Dual Mechanisms of CYP3A Protein Regulation by Proinflammatory Cytokine Stimulation
in Primary Hepatocyte Cultures

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Running Title: Cytokines and CYP3A degradation

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Number of text pages: 34 Number of tables: 0
Number of figures: 7 References: 31
Words in abstract: 245 Words in introduction: 402
Words in discussion: 1431

Abbreviations: CYP, cytochrome P450; IL, interleukin; LPS, bacterial lipopolysaccharide; PB, phenobarbital; NFκB, nuclear factor kappa B; PXR, pregnane X receptor; Dex, dexamethasone; PCN, pregnenolone-16 alpha- carbonitrile; CAR, constitutive androstane receptor; DDEP, 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine; L-NIL, L-N6-(1-Iminoethyl)lysine (L-NIL); NOC-18, (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; GSNO, N-(N-L-γ-glutamyl-S-nitroso-L-cysteiny1)- glycine; L-NAME, Nω-Nitro-L-arginine methyl ester hydrochloride; NMA, N^G-Methyl-L-arginine; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde phosphate dehydrogenase; NOx, nitrate plus nitrite; IRP2, iron regulatory protein-2
ABSTRACT

While many cytochrome P450 enzymes are transcriptionally suppressed by inflammatory stimuli, down-regulation of CYP2B protein by the inflammatory cytokine interleukin (IL)-1β is nitric oxide (NO) dependent, and occurs via polyubiquitination and proteasomal degradation. Here, we used iTRAQ proteomic analysis to search for other proteins that are potentially down-regulated by cellular NO in cultured rat hepatocytes, and identified CYP3A1 as one such protein. Therefore, we examined whether or not CYP3A proteins, like CYP2B, undergo NO- and proteasome-dependent degradation in response to cytokine treatment of rat hepatocytes. In cultured rat hepatocytes treated with phenobarbital (PB), IL-1β-stimulation failed to down-regulate CYP3A1 mRNA within 24 h of treatment, whereas CYP3A protein was down-regulated to 40% of control within 6 h, demonstrating the post-transcriptional down-regulation of CYP3A1 protein. The down-regulation of CYP3A after 9 h of stimulation by IL-1β was attenuated by inhibitors of NOS and of the proteasome, demonstrating NO- and proteasome-dependent down-regulation at earlier time points. However, the down-regulation of CYP3A evoked by IL-1β measured 24 h after stimulation was not affected by the inhibition of NOS or by proteasomal inhibitors, demonstrating that CYP3A1 down-regulation at later time points is NO- and proteasome-independent. IL-6, which did not evoke NO production nor affect CYP3A1 mRNA within 24 h, produced a delayed proteasome-independent down-regulation as well. Taken together, these observations demonstrate a novel dual mode of posttranscriptional CYP3A down-regulation by cytokines; NO- and proteasome-dependent at earlier time points, and NO- and proteasome-independent at later times.
INTRODUCTION

Cytochrome P450s (CYPs) are heme-thiolate enzymes whose carbon monoxide-bound forms have peak spectral absorbances of 450 nm. Two-thirds of the human CYP gene superfamily are important in the biosynthesis or catabolism of endogenous substances such as steroid hormones, sterols, and fatty acids. The other third are involved in metabolism of xenobiotics, and most of them are mainly expressed in liver. However, a subset of CYPs are also expressed to some degree in extra-hepatic tissues (Ding and Kaminsky, 2003). Cancer cells also express various CYP enzymes (Rooney et al., 2004). The enzyme activities and the expression levels of many CYPs are down-regulated during inflammation and infection, causing decreased metabolic clearance of CYP substrates, elevation of plasma drug levels, and drug toxicity (Aitken et al., 2006). Proinflammatory cytokine treatments of primary human and rodent hepatocytes recapitulate many of the in vivo effects of inflammation of hepatic CYP enzymes (Aitken et al., 2006; Aitken and Morgan, 2007).

Nitric oxide, NO is a short-lived cellular messenger with important roles in cell signaling. Inducible NO synthase (iNOS, NOS2) is induced during infection and inflammation via nuclear factor kappa B (NFκB) activation, and NFκB activation is dependent on proteasomal activity (Palombella et al., 1994). Bacterial endotoxin (LPS) or certain proinflammatory cytokine treatments cause NOS2 induction and NO production in vivo and in cultures of various cell lines (Geller et al., 1994). We reported that CYP2B1 is down-regulated in primary cultures of rat hepatocytes by two independent mechanisms in response to LPS: NO-independent mRNA suppression at lower concentrations, and NO-dependent protein suppression at higher
concentrations (Ferrari et al., 2001). More recently we discovered that NO-dependent CYP2B1 degradation proceeds via polyubiquitination and the proteasomal degradation pathway (Lee et al., 2008).

While the transcriptional regulation of CYP genes by inflammatory factors has been relatively well-characterized, posttranscriptional regulation of CYP proteins is less well understood. Therefore, the purpose of this study was to identify other hepatic proteins that undergo NO-dependent degradation in a manner similar to that which we described for CYP2B proteins (Lee et al., 2008). Using the iTRAQ-based proteomic approach (Zieske, 2006), we identified CYP3A1 as a protein whose levels were increased in hepatocytes treated with interleukin (IL)-1β plus the NOS inhibitor NMA, compared to hepatocytes treated with IL-1β alone. Subsequent experiments revealed that CYP3A protein, like CYP2B proteins, undergoes NO-and proteasome-dependent degradation in response to IL-1β treatment in PB-treated rat hepatocytes. However, these studies also revealed a slower mechanism of post-transcriptional down-regulation of CYP3A by IL-6, or IL-1β, that is both NO- and proteasome-independent.
MATERIALS AND METHODS

Materials and Reagents. Interleukin (IL)-1β and IL-6 were purchased from R&D systems (Minneapolis, MN). L-N6-(1-Iminoethyl)lysine (L-NIL), (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (NOC-18), and N-(N-L-γ-glutamyl-S-nitroso-L-cysteinyl)-glycine (GSNO) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), NG-Methyl-L-arginine (NMA), PB, Dex (water-soluble), Williams media, Krebs-Ringer buffer, collagenase IV, and other general chemicals were acquired from Sigma (St Louis, MO). Insulin solution was acquired from Invitrogen (Carlsbad, CA). Antibodies to CYP2B1 were kindly provided by Dr. James Halpert (University of California, San Diego, CA) and anti-CYP3A2 antibody was purchased from Daiichi Pure Chemicals (Japan). DDEP was kindly provided by Dr. M. Almira Correia (University of California, San Francisco, CA).

Rat Hepatocyte Isolation and Treatment. Rat hepatocytes were isolated by a two-step \textit{in situ} collagenase perfusion procedure as previously described (Lee et al., 2008). After male Sprague-Dawley rats (230-280 g) were anesthetized by ketamine/xylazine solution, the liver was perfused with Krebs-Ringer bicarbonate buffer and then with 0.3 mg/ml collagenase type IV for 10 min. Cells were plated on collagen plates with plating medium containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and three hours later, cells were overlaid with new medium containing Matrigel (0.23 mg/ml, BD Biosciences, San Jose, CA). Next day, the medium was changed to CYP2B1 medium (Williams E containing 10mM Hepes pH 7.4, 10 nM insulin, 25 nM Dex and 10mg/ml Pen/Strep). Cultures were maintained for 6 to 7 days at 37°C in 5% CO\textsubscript{2} with regular media changes. Beginning on day 4, cells were treated for 2 days with 1 mM PB to induce
CYP3A expression, and PB was present for the rest of the experiment. At the conclusion of the experiment, media were removed and reserved for NO assay by the Griess reaction (Lee et al., 2008).

Protein Extraction and Immunoblotting. Hepatocytes were harvested with a cell scraper, after which the cells were incubated on the ice in phosphate buffered saline (PBS) with 1 mM EDTA for at least 20 min to remove Matrigel, and then were collected by centrifugation at 1000g for 5 min. To extract total protein, cells in lysis buffer (50 mM Tris, pH7.5; 0.1% SDS; 0.5% NP40; 1 mM EDTA; protease inhibitor cocktail, Sigma) were sonicated briefly for 10 sec and then centrifuged for 10 min at 11,000 g. The supernatant (total cell lysate) was used for SDS-PAGE and immunoblotting. SDS-PAGE and Western blotting were carried out as described previously (Ferrari et al., 2001). Anti-CYP2B1 antibody, (diluted 1:20,000) and anti-CYP3A2 antibody (diluted 1:10,000) were incubated overnight at 4 °C and then horseradish peroxidase (HRP)-conjugated secondary antibody was incubated for 1 hr at room-temperature. Chemiluminescence was detected with ECL substrate (Pierce Biotech) on X-ray film. The protein loads used in the Western blot assays were chosen to be within the range that gave a linear correlation of band densities with amount of applied antigen, as determined in optimization experiments. The intensities of bands were measured by densitometry and Kodak imaging software.

iTRAQ-based scan for NO-regulated proteins Four-day old primary rat hepatocytes cultured on 150 mm plates were treated for two days with 1 mM PB and then treated with 5 ng/ml IL-1β or IL-1β + NMA (300 μM) in the continued presence of PB for 9 hours. Total cell lysates were prepared as described above, except that an additional wash step with PBS/EDTA was included to remove
any residual Matrigel from the cells. Total cell lysates were precipitated with ice-cold acetone for 2 h at -20 °C, followed by centrifugation at 10,000g for 2 min. For each treatment condition, proteins from three 150 mm culture plates were pooled. After washing the precipitates, the lysates were redissolved in 20 mM potassium phosphate buffer, pH 7.4 containing 1% SDS. SDS was removed on BioGel P-6 desalting columns (Bio-Rad, Hercules, CA). Samples were analyzed by SDS-PAGE and Coomassie staining to ensure that there were no gross differences between the samples prior to iTRAQ analysis. Two identical aliquots of each sample (IL-1 or IL-1 plus NMA) were subjected to iTRAQ analysis by the Emory Microchemical and Proteomics Facility. The samples (0.1 mg protein per 77 μl each) reconstituted in 0.1 M triethylammonium bicarbonate (pH 8.5) containing 1mM EDTA, 0.1% SDS, and 0.1% Triton X-100, were reduced with 5 mM tris-2-carboxyethyl phosphine (60 °C, 1 h) followed by oxidation with 10 mM methyl methanethiosulfonate (25 °C, 10 min). The samples were then digested with 10 μg of sequencing grade trypsin (Promega, Madison, WI) for 16 h at 37 °C, and checked by SDS-PAGE to ensure adequate and equal digestion. The samples were dried in a SpeedVac evaporator and reconstituted in 30 μl of dissolution buffer followed by acylation with one of the four iTRAQ™ reagents having mass tags of 114, 115, 116 or 117 (1 h at 25 °C) following the manufacturer’s standard protocol (Applied Biosystems, Foster City, CA). The four iTRAQ™ labeled samples were then diluted 10-fold with cation exchange loading buffer (10 mM potassium phosphate in 25% acetonitrile, pH 3.0) and the peptides were fractionated on a cation exchange column (Applied Biosystems, POROS 50 HS, 50-um particle size, 4.0 mm x 15 mm) using step-wise elution with increasing concentrations of KCl in the loading buffer: 5 mM, 10 mM, 20 mM, 40 mM, 60 mM, 120 mM, and 350 mM KCl. Each of the seven cation exchange fractions was analyzed by nano-LC-ESI-TOF MS/MS using the Applied Biosystems model QSTAR-XL mass spectrometer interfaced with the
Dionex (Sunnyvale, CA) model U3000 nanobore HPLC equipped with an autosampler. The peptides were fractionated on a Dionex PepMap100 C18 reversed-phase column (100 Å, 3 μm particle size, 0.075 x 150 mm) with initial trapping using the matching phase Dionex trap cartridge (0.3 x 5 mm). The elution of peptides was effected using a linear gradient of acetonitrile (2% v/v to 70% v/v in 140 min including 20 min equilibration) at a flow rate of 180 nl/min during the core of the run. The MS/MS spectra of the five most intense peaks with two to four charges in the MS scan were automatically acquired in information-dependent acquisition mode. MS/MS spectra were extracted and searched against rat non-redundant protein database (NCBI) using ProQuant (Applied Biosystems) and MASCOT (Matrix Science, London, UK) algorithms.

RNA extraction and Real-Time RT-PCR. Total RNA was extracted from 6-well plates with TRIzol® Reagent (Invitrogen) according to the manufacturer’s instruction. Two micrograms of total RNA were used for cDNA synthesis with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), and real-time PCR was carried out with SYBR® GREEN PCR Master Mix (Applied Biosystems) using ABI 7300 RT-PCR equipment. Glyceraldehyde 3-phosphatase dehydrogenase (GAPDH) mRNA was used as the normalization control. The primers described by Hoen et al (2000) were used for CYP3A isoform measurements by real-time PCR: The GAPDH primers used were TTCAACGGGCACAGTCAAG and CACACCCATCACAAACAT. Analysis of real-time PCR were carried out by the ΔΔ Ct method (Livak and Schmittgen, 2001).

Cellular CYP3A Activity Assay. CYP3A activity was assayed by using the P450-Glo™ CYP3A4 assay system with Luciferin-PPXE as a substrate of CYP3A following the manufacture’s direction (Promega, Madison, WI). According to the supplier, Luciferin-PPXE shows good
selectivity for rat CYP3As over other P450s, although its specificity has not been rigorously established. For cellular assays, hepatocytes were incubated with substrate (1 ml of media containing 25 μM Luciferin-PPXE/well in 6-well plates, or 0.5 ml/well in 12-well plates) for 1 hr, and then media were collected for luciferin assay. Equal volumes of the supernatant and Luciferin Detection Reagent (Promega) were mixed and 20 minutes later luciferin was measured on a SpectraMax L luminometer (Molecular Devices, Sunnyvale, CA).
RESULTS

Identification of CYP3A1 as a NO-regulated protein in cultured rat hepatocytes by iTRAQ proteomic scan. iTRAQ is a method to analyze the differential expression or regulation of proteins in cells. Cellular proteins from 2-4 different samples (e.g. control and treated) are digested with e.g. trypsin, and then labeled on amine groups with tags of identical mass that, upon fragmentation in MS/MS, generate four different reporter ions. The samples are mixed together and then, following purification and resolution by liquid chromatography, the differentially labeled peptide fragments can be separated, identified and the relative amounts of the peptide in the four samples are quantified by MS/MS (Zieske, 2006). Because all peptides are labeled, the ratios of the different mass tags can usually be obtained for several peptides from the same protein, increasing accuracy of the calculations and confidence of the predictions.

To identify proteins whose levels are rapidly affected by NO in response to cytokines, primary hepatocytes were treated with IL-1β or IL-1β plus the NOS inhibitor NMA for 9 h, and total cell lysates were used for iTRAQ analysis. Proteins whose levels differ in the two groups are candidates for being regulated by cellulary generated NO. From the iTRAQ data we identified 229 proteins at a confidence level of 95% (data not shown). We then searched for proteins whose abundance differed between the two samples. One such protein was CYP2B, which is known to be regulated by NO (Lee et al., 2008). Its relative abundance, calculated from the peptides FSLATMR and YFPGAHR (contained in both CYP2B1 and 2B2), was increased by a factor of 1.6 by NMA treatment (data not shown). Another protein identified in this manner was CYP3A1 (Fig. 1). Peptides ALLSPTFTSGR (CYP3A1 and 3A2 specific) and GSVVMIPSYALHR
(CYP3A1 specific) were identified and assigned to CYP3A1. The mass chromatograms for these peptides are shown in Fig. 1, in which the samples labeled with the higher mass tags (116 and 117; corresponding to cells treated with IL-1β + NMA) were almost twice as abundant as those labeled with the lower mass tags (114 and 115; IL-1β alone). These data suggested that CYP3A1 might be down-regulated by NO.

**CYP3A expression in PB-treated rat hepatocytes.** Because the proteomic scan identified CYP3A1 as a protein likely to be regulated by NO, we examined in detail the CYP3A1 regulation by NO in the primary cultures. In the rat, five CYP3A isoforms have been identified (Hoen et al., 2000): CYP3A1, 3A2, 3A9, 3A18, and 3A23. However CYP3A23 encodes active CYP3A1 protein and was suggested to be the same gene (Hoen et al., 2000). CYP3A1 and 3A2, the major rat liver isoforms of CYP3A, are inducible via pregnane X receptor (PXR) and/or constitutive androstane receptor (CAR) activation (Guengerich, 1999). In cultured primary hepatocytes without an inducer, it was difficult to detect CYP3A protein in total cell lysates by immunoblotting (Fig. 2A). Therefore, to study the down-regulation of CYP3A proteins, we treated primary hepatocytes with 1 mM PB for 2 days to induce CYP3A mRNAs and proteins. Several studies have reported CYP3A regulation by PB treatments in rat primary cultures (Brown et al., 1997; Joannard et al., 2000), but the effect of PB on all four rat isoforms of CYP3A has not been reported in these cultures. CYP3A protein expression was greatly enhanced by PB treatment (Fig. 2A). In our system, CYP3A9 was not detectable in control or PB treated samples. CYP3A2 showed the highest fold increase (285-fold) by PB treatment (Fig. 2B), followed by CYP3A1 (98-fold) and CYP3A18 (14-fold). Even though CYP3A2 mRNA showed the highest fold increase, the absolute Ct values indicated that CYP3A1 mRNA was 100-fold and 18-fold more abundant.
than CYP3A2 and CYP3A18, respectively, in the PB-treated cells (Supp. Fig. 1A). Based on the Ct values and our iTRAQ data, we suggest that the CYP3A protein we detect in the PB-treated primary cultures is CYP3A1, even allowing for possible small differences in amplification of the different 3A transcripts in the PCR reaction. CYP3A activity in live cultures was measured using the P-450 Glo® assay system with luciferin-PPXE as a CYP3A substrate. Although the specificity of this substrate for rat CYP3A proteins has not been proven, PB treatment of the cells resulted in about a 20-fold increase in CYP3A activity (Suppl Fig. 1B), which is well correlated with CYP3A protein level.

Post-transcriptional Down-regulation of CYP3A. Rat hepatocytes treated with PB for 2 days were stimulated with IL-1β for various times. Hepatocyte CYP3A protein levels started to decline within 6 h to 40% of control level, and continued to decline to 20% of control levels at 24 h, while the mRNA of CYP3A1 was not significantly affected at any time point (Fig. 3A & B). NO₂ and NO₃ (NOx) species were detectable in culture medium within 6 h and achieved their maximum levels at 24 h (Suppl. Fig. 2A). The data demonstrate that CYP3A protein is down-regulated post-transcriptionally by IL-1β stimulation, since protein down-regulation occurs prior to mRNA down-regulation.

IL-6 is another proinflammatory cytokine known to regulate CYP expression, which does not induce NOS2. Treatment of hepatocytes with IL-1β, but not IL-6, stimulated NO production (Suppl Fig. 2B). In agreement with our previous findings (Lee et al., 2008), CYP2B proteins were down-regulated only in IL-1β stimulated cells or IL-1β and IL-6 co-treated samples which were producing NOx in the culture media (Fig. 4A). In sharp contrast, CYP3A protein was down-
regulated by both cytokines, (Fig. 4A) although their effects were not additive. CYP2B1 or CYP3A1 mRNAs were not down-regulated within 18 h of any treatment (Fig. 4A). IL-6 treatment did not down-regulate CYP3A protein within 9 h, but resulted in CYP3A down-regulation to 25% of control at 24 h (Fig. 4B).

**NO-dependent and NO-independent CYP3A Down-regulation.** To confirm our iTRAQ data suggesting that CYP3A1 is an NO-regulated protein, primary hepatocytes were treated with IL-1β for 9 or 24 h with or without the NOS inhibitor NMA. NMA (100 μM) treatment resulted in blockade of NO production in media (data not shown). As shown previously (Lee et al., 2008), IL-1β-stimulated CYP2B down-regulation was prevented by NMA treatment at both 9 and 24 h (Fig. 5). IL-1β stimulation caused CYP3A down-regulation to less than 30% of control at 24 h: this down-regulation was not blocked by NMA co-treatment and is therefore NO-independent (Fig. 5). However at 9 h CYP3A was down-regulated by IL-1β to 40% of control levels and this down-regulation was partially inhibited by NMA co-treatment (Fig. 5), demonstrating that IL-1β-stimulated CYP3A down-regulation is at least partially NO-dependent at earlier time points.

Other NOS inhibitors. L-NIL or L-NAME (100 μM each) each effectively blocked NO production, and also blocked the down-regulation of CYP3A protein caused by 9 h of IL-1β-stimulation (Suppl Fig. 3). Furthermore, treatment with the chemical NO donors, GSNO and NOC-18 mimicked the cellular effect of NO, resulting in 50% down-regulation of CYP3A protein within 8 h in the primary hepatocytes (Suppl. Fig. 4). Taken together, our data indicate that IL-1β-stimulated CYP3A down-regulation is NO-dependent at earlier time points (9 h), but not at later times (24 h).
We also measured CYP3A activity in the cultures using luciferin-PPXE. IL-1β stimulation in the hepatocytes resulted in down regulation of CYP3A activity to 35% and 22% of control within 9 and 24 h, respectively (Suppl. Fig. 5). These data are well correlated with the above data on CYP3A protein levels, suggesting that protein down-regulation is causing the reduced CYP3A activity in the hepatocytes (with the caveat that this substrate may not be specific for CYP3As in rat).

Proteasome-dependent and -independent down-regulation of CYP3A. We previously showed that NO-dependent CYP2B down-regulation occurs via proteasomal degradation (Lee et al., 2008). Therefore, we examined whether or not NO-dependent CYP3A down-regulation is proteasome dependent. In a control experiment, we ascertained that MG132, a proteasome inhibitor, effectively inhibited CYP3A degradation caused by the suicide substrate DDEP (Suppl Fig 6A) (Wang et al., 1999). We also determined that that MG132 effectively blocked the proteasome activity in our cultures, using Suc-LLVY-luciferin as a proteasome substrate. (Suppl. Fig. 6B).

Next, we stimulated the hepatocytes with IL-1β to allow for NO production, and added MG132 three hours later. Cells were harvested 9 h after IL-1β treatments for measurement of CYP3A protein levels. This three hour delay in MG132 treatment is necessary because NOS2 induction requires proteasome activity for its transcriptional activation via NF-κB. As shown previously (Lee et al., 2008), this design permitted NO production to a level that was 60 % of that of cells treated with IL-1β alone (Fig. 6A). MG132 treatment completely blocked IL-1β-evoked down-regulation of CYP3A (Fig. 6A), suggesting that the NO-dependent CYP3A down-regulation by IL-1 stimulation is proteasome-dependent. Treatment of the hepatocytes for 12 h with the NO
donor NOC-18 (500 μM) resulted in the down-regulation of CYP3A to 25% of control levels, and this was blocked by co-treatment with bortezomib (Fig. 6B), a proteasome inhibitor used to treat cancer patients with multiple myeloma. Bortezomib effectively inhibited proteasome activity in the hepatocyte cultures (Suppl. Fig. 6B). Taken together, our data demonstrate that NO-evoked CYP3A down-regulation is proteasome dependent.

We then examined whether the NO-independent (slower) down-regulation of CYP3A by IL-1β is proteasome-dependent. PB-treated primary hepatocytes were treated for 24 h with IL-1β with or without addition (3 h delay) of the proteasome inhibitors, MG132 and gliotoxin. NOx production in cells receiving the delayed proteasome inhibitor treatment was only about half of that with IL-1β alone (Fig. 7A), but this may not be important because in this experiment we are studying an NO-independent mechanism. IL-1β treatment resulted in the down-regulation of CYP2B and CYP3A (Fig. 7A). The down-regulation of CYP2B, which is NO-dependent even at 24 h, was completely blocked by the proteasome inhibitors, MG132 and gliotoxin. However, CYP3A down-regulation was not blocked by these treatments (Fig. 7A), suggesting that NO-independent CYP3A down-regulation is also proteasome-independent. E64-d, a membrane permeable calpain inhibitor, did not inhibit the IL-1β evoked CYP2B down-regulation (Fig. 7A). Although E64-d alone suppressed the expression level of CYP3A protein to some extent, IL-1β-stimulated CYP3A down-regulation was partially inhibited by E64-d treatment, suggesting that calpain might be involved in the NO-independent down-regulation of CYP3A by cytokines. The NO-independent down-regulation of CYP3A proteins by IL-6 is also proteasome-independent, because it was not blocked by bortezomib (10 μM) co-treatment (Fig. 7B).
DISCUSSION

This work demonstrates that iTRAQ proteomic analysis can be used to identify novel modes of hepatic cytochrome P450 regulation. Using this technique, we identified CYP3A1 as a protein whose expression was higher in hepatocytes treated with IL-1β and a NOS inhibitor than without the inhibitor, suggesting that it might be regulated by NO. The evidence for NO-dependent down-regulation of CYP3A at earlier time points of IL-1β stimulation includes; 1) NOS2 inhibitors, NMA, L-NAME, and L-NIL, all block the effect. 2) IL-6, which did not produce NO within 24 h, caused much slower CYP3A down-regulation. 3) NO-donors, NOC-18 and GSNO, down-regulated CYP3A, mimicking the effect of cellular NO. Nitric oxide-dependent CYP3A down-regulation is proteasome dependent in that proteasome inhibitors block the IL-1β- or NO donor-evoked down-regulation. However, IL-6, or IL-1β at later time points act to down-regulate CYP3A by slower post-transcriptional pathways that are not blocked by NOS inhibitors. This NO-independent CYP3A down-regulation by IL-1β or IL-6 was also insensitive to proteasome inhibitors. This was not due to a lack of efficacy of the inhibitors, because CYP2B down-regulation was blocked. Thus, CYP3A protein exhibits novel dual regulation mechanisms by cytokines.

How cellular NO can trigger the degradation of CYP3As remains to be determined. Proteasome-dependent degradation stimulated by cellular NOS-derived NO, or chemical NO donors, has been reported for several other proteins, e.g. neutral ceramidase in rat renal mesangial cells (Franzen et al., 2002), iron regulatory protein-2 (IRP2) in RAW 264.7 cells (Kim et al., 2004), and insulin receptor substrate-1 in murine myotubes (Sugita et al., 2005). NO (or reactive
nitrogen species) also can modify amino acid residues, cysteine or tyrosine, of proteins by S-nitrosylation and tyrosine nitration. Moreover, S-nitrosylation of IRP2 triggers its poly-ubiquitination proteasomal degradation, and mutation of cysteine (C178S) of IRP2 prevents NO-mediated degradation (Kim et al., 2004). We showed that CYP2B1 can be S-nitrosylated by GSNO treatment \textit{in vitro} and that this treatment increases poly-ubiquitination of the protein (Lee et al., 2008). We have confirmed that CYP3A can also be S-nitrosylated by GSNO treatment \textit{in vitro} (not shown). It is not clear yet how NO can stimulate proteasome-dependent protein down-regulation of CYP, but we speculate that S-nitrosylation or tyrosine nitration of CYP3A1 and CYP2B1, like IRP2, is necessary for their recognition by specific ubiquitin E3 ligases that target them for proteasomal degradation. However, NO can bind cytochrome P450 heme reversibly and irreversibly (Wink et al., 1993), and Hollenberg’s laboratory showed that tyrosines of CYP2B1 and CYP2E1 and heme of CYP2B1 are modified \textit{in vitro} by peroxynitrite treatment, resulting in reduced activity of CYP2B1, but not CYP2E1 (Lin et al., 2007). These modifications also deserve mechanistic interrogation for their roles in CYP degradation.

However, unlike CYP2B1, CYP3A is degraded by a second, novel proteasome- and NO-independent mechanism. Both cytokines IL-1 and IL-6 treatments showed CYP3A down-regulation that was not blocked by proteasome inhibitors. We are currently working to elucidate the common downstream mechanisms that are responsible for the down-regulation. The observation that CYP3A degradation was attenuated by a calpain inhibitor deserves further study into the role of calpain proteases. Correia and co-workers have elegantly documented the CYP3A degradation pathway in the normal or suicide substrate-modified states, showing that both occur via ubiquitin-dependent proteasomal degradation through the endoplasmic reticulum-associated
degradation (ERAD) pathway in primary rat hepatocytes (Wang et al., 1999; Correia et al., 2005; Faouzi et al., 2007). However, our observation of a proteasome-independent down-regulation by cytokines was not due to a lack of efficacy of proteasome inhibition, because CYP2B1 degradation was clearly inhibited in the same cultures, and we showed that rapid DDEP-mediated CYP3A down-regulation was suppressed by MG132 in our cells. Cells co-treated with IL-6 and bortezomib showed a higher level of CYP3A compared to the IL-6 alone treated sample, but the difference was not significant (P<0.05) statistically. This tendency might reflect the degradation of the native CYP3A through the proteasomal pathway.

Zangar et al. (2003) reported that basal CYP3A protein levels are slightly suppressed by proteasome inhibitors lactacystin (20 µM) and MG132 (200 µM) in primary cultured rat hepatocytes. Noreault-Conti et al. (2006) found that proteasome inhibitors reduced Dex-inducible 3A1 expression via both pretranslational and translational mechanisms. Consistent with these findings, our data also showed that CYP3A levels were slightly suppressed by 24 h of proteasome inhibitor treatments alone, such as MG132 and gliotoxin (Fig. 7A), and bortezomib (Fig. 7B). The reason why these agents affect CYP3A and not CYP2B enzymes is not known. Similarly, we do not know how NMA alone down-regulates CYP3A protein after 24 h in culture (Fig. 5). Nevertheless, no effect of NMA or the other inhibitors L-NAME and NIL on CYP3A protein occurred at 9h, when these agents blocked down-regulation by IL-1β.

If CYP3A degradation is NO-dependent at early time points after IL-1β stimulation, how does it later become NO-independent? NOx measurement by the Griess reagent represents accumulation of NO₂ plus NO₃ in the media. We found that NOx started to be detected within 6 h,
and continuously accumulated up to 24 h, but the rate of accumulation was reduced after 18 h (data not shown), suggesting the actual NO radical production by NOS2 in the culture by IL-1β stimulation peaks at earlier time points. Thus, we hypothesize that CYP3A is degraded via the NO-dependent pathway when NO radicals are produced at higher rates (earlier time point, within 9 h) and then the degradation signal is changed to the NO-independent mode when the NO radical production is reduced and cells produce anti-oxidants such as glutathione (GSH) and thioredoxin (Haendeler et al., 2004) to maintain homeostasis in the cell. That both proteasomal and calpain pathways can participate in the degradation of the same protein, and that degradation pathways can change under different conditions has been shown for several proteins, e.g. hypoxia-inducible factor 1α (Zhou et al., 2006), c-Fos, c-Jun and p53 (Salvat et al., 1999).

Hepatic CYP3A enzymes are down-regulated by infection and inflammation in vivo. LPS injection caused the down-regulation of CYP3A1 mRNA by 80% in rat liver (Fang et al., 2004). We also reported that LPS injection down-regulated CYP3A2 mRNA more rapidly than CYP3A protein in rat liver (Sewer and Morgan, 1998), and these effects were not blocked by the NOS inhibitor aminoguanidine. This is in apparent opposition to the results we present here. The model we used, of primary rat hepatocytes cultured with Matrigel, is accepted as the best cell model to study CYP regulation in rat liver, and dexamethasone-treated hepatocytes have been employed to demonstrate the proteasome-dependent degradation of native and inhibitor-modified CYP3As (Faouzi et al., 2007). The reported down-regulation of CYP3A1 and CYP3A2 mRNAs in vivo could reflect direct effects of LPS and/or additive or synergistic effects of cytokines in vivo. The NO-independent down-regulation of CYP3A mRNAs in vivo could be obscuring the NO-dependent down-regulation of the protein. In any case, the lack of effect of the cytokines on...
CYP3A1 mRNA allowed us to study the regulation of the protein against a relatively stable mRNA background. This allowed us to validate the iTRAQ approach for identification of proteins regulated in a specific way, and to uncover a novel proteasome-independent mode of CYP3A regulation as well. Interestingly, in the absence of cytokine stimulation the levels of CYP3A protein tended to increase in the cultures during the 24 h after the medium change, whereas CYP3A1 mRNA declined slightly. The reason for this is unclear, but it may reflect different stabilities of the CYP3A proteins and mRNAs.

The relevance of these findings to human CYP3As is uncertain. CYP3A4 mRNA is down-regulated by proinflammatory cytokine treatments in primary human hepatocytes (Aitken and Morgan, 2007; Aitken et al., 2008), but neither its mRNA or protein down-regulation are NO-dependent. The NO-responsiveness of human CYPS 3A5 and 3A7 remains to be determined.

In summary, we have validated iTRAQ technology as an approach to study multiple hepatocyte proteins regulated by similar pathways in hepatocytes, and thereby we demonstrated that NO-regulated proteasomal degradation of CYP proteins is not limited to CYP2B1, but also pertains to CYP3A proteins as well. Moreover, the mechanism of cytokine-evoked degradation of rat CYP3As undergoes a temporal switch from NO- and proteasome-dependent to NO- and proteasome-independent. Our results suggest that yet more CYP enzymes, involved in metabolism of drugs or physiological substrates, may undergo NO-dependent degradation during inflammatory disease states or treatment with NO-releasing drugs, with obvious consequences for drug efficacy and/or toxicity.
ACKNOWLEDGMENTS

We thank Dr. Almira Correia, University of California San Francisco for the generous gift of DDEP, Malim Raynor for expert technical assistance, and Dr. Keith Wilkinson, Dept. of Biochemistry, Emory University for helpful discussions.
REFERENCES


FOOTNOTES

This work was supported by grants from the National Institutes of Health [GM069971,T32ES01287] and from NIH-NCRR [02878, 12878 and 13948].

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FIGURE LEGENDS

Figure 1. CYP3A1 peptide quantification by iTRAQ labeling and mass spectrometry. Duplicate aliquots of pooled lysates from hepatocytes treated with IL-1β or IL-1β/NMA were digested with trypsin and the peptides were labeled with iTRAQ reagent 114 and 115 (for IL-1β) or 116 and 117 (for IL-1β/NMA) as described in the text. Following cation exchange column separation, fractions were analyzed by nano-LC-ESI-TOF MS/MS as described in Materials and Methods. The graphs show the relative intensities of the mass tags on the two identified CYP3A1 peptides, indicating the relative abundance of CYP3A1 in the different samples.

Figure 2. Effect of PB treatment on CYP3A expression. Four-day old primary rat hepatocytes were cultured with fresh medium with (PB) or without (Con) 1 mM PB for an additional 2 days. Cells were cultured with an additional media change for 4 hours, and then harvested for RNA and protein extraction. A. Total cell lysates (20 μg protein) from three different culture plates were separated on SDS-PAGE and immunoblotting was carried out with anti-CYP3A2 antibody. 20 μg of post-mitochondrial supernatants from untreated and PB-treated (50 mg/kg, 24h) male rats (3 rats/group) are shown for comparison. B. Levels of CYP3A mRNAs in hepatocytes were measured by real-time RT-PCR, comparing control to 1 mM PB treatments.

Figure 3. Time course of the effects of IL-1β on hepatic CYP3A proteins in primary rat hepatocyte cultures. After 48 hr treatment with 1 mM PB, hepatocytes were treated with fresh medium containing PB without (Con) with or IL-1β (10 ng/ml). Cells were harvested at indicated time points from 6-well plates and 60 mm cell culture dishes for RNA or protein extraction,
respectively.  A. Western blots of CYP3A proteins.  B. Levels of CYP3A1 mRNA were assayed by RT-PCR.  CYP3A protein levels were quantified from the Western blots.  Error bars represent standard errors of the mean.  * Significantly different from control group mean (p<0.05; n=3), T-test.

Figure 4. Down-regulation of hepatocyte CYP3A proteins by IL-6.  After 48 hr treatment with 1 mM PB, hepatocytes were treated with fresh medium containing 1 mM PB and the indicated treatments (Con, no addition; IL-1β, 5 ng/ml; IL-6, 10 ng/ml).  After 18 h of treatment cells were harvested for RNA and protein analysis.  A. Upper panel, immunoblot of CYP3A and CYP2B1 from the indicated treatments.  Lower panel, quantification of immunoblots and steady state mRNA levels of CYP3A1 and CYP2B1 determined by quantitative RT-PCR.  B. After 48 hr treatment with 1 mM PB, hepatocytes were treated with fresh medium containing 1 mM PB with IL-6 (10 ng/ml) for the indicated times. Cells were harvested and assayed by Western blotting.  Error bars represent standard errors of the mean.  * Significantly different from control group mean (p<0.05; n=3), T-Test.

Figure 5. Temporal switch of the mechanism of CYP3A down-regulation by IL-1.  After treating cells for 2 days with 1 mM PB, they were treated with IL-1β (5 ng/ml) with or without NMA (300 μM).  CYP protein levels were measured by immunoblotting 9h or 24 h later as shown.  Upper panel, immunoblots of CYP3A and CYP2B1.  Lower panel, quantification of immunoblots.  Error bars represent standard errors of the mean.  a. significantly different from control group mean; b, significantly different from IL-1β-treated group (p<0.05, n=3), one-way ANOVA and Tukey’s post-hoc test.
Figure 6. Role of the proteasome in the NO-dependent and -independent pathways. Cells were treated with 1 mM PB for 2 days to increase CYP3A expression, and then treated for various times with the indicated agents. CYP3A protein levels were measured by immunoblotting. In each panel, the graphs represent the quantification of the immunoblotting data. A. Cells were treated for 9 h with IL-1β (5 ng/ml) +/- MG132 (10 μM). MG132 (10 μM) was added 3 h after IL-1β. B. Cells were treated with NOC-18 (500 μM), NOC-18/Bort (bortezomib, 10 μM), or Bort (10 μM) for 12 h. Error bars represent standard errors of the mean. a, significantly different from control group mean; b, significantly different from IL-1β group (p<0.05, n=3), one-way ANOVA and Tukey’s post-hoc test.

Figure 7. Proteasome independence of the NO-independent pathway. A. Cells were treated for 24 h with indicated treatments: IL-1β (5 ng/ml), MG132 (10 μM); E64-d (10 μM); Glio (gliotoxin, 1 μM); MG132 or Glio were added 3 h after IL-1β to provide time for activation of NFκB in co-treated cells. CYP3A and CYP2B protein were assayed by Western blotting. B. Cells were treated with IL-6 (10 ng/ml), IL-6/Bort (10 μM), or Bort (10 μM) for 24 h. In each panel, the graphs represent the quantification of the immunoblotting data. Error bars represent standard errors of the mean. a, significantly different from control group mean; b, significantly different from IL-1β group (p<0.05, n=3), one-way ANOVA and Tukey’s post-hoc test.
Fig. 2
Fig 7

A

Relative Levels (% control)

Control | IL-1 | MG132 | E64d | Glio | MG132 | E64d | Glio

CYP2B
CYP3A
NOx

Treatments (24 h)

B

Control | IL-6 | IL-6/Bort | Bort

CYP3A protein (% control)

Control | IL-6 | IL-6/Bort | Bort

Treatments (24 h)