Arsenite decreases CYP3A4 and RXRα in primary human hepatocytes


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ABBREVIATIONS: CYP, cytochrome P450; Rif, rifampicin; PB, sodium phenobarbital;
RXRα, retinoid X receptor alpha; PXR, pregnane X receptor; CAR, constitutive
androstane/active receptor; As, sodium arsenite; DEX, dexamethasone; FeNTA, ferric
nitrilotriacetic acid; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate
dehydrogenase; TBARS, thiobarbituric acid reactive substances
Abstract

Arsenic is a naturally occurring, worldwide contaminant implicated in numerous pathological conditions in humans, including cancer and several forms of liver disease. One of the contributing factors to these disorders may be the alteration of cytochrome P450 (CYP) levels by arsenic. CYPs are involved in the oxidative metabolism and elimination of numerous toxic chemicals. CYP3A4, a major CYP in humans, is involved in the metabolism of half of all currently used drugs. Acute exposure to arsenite decreases the induction of CYP1A1/2 proteins and activities in cultured human hepatocytes, as well as CYP3A23 in cultured rat hepatocytes. Here, in primary cultures of human hepatocytes, we assessed the effects of acute arsenite exposure on CYP3A4 and several transcription factors involved in CYP3A4 expression. The concentrations of arsenite used in these studies were non-toxic to the hepatocytes and failed to elicit an oxidative response. Treatment with arsenite in the presence of CYP3A4 inducers, rifampicin (Rif) or phenobarbital (PB), caused major decreases in CYP3A4 mRNA, protein and activity. In addition, the levels of CYP3A4 in untreated cells were decreased following arsenite treatment. Transcription of the CYP3A4 gene is primarily regulated by heterodimers of the retinoid X receptor alpha (RXRα) and the pregnane X receptor (PXR). We found that arsenite failed to affect expression of PXR or the transcription factor Sp1, yet caused a significant decrease in PXR responsiveness to Rif. Arsenite caused a large decrease in nuclear RXRα protein, and, to a lesser extent, RXRα mRNA. These results suggest that arsenite inhibits both untreated and induced CYP3A4 transcription in primary human hepatocytes by decreasing the activity of PXR, as well as expression of the nuclear receptor RXRα.
Arsenic was recognized as one of the first human carcinogens when an association between medicinal arsenic compounds and skin cancer was observed in the 1800’s (Hutchinson, 1888). Arsenic is a naturally occurring element found within the Earth’s crust and, therefore, is an environmental contaminant in many areas throughout the world. In recent years, environmental exposure to arsenic has been implicated in the occurrence of various cancers and numerous health problems (Yoshida et al., 2004). One target of arsenic appears to be the liver, as hepatocellular carcinoma, fibrosis and cirrhosis, as well as biliary occlusion are associated with arsenic exposure (Ferrini et al., 1997; Runge et al., 2000).

A target of arsenite in liver cells may be the CYPs, which are gene families of hemoproteins that catalyze the oxidation of many endogenous and exogenous lipophilic chemicals (Nelson et al., 1996). Exposure to organic toxic chemicals can result in increases in CYPs involved in their metabolism, a response that may have evolved in order to protect against the toxicity of such chemicals (Whitlock, 1999). In rodents, acute exposure to arsenite reduces both basal and induced levels of some hepatic CYPs, as measured spectrally or enzymatically (Albores et al., 1992; Sardana et al., 1981). In primary cultures of rat hepatocytes, arsenite has been found to decrease induction of CYPs 1A1/2, 2B1 and 3A23 (Jacobs et al., 1999). The effect of arsenite to decrease CYPs can have major implications in human health, by altering the metabolism and elimination of toxic chemicals, as well as drugs, that are substrates for such CYPs.

It is generally accepted that CYP3As are the most abundant CYP proteins in the human liver, accounting for between 30% and 60% of the total cytochrome P450 content, with CYP3A4 being the major hepatic CYP3A present (Shimada et al., 1994; Wrighton et al., 1990). In humans,
CYP3A proteins are involved in the metabolism of 45-60% of all currently used drugs (Evans and Relling, 1999; Li et al., 1995). Therefore, variability in CYP3A4 expression would be expected to have a profound effect on the efficacy and safety of drugs that have a narrow therapeutic index and are metabolized by CYP3As. In addition to being a worldwide contaminant, arsenic, in the form of arsenic trioxide, is a first-line treatment for cancers such as multiple myeloma (Rousselot et al., 2004) and acute promyelocytic leukemia (Evens et al., 2004). Arsenic-mediated decreases in the de novo synthesis of CYP3A may compromise the metabolism or effectiveness of other drugs administered to these patients. On the other hand, arsenic-mediated decreases in CYP3A could prove advantageous in prolonging the half-life of co-administered drugs in which the parent drug is active.

CYP gene activation is induced by many natural and environmental compounds. The xenobiotic–mediated induction of CYP3A4 is regulated primarily by PXR (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). However, Goodwin et al. (2002a) have recently reported that the constitutive androstane/active receptor (CAR) can transactivate expression of the CYP3A4 gene in HepG2 cells. Rif has been shown to activate human PXR, but not CAR, in transfection studies using CV-1 cells (Moore et al., 2000). CAR and PXR are members of the nuclear receptor superfamily and form heterodimers with RXRα. PXR is activated by a number of xenobiotics and steroids (Goodwin et al., 2002b) and regulates CYP3A gene induction by targeting specific response elements present in the regulatory region of CYP3A (Goodwin et al., 2002b; Sueyoshi and Negishi, 2001). Although PXR and CAR function as heterodimers with RXRα, little is known about the role of RXRα or RXRα-selective ligands in CYP regulation.
We have previously reported that 5 μM arsenite decreases PB-induced CYP3A23 protein, with little to no decrease in CYP3A23 mRNA, in primary cultures of rat hepatocytes (Jacobs et al., 1999). Arsenite has also been shown to decrease CYP1A1 and CYP1A2 expression in primary human hepatocytes (Vakharia et al., 2001), benzo[a]pyrene-induced CYP1A1 and CYP1B1 expression in T-47D human breast cancer cells (Spink et al., 2002), and benzo[k]fluoranthene-mediated induction of CYP1A1 mRNA in HepG2 cells (Bessette et al., 2005). Although the mechanisms underlying arsenite-mediated decreases in CYPs have yet to be identified, several hypotheses have been generated, including transcriptional and post-translational events (Spink et al., 2002; Vernhet et al., 2003).

In the present study, we examined the effect of low concentrations of arsenite (2.5 or 5 μM) on untreated and Rif- or PB-induced expression of CYP3A4, as well as on several transcription factors involved in CYP3A4 expression. We found that arsenite abolished the untreated and induced expression of CYP3A4 mRNA and protein, as well as associated catalytic activity. Furthermore, arsenite caused a dramatic decrease in nuclear RXRα protein, while having no effect on expression of PXR, or the transcription factor Sp1. Arsenite also caused a significant decrease in the responsiveness of PXR to inducer. These results suggest that arsenite is acting at the level of CYP3A4 transcription, by decreasing expression of RXRα and activity of PXR.
Materials and Methods

Chemicals. Sodium arsenite (As), Rif, and PB were obtained from Sigma (St. Louis, MO). L-[^14C(U)]-leucine was from DuPont NEN Research Products (Boston, MA). Williams E powder was from GIBCO Laboratories (Grand Island, NY). Polyclonal antibody to human CYP3A4 was purchased from Chemicon International (Temecula, CA). Polyclonal antibodies to human Sp1 and PXR were purchased from Active Motif (Carlsbad, CA), and polyclonal antibody to human RXRα was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alkaline phosphatase-conjugated goat anti-rabbit antibody was purchased from Bio-Rad (Hercules, CA).

Human Hepatocyte Culture. Human hepatocytes were prepared from livers not used for whole organ transplant within 24 h of procurement. Information on the liver donors is provided in Table 1. Hepatocytes were isolated by a three-step collagenase perfusion technique, as described previously (Strom et al., 1996), and plated at a cell density of 2 x 10^6 cells per well in 6-well plates previously coated with type I collagen. The hepatocytes were maintained in Williams E medium supplemented with 10^{-7} M dexamethasone (DEX), 10^{-7} M insulin, 100 units/ml penicillin G, 100 µg/ml streptomycin, 1 mM ascorbate, 10^{-7} M selenium, 20 mM Hepes, and were kept at 37°C in a humidified incubator with 95% air/5% CO_2. Following 24-48 h in culture, cells either remained untreated or were exposed to 10 µM Rif or 2 mM PB for 24 h, with or without 2.5, 5 or 10 µM arsenite or 20 µM ferric nitrilotriacetic acid (FeNTA), as indicated in the figure legends. Nuclear and cytoplasmic extracts were prepared from hepatocytes, as described (Pascussi et al., 2000a). PB was dissolved in saline. As (made fresh) and FeNTA were dissolved in water. Rif and testosterone were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in culture medium was below 1 µl/ml.
Rat Hepatocyte Culture and Transfection. Primary cultures of rat hepatocytes were transfected with pGL3CYP3A23, a luciferase reporter construct containing −1360 to +82 base pairs of the 5′ upstream region of rat CYP3A23 (Burger et al., 1992) and pCMX-PL2-hSXR, an expression vector containing the complete protein-coding region of human PXR (steroid and xenobiotic receptor [SXR]) (Blumberg et al., 1998). Cells were prepared from mature male Fisher 344 rats (200 to 250g), as previously described (Sinclair et al., 1990). The yield was approximately 350 x 10^6 cells per liver. Cells were plated onto tissue culture plates previously coated with collagen, and maintained in Williams E medium containing 10^-7 M DEX, 10^-7 M insulin, 10^-7 M selenium, 100 units/ml penicillin G, 100 µg/ml streptomycin and 0.3 mM ascorbate. Forty-eight hours after isolation, transfection of the plasmid DNA was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Transfections were performed in fresh medium using 2 x 10^6 cells, 500 ng of pGL3CYP3A23, 100 ng of pCMX-PL2-hSXR and 50 ng of pRL-tk (Promega, Madison, WI) as an internal control. After 6 h, the medium was changed, and fresh medium containing Matrigel (0.2 mg/ml) was added. The following day, chemicals were added in fresh medium. Cells were harvested in Passive Lysis Buffer (Promega) 18 h after treatments and Firefly and Renilla luciferase levels in cell extracts were analyzed according to the manufacturer's directions (Promega).

Protein Synthesis. Protein synthesis was determined in intact cells by the incorporation of [14C]-leucine (specific radioactivity, 0.1 mCi/mmol, 0.2 µCi/plate) into acid-precipitable material
in 1 h, as described (Kostrubsky et al., 1997). The rates of protein synthesis are expressed relative to cells treated with inducer (Rif) alone.

**RNA Isolation and Invader® RNA Analysis.** Total RNA was extracted from cells using guanidinium isothiocyanate lysis buffer and cesium chloride ultracentrifugation, as described (Hamilton et al., 1988). CYP3A4 mRNA was quantified using the CYP3A4 Invader® RNA assay kit, according to instructions provided by the manufacturer (Third Wave Technologies; Madison, WI). This assay is specific for CYP3A4, and uses a fluorescence resonance energy transfer (FRET)-based signal amplification method that is based on an enzyme-substrate reaction using Cleavase® enzymes. These enzymes cut only the specific structure on targeted mRNA that is formed during the Invader® process. Primary reactions (20 µl) were performed in 96-well microplates (MJ Research, Waltham, MA), and to each well, 5 µl primary reaction components (10 mM MOPS, pH 7.5, 0.05% Tween-20, 0.05% Nonidet P-40 (NP-40), 4% polyethylene glycol (PEG), 100 mM KCl, 12.5 mM MgSO₄, 2 ng/µl Tth 5'-nuclease, 0.8 µM probe, 0.5 µM invasive oligonucleotide and 0.3 µM stacking oligonucleotide), 5 µl standard or RNA sample, and 10 µl mineral oil were added. Microplates were incubated at 60 °C for 1 h. To initiate the secondary reaction, 5 µl of secondary reaction components (10 mM MOPS, pH 7.5, 0.05% Tween-20, 0.05% NP-40, 20 mM MgSO₄, 0.67 µM FRET oligonucleotide, 0.1 µM secondary reaction template and 2.1 µM arrestor oligonucleotide) were added to each well and incubated at 60 °C for 1 h. Microplates were read directly in a SpectraMax Gemini XS (Molecular Devices; Sunnyvale, CA) using SOFTmax Pro Software (Molecular Devices) at 485 nm excitation and 530 nm emission. A standard curve for CYP3A4 mRNA was generated in each assay using purified CYP3A4 mRNA provided by the manufacturer. Expression levels
(CYP3A4 mRNA molecules/ng total RNA) were calculated using net signal values from the
CYP3A4 standard curve equation and reported as fold induction over control. Each sample was
assayed in triplicate using a range of 1-50 ng total RNA per reaction.

**RT-PCR.** Human PXR, Sp1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNAs
were measured by RT-PCR using the RNA PCR kit (TAKARA Co., Kyoto, Japan), according to
the manufacturer’s instructions. RXR\(\alpha\) expression levels were measured by RT-PCR using the
SuperScript First-Strand Synthesis (Invitrogen) and TAKARA PCR Amplification kits. The
primer sequences were as follows: PXR sense: 5’-TCCGGAAGATCTGTGCTCTCT-3’,
antisense: 5’-AGGGAGATCTGGTCCTCGAT-3’, Sp1 sense: 5’-AATTCAAGGCTGCGGAC-3’,
antisense: 5’-CCATGGAGACCAAGCTGAGC-3’, RXR\(\alpha\) sense: 5’-
TTCGCTAAGCTCTTCCTCAT-3’, antisense: 5’-ATAAGGAAGGTGTCAATGGG-3’, GAPDH
sense: 5’-GGTCGGAGTCAACGGATTTGGTCG-3’, antisense: 5’-
CAAGTTTGCATGGATGACC-3’. Amplification was carried out in a Bio-Rad iCycler with
initial denaturation at 94-95 °C for 2 min, followed by 16-24 cycles of 94 °C for 30 sec, 60-62
°C for 30 sec and 72 °C for 30 sec. PCR samples were electrophoresed on agarose gels and
visualized by ethidium bromide. The number of PCR cycles resulting in PCR products in the
linear phase of the amplification curve was determined. The primers were designed to cross
exon junctions and PCR products amplified from genomic DNA templates were not seen. PCR
products were sequenced to verify integrity. In some experiments, the resulting gels were
quantitated by densitometry, using Adobe Photoshop with an HP Precision Scanner and
OneDScan software (Scanalytics, Fairfax, VA).
Western Blot. CYP3A4 and Sp1 protein levels were measured in cell sonicates, and PXR and RXRα protein levels were analyzed in nuclear and cytoplasmic fractions. Briefly, proteins were separated by electrophoresis in SDS-PAGE (10% acrylamide) and transferred to nitrocellulose electrophoretically at 100V for 1 h. The nitrocellulose sheets were blocked overnight at 4 °C in phosphate-buffered saline containing 5% non-fat dry milk and 0.3% Tween-20, incubated for 1 h at room temperature (CYP3A4) or overnight at 4 °C (Sp1, PXR, RXRα) with primary antibodies, and exposed for 1 h to an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. The resulting blots were quantitated by densitometry, using Adobe Photoshop with an HP Precision Scanner and OneDScan software (Scanalytics, Fairfax, VA).

CYP3A4 Enzymatic Activity. The activity of CYP3A4 was measured by formation of 6β-hydroxytestosterone in intact cultured hepatocytes, as described (Kostrubsky et al., 1999). Briefly, after a 24 h exposure to chemicals, the culture media was replaced with fresh Williams E medium containing 200 µM testosterone. The cells were incubated for 30 min at 37 °C and 6β-hydroxytestosterone was measured in the media by HPLC, as described (Kostrubsky et al., 1999).

Additional Assays. Proteins were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Lipid peroxidation in hepatocytes and media was measured by the generation of thiobarbituric acid reactive substances (TBARS), as described (Shedlofsky et al., 1983).
Statistical Analyses. Data are presented for representative experiments that were repeated 2 or more times. Within each experiment, treatments were performed in duplicate or triplicate, as indicated in the figure legends. In triplicate treatments, results were analyzed either by a Student’s $t$ test or by ANOVA, followed by a Student-Neuman-Kuels multiple comparisons test. A $p$ value of <0.05 was taken to indicate significance.
Results

Effect of Arsenite on Induction of CYP3A4 mRNA, Immunoreactive Protein and Enzyme Activity in Primary Human Hepatocytes. Vakharia et al. (2001) found that 5 µM arsenite decreased induction of CYPs 1A1 and 1A2 in primary cultures of human hepatocytes with no effect on cell viability, as measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Therefore, we investigated the effect of 2.5 and 5 µM arsenite on induction of CYP3A4 by Rif and PB. Neither of these doses of arsenite, in the presence of 10 µg/ml Rif, was toxic to the cells, as evidenced by no decrease in total protein synthesis (Fig. 1A). In contrast, a higher dose of arsenite (10 µM) caused a 30% decrease in protein synthesis (results not shown), and therefore, was not used in later studies.

We examined three parameters in investigating the effect of arsenite on induction of CYP3A4 by Rif or PB: mRNA, protein and enzyme activity. CYP3A4 mRNA levels were measured using the highly sensitive and specific CYP3A4 Invader® RNA assay, which does not detect the other forms of human CYP3A (de Arruda et al., 2002). Inducibility of CYP3A4 by Rif and PB was variable among human cultures from different donors, as previously reported for CYPs in human hepatocyte cultures (Daujat et al., 1991; Kocarek et al., 1995; Madan et al., 2003). Figure 1B demonstrates that Rif induced expression of CYP3A4 mRNA, and that arsenite (2.5 or 5 µM) abolished induction of CYP3A4 mRNA in three separate human hepatocyte cultures. In order to assess whether these diminished CYP3A4 mRNA levels would have an impact on the amount of immunoreactive protein formed, as well as associated catalytic activity, CYP3A4 protein and activity levels were determined. In a representative experiment, treatment with 5 µM arsenite for 24 h caused a 75% decrease in CYP3A4 protein compared to Rif treatment alone (Fig. 1C).
CYP3A4 catalytic activity was measured by determining the 6β-hydroxylation of testosterone by intact cells (Kostrubsky et al., 1999). The fold increases in CYP3A4 activity did not correlate directly with the increases in CYP3A4 mRNA (Fig. 1D vs. 1B). However, low-dose arsenite (2.5 or 5 µM) caused significant decreases in CYP3A4 activity in all three cultures (Fig. 1D). Thus, Rif-mediated induction of CYP3A4 mRNA, protein and enzyme activity was abolished by simultaneous treatment with arsenite.

We next investigated whether arsenite decreases CYP3A4 induction by PB, which is capable of inducing CYP3A4 through PXR or CAR (Goodwin et al., 2002a; Moore et al., 2000), in contrast to Rif, which induces CYP3A4 through PXR (Moore et al., 2000). In two human hepatocyte cultures that were highly responsive to PB-mediated induction of CYP3A4 (Fig. 2A insert; HH 994 and HH 1051), arsenite decreased the induction of CYP3A4 mRNA (Fig. 2A). Although two other hepatocyte cultures were much less responsive to PB-mediated induction of CYP3A4 (Fig. 2A; HH 845 and HH 888), arsenite still caused a significant decrease in CYP3A4 mRNA. PB-induced CYP3A4 protein (Fig. 2B) and catalytic activity (Fig. 2C) were also decreased when arsenite was included in the treatment. These results show that arsenite decreases both Rif- and PB-mediated induction of CYP3A4 at the level of mRNA, protein, and activity.

**Effect of Arsenite on Expression of CYP3A4 mRNA, Immunoreactive Protein and Enzyme Activity in Untreated Primary Human Hepatocytes.** We next investigated whether arsenite affects expression of CYP3A4 in untreated human hepatocytes. Similar to our findings with induced CYP3A4 mRNA levels, arsenite caused a significant decrease in levels of CYP3A4 mRNA in hepatocytes not treated with inducers (Fig. 3A). Likewise, CYP3A4 protein (Fig. 3B)
and catalytic activity (Fig. 3C) were diminished following 24 h treatment with 5 µM arsenite. Therefore, in addition to interfering with the induction of CYP3A4 mRNA, arsenite suppressed the expression of CYP3A4 in cells that were not co-treated with inducers.

**Effect of Arsenite on Expression of PXR and Sp1 in Primary Human Hepatocytes.**

CYP3A4 mRNA expression in untreated hepatocyte cultures, as well as following treatment with inducers, was significantly affected by arsenite (Figs. 1B, 2A, 3A), a finding that could result from inhibition of transcription of the CYP3A4 gene. Therefore, we examined whether arsenite affects expression of a nuclear receptor critical to CYP3A4 expression, PXR, and the transcription factor Sp1. The proximal promoter of CYP3A4 contains an interaction site for Sp1, which is capable of affecting both the basal- and xenobiotic-mediated activation of gene expression (Bombail et al., 2004). Analyses by RT-PCR show that a 24 h treatment with 2.5 or 5 µM arsenite did not affect PXR or Sp1 mRNA levels (Fig. 4A). PXR protein was detected in both the cytoplasm and the nucleus (Fig. 4B). None of the treatments altered the cellular localization of PXR, including arsenite alone, Rif alone, or both in combination (Fig. 4B). These treatments also failed to affect the levels of PXR and Sp1 protein (Fig. 4B).

**Effect of Rifampicin and Arsenite on Activity of Human PXR in Primary Rat Hepatocytes.**

To determine if the decrease in CYP3A4 mRNA was due to inhibition of PXR activity, we investigated the effect of arsenite on expression of a CYP3A23 reporter construct in primary rat hepatocytes. This expression plasmid is responsive to Rif in rat hepatocytes only when human PXR is expressed (Xie et al., 2000a). The cultures were transiently transfected with a Firefly luciferase reporter vector containing nucleotides -1360 to +82 of the 5' upstream region of rat
CYP3A23 (pGL3CYP3A23) (Burger et al., 1992). These cultures were co-transfected with pCMX-PL2-hSXR, an expression vector containing the complete protein-coding region of human PXR (Blumberg et al., 1998), as well as a Renilla luciferase plasmid as an internal control for transfection efficiency. Figure 5A shows that Rif is not able to increase reporter gene expression in the absence of human PXR, but upon co-transfection of a vector containing human PXR, Rif caused a 2-fold increase in reporter gene expression. When arsenite was also included, reporter gene expression was decreased by approximately 75% (Fig. 5B), indicating an effect of arsenite to decrease the activity of human PXR.

**Effect of Arsenite on RXRα Expression in Primary Human Hepatocytes.** We next investigated the effect of arsenite on expression of the nuclear receptor RXRα. In contrast to PXR, RXRα protein was detected in nuclear fractions, but not in cytoplasmic fractions (Fig. 6A). Figure 6A shows that a 24 h treatment with arsenite caused a dramatic decrease in nuclear RXRα protein levels. Similar results were observed in cultures from five separate livers (results not shown). Arsenite-mediated decreases in RXRα were observed in untreated as well as Rif-treated cells. To determine whether arsenite decreased RXRα protein by decreasing RXRα mRNA, we measured RXRα mRNA by RT-PCR. Figure 6B shows that arsenite decreased expression of RXRα mRNA, however the extent of the decrease was less than that of the protein (Fig. 6A). These results suggest that the effect of arsenite to decrease RXRα may contribute to diminished transcription of CYP3A4 following treatment with arsenite.

**Effect of Arsenite on Oxidative Stress in Primary Human Hepatocytes.** Exposure to arsenite (1 or 10 µM) for 24 h was reported to cause oxidative stress in human gastric cancer cells, as
measured by the formation of reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS) (Chen et al., 2002). To determine whether 5 µM arsenite causes oxidative stress in primary cultures of human hepatocytes, we measured the generation of lipid peroxides as an indicator of the oxidative response. Exposure of hepatocytes to 20 µM FeNTA, an iron compound known to cause the formation of ROS (Morel et al., 1990), for 24 h, caused a 3- to 4-fold increase in lipid peroxidation (Fig. 7), as determined by the generation of TBARS. In contrast, arsenite (2.5 or 5 µM), in combination with Rif (10 µM), did not cause an increase in lipid peroxidation (Fig. 7).
Discussion

Arsenic is a natural contaminant that may cause human liver diseases by altering hepatic levels of CYPs. We investigated the effect of arsenite on the expression of CYP3A4 in primary cultures of human hepatocytes. We found that simultaneous exposure to arsenite and inducers of CYP3A4 for 24 h completely prevented any increase in CYP3A4 mRNA, protein and activity (Figs. 1 and 2). In addition, arsenite decreased expression of CYP3A4 in untreated hepatocyte cultures (Fig. 3). These changes occurred at concentrations of arsenite (2.5 and 5 µM) that did not cause toxicity (Fig. 1A). Although these concentrations of arsenite have been reported to stimulate the formation of ROS and TBARS in cultures of other cell types (Chen et al., 2002), 5 µM arsenite failed to increase lipid peroxidation in human hepatocytes (Fig. 7). Importantly, we show that arsenite caused a major reduction in the activity of PXR (Fig. 5B) and nuclear protein levels of RXRα (Fig. 6A), with no effect on PXR or Sp1 expression (Fig. 4). The overall findings suggest that arsenite acts at the level of transcription by inhibiting PXR activity and lowering the amount of RXRα available for expression of CYP3A4.

Transcriptional regulation of CYPs is complex. PXR and CAR have both been shown to regulate gene expression of CYP3A4, depending on the inducer (Bertilsson et al., 1998; Blumberg et al., 1998; Goodwin et al., 2002a; Lehmann et al., 1998). Both PB and Rif can activate human PXR (Moore et al., 2000). PB has recently been shown to cause translocation of CAR to the nucleus in primary human hepatocyte cultures (Assenat et al., 2004), indicating that PB can also activate human CAR. We investigated whether arsenite affects Rif- and PB-mediated induction of CYP3A4 in primary human hepatocytes. Both Rif- and PB-induced CYP3A4 mRNA levels were abolished by arsenite (Figs. 1B and 2A), suggesting that arsenite
affects PXR- and possibly CAR-mediated transcription of CYP3A4. One possibility is that arsenite targets a factor common to both receptors. RXRα heterodimerizes with PXR and CAR to transactivate target genes in a ligand-dependent manner (Xie et al., 2000b). Our findings that arsenite decreased nuclear protein levels of RXRα in both untreated and Rif-induced hepatocytes (Fig. 6A) may account for the decreased expression of CYP3A4. The decrease in RXRα protein (Fig. 6A) was far greater than the decrease in RXRα mRNA following treatment with arsenite (Fig. 6B). This suggests that arsenite not only decreases the expression of RXRα mRNA, but also has a post-transcriptional effect. One possibility is that arsenite enhances the degradation of RXRα protein. For example, it has been reported that tetradecanoylphorbol-1, 3-acetate (TPA) can induce the degradation of RXRα in gastric cells via the proteasome pathway (Ye et al., 2003). We are currently investigating whether protein degradation plays a role in the arsenite-mediated decrease in RXRα protein.

Our findings that arsenite decreased nuclear RXRα protein (Fig. 6A) and CYP3A4 mRNA (Figs. 1B, 2A, 3A) are similar to a recent report that lipopolysaccharide (LPS) decreases RXRα protein levels in livers of mice, in parallel with decreases in Cyp3a11 mRNA (Ghose et al., 2004). Nuclear translocation affects gene expression by some nuclear receptors. In that study, LPS changed the localization of RXRα from the nucleus into the cytoplasm (Ghose et al., 2004). In our studies, arsenite did not change the RXRα localization at 24 h (Fig. 6A), but may have had an effect at an earlier time. Nuclear translocation of PXR in response to ligand has been reported to occur following treatment of mice with 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) (Squires et al., 2004). A time course of PCN treatment in mice demonstrated that the increase in hepatic nuclear PXR protein was transient, returning to basal levels by 17 h (Squires et al., 2004).
et al., 2004). This decrease may have been caused by decreases in the hepatic level of inducer over time. In our studies, a 24 h treatment with Rif had no effect on nuclear localization of PXR (Fig. 4B), even though CYP3A4 mRNA was induced. Since these cells were isolated from livers of humans pre-exposed to numerous drugs (Table 1), including many CYP3A4 inducers, PXR may have already been activated and translocated to the nucleus. Arsenite had no effect on nuclear levels of PXR (Fig. 4B). Since the cultured hepatocytes were continuously exposed to inducer (Rif) for 24 h, arsenite may have had a transient effect on the accumulation of PXR protein in the nucleus. Nevertheless, our results do demonstrate that after 24 h of treatment with inducer, when CYP3A4 mRNA levels are increased, arsenite does not affect CYP3A4 transcription by causing a translocation of PXR or RXRα from the nucleus to the cytoplasm.

It is well known that arsenite interacts with critical cysteine residues of many intracellular proteins (Del Razo et al., 2001). Therefore, arsenite may also bind to the nuclear receptors involved in expression of CYP3A4, resulting in decreases in their ability to bind ligands or other transcription factors required for upregulation of CYP3A4. Our finding that arsenite caused a large decrease in the responsiveness of human PXR to Rif (Fig. 5B) supports this hypothesis. However, if the arsenite-mediated decreases in RXRα expression observed in cultured human hepatocytes also occurred in cultured rat hepatocytes, the decrease in human PXR activity (Fig. 5B) may arise from an insufficient amount of RXRα for heterodimerization. Therefore, we plan to investigate whether arsenite decreases RXRα in primary rat hepatocytes. Furthermore, arsenite has been shown to down-regulate telomerase gene expression in human leukemia cells, partly through inhibition of the DNA binding activity of Sp1, which contains redox-sensitive cysteine residues (Chou et al., 2001). Although we found that arsenite does not decrease levels
of Sp1 in cultured human hepatocytes (Fig. 4), arsenite may inhibit the ability of Sp1 to bind DNA. Additionally, there are numerous other transcription factors that may be affected by arsenite treatment. For example, hepatocyte nuclear factor 3 (HNF3), HNF4 and CCAAT/enhancer binding protein (C/EBPα) have been shown to play a role in the basal or xenobiotic-mediated regulation of CYP3A4 transcription (Bombail et al., 2004; Jover et al., 2001). Therefore, further work is needed in order to establish whether arsenite affects expression or activity of these or other transcription factors that are involved in CYP3A4 regulation.

In human hepatocyte cultures, glucocorticoids increase the level of CAR and PXR mRNAs and proteins, leading to the potentiation of xenobiotic-mediated induction of CYP3A4 (Pascussi et al., 2000a,b). Since we found that arsenite does not affect PXR mRNA or protein levels (Fig. 4), it is likely that the action of arsenite on expression of CYP3A4 is independent of the glucocorticoid receptor (GR). In contrast, arsenite has been shown to inhibit GR-mediated transcription in hepatoma cells (Bodwell et al., 2004; Kaltreider et al., 2001). However, another study demonstrated that exposure to arsenite leads to transactivation of GR elements in mouse epidermal JB6 cells (Huang et al., 2001). Therefore, the effect of arsenite may depend on the cell type.

In our previous studies, using primary rat hepatocytes, treatment with arsenite (5 µM) decreased DEX-, but not PB-mediated induction of CYP3A23 mRNA even though arsenite decreased both DEX- and PB-mediated increases in CYP3A protein (Jacobs et al., 1999). However, the extent of the decrease in CYP3A23 mRNA induced by DEX was small (25%) compared to the much
larger decreases (up to 95%) observed with CYP3A4 mRNA in human hepatocytes (Figs. 1B and 2A). Therefore, it appears that CYP3A transcriptional regulation in human hepatocytes is more sensitive to arsenite than in rat hepatocytes.

In summary, we have shown that low doses of arsenite (2.5 or 5 \mu M) prevented induction of CYP3A4 mRNA, protein, and catalytic activity in primary cultures of human hepatocytes. Arsenite also decreased expression of CYP3A4 in hepatocytes not treated with inducers. The substantial decreases in CYP3A4 mRNA suggest that the mechanism of action of arsenite involves suppressed transcription of the CYP3A4 gene. This possibility is supported by our findings that arsenite caused a dramatic decrease in nuclear protein levels of RXR\(\alpha\) and responsiveness of PXR to inducer. We are currently evaluating the mechanism by which arsenite decreases RXR\(\alpha\) and the effect of arsenite on other genes regulated by this critical nuclear receptor.
References


and CYP3A4 but not of CYP2C9, CYP2C19, multidrug resistance (MDR-1) and multidrug resistance associated protein (MRP-1) by prototypical inducers in human hepatocytes.


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Footnotes

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Figure Legends

Figure 1. Effect of sodium arsenite on toxicity and rifampicin-induced CYP3A4 mRNA, protein and activity in primary human hepatocytes. Hepatocytes were either untreated (Control) or treated with Rif (10 µM), alone or in combination with As (2.5 or 5 µM) for 24 h, as indicated in the figures. (A) Toxicity was tested in Rif-treated cells using the incorporation of 14C leucine into protein as an indicator, as described (Kostrubsky et al., 1997). Values represent the mean ± range/SD of duplicate or triplicate determinations in hepatocytes from two representative donors (HH 789, HH 1142). (B) Total RNA was isolated and CYP3A4 mRNA was measured using the Invader® RNA Assay, as described in Materials and Methods. Values are expressed relative to control levels (“fold induction”) and represent the mean ± SD of duplicate or triplicate treatments assayed in triplicate. The absolute values (molecules/ng total RNA) in untreated cells were: HH 840, 5943 ± 995; HH 841, 5774 ± 1998; HH 893, 30741 ± 4395. (C) CYP3A4 protein (HH 1122) was assayed in cell sonicates by SDS-PAGE using a polyclonal antibody which recognizes human CYP3A4 protein. Values represent the mean ± range of duplicate treatments. (D) Testosterone 6β-hydroxylation was measured, as described (Kostrubsky et al., 1999). Each value represents the mean ± SD of triplicate treatments. N = not determined. ***p<0.001 vs. Rif.

Figure 2. Effect of sodium arsenite on phenobarbital-induced CYP3A4 mRNA, protein and activity in primary human hepatocytes. Hepatocytes were either untreated (Control) or treated with PB (2 mM), alone or in combination with As (2.5 or 5 µM) for 24 h, as indicated in the figures. (A) Total RNA was isolated and CYP3A4 mRNA was measured using the Invader® RNA Assay, as described in Materials and Methods. Values are expressed relative to control
levels ("fold induction") and represent the mean ± SD of duplicate or triplicate treatments assayed in triplicate. The absolute values (molecules/ng total RNA) in untreated cells were: HH 845, 9664 ± 752; HH 888, 5120 ± 1742; HH 994, 479 ± 247; HH 1051, 336 ± 193. (B) CYP3A4 protein (HH 888) was assayed in cell sonicates by SDS-PAGE using a polyclonal antibody which recognizes human CYP3A4 protein. Values represent the mean ± range of duplicate treatments. (C) Testosterone 6β-hydroxylation was measured, as described (Kostrubsky et al., 1999). Each value represents the mean ± SD of triplicate treatments. N = not determined. *p<0.05 vs. PB. **p<0.01 vs. PB. ***p<0.001 vs. PB.

Figure 3. Effect of sodium arsenite on CYP3A4 mRNA, protein and activity in untreated human hepatocytes. Hepatocytes were either untreated (Control) or treated with 2.5 or 5 µM As for 24 h, as indicated in the figures. (A) Total RNA was isolated and CYP3A4 mRNA was measured using the Invader® RNA Assay, as described in Materials and Methods. Values are expressed relative to control levels ("fold over control") and represent the mean ± SD of duplicate treatments assayed in triplicate. The absolute values (molecules/ng total RNA) in untreated cells were: HH 889, 10077 ± 1473; HH 1051, 230 ± 70. (B) CYP3A4 protein (HH 1051) was assayed in cell sonicates by SDS-PAGE using a polyclonal antibody which recognizes human CYP3A4 protein. Values represent the mean ± SD of triplicate treatments. (C) Testosterone 6β-hydroxylation was measured, as described (Kostrubsky et al., 1999). Each value represents the mean ± SD of triplicate treatments. N = not determined. *p<0.05 vs. Control. **p<0.01 vs. Control. ***p<0.001 vs. Control.
Figure 4. Effect of sodium arsenite on PXR and Sp1 expression in primary human hepatocytes. Hepatocytes were either untreated (Control) or treated with 10 µM Rif and 2.5 or 5 µM As for 24 h, as indicated in the figures. (A) Total RNA was isolated and PXR and Sp1 RNA (HH 893) measured using RT-PCR, as described in Materials and Methods. (B) PXR protein (HH 1142) was assayed in cytoplasmic and nuclear extracts by SDS-PAGE using a polyclonal antibody that recognizes human PXR protein. Sp1 protein (HH1122) was assayed in cell sonicates by SDS-PAGE using a polyclonal antibody that recognizes human Sp1 protein.

Figure 5. Effect of rifampicin and sodium arsenite on human PXR activity in primary rat hepatocytes. After 48 h in culture, primary rat hepatocytes were transfected with 500 ng of pGL3CYP3A23, 0 or 100 ng of pCMX-PL2-hSXR and 50 ng of pRL-tk, as described in Materials and Methods. The following day, treatment chemicals, 10 µM Rif (A) or 10 µM Rif with or without 5 µM As (B), were added in fresh medium. Cells were harvested 18 h after treatments and cell extracts analyzed for Firefly and Renilla luciferase. Firefly luciferase values were normalized to Renilla luciferase values in the same samples and are expressed as the ratio Firefly/Renilla. Each value represents the mean of triplicate treatments with the SD indicated by the vertical bar. A and B represent two separate experiments. *p<0.05 vs. Rif. ***p<0.001 vs. Rif.

Figure 6. Effect of sodium arsenite on RXRα expression in primary human hepatocytes. Hepatocytes were either untreated (Control) or treated with 10 µM Rif and 5 µM As for 24 h, as indicated in the figures. (A) RXRα protein (HH 1174) was assayed in cytoplasmic and nuclear extracts by SDS-PAGE using a polyclonal antibody that recognizes human RXRα protein.
Values represent the mean ± range of duplicate treatments for nuclear RXRα protein. (B) Total RNA was isolated and RXRα RNA measured using RT-PCR, as described in Materials and Methods. Values represent the mean ± SD of samples from three human cultures (HH 889, HH 893, HH 1183). A representative gel (HH 1183) of the RT-PCR products is shown below the graph.

**Figure 7.** Effect of rifampicin, sodium arsenite, and ferric nitrilotriacetic acid on lipid peroxidation in primary human hepatocytes. Following 24 h with no treatment (Control), or the chemicals indicated, TBARS were measured in media and cells, as described (Shedlofsky et al., 1983). Values represent the mean ± SD of triplicate treatments. ***p<0.001 vs. Control.
### Clinical Characteristics of Human Hepatocyte Donors

<table>
<thead>
<tr>
<th>Donor (HH #)</th>
<th>Age (years)</th>
<th>Sexa</th>
<th>Raceb</th>
<th>Cause of Deathc</th>
<th>Drug Historyd</th>
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<tr>
<td>789</td>
<td>36</td>
<td>M</td>
<td>C</td>
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<td>smoker, smoker, cefazolin, vecuronium bromide, vitamin K, enalapril, metoclopramide, pentobarbital, dopamine, norepinephrine</td>
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<td>F</td>
<td>C</td>
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</tr>
</tbody>
</table>

a M, male; F, female
b C, Caucasian; H, Hispanic; AA, African American
c ICH, intracranial hemorrhage; CVA, cerebrovascular accident
d Drinker, consumer of at least 50g ethanol/day; Smoker, user of more than 1/2 pack of cigarettes/day.
Figure 1

A. Graph showing the percentage of protein synthesis (% protein synthesis) vs. arsenite concentration (μM) for HH 789 and HH 1142.

B. Bar chart showing CYP3A4 mRNA (fold induction) for HH 840, HH 841, and HH 893.

C. Bar chart showing CYP3A4 protein (arbitrary units) for control, rifampin (Rif), and rifampin/arsenic (Rif/5 μM As).

D. Bar chart showing 6β-hydroxytestosterone (pmol/min/mg protein) for HH 840, HH 841, and HH 893.

- Control
- Rif
- Rif/2.5 μM As
- Rif/5 μM As

* * * indicates statistical significance.
Figure 3

A.

CYP3A4 mRNA (fold over control)

- **HH 889**
  - Control
  - 2.5 μM As
  - 5 μM As

- **HH 1051**
  - Control
  - 2.5 μM As
  - 5 μM As

B.

CYP3A4 Protein (arbitrary units)

- **Control**
- **5 μM As**

C.

6β-hydroxycortisol (pmol/min/mg protein)

- **HH 888**
  - Control
  - 5 μM As

- **HH 1011**
  - Control
  - 5 μM As
Figure 7

TBARS Accumulation (nmol/plate)

- Control
- Rif
- Rif/2.5 μM As
- Rif/5 μM As
- FeNTA

HH 1121

HH 1122

***