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Nitric oxide-regulated proteolysis of human CYP2B6 via the ubiquitin-proteasome system

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Abstract

We showed previously that rat cytochrome P450 CYP2B1 undergoes NO-dependent proteasomal degradation in response to inflammatory stimuli, and that the related human enzyme CYP2B6 is also down-regulated by NO in primary human hepatocytes. To investigate the mechanism of CYP2B6 down-regulation, we made several cell lines (HeLa and HuH7 cells) in which native CYP2B6 or CYP2B6 with a C-terminal V5 tag (CYP2B6V5) are expressed from a lentiviral vector with a cytomegalovirus promoter. Native CYP2B6 protein was rapidly down-regulated in HeLa cells within 3 h of treatment with the NO donor (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazene-1,1,2-triolate, while its mRNA level was not down-regulated. Treatment of the cells with the NO donor (Z)-1-[N-(3-aminopropyl)-N-(3-ammoniopropyl)amino]diazene-1,1,2-triolate also resulted in rapid down-regulation of CYP2B6 activity, measured as the formation of 7-hydroxy-4-trifluoromethylcoumarin, as well as 2B6 protein in the CYP2B6 HeLa cell line. CYP2B6V5 was also down-regulated by NO donors in HuH7 cells. Down-regulation was observed in the presence of cycloheximide, demonstrating that this occurs via a post-translational mechanism. We generated a HeLa cell line expressing both CYP2B6V5 and human nitric oxide synthase 2 (NOS2), the latter under positive control by tetracycline. The cellular NO produced by doxycycline treatment also effectively down-regulated CYP2B6 protein, which was blocked by the co-treatment with the NOS2 competitive inhibitor L-N^G-nitroarginine methyl ester (L-NAME). We next investigated the proteolytic enzymes responsible for NO-dependent CYP2B6 degradation. Neither calpain inhibitors (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal, carbobenzoxy-valinyl-phenylalaninal), nor lysosomal protease inhibitors (3-methyladenine and chloroquine) inhibited the NO dependent CYP2B6V5 down-regulation. The proteasome inhibitors MG132 and bortezomib attenuated, but did not completely block the NO-induced down-regulation in the HuH7 cell line. However, when cells were co-treated with NO donor and proteasome inhibitors, high molecular mass species could be detected on native CYP2B6 as well as CYP2B6V5 Western blots. Further investigation demonstrated that CYP2B6 protein was polyubiquitinated and this was dramatically enhanced by co-treatment with NO donor and bortezomib. Taken together, our data demonstrate that CYP2B6 is down-regulated in an NO-dependent manner via ubiquitination and proteasomal degradation.

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Graphical abstract

Keywords

Nitric oxide; cytochrome P450; CYP2B6; inflammation; cytokines; protein degradation

Introduction

Khatsenko et al. [1] and Stadler et al [2] provided the first evidence that the down-regulation of certain hepatic drug-metabolizing cytochrome P450 (P450) enzymes under inflammatory conditions was mediated by nitric oxide generated from inducible nitric oxide synthase (NOS2). This was supported by studies in short-term cultures of rat hepatocytes showing that NOS inhibitors attenuated the suppression of CYP1A2, 2B, 2C11 and 3A2 by a cytokine mixture or individual cytokines [3]. In the same study, CYP2B protein was the most sensitive to down-regulation by the NO donor (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (NOC18). Various additional animal studies have suggested a role of NO in the down-regulation of P450 mRNAs [4], proteins [4] [5, 6] and/or activities [4, 6-9] in in vivo models of inflammation or infection, based on attenuation of the down-regulation by NOS inhibitors. Moreover, infusion of the organic nitrates glyceryl trinitrate or isosorbide dinitrate for 48 h caused down-regulation of multiple P450 proteins in rat livers [10]. However, we found that effective inhibition of NOS did not impair the down-regulation of CYP2C11 or CYP3A2 mRNA or proteins in rats treated with bacterial lipopolysaccharide [11].

Discordant findings regarding the role of NO in P450 down-regulation in vivo, even in the same model [4, 11], likely reflect the fact that multiple mechanisms, both transcriptional and post-transcriptional, control the down-regulation of hepatic P450 enzymes in an isoform-specific manner [12]. Cell culture studies provide a more tractable model to study the contributions of NO to the regulation of specific P450enzymes. As such, we demonstrated that NO is responsible for the down-regulation of CYP2B enzymes by bacterial lipopolysaccharide [13] and IL-1 [14] in cultured rat hepatocytes, confirming the original findings of Khatsenko et al [1] and Carlsson and Billings [3]. Notably, down-regulation of CYP2B mRNA by the same agents is slower and NO-independent [13, 14]. Conversely,

CYP2C11 mRNA and protein down-regulation by IL-1 was independent of NO generation [15]. More recently, we used proteomic techniques to identify rat CYP3A1 and CYP2C22 as NO-regulated proteins [16, 17]. While NO-evoked degradation of CYP2B1 proceeds via the canonical ubiquitin (Ub)-proteasome pathway [14], CYP2C22 degradation was insensitive to proteasomal or lysosomal inhibition suggesting the participation of another proteolytic system in this regulation [17].

Together, the above results demonstrate that rodent P450 enzymes are differentially sensitive to NO-mediated regulation. However, there is less known about the sensitivity of human P450s to NO-mediated degradation. Studies in HepG2 cells demonstrated that CYP2D6 was suppressed by NO donors via inhibition of hepatocyte nuclear factor-4, which controls CYP2D6 transcription [18]. NO donor compounds impaired the induction of CYP3A4 mRNA by 1,25-dihydroxyvitamin D3 in Caco-2 colon carcinoma cells [19], apparently via induction of the oncogenic transcription factor *c-myc* [20]. CYP3A4 expressed in another colon carcinoma cell line was down-regulated by ceramide in an NO-dependent manner, and this could be mimicked with an NO donor [21]. We found that, whereas NOS inhibition failed to affect down-regulation of CYP3A4 mRNA or protein by cytokines in cultured primary human hepatocytes, the NO donor NOC18 produced a 50% down-regulation of CYP3A4 mRNA after 24 h of treatment [22]. Furthermore, we showed that down-regulation of CYP2B6 protein by cytokines, but not of its mRNA, was attenuated by NOS inhibition [22]. NOC18 preferentially down-regulated CYP2B6 protein compared to its mRNA [22].

The above findings demonstrated that, like rat CYP2Bs, human CYP2B6 protein is suppressed by NO independently of any effect on its mRNA. To study the mechanisms by which NO achieves this effect, we established lentivirally-transduced HeLa and human hepatoma cell lines expressing CYP2B6. Here, we demonstrate that CYP2B6 protein is more sensitive to NO-stimulated protein degradation than rat CYP2B proteins, whereas CYP3A4 is relatively refractory. Surprisingly, although CYP2B6 is ubiquitinated in response to NO exposure, its down-regulation is only partially inhibited by proteasome inhibitors.

Materials and Methods

Reagents

(Z)-1-[N-(3-aminopropyl)-N-(3-ammoniopropyl)amino]diazene-1-ium-1, 2-diolate (DPTA NONOate), 2,2'-(Hydroxynitrosohydrazino)bis-ethanamine (NOC18), S-Nitrosoglutathione (GSNO), 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride (SIN-1), (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazene-1-ium-1,2-diolate (Spermine NONOate), L-alanyl-N-[(1S)-3-chloro-2-oxo-1-(phenylmethyl)propyl]-L-alaninamide (AAF) and N-acetyl-L-leucyl-N-[(1S)-1-formylpentyl]-L-leucinamide (ALLN) were purchased from Cayman Chemicals (Ann Arbor, MI). N-[(1S)-1-[(1-formyl-2-phenylethyl)amino]carbonyl]-2-methylpropyl]-carbamic acid, phenylmethyl ester (Cal III) were from Calbiochem/EMD Millipore, Billerica, MA. Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) was from Boston Biochemicals (Cambridge, MA) and bortezomib was from LC laboratories (Woburn, MA). FBS was purchased from Atlanta Biologicals (Flowery Branch, GA). Dulbecco's Modification of Eagle's Medium (DMEM) and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA). Opti-MEM media, SuperSignal West Pico

Chemiluminescent Substrate were from ThermoFisher Scientific, Grand Island, NY. RNA-Bee isolation reagent was from Tel-Test, Friendswood, TX. 3-methyladenine (3-MA) was from ARCOS Organics, a ThermoFisher Brand. 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) and 7-hydroxy-4-trifluoromethylcoumarin (7-HFC), cycloheximide, chloroquine (CQ), N^ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), polybrene, and chemicals whose procurement is not specified were from Sigma-Aldrich (St. Louis, MO). CYP2B6 Supersomes (Cat #456210) were from BD Biosciences, San Jose, CA.

Antibodies to CYP2B1 were kindly provided by Drs. James Halpert (University of California, San Diego). CYP2B6 (WB-2B6-Pep, Cat# 458226), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cat# MAB374) and V5-tag antibodies (Cat# V8012) were from BD Biosciences (San Jose, CA), Millipore (Billerica, MA), and Sigma-Aldrich (St. Louis, MO), respectively. The monoclonal anti-hemagglutinin (HA) antibody was from Santa Cruz Biotechnology, Dallas, TX (Cat# sc-7392). IRDye® 680RD Goat anti-Rabbit IgG and IRDye® 800CW Goat anti-Mouse IgG were from LI-COR Biosciences, Lincoln, NE. Anti-V5-tag mAb-Magnetic Beads were obtained from MBL International, Woburn, MA.

Cloning

All clones generated were confirmed by sequencing. CYP2B6 cDNA was amplified by PCR from a plasmid provided by Dr. James Halpert [23], using primers with XbaI and SalI sites on their 5' ends, and subcloned into a lentiviral vector Lv-CMV-GFP-U3Nhe [24] after removing the GFP insert, which was provided by Emory Viral Vector Core. The plasmid pLenti6.2/V5-DEST for CYP2B6V5 expression (V5-tag attached on c-terminus) was obtained from the Arizona State/DNASU plasmid repository, <https://dnasu.org/DNASU/Home.do>. A human CYP3A4 clone was also procured from DNASU. A human NOS2 cDNA clone was obtained from transOMIC technologies Inc. (Huntsville, AL) and subcloned into a tetracycline inducible plasmid pLIX_403 (a gift from David Root; Addgene plasmid # 41395) using NheI and SalI restriction sites and named pLIX-hNOS2.

Lentivirus production and infection

For native CYP2B6 expression, we used a lentiviral packaging system, consisting of pCMV R8.92 (Emory Viral Core) containing the human immunodeficiency virus 1 (HIV1) gag/pol, tat and rev genes, and the pantropic packing vector pVSVG (Emory Viral Core). Human embryonic kidney HEK293T cells were grown in high-glucose DMEM containing 10% v/v FBS and 1% penicillin/streptomycin, to 60-70% confluency on 6-well, collagen coated plates. On the day of transfection, the medium was changed to antibiotic-free DMEM. The transfection mixture was prepared by combining 0.625 µg of pCMV R8.92, 0.42 µg of pVSVG and 0.84 µg of a CYP expression vector with Opti-MEM medium. Transfections were carried out with Lipofectamine 2000 using the manufacturer's protocol. The cells were incubated at 37 °C under 10% CO₂ for 18 h, and the medium was replaced with 3 ml/well of virus harvest medium (10% FBS/1% penicillin-streptomycin/DMEM). The cells were further incubated under 5% CO₂, and virus-containing supernatants were collected at 48h and 72h post-transfection. The combined viral supernatants were passed through a 0.45 µm filter and stored at -80°C. For CYP2B6V5 expression, a second generation lentiviral packaging system consisting of pMD2.G and psPAX2 (gifts from

Didier Trono; Addgene plasmid # 12259 and # 12260, respectively) was used and followed virus production protocol from Addgene (<https://www.addgene.org/tools/protocols/plko/>).

HeLa cells were obtained from the American Type Culture Collection, and Dr. Arash Grakoui of Emory University kindly provided HuH7 cells. The cells were verified by short tandem repeat analysis by the Emory Integrated Genomics Core facility. Cells were cultured in DMEM containing 10% FBS in a 5% CO₂ incubator. The frozen lentivirus was thawed at room temperature. Two µl of 4 µg/mL polybrene was added to 1mL of viral media for a final concentration of 8 µg/mL, and this was added to the 6-well plated cells (HeLa or HuH7) at 60 to 80% confluency. The plate was swirled gently and then incubated at 37°C in 5% CO₂. After 24h, the medium was replaced with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin.

Cell-based CYP2B6 Activity Assay and NO assay

Cell-based CYP2B6 activity was measured with 7-EFC as a substrate [25]. Cells were grown to over 90% confluence in 12 or 24-well plates. The cells were incubated with 1 ml of 50 µM 7-EFC in CYP Assay buffer (1 mM Na₂HPO₄, 137 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM Hepes, pH 7.4) for 10 min at 37°C, and 50 µl aliquots of the reaction mixture were transferred to 96-well assay plates. After adding 100 µl of quenching solution (0.25 M Tris in 60% (v/v) acetonitrile), the fluorescence of 7-EFC was measured (Ex, 395 nm; Em, 500 nm) on a Spectra Max M2 plate reader (Molecular Devices, Sunnyvale, CA). Cells displaying high activity were further tested for CYP2B6 protein expression by Western blotting as described below.

To evaluate NO production from cell line containing the pLIX-hNOS2 plasmid, cells were treated with 10 µM doxycycline for various times and then media were harvested for assay of nitrite+nitrate concentrations via the Griess reaction [26].

Western Blotting

Cells (90 to 100% confluence) were harvested with cell lysis buffer containing 50 mM Tris-Cl, pH 7.5, 0.1% SDS, 1% NP-40, 1 mM EDTA, and a protease inhibitor cocktail (Sigma-Aldrich P8340). The lysates were centrifuged at 12,000 × g for 5 min and the supernatants were collected. Equal amounts of protein measured by the bicinchoninic acid method (ThermoFisher Scientific, Grand Island, NY) were loaded and resolved on SDS-PAGE, and blotted on nitrocellulose paper (Bio-Rad, Hercules, CA). CYP2B6 protein in total cell lysates was measured by incubating the membrane with either anti-CYP2B6 peptide antibody (WB-2B6-Pep 1:1,000) or anti-CYP2B1 (1:10,000) at 4 °C overnight. V5-tagged CYP2B6 and CYP3A4 were detected with a V5 antibody (1:10,000). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10,000) antibody was included in the incubations as a loading control. Bound antibodies were visualized by one of two different methods. Earlier experiments used chemiluminescence detection, while infrared (IR) fluorescence, which gives more quantitative measurements, was used in later experiments. For chemiluminescence detection, the blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase, and the blots were developed with SuperSignal West Pico Chemiluminescent Substrate. Blots were digitized on a Bio-Rad

(Hercules, CA) imager Gel Doc XR+ and band intensities were quantified using Bio-Rad Image Laboratory software version 4.0. Because of unequal GAPDH and P450 expression in the cells, different film exposures of the same blots were used to quantify the two proteins in order to avoid nonlinearity from overexposure. For IR fluorescence detection, blots were incubated with IRDye® 680RD Goat anti-Rabbit IgG and IRDye® 800CW Goat anti-Mouse IgG (1:10,000 dilution) for 1h and the blots were analyzed with an Odyssey® Fc Imaging System (LI-COR Biosciences) to detect fluorescence signals. Fluorescence intensity was measured using Image Studio™ software (LI-COR Biosciences). Note that some blots are presented with their aspect ratio altered (width increased relative to height) to reduce the space taken up by the figures.

To combine data from multiple experiments, we first calculated the ratio of the V5 signal to that of the control gene (GAPDH) for each sample. This was then divided by the mean of the ratios for all the samples on the blot.

$$\text{For sample } x, \text{ normalized value } v = \frac{\text{CYP2B6}_x}{\text{GAPDH}_x} * \frac{n}{\sum \left(\frac{\text{CYP2B6}_n}{\text{GAPDH}_n} \right)}$$

The resulting values were used to calculate group means for each experiment. The group means for the individual experiments were used to calculate the mean and SD for the combined data, and the results were then scaled to a control group mean value of 100.

Ubiquitination of CYP2B6 in HeLa Cells

HeLa- or HuH7-CYP2B6V5 cells were cultured in DMEM containing 10% FBS in a 5% CO₂ incubator. Plasmid pCDMA3.1-HA-Ub [27] wild type, expressing HA-tagged Ub was transfected into HeLa or HuH7 cells using Lipofectamine 2000 (Invitrogen) by the manufacturer's instructions. Briefly, 1 µg of pCDMA3.1-HA-Ub was mixed with 2 µl of Lipofectamine 2000 in Opti-MEM media and applied dropwise onto 80 to 90% confluence HeLa or HuH7 cell cultures on 6-well plates. After 24 h, cells were treated with DPTA and/or proteasome inhibitors for the indicated times. After harvesting of cells, total cell lysates were subjected to immunoprecipitation to pull down CYP2B6V5 protein. 75 µl of Anti-V5-tag mAb-Magnetic Beads (MBL International, Woburn, MA) was added to the supernatant and incubated overnight at 4°C with continuous mixing. After extensive washing of the bead mixture, CYP2B6V5 protein was released by SDS-loading buffer and subjected to SDS-PAGE. After SDS-PAGE and blotting, the membrane was incubated with monoclonal anti-HA antibody (1:1000 dilution) at 4 °C overnight, and goat anti-mouse IgG labeled with horseradish peroxidase was incubated as a secondary antibody. The ubiquitination signal was detected with SuperSignal West Pico Chemiluminescent substrate.

Quantitative PCR

RNA-Bee isolation reagent was used to prepare total RNA from the cultured cells, according to the manufacturer's instructions. cDNA synthesis from total RNA was performed using the SuperScript First-Strand Synthesis System kit (Invitrogen). Realtime RT-PCR was used to measure the relative expression of P450 mRNAs, using the ABI PRISM 7000 Sequence

Detection System and SyBr® Green Master Mix reagent (Applied Biosystems, Bedford, MA) as described [22]. The following sequences were used for real time PCR amplification of the cDNAs: 2B6 (atg ggg cac tga aaa aga ctg a; aga ggc ggg gac act gaa tga c) and GAPDH (gga cca cca gcc cca gca aga g; gag gag ggg aga ttc agt gtg gtg). Values were normalized to GAPDH mRNA by the Ct method and the expression level in control samples was arbitrarily set to 1.

Statistical analyses

Data are expressed as the mean $\pm$ standard deviation (SD). Differences between groups were calculated either by t-test or by one way analysis of variance followed by an appropriate post-hoc test using GraphPad Prism software (La Jolla, CA), as dictated by the experimental design. Experiments for which no statistical comparisons were performed are deemed exploratory, and in such cases the SD, if shown, are calculated from replicate cell culture wells within a single experiment.

Results

Down-regulation of CYP2B6 proteins in lentivirally transduced cells

To study CYP2B6 down-regulation, we generated HeLa and HuH7 cell lines expressing native CYP2B6 or CYP2B6 with a C-terminal V5 tag (CYP2B6V5) proteins. First we confirmed the expression of native CYP2B6 protein in the HeLa cell line (HeLa-2B6) by detecting native 2B6 protein with an anti-2B6 peptide antibody as well as anti-2B1 antibody (data not shown). CYP2B6 Supersomes (microsomes from insect cells expressing CYP2B6) were used as a positive control for the protein. Cellular CYP2B6 activity was measured using 7-EFC as a substrate. Once expression and activity were confirmed, we used the anti-2B1 antibody to detect native 2B6 protein. In this study, we used two NO donors that release NO at different rates. NOC18 decays with a half-life of 20 h, whereas DPTA releases NO with a half-life of only 3h [28]. Treatment of HeLa cells expressing CYP2B6 (HeLa-2B6) with 500 μ M NOC18 resulted in near-complete suppression of CYP2B6 within 3 h (Fig. 1) that lasted for the duration of the experiment (24 h), while the CYP2B6 mRNA expression was not affected at 3 and 6 h, and actually increased at later time points. CYP2B6 protein levels and activity declined to 17% of control after only 1 h of treatment with 500 μ M DPTA (Fig. 2A, B). By comparison, rat CYP2B1 down-regulation by these donors was much slower [14]. The EC₅₀ for down-regulation of CYP2B6 by NOC18 (6 h, data not shown) and DPTA (4 h, Fig. 2C) were <25 μ M and 10 μ M, respectively. To test the specificity of CYP2B6 down-regulation by NO, we also expressed CYP3A4 in HeLa cells and found it to be relatively refractory to NO donor treatment (supplemental Fig. S1A), consistent with our findings in cultured human hepatocytes [22].

Down-regulation of V5-tagged CYP2B6V5 protein

Since CYP2B6-specific antibodies were difficult to obtain (see additional band in Fig. 1), we generated HeLa or HuH7 cell lines expressing V5-tagged CYP2B6 (HeLa- or HuH7-2B6V5) and examined the regulation of the CYP2B6V5 protein. As seen in Fig. 3, CYP2B6V5 protein was also down-regulated by DPTA in a time and dose dependent manner although the V5-tagged protein was slightly less sensitive than the native enzyme

(Fig 1). We also determined that the activity of CYP2B6V5 was similar to that of native 2B6 (data not shown).

To gain possible insight into the mechanism of NO regulation, we examined the effects of other NO donors on CYP2B6V5 down-regulation. Spermine NONOate, like DPTA and NOC18 is a pure NO donor [28, 29], releasing NO radicals in a time dependent manner. The half-life of spermine NONOate is 37 min. GSNO is considered to nitrosylate cysteine residues [30] and SIN-1 is suggested to nitrate tyrosine residues via peroxynitrite production [31]. As seen in Fig. 4A, the pure NO donors were the most efficacious in down-regulation of CYP2B6V5 protein and its activities, whereas GSNO had little effect and SIN-1 had no effect on CYP2B6 protein or its activity. In contrast, CYP3A5V5 expressed in HeLa cells was not affected by any NO donor treatments (Fig. S1B).

Since our data until now were generated by chemical NO donors, we examined the effects of physiologically-produced NO on the down-regulation of CYP2B6V5 protein. We transduced the doxycycline inducible pLIX_403-hNOS2 lentivirus into HeLa cells and the infected cells were selected with puromycin. The cells produced NO (as measured by NO₂ and NO₃ formation) in a time and dose dependent manner (data not shown). We then treated the cells with doxycycline with or without L-NAME, a competitive inhibitor of NOS2. As seen in Figs. 4B and S2, doxycycline treatment resulted in NO generation in the cell and the NO production was blocked by L-NAME co-treatment. The CYP2B6V5 protein was down-regulated upon doxycycline treatment and the down-regulation was reversed by co-treatment with L-NAME, demonstrating that the CYP2B6 protein down-regulation is NO-dependent. Since CYP2B6V5 was down-regulated similarly by chemical NO donors and NO formed by hNOS2, we carried out the remainder of experiments with chemical NO donors.

Post-translational down-regulation of CYP2B6

NOC18 treatment resulted in the down-regulation of 2B6 protein, whereas the mRNA of 2B6 (Fig. 1A) or CYP2B6V5 mRNA (data not shown) were not down-regulated, suggesting post-translational down-regulation of 2B6 protein by NOC18. To support these data, we treated a HuH7-2B6V5 cell line with the protein synthesis inhibitor cycloheximide 30 min prior to, and during DPTA treatment. The 2B6V5 protein was rapidly down-regulated by 50% within 2h and further down-regulated to 40% of control within 4h of DPTA treatment in the presence of cycloheximide (Fig. 5), confirming post-translational down-regulation of CYP2B6 by NO.

Proteases involved in NO-evoked degradation of CYP2B6

Because rat CYP2B1 degradation in response to NO is blocked by proteasome inhibitors [14], we examined the effects of inhibitors of the proteasomal and other protein degradation pathways on NO-evoked CYP2B6 down-regulation in HeLa and HuH7 cells. HuH7-2B6V5 cells were treated with DPTA, or DPTA and cell permeable protease inhibitors. DPTA treatment resulted in down-regulation of 2B6V5 protein to approximately 35% of control and the down-regulation was partially but significantly attenuated by the co-treatment of proteasome inhibitors, either bortezomib or MG132 (Fig. 6A). Since proteasome inhibition failed to completely block CYP2B6 down-regulation, we examined the effects of inhibitors

of other cellular proteases. Inhibition of lysosomal function via 3-MA or chloroquine (CQ) had no effect on the down-regulation by DPTA in HuH7 cells (Fig. 6A, B). Similarly, the calpain protease inhibitors ALLN and Cal III also had no effect on CYP2B6 down-regulation (Fig. 6B).

We found the same results in HeLa-2B6 or HeLa-2B6V5 cell lines, in that proteasome inhibitors bortezomib or MG132 did not completely block NO-evoked down-regulation, but produced only partial effects (data not shown). We suspected that other cellular protein degradation pathways might be involved in the degradation. Tripeptidyl peptidase (TPP) II is a serine peptidase complex that can perform some of the functions of the proteasome [32]. Therefore, we treated HeLa cells with the TPP inhibitor AAF in the presence or absence of proteasome inhibitors. While AAF appeared to have little effect on its own, in combination with proteasome inhibitors it did not further inhibit CYP2B6 down-regulation (Fig. 7). Notably, in all cultures treated with DPTA plus a proteasome inhibitor (but not in the absence of DPTA or with DPTA + AAF), higher molecular weight bands reactive with the CYP2B6 antibody were observed, especially when the blot was over-exposed (Fig. 7).

This observation prompted us to reanalyze our data in Fig. 6A. By increasing the gain on the fluorescence signals of these blots we were able to visualize and quantify the fluorescence signals of the higher molecular mass (HMM) species (three independent experiments) (Fig. 8). The co-treatments of DPTA and bortezomib or MG132 caused more than 10-fold increases of HMM species, while DPTA or proteasome inhibitors (bortezomib or MG132) treatments alone resulted in 1.5-fold to 2.3-fold increases of HMM, respectively (Fig. 8B). This data suggests that NO-evoked 2B6 degradation occurs via the proteasomal pathway.

Polyubiquitination of CYP2B6V5 protein by NO

To examine whether or not the observed HMM species are indeed due to polyubiquitination of CYP2B6V5, after transient transfection of the pCMV-HA-Ub plasmid into HeLa-2B6V5 cells, the cells were treated with DPTA (100 μ M) or DPTA/Bortezomib (10 μ M) for 2h and then the CYP2B6V5 protein was immunoprecipitated using anti-V5 magnetic beads. Precipitated CYP2B6V5 complexes were subjected to SDS-PAGE and polyubiquitination was detected by immunoblotting with an anti-HA antibody. As seen in Fig. 9, co-treatment of DPTA and bortezomib significantly increased the polyubiquitination signal compared to those of control, DPTA-treated, or bortezomib-treated cells. Taken together our data suggest that NO-evoked CYP2B6 degradation occurs via a ubiquitin-dependent proteasomal mechanism.

Discussion

Here we demonstrated that CYP2B6 protein is down-regulated by NO originating either from chemical NO donors or of biological origin. We showed that both native CYP2B6 and V5-tagged CYP2B6 are down-regulated by NO in a time and dose-dependent manner. Moreover, degradation of CYP2B6 by NO occurs via the ubiquitin-proteasomal pathway.

Previously, we showed that rat P450 enzymes CYP2B1 [14], CYP3A [16], and CYP2C22 [17] are down-regulated by NO in rat primary hepatocytes, and that CYP2B1 and CYP3A

degradation are blocked by proteasome inhibitors whereas CYP2C22 degradation is insensitive. These findings clearly demonstrate that the pathways of NO-dependent degradation differ among different P450 enzymes. Although we reported previously that human CYP2B6 [22] is also down-regulated by NO in primary human hepatocytes, the pathways involved were unknown until now. Here, we discovered that, although NO-evoked down-regulation of CYP2B6 was only partially attenuated by proteasome inhibitors, CYP2B6 down-regulation occurs via ubiquitination and proteasome degradation. This conclusion is supported by three lines of evidence: 1) the proteasomal inhibitors MG132 and bortezomib partially attenuated NO-evoked CYP2B6 down-regulation (Fig. 8); 2) HMM signals are enhanced by the co-treatment of DPTA and bortezomib (Fig. 4D); and 3) levels of CYP2B6 polyubiquitination are greatly enhanced by treatment with DPTA and bortezomib (Fig. 9). We were only able to detect significant HMM species with DPTA/proteasome inhibitor co-treatment (Figs. 7,8), indicating that the degradation of CYP2B6 occurs rapidly once the protein is ubiquitinated. The reason that proteasome inhibition did not completely rescue levels of unmodified CYP2B6 is likely because proteasome inhibition does not prevent the formation and accumulation of HMM species. Here, we also found that compared to the rat P450s, human CYP2B6 protein was down-regulated significantly faster by NO donor treatments (12 to 24 h in rat hepatocytes vs. 1- 2 h in HeLa and HuH7) and by lower concentrations of NO donors (150 to 500 μ M vs 50 μ M), suggesting that human 2B6 protein is significantly more susceptible to NO than CYP2B1 and rat CYP3As. CYP3A4, on the other hand, is resistant to NO-mediated degradation in both human hepatocytes and HeLa cells (Fig. S1).

Because HuH7 and HeLa cells do not produce significant induction of NOS2 in response to inflammatory stimuli, we engineered cell lines to produce hNOS2 and NO in response to doxycycline, and the NO production was blocked by the NOS inhibitor L-NAME (Fig. 4B). Importantly, the levels of NO₂ + NO₃ (50 μ M) detected in the media of cells treated with doxycycline were similar to levels we observe in cultured human hepatocytes stimulated with a cytokine mixture (20 to 70 μ M), [22] and unpublished data.

NO-stimulated protein degradation occurs for several non-P450 proteins, such as iron regulatory protein 2 (IRP2) [33], insulin receptor substrate (IRS) -1 and -2 proteins [34][35], mouse double minute 2 homolog (Mdm2) [36], O⁶-alkylguanine-DNA alkyltransferase [37], serine/threonine-protein kinase STK11 [38], cyclin-dependent kinase 5 [39], and caspase 8 inhibitory protein [40]. In the case of IRP2, opposite effects have been reported. IRP2 was degraded by nitric oxide generated by LPS and INF γ stimulation [33] in RAW 264.7 cells, whereas treatment with S-nitroso-N-acetyl-penicillamine was reported to inhibit the degradation of IRP2 [41] in mouse B6 fibroblasts or human H1299 lung cancer cells. Those differences might have resulted from different cell types or different kinetics or concentrations of NO in the cells. The protein regulation mechanisms elicited by NO are also diverse. For example phosphorylation of IRS-2 protein is required for NO-dependent degradation [35], and IRP2 and caspase 8 inhibitory protein undergo NO-dependent S-nitrosylation before proteasomal degradation [42][40]. NO can affect not only protein degradation but also protein function. Parkin and phosphatase and tensin homolog proteins are S-nitrosylated and inhibited by NO but their protein levels are unchanged [43]. Here we showed that NO can reduce CYP2B6 activity and protein levels (Fig. 1B). NO is well known

to inhibit P450 proteins via nitrosation of the heme moiety [44, 45]. The Hollenberg group identified nitration of tyrosine residues 354, 244, 268, and 380 in CYP2B6 by *in vitro* peroxyxynitrite treatment in vitro with concomitant inhibition of its activity [46]. We tried but could not identify an amino acid residue modification caused by DPTA treatment in the cells. This may be due to rapid degradation of CYP2B6 protein once it is modified, as the kinetics of inhibition and down-regulation of CYP2B6 protein and activity by DPTA were almost identical (Fig. 1B).

We noted that the patterns of HMM species (measured by blot overexposure) and polyubiquitination (measured by an antibody to HA-Ub) were different. The HMM species show smear band patterns from MW 65 KD to 170 KD (Figs. 7 and S2), while the polyubiquitination pattern started at 70 KD and the level of signal was much higher in the very high molecular weight range (>130 kD) (Figs. 8 and S2). This is likely due to the fact that the higher mass complexes will have many more HA epitopes attached, resulting in stronger signals in that region. On the other hand, the v5 signal should be the same for each CYP2B6 molecule regardless of how many HA-Ub units are attached.

Conclusions

Our data demonstrate that, like its rat counterpart CYP2B1, human CYP2B6 undergoes Ub-dependent proteasomal degradation in response to pathophysiological levels of NO. CYP2B6 metabolizes a number of drugs including artemisinin, bupropion, cyclophosphamide, efavirenz, ketamine, and methadone and is a major contributor to bioactivation of the anticancer and immunosuppressant prodrug cyclophosphamide [50]. Since our data showed that both pharmacological NO donors and cellular NO as is produced during infection and inflammation can cause CYP2B6 degradation, one should consider drug-drug interactions (such as with NO releasing drugs) as well as decreased CYP2B6 activity under conditions of infectious and inflammatory diseases when CYP2B6-metabolized drugs are administered to a patient. Further work is needed to determine which other human P450 proteins are subject to this regulatory mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

AAF	L-alanyl-N-[(1S)-3-chloro-2-oxo-1-(phenylmethyl)propyl]-L-alaninamide, mono trifluoro-acetate
ALLN	N-acetyl-L-leucyl-N-[(1S)-1-formylpentyl]-L-leucinamide
Cal III	N-[(1S)-1-[(1-formyl-2-phenylethyl)amino]carbonyl]-2-methylpropyl]-carbamic acid, phenylmethyl ester
CQ	chloroquine
DMEM	Dulbecco's modified Eagle's medium
DPTA	(Z)-1-[N-(3-aminopropyl)-N-(3-ammoniopropyl)amino]diazen-1-ium-1, 2-diolate
7-EFC	7-ethoxy-4-trifluoromethylcoumarin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSNO	S-nitrosoglutathione
HA	hemagglutinin
HMM	high molecular mass

7-HFC	7-hydroxy-4-trifluoromethylcoumarin
IRP2	iron regulatory protein 2
IRS	insulin receptor substrate
L-NAME	L-N ^G -nitroarginine methyl ester
3-MA	3-methyladenine
MG132	carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
P450	cytochrome P450
NOC18	(Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate
NOS	nitric oxide synthase
Pen-Strep	Penicillin-Streptomycin
SIN-1	5-amino-3-(4-morpholiny)-1,2,3-oxadiazolium chloride
Spermine NONOate	(Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazene-1-ium-1,2-diolate
SD	standard deviation
TPP	tripeptidyl peptidase
Ub	ubiquitin

Highlights

- Cytochrome P450 2B6 is downregulated by nitric oxide from NOS2 or NO donors
- Occurs in presence of cycloheximide, indicating NO-mediated protein degradation.
- Down-regulation is partially inhibited by proteasome inhibitors
- Ubiquitin-CYP2B6 conjugates accumulate in presence of NO and proteasome inhibition

Figure 1.

Time course of CYP2B6 down-regulation by NOC18. CYP2B6 HeLa cells were incubated for the indicated times with 500 μ M NOC18, and cells were harvested for assay of CYP2B6 protein or mRNA levels. The upper panel shows the Western blot probed with anti-CYP2B1, which detects an additional non-specific band in these cells. Exploratory time course and dose dependence experiments were repeated several times, and a sample experiment is shown here. Because the experiments were not identical they could not be combined. Values are the means $\pm$ SD of determinations on four different cell culture wells. Absence of a visible error bar indicates that the error is smaller than the symbol size.

Figure 2.

Time and dose dependency of CYP2B6 down-regulation by DPTA. CYP2B6 HeLa cells were incubated for the indicated times with DPTA, and cells were harvested for assay of CYP2B6 protein levels. A. These are exploratory time course and dose dependence experiments which were repeated several times with similar results. Sample experiments are shown here. Because the experiments were not identical they could not be combined. Values are the means $\pm$ SD of determinations on four different cell culture wells. Absence of a visible error bar indicates that the error is smaller than the symbol size. A, Time course of CYP2B6 protein expression and cellular CYP2B6 catalytic activity after exposure to 500 μ M DPTA. B, Concentration dependence of CYP2B6 protein levels after exposure to DPTA for 4 h.

Fig. 3.

Down-regulation of CYP2B6V5 in HuH7 cells. HuH7 cells were transduced with pLX304-2B6V5 lentivirus and CYP2B6V5-expressing cells were selected with blasticidin (5 $\mu\text{g}/\text{ml}$) to generate the HuH7-CYP2B6V5 cell line. After treatment with DPTA, total cell lysates were used for immunoblotting with an antibody to the V5 tag. Values in panels are the means $\pm$ SD of 3 independent experiments. A, IR fluorescence Western blot showing time- and dose-dependence of CYP2B6V5 down-regulation. For the print edition, the image has been converted from red/green to grayscale. B, Quantitation of data from three independent experiments. Concentration dependence was determined at the 4h time point, and time dependence was determined using 500 μM DPTA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from zero time or zero concentration control group, Dunnett's test.

Fig. 4.

Down-regulation of CYP2B6V5 by NO donors and endogenous NO in HuH7 cells. A, Effect of different NO donors. HuH7-2B6V5 cells were treated with DPTA, 500 μ M for 4 h; NOC18, 500 μ M for 6h; spermine NONOate (SpNO), 500 μ M for 2h; GSNO, 500 μ M for 2h; or SIN-1, 500 μ M for 2h. Concentrations and times were chosen based on experimental trials. After incubation, 7-HFC formation was measured for CYP2B6 activity, then total cell lysates were harvested for IR fluorescence immunoblotting. For the print edition, the images have been converted from red/green to grayscale. Activities were measured in only two independent experiments, therefore no statistical tests were performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from untreated control group, Dunnett's test, $n = 3$. B, Down-regulation of CYP2B6 protein by endogenously generated NO. HeLa cells transduced with lentiviruses expressing CYP2B6V5 and tetracycline inducible NOS2 genes, were selected with blasticidin (10 μ g/ml) and puromycin (10 μ g/ml). Cells were treated with doxycycline, 10 μ M and/or L-NAME, 200 μ M for 18 h. Media were harvested for assay of NO production. a, significantly different from control; b, significantly different from Dox, $P < 0.05$. ANOVA and Tukey's test. This is a single experiment, in which SD values represent variation from 3 separate wells in a culture dish. Down-regulation of CYP2B6 by Dox and its reversal by L-NAME were observed in several separate experiments (see Fig S2).

Fig. 5.

Posttranslational down-regulation of CYP2B6. HuH7-CYP2B6V5 cells were treated with cycloheximide (CHX, 100 μ g/ml) prior to the addition of 500 μ M DPTA or medium. After the indicated times, total cell lysates were harvested for IR fluorescence immunoblotting. For the print edition, the image has been converted from red/green to grayscale. Values are mean $\pm$ SD of 3 independent experiments. ** $P < 0.01$, *** $P < 0.001$, significantly different from CHX alone, t-test.

Fig. 6.

Effects of proteolytic enzyme inhibitors on CYP2B6 down-regulation. A, HuH7-CYP2B6V5 cell lines were treated with DPTA, 500 μ M; proteasome inhibitors MG132 or bortezomib (Bort) 10 μ M; or autophagic inhibitor 3-MA (20 μ M) for 4 h. Total cell lysates were subjected to IR fluorescence immunoblotting with anti-V5 antibody. For the print edition, the image has been converted from red/green to grayscale. B, HuH7-CYP2B6V5 cell lines were treated with DPTA, 500 μ M; calpain protease inhibitors ALLN or Cal III, 10 μ M respectively; or lysosomotropic inhibitor chloroquine (CQ, 100 μ M) for 4 h. Total cell

lysates were subjected to immunoblotting with anti-V5 antibody. Values are mean $\pm$ SD. a, significantly different from control; b, significantly different from DPTA, $P < 0.05$. ANOVA and Sidak's test ($n=3$).

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Fig. 7.

Formation of high molecular mass (HMM) species of CYP2B6 in the presence of proteasome inhibitors. Huh7-CYP2B6 cells were treated with DPTA (500 μ M) in the presence or absence of proteasome inhibitors (10 μ M) or the tripeptidyl peptidase II (TPP) inhibitor AAF (50 μ M) for 2 h, and Western blots were carried out with the CYP2B6-specific peptide antibody and chemiluminescent detection. The gel is overexposed to allow visualization of the HMM species. This is a single experiment that was not repeated.

Fig. 8.

Formation of HMM species of CYP2B6V5. The blot from Fig 6A is shown with the gain increased to allow visualization of HMM species. The graph is from quantitative measurement of HMM species in three independent experiments. The 70 kD marker was located by increasing the gain on the image. Values are mean $\pm$ SD. a, significantly different from control; b, significantly different from DPTA, $P < 0.05$. ANOVA and Sidak's test ($n=3$).

Fig. 9.

Ubiquitination of CYP2B6 protein. HeLa cells expressing CYP2B6V5 were transfected with a pCMV-HA-Ub plasmid. 24h post-transfection, cells were treated with DPTA or DPTA plus bortezomib (Bort) for 2h. Total lysate proteins were subjected to immunoprecipitation with anti-V5 magnetic beads. After SDS-PAGE of immunoprecipitates, immunoblotting was carried out with anti-HA or anti-2B1 antibodies with chemiluminescent detection. A, Western blot of the pooled samples. B, Blot of anti-v5 immunoprecipitated samples. This experiment was repeated 3 times with similar results.