Nitric oxide and interleukin-1β stimulate the proteasome-independent degradation of the retinoic acid hydroxylase CYP2C22 in primary rat hepatocytes

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Abbreviations: AhR, aryl hydrocarbon receptor; ALLM, N-acetyl-Leu-Leu-methionine-aldehyde; atRA, all-trans retinoic acid; Boc-LM-CMAC, BOC-L-Leucyl-L-Methionine amide; CAR, constitutive androstane receptor; dipropylethenetriamine NONOate; DPTA; E64d, 2S,3S-trans-(Ethoxycarbonyloxirane-2-carbonyl)-L-leucine-(3-methylbutyl) amide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1, interleukin-1; iTRAQ, isobaric tags for relative and absolute quantitation; L-NAME, Nω-Nitro-L-arginine methyl ester; MDL-28170, Z-Val-Phe-aldehyde; NOC-18, 3,3-bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene; NO, nitric oxide; NOS2, inducible nitric oxide synthase; NOx, nitrate + nitrite; 4-OH-RA, 4-hydroxyretinoic acid; P450, cytochrome P450; PCN, pregnenolone 16α-carbonitrile; PPARα, peroxisome proliferator-activated receptor-α; PXR, pregnane X receptor; RAR, retinoic acid receptor; RT, reverse transcriptase
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ABSTRACT

CYP2C22 was recently described as a retinoic acid-metabolizing cytochrome P450 (P450) enzyme whose transcription is induced by all-trans retinoic acid (atRA) in hepatoma cells (Qian, L et al (2010) J Lipid Res 51: 1781). We identified CYP2C22 as a putative nitric oxide (NO)-regulated protein in a proteomic screen, and raised specific polyclonal antibodies to CYP2C22 to study its protein expression. We found that CYP2C22 is a liver specific protein that was not significantly induced by activators of the pregnane X receptor, constitutive androstane receptor or peroxisome proliferator-activated receptor-α, but was down-regulated to <25% of control by the aryl hydrocarbon receptor agonist β-naphthoflavone in cultured rat hepatocytes. CYP2C22 protein and its mRNA both were induced by atRA in hepatocytes, with EC₅₀s of 100-300 nM, while the maximal extent of mRNA induction was twice that of the protein. CYP2C22 protein, but not its mRNA was rapidly down-regulated in hepatocytes by interleukin-1 (IL-1) or NO-donating compounds, and the down-regulation by IL-1 was blocked by inhibition of NO-synthases. The NO donor dipropylenetriamine NONOate (DPTA) reduced the half-life of CYP2C22 from 8.7 h to 3.4 h in the presence of cycloheximide, demonstrating that NO-dependent down-regulation is due to stimulated proteolysis. No intermediate degradation products were detected. However, this degradation was insensitive to inhibitors of calpains or the canonical proteasomal or lysosomal pathways, indicating that NO-dependent degradation of CYP2C22 proceeds via a novel pathway.
INTRODUCTION

Nitric oxide (NO) has diverse physiological roles, including vasodilation and blood pressure regulation (Palmer et al., 1987), neuronal communication (Dawson et al., 1992), and pathogenic cell killing (Green et al., 1990). In the latter case, NO is produced at high concentrations by the inducible nitric oxide synthase (NOS2) (Nussler and Billiar, 1993), whose expression is induced via activation of toll-like receptors and cytokine receptors in cells of the innate immune system as well as in hepatocytes and other cell types (Nussler and Billiar, 1993).

Activation of the immune system results in the down-regulation of many drug metabolizing enzymes (Aitken et al., 2006) and transporters (Cressman et al., 2012) in the liver, notably enzymes of the cytochrome P450 (P450) superfamily (Aitken et al., 2006). Stadler et al. (Stadler et al., 1994) first suggested that this down-regulation of P450 proteins and/or mRNAs by inflammatory mediators could be due to NO produced via NOS2 induction. While subsequent studies have revealed that this is not true for all, or even perhaps most P450s (Sewer and Morgan, 1997; Sewer et al., 1998; Sewer and Morgan, 1998), various studies have suggested a role for NO in the regulation of CYP2B enzymes (Khatsenko et al., 1993; Carlson and Billings, 1996; Khatsenko et al., 1997). We have shown that NO produced by interleukin-1β (IL-1β) or lipopolysaccharide stimulation of hepatocytes causes ubiquitin-dependent proteasomal degradation of the rat phenobarbital-inducible P450 CYP2B1 (Ferrari et al., 2001; Lee et
al., 2008; Sun et al., 2012). The related human enzyme CYP2B6 also undergoes down-regulation at the protein level in response to NO (Aitken et al., 2008).

The mechanism whereby NO triggers the degradation of CYP2B1 is unknown. Both iron regulatory protein 2 (Kim et al., 2004) and alkylguanine DNA-alkyltransferase (Wei et al., 2010) undergo proteasomal degradation triggered by S-nitrosylation of critical cysteines. Although CYP2B1 can be nitrosylated by NO donors in vitro (Lee et al., 2008), changes in nitrosylation state of the protein have not been demonstrated in a cellular context. As another approach, we have been searching for other P450 enzymes that undergo NO-dependent degradation. We described earlier an isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic approach to this problem, and we used it to identify rat CYP3A enzymes as additional NO targets (Lee et al., 2009). The primary objective of the current study was to investigate the regulation by NO of an additional P450 enzyme identified in the same proteomic study, CYP2C22.

During the course of the current work, CYP2C22 was identified as a putative Vitamin A-regulated gene by microarray analysis of livers from Vitamin A-sufficient and -deficient rats (McClintick et al., 2006). Qian et al replicated this observation using in situ hybridization (Qian et al., 2010), and they demonstrated via reverse transcriptase (RT)-PCR that CYP2C22 mRNA is expressed predominantly in liver. CYP2C22 mRNA was induced by all-trans-retinoic acid (atRA) and the synthetic retinoic acid receptor (RAR) agonist Am580 in H-4-II-E rat hepatoma cells (Qian et al., 2010). Transient transfection of luciferase reporter constructs were used to identify a retinoic acid response element
in the distal promoter region of the CYP2C22 gene. Moreover, CYP2C22 expression in HEK293T cells conferred higher retinoic acid metabolizing activity upon those cells (Qian et al., 2010), identifying CYP2C22 as a retinoic acid hydroxylase. Therefore, a second objective of our work was to confirm the regulation of CYP2C22 by retinoids at the protein expression level, and to investigate the regulation of this enzyme by classical inducers of hepatic P450s.

In this manuscript, we describe the preparation of specific polyclonal antibodies to CYP2C22, and use them to investigate the regulation of this poorly understood enzyme by nitric oxide, IL-1β and atRA, as well as by prototypical nuclear receptor agonists.

MATERIALS AND METHODS

Materials

IL-1β was purchased from R & D systems (Minneapolis, MN), and (Z)-1-[N-(3-aminopropyl)-N-(3-ammoniopropyl)amino]diazen-1-ium-1,2-diolate (DPTA NONOate, DPTA) and 3,3-bis(amo:noethyl)-1-hydroxy-2-oxo-1-triazene (NOC-18) were from Cayman Chemical (Ann Arbor, MI). L-N\textsuperscript{G}-Nitroarginine methyl ester (L-NAME), chloroquine, Williams’ Medium E, Krebs-Ringer buffer, collagenase, atRA, protease inhibitor cocktail and other general chemicals were acquired from Sigma-Aldrich (St. Louis, MO). NADPH was from CalBiochem, Darmstadt, Germany and Brefeldin A, N-acetyl-Leu-Leu-methionine-aldehyde (ALLM), 2S,3S-\textit{trans}-(Ethoxycarbonyloxirane-2-
carbonyl)-L-leucine-(3-methylbutyl) amide (E64d) and Z-Val-Phe-aldehyde (MDL-28170) were obtained from Enzo Life Sciences (Farmingdale NY). Proteasome inhibitors MG132 and bortezomib were purchased from Boston Biochemicals (Cambridge, MA) and LC Laboratories (Woburn, MA), respectively. The calpain substrate 7-Amino-4-Chloromethylcoumarin, t-BOC-L-Leucyl-L-Methionine amide (Boc-LM-CMAC) was purchased from Molecular Probe (Grand Island, NY). 4-OH-RA was synthesized as described previously (Lutz et al., 2009; Shimshoni et al., 2012). Anti-CYP2B1 was kindly provided by Dr. James Halpert (University of California, San Diego, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was acquired from Millipore (Billerica, MA), and anti-NOS2 and anti-Annexin VI antibodies were purchased from Santa Cruz (Santa Cruz, CA) respectively. Anti-CYP2C11 antibody was generated in our laboratory as described previously (Morgan et al., 1985).

**Antibody production and peptide blocking experiment**

Peptide synthesis and antibody production were carried out by Open Biosystems, Inc (Huntsville, AL). The CYP2C22-specific peptide corresponds to residues 366 to 390 (24 amino acids, PRKTTQDVEFRGYHIPKGTSVMAC). Antibodies were raised in two rabbits, which were bled to measure antibody production after each injection. After the third injection of the peptide, a final bleed was performed, and then CYP2C22 antisera were prepared. To determine the specificity of the CYP2C22 antibody, various amounts of the immunogenic CYP2C22-specific peptide were immobilized by adsorption on nitrocellulose paper, which was then blocked with 5% non-fat dry milk for 1 h. After washing 3 times with washing buffer (10 mM potassium phosphate, pH7.4;
0.05% Tween 20; 1.15% KCl; 0.02% sodium azide), 5 ml of 1,000-fold diluted polyclonal antibody sera were incubated overnight with the nitrocellulose paper at 4 °C to immobilize CYP2C22-specific antibody. Unbound antibody solution was then taken for Western blotting to determine the specificity of antibody.

**Rat hepatocyte isolation, treatment and harvesting**

Rat hepatocytes were isolated by a two-step *in situ* collagenase perfusion procedure (Lee et al., 2008) with minor modifications, which are described below. The procedure was approved by the Emory University Institutional Animal Care and Use Committee. Male F344 rats (200–250 g) were anesthetized with ketamine-xylazine solution, and then the liver was perfused with 300–400 ml of Krebs-Ringer bicarbonate buffer for 8–10 min. This was followed by perfusion with 0.3 mg/ml collagenase type IV (Sigma) for 10 min. The liver was harvested and gently chopped to release cells, which were filtered through 90- and 200-μm meshes. Hepatocytes were cultured under conditions, and treated at time points, that yield optimal constitutive (Liddle et al., 1992) and phenobarbital-inducible (Sidhu et al., 1993) cytochrome P450 expression. Cells were plated on collagen plates with plating medium containing 10% FBS, and 3 h later cells were overlaid with new media containing Matrigel (0.234 mg/ml, BD Biosciences). After 24 h the medium was changed to Williams E medium containing 10 mM Hepes, pH 7.4, 10 nM insulin, 25 nM dexamethasone, and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Cultures were maintained for 5-6 days at 37 °C in 5% CO₂ with regular media changes. At the conclusion of the experiment, media were removed and reserved for an NO assay using the Griess reaction (Green et al., 1982). The cells
were harvested with a cell scraper, then incubated on ice at 4°C in phosphate-buffered saline with 1 mM EDTA for at least 20 min to remove Matrigel. For preparation of total cell lysates, cells were pelleted at 1000 x g for 5 min at 4°C, then lysed by the addition of ice-cold 50 mM Tris Cl buffer, pH 7.5, containing 0.1% SDS, 0.5% Nonidet P-40, 1 mM EDTA and a protease inhibitor mixture and sonication for 10 s. After centrifugation for 10 min at 11,000 x g at 4°C, the supernatant was used for SDS-polyacrylamide gel electrophoresis and immunoblotting.

2.4. Preparation of rat liver S9 and microsomal fractions, and immunoblotting

Rat livers were homogenized in ice-cold homogenization buffer (0.1 M potassium phosphate buffer, pH 7.4, containing 0.125 M potassium chloride, 1.0 mM EDTA, and a protease inhibitor mixture (aprotinin, bestatin, E-64, leupeptin and pepstatin (Sigma P83400)) and then centrifuged at 10,000 × g at 4°C for 20 min to obtain the S9 fraction. For preparation of microsomes, the S9 fraction was centrifuged at 160,000 × g for 45 min at 4°C. The supernatant from ultracentrifugation was considered the cytosolic fraction, and the pellet was washed with cold microsome storage buffer (0.25 M sucrose containing protease inhibitor mixture) and homogenized in the same buffer.

SDS-PAGE and Western blotting were carried out as described by Lee et al. (Lee et al., 2008). Equal amounts of each sample, typically 30-40 μg of protein, were loaded on the gels. Typically, Western analysis was carried out on freshly prepared lysates, although occasional experiments were done on lysates stored at -20°C. In our experience, lysate proteins are stable in the presence of the protease inhibitor cocktail, even after storage overnight at room temperature. Each experiment was performed
using cells from a single animal, with at least three separate cell incubation replicates per treatment group. Primary antibodies (rat CYP2C22 antibody, diluted 1:3,000; anti-GAPDH, 1:10,000 dilution; anti-Annexin VI, 1:3000) were incubated overnight at 4 °C, the blots were washed, and then horseradish peroxidase-conjugated goat-anti-rabbit IgG was incubated for 1 h at room temperature. Chemiluminescence was detected with ECL substrate (Thermo Scientific, Rockford, IL) on X-ray film. All assays were performed within a linear range, and the intensity of stained bands was measured by Bio-Rad imaging software (Image Lab ver. 4.0; Hercules, CA).

**Stable expression of CYP2C22 in HuH7 Cells**

CYP2C22 cDNA (kindly provided by Dr. A. Catherine Ross, Pennsylvania State University (Qian et al., 2010) was cloned into pLV-CMV-GFP-U3Nhe by replacing the green fluorescent protein (GFP) gene and the new construct was named Lv-CMV-2C22. Lentivirus was generated with a second generation vector system as described by Naldini et al (Naldini et al., 1996). Lv-CMV-2C22 or pLV-CMV-GFP-U3Nhe along with pCMVΔR8.92 and pVSVG plasmids were transiently transfected into Hek293T cells using Fugene HD (Promega, Madison, WI). Transfection efficiency was assessed by fluorescence microscopy of GFP expression. The lentiviruses were harvested 48 h and 72 h after transfection. The media containing virus were filtered through 0.45-μm cellulose acetate filters, and the viruses were used to transduce HuH7 cells as described (http://www.addgene.org/tools/protocols/plko/). Expression of CYP2C22 protein was determined by immunoblotting.
**RNA Extraction and reverse transcriptase-real-time PCR (RT-qPCR)**

Total RNA was extracted with RNA-Bee reagent (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. One microgram of total RNA was 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 Eppendorf Mastercycler Realplex PCR equipment (Hauppauge, NJ). Each reaction contained the equivalent of 50 ng of reversed transcribed RNA. Primers were annealed at 60°C, and primer extension was for 30 seconds at 72 °C for each cycle. The primers used are as follows: CYP2C11 (GAATTGCATGGATCCAGCCCTAGTCC, GTCGACAATCAGATGAGAGCTTAG), CYP 2C22 (TCACACCGGCTACCAACCCT, CTGTGGGTCATGGAGAGCTG), GAPDH (TGCCAAGTATGATGACATCAAGAAG, AGCCCAGGATGCCCTTTAGT), CYP26B1 (TTAGGGCTTGGAGTTGGT, AACGTTGCCATACTTCTCGC), and CYP26A1 (GTGCCAGTGATTGCTGAAGA, GGAGGTGTCCTCTGGATGAA). Melting curves were routinely checked to ensure a single PCR product. Relative expression of mRNAs was calculated by the ΔΔCt method as described by Livak and Schmittgen (Livak and Schmittgen, 2001). GAPDH mRNA was used as the normalization control.

**Hepatocyte retinoic acid metabolism**

All incubations were done under red light, and were conducted as duplicates of each of the biological triplicates in the treatment group. The incubations contained 60 μg of rat hepatocyte S9 protein, 1 mM NADPH and 2 μM atRA in 100 μl of 100 mM potassium phosphate buffer (pH 7.4). After 5 minutes at 37 °C, the reactions were
initiated by the addition of NADPH and allowed to proceed at 37 ºC for 20 mins. A control incubation without NADPH was conducted for each group to assure that 4-OH-RA formation was NADPH dependent. The reactions were terminated with 100 µl ice cold acetonitrile containing the internal standard (13-cis-4oxo-RA-d₃, Toronto Research Chemicals, ON, CA).

The formation of 4-hydroxy retinoic acid (4-OH-RA) from atRA was measured using an AB Sciex API 5500 QTrap (Foster City, CA) mass spectrometer equipped with an Agilent UHPLC 1290 (Palo Alto, CA) and Agilent Zorbax Eclipse Plus C18 (3.5 µm, 2.1 mm x 100 mm) column heated to 40 ºC. The analytes were separated using a flow rate of 300 µL/minute and the following linear gradient and a mobile phase combination of water (A) and acetonitrile (B): 0 minutes: 90% A, 10%B; 3 minutes: 90% A, 10% B; 3.5 minutes: 50% A, 50% B; 7.5 minutes: 15% A, 85% B; 7.6 minutes: 5% A, 95% B; 10 minutes: 5% A, 95%B; 10.1 minutes: 90% A, 10% B; 11.5 minutes: 90% A, 10% B. The 4-OH-RA and the internal standard were detected using negative ion APCI and m/z transitions of 315.1 → 253.2 for 4-OH-RA and 316.0→272.0 for 13cis-4oxo-RA-d₃. The compound-specific MS/MS parameters for 4-OH-RA were DP: -40, EP: -8, CE: -23, CXP: -19 and for 13cis-4oxo-RA-d₃ DP: -15, EP: -10, CE: -10, and CXP: -5. The data was analyzed using Analyst software and the 4-OH-RA peak area was normalized to the peak area of the 13cis-4oxo-RA-d₃ internal standard. The percent activity remaining of 4-OH-RA formation in each incubation was calculated by comparing to the control treatment group.
Cellular calpain activity assay

Calpain activity was measured using a fluorescence-based assay with Boc-LM-CMAC as substrate (Rosser et al., 1993). Assay buffer (1 mM Na$_2$HPO$_4$, 137 mM NaCl, 5 mM KCl, 0.5 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM glucose, and 10 mM Hepes, pH 7.4; (Donato et al., 2004)) containing Boc-LM-CMAC (25 μM) was added to fully confluent CYP2C22 lentivirus-transduced HuH7 cells grown in 12-well plates, and incubated for 20 min. CMAC released from the cell due to calpain cleavage of the substrate was measured in the media with a fluorometer (Excitation $\lambda$, 380 nm; Emission $\lambda$, 455 nm).

Statistical analyses.

Data are expressed as the mean ± standard error of the mean. Differences between groups were calculated either by T-test or by one-way ANOVA followed by Dunnett’s test or Tukey’s test, as appropriate for each experimental design.

RESULTS

Discovery of CYP2C22 as a putative NO-regulated protein.

We described previously how we used iTRAQ proteomic technology to identify proteins that were rapidly down-regulated in a NO-dependent manner by interleukin-1β (IL-1β) in cultured rat hepatocytes (Lee et al., 2009). From 229 total proteins identified, three were predicted to be regulated by NO. Two of them, CYP2B1/2 and CYP3A1/2 have been demonstrated to undergo NO-dependent proteasomal degradation in response to IL-1 and NO donors (Lee et al., 2008; Lee et al., 2009). The third protein
was CYP2C22, which was identified from peptides KLPPGPTPLPIFGNILQVGVK and RFSLMVLRL, having average abundances that were 1.9-fold lower in the samples exposed to NO (data not shown).

**CYP2C22 antibodies and their characterization**

As discussed earlier, CYP2C22 has been described as a RAR-regulated mRNA in rat liver in vivo and in rat hepatoma cells, although nothing is known about its protein expression. Alignment of the amino acid sequence of CYP2C22 with those of other rat CYP2C subfamily members revealed the region from amino acids 366 to 390 to be one of greatest divergence, and therefore this peptide was used to raise antibodies in rabbits. As shown in Fig. 1A, the antibodies from rabbit #96 recognized a single protein band in rat hepatocyte lysates, rat liver S9 fraction and rat liver microsomes, and this was abolished by preincubation of the serum with the peptide antigen bound to nitrocellulose paper. Antibodies from rabbit #97 recognized the same band, and upon overexposure two minor bands of lower molecular weight. Preincubation of #97 serum with the antigen peptide abolished all of the bands (even longer exposure revealed one faint band of the same MW as CYP2C22, which was only seen in liver S9 fractions). The #97 antibody was selected for use going forward, as it had a higher titer. The #96 and #97 antibodies failed to recognize rat CYP2B1, 2C6, 2C11, 2C12, 2D1, 2D2 or 3A2 (Fig. 1B), or human CYPs 1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, or 3A4 (not shown). Together, these experiments establish the specificity of the antibodies for CYP2C22 in rat liver microsomes and hepatocyte lysates.
Tissue-specific expression.

While CYP2C22 mRNA was reported to be restricted to rat liver (with very low-level expression in brain (Qian et al., 2010)), the expression of CYP2C22 protein is not known. Western blotting revealed that CYP2C22 protein expression is restricted to the liver, with undetectable levels in brain, lung, heart, spleen, kidney or small intestine (Fig. 1C), mRNA expression in these tissues was at least 1,000-fold lower than liver (Fig. 1D). CYP2C11 expression, included for comparison, was also liver-specific.

Regulation by atRA

CYP2C22 protein was significantly induced 1.8-fold within 6 h of 5 μM atRA treatment of cultured hepatocytes, reaching 3.2-3.5-fold of control at 18-24 h of incubation (Fig. 2A,B). CYP2C22 mRNA was rapidly induced by atRA, by 3.6-fold at 6 h and reaching a maximum of 7.4-fold within 12 h (Fig. 2B). The induction of CYP2C22 protein lagged only slightly behind of its mRNA. atRA induced CYP2C22 protein with an approximate EC₅₀ in the 10 - 100 nM range (Fig. 2C). CYP2C22 mRNA was induced by atRA in a similar concentration range. Induction of the mRNA for the known RAR target genes CYP26B1 and CYP26A1 had similar kinetics to those of CYP2C22, although their maximal extent of induction were much higher at 480- and 74-fold, respectively (Fig. 2B). Induction of the CYP26 mRNAs was only significant at atRA concentrations >1 μM (Fig. 2D). Again, the magnitude of CYP26B1 induction was about 10-fold higher than that of CYP26A1.

Regulation by nuclear receptor agonists
We next asked whether CYP2C22 expression is regulated by any of the xenobiotic receptors that regulate many of the other drug metabolizing P450s. Cultured hepatocytes were incubated for 3 days with 40 μM β-naphthoflavone (an aryl hydrocarbon receptor (AhR) agonist), 1 mM phenobarbital (a constitutive androstane receptor (CAR) activator), 20 μM dexamethasone (a pregnane X receptor (PXR) agonist), 25 μM pregnenolone 16α-carbonitrile (PCN, a PXR agonist) or 10 μM clofibrate (a peroxisome proliferator-activated receptor-α (PPARα) agonist). We used concentrations that we and others determined previously were sufficient to induce prototypic target genes in rat hepatocytes.

PCN caused significant induction of CYP2C22 mRNA after 3 days, while dexamethasone, and clofibrate trended in the same direction. However, none of these agents affected the levels of CYP2C22 protein (Fig. 3A, B). Phenobarbital had no effect on CYP2C22 mRNA or protein, whereas β-naphthoflavone down-regulated both mRNA and protein to 38% and 24% of control levels, respectively (Fig. 3A, B). The effects of β-naphthoflavone and clofibrate on CYP2C22 protein were further investigated with respect to time course (results not shown). The down-regulation by β-naphthoflavone was already manifested after 24 h of treatment, whereas clofibrate produced a small (12%) but significant suppression at 3 days only.

**Down-regulation of CYP2C22 by IL-1β via NO generation**

Incubation of rat hepatocytes with 5 ng/ml IL-1β resulted in a down-regulation of CYP2C22 protein, which became significant within 8 h of treatment and reached a nadir
at 12 h (Fig. 4A,B). This corresponded with the time course of induction of NOS2, and the detection of nitric oxide products (NOx) in the culture medium. Notably, NOS2 expression and the rate of NOx production declined significantly between 12 and 24 h after treatment, during which time CYP2C22 protein expression began to recover. The rapid decline in CYP2C22 protein expression occurred in the absence of any significant effect of IL-1β on CYP2C22 mRNA levels (Fig. 4C).

The NOS inhibitor L-NAME effectively blocked IL-1β-evoked NO production in the hepatocytes, and at the same time blocked the down-regulation of CYP2C22 (Fig. 4D-F). We performed this experiment with and without phenobarbital pretreatment of the cells, because our proteomic experiment in which we identified CYP2C22 as a putative NO-regulated protein was performed in phenobarbital-treated cells. The same results were seen regardless of the pretreatment (Fig. 4D-F).

**An NO donor mimics the effect of IL-1β inhibits retinoic acid metabolism in hepatocytes**

NOC18 is an NO donor that releases NO with a half-life of approximately 20 h at 37°C (Hrabie et al., 1993), which we have demonstrated to cause the down-regulation of CYP2B and CYP3A proteins in rat hepatocytes (Lee et al., 2008) (Lee et al., 2009). The time courses of the effects of NOC18 on CYP2C22 mRNA and protein was investigated. While levels of both CYP2C22 mRNA and protein changed over time in the control cells, NOC18 caused a marked down-regulation of CYP2C22 protein at every time point studied (3 – 24 h), while producing no effect on the corresponding
mRNA levels (Fig. 5A,B). Almost complete suppression of CYP2C22 was achieved with 1 mM NOC 18, and the IC₅₀ was approximately 300-500 μM (Fig. 5C, D).

Since CYP2C22 is a retinoic acid-metabolizing enzyme in rat liver, we next determined the effect of NO donor treatment on the retinoic acid metabolizing activities of hepatocytes. Cells were treated with 5 ng/ml IL-1β or 500 μM NOC18 for 12 h, or 500μM DPTA for 6 h, then the cells were harvested and S9 fractions were prepared. The treatments decreased atRA 4-hydroxylation by 60-80% indicating significantly decreased CYP2C22 activity (Fig. 5E). The efficacies of these treatments on CYP2C22 protein levels were verified by Western blotting (Fig. 5F), and the declines in CYP2C22 protein contents of the microsomes were correlated with comparable reductions in retinoic acid metabolism (Fig. 5E, F). The decreased CYP2C22 protein was reflected in significantly decreased atRA hydroxylation. We note a faint lower molecular weight band detected by the CYP2C22 antibody in this experiment. Its identity is unknown, and it is only detected in some experiments.

**Stimulation of CYP2C22 protein degradation by NO donors**

CYP2C22 protein down-regulation by NOC18 was maintained in the presence of the protein synthesis inhibitor cycloheximide (Fig. 6A,B). A time course analysis with DPTA as the NO donor showed that the DPTA reduced the half-life of CYP2C22 protein from 8.7 h to 3.4 h (Fig. 6C).
To determine the effect of proteasome inhibition on CYP2C22 down-regulation, we performed the experiments in the presence of 1 mM phenobarbital to allow for expression of CYP2B1, which we have shown is degraded via the proteasome following IL-1β or NOC-18 exposure of the cells. Hepatocytes were treated with the proteasome inhibitors MG132 or bortezomib 4 h after initiation of IL-1 treatment of the cells. As we demonstrated previously, these treatments effectively blocked CYP2B1 protein down-regulation (Fig. 7A,B) while not affecting NO production by the cells (Fig. 7C). However, proteasome inhibition failed to block the down-regulation of CYP2C22 (Fig. 7A,B). We verified that proteasome inhibitors also did not block CYP2C22 down-regulation by IL-1β in the absence of phenobarbital (data not shown). Proteasome inhibition also failed to block the down-regulation of CYP2C22 by DPTA (Fig. 7D). Brefeldin A (inhibits endoplasmic reticulum to Golgi transport as well as endosomal trafficking (Lippincott-Schwartz et al., 1991) (Strous et al., 1993)), also failed to affect CYP2C22 down-regulation (Fig. 7D).

In experiments not shown, we tested several different calpain and lysosomal protease inhibitors for their abilities to block the IL-1, NOC18 or DPTA-evoked degradation of CYP2C22 in hepatocytes at various time points, and were unable to detect any significant effect. We also tested whether oxidative signals other than NO could down-regulate CYP2C22 and CYP2B1. Phenobarbital-induced hepatocytes were treated with 5 mM H2O2, NO donors or IL-1β. The NO donors and IL-1β down-regulated the P450 proteins as seen previously, but H2O2 failed to do so in the presence or absence of the catalase inhibitor aminotriazole (Fig. 7E, F).
To investigate further the proteolytic enzymes responsible for CYP2C22 down-regulation, we established a HuH7 cell line expressing CYP2C22 using a lentiviral vector. As shown in Fig 8A, DPTA down-regulated CYP2C22 protein expression to 30% of control in these cells, but the calpain inhibitors E64d, MDL-2180 or ALLM failed to attenuate this down-regulation. MDL-2180 or ALLM effectively inhibited calpain activity, whereas E64d was less effective (Fig. 8B). The lysosome inhibitors chloroquine and NH₄Cl also failed to block CYP2C22 down-regulation, but effectively inhibited lysosomal degradation of Annexin VI (Cuervo et al., 2000) as observed by a significant increase in Annexin VI protein in the cells (Fig. 8C).

DISCUSSION

Our data establish CYP2C22 as a third P450 protein targeted by NO-mediated degradation in rat hepatocytes, based on the prevention of IL-1β-mediated suppression by NOS inhibitors; suppression of protein expression in the absence of significant effects on the cognate mRNA; and, its rapid down-regulation by NO donors in the presence of the protein synthesis inhibitor cycloheximide. Using DPTA, an NO donor with a chemical half-life in aqueous solution of 3 h (Hrabie et al., 1993), the half-life of CYP2C22 was reduced by 60% to 3.4 h. While the kinetics of down-regulation could be fitted to an exponential curve (R=0.964), the data gave some indication of a biphasic decay (Fig 6C). If anything, therefore, the estimate of 3.4 h may underestimate the initial rapid decay of the protein. Furthermore, we demonstrate that this effect is
specific to NO and is not reproduced by oxidant stress caused by application of hydrogen peroxide in the presence or absence of catalase inhibition.

The down-regulation of CYP2C22 by IL-1β and the NO donors DPTA and NOC18 was accompanied by similar changes in the retinoic acid hydroxylase activities of the cells, supporting the reported finding of CYP2C22 as a retinoic acid hydroxylase (Qian et al., 2010). This significant role of CYP2C22 in atRA metabolism is most likely due to the fact that CYP26 enzyme expression is low in the absence of atRA treatment as shown in human hepatocytes and HepG2 cells (Tay et al., 2010). The remaining atRA hydroxylation activity in the IL-1 and NO donor- treated hepatocytes could be due to the contribution of CYP26 enzymes to atRA hydroxylation.

Unlike CYP2B1 (Lee et al., 2008) (Sun et al., 2012) and CYP3A (Lee et al., 2009) however, the down-regulation of CYP2C22 by NO could not be inhibited by proteasome inhibitors. Lysosomal degradation is the main route of degradation of CYP2B and some other P450 enzymes in their native states (Ronis et al., 1991) (Roberts, 1997) (Liao et al., 2005), but we found that the lysosomal inhibitors NH₄Cl and chloroquine; as well as three different calpain inhibitors all failed to inhibit CYP2C22 down-regulation. Therefore, we conclude that CYP2C22 degradation in response to NO proceeds via a novel pathway. The protein is not being trafficked to a different cellular compartment, since whole cellular lysates were used. Also, we have not detected the appearance of any lower molecular weight bands during CYP2C22 degradation, even on overexposed blots from cells treated with proteasome inhibitors.
The possibility that CYP2C22 was being cleaved in the antigenic domain (amino acids 366-390) was also investigated. In experiments not shown, we determined that a different polyclonal antibody raised to the entire CYP2C11 protein recognizes a protein in rat hepatocytes that undergoes rapid NO-dependent down-regulation similar to that of CYP2C22. It has been documented that CYP2C22 expression increases when hepatocytes are cultured, while CYP2C11 declines (Emi et al., 1990). Since we have determined that CYP2C11 is not an NO-responsive protein (Sewer and Morgan, 1997) (Lee et al., 2009), we conclude that this CYP2C11 antibody detects primarily CYP2C22 in these cultures. Using this polyclonal CYP2C antibody, we were also unable to detect discreet cleavage products in overexposed blots from cells treated with proteasome inhibitors and NO donors. Clearly the CYP2C11 antibody recognizes additional CYP2C epitopes to the specific CYP2C22 antibody. Therefore, we conclude it to be unlikely that the disappearance of the protein is due to a peptidase making a discreet cut between amino acids 366-390.

CYP2C11 and CYP2C22 share 61% amino acid identity (298/489), yet CYP2C22 undergoes NO-stimulated degradation whereas CYP2C11 does not. The contrasting behaviors of these highly related proteins may provide a strategy from which to identify structural features that confer response to NO. As mentioned above, cysteine nitrosylation sensitizes iron regulatory protein 2 and alkylguanine DNA-alkyltransferase (Kim et al., 2004) (Wei et al., 2010) to proteasomal degradation. On the other hand, tyrosine nitration of CYP2B1 on Y190 is causal for its catalytic inactivation by
peroxynitrite (Lin et al., 2005), and nitration could possibly contribute to CYP2C22 degradation. CYP2C22 has four cysteine residues (C13, C55, C164 and C389) and six tyrosine residues (Y78, Y151, Y219, Y223, Y265 and Y315) that are not present in CYP2C11. Human CYP2C8, 2C9 and 2C19 share 60.7, 62.0 and 61.6% amino acid identity with CYP2C22. C164 and C389 are present in all three human CYP2Cs, and C13 is present in CYP2C9 and 2C19. None of the six tyrosines absent from 2C11 are present in the human CYP2C enzymes. The contributions of these residues to NO-mediated degradation are currently under investigation. However, it must also be considered that larger structural features could be involved in the susceptibility to degradation.

Using antibodies specific to CYP2C22, we demonstrated that its tissue distribution is the same as reported previously for its mRNA specificity (Qian et al., 2010). In a rat hepatoma cell line, CYP2C22 mRNA was induced approximately 10-fold by 100 nM – 1μM atRA, while higher concentrations were not tested (Qian et al., 2010). Our data in primary hepatocytes is in good agreement with this finding. Interestingly CYP2C22 mRNA and CYP26B1 levels began to decline between 12 h and 24 h of treatment, possibly reflecting increased atRA metabolism due to the induction of these proteins (Isoherranen, N et al, unpublished data). As a consequence, CYP2C22 protein levels never reached the same magnitude of induction as CYP2C22 mRNA. Nevertheless, CYP2C22 protein began to be induced only 3 h after the mRNA was first seen to increase, consistent with our determination of a half-life of only 8.7 h for the protein in the absence of NO.
Westin et al found that the closely related CYP2C7 mRNA was induced by atRA in rat hepatocytes and was partially dependent on atRA metabolism (Westin et al., 1993) (Westin et al., 1997). However, significant induction was only observed at atRA concentrations >1μM in hepatocytes (Westin et al., 1993), whereas CYP2C22 protein and mRNA induction can be detected with 100nM atRA. Together with the data of Qian et al. (2010), this suggests that CYP2C22 induction, by contrast to CYP2C7, proceeds via a classic RAR mechanism. Interestingly, Qian et al. (2010) also found that human CYP2C9 is induced by atRA in human hepatocytes. This perhaps raises the question of whether CYP2C22 and CYP2C9 might share other regulatory mechanisms, such as stimulated degradation by NO.

From our experiments in cultured rat hepatocytes, CYP2C22 protein is not significantly regulated by activators of the receptors PXR, CAR or PPARα, which are important in the regulation of many xenobiotic-metabolizing P450 enzymes. Although Emi et al concluded from qualitative analysis of Northern blots that phenobarbital and the AhR agonist 3-methylcholanthrene did not induce hepatic CYP2C22 mRNA in vivo (Emi et al., 1990), closer inspection of their Northern blots suggests that levels were slightly reduced by 3-methylcholanthrene. Supporting this interpretation, our data show a strong and reproducible down-regulation by β-naphthoflavone of CYP2C22 mRNA and protein levels in rat hepatocytes. The potent AhR agonist tetrachlorodibenzo-p-dioxin causes depletion of hepatic retinoid stores, possibly via increased metabolism by AhR-responsive P450 and UDP-glucuronosyltransferase enzymes (Novak et al., 2008).
Further research will be needed to determine if such a mechanism could explain the rapid down-regulation of CYP2C22 mRNA by β-naphthoflavone.

We showed previously that, like rat CYP2B1, human CYP2B6 protein undergoes NO-dependent down-regulation in human hepatocytes (Aitken et al., 2008). In addition to CYP2C22, Qian et al showed the related human CYP2C9 mRNA to be regulated by atRA (Qian et al., 2010). Whether or not any of the human CYP2C enzymes are regulated by nitric oxide is a subject of ongoing investigation. However, until the mechanisms of NO-mediated down-regulation are better understood, it is not possible to predict the susceptibility of individual forms from amino acid sequence or indeed from tertiary structures.

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REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Fig. 1. CYP2C22 antibody specificity and liver-specific expression of the enzyme. Polyclonal antibodies were raised to a peptide containing amino acids 366-190 of CYP2C22. A, Western blots of various hepatic fractions incubated with a 1:3,000 dilution of anti-CYP2C22 serum #96 or a 1:3,000 dilution of antiserum #97. Prior to addition to the blot, the diluted antisera were incubated overnight at 4°C with the indicate amounts of antigenic peptide adsorbed to nitrocellulose paper. Lane 1: total cell extract from primary rat hepatocytes (34 μg); lane 2: rat liver S9 fraction (40 μg); lanes 3,4: rat liver microsomes (5 and 15 μg, respectively). B, Western blots using CYP2C22 antiserum #96 (1:1,000 dilution) or #97 (1:3,000 dilution) or anti-CYP2B1 (1:5,000 dilution) against insect cell microsomes expressing rat P450 enzymes (1.5 μg protein) as indicated. C, Western blot using CYP2C22 antiserum #97 (1:3,000 dilution) against microsomes from various tissues isolated from a 230 g male Sprague-Dawley rat. This blot is representative of three blots from different animals. D, CYP2C22 and 2C11 mRNA expression in the same tissues described in panel C, measured by RT-qPCR. Relative mRNA expression is expressed as a percentage of the liver values which were arbitrarily set at 100. Values are means ± SEM of values from 3 different animals.

Fig. 2. Regulation of CYP2C22 mRNA and protein by atRA in cultured rat hepatocytes. Hepatocytes were cultured for 4 days prior to the addition of atRA. A, Western blot showing relative levels of CYP2C22 protein in hepatocytes treated with atRA. atRA (5 μM) was added to the cells at different times so that all samples were harvested at the
same time. Control cells (0 h) were untreated and harvested at the same time as the treated cells. B, Quantitative analysis of the data in panel A and levels of CYP2C22 and CYP26 mRNAs in cells from the same experiment (n=3 independent samples). *, significantly different from 0 h control group, p<0.05, Dunnett’s test. C, Concentration dependencies of CYP22C and 2C26 induction. Hepatocytes were incubated with the indicated concentration of atRA for 24 h. The points on the y-axis indicate cells that were treated with vehicle (DMSO) only. *, significantly different from untreated control group, p<0.05, Dunnett’s test.

Fig. 3. Effect of prototypic inducers of drug metabolizing enzymes on CYP2C22 expression. Rat hepatocytes in sandwich cultures were treated with media (Con), 40 μM β-napthoflavone (βNF), 1mM phenobarbital (PB), 20 μM dexamethasone (Dex), 25 ChoonμM pregnenolone 16α-carbonitrile (PCN), or 10 μM clofibrate (Clo) for 72 h, after which the cells were harvested and analyzed for CYP2C22 mRNA and protein expression. A, Western blot of representative samples from each treatment group. B, Quantitative analysis of individual samples (n=3). Values are expressed relative to the control group. *, significantly different from control, p<0.05, Dunnett’s test.

Fig. 4. NO-dependent, down-regulation of CYP2C22 protein. A, Time courses of regulation of CYP2C22 and NOS2 proteins in hepatocytes treated with 5 ng/ml IL-1. B, Quantitative analysis of the data in panel A. Levels of nitrate + nitrite (NOx) in the media are also included. *, significantly different from zero time control, p<0.05, Dunnett’s test. C, Post-transcriptional regulation of CYP2C22 by IL-1. Hepatocytes
were treated with 5 ng/ml IL-1 and harvested at the indicated times for measurement of CYP2C22 mRNA and protein levels. * Significantly different from control cells at same time point, p<0.05, t-test. D, Inhibition of CYP2C22 protein down-regulation by L-NAME. Hepatocytes were incubated for 12 h with control medium (Con), IL-1 (5 ng/ml), 100 μM L-NAME or both. In the lower panel, cells were treated with 1 mM phenobarbital (PB) for 48 h prior to and during incubation with IL-1. E, Quantitative analysis of the data in panel D (n=3). a, significantly different from control; b, significantly different from IL-1 treated cells; p<0.05 ANOVA and Tukey’s test. F, Levels of nitrate + nitrite (NOx) in the media of the treated cells from panels D and E.

Fig. 5. Effects of NO donors on CYP2C22 expression and retinoid metabolism in hepatocytes. A, Western blot showing time course of down-regulation of CYP2C22 protein by the NO donor NOC18 in cultured hepatocytes. B, Time courses of down-regulation of CYP2C22 mRNA and protein by NOC18. Cells were treated with 500 μM NOC18 or vehicle and harvested at the indicated times. CYP2C22 data are from the blots shown in panel A. *, significantly different from timed control, p<0.05 (n=3), t-test. C, Western blot showing concentration dependence of the NOC18 effect. Cells were treated for 12 h with the indicated concentration. D, Quantitative analysis of the data in panel C. *, significantly different from zero time control, p<0.05, Dunnett’s test (n=3). E, Effect of NO donors and IL-1 on retinoic acid metabolizing activities of rat hepatocytes. Hepatocytes were treated with medium (control), 5 ng/ml IL-1 for 12 h, 500 μM NOC18 for 12 hr, or 500 μM DPTA for 6 hr. The cells were harvested and the formation of 4-OH-RA from aPRA was measured in S9 fractions of the cells. The activities are shown
relative to control cells. *, significantly different from control, p<0.05, Dunnett’s test (n=4).

F. Western blot showing CYP2C22 expression in the samples from Panel E.

Fig. 6. Stimulated degradation of CYP2C22 protein. A, Western blot of lysates from cells treated for 12 h with 500 μM NOC18 or medium (Con) in the presence (CHX) or absence (Mock) of 20 μg/ml cycloheximide. B, Quantitative analysis of the data in panel A. *, significantly different control, p<0.05, t-test (n=3). C. Cycloheximide chase experiment. Hepatocytes were treated with medium (Con) or 500 μM DPTA for 2, 4, 6 or 8 h in the presence or absence of 20 μg/ml cycloheximide, and CYP2C22 levels were measured by Western blotting (n=3). Exponential curve fitting was performed using Kaleidagraph software (Synergy Software, Reading, PA).

Fig. 7. Lack of effect of proteasome, lysosome and calpain inhibitors. A, Western blot showing the effect of proteasome inhibitors on CYP2C22 down-regulation by IL-1. Three day-old hepatocytes were pretreated with 1 mM PB, which was present for the rest of the experiment. After two days of PB induction, the cells were treated with IL-1 (5 ng/ml) or control medium (Con). 4 h later, bortezomib (Bort, 10 μM), MG132 (MG, 20 μM), or control media were added. The cells were harvested 12 h after IL-1 addition, and relative expression of CYP2C22 and CYP2B proteins were measured. B, quantitative analysis of the data in panel A. a, significantly different from control; b, significantly different from IL-1 treated cells; p<0.05, ANOVA and Tukey’s test (n=3). C, Nitric oxide production by the cells in the experiment depicted in panels A and B. D, Effect of proteasome inhibitors and Brefeldin A on CYP2C22 degradation stimulated by
an NO donor. Hepatocytes were treated with medium (Con) or 500 μM DPTA for 5 h in the presence or absence of bortezomib (10 μM), MG132 (20 μM), or brefeldin A (Bre, 10 μM). E, Effect of hydrogen peroxide on CYP2C22 and CYP2B expression.

Hepatocytes were pretreated with phenobarbital as described for panel A, and then treated with control medium (Con) or H₂O₂ (5 mM) with or without aminotriazole (AT, 25 mM) for 5 h, or with NOC18 (500 μM, 12 h), DPTA (500 μM, 6 h), or IL-1 (5 ng/ml, 12 h). The cells were harvested at the same time and the levels of CYP2C22 and CYP2B proteins were assessed by Western blotting. F, Quantitative analysis of the data in panel E. *, significantly different from control, p<0.05 Dunnett’s test (n=3).

Fig. 8. Down-regulation of lentiviral-expressed CYP2C22 by NO in HuH7 cells is not attenuated by inhibitors of calpain or lysosomal degradation. A, Effect of calpain inhibitors on CYP2C22 down-regulation. Fully confluent 3-day old HuH7 cells stably expressing CYP2C22 in 12-well plates were treated with DPTA (500 μM) and /or E64d (10 μM), MDL 28170 (MDL, 10 μM), or ALLM (10 μM) for 4 h and total cell lysates were harvested for CYP2C22 and GAPDH immunoblotting. a, significantly different from control, untreated cells; b, significantly different from cells treated with inhibitor alone, p<0.05, ANOVA and Tukey’s test (n=3). B, Inhibition of calpain activity. Cells cultured as described in panel A were treated with control media or calpain inhibitors E64d (10 μM), MDL 28170 (MDL, 10 μM), and ALLM (10 μM) for 2 h. The media were exchanged for assay buffer containing the calpain substrate Boc-LM-CMAC (25 μM), and fluorescence in the media was measured after 20 min of incubation. *, significantly different from control; p<0.05, ANOVA and Dunnett’s test (n=4). C. Effect of
lysosomotropic agents. To study lysosome-dependent degradation of Annexin VI, cells were first deprived of serum for 4 h to enhance the expression of Annexin VI, then serum was added to stimulate its degradation. DPTA (500 μM) and/or chloroquine (CQ, 100 μM) or NH₄Cl (20 mM) were added concomitantly with the serum, and the cells were incubated for an additional 4 h. Total cell lysates were harvested for Western blotting. a, significantly different from control, untreated cells; b, significantly different from cells treated with inhibitor alone, p<0.05, ANOVA and Tukey’s test (n=3).
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