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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.

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)

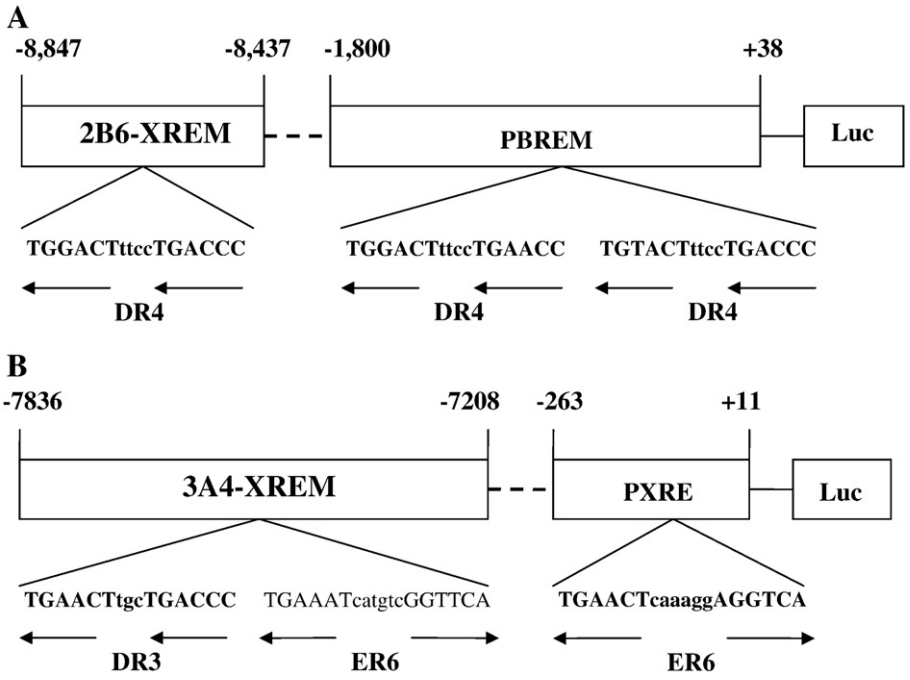

Fig. 1. Map of (A) *CYP2B6*-luciferase and (B) *CYP3A4*-luciferase constructs. The CYP2B6-luc contained the proximal 1.8 kb PBREM (with two DR4 motifs) and the distal 410 bp XREM (with a DR4 motif) of the *CYP2B6* promoter. The *CYP3A4*-luc contained the proximal (+11 to −263) PXRE with an ER6 motif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