulfan-α in ethanol, diluting with peanut oil, and then driving off the ethanol under a stream of CO₂. Peanut oil alone served as the vehicle control. Male mice were used in all experiments. Mice were treated with endosulfan by oral gavage (0.1 mL/10 g body weight) for seven days at doses of 0, 1.25 or 2.5 mg/kg/day. Dex (5 mg/kg/day) or rifampicin (12.5 mg/kg/day) were given by oral gavage for 2 days and used as positive control agents to induce CYP3A activity. On the day after the last
treatment, all mice were injected (ip.) with tribromoethanol (TBE, 400 mg/kg), which is metabolized by CYP3A in mice, as previously described (Xie et al., 2000). Ophthalmic ointment was placed on the eyes to prevent dehydration (due to loss of blink reflex). Each animal was placed on a heating pad (37 °C) and the duration of anesthesia was noted as the time from unconsciousness to the onset of recovery, with recovery measured as the ability of the animal to right itself from dorsal recumbency.

Statistical analysis. Statistical analysis of in vitro studies was performed using JMP software, version 6.0.0 (SAS Institute, Inc., Cary, NC). Analysis was done using ANOVA followed by comparisons of the treatment means with control (untreated) using Dunnett’s method with a significance level of 0.05. Statistical analysis of in vivo studies was performed using SAS version 9.1 for Windows (SAS Institute, Inc., Cary, NC). Analysis of variance was performed using the General Linear Models procedure and LSD post-hoc all-pairwise t tests with a minimum level of significance of p < 0.05.

Results

Endosulfan induction of CYP3A4 and CYP2B6 promoter activity

To assess the dose-dependent induction of CYP2B6 and 3A4 promoter activity by endosulfan and to determine whether the mechanism(s) of induction involved specific receptors a series of transient transfection experiments were done using the HepG2 human hepatoma cell line. To determine whether endosulfan is able to activate hPXR and induce CYP2B6 and CYP3A4 promoter activity, reporter assays were performed. HepG2 cells were transiently transfected with pSG5-hPXR expression vector and CYP2B6-Luc or CYP3A4-Luc. Transfected cells were treated with the known hPXR agonist rifampicin, which is the prototypical inducer of CYP3A4 and has been shown to induce CYP2B6 (Goodwin et al., 2001; Wang et al., 2003; Lemaire et al., 2004). Rifampicin (10 µM), in the presence of hPXR, induced CYP2B6 and CYP3A4 promoter activities by 16-fold and 11-fold, respectively, over control (Fig. 2A and B). In comparison, endosulfan-α at 10 µM induced CYP2B6 (Fig. 2A) and CYP3A4 (Fig. 2B) promoter activities by 16-fold and 11-fold, respectively, over control. The lowest dose for which significant induction of CYP2B6 promoter activity was seen was 1 µM endosulfan-α, and no significant induction of CYP3A4 was seen at the 0.1, 0.01, and 1 µM concentrations. Interestingly, the metabolite endosulfane sulfate (10 µM) also induced CYP2B6 and CYP3A4 promoter activities by 12-fold and 6-fold, respectively, over control. In the absence of hPXR, rifampicin, endosulfan-α and its metabolite endosulfane sulfate did not induce CYP2B6 and CYP3A4 promoter activity (data not shown).

![Fig. 2. Endosulfan-α induces CYP2B6 and CYP3A4 promoter activity.](image1)

![Fig. 3. Endosulfan-α induction of the CYP2B6 promoter activity via mCAR and hCAR-3.](image2)
In light of a report that endosulfan-β is metabolized primarily by CYP3A4 and CYP3A5, the ability of endosulfan-β and technical-grade endosulfan to induce CYP3A4 promoter activity was investigated to determine, whether similarly to endosulfan-α, these compounds could activate PXR (Lee et al., 2003). Our results showed that technical-grade endosulfan (10 µM) and endosulfan-β (10 µM) induced the CYP3A4 promoter to a level similar to that measured with endosulfan-α (Fig. 2C).

Endosulfan activates CAR and weakly induces CYP2B6 promoter activity

To determine if endosulfan is able to activate CAR and induce CYP2B6 promoter activity, similar reporter assays were performed. Faucette et al., 2006, reported that CYP2B6 and CYP3A4 are non-selectively induced by PXR, but CYP2B6 is preferentially induced (over CYP3A4) by CAR. Hence, we examined whether endosulfan-α induction of CYP2B6 is CAR-mediated. HepG2 cells were transiently transfected with mCAR expression and CYP2B6-Luc plasmids. The cells were treated with androstenol (4 µM) alone, both androstenol (4 µM) and TCP080P (0.25 µM), or both androstenol (4 µM) and endosulfan-α (10 µM). Endosulfan-α, in the presence of mCAR and the transcriptional repressor androstenol, only weakly reversed androstenol repression and induced CYP2B6 promoter activity 3-fold over androstenol-treated HepG2 cells (Fig. 3A). The positive control TCP080P, strongly reversed androstenol repression and induced CYP2B6 promoter activity by 14-fold. The ability of endosulfan to activate human CAR was next investigated. Endosulfan-α activated the human CAR-3 isoform and induced CYP2B6-Luc activity 3-fold at the highest dose of endosulfan-α, which was similar to the positive control CITCO (Fig. 3B).

Assessment of cytotoxicity in HepG2 cells

Cytotoxicity has been shown to cause underestimation of CYP induction in promoter reporter activity assays in studies of various PXR ligands using the HepG2 cell line (Vignati et al., 2004). The release of adenylate kinase was used as a measure of cytotoxicity following endosulfan-α exposure to determine if cytotoxicity could impact our assessment of CYP promoter activity induction. After 24 h, no significant cytotoxicity was detected for HepG2 cells exposed to endosulfan at concentrations up to 100 µM (Fig. 4A). Therefore, endosulfan-mediated cytotoxicity was not a concern for the transient transfection reporter assay studies conducted here. Significant cytotoxicity was observed for HepG2 cells after 48 and 72 h of exposure to endosulfan at 50 µM or higher concentrations (Fig. 4A).

Caspase-3/7 activity is one of the important markers of the cellular apoptotic process. In order to further assess cytotoxicity, and determine whether endosulfan-α mediated cell death was triggered through this apoptotic pathway, cultured HepG2 cells were exposed to increasing concentrations of endosulfan-α (1 to 100 µM) for 24, 48 and 72 h and caspase-3/7 activity was measured. Time- and dose-dependent induction of caspase-3/7 activity was noted from 1 to 12.5 µM endosulfan-α, and decreased from 25 to 100 µM endosulfan-α. The maximum induction was ∼4-fold above solvent treated control at 72h (Fig. 4B).

Endosulfan increases CYP3A4 and CYP2B6 protein levels in human hepatocytes

Freshly isolated human hepatocytes were obtained from three individuals to assess the concentration-dependent ability of endosulfan-
α to increase the protein levels of CYP2B6 and CYP3A4. Western blots were performed using human hepatocyte protein samples. Endosulfan-α increased CYP2B6 protein levels in primary human hepatocytes in a dose-dependent manner. Endosulfan-α increased CYP2B6 1.8-, 3.2-, 3.3-, and 7.4-fold at 1, 5, 10, and 50 µM and increases due to 10 µM endosulfan was greater than the positive controls of 10 µM rifampicin or 100 µM phenobarbital (Fig. 5). CYP3A4 protein levels were increased 2.7 and 4.9-fold by 10 and 50 µM endosulfan-α and no significant change was measured at 0.1, 1, and 5 µM concentrations (Fig. 6).

Assessment of cytotoxicity in human hepatocytes

In endosulfan-treated human hepatocytes, no significant cytotoxicity was observed based on levels of adenylate kinase release after 24 h exposure to endosulfan below 50 µM, whereas cytotoxicity was observed at 6.25 µM and higher concentrations after 48 and 72 h of exposure (Fig. 7A). To determine if caspase-3/7 activity was increased similar to HepG2 cells, freshly isolated human hepatocytes were exposed similarly to increasing concentrations of endosulfan-α (1 to 100 µM). Additionally, hepatocytes exposed to 100 µM endosulfan-α were treated with the specific caspase 3/7 inhibitor Z-DEVD-FMK. Results indicated that endosulfan at 50 and 100 µM significantly induced caspase-3/7 activity ~4-fold at 24, 48 and 72 h, however induction of caspase-3/7 activity reached a plateau at 72 h (Fig. 7B). Z-DEVD-FMK completely abrogated the endosulfan-induced caspase-3/7 activity, confirming the endosulfan-mediated induction of caspase-3/7 (Fig. 7B). Hepatocytes were treated with doses of endosulfan-α within the range that we had observed cytotoxicity in experiments measuring CYP protein induction, but based on previous findings from our laboratory this level of cytotoxicity would not compromise CYP protein induction (Das et al., 2006).

Endosulfan increases CYP3A metabolism activity in vivo

The ability of endosulfan to induce CYP3A in vivo was investigated in wild-type, PXR-null, and humanized PXR mice. Sleep times in these animals were measured after anesthesia with the sedative tribromoethanol (TBE), a CYP3A substrate. Reduced sleep times indicates induction of CYP3A activity and increased metabolism of TBE (Xie et al., 2000). In wild-type mice, endosulfan treatment resulted in statistically significant and dose-dependent reduction in sleep times to approximately 60% of control at the highest dose, 2.5 mg/kg/day (p<0.05) (Fig. 8A). Similarly, in PXR-humanized mice, a statistically significant and dose-dependent decrease in sleep times was seen with endosulfan exposure to approximately 54% of control at the highest dose, 2.5 mg/kg/day (p<0.05) (Fig. 8C). By contrast, in mice lacking expression of a functional PXR, no significant changes in sleep times were seen following endosulfan treatment (Fig. 8B). Exposure to Dextromethorphan (5 mg/kg/day for 2 days) (Fig. 8D) or rifampicin (12.5 mg/kg/day for 2 days) (Fig. 8E) resulted in a similarly significant reduction of TBE-induced sleep times in wild type and hPXR mice, respectively, whereas no change in TBE-induced sleep times was observed in PXR-null mice treated with either of the positive control agents (Fig. 8F). Western blot of endosulfan-treated mice confirmed an increase in CYP3A11 protein levels in wild-type PXR and PXR-humanized mice, but no change in CYP3A11 was seen in PXR-null mice (Fig. 9). These data suggest that PXR is necessary for induction of CYP3A by endosulfan in vivo.

Discussion

We have demonstrated here that endosulfan-α can significantly induce the CYP2B6 (16-fold) and CYP3A4 (10-fold) promoter activities in a concentration-dependent manner by activating hPXR in transfected HepG2 cells (Fig. 2A and B). The minimal endosulfan-α concentration required was 1 µM for CYP2B6 and 10 µM for CYP3A4. These results are in agreement with those of Coumoul et al. (2002) and Lemaire et al. (2004) who also used transfected HepG2 cells, although no dose-response data were shown, and we observed significant induction of CYP2B6 promoter activity with 1 µM endosulfan-α. Importantly, CYP2B6 and 3A4 promoter activation by endosulfan-α was observed at concentrations below those that caused cytotoxicity or apoptosis (Fig. 4). Also demonstrated here, but previously unreported, was the
Fig. 8. In vivo reduction of induction sleep time by endosulfan-α is dependent on PXR. Male wild type (Wt Type, Panel A), PXR knockout (PXR-null, Panel B), and human PXR (hPXR, Panel C) mice were treated daily with vehicle (peanut oil) or varying doses of endosulfan-α for 7 days. On day eight mice were injected with the sedative and CYP3A substrate tribromoethanol (TBE) (400 mg/kg), and the sleep time (the duration of unconsciousness) was measured. As positive controls, wild type mice were treated with dexamethasone (Dex) (Panel D) and hPXR (Panel E) were treated with rifampicin (Rif) to induce CYP3A activity and hPXR-null mice were treated with both agents (Panel F). The data presented represent means ± SEM for 2–4 combined experiments per mouse strain (n = 7–26 animals per treatment group). The means with different letters were found to be significantly different (p ≤ 0.05).

Fig. 7. Endosulfan cytotoxicity to human hepatocytes. Dose and time-dependent effects of endosulfan-α on (A) adenylate kinase activity and on (B) caspase-3/7 activity in primary human hepatocytes was measured as Relative Luminescence Units (RLUs). Data are expressed as means relative to untreated controls ± standard deviation from six determinations. Statistical significance was determined by ANOVA, followed by comparisons of the treatment means with control using Dunnett’s method, *p < 0.05.
concentrations of 50

Human hepatocytes may be more sensitive to endosulfan-

These results suggest that the increase in CYP2B6 protein levels in

CYP2B6 protein levels were observed at 1 µM of endosulfan-

CYP3A4 protein levels was 10 µM (Fig. 6), signi

whereas the lowest levels of endosulfan-

approximately 2.6- and 1.7-fold, respectively. A similar level of increase for

representative western blot analysis is shown.

was visualized using a rat anti-CYP3A1 antibody and ECL kit (Amersham). A

fractions were prepared for CYP3A11 and

hPXR-mice with 2.5 mg/kg, endosulfan-

activation than CAR activation.

endosulfan induction is more likely to be mediated thorough PXR

by a reduction in TBE-induced sleep times by approximately 50%. TBE

and hPXR-transgenetic mice relative to mPXR-null mice, as measured

with a decline in adenylate kinase, CYP3A4 protein elevations were

observed (Das et al., 2006). At these high concentrations associated

with a decline in adenylate kinase, CYP3A4 protein elevations were

found to be compromised, and therefore in these studies hepatocytes were

not treated with 100 µM endosulfan in experiments measuring induction of CYP3A4 and 2B6 protein levels.

Recent studies have indicated that endosulfan causes oxidative
damage and cytotoxicity in HepG2 cells with an IC50 value of 49 µM
(Sohn et al., 2004). Our studies indicate that adenylate kinase was

highest at 50 µM and 100 µM in human primary hepatocytes and

HepG2 cells, respectively. Endosulfan-α also significantly increased
caspase-3/7 activity in HepG2 and primary hepatocytes. However, the
difference in the potential of endosulfan to increase caspase-3/7
activity in HepG2 and primary hepatocytes was likely due to their basic characteristic differences. Our previous findings determined that significant increases in caspase-3/7 activities did not compromise CYP increases due to the pesticide fipronil, but decreases in activity at higher concentrations of fipronil resulted in a loss of CYP induction (Das et al., 2006). A decrease in endosulfan-α induced caspase-3/7 activity was not observed at the highest dose and activity was completely abrogated with the caspase inhibitor Z-DEVD-FMK. Unlike the recent findings concerning testicular toxicity and tissue damage, the present data suggest that endosulfan mediated cytotoxicity in human hepatocytes may be occurring through processes of apoptosis (Jaiswal et al., 2005; Antherieu et al., 2007).

Using both wild type and mPXR-null/hPXR-transgenic mice, we showed (Fig. 8) that endosulfan-α acts as an agonist for PXR and upregulates CYP3A activity. At 2.5 mg/kg/day, endosulfan-α exposure caused a significant upregulation of TBE clearance in wild type and hPXR-transgenic mice relative to mPXR-null mice, as measured by a reduction in TBE-induced sleep times by approximately 50%. TBE is a CYP3A substrate (Xie et al., 2000), and increased TBE clearance most likely occurred through induction of mouse CYP3A11 and western blot analysis determined CYP3A11 levels increased in wild type and hPXR mice. TBE metabolism has been correlated with CYP3A11 and multi-drug resistance protein expression levels (Huang et al., 2007). Moreover, because no difference in sleep times was observed between vehicle treated control and endosulfan treated mPXR-null mice, these results suggest that reduced sleep times in PXR intact mice is independent of mCAR activation. Together, these findings suggest that endosulfan exposure may increase the metabol-

ism of CYP3A substrates.
In summary, we present evidence that endosulfan activates hPXR, but only weakly activates the mCAR and hCAR-3, and subsequently endosulfan induces CYP3A4 and CYP2B6 promoter activity. Endosulfan was also found to elevate CYP3A4 and CYP2B6 protein expression in human hepatocytes in a dose dependent manner. Endosulfan also was found to cause hepatic toxicity via an apoptotic process. Furthermore, in vivo studies using mouse models have demonstrated that endosulfan increase of CYP3A4 metabolism is dependent on PXR. Data presented here indicate endosulfan elevated levels of CYP3A4 and 2B6, enzymes that our previous studies had found were responsible for human liver metabolism of endosulfan (Casabar et al., 2006). Endosulfan regulation of CYP3A4 and 2B6 may cause detrimental metabolic interactions with endogenous and exogenous CYP3A4 or 2B6 substrates.

Conflict of interest statement
The authors declare that there are no conflicts of interest related to this work.

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