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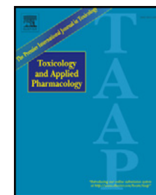

Endosulfan induces CYP2B6 and CYP3A4 by activating the pregnane X receptor

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ABSTRACT

Endosulfan is an organochlorine pesticide commonly used in agriculture. Endosulfan has affects 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.

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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 spermatozoa, 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 hemeproteins 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

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[1] This paper is dedicated to the living memory of our friend and colleague who was lost to us in a tragic car accident.

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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 detoxicification 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 detecting 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; Lemaire et al., 2004). To date, no studies have yet examined endosulfan's activity with regard to CAR.

The aim of the present study was to determine the dose-response relationship for induction of *CYP2B6* and *CYP3A4* by endosulfan and determine the dependence of induction on PXR or CAR. We report that endosulfan-α, (1) induces *CYP2B6* and *CYP3A4* promoter activity and increases protein expression in a dose and PXR-dependent manner using both HepG2 cells and human hepatocytes, and (2) dose-dependently causes reduced anesthetic-induced sleep times in mice, also in a PXR-dependent manner. Together, these findings demonstrate that endosulfan strongly activates human PXR *in vitro* and *in vivo* and can affect the CYP2B6 and CYP3A4 metabolizing pathways.

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 (TCPOBOP) were obtained from Sigma-Aldrich (St. Louis, MO). Androstenol was dissolved in ethanol and TCPOBOP was dissolved in DMSO, with dilutions dissolved in ethanol. 6-(4-Chlorophenyl)imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (Citco) was purchased from BIOMOL 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 IRDye680 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. 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)

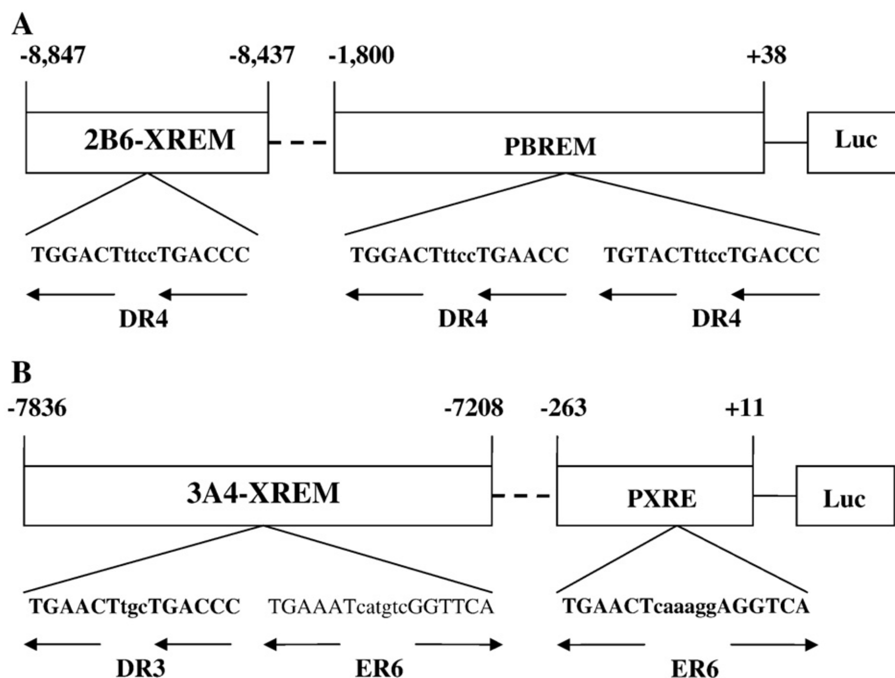

Fig. 1. Map of (A) *CYP2B6*-luciferase and (B) *CYP3A4*-luciferase constructs. The CYP2B6-luc contained the proximal 1.8 kb PBREM (with two DR4 motifs) and the distal 410 bp XREM (with a DR4 motif) of the *CYP2B6* promoter. The *CYP3A4*-luc contained the proximal (+11 to −263) PXRE with an ER6 motif and the distal (−7.2 kb to −7.8 kb) XREM with an ER6 and a DR3 motif.

luciferase reporter vector was utilized to construct a reporter plasmid that contained the proximal PBREM (with two DR4 motifs) and the distal XREM (with a DR4 motif) of the CYP2B6 promoter (Fig. 1A). The distal XREM and proximal PBREM regions were PCR-amplified from human genomic DNA (Promega Corp.) using the High fidelity PCR system (Roche Diagnostics, Indianapolis, IN). For the XREM, the forward primer 5'-CGGGGTACCCTTTCTCCATCCACAAAATCG-3' (with *Kpn*I restriction site) and the reverse primer 5'-CGCTCGAGGATGCTGATTCAGGGAATCCA-3' (with *Xho*I restriction site) were used and for the PBREM, the forward primer 5'-GAAGATCTCTGCAATGAGCAC CCAATCTT-3' (with *Bgl*II restriction site) and the reverse primer 5'-CCCAAGCTTCTGCAC CCTGCTGCAGCCTCC-3' (with *Hin*dIII restriction site) were utilized. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). The resulting 410 bp (XREM) and 1.8 kb (PBREM) products were cloned into the pGL4.10 to create the pGL4-XREM-PBREM plasmid containing the two responsive regions of the CYP2B6 promoter and the major transcriptional start site for CYP2B6 (Fig. 1A) (Zukunft et al., 2005). DNA ligation and gel purification systems were purchased from Promega Corp. The *CYP3A4*-luciferase construct contains a proximal (−263 to +11) ER6 motif and a distal xenobiotic response element (XREM), which contains a DR3 motif (Fig. 1B) in the pGL3-basic (Promega Corp.) plasmid was constructed by Jean Marc Pascussi (INSERM, France) following methods described by Goodwin et al. (1999). Plasmid DNA was prepared using purification kits purchased from Qiagen (Valencia, CA).

Cell culture, transfection, and luciferase assays. The human liver carcinoma cell line HepG2 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and transient transfections were done using cells passaged seven times or less. HepG2 cells do not express significant levels of functional hPXR and CAR. HepG2 cells were maintained in Eagle's minimal essential medium (EMEM) (MediaTech, Inc., Herdon, VA), with 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin–streptomycin, 1% sodium pyruvate, and 1% non-essential amino acids. Prior to transfection, HepG2 (3×10^5 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 *CYP2B6* pGL4-XREM-PBREM-luc or *CYP3A4*-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 androstenol to block mCAR constitutive activity as described by Sueyoshi et al. (1999). Treatments included androstenol alone, or both androstenol and 0.25 µM TCPOBOP, or both androstenol 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® (Lonza, Rockland, ME) and Caspase-Glo®-3/7 (Promega Corp, Madison, WI) assay systems. Luminescence produced by luciferase is proportional to adenylate 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 (1.5×10^6/well) in 6-well culture plates coated with Collagen Type I and overlayed with Matrigel™ (Hamilton et al., 2001). The hepatocytes were equilibrated in a humidified incubator at 5% CO_2/95% 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 either 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 pooled 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 0.1 mM EDTA at pH 7.5) and sonicated twice for 30 s. S9 protein fraction was prepared by centrifuging the sample at 9000*g* for 15 min. The protein concentrations of S9 samples were measured using the Bio-Rad protein assay (BioRad, Hercules, CA).

Western blotting was conducted to determine levels of CYP2B6 and CYP3A4 protein in primary hepatocytes treated with different doses of endosulfan-α. Eight percent tris-glycine pre-cast gels (Invitrogen, Carlsbad, CA) were used for electrophoresis of protein samples (S9 fractions), which were then transferred onto a nitrocellulose membrane by electroblotting. CYP3A4, CYP2B6, or β-actin were detected using primary antibodies followed by incubation with secondary goat anti-mouse IRDye680 fluorescently labeled, goat anti-rabbit IRDye800, or horseradish peroxidase-conjugated anti-rabbit secondary antibodies. All primary antibodies were incubated overnight at 4 °C in tris buffered saline (TBS) or phosphate buffered saline (PBS) with Tween and 1% nonfat dry milk. Immunoblots were visualized using chemiluminescence detection [Enhanced chemiluminescence, GE Healthcare Biosciences (Piscataway, NJ)] or with a LiCOR Odyssey Infrared Imaging System. Densitometric analysis of immunoreactive protein bands was performed using a Kodak Image Station 440 CF with Kodak Molecular Imaging Software (Rochester, NY). The integrated intensity of CYP3A4 protein and β-actin levels was determined by LiCOR Odyssey Imaging Software (Lincoln, NE).

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 C57Bl6/J 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 CO_2. 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 $p<0.05$.

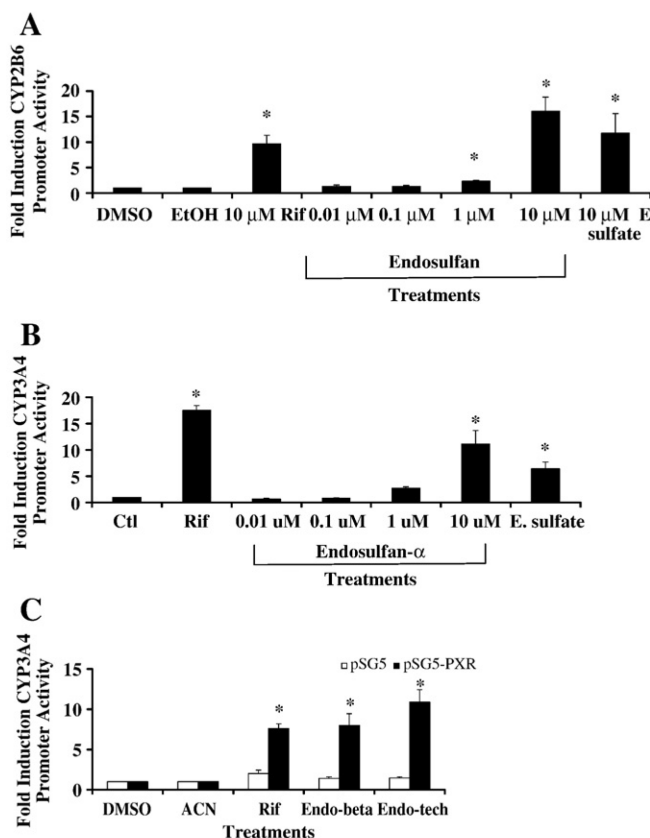

Fig. 2. Endosulfan-α induces *CYP2B6* and *CYP3A4* promoter activity. (A) HepG2 cells were transfected with *CYP2B6*-Luciferase reporter, pSG5-hPXR, and β-gal plasmids and treated with controls dimethyl sulfoxide (DMSO), ethanol (EtOH), 10 µM rifampicin (Rif), or increasing doses of endosulfan-α or the metabolite 10 µM endosulfan sulfate (E. sulfate). (B) HepG2 cells were transfected with CYP3A4-Luciferase reporter, pSG5-hPXR, and β-gal plasmids and treated with controls dimethyl sulfoxide (DMSO), ethanol (EtOH), 10 µM rifampicin (Rif), or increasing doses endosulfan-α or the metabolite 10 µM endosulfan sulfate (E. sulfate). (C) HepG2 cells were transfected with *CYP3A4*-Luciferase reporter, pSG5 or pSG5-hPXR, and β-gal plasmids and treated with the controls dimethyl sulfoxide (DMSO), acetonitrile (ACN), or 10 µM rifampicin (Rif), endosulfan-β, or endosulfan-technical grade (Endo-tech). Data shown are means of three independent experiments done in triplicate. Measured luciferase activity was normalized to β-galactosidase activity. The results are expressed as mean fold induction over vehicle treated control ± SD ($n=9$). Statistical significance was determined by ANOVA, followed by comparisons of the treatment means with control using Dunnett's method, $^{*}p<0.05$.

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 10-fold and 17-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 endosulfan 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 endosulfan sulfate did not induce *CYP2B6* and *CYP3A4* promoter activity (data not shown).

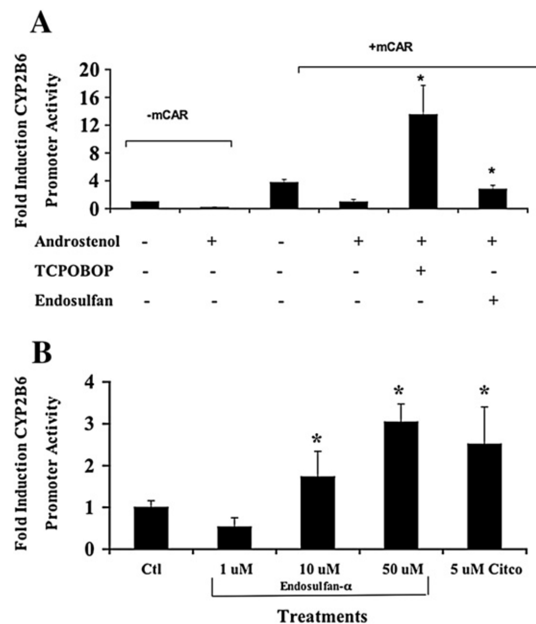

Fig. 3. Endosulfan-α induction of the *CYP2B6* promoter activity via mCAR and hCAR-3. (A) HepG2 cells were transfected with *CYP2B6*-Luciferase reporter, pSG5 or pSG5-mCAR, and β-gal plasmids and treated with the controls dimethyl sulfoxide (DMSO), androstenol, androstenol and TCPOBOP, or androstenol and endosulfan-α. Data shown are means of two independent experiments done in triplicate. Measured luciferase activity was normalized to β-galactosidase activity. The results are expressed as mean fold induction over vehicle treated control ± SD ($n=6$). (B) HepG2 cells were transfected with *CYP2B6*-Luciferase reporter, hCAR-3 and β-gal plasmids and treated with dimethyl sulfoxide control (Ctl) or the positive control 6-(4-Chlorophenyl) imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (Citco), or increasing doses of endosulfan-α. Data shown are means of two–three independent experiments done in triplicate. Measured luciferase activity was normalized to β-galactosidase activity. The results are expressed as mean fold induction over vehicle treated control ± SD ($n=6$–9). Statistical significance was determined by ANOVA, followed by comparisons of the treatment means with androstenol control (4A) or DMSO control (4B) using Dunnett's method, $^{*}p<0.05$.

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 TCPOBOP (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 TCPOBOP, 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-

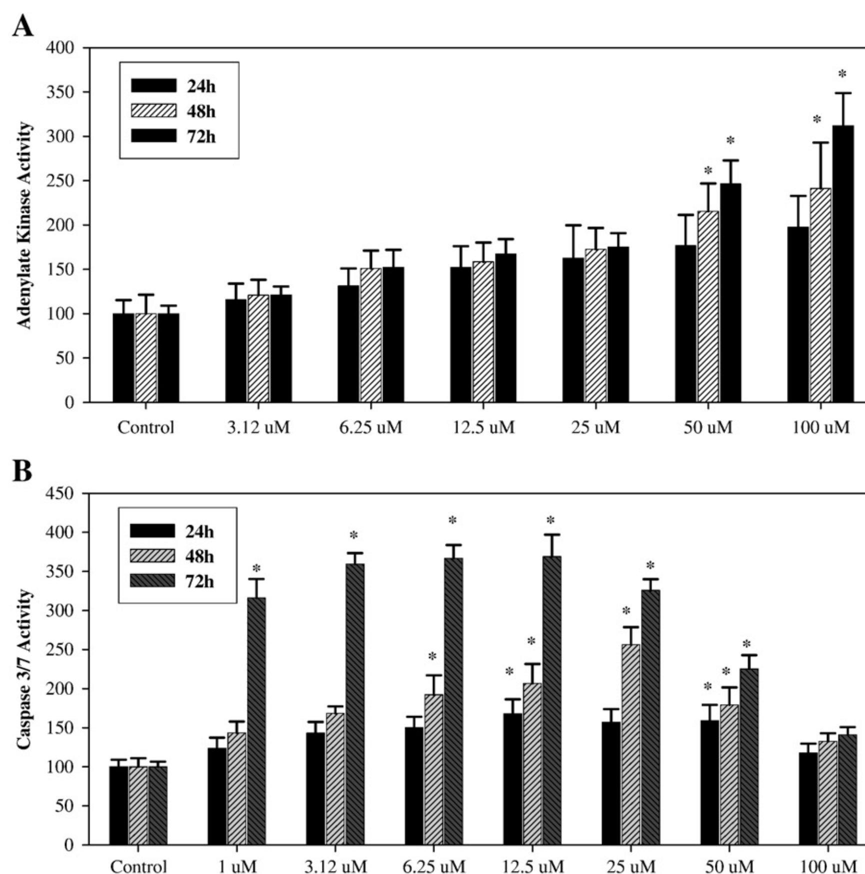

Fig. 4. Endosulfan cytotoxicity to the HepG2 cell line. Dose and time-dependent effects of endosulfan-α on (A) adenylate kinase activity and on (B) caspase-3/7 activity in HepG2 cells was measured as Relative Luminescence Units (RLUs). Data are expressed as means relative to untreated controls ± standard deviation from six determinations. Statistical significance was determined by ANOVA, followed by comparisons of the treatment means with control using Dunnett's method, *$p<0.05$.

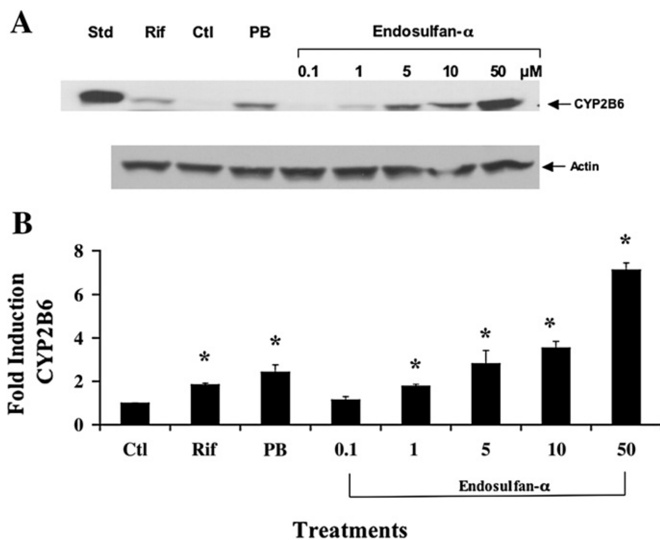

Fig. 5. Endosulfan-α increased CYP2B6 protein levels in human hepatocytes. (A) Human hepatocytes from three individual hepatocyte donors were cultured and treated for 2 consecutive days with vehicle control (Ctl), 10 μM rifampicin (Rif), 50 μM phenobarbital (PB), or increasing doses of endosulfan. Cells were harvested and S9 fraction was prepared for CYP2B6 and β-actin immunoblotting. (B) The integrated intensity of CYP2B6 and β-actin was determined to quantitate the relative induction of CYP3A4 protein and is represented as fold induction over vehicle treated control ± SD ($n = 3$). Statistical significance was determined by ANOVA, followed by comparisons of the treatment means with control using Dunnett's method, *$p<0.05$.

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).

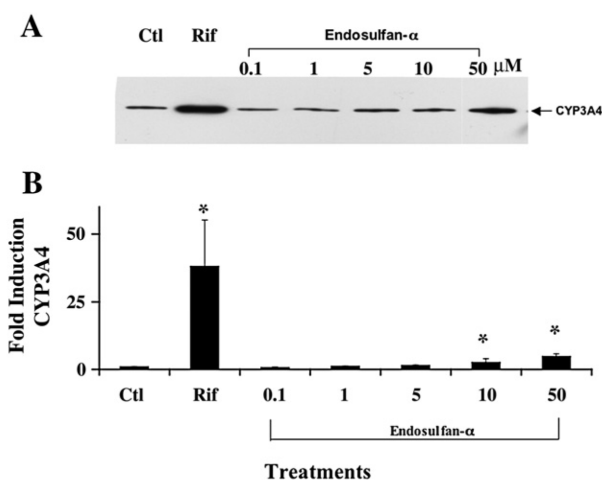

Fig. 6. Endosulfan-α increased CYP3A4 protein levels in human hepatocytes. (A) Human hepatocytes from three individual hepatocyte donors were cultured and treated for 2 consecutive days with vehicle control (Ctl), 10 μM rifampicin (Rif), or increasing doses of endosulfan-α. Cells were harvested and S9 fraction was prepared for CYP3A4 and β-actin immunoblotting. (B) The integrated intensity of CYP3A4 and β-actin was determined to quantitate the relative induction of CYP3A4 protein and is represented as fold induction over vehicle treated control ± SD ($n = 3$). Statistical significance was determined by ANOVA, followed by comparisons of the treatment means with control using Dunnett's method, *$p<0.05$.

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 Dex (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

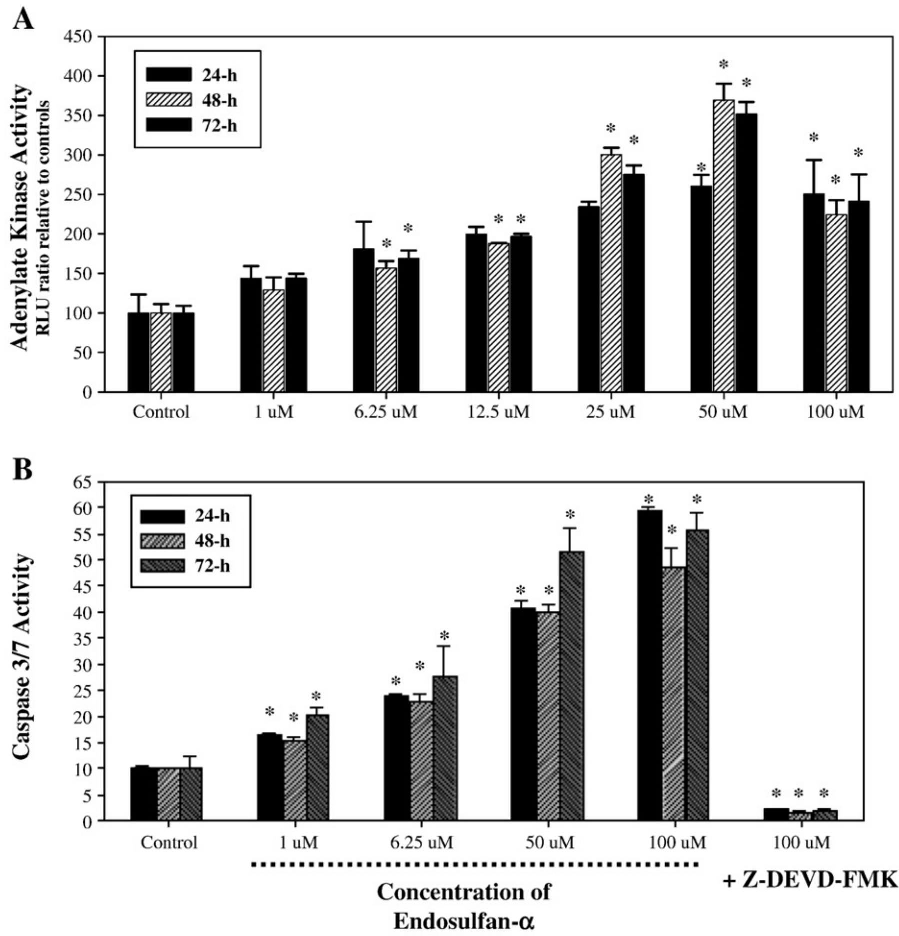

Fig. 7. Endosulfan cytotoxicity to human hepatocytes. Dose and time-dependent effects of endosulfan-α on (A) adenylate kinase activity and on (B) caspase-3/7 activity in primary human hepatocytes was measured as Relative Luminescence Units (RLUs). Data are expressed as means relative to untreated controls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

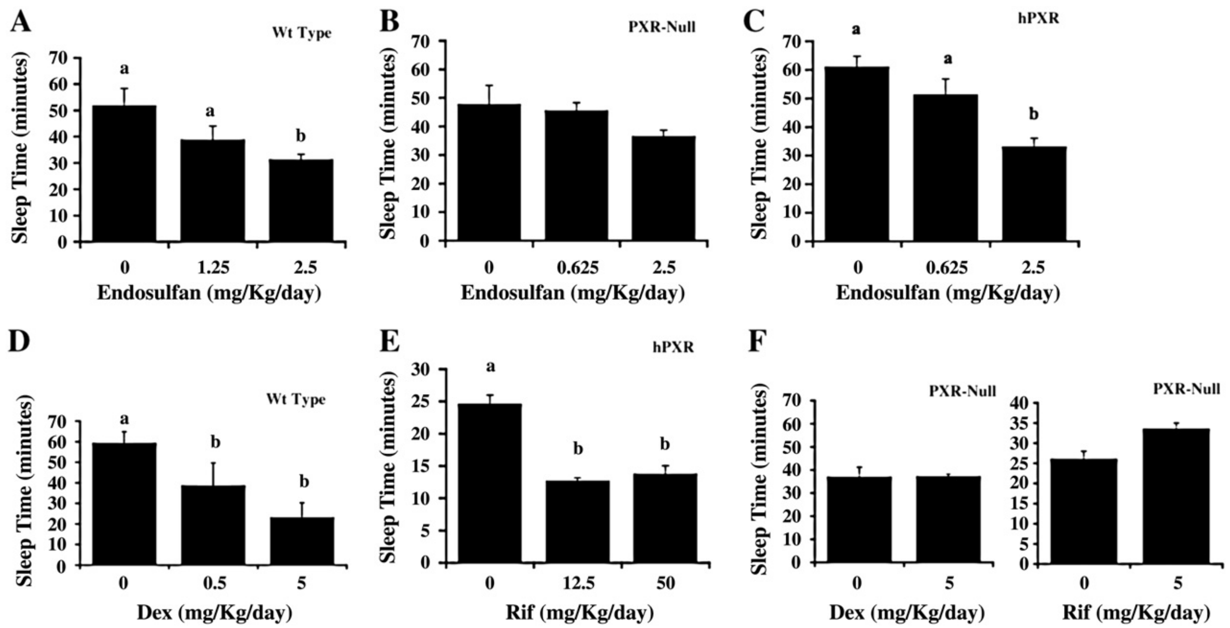

Fig. 8. *In vivo* reduction of induction sleep time by endosulfan-α is dependent on PXR. Male wild type (Wt Type, Panel A), PXR knockout (PXR-null, Panel B), and human PXR (hPXR, Panel C) mice were treated daily with vehicle (peanut oil) or varying doses of endosulfan-α for 7 days. On day eight mice 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 ($p \leq 0.5$).

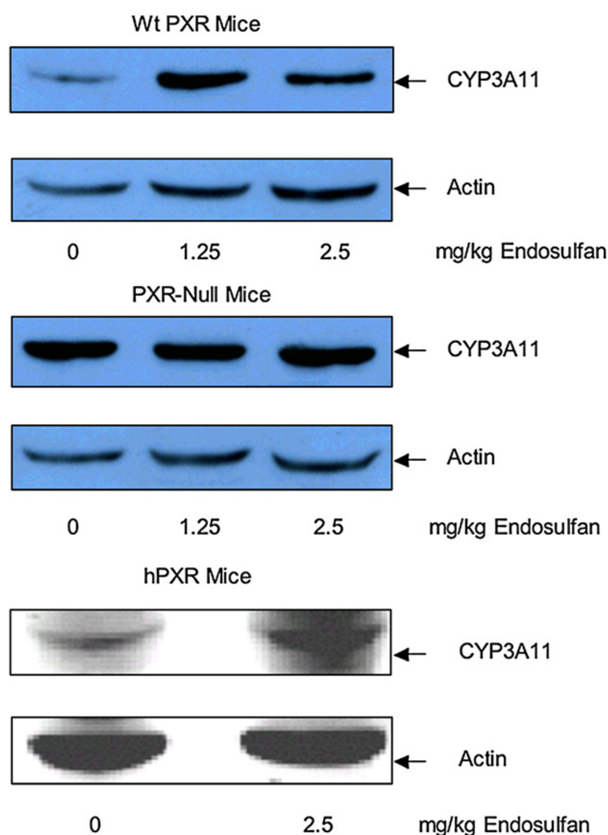

Fig. 9. Endosulfan-α increased CYP3A11 protein levels in Wt PXR and hPXR mice. Liver tissues from Wt Type PXR and PXR-Null mice treated with 0, 1.25, and 2.5 mg/kg, or hPXR-mice with 2.5 mg/kg, endosulfan-α in sleep studies were isolated and S9 fractions were prepared for CYP3A11 and β-actin immunoblotting. CYP3A11 protein was visualized using a rat anti-CYP3A1 antibody and ECL kit (Amersham). A representative western blot analysis is shown.

CAR-dependent weak induction (3-fold) of *CYP2B6* promoter activity (Fig. 3). Either mouse CAR or human CAR was sufficient to significantly upregulate *CYP2B6* promoter activity in the presence of 10 μM endosulfan-α, the lowest concentration that did so. Previous studies in our laboratory also demonstrated that endosulfan-α is primarily metabolized by CYP2B6, with a minor contribution by CYP3A4 (Casabar et al., 2006). Interestingly, as shown in this current communication, endosulfan-α induced *CYP2B6* and *CYP3A4* promoter activity, and these results suggest that endosulfan-α regulates its own metabolism by increasing CYP2B6 and CYP3A4 protein levels, which are the main enzymes responsible for its metabolism. These results also suggest that endosulfan induction is more likely to be mediated thorough PXR activation than CAR activation.

Using primary human hepatocytes, we found that endosulfan-α elevated CYP2B6 and CYP3A4 protein expression in a concentration dependent manner. These results support findings of Lemaire et al. (2004), although no dose–response data were shown. In their study, endosulfan-α upregulated levels for CYP2B6 and CYP3A4 by approximately 2.6- and 1.7-fold, respectively. A similar level of increase for these enzymes was observed in the present study. In addition, whereas the lowest levels of endosulfan-α required to upregulate CYP3A4 protein levels was 10 μM (Fig. 6), significant increases in CYP2B6 protein levels were observed at 1 μM of endosulfan-α (Fig. 5). These results suggest that the increase in CYP2B6 protein levels in human hepatocytes may be more sensitive to endosulfan-α than the increases seen in CYP3A4 protein levels.

The concentrations of endosulfan within the 1–10 μM range used in these studies are relevant to human exposure levels, but concentrations of 50–100 μM are most likely above relevant concentrations. In animal studies, endosulfan and its metabolites were concentrated as much as ten times higher in liver tissues than in blood (Nicholson and Cooper, 1977; Nath et al., 1978; Hoechst, 1986). A study of environmentally exposed children reported significantly higher serum levels of endosulfan-α (1.0×10^{-8} M), endosulfan-β (4.3×10^{-9} M), and total endosulfan (1.8×10^{-8} M) as compared to a control population of children (Saiyed et al., 2003). In these chronically exposed children, decreased testosterone levels and delayed male sexual maturity was observed, though testing for other environmental contaminants that could be responsible for the observed effects was not done. Similarly, a study of the general population of southern Spain detected endosulfan-α, endosulfan-β, or its metabolites in the serum at concentrations reaching 4.8×10^{-8} M, 1.7×10^{-8} M, and 3.58×10^{-7} M, respectively (Carreno et al., 2007).

In the present study our assessment of cytotoxicity determined that levels of adenylate kinase increased in a dose dependent manner due to endosulfan-α treatment in both HepG2 and human hepatocytes. These findings indicate that using endosulfan-α up to 50 μM in HepG2 transfection studies of *CYP* promoter activity did not compromise cell viability at the twenty-four h time point. At 100 μM of endosulfan primary hepatocytes lost their ability to make ATP and release adenylate kinase. Our previous findings using hepatocytes had determined that with varying concentrations of the pesticide fipronil, adenylate kinase activity increased to a peak level and at the highest concentrations of fipronil a decline in adenylate kinase production was 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 IC_{50} 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-transgenetic 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 metabolism 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 CYP3A 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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