THE EFFECT OF CYCLOPHOSPHAMIDE WITH AND WITHOUT DEXAMETHASONE ON CYTOCHROME P450 3A4 AND 2B6 IN HUMAN HEPATOCYTES

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(Received June 4, 2001; accepted March 28, 2002)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

The purpose of this study was to characterize the concentration-response effects of cyclophosphamide (CPA) with and without dexamethasone (DEX; 10 μM) on the expression of CYP3A4 and CYP2B6 in cultured human hepatocytes at concentrations representative of standard- and high-dose CPA therapy (25 to 750 μM). CPA produced concentration-dependent increases in CYP3A4 and CYP2B6 activity and immunoreactive protein that peaked at 250 and 125 μM, respectively, and declined thereafter. The inductive effect of CPA alone and in combination with DEX was greater in magnitude for CYP2B6 compared with CYP3A4. To further examine the inductive effect of CPA on CYP3A4, CPA (250 μM) and DEX (10 μM) alone and in combination were examined in 10 hepatocyte preparations. The combination of CPA and DEX yielded higher rates of 6β-hydroxytestosterone formation than either agent alone. However, the effect was less than additive in human hepatocyte cultures with relatively high baseline CYP3A4 activity and additive or synergistic in human hepatocyte cultures with relatively low baseline CYP3A4 activity. Induction index was highly correlated with CYP3A4 baseline activity for both CPA (r² = 0.75) and CPA plus DEX (r² = 0.85). To investigate the potential mechanism for CPA-induced increases in CYP3A4 activity, the ability of CPA alone and in combination with DEX to activate pregnane X receptor (PXR) was explored using transient transfection assays. CPA produced a dose-dependent increase in PXR activation that was maximal at the highest CPA concentration studied (500 μM). The addition of DEX to CPA resulted in a minor increase in PXR activation compared with CPA alone. These results indicate that CPA alone and in combination with DEX differentially induces the expression of CYP3A4 and 2B in a concentration-dependent manner, which may be mediated partially through activation of PXR. The impact of these effects on the efficacy and toxicity of CPA therapy warrants further investigation.

Cyclophosphamide (CPA) is an alkylating agent prodrug widely used over a large dose range in the treatment of malignancies and autoimmune disorders. CPA is extensively metabolized by cytochrome P450 (P450) pathways via 4-hydroxylation and N-dechloroethylation, with only 10 to 30% of a dose excreted in the urine as unchanged parent compound (Sladek, 1988; Moore, 1991). The predominant pathway, 4-hydroxylation, yields the active alkylating moiety and an equimolar amount of the urototoxic byproduct acrolein. N-dechloroethylation generates dechloroethylCPA and the nephrotoxic and neurotoxic byproduct chloroacetaldehyde (Fleming, 1997).

This work was supported in part by a Hollingsworth Faculty Scholarship awarded to Celeste Lindley by the School of Pharmacy, University of North Carolina at Chapel Hill, and by a Food and Drug Administration Center for Drug Evaluation and Research contract 223-97-3004.

1 Abbreviations used are: CPA, cyclophosphamide; P450, cytochrome P450; PXR, pregnane X receptor; DEX, dexamethasone; HBUP, hydroxybupropion; HPLC, high-performance liquid chromatography; DMEM, Dulbecco’s modified Eagle’s medium.

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The major P450 enzymes responsible for 4-hydroxylation of CPA appear to be CYP2B6, CYP3A4, and CYP2C (Chang et al., 1993; Roy et al., 1999; Huang et al., 2000). Among these enzymes, CYP2B6 was identified as the P450 isozyme with the highest intrinsic clearance for CPA 4-hydroxylation (Roy et al., 1999). This is in contrast to the findings of another group of investigators who found no evidence for a quantitatively significant amount of formation of 4-hydroxyCPA from CPA by CYP2B6 in a panel of 5 human liver microsomes (Ren et al., 1997). CYP2B6 contribution to CPA activation has been shown to vary significantly among liver samples due to differences in the expression of CYP2B6 protein levels in individual livers (Roy et al., 1999; Huang et al., 2000). Huang et al. (2000) reported that CPA 4-hydroxylation ranged from 0.1 to 0.6 nmol/min/mg of protein in a panel of 17 human liver samples and that CYP2B6 contributed to an average of 45% of total CPA 4-hydroxylation (range 10–70% depending on the CYP2B6 content of the liver), compared with 25 and 12% for CYP3A4 and CYP2C9, respectively. CYP3A4 was shown recently to be the P450 enzyme exclusively involved in the N-dechloroethylation of CPA (Huang et al., 2000). Given the high capacity of CYP2B6 for CPA 4-hydroxylation and negligible formation of dechloroethylCPA, patients with relatively high CYP2B6 activity would be expected to experience the greatest therapeutic response with the least amount of nonmyelosuppressive toxicity from an
administered CPA dose. In contrast, CYP3A4 produces relatively equivalent amounts of active and inactive CPA metabolites, and therefore patients with relatively high CYP3A4 activity would be expected to experience good therapeutic response at the expense of toxicity.

A high degree of interpatient variability has been reported in the pharmacokinetics and systemic exposure of CPA in children and adults with cancer (Moore et al., 1988; Yule et al., 1996; Ren et al., 1998; Yule et al., 1999a). This variability could partially reflect differences in the expression of individual P450 isoforms. The CYP3A subfamily is the most abundantly expressed in human liver and small intestine, and interindividual differences in CYP3A expression exceed 50-fold in some populations (Watkins, 1995). A recent report characterizing the genetic basis of polymorphic CYP3A5 expression found that CYP3A5 was expressed in 60% of livers of African Americans compared with 33% for Caucasians, and that, when present, represented at least 50% of total hepatic CYP3A content (Kuehl et al., 2001). Although previous work focused on the relative importance of CYP3A4 to the CYP3A subfamily, CYP3A5 may be the more important contributor to interindividual and ethnic differences in metabolic clearance of a large number of CYP3A substrates. In addition, recent studies using improved immunonquantitative techniques have reported a greater frequency of detection and a higher contribution of CYP2B6 to total P450 content than previously estimated. In addition, 20- to 250-fold variability (Code et al., 1997; Stresser and Kupfer, 1999) and ethnic differences (Shimada et al., 1994; Kim et al., 1997) in CYP2B6 expression have been recognized.

Other sources of variation include concomitant treatment with P450 inhibitors (Yule et al., 1999b) or prior treatment with P450 inducers including dexamethasone (Yule et al., 1996) and anticonvulsants (Slattery et al., 1996). CPA itself has been shown to induce its own metabolism manifested by an increase in clearance following repeated administration of high doses at 24-h intervals (Chen et al., 1995; Slattery et al., 1996; Busse et al., 1997). CPA autoinduction appears to be through induction of P450 isoforms. Exposure of cultured human hepatocytes to 250 μM CPA resulted in increased 6β-hydroxylation of testosterone and CYP3A4 protein (Chang et al., 1997). However, no increase in CYP2B6 protein was detected with this same treatment. In contrast, Gervot et al. (1999) reported induction of CYP2B6 protein and mRNA in hepatocytes treated with 1 mM CPA. To date, no studies have reported whether CPA-induced increases in P450 activity occur through activation of the pregnane X receptor (PXR), which has been identified as the primary mediator of drug-induced increases in CYP3A4 expression (Lehmann et al., 1998; Goodwin et al., 1999).

Although dexamethasone (DEX) is frequently coadministered with CPA as an antiemetic agent, information regarding the effect of this CYP3A4 inducer on CPA autoinduction is not available. Our laboratory has previously demonstrated significant increases in CYP3A4 activity with DEX administration in healthy volunteers and in microsomes isolated from human hepatocytes (McCune et al., 2000). CYP3A4 induction was highly variable, and the induction ratio (-fold increase in testosterone 6β-hydroxylase activity compared with control) was inversely related to baseline activity, with the greatest increase in activity observed in healthy volunteers and human hepatocytes with the lowest baseline CYP3A4 activity (McCune et al., 2000). In hepatocytes, 2 to 250 μM DEX resulted in an average 1.7- to 6.9-fold increase in CYP3A4 activity, respectively. In contrast, a similar concentration range of DEX resulted in less than 2-fold induction of CYP2B6 activity in human hepatocytes (Faucette et al., 2001).

The purpose of this study was to characterize the concentration-response effects of CPA on the expression of CYP2B6 and CYP3A4, the major CPA-metabolic activator and deactivator, respectively, in primary cultures of human hepatocytes. In addition, the influence of factors such as coadministration of DEX and baseline CYP2B6 and CYP3A4 activities on the extent of the autoinduction of CPA was evaluated. Finally, the capacity of CPA and DEX, alone and in combination, to activate PXR was explored using transient transfection assays.

### Materials and Methods

**Chemicals.** NADP+, potassium phosphate, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, magnesium chloride, EDTA, triethylamine, cortisone, bupropion, and cyclophosphamide were purchased from Sigma-Aldrich (St. Louis, MO). Hydroxybupropion (HBUP) was graciously supplied by Glaxo Wellcome Inc. (Research Triangle Park, NC). Testosterone and 6β-hydroxytestosterone were purchased from Steraloids Inc. (Newport, RI). Formic acid was obtained from Fisher Scientific (Fair Lawn, NJ), and HPLC-grade acetonitrile and methanol were purchased from J. T. Baker, Mallinckrodt Baker Inc., Div (Phillipsburg, NJ). All other chemicals were of the highest grade commercially available.

**Hepatocyte Isolation and Cell Culture.** All hepatic tissues were obtained through qualified medical staff, with donor consent and with the approval of the University of North Carolina Hospitals Institutional Review Committee. Hepatocytes were isolated from human liver tissue procured through the Department of Surgery, University of North Carolina at Chapel Hill School of Medicine or nontransplantable donor livers using a modified two-step collagenase digestion method. Prior to perfusion of the tissue, the outer capsule on the cut surface was reconstructed using medical grade adhesive. This technique permitted the use of lower flow rates and enhanced both blood clearance and digestion of the liver. Encapsulated liver tissue (15–100g) was perfused with calcium-free buffer containing 5.5 μM glucose, 0.5 mM EGTA, 0.05% ascorbic acid, and 0.5% bovine serum albumin for 10 to 15 min at a flow rate of 14 to 18 ml/min, followed by Dulbecco’s modified Eagle’s medium (DMEM) containing 0.5% bovine serum albumin, ascorbic acid (0.05%), and collagenase (0.4–0.8 mg/ml) for 15 to 20 min at a flow rate of 28 to 32 ml/min.

Hepatocytes were dispensed from the digested liver in DMEM supplemented with 5% fetal calf serum, insulin (4 μg/ml), and dexamethasone (1.0 μM), passed through a series of fluorocarbon filters (1000, 400, and 70 μm mesh), and washed by low-speed centrifugation (70g, 4 min). Cell pellets were resuspended in supplemented DMEM and 90% isotonic Percoll and centrifuged at 100g for 5 min. The resulting pellets were resuspended in fresh medium and washed once by low-speed centrifugation. Hepatocytes were resuspended in supplemented DMEM, and viability was determined by trypan blue exclusion. Cell yields and viability varied between 10 to 30 million cells per gram of wet tissue, and 75 to 95%, respectively.

Hepatocytes were cultured according to methods previously described by LeChuyse et al. (1996, 2000). In most cases, 4 × 10⁶ Hepatocytes were added to 60-mm culture dishes precoated with collagen in 3 ml of supplemented DMEM and allowed to attach for 2 to 3 h at 37°C in a humidified incubator with 95%:5% air:CO₂. After cell attachment, culture dishes were gently swirled, and medium containing unattached cells was aspirated and replaced with modified Chee’s medium containing 0.1 μM dexamethasone, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, and 6.25 ng/ml selenium, (ITS+; BD Biosciences, Bedford, MA) (LeChuyse et al., 2000). Medium was changed on a daily basis thereafter. Unless otherwise specified, primary cultures of human hepatocytes were maintained for 2 to 3 days before initiating experiments with inducers. Final concentrations of CPA, DEX, and rifampin were cyclophosphamide (10, 50, 125, 250, 375, 500, 750, 1000, 1500, and 2000 μM); dexamethasone (2, 10, 50, 100, and 250 μM); cyclophosphamide (25, 125, 250, 375, 500, and 750 μM) with dexamethasone (10 μM); and rifampin (10 μM) (positive control). Stock solutions of rifampin and DEX were prepared in dimethyl sulfoxide and added to cultures at a final volume of 0.1%. Stock solutions of CPA were prepared in sterile water at a final concentration of 120 mM, and dimethyl sulfoxide was added to cultures at a final concentration of 0.1%. Inducers were added every 24 h for 72 h in fresh medium. At the end of the treatment period, hepatocytes were harvested and microsomes prepared as previously described (LeChuyse et al., 2000).
Microsomal Assays. Microsomal CYP3A4 and CYP2B6 activities were determined by measuring the formation rate of 6β-hydroxytestosterone from testosterone and HBUP from bupropion, respectively (Faucette et al., 2000). Formation rates were determined in duplicate with microsomes prepared from individual treatment groups of hepatocyte cultures (n = 3). Preliminary experiments in pooled microsomes were conducted to identify microsomal protein amounts and incubation times resulting in linear rates of 6β-hydroxytestosterone and HBUP formation.

Microsomal assays were conducted with 0.1 mg of microsomes, 250 μM testosterone, or 500 μM bupropion, 50 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 3 mM magnesium chloride, 1 mM NADPH, 5 mM glucose 6-phosphate, and 1 U/ml glucose-6-phosphate dehydrogenase. Total incubation volumes for the testosterone and bupropion assays were 0.25 and 0.2 ml, respectively. Testosterone and bupropion stock solutions were prepared in methanol and added to individual reaction mixtures in volumes not exceeding 1% of total incubation volumes. Internal standards for the testosterone and bupropion assays were cortisol (75 μM) and tripolidine (20 μg/ml), respectively.

Reactions in microsomes were initiated at 37°C by addition of NADPH-regenerating system and proceeded for 12.5 min (testosterone) or 30 min (bupropion) prior to termination of reactions with 100 μl of a mixture containing ice-cold acetonitrile and internal standard. On completion of the reaction, incubation mixtures were vortexed and centrifuged at 3000 rpm for 5 min to remove precipitated protein. The amount of 6β-hydroxytestosterone and HBUP in the resulting supernatant fraction was determined by reverse-phase HPLC (Pearce et al., 1996; Faucette et al., 2000).

HPLC Analysis. The HPLC system for quantitation of 6β-hydroxytestosterone and cortisol (internal standard) consisted of a Hewlett Packard 1090L model system (Hewlett Packard, Palo Alto, CA) with UV detection set at 245 nm. 6β-Hydroxytestosterone, and cortisol peaks were separated and resolved at 40°C on a Supelcosil LC-18, 150 × 4.6 mm, 5 μm column proceeded by a Pelliguard LC-18 2 cm guard column (Sigma-Aldrich). Mobile phases A (64% water, 35% methanol, 1% acetonitrile) and B (18% water, 80% methanol, 2% acetonitrile) were pumped over a gradient at a flow rate of 1 ml/min. Retention times for 6β-hydroxytestosterone and cortisol were 4.3 and 5.5 min, respectively. The HPLC system for detection of HBUP and the internal standard tripolidine consisted of a Hewlett Packard 1100 liquid chromatograph (Hewlett Packard, Avondale, PA) connected to a Hewlett Packard model 1100 UV detector set at 214 nm. Peaks of interest were separated on a 5 μm, 4.6 mm, 5 μm Supelcosil Symmetry LC-18 guard column (Sigma-Aldrich) connected to a Hewlett Packard 1100 liquid chromatograph (Hewlett Packard, Avondale, PA) connected to a Hewlett Packard model 1100 UV detector set at 214 nm. Peaks of interest were separated on a 5 μm Symmetry LC-18 guard column (Sigma-Aldrich). Mobile phases A (0.25% triethylamine, 15% acetonitrile, and 0.1% formic acid) and B (100% acetonitrile) were pumped over a gradient at a flow rate of 1 ml/min. Retention times for 6β-hydroxytestosterone and cortisol were 7.5 and 24.5 min, respectively.

Calibration standards of 6β-hydroxytestosterone and hydroxybupropion were prepared by addition of a known amount of metabolite to microsomal incubation mixture components. 6β-Hydroxytestosterone or HBUP picomoles were calculated from peak area or height ratios, respectively, using least-squares linear regression, with weighting by the reciprocal of the squared standard concentrations. Interday coefficients of variation ranged from 6 to 28% for 6β-hydroxytestosterone standards. Lower limit of detection was 180 pmol for 6β-hydroxytestosterone. Hydroxybupropion interday coefficients of variation for calibration standards ranged from 22% for the lowest standard to 13% for the highest standard. The lower limit of quantitation was 20 ng/ml.

Cotransfection Assays. HuH7 cells were seeded in 24-well plates (50,000/well) in high glucose DMEM supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone Laboratories Inc., Logan, UT). Transfection mixes contained 100 ng of hFXR expression vector (pCMV-SPORT; Invitrogen, Carlsbad, CA), 100 ng of firefly luciferase reporter plasmid (CYP3A4 ERα2-GL3-Promoter Vector; Invitrogen), and 10 ng of Renilla luciferase reporter vector (pRL-TK Vector; Invitrogen) as internal control. Transfections were performed with Effectene (QIAGEN, Inc., Valencia, CA). Drug dilutions were prepared in phenol red-free medium supplemented with 10% charcoal-stripped, delipidated calf serum (Sigma-Aldrich). Cells were incubated for 24 h in the presence of drugs, and cell extracts were prepared in lysis buffer (Promega Corp., Madison, WI). Reporter activity was determined using the dual-luciferase reporter assay system, essentially according to the manufacturer’s instructions (Promega).

Western Immunoblotting. Western immunoblotting was carried out as described previously by Parkison and Gemzik (1991). Briefly, equal amounts of microsomal protein (10 μg for CYP3A4, 20 μg for CYP2B6) were loaded onto polyacrylamide gels. Proteins were transferred onto nylon membranes, and immunoblots were incubated with primary antibody overnight at room temperature. Levels of immunoreactive CYP3A4 protein were detected with antibodies against CYP3A4-specific peptide at a 1:1500 dilution. Levels of immunoreactive CYP2B6 proteins were detected with antibodies against CYP2B6-specific peptide at a 1:500 dilution as previously described (Faucette et al., 2000). Secondary labeling was achieved with alkaline phosphatase-conjugated goat affinity purified antibody to rabbit IgG (INC/Cappel, Aurora, OH), diluted 1:1000, and incubated for 3 h at room temperature. Blots were developed with 5-bromo-4-chloro-indolyl-phosphatase and nitroblue tetrazolium as substrate solution (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD).

Results

Cyclophosphamide and Dexamethasone Concentration-Dependent Effects on CYP3A4 Activity. The concentration-dependent effects of CPA on CYP3A4 activity in 10 microsomal preparations of human hepatocytes treated with various concentrations of CPA (2 to 1500 μM) were examined in a series of preliminary experiments (data not shown). CYP3A4 activity, expressed as -fold induction over control 6β-hydroxytestosterone formation, was increased by 1.5-fold at 250 μM CPA concentration, peaked at 3.5-fold at 250 μM, and gradually declined at concentrations greater than 250 μM. Significant variability was observed in the extent of induction, and CPA concentration at which maximal inductive effects occurred was observed. For the composite data, the concentration at which EC50 was achieved was 40 μM, and the maximum effect was 3.5-fold induction over control. Microsomal protein concentration and lactate dehydrogenase leakage in hepatocyte cell culture treated with concentrations of CPA greater than 250 μM were not different from those treated with <250 μM (data not shown), suggesting that a cytotoxic effect of CPA on hepatocytes was not an explanation for the decrease in percent induction in CYP3A4 activity observed at higher CPA concentrations.

Treatment of hepatocyte preparations with dexamethasone resulted in a highly variable concentration-dependent increase in CYP3A4 activity among the 10 hepatocyte preparations. Average increases in CYP3A4 activity of 1.7-, 1.9-, 3.9-, 6.9-, and 6.6-fold were observed after exposure to dexamethasone at 2, 10, 50, 100, and 250 μM DEX, respectively. This data has been previously reported in detail (McCune et al., 2000). For the composite data, the concentration at which 50% of the maximum effect was achieved was 51 μM, and the maximum effect was 6.6-fold induction over control. Variability in extent of CYP3A4 induction was primarily the result of baseline differences in CYP3A4 activity rather than to differences in maximum CYP3A4 activity achieved, as was previously described by McCune et al. (2000).

Effects of Cyclophosphamide (250 μM), Dexamethasone (10 μM) Alone and Cyclophosphamide (250 μM) with Dexamethasone (10 μM) on CYP3A4 Expression. Figure 1 illustrates the CYP3A4 activity in microsomes prepared from 10 human hepatocyte cultures treated with dimethyl sulfoxide (control), DEX (10 μM), CPA (250 μM), and DEX (10 μM) plus CPA (250 μM) for 72 h. The concentration of CPA (250 μM) chosen was that which produced maximal induction of CYP3A4 activity in preliminary experiments with CPA. The DEX concentration (10 μM) chosen represents a concentration approximately 5-fold higher than the maximum concentration observed following a 20-mg oral dose of DEX (Brophy et
al., 1983) and a point on the upward curve of CYP3A4 induction observed with DEX. CPA concentrations/11021 250 /H9262 M are commonly achieved in cancer patients receiving standard CPA doses (i.e., 1 g/m²), whereas >250 /H11022 M are observed in cancer patients receiving CPA as myeloablative therapy (Moore et al., 1988; Slattery et al., 1996; Yule et al., 1996; Ren et al., 1998; Busse et al., 1999; Yule et al., 1999b). Rifampin (10/9262 M) was used as a positive control in all hepatocyte preparations. Previous dose-response studies have revealed that maximal increases in CYP3A4 are attained at rifampin concentrations greater than 2/9262 M (Sahi et al., 2000).

When administered as single agents, CPA and DEX produced approximately equivalent increases in CYP3A4 activity, expressed as rates of 6β-hydroxytestosterone formation in picomoles per minute per milligram of protein, in microsomes isolated from human hepatocytes with control CYP3A4 activity/11022 1500 pmol/min/mg of protein (Fig. 1). Mean CYP3A4 activity (±S.D.) of CPA (250 /H9262 M) and DEX (10 /H9262 M) were 5348 (1559) and 4879 (1638) pmol/min/mg of protein, respectively, in microsomes prepared from hepatocyte cultures with control CPA3A4 activity/11021 1500 pmol/min/mg of protein. CPA (250 /H9262 M) produced significantly greater increases in CYP3A4 activity than DEX (10 /H9262 M) with a mean (±S.D.) CYP3A4 activity of 9761 (3161) (data not shown).

The combination of 250 /H9262 M CPA and 10 /H9262 M DEX yielded a higher rate of 6β-hydroxytestosterone formation than either agent alone in all 10 hepatocyte preparations. The combination of CPA and DEX produced at least additive effects in microsomes from hepatocyte preparations with baseline CYP3A4 activity <1500 pmol/min/mg of protein. However, a less than additive effect was observed in microsomes prepared from human hepatocytes with higher baseline CYP3A4 activity. Induction index, expressed as -fold induction over control, was highly correlated to CYP3A4 baseline activity for both CPA ($r^2 = 0.75$) and the combination of CPA and DEX ($r^2 = 0.85$).

**Cotransfection Assays.** To determine whether the induction of CYP3A4 by CPA and DEX was mediated through activation of the PXR, the concentration-dependent effects of CPA (25–500 /H9262 M) alone and in combination with DEX (10 /H9262 M) were examined in HUH7 cells cotransfected with a hPXR expression vector and a reporter gene

**FIG. 1.** Effect of CPA and DEX alone and in combination on the rate of 6β-hydroxytestosterone formation CYP3A4 expression.

Hepatocyte cultures were treated for 3 days with 0.1% dimethyl sulfoxide (control), 250 /H9262 M CPA, 10 /H9262 M DEX, or both CPA (250 /H9262 M) and DEX (10 /H9262 M), as indicated under Materials and Methods. Microsomes were subsequently prepared and analyzed for CYP3A4 enzyme activity based on the 6β-hydroxylation of testosterone.

**FIG. 2.** Effects of CPA on PXR activation in the absence and presence of DEX. HUH7 cells were cotransfected with expression plasmids for hPXR and the (CYP3A4 ER 6 ) 2 -tk-luciferase reporter. Cells were treated for 24 h with each compound at final concentrations indicated, and cell extracts were subsequently assayed using the dual-luciferase reporter assay system as described under Materials and Methods. Data represent the mean ± S.E. from triplicate wells. * , p ≤ 0.05 compared with CPA-0 activity.
A construct containing multiple copies of the CYP3A4 proximal PXRE ([ER_{6}]_2) (Lehmann et al., 1998). Figure 2 shows the fold induction over control value for CPA (25–500 μM) with and without DEX (10 μM). Rifampin (10 μM) was included as a positive control. The high basal PXR activity is due to the fact that the ER{sub 6} reporter plasmid that was used contains the SV40 promoter, which is quite strong in the transfected cells. The results demonstrate that CPA produced a dose-dependent increase in PXR activation that was maximal at the highest CPA concentration studied (CPA 500 μM), and maximum activation observed was approximately 65% of rifampin activation. The addition of DEX to CPA resulted in only minor increases in PXR activation over CPA alone. PXR activation was approximately additive with respect to the effect of CPA and DEX alone. In separate experiments, CPA was more potent in activating PXR than other known inducers of CYP3A4 (DEX and phenytoin) when administered in equimolar amounts (data not shown). To our knowledge, this is the first report demonstrating that direct activation of PXR by CPA may be a possible molecular mechanism responsible for the autoinduction of CPA metabolism.

**Concentration-Dependent Effects of Cyclophosphamide with and without Dexamethasone on CYP3A4 and CYP2B6 Activity and Protein Expression.** To further characterize the apparent enhancement of CPA-mediated induction of CYP3A4 by DEX and to investigate potential inductive effects on CYP2B6, CPA alone and in combination with DEX (10 μM) was examined in three separate human hepatocyte preparations (HL092, HL093, HL100) over the range of CPA concentrations (25–750 μM) observed in patients receiving standard and high-dose CPA regimens. As observed in the prior experiments, CPA produced concentration-dependent increases in CYP3A4 activity that peaked at 250 μM and declined thereafter (Fig. 3A). DEX enhanced CPA induction of CYP3A4 activity, and this was particularly apparent at the low end of the CPA concentration range. CPA also produced concentration-dependent increases in CYP2B6 activity that peaked at 125 μM and were greater in magnitude than the effect of CPA on CYP3A4 activity (Fig. 3B). DEX

**Fig. 3.** Effect of CPA alone and in combination with DEX (10 μM) on CYP3A4 (3A) and CYP2B6 (3B) activity.
enhanced the inductive effect of CPA on CYP2B6 activity and, again, this was particularly apparent at the low end of the CPA dose-response curve. DEX plus CPA resulted in greater fold induction of CYP2B6 activity compared with CYP3A4. Corresponding increases in CYP3A4 and CYP2B6 immunoreactive protein as assessed by Western immunoblot and densitometric analysis of microsomes prepared from HL092 and HL100 confirmed CPA and CPA plus DEX induction of CYP3A4 and CYP2B6 activity (Figs. 4 and 5).

**Discussion**

Overall, these findings confirm and extend earlier reports showing CPA induction of CYP3A4 (Chang et al., 1997) and CYP2B6 (Gervot et al., 1999) in human hepatocyte to cultures. Specifically, CPA produced variable concentration-dependent changes in CYP3A4 activity that were inversely correlated with CYP3A4 baseline activity. CPA (250 μM) alone resulted in greater formation rates of 6β-hydroxytestosterone than DEX (10 μM) alone in microsomes from human hepatocytes with baseline CYP3A4 activity <1500 pmol/min/mg of protein; however, CPA alone and DEX alone produced approximately equivalent increases in testosterone 6β-hydroxylase activity in microsomes from hepatocytes with higher CYP3A4 baseline activity. Neither CPA nor DEX was as potent as the prototypical inducer rifampin at inducing CYP3A4 activity. At the concentrations tested, CPA produced greater increases in CYP2B6 than CYP3A4 activity in the three hepatocyte preparations studied. CPA appeared to reach maximum inductive effects on both CYP3A4 and CYP2B6 in concentrations in the range of 125 to 250 μM. Microsomal protein and lactate dehydrogenase activity remained relatively constant at high versus low CPA concentrations, indicating that a cytotoxic effect of higher concentrations of CPA in hepatocyte cell cultures was not the basis for this decline (data not shown). The importance of discordant effects of low and high CPA concentrations on CYP3A4 and CYP2B6 activity warrants further investigation as the concentration range investigated in cell culture was chosen to encompass the concentration range.
range achieved in adult patients treated with standard and high-dose CPA regimens.

DEX enhanced the CYP3A4- and CYP2B6-inductive effect of CPA beyond that observed for CPA alone and DEX alone in microsomes prepared from cultured hepatocytes. However, in most cases, the increase in induction observed with CPA plus DEX was not additive. Increases in CYP3A4 activity with CPA and DEX were additive or synergistic in hepatocyte preparations with low baseline CYP3A4 activity. The amount of augmentation of enzyme activity was also concentration-dependent inasmuch as the greatest effects of DEX (10 μM) on both CYP3A4 and CYP2B6 activities were seen at the low end of the CPA range used for concentration-response experiments. DEX produced greater augmentation of the inductive effect of CPA on CYP2B6 compared with CYP3A4.

The results from the transient transfection assays show that CPA is capable of activating PXR, suggesting that this may be the predominant mechanism of the observed autoinduction exhibited by this drug in vivo. However, the dose response for PXR activation by CPA does not appear to be sufficient to account entirely for the extent of induction that is observed in primary hepatocyte cultures. This could be due to a number of factors including the involvement of other nuclear receptors or the formation of active metabolites of CPA that are more efficacious activators of PXR in primary cell culture systems. DEX enhanced the inductive effect of CPA far less in this model compared with human hepatocyte.

The distinct dose-responses for CPA induction of 3A4 versus 2B6 may also be explained by the differential involvement of other nuclear receptors known to play a role in the regulation of these.
two genes, namely CAR and GR (Sueyoshi et al., 1999; Pascussi et al., 2000; Schuetz et al., 2000). Coregulation of CYP3A and CYP2B has been shown to occur by direct, but differential, binding of PXR and CAR to the same response elements in the CYP3A and CYP2B promoters (e.g., DR3/DR4) (Moore et al., 2000; Xie et al., 2000; Goodwin et al., 2001). Differential regulation of two separate genes can also occur if one gene is partially regulated by a repressor element that must first be overcome before up-regulation occurs. We would hypothesize that one of these, if not both, events is the predominant mechanism that determines the differences we observe. However, Pascussi et al. (2000) have shown that the glucocorticoid receptor can indirectly effect the regulation of these genes by modulating the levels of the receptors themselves. In addition, we and others have observed that the basal levels of 3A and 2B expression are differentially regulated by activation of the GR. For example, 3A basal expression is increased in a dose-dependent fashion by increasing levels of dexamethasone whereas basal levels of 2B are not. This, in effect, affects the overall -fold induction profile that is observed for each respective gene by inducers such as rifampicin. Thus, the regulation of these two genes is much more complicated than first meets the eye; the evidence suggests that PXR is a predominant factor in the regulation of both.

The importance of these findings to cancer patients receiving various doses of CPA is not clear. Although CYP3A4 activates CPA to its alkylating moiety, it has also recently been shown the P450 isoform responsible for the N-dechloroethylation and production of toxic byproducts (Roy et al., 1999). Therefore, induction of CYP3A4 may ultimately benefit or harm patients receiving this agent. Roy et al. (1999) demonstrated that CPA was the major P450 isoform responsible for the metabolic activation of cyclophosphamide and ifosfamide. Therefore, induction of these two genes would thus favorably impact the metabolic disposition of CPA.

References


Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, and Negishi M (1999) The repressed nuclear...


