ABSTRACT

COOPER, BETH WASILAK. Glucocorticoid Regulation of the Pregnane X Receptor is a Key Determinant in the Magnitude of CYP3A Induction by Xenobiotics. (Under the direction of Andrew D. Wallace.)

The purpose of this research was to investigate the potential for glucocorticoid regulation of the pregnane X receptor (PXR) to be a key determinant in the magnitude of cytochrome P40 3A4 (CYP3A4) induction by different classes of xenobiotics. We hypothesized that the magnitude of CYP3A4 induction by xenobiotics is dependent on the expression level of PXR and may be significantly higher in the presence of glucocorticoids. Glucocorticoids, such as dexamethasone (Dex), have been shown to positively regulate PXR expression by a mechanism involving activation of the glucocorticoid receptor (GR) and play a critical role in CYP3A induction. Previous work in this area led to a proposed two-stage model of CYP3A induction involving GR-mediated induction of PXR expression by glucocorticoids and subsequent PXR-mediated induction of CYP3A by PXR agonists. Most of the clinical implications of PXR action focus on its role as the primary regulator of inducible CYP3A expression. Activation of PXR by clinical drugs, herbal remedies and vitamin supplements has been linked to potentially dangerous drug-drug interactions. CYP3A4 is involved in the metabolism of greater than 60% of all pharmaceutical agents and induction of CYP3A4 expression and activity can result in altered drug metabolism and may affect their duration of action, efficacy, toxicity and resulting side effects. To test our hypothesis, we utilized two different classes of xenobiotics that were either previously shown to activate PXR or speculated to be agonists for PXR. Additionally, we chose classes of xenobiotics for which there was relevant human exposure concomitant with exposure to

glucocorticoids. In the studies presented in Chapter 1, we focused on the phthalate ester di-2-ethylhexyl-phthalate (DEHP) and its primary metabolite mono-2-ethylhexyl-phthalate (MEHP). In Chapter 2, we utilized the anti-cancer drugs, paclitaxel and docetaxel; both members of the taxane class of chemotherapeutics.

As shown in Chapter 1, DEHP and MEHP activated PXR and were capable of inducing *CYP3A4* promoter reporter activity in a dose-dependent manner in the HepG2 human hepatocellular carcinoma cell line. Additionally, utilizing the rat hepatoma cell line, H4IIE-C3, concomitant exposure to phthalates and Dex resulted in significantly enhanced *CYP3A4* promoter activity, well above that observed with any individual treatment. This synergistic effect was significantly abrogated by addition of the GR antagonist RU486, further elucidating the role of GR activation in this two-stage process. Utilizing primary human hepatocytes, we observed that Dex induced PXR protein expression in a dose-dependent manner and that CYP3A protein was highly induced by Dex and DEHP co-administration. Finally, to demonstrate the functional significance of CYP3A4 protein induction, we identified enhanced 6-β-hydroxytestosterone (6β-OHTST) formation from testosterone in human hepatocytes treated with phthalates and Dex.

As shown in Chapter 2, paclitaxel and docetaxel dose-dependently induced PXR-mediated transcription of the *CYP3A4* promoter reporter in HepG2 cells. Concomitant administration of the GR agonist Dex with paclitaxel or docetaxel resulted in synergistic induction of *CYP3A4* promoter activity in H4IIE-C3 cells; far exceeding any induction observed with individual treatments. Additionally, CYP3A protein expression was highly induced in human hepatocyte cultures by co-administration of dexamethasone and paclitaxel.

We conclude that concomitant exposure to glucocorticoids and PXR agonists results in highly induced CYP3A4 expression and activity. Glucocorticoid regulation of PXR heavily influences the magnitude of CYP3A4 induction in response to xenobiotics and co-exposure to these compounds should be further evaluated. Enhanced CYP3A4 enzymatic activity may lead to alterations in metabolism, resulting in deleterious drug-drug interactions, accelerated metabolism and clearance of important pharmaceuticals, bioactivation of some drugs and enhanced testosterone metabolism. Understanding the role of glucocorticoid regulation of PXR as a key determinant in the magnitude of CYP3A4 induction by xenobiotics will provide insight into possible adverse drug effects in susceptible populations.

GLUCOCORTICOID REGULATION OF THE PREGNANE X RECEPTOR IS A KEY DETERMINANT IN THE MAGNITUDE OF CYP3A INDUCTION BY XENOBIOTICS

by

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DEDICATION

I would like to dedicate this work to my loving family. To my husband Isaac, your continued love and support carry me through each day. I am so fortunate to have you by my side through all of life's challenges. To my wonderful parents, I'm the woman I am today because of you. I could never thank you enough for all that you have given me in life.

BIOGRAPHY

On April 30th, 1979 Beth Cooper was born Beth Ann Wasilak to proud parents Phil and Karen Wasilak. She grew up in Derry, a mid-sized town nestled in southeastern NH where loyalty to the Boston Red Sox and the New England Patriots runs deep. When Beth was 5 years old, she asked for a little sister and in October of 1984, the family grew as her parents welcomed a son named Jason. That was Beth's first, valuable lesson in life; you don't always get what you ask for. Growing up, Beth spent time camping on the Maine coast and playing in the lake and spotting moose at camp in Rumford, Maine with her family. From a very young age, Beth showed great compassion for animals and spent lots of time with her cat and very best friend, Sunshine. Beth attended high school at Pinkerton Academy in Derry, where her interest in science developed. After graduating in 1997, Beth enrolled at the University of New Hampshire as an Animal Sciences major with a focus in pre-vet medicine. At some point during her first year of college, Beth let go of her dream to become a veterinarian and changed her degree focus to bioscience & technology. After taking on an undergraduate research project, Beth's interest in working at the lab bench grew and she pondered the possibility of graduate school. It wasn't until she took an endocrinology class her senior year and learned about endocrine disruption, that Beth's interest in the field of toxicology was piqued. After graduating from UNH in 2001, Beth took what ended up to be two years off from school and worked as a laboratory technician in the R&D department at Stonyfield Farm, Inc. in Londonderry, NH. Although she enjoyed working for an environmentally conscious company, making yogurt wasn't really her thing, so Beth decided to pursue graduate school and got accepted to the Toxicology department at North Carolina

State University. In 2003, Beth and her fiancé Isaac said good-bye to winters in NH and moved to Raleigh, NC. On May 12th, 2006, Beth Wasilak became Beth Cooper when she and Isaac wed in an intimate ceremony at the historic Tucker House in Raleigh, NC. Beth and Trooper Cooper share their home in Knightdale, NC with their two fancy felines, Wicket and Bailey.

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INTRODUCTION

The superfamily of nuclear receptors

Nuclear hormone receptors comprise a superfamily of ligand-activated transcription factors, that when bound to specific sequences of DNA, act as switches to regulate gene transcription. These receptors play important roles in development, reproduction, metabolism and maintaining homeostasis. Members of the family share a common structure consisting of a highly conserved central DNA-binding domain (DBD) and a less conserved carboxyterminal ligand-binding domain (LBD). Additionally, these receptors contain similar subdomains for receptor dimerization, nuclear translocation and binding of nuclear receptor co-regulators. Ligand activated nuclear receptors regulate the expression of target genes by binding to specific promoter or enhancer regions containing DNA response elements with one or two core half-sites, separated by a varying number of nucleotides.

Presently, the human nuclear receptor family has 48 members. Within the superfamily of nuclear receptors there are several subfamilies that can be classified based on their dimerization and DNA-binding properties. Class I includes the known steroid hormone receptors which function as ligand induced homodimers and bind hormone response elements composed of two DNA half-sites organized as a palindrome with a three nucleotide spacer. Members of this class include the estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Class II receptors are the nonsteroid hormone receptors which preferentially bind as heterodimers with the 9-*cis* retinoic acid receptors (RXRs) to two DNA half-sites organized as direct repeats. Members of this class include those for thyroid hormone (TR), retinoic acid (RAR) and vitamin D (VDR). A third subclass contains the orphan receptors for which

no known endogenous ligands have been identified. This class of receptors bind primarily to direct repeats as homodimers or bind extended core sites as monomers (Glass, 1994; Mangelsdorf and Evans, 1995). The orphan receptors were cloned based on their high degree of homology to classic steroid receptors, but physiological ligands have not been identified for all of these receptors (Kliewer *et al.*, 2002). The orphan subfamily is now known to include "adopted" receptors for fatty acids, bile acids, cholesterol metabolites and lipophilic xenobiotics.

Identification of the pregnane X receptor

The pregnane X receptor (PXR) (NR1I2), also known as the steroid and xenobiotic receptor (SXR), is a member of the nuclear receptor subfamily. Credit for the cloning and discovery of PXR is attributed to three different groups that simultaneously identified PXR cDNA clones from human (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998) and mouse (Kliewer *et al.*, 1998) genomic libraries. Since then, monkey, dog, rabbit and rat PXR have been cloned, as well as similar receptors in chicken and fish. Across species, the DBDs of mammalian PXRs are highly conserved, sharing more than 95% amino acid identity, but the LBDs of the PXRs are more divergent than those of other nuclear receptors (Kliewer *et al.*, 2002). PXR is highly expressed in liver, colon and small intestine; tissues associated with detection and metabolism of xenobiotics and potentially toxic endobiotics. Additionally, PXR mRNA has also been detected in kidney, stomach, fetal liver, thalamus, spinal cord, lung, uterus, ovary, adrenal gland, heart and bone marrow (Lamba *et al.*, 2004; Zhang *et al.*, 1999) as well as normal and neoplastic breast tissue (Dotzlaw *et al.*, 1999).

In the mouse, two PXR isoforms have been identified as PXR.1 and PXR.2, a splice variant of PXR.1. PXR.2 is identical to PXR.1 except for a deletion of 123 nucleotides, resulting in deletion of a single exon. PXR.2 cDNA encodes a 390 amino acid protein that lacks a 41 amino acid region in the LBD of PXR.1 (Kliewer *et al.*, 1998). In humans, three PXR isoforms have been identified: hPXR.1, hPXR.2 and hPXR.3 (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Lehmann *et al.*, 1998). Human PXR.1 and hPXR.2 are encoded by two different mRNAs containing divergent 5' untranslated regions. While hPXR.1 protein has 434 amino acids, hPXR.2 has an additional 39 amino acids at the N-terminal region. Human PXR.3 protein is identical to hPXR.1, but contains a deletion of 37 amino acids in the LBD; a result of mRNA alternative splicing.

Functional characteristics of PXR

PXR is distinct in the nuclear receptor superfamily because it possesses a large ligand binding pocket with a cavity volume that is substantially larger than that of other nuclear receptors. The large volume of the pocket is due in part to the presence of a five-stranded β-sheet, while other nuclear receptors have only 2 or 3 strands in their structure (Watkins *et al.*, 2002; Watkins *et al.*, 2001). Additionally, a flexible loop structure with the ability to expand and contract provides PXR with the ability to accommodate both small and large structurally diverse ligands (Watkins *et al.*, 2002; Watkins *et al.*, 2001). Mapping the three-dimensional structure of the PXR LBD by x-ray crystallography has provided insight into the molecular basis for the remarkable promiscuity that characterizes PXR ligand binding properties. Species-specific differences in ligand binding exist as the LBDs of the PXRs vary considerably in sequence homology. A small number of amino acid differences have been

shown to be responsible for species specific ligand binding differences between rodents and humans (Jones *et al.*, 2000; Watkins *et al.*, 2001).

Nuclear receptors primarily act through direct association with specific DNA sequences known as hormone response elements. The nonsteroid hormone receptors bind to response elements composed of two copies of the consensus binding motif AG(G/T)TCA (Mangelsdorf *et al.*, 1995). Like most nonsteroid and orphan nuclear receptors, PXR binds to DNA elements in the promoter regions of genes as a heterodimer with RXRα. The response elements recognized by the PXR/RXR heterodimer can be arranged as direct repeats with 3 to 5 nucleotide spacers separating the binding sites (DR-3, DR-4 and DR-5 elements), as well as everted repeats separated by 6 or 8 bases (ER-6 and ER-8 elements) (Carnahan and Redinbo, 2005; Kliewer *et al.*, 2002). These elements have been identified in the promoter regions of PXR responsive genes.

The list of genes regulated by PXR is extensive and growing rapidly. Ligand-activated PXR has been shown to stimulate the expression of genes involved in the oxidation, conjugation and elimination of xenobiotics as well as genes that are central to cholesterol and bile acid metabolism and excretion. The first gene products shown to be regulated by PXR were members of the cytochrome P450 3A family (CYP3A). Additional phase I genes regulated by PXR include members of the CYP2B and CYP2C families. Phase II genes upregulated by PXR agonists include the glutathione-S-transferases, sulfotransferases and UDP-glucuronosyltransferases. These genes are involved in conjugating xenobiotics. Hepatic transporters important for Phase III elimination of pharmaceuticals and other xenobiotics are also regulated by PXR and include Oatp2, MRP2 and MDR1 (Carnahan and Redinbo, 2005; Kliewer *et al.*, 2002). Overall, PXR plays a central role in coordinating and

regulating the many genes involved in the process of drug metabolism and elimination of xenobiotics from the body.

CYP3A4: Target gene regulation by PXR

Perhaps the most important and well studied of the PXR-regulated genes is that of CYP3A4. As a member of the cytochrome P450 superfamily of heme-dependent monooxygenases, CYP3A4 catalyzes the first oxidative step in the detoxification of lipophilic chemicals (Nelson *et al.*, 1996). Members of the CYP3A subfamily are the most abundant in human liver and intestine, tissues that are routinely exposed to xenobiotics, and they demonstrate broad substrate specificity (Guengerich, 1999; Wrighton *et al.*, 2000). CYP3A family members are highly inducible by xenobiotics and many of the chemicals that induce CYP3A expression are also substrates for CYP3A enzymes; resulting in a regulatory mechanism for amplifying the detoxification response to xenobiotic insult (Denison and Whitlock, 1995; Quattrochi and Guzelian, 2001).

Most of the clinical implications of PXR action focus on its role as the primary regulator of inducible CYP3A expression. CYP3A4 alone is involved in the metabolism of greater than 50% of all pharmaceutical agents. Activation of PXR by clinical drugs, herbal remedies and vitamin supplements has been linked to potentially dangerous drug-drug interactions. Induction of CYP3A4 can result in altered drug metabolism and may affect the duration of action of drugs, their efficacy, toxicity and side effects. A well known example of drug-drug interaction is the increased incidence of failure of oral contraceptives in women who receive the antibiotic rifampicin, a known PXR agonist. The PXR-mediated induction of CYP3A4, as well as UDP-glucuronosyltransferase, by rifampicin resulted in enhanced

clearance of the pharmaceutical hormone levels in patients taking oral contraceptives (LeBel *et al.*, 1998). Another well documented example involves the activation of PXR by hyperforin, a component of the herbal anti-depressant St. John's wort (Moore *et al.*, 2000; Wentworth *et al.*, 2000). The use of St. John's wort has been shown to induce CYP3A4 expression and enhance the metabolism of various therapeutic agents including the immunosuppressant cyclosporin, the anticoagulant warfarin and the antiviral indinavir, used to treat HIV (Breidenbach *et al.*, 2000; Ernst *et al.*, 1998; Piscitelli *et al.*, 2000; Ruschitzka *et al.*, 2000). Taken together, these observations indicate that potentially dangerous drug-drug and supplement-drug interactions may result from activation of PXR. The availability of PXR assays has created an opportunity in the drug development process to screen for CYP3A inducers before human clinical testing.

Because PXR plays such a pivotal role in regulating metabolic genes, and as such has been deemed the master regulator of metabolism, it is important to understand how PXR itself is regulated. Very little is known about PXR gene expression and how it is regulated. The PXR promoter region and the transcription factors driving its expression have not been investigated. Pascussi and coworkers (2001) demonstrated that low doses of the synthetic glucocorticoid dexamethasone (Dex) are able to induce PXR mRNA expression in human hepatocyte cultures. It has been hypothesized that this effect is mediated by the glucocorticoid receptor (GR) and putative GR responsive elements in the PXR promoter have been identified (Kurose *et al.*, 2005). Additionally, two groups have demonstrated a synergistic induction of CYP3A promoter activity and mRNA expression after concomitant treatment with Dex and PXR agonists (Huss and Kasper, 2000; Pascussi *et al.*, 2001). These findings have led to a proposed two-stage model of CYP3A induction involving GR-

mediated induction of PXR expression by Dex and subsequent PXR-mediated induction of CYP3A by PXR ligands. This model serves as the basis of our hypothesis, which states that glucocorticoid regulation of PXR may be a key determinant in the magnitude of CYP3A4 induction by xenobiotics.

Phthalate ester plasticizers

In Chapter 1 we report on our investigation of the effect of co-exposure to phthalate esters and glucocorticoids on CYP3A4 expression and activity. Phthalate esters, including di-2-ethylhexyl phthalate (DEHP), are widely used plasticizers that confer durability and flexibility to otherwise rigid polyvinyl chloride (PVC) products. They are extensively used in medical products such as IV fluid bags, blood bags and medical tubing. Since these plasticizers are not chemically bound to the plastic matrix, they can leach out of PVC when in contact with lipophilic substances, blood and many therapeutic drug formulations (Tickner *et al.*, 2001). Although there is daily ambient exposure to DEHP in the general population, hospital patients are uniquely exposed to high DEHP levels. Exposure to phthalates has been linked to a variety of adverse effects including hepatotoxicity, teratogenicity and endocrine disrupting effects resulting from decreased steroidogenesis (Foster *et al.*, 2001; Tickner *et al.*, 2001). In rodent models, *in utero* exposure to DEHP resulted in epididymal and testicular lesions in male offspring (Gray *et al.*, 1999) while human prenatal phthalate exposure has been linked to developmental abnormalities including decreased anogenital distance in males (Swan *et al.*, 2005).

Although many of the adverse effects associated with phthalate exposure are mediated by PPARα (Lapinskas *et al.*, 2005), there is some evidence to show that exposure

to DEHP and the primary metabolite MEHP may affect PXR-mediated CYP3A expression (Fan *et al.*, 2004; Kim *et al.*, 2003). Additionally, pharmacological doses of glucocorticoids such as Dex are routinely administered to premature infants to aid in lung development as well as being used in the treatment of immune disorders and as part of cancer chemotherapy regimes. We were interested in understanding how Dex and DEHP co-exposure would alter CYP3A4 expression and enzymatic activity in this susceptible subpopulation. We suggest that the magnitude of CYP3A4 induction by phthalates is dependent on the expression of PXR and may be significantly higher in the presence of glucocorticoids.

Taxane chemotherapeutic drugs

In Chapter 2 we investigated a similar hypothesis, but in this study we were interested in the effects of concomitant exposure to glucocorticoids and chemotherapeutic taxanes on CYP3A4 expression. Paclitaxel (Taxol®) and docetaxel (Taxotere®) are semisynthetic chemotherapeutic drugs known as taxanes. Collectively, these agents are used to effectively treat various malignancies; including ovarian, breast, head and neck, lung and prostate cancer. These agents share a unique mechanism of action by promoting the polymerization of microtubules, resulting in cell cycle arrest, cellular apoptosis and cytotoxicity (Herbst and Khuri, 2003; Horwitz, 1994; Orr *et al.*, 2003). For paclitaxel and docetaxel, the major route of elimination is hepatic metabolism by cytochrome P450s followed by biliary excretion. It has been shown that paclitaxel is metabolized by CYP2C8 and CYP3A4 isoforms (Cresteil *et al.*, 1994; Harris *et al.*, 1994; Rahman *et al.*, 1994) while docetaxel is primarily metabolized by CYP3A4 (Marre *et al.*, 1996). Paclitaxel and docetaxel are administered by intravenous infusion and Dex pretreatment is recommended for all chemotherapeutic treatments with

these agents to prevent anaphylaxis and hypersensitivity reactions. We were interested in understanding how co-administration of Dex and paclitaxel or docetaxel would alter CYP3A4 expression and enzymatic activity in this susceptible subpopulation. For cancer patients receiving chemotherapeutic treatment with the taxanes, enhanced CYP3A4 activity may have deleterious effects on therapeutic blood levels as CYP3A4 is responsible for the oxidative metabolism of these agents. This may result in altered pharmacokinetics and decreased efficacy of these important, potentially life-saving drugs.

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CHAPTER 1

Phthalate Induction of CYP3A4 is Dependent on Glucocorticoid Regulation of PXR Expression

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ABSTRACT

Cytochrome P450 3A4 (CYP3A4), the most abundant of the phase I enzymes expressed in the liver, is responsible for oxidative metabolism of more than 60% of all pharmaceuticals. CYP3A4 is highly inducible by xenobiotics that activate pregnane X receptor (PXR), and enhanced 3A4 activity has been implicated in adverse drug interactions. Recent evidence has suggested that the widely used plasticizer, di-2-ethylhexyl phthalate (DEHP), and its primary metabolite MEHP may act as agonists for PXR. Hospital patients are uniquely exposed to high levels of DEHP as well as being administered glucocorticoids. Glucocorticoids positively regulate PXR expression in a glucocorticoid receptor (GR)-mediated mechanism. We suggest that the magnitude of CYP3A4 induction by phthalates is dependent on the expression of PXR and may be significantly higher in the presence of glucocorticoids. DEHP and MEHP induced PXR-mediated transcription of the *CYP3A4* promoter in a dose-dependent fashion. Co-exposure to phthalates and dexamethasone (Dex) resulted in enhanced *CYP3A4* promoter activity; furthermore, this induction was abrogated by the GR antagonist, RU486. Dex induced PXR protein expression in a dose-dependent manner in both human hepatocytes and liver-derived rat cell line. Additionally, CYP3A4 protein was highly induced by Dex and DEHP co-administration in human hepatocyte cultures. Finally, enhanced 6-β-hydroxytestosterone (6β-OHTST) formation in Dex and phthalate co-treated human hepatocytes confirmed CYP3A4 enzyme induction. Concomitant exposure to glucocorticoids and phthalates resulting in enhanced metabolic activity of CYP3A4 may play a role in altered efficacy of pharmaceutical agents as well as endocrine disruption. Understanding the role of glucocorticoid regulation of PXR as a key determinant

in the magnitude of CYP3A4 induction by xenobiotics may provide insight into possible adverse drug effects in a sensitive population.

INTRODUCTION

Di-2-ethylhexyl phthalate (DEHP) is a widely used plasticizer for medical products such as IV fluid bags, blood bags and medical tubing. Phthalate plasticizers are not chemically bound to the plastic matrix and leach out of PVC products over time and with use. Lipophilic substances and many therapeutic drug formulations actually enhance the extraction of DEHP from plastics (Tickner *et al.*, 2001). Phthalates are ubiquitous environmental contaminants with human exposure to DEHP occurring by various routes. Hospital patients are uniquely exposed to high DEHP levels. Concern has been raised in the pediatric medical setting due to reports indicating that hospitalized neonates receiving intensive therapeutic interventions may have exposure levels up to three orders of magnitude above ambient exposures for the general population (Shea, 2003). Research has linked DEHP or its metabolites to a variety of adverse effects including hepatotoxicity, nephrotoxicity, teratogenicity and testicular toxicity. The developing animal is highly susceptible to the endocrine disrupting effects of phthalates, and studies have shown that exposure to high levels of DEHP causes testicular dysgenesis syndrome (Foster *et al.*, 2001; Tickner *et al.*, 2001). The National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) recently released an updated evaluation of the scientific evidence that DEHP is a reproductive or developmental toxicant (Kavlock *et al.*, 2006).

It is well established that the peroxisome proliferative and hepatocarcinogenic effects of phthalates are mediated by the nuclear receptor, peroxisome proliferator-activated receptor (PPARα). Research showing that DEHP-induced renal and testicular toxicities may also be mediated in a PPARα-independent fashion suggests that other signaling pathways may play a

role in eliciting the toxicological effects of phthalates(Ward *et al.*, 1998). Hurst and Waxman (2004) showed that the nuclear receptor pregnane X receptor (PXR) is activated by MEHP and other phthalate monoesters. PXR, also know as the steroid and xenobiotic receptor (SXR), is considered to be a master regulator of xenobiotic metabolism, capable of binding response elements in the promoter regions of many key metabolic genes.

PXR, a member of the nuclear receptor superfamily, has a unique flexible ligand binding pocket able to bind a large spectrum of structurally diverse compounds known to induce target gene expression (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Kliewer *et al.*, 2002; Kliewer *et al.*, 1998). With the identification of PXR, a novel signaling pathway for CYP3A induction by xenobiotics and endogenous steroids was discovered. Cytochrome P450 3A4 (CYP3A4), the most abundant Phase I enzyme in adult human liver, is responsible for the oxidative metabolism of more than 60% of pharmaceuticals and other xenobiotics (Li *et al.*, 1995). CYP3A4 gene expression is highly inducible by a variety of xenobiotics, resulting in altered drug metabolism and drug-drug interactions in addition to enhanced metabolism of endogenous substrates such as testosterone. Xenobiotic response elements in both the proximal promoter and distal enhancer regions of the CYP3A4 gene have been identified as PXR binding sites.

Glucocorticoids are steroid hormones, essential for normal growth and development, for liver and immune functions, and in mediating stress responses. The actions of glucocorticoids are mediated by binding to the glucocorticoid receptor (GR), a member of the superfamily of nuclear receptors. One of the most potent and widely prescribed synthetic glucocorticoids is dexamethasone (Dex), which is used in the treatment of cancer and immune disorders, and to prevent postnatal complications due to mechanical ventilation.

Additionally, circulating levels of endogenous glucocorticoids will rise in response to stress. Low concentrations of glucocorticoids, such as Dex, play a critical role in *CYP3A4* expression and induction through a process involving GR-mediated PXR mRNA accumulation (Pascussi *et al.*, 2000). These findings have led to a proposed two-stage model of CYP3A induction involving GR-mediated induction of PXR expression by glucocorticoids and subsequent PXR-mediated induction of CYP3A by PXR ligands (Huss and Kasper, 2000; Pascussi *et al.*, 2001). Based on this model, we suggest that the magnitude of CYP3A4 induction by phthalates is dependent on the expression of PXR and may be significantly higher in the presence of glucocorticoids.

We show that DEHP and the primary metabolite MEHP activated PXR and are capable of inducing *CYP3A4* promoter reporter activity. Additionally, co-exposure to phthalates and Dex resulted in enhanced *CYP3A4* promoter activity and this effect was abrogated by the GR antagonist RU486. Utilizing primary human hepatocytes, we observed that Dex induced PXR protein expression in a dose-dependent manner and that CYP3A4 protein was highly induced by Dex and DEHP co-administration. Finally, to demonstrate the functional significance of CYP3A4 protein induction, we identified enhanced 6-β-hydroxytestosterone (6β-OHTST) formation from testosterone by human hepatocytes treated with phthalates and Dex. Co-exposure to glucocorticoids and phthalates resulting in enhanced metabolic activity of CYP3A may play a role in endocrine disruption as well as altered efficacy of pharmaceutical agents.

MATERIALS AND METHODS

Chemicals:

1,4-pregnadien-9 -fluoro-16 -methyl-11, 17, 21-triol-3, 20-dione (dexamethasone), 4-androsten-17β-ol-3-one (testosterone) and 4-androsten-6β, 17β-diol-3-one (6β-OHTST) were purchased from Steraloids, Inc. (Newport, RI). Rifampicin, Mifepristone (RU486), DEHP and dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Mono-2-ethylhexyl phthalate (MEHP) was purchased from TCI America (Portland, OR). All cell culture media and supplements were purchased from Mediatech, Inc. (Herndon, VA) unless otherwise noted. All primary hepatocyte culture medium and reagents were GIBCO brand, purchased from Invitrogen Corp. (Carlsbad, CA).

Plasmids:

The plasmid CYP3A4-luc, a kind gift of Dr. Jean-Marc Pascussi, contains -7836/-7208 nt of the distal enhancer region (XREM) cloned 5' of -263/+11 nt of the proximal promoter of the *CYP3A4* gene cloned into the pGL3 reporter vector (Promega, Madison, WI). The plasmid hPXR-pSG5, a kind gift of Dr. Steven Kliewer, contains hPXR.1 cloned into the pSG5 expression vector (Stratagene, La Jolla, CA). The control vector pSV-Beta-galactosidase was purchased from Promega (Madison, WI).

Cell Culture and Transactivation Experiments:

The human hepatocellular carcinoma, HepG2, cell line and the rat hepatoma H4IIE-C3 cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured according to ATCC recommendations. HepG2 cells were transfected in Opti-

Mem I reduced serum medium (Invitrogen Corp.) using *Trans*IT-LT1 reagent (Mirus Bio Corp., Madison, WI) according to the manufacturer's recommendations. The cells were incubated with transfection reagent for 4-6 hrs, then the Opti-MEM medium was removed, and cells were rinsed once and incubated overnight in Cellgro Complete medium. For transfection of the H4IIE-C3 cell line, plated cells were washed with Opti-MEM and transfected using Lipofectamine reagent enhanced by Plus reagent (Invitrogen Corp.) according to the manufacturer's recommendations. The cells were incubated with transfection reagents for 3 hrs before being rinsed once and incubated overnight in complete medium supplimented with 10% dextran-charcoal stripped FBS.

Analysis of Luciferase Activity:

Following transfection, cells were allowed to recover overnight in complete medium then treated with vehicle or specified compound. After 48 hrs of treatment, cells were rinsed with PBS and harvested in 1x Reporter Lysis Buffer from the Luciferase Assay System (Promega Corp.). Cell lysates were prepared according the manufacturer's recommended protocol and luciferase activity was measured using a TD-20/20 Luminometer by Turner Designs, Inc. (Sunnyvale, CA). As an internal transfection control, β-galactosidase assays were performed on cell lysates according to the recommended protocol in the β-Galactosidase Enzyme Assay System (Promega). Luciferase activity was normalized to either β-galactosidase activity or to total protein as indicated and then transformed to fold induction over control.

Human Hepactocyte Culture:

Freshly isolated human hepatocytes plated with Matrigel overlay were received from ADMET (RTP, NC), CellzDirect (Pittsboro, NC) or Cambrex (Walkersville, MD). In primary cultures of human hepatocytes, Matrigel overlay aids in maintaining differentiated characteristics of the cells as well as facilitating the induction of xenobiotic metabolizing enzymes (Gross-Steinmeyer *et al.*, 2005). Cells were cultured for 2-3 days in William's E medium with ITS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and free of dexamethasone. Medium was changed every 24 hrs during culture and treatment periods. For western blotting, hepatocytes were treated for 2 consecutive days and cellular lysates were prepared as described below. For testosterone hydroxylase assays, hepatocytes were treated for 3 consecutive days. LeCluyse et al. (2000) demonstrated that maximal activity of CYP3A4 was achieved after 3 days of exposure to inducing agents. The medium was aspirated 24 hrs following the final treatment and cells were rinsed 2 times and then incubated with 100 μM testosterone substrate in fresh medium for 30 min. Medium was removed and combined with equal volume of methanol, vortexed briefly and centrifuged at 21,000 x g for 5 min. The supernatant was used for subsequent HPLC analysis.

Analysis of 6β-OHTST by HPLC:

The generation of metabolites was analyzed using a Waters 2695 HPLC system equipped with a 2996 photodiode array (PDA) detector (Waters, Milford, MA). The HPLC system was equipped with a degasser and an autoinjector and Waters Empower software ver. 5.0 was utilized for data collection and analysis. The mobile phase for pump A was 5% tetrahydrofuran, 95% water and for pump B was 100% methanol. The flow rate was 0.5

ml/min and a gradient system was employed as described previously (Usmani *et al.*, 2003). Metabolites were separated on an Ultracarb column [Ultracarb 5μ, 150 x 4.6 mm, ODS (30)] (Phenomenex, Rancho Palos Verdes, CA) and detected at 240 nm. 6β-OHTST was detected at the retention time of 15.8 min as determined by the use of a 6β-OHTST standard.

Western Blot Analysis:

For PXR detection, cellular lysates were prepared by resuspending and sonicating the cell pellet in NP-40 Lysis Buffer (25 mM HEPES, 10 mM EDTA, 10 mM EGTA, 3 mM $MgCl_2$, 10% glycerol, 20 mM sodium molybdate and 0.5% IGEPAL) containing protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Indianapolis, IN). For CYP3A4 enzyme detection, S9 fractions were obtained after centrifugation of cell lysates at 19,000 x g. All samples were prepared in Laemmli Buffer containing β-mercaptoethanol and boiled for 5 min. Protein samples were resolved on 8% Novex Tris-Glycine gels (Invitrogen Corp.) and transferred to nitrocellulose membrane. Immunoreactive PXR was detected using an anti-hPXR epitope-specific polyclonal antibody (1:500) raised in rabbits (Covance) followed by detection with horseradish peroxidase-conjugated anti-rabbit secondary antibody. CYP3A4 was detected using a monoclonal mouse anti-CYP3A antibody (Gentest, BD Biosciences) (1:500) and horseradish peroxidase-conjugated anti-mouse secondary antibody. β-actin was detected using rabbit anti-β-actin primary antibody (Sigma). All primary antibodies were incubated overnight at 4º C in TBS with Tween and 1% non-fat dry milk. Immunoblots were visualized using chemiluminescence detection (ECL, GE Healthcare). Densitometric analysis of immunoreactive protein bands was performed using a Kodak Image Station 440 CF with Kodak Molecular Imaging Software.

Statistical Analysis:

All statistical analysis was performed using JMP software, version 6.0.0 (SAS Institute, Inc., Cary, NC). Comparison of means between all treatment groups were made using ANOVA followed by Student's t test with a significance level of 0.01. Comparisons between treatment and control groups were made using ANOVA followed by Dunnett's multiple comparison test with a significance level of 0.05.

RESULTS

It is well established that PXR-mediated induction of CYP3A4 can result in altered metabolism of pharmaceuticals and endogenous steroids, which may lead to adverse drug interactions. The ability of glucocorticoids to positively regulate PXR expression may be a key determinant in the magnitude of CYP3A4 induction by xenobiotics, such as the phthalate esters DEHP and MEHP. To investigate the ability of DEHP to activate human PXR and induce *CYP3A4* promoter activity, HepG2 cells were transiently transfected with expression plasmid containing PXR or empty vector and luciferase reporter containing a portion of the *CYP3A4* promoter with three PXR binding sites (Fig. 1A). In the presence of PXR, 10 μM DEHP induced *CYP3A4* promoter activity 22-fold above that of vehicle control while rifampicin (Rif), the prototypical ligand for hPXR (Zhang *et al.*, 1999), induced the *CYP3A4* promoter 28-fold over control. The *CYP3A4* promoter was not activated in the absence of PXR for any of the treatment groups.

DEHP is rapidly metabolized after all routes of uptake; the first step being cleavage of DEHP into the monoester MEHP, which is then further metabolized by various oxidation reactions (Albro and Thomas, 1973; Schmid and Schlatter, 1985). We investigated the ability of the primary metabolite, MEHP, to activate PXR and induce *CYP3A4* promoter activity. HepG2 cells were utilized in transient transfection studies with the expression plasmid for PXR and *CYP3A4*-luciferase reporter. Both DEHP and MEHP induced *CYP3A4* promoter activity in a dose-dependent manner in the presence of PXR, with maximal activation at 25 μM for both compounds (Fig. 1B). Neither DEHP nor MEHP activated the promoter construct in the absence of PXR (data not shown). At each concentration, DEHP induction of *CYP3A4* was greater than MEHP. Since patients undergoing multiple medical

procedures are uniquely exposed to high levels of phthalates and many are also administered glucocorticoids, such as dexamethasone, we evaluated the effects of co-exposure to Dex and DEHP or MEHP on *CYP3A4* promoter activity. Transient transfection of rat hepatoma H4IIE-C3 cells was performed using the *CYP3A4*-luciferase reporter. H4IIE-C3 cells are glucocorticoid responsive and express PXR. The *CYP3A4*-luciferase reporter was induced approximately 10-fold by 10 μM DEHP and 5-fold by 0.1 μM Dex, but co-treatment with Dex and DEHP dramatically induced *CYP3A4* activity 65-fold over vehicle treated control (Fig. 2A). Co-exposure to 0.1 μM Dex and 10 μM MEHP co-exposure resulted in 12-fold induction of *CYP3A4* promoter activity over vehicle treated control (Fig. 2B). This led us to hypothesize that GR plays a critical role in the induction of CYP3A4 by glucocorticoids and phthalates. To further elucidate the role of GR in this process, transfected H4IIE-C3 cells were treated with the glucocorticoid antagonist RU486. When 0.1 μM RU486 was administered in addition to Dex and DEHP, *CYP3A4* promoter activity was reduced more than 50% to approximately 30-fold over vehicle treated control (Fig. 3A). Additionally, when 0.1 μM RU486 was administered in conjunction with Dex and MEHP, *CYP3A4*-luciferase reporter activity was reduced to 5-fold over control; less than half of the induction seen with Dex and MEHP co-administration (Fig. 3B). The GR antagonist RU486 effectively abrogates the coordinate Dex and phthalate induction of *CYP3A4* promoter activity.

It's been shown that submicromolar concentrations of Dex increase PXR mRNA expression (Pascussi *et al.*, 2000); however, the effect on PXR protein is unknown. To evaluate the ability of low-dose Dex to induce PXR protein expression we utilized H4IIE-C3 cells and freshly isolated human hepatocytes. Western blot analysis was performed on

cellular lysates prepared after 48 hrs of treatment with increasing doses of Dex (Fig. 4A-B). In H4IIE-C3 rat hepatoma cells, rPXR protein expression was enhanced by 0.01 μM and 0.1 μM Dex in a dose-dependent manner, over that of basal expression (Fig. 4A). Human PXR protein was induced over control in all Dex treated samples in a dose-dependent manner (Fig. 4B).

After observing that Dex and DEHP coordinately induced *CYP3A4* promoter activity in transactivation assays, we were interested in evaluating the effect of co-treatment with Dex and DEHP on CYP3A4 protein expression. Freshly isolated human hepatocyte cultures were treated with DEHP in the presence or absence of Dex and Western blot analysis was performed on cellular lysates to visualize the levels of CYP3A4 protein (Fig. 5A). As indicated by densitometric analysis, treatment with Dex alone induced CYP3A4 protein approximately 7-fold over control, while DEHP alone had no effect on 3A4 protein levels (Fig. 5B). However, in the presence of Dex, DEHP induced CYP3A4 protein to 14-fold over basal expression level.

To assess if changes observed in CYP3A4 protein expression resulted in altered CYP3A4 enzymatic activity, testosterone was used as a specific substrate probe. The utility of measuring testosterone 6β-hydroxylase activity in intact human hepatocyte cultures to monitor CYP3A4 activity has been demonstrated previously (Fayer *et al.*, 2001; Kostrubsky *et al.*, 1999). Formation of the 6β-OHTST metabolite can be correlated to CYP3A4 enzymatic activity (LeCluyse *et al.*, 2000; Waxman *et al.*, 1988). CYP3A4 activity was strongly induced by 10 μM Rif, a known inducer of CYP3A4 (Fig. 6). This effect was greatly enhanced by the presence of Dex. DEHP and MEHP had not effect at 50 and 500 μM

when administered in the absence of Dex (Fig. 6). However, co-administration of Dex resulted in a phthalate dose-dependent increase in 6β-OHTST formation.

DISCUSSION

We have investigated the effects of concomitant exposure to submicromolar concentrations of dexamethasone and phthalate esters, DEHP and MEHP, on CYP3A4 expression and activity. Enhanced CYP3A4 activity has been implicated in cases of drug-drug interactions, decreased efficacy of pharmaceutical agents, and increased toxicity via bioactivation of therapeutics. DEHP and MEHP fall in the broad category of peroxisome proliferators, exhibiting many of their hepatotoxic effects through activation of the peroxisome proliferator-activated receptor α (PPARα) (Lee *et al.*, 1995; Maloney and Waxman, 1999; Ward *et al.*, 1998). PPARα is a member of the nuclear receptor family and is expressed in numerous tissues important in lipid metabolism (Braissant *et al.*, 1996). Studies have shown that exposure to peroxisome proliferators leads to alterations in expression of steroidogenic enzymes involved in the conversion of cholesterol to testosterone (Lehmann *et al.*, 2004) as well as increased expression of cytochrome P450s responsible for metabolism of testosterone, including members of the CYP2C and CYP3A families (Fan *et al.*, 2004; Kim *et al.*, 2003). However, evidence that PPARα null mice develop renal and testicular toxicity in response to DEHP exposure (Ward *et al.*, 1998) presents the possibility that phthalates may elicit toxicity through additional signaling pathways involving other nuclear receptors.

Similar to PPARα, PXR is a member of the nuclear receptor superfamily. It is involved in regulation of metabolic genes and is expressed in liver, kidney, and intestinal tissues. PXR binds a variety of response elements in promoters of genes involved in transport, metabolism and elimination of bile acids, steroids and xenobiotics; most notably, CYP3A4 (Kliewer *et al.*, 2002). The most abundant P450 in adult human liver, CYP3A4

plays a pivotal role in drug metabolism and maintenance of endogenous steroid hormone levels. PXR responsive regions within the *CYP3A* gene promoter confer high inducibility by xenobiotics (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Kliewer *et al.*, 1998). Such induction leads to accelerated metabolism of pharmaceuticals and altered pharmacokinetic and pharmacodynamic properties, as well as enhanced testosterone metabolism and endocrine disrupting effects.

The results of our initial HepG2 cell reporter assays demonstrated that DEHP, as well as the monoester metabolite MEHP; activated PXR-mediated transcription of the *CYP3A4* target gene. The ability of MEHP and other phthalate monoesters to activate mPXR and hPXR was demonstrated previously (Hurst and Waxman, 2004), but the ability of the parent compound DEHP to act as a PXR ligand has not been well studied. We have shown here that DEHP is actually more efficacious than MEHP at activating hPXR, reaching maximal induction of the *CYP3A4*-luciferase reporter at 25 μM. In human colon carcinoma cells, DEHP was shown to activate PXR-mediated transcription of the *MDR1* gene promoter. Additionally, the LBD of human PXR associated with the receptor interacting domain of the co-activator SRC-1 in the presence of 10 μM DEHP in mammalian two-hybrid assays (Takeshita *et al.*, 2006). Taken together, these data suggest that DEHP and MEHP may be ligands for PXR and may initiate PXR-mediated transcription of target genes.

Historically, CYP3A family members across species have demonstrated responsiveness to glucocorticoids, but the Dex responsive sites in *CYP3A* promoters do not contain glucocorticoid responsive elements, and the glucocorticoid receptor (GR) does not associate with these elements (Huss *et al.*, 1996; Quattrochi *et al.*, 1995). Additionally, the time course of *CYP3A* induction following glucocorticoid treatment does not follow that of

other GR-regulated genes, suggesting that the regulation of *CYP3A* induction is a secondary effect of GR activation (Schuetz *et al.*, 1984). Pascussi and coworkers (2000) demonstrated that at high concentrations, Dex can act as a ligand for human PXR and subsequently induce CYP3A4, while treatment with the anti-glucocorticoid, RU486, was shown to inhibit CYP3A4 protein expression. Pre-treatment of human hepatocytes with cycloheximide was shown to inhibit PXR-mediated *CYP3A4* gene expression resulting from treatment with submicromolar concentrations of Dex, but had no effect on PXR mRNA accumulation (Pascussi *et al.*, 2000). These findings led to a proposed two-stage model of CYP3A induction involving GR-mediated induction of PXR expression by low-dose Dex and subsequent PXR-mediated induction of CYP3A by PXR ligands (Huss and Kasper, 2000; Pascussi *et al.*, 2001).

We have demonstrated here that *CYP3A4* promoter activity is significantly increased when DEHP and MEHP are administered in the presence of Dex. This induction of *CYP3A4* transcription is strongly abrogated by co-administration of the GR antagonist, RU486, further confirming that GR activation is necessary for synergistic CYP3A4 induction by glucocorticoid and PXR agonist co-exposure. The H4IIE-C3 rat hepatoma cell line utilized in these reporter assays is an excellent model for evaluating the combined effects of GR and PXR activation. These cells posses a functional GR as well as PXR (Huss and Kasper, 1998), so we were able to measure the effects of our treatments on the endogenous receptors and minimize the artificial nature of a cell-based reporter assay. Huss and Kasper (2000) demonstrated synergistic induction of the rat *CYP3A23* promoter in H4IIE cells by co-treatment with 10 μM pregnenolone-16α-carbonitrile (PCN), an agonist for rat PXR, and 0.1

μM Dex. The resulting transcriptional activity far exceeded that reached with either individual treatment, and our current data agree nicely with their previous findings.

Submicromolar concentrations of Dex increase PXR mRNA expression in human hepatocytes via enhanced transcription of PXR (Pascussi *et al.*, 2000). We have shown that similar concentrations of Dex induced rat PXR protein expression as well as human PXR protein expression in freshly isolated human hepatocytes. In both cases, a dose-dependent increase in PXR protein was observed. Evidence that GR positively regulates the expression of PXR may account for the synergistic effect of Dex and PXR activators on CYP3A induction. In cultured human hepatocytes, concomitant addition of Dex enhanced rifampicin- and clotrimazole-dependent CYP3A4 mRNA induction with maximal induction at 0.1 μM Dex, a concentration known to fully activate the GR (Pascussi *et al.*, 2000). Additionally, we demonstrated that human hepatocyte cultures co-exposed to DEHP and Dex have highly induced CYP3A4 protein levels when compared to basal CYP3A4 expression or to resulting CYP3A4 levels from either individual treatment. Interestingly, there is a slight decrease in CYP3A4 protein due to DEHP treatment alone. This was unexpected based on the induced *CYP3A4* promoter activity that we observed due to DEHP treatment in our reporter assays. We hypothesize that due to low basal PXR expression in human hepatocytes, endogenous CYP3A4 levels are actually negatively regulated and GR stimulation is needed to increase PXR expression in order to see any effect on CYP3A4 by PXR agonists. It is also possible that DEHP binding to PXR LBD causes conformational changes that alter interactions of PXR with specific co-regulators. The addition of Dex and activation of GR may recruit necessary co-activators to the transcriptional assembly, thus promoting PXR stimulation of *CYP3A4* transcription. Additionally, PXR has been shown to

interact with co-repressors in the presence of ligand, resulting in reduced *CYP3A4* promoter activity (Takeshita *et al.*, 2002).

The significance of enhanced CYP3A4 protein expression can be demonstrated by measuring functional increases in testosterone hydroxylase activity and formation of the 6β-OHTST metabolite in human hepatocyte cultures. We have shown here that DEHP and MEHP, as well as rifampicin, dramatically enhanced 6β-OHTST production when co-administered with Dex in human hepatocytes. Our finding that both phthalates did not induce testosterone hydroxylase activity when given in the absence of Dex was expected, since DEHP did not induce CYP3A4 protein levels when given alone.

DEHP is a ubiquitous environmental contaminant resulting in daily ambient exposure for the general population, but various sub-populations are exposed to extremely high levels of this phthalate ester and its primary metabolite MEHP. These populations are subject to additional risk when receiving pharmaceutical doses of glucocorticoids, or even in times of stress when circulating glucocorticoid levels spike. These findings implicate DEHP and MEHP as being involved in induction of enhanced steroid metabolism. This presents a two-pronged effect on steroid hormone levels when you consider the previously established role of these phthalates in decreasing steroidogenesis pathways. Additionally, the alteration of drug metabolism as a result of CYP enzyme induction may be responsible for changes in the efficacy and toxicity of pharmaceutical drugs as well as detrimental drug-drug interactions. The data presented here advance our understanding of how concomitant exposure to glucocorticoids and phthalate esters may alter the expression level and function of CYP3A4, a key player in phase I metabolism.

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FIGURE LEGENDS

Fig. 1 **Phthalates induce PXR-mediated *CYP3A4* promoter activity.**

A, HepG2 cells were transiently transfected with 0.1 μg pSG5 empty vector (*open bars*) or 0.1 μg pSG5-hPXR (*closed bars*), 1.0 μg *CYP3A4*-luc reporter and 0.1 μg β-gal plasmid. After 48 h treatment with DMSO vehicle control, 10 μM Rifampicin (Rif) or 10 μM DEHP, cellular lysates were collected and luciferase and β-galactosidase activity measured. The results are expressed as mean fold induction ± standard error (SE), (n=3). Statistical significance was determined by ANOVA, Dunnett's mean comparison test when compared to ctrl with hPXR; * $p<0.0001$. The data is a single representative of triplicate independent experiments. B, HepG2 cells were transiently transfected with 1.0 μg *CYP3A4*-luc reporter, 0.1 μg pSG5-hPXR and 0.1 μg β-gal plasmid. Cells were treated for 48 h with indicated concentrations of DEHP (*open bars)* or MEHP *(closed bars)*. Measured luciferase activity was normalized to β-galactosidase activity. The results are expressed as mean fold induction over vehicle treated control (data not shown) ± SE, (n=3). Statistical significance was determined by ANOVA, Dunnett's mean comparison test, * $p<0.0001$, **$p=0.0005$ (see fig).

Fig. 2 ***CYP3A4* promoter activity is significantly induced by concomitant exposure to Dex and phthalates**. H4IIE-C3 cells were transiently transfected with 1.0 μg *CYP3A4*-luc reporter and treated for 48 h with 10 μM DEHP or MEHP (as indicated in A or B) either alone or in the presence of 0.1 μM Dex. Controls were treated with DMSO vehicle. Cellular lysates were collected and measured luciferase activity was normalized to total protein per well. The results are expressed as mean fold induction over control ± SE (n=3). Statistical significance was determined by ANOVA, Student's t test, * $p<0.0001$.

Fig. 3 **RU486 abrogates the *CYP3A4* promoter induction from Dex and phthalate co-exposure.** H4IIE-C3 cells were transiently transfected with 1.0 μg *CYP3A4*-luc reporter and treated for 48 h with 10 μM DEHP or MEHP (as indicated in A or B) either alone or in the presence of 0.1 μM Dex and 0.1 μM RU486. Controls were treated with DMSO vehicle. Cellular lysates were collected and measured luciferase activity was normalized to total protein per well. The results are expressed as mean fold induction over control ± SE (n=3). Statistical significance was determined by ANOVA, Student's t test, $p<0.0001$. Treatments not connected by the same letter are significantly different. The data is a single representative of triplicate independent experiments.

Fig. 4 **Dex dose-dependently increases PXR protein levels.**

A, H4IIE-C3 cells were treated for 48 h with varying doses of Dex. Cells were harvested and protein was collected for Western blot analysis using 50 μg of total protein. Membranes were stripped and re-probed for β-actin as a loading control. B, Freshly isolated human hepatocytes in culture were treated for 2 consecutive days with varying doses of Dex. Cells were harvested and protein was collected for Western blot analysis using 7.5 μg total protein. This blot is representative of human hepatocytes from one individual. Triplicate independent experiments were performed with human hepatocytes from additional individuals.

Fig. 5 **CYP3A4 protein is highly induced by Dex and DEHP co-administration.**

A, Human hepatocytes were cultured in Dex free medium and treated for three consecutive days with 5 μM DEHP either alone or in conjunction with 0.1 μM Dex. Cells were harvested

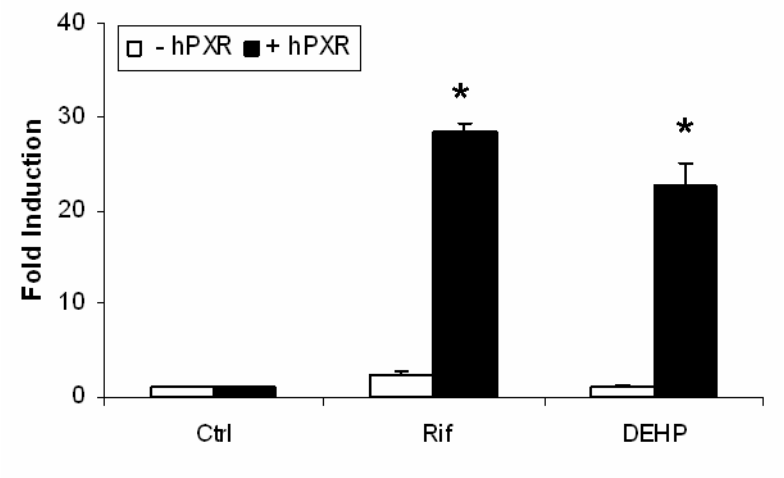
and S9 fraction prepared for CYP3A4 immunoblotting using 4.5 μg protein. B, Densitometric analysis was performed to quantitate the relative induction of CYP3A4 protein and is represented as fold induction over vehicle treated control.

Fig. 6 **6β-OHTST metabolite formation is dramatically enhanced by Dex and phthalate co-administration.**

Testosterone hydroxylase assays were performed on human hepatocyte cultures treated for 3 consecutive days with 10 μM Rifampicin (Rif) or indicated concentrations of DEHP and MEHP either in the absence of Dex *(striped bars)* or in the presence of 0.1 μM Dex *(closed bars)*. This data represents one individual batch of human hepatocytes but is a representative of three independent experiments performed with additional individuals.

FIGURES

A



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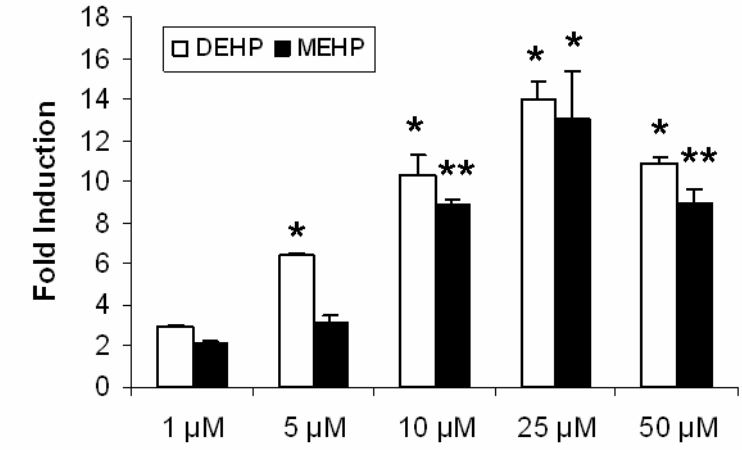
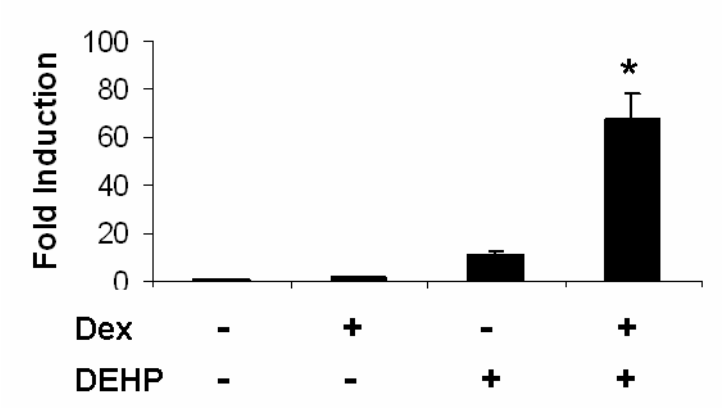


Fig. 1 Phthalates induce PXR-mediated *CYP3A4* promoter activity.

A



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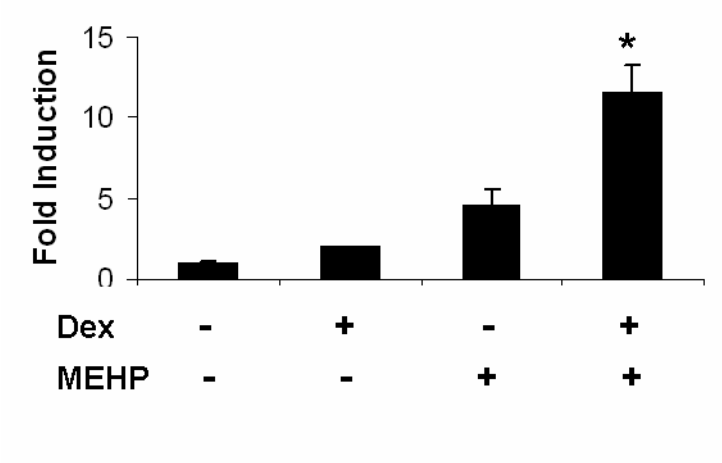
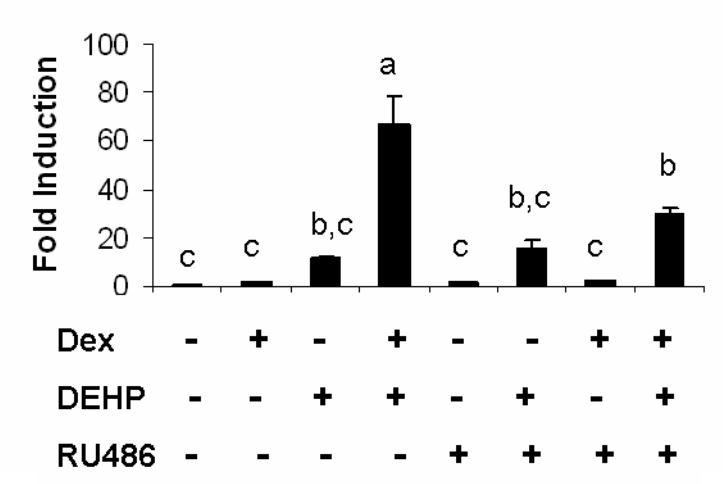


Fig. 2 *CYP3A4* promoter activity is significantly induced by concomitant exposure to Dex and phthalates.

A



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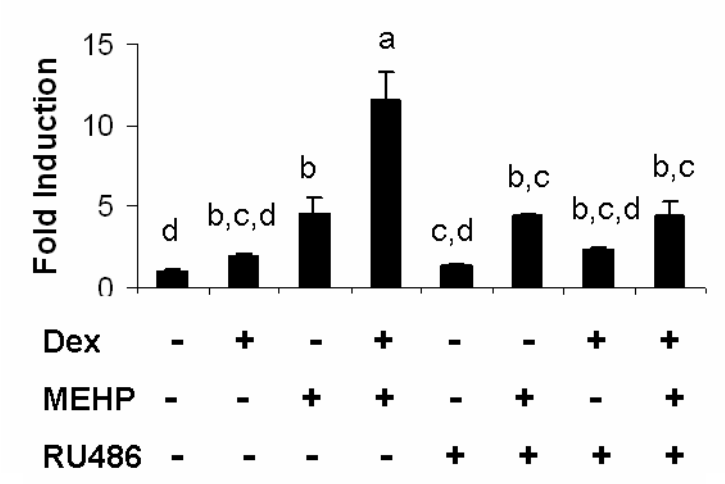
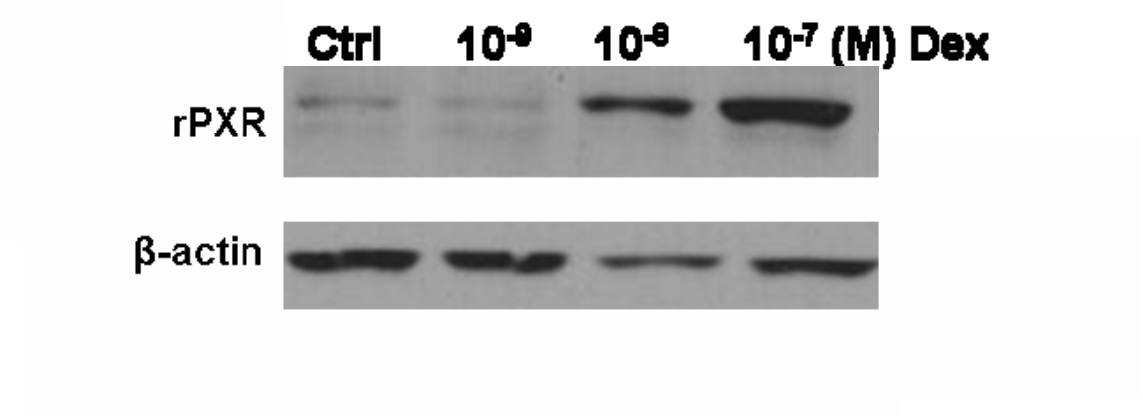


Fig. 3 RU486 abrogates the *CYP3A4* promoter induction from Dex and phthalate co-exposure.

A



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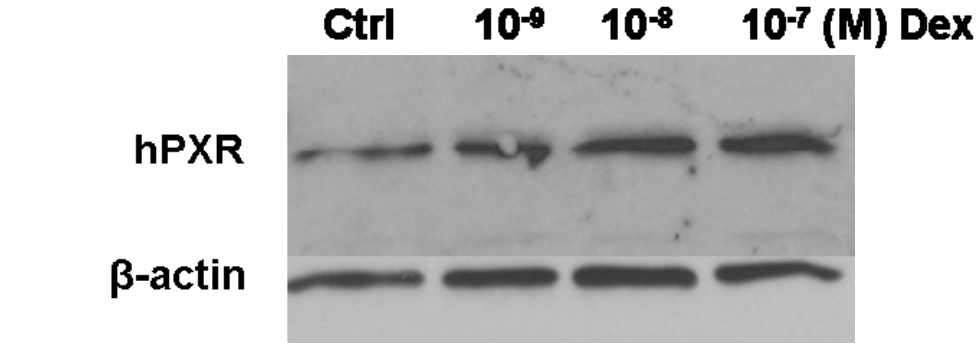
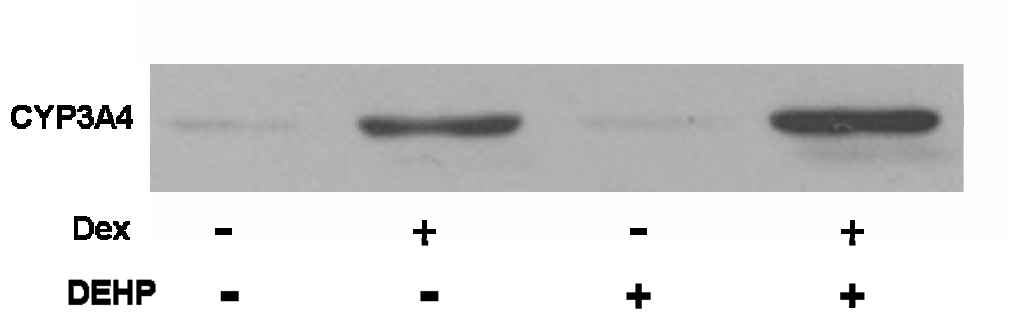


Fig. 4 Dex dose-dependently increases PXR protein levels.

A



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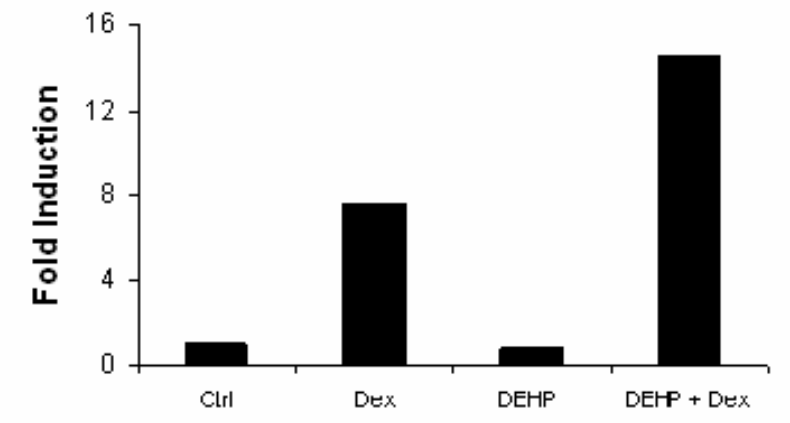

Fig. 5 CYP3A4 protein is highly induced by Dex and DEHP co-administration.

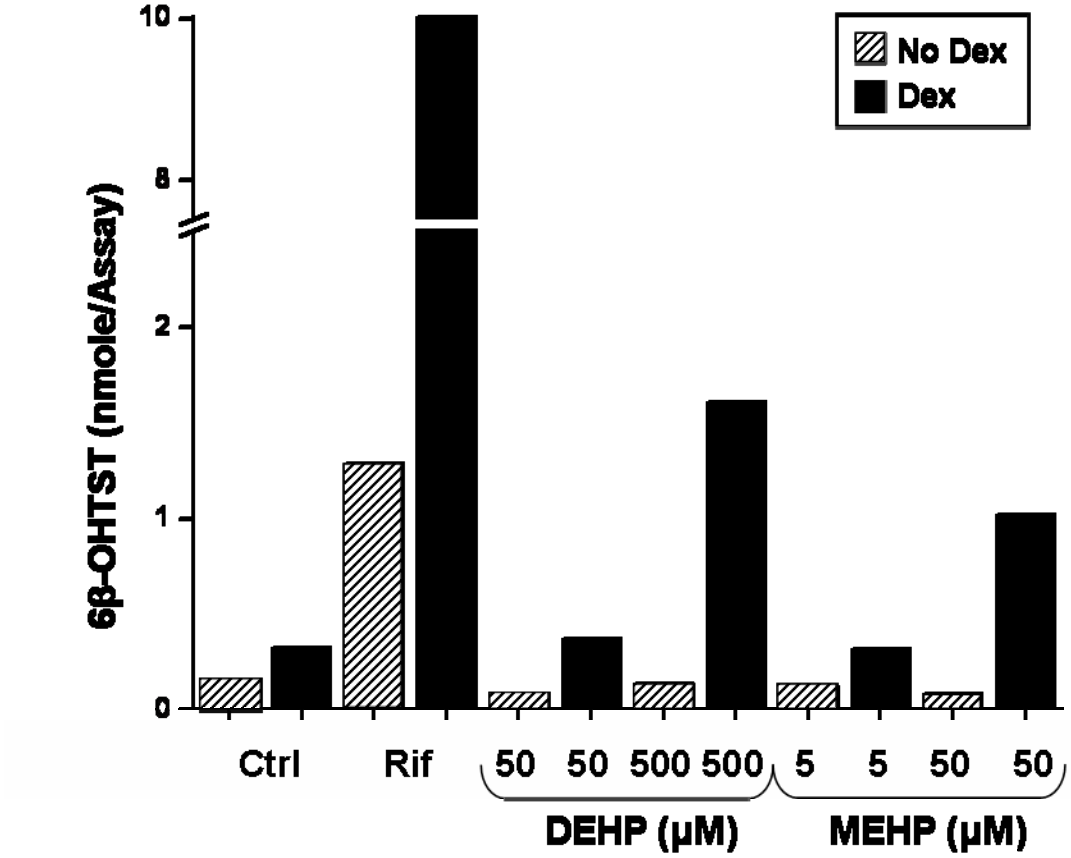

Fig. 6 6β-OHTST metabolite formation is dramatically enhanced by Dex and phthalate co-administration.

CHAPTER 2

Glucocorticoid Regulation of PXR Influences Paclitaxel and Docetaxel Dependent Induction of CYP3A4

ABSTRACT

Paclitaxel and docetaxel are chemotherapeutic drugs known as taxanes that promote microtubule stabilization and are used in the treatment of various malignancies. These compounds have been shown to activate the pregnane X receptor (PXR) and induce cytochrome P450 3A4 (CYP3A4); a key phase I enzyme that plays a role in the metabolism of these drugs. Administration of paclitaxel and docetaxel to chemotherapy patients is preceded by treatment with glucocorticoids to prevent anaphylaxis. Glucocorticoids have been shown to positively regulate the expression of PXR in a glucocorticoid receptor (GR)-mediated mechanism. We suggest that the magnitude of CYP3A4 induction by paclitaxel and docetaxel is dependent on the expression of PXR and may be significantly higher in the presence of glucocorticoids. PXR-dependent transcription of the *CYP3A4* promoter was significantly induced in a dose-dependent manner by paclitaxel and docetaxel treatment. Concomitant treatment with the GR agonist dexamethasone and paclitaxel or docetaxel resulted in synergistic induction of *CYP3A4* promoter activity, far exceeding that observed with individual treatments. Additionally, CYP3A protein expression was enhanced in human hepatocyte cultures by co-administration of dexamethasone and paclitaxel. Concomitant clinical administration of Dex and paclitaxel or docetaxel may alter the metabolic activity of CYP3A4, possibly resulting in decreased efficacy and enhanced clearance of these and other

important drugs. Understanding the role of PXR regulation by glucocorticoids and how this influences the magnitude of CYP3A4 induction by taxanes may provide insight into additional susceptibility for cancer chemotherapy patients.

INTRODUCTION

Paclitaxel (Taxol®) and docetaxel (Taxotere®) are semisynthetic chemotherapeutic drugs known as taxanes. Collectively, these agents are used to effectively treat various malignancies; including ovarian, breast, head and neck, lung and prostate cancer. These agents share a unique mechanism of action by promoting the polymerization of microtubules, resulting in cell cycle arrest, cellular apoptosis and cytotoxicity (Herbst and Khuri, 2003; Horwitz, 1994; Orr *et al.*, 2003). For paclitaxel and docetaxel, the major route of elimination is hepatic metabolism by cytochrome P450s followed by biliary excretion (Vaishampayan *et al.*, 1999). It has been shown that paclitaxel is metabolized by CYP2C8 and CYP3A4 isoforms (Cresteil *et al.*, 1994; Harris *et al.*, 1994; Rahman *et al.*, 1994) while docetaxel is primarily metabolized by CYP3A4 (Marre *et al.*, 1996). In humans, members of the CYP3A subfamily are highly expressed in the liver and intestine and contribute to the oxidative metabolism of pharmaceuticals and other xenobiotics (Li *et al.*, 1995). Enhanced metabolic activity of CYP3A4 can alter the efficacy of pharmaceutical agents and result in adverse drug-drug interactions. The pregnane X receptor (PXR), a member of the nuclear receptor family, mediates induction of CYP3A4 by a variety of xenobiotics (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Lehmann *et al.*, 1998; Nallani *et al.*, 2001). Working as a ligand activated transcription factor, PXR regulates genes involved in the oxidation, conjugation and elimination of xenobiotics.

Recently, paclitaxel and docetaxel were shown to activate PXR and induce CYP3A expression and activity in rat and human hepatocytes (Nallani *et al.*, 2001; Nallani *et al.*, 2004; Synold *et al.*, 2001). The mechanisms regulating the expression of PXR have not been thoroughly investigated, but it has been shown that submicromolar concentrations of the

glucocorticoid receptor agonist, dexamethasone (Dex) increase PXR mRNA expression (Pascussi *et al.*, 2000) . Additionally, synergistic increases in CYP3A have been observed by concomitant treatment with Dex and prototypical PXR agonists (Huss and Kasper, 2000; Pascussi *et al.*, 2001). Dex is a potent and widely prescribed synthetic glucocorticoid used in the treatment of cancer and immune disorders. Paclitaxel and docetaxel are administered by intravenous infusion and Dex pretreatment is recommended for all chemotherapeutic treatments with these agents to prevent anaphylaxis and hypersensitivity reactions (Bristol-Myers Squibb Company, 2003; Aventis Pharmaceuticals Inc., 2003). Glucocorticoid regulation of PXR expression may be a key determinant in the magnitude of CYP3A induction by paclitaxel and docetaxel. We suggest that the magnitude of CYP3A4 induction by taxanes is dependent on the expression of PXR and may be significantly higher in the presence of glucocorticoids.

We show that paclitaxel and docetaxel activated hPXR and induced *CYP3A4* promoter activity in a dose-dependent fashion. Additionally, treatment with paclitaxel and docetaxel in the presence of Dex resulted in significantly enhanced *CYP3A4* promoter activity. Utilizing primary human hepatocytes, we observed that CYP3A4 protein expression was highly induced by concomitant paclitaxel and Dex administration. Co-exposure to glucocorticoids and taxane chemotherapeutic agents resulting in enhanced CYP3A4 expression and activity may play a role in altered efficacy of these drugs; possibly putting cancer chemotherapy patients at additional risk.

MATERIALS AND METHODS

Chemicals:

Dexamethasone and pregnenolone-16α-carbonitrile (PCN) were purchased from Steraloids, Inc. (Newport, RI). Rifampicin, paclitaxel and dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Docetaxel was purchased from Toronto Research Chemicals, Inc. (New York, Ontario, Canada). All cell culture media and supplements were purchased from Mediatech, Inc. (Herndon, VA) unless otherwise noted. All primary hepatocyte culture medium and reagents were GIBCO brand, purchased from Invitrogen Corp. (Carlsbad, CA).

Plasmids:

The plasmid CYP3A4-luc, a kind gift of Dr. Jean-Marc Pascussi, contains -7836/-7208 nt of the distal enhancer region (XREM) cloned 5' of -263/+11 nt of the proximal promoter of the *CYP3A4* gene cloned into the pGL3 reporter vector (Promega, Madison, WI). The plasmid hPXR-pSG5, a kind gift of Dr. Steven Kliewer, contains hPXR.1 cloned into the pSG5 expression vector (Stratagene, La Jolla, CA). The control vector pSV-β-galactosidase was purchased from Promega (Madison, WI).

Cell Culture and Transactivation Experiments:

The human hepatocellular carcinoma, HepG2, cell line and the rat hepatoma H4IIE-C3 cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured according to ATCC recommendations. HepG2 cells were transfected in Opti-Mem I reduced serum medium (Invitrogen Corp.) using *Trans*IT-LT1 reagent (Mirus Bio

Corp., Madison, WI) according to the manufacturer's recommendations. The cells were incubated with transfection reagent for 4-6 hrs, then the Opti-MEM medium was removed, and cells were rinsed once and incubated overnight in Cellgro Complete medium. For transfection of the H4IIE-C3 cell line, plated cells were washed with Opti-MEM and transfected using Lipofectamine reagent enhanced by Plus reagent (Invitrogen Corp.) according to the manufacturer's recommendations. The cells were incubated with transfection reagents for 3 hrs before being rinsed once and incubated overnight in complete medium supplimented with 10% dextran-charcoal stripped FBS.

Analysis of Luciferase Activity:

Following transfection, cells were allowed to recover overnight in complete medium then treated with vehicle or specified compound. After 48 hrs of treatment, cells were rinsed with PBS and harvested in 1x Reporter Lysis Buffer from the Luciferase Assay System (Promega Corp.). Cell lysates were prepared according the manufacturer's recommended protocol and luciferase activity was measured using a TD-20/20 Luminometer (Turner Designs, Inc., Sunnyvale, CA). As an internal transfection control, β-galactosidase assays were performed on cell lysates according to the recommended protocol in the β-Galactosidase Enzyme Assay System (Promega). Luciferase activity was normalized to β-galactosidase activity and then transformed to fold induction over control.

Human Hepactocyte Culture:

Freshly isolated human hepatocytes plated with Matrigel overlay were received from ADMET (RTP, NC), CellzDirect (Pittsboro, NC) and Cambrex (Walkersville, MD). In

primary cultures of human hepatocytes, Matrigel overlay aids in maintaining differentiated characteristics of the cells as well as facilitating the induction of xenobiotic metabolizing enzymes (Gross-Steinmeyer *et al.*, 2005). Cells were cultured for 2-3 days in William's E medium with insulin, transferrin and selenium (ITS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and free of dexamethasone. Medium was changed every 24 hrs during culture and treatment periods. For western blotting, hepatocytes were treated for 2 consecutive days and cellular lysates were prepared as described below.

Western Blot Analysis:

For CYP3A enzyme detection, S9 fractions were obtained after centrifugation of cell lysates at 19,000 x g. All samples were prepared in Laemmli Buffer containing β-mercaptoethanol and boiled for 5 min. Protein samples were resolved on 8% Novex Tris-Glycine gels (Invitrogen Corp.) and transferred to nitrocellulose membrane. CYP3A was detected using a monoclonal mouse anti-CYP3A antibody (Gentest, BD Biosciences) (1:1000) incubated overnight at 4º C in TBS with Tween and 1% non-fat dry milk followed by horseradish peroxidase-conjugated anti-mouse secondary antibody. Immunoblots were visualized using chemiluminescence detection (ECL, GE Healthcare, Piscataway, NJ).

Statistical Analysis:

All statistical analysis was performed using JMP software, version 6.0.0 (SAS Institute, Inc., Cary, NC). Comparisons between treatment and control groups were made using ANOVA followed by Dunnett's method of multiple mean comparisons with a significance level of 0.01.

RESULTS and DISCUSSION

CYP3A4 is responsible for the oxidative metabolism of more than 60% of pharmaceuticals, including paclitaxel and docetaxel. PXR-mediated induction of CYP3A4 by xenobiotics may result in enhanced clearance and decreased efficacy of these and other important therapeutic agents. To investigate the ability of paclitaxel and docetaxel to activate hPXR and induce *CYP3A4* promoter activity, HepG2 cells were transiently transfected with expression plasmid containing PXR or empty vector and luciferase reporter containing a portion of the *CYP3A4* promoter with three PXR binding sites (Fig. 1). In the presence of hPXR, 10 μM paclitaxel induced *CYP3A4* promoter activity 27-fold above that of vehicle control while 10 μM rifampicin (Rif), the prototypical ligand for hPXR (Zhang *et al.*, 1999), induced the *CYP3A4* promoter 26-fold over control. *CYP3A4* promoter activity was induced 9-fold by 10 μM docetaxel treatment. The *CYP3A4* promoter was minimally activated in the absence of hPXR by Rif and Paclitaxel treatments; this may be due to activation of endogenous PXR expressed in the HepG2 cell line. Previous studies demonstrated that paclitaxel activated mouse PXR and induced CYP3A4 promoter activity in CV-1 cells (Nallani *et al.*, 2003). Both paclitaxel and docetaxel induced *CYP3A4* promoter activity in a dose-dependent manner, albeit paclitaxel appeared to be a better agonist for hPXR than docetaxel (Fig. 2). This agrees with findings by Nallani and coworkers (2004) which demonstrated that relative to rifampicin and paclitaxel, docetaxel was a weak hPXR activator.

Paclitaxel and docetaxel administration is routinely preceded by treatment with glucocorticoids, such as Dex, to prevent anaphylaxis and hypersensitivity reactions.

Additionally, glucocorticoids are regularly prescribed for cancer chemotherapy patients to help ease joint pain and nausea. It is known that glucocorticoid receptor (GR) activation by Dex positively regulates PXR mRNA and plays a pivotal role in CYP3A induction (Pascussi *et al.*, 2000). Additionally, we have shown that Dex treatment induces PXR protein expression (manuscript in preparation). For these reasons, we evaluated the effects of concomitant Dex and taxane exposure on *CYP3A4* promoter activity using rat hepatoma H4IIE-C3 cells that were transiently transfected with a *CYP3A4*-luciferase reporter. H4IIE-C3 cells are glucocorticoid responsive and express a functional PXR (Huss and Kasper, 1998); which make them an excellent model for evaluating the combined effects of GR and PXR activation. PCN, an agonist for rat PXR, was used as a positive control in these experiments. Paclitaxel and docetaxel treatments of H4IIE-C3 cells activated rat PXR and induced the *CYP3A4* promoter reporter 6-, 6-, and 13-fold respectively, in the absence of Dex (Fig. 3). However, in the presence of 0.1 μM Dex, *CYP3A4* promoter activity was induced 22-fold over control by 10 μM paclitaxel and 70-fold by 10 μM docetaxel. PCN and Dex co-treatment resulted in 17-fold induction of *CYP3A4* promoter activity. Huss and Kasper (2000) demonstrated synergistic induction of the rat *CYP3A23* promoter in H4IIE cells by co-treatment with 10 μM PCN and 0.1 μM Dex and the resulting transcriptional activity far exceeded that reached with either individual treatment. Our current, clinically relevant data expand upon their previous findings.

After observing that Dex and taxanes coordinately induced *CYP3A4* promoter activity in cell-based reporter assays, we evaluated the effect of co-administration of Dex and paclitaxel on CYP3A protein expression. Freshly isolated human hepatocyte cultures were treated with 5 μM paclitaxel, alone or in conjunction with 0.1 μM Dex and Western blot

analysis was performed on S9 fractions to visualize the levels of CYP3A protein (Fig. 4). Human hepatocyte cultures co-exposed to paclitaxel and Dex had highly induced CYP3A protein levels when compared to basal CYP3A expression or to induced CYP3A levels from either individual treatment. We did not observe any induction of CYP3A protein in the samples treated with paclitaxel alone. This actually differs from previous findings that demonstrated CYP3A mRNA and protein induction in human hepatocyte cultures treated with paclitaxel (Kostrubsky *et al.*, 1999). Increases in CYP3A protein expression are highly correlated with enhanced testosterone hydroxylase activity, a specific marker for CYP3A4 enzymatic activity.

Paclitaxel, when co-administered with Dex, is able to highly induce CYP3A protein expression; coupled with the role that CYP3A4 plays in the metabolism of this and other compounds present a confounding situation where paclitaxel and docetaxel potentially modulate their own metabolism and elimination. Enhanced CYP3A4 enzymatic activity may result in altered metabolism of these taxanes and other co-administered drugs, resulting in decreased half-life and efficacy of the drugs. Previous studies have demonstrated that administration of Dex to mice resulted in decreases in serum docetaxel concentrations; attributed to induction of CYP3A (Kamataki *et al.*, 1998). Most antineoplastic agents have narrow therapeutic indices and minor changes in plasma levels may have significant influence on the drug pharmacodynamics. Alterations in the pharmacokinetics and pharmacodynamics of paclitaxel and docetaxel may increase the possibility for adverse drug effects in cancer chemotherapy patients. Glucocorticoid regulation of PXR heavily influences the magnitude of CYP3A4 induction in response to xenobiotics. The data presented here advance our understanding of how concomitant clinical administration of Dex

and taxanes may alter the metabolic activity of CYP3A4 with therapeutic consequences for cancer chemotherapy patients.

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FIGURE LEGENDS

Fig. 1 **Taxane treatment induces PXR-mediated *CYP3A4* promoter activity.** HepG2 cells were transiently transfected with 0.1 μg pSG5 empty vector (*open bars*) or 0.1 μg pSG5-hPXR (*closed bars*), 1.0 μg *CYP3A4*-luc reporter and 0.1 μg β-gal plasmid. After 48 h treatment with DMSO vehicle control, 10 μM Rifampicin (Rif), 10 μM paclitaxel or 10 μM docetaxel, cellular lysates were collected and luciferase and β-galactosidase activity measured. The results are expressed as mean fold induction ± standard error (SE), (n=3). Statistical significance was determined by ANOVA, Dunnett's mean comparison test when compared to ctrl with hPXR; * $p<0.001$, ** $p=0.01$ (see fig).

Fig. 2 **Taxane treatment induces *CYP3A4* promoter activity in a dose-dependent fashion.** HepG2 cells were transiently transfected with 1.0 μg *CYP3A4*-luc reporter, 0.1 μg pSG5-hPXR and 0.1 μg β-gal plasmid. Cells were treated for 48 h with indicated concentrations of paclitaxel (*open bars)* or docetaxel *(closed bars)*. Measured luciferase activity was normalized to β-galactosidase activity. The results are expressed as mean fold induction over vehicle treated control (data not shown) ± SE, (n=3). Statistical significance was determined by ANOVA, Dunnett's mean comparison test, * $p<0.001$

Fig. 3 ***CYP3A4* promoter activity is significantly induced by concomitant exposure to Dex and taxanes**. H4IIE-C3 cells were transiently transfected with 1.0 μg *CYP3A4*-luc reporter and treated for 48 h with 10 μM of the indicated treatments either in the absence of Dex (*open bars*) or in the presence of 0.1 μM Dex (*closed bars*). Controls were treated with

DMSO vehicle. Cellular lysates were collected and measured luciferase activity was normalized to total protein per well. The results are expressed as mean fold induction over control ± SE (n=3). Statistical significance was determined by ANOVA, Dunnett's mean comparison test when compared to ctrl in the absence of Dex, * $p<0.001$. The data is a single representative of duplicate independent experiments.

Fig. 4 **CYP3A protein is highly induced by Dex and paclitaxel co-administration.** Human hepatocytes were cultured in Dex free medium and treated for three consecutive days with 5 μM paclitaxel either alone or in conjunction with 0.1 μM Dex. Cells were harvested and S9 fraction prepared for CYP3A immunoblotting using 8 μg protein. This data represents one individual batch of human hepatocytes but is a representative of two individuals.

FIGURES

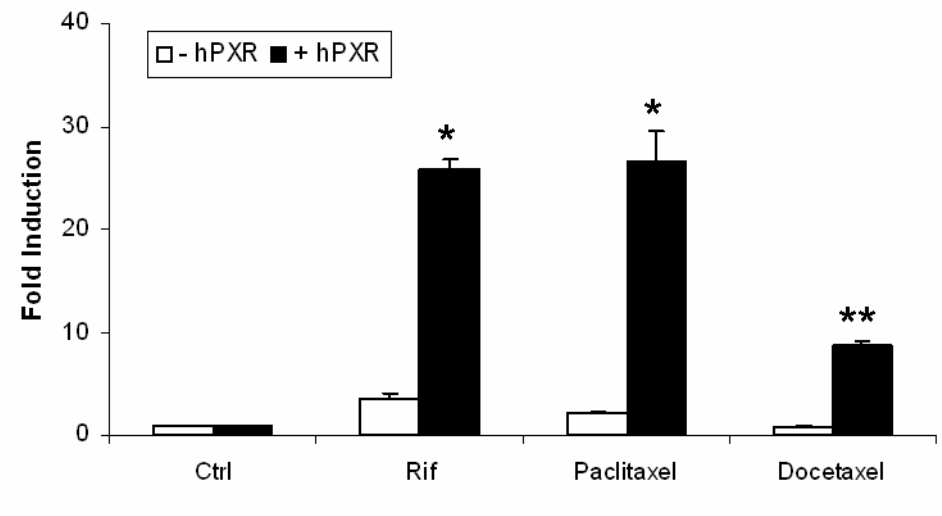

Fig. 1 Taxane treatment induces PXR-mediated *CYP3A4* promoter activity.

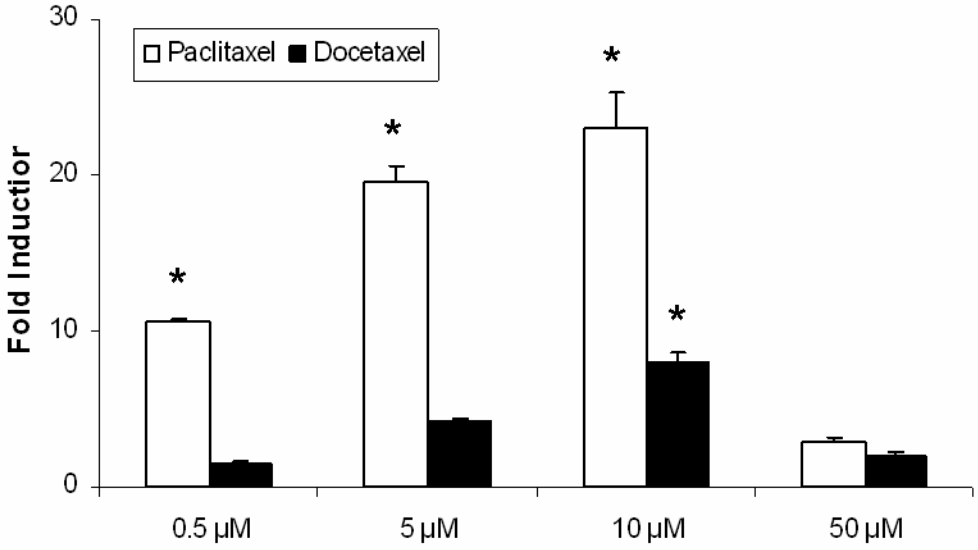

Fig. 2 Taxane treatment induces *CYP3A4* promoter activity in a dose-dependent fashion.

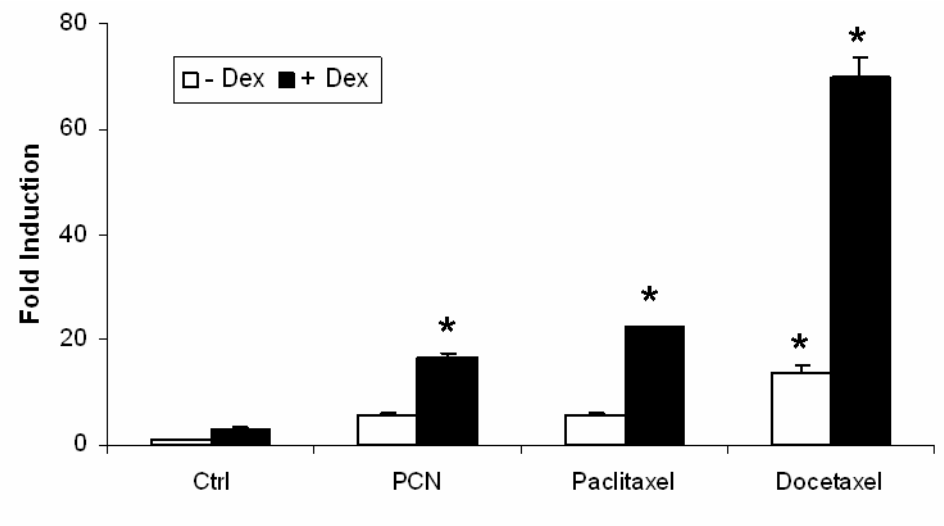

Fig. 3 ***CYP3A4*** **promoter activity is significantly induced by concomitant exposure to Dex and taxanes**.

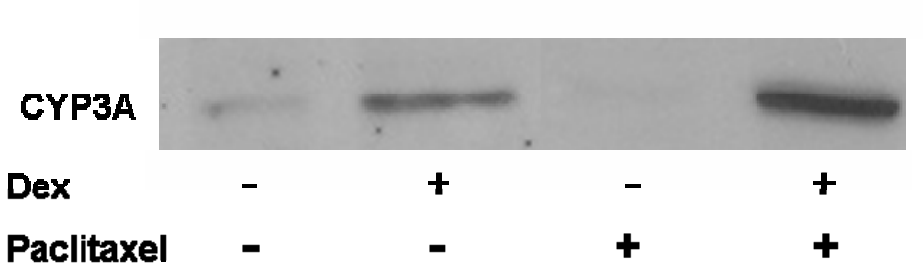

Fig. 4 CYP3A protein is highly induced by Dex and paclitaxel co-administration.

CONCLUSION

The clinical significance of PXR activation and subsequent enhanced CYP3A4 enzymatic activity is well established. Induction of CYP3A4 leads to alterations in metabolism of pharmaceuticals and endogenous steroid hormones. This may result in drug-drug interactions, bioactivation and enhanced toxicity of some drugs, decreased efficacy and duration of therapeutic agents and endocrine disrupting effects. Activation of the promiscuous PXR has become so important in the drug development process that many drug candidates are now routinely screened to determine if they are PXR agonists. Glucocorticoids are known to positively regulate PXR expression and play a critical role in the extent to which PXR target genes are induced. Endogenous circulating levels of glucocorticoids fluctuate regularly and increase during times of stress. Additionally, synthetic glucocorticoids are routinely administered for many therapeutic uses, including in treatment of immune disorders, in chemotherapeutic treatment regimes and to aid lung development of premature infants. Because the body is continuously exposed to xenobiotics that are either known PXR agonists or have the potential to activate PXR, it is important to consider the influence of endogenous and exogenous glucocorticoids when estimating induction of PXR target genes. If the levels of PXR are increased due to glucocorticoids, then the response to PXR agonists will be significantly amplified and alterations in metabolism and elimination rates will be much greater than predicted. Glucocorticoid receptor-mediated regulation of PXR may influence the magnitude of CYP3A4 induction by PXR agonists, including phthalate esters, taxane chemotherapeutics and any number of other exogenous chemicals. We have shown that concomitant exposure to glucocorticoids and each of these PXR agonists resulted in enhanced CYP3A4 promoter activity as well as

increased CYP3A4 protein expression and enzymatic activity as demonstrated by 6β-OHTST metabolite formation. The data presented here advance our understanding of how concomitant exposure to glucocorticoids and PXR agonists may alter the expression level and function of CYP3A4, a key player in phase I metabolism. Understanding the role of glucocorticoid regulation of PXR as a key determinant in the magnitude of CYP3A4 induction by xenobiotics will provide insight into possible adverse drug effects in sensitive populations.

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