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Nitric oxide stimulates cellular degradation of human CYP51A1, the highly conserved lanosterol 14 α -demethylase

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Abstract

Nitric oxide (NO) is known to down-regulate drug metabolizing cytochrome P450 enzymes in an enzyme-selective manner. Ubiquitin-proteasome dependent and independent pathways have been reported. Here we studied the regulation of expression of human CYP51A1, the lanosterol 14 α -demethylase required for synthesis of cholesterol and other sterols in mammals, which is found in every kingdom of life. In Huh7 human hepatoma cells, treatment with NO donors caused rapid post-translational down-regulation of CYP51A1 protein. Human nitric oxide synthase (NOS) – dependent down-regulation was also observed in cultured human hepatocytes treated with a cytokine mixture and in Huh7 cells expressing human NOS2 under control of a doxycycline-regulated promoter. This down-regulation was partially attenuated by proteasome inhibitors, but only trace levels of ubiquitination could be found. Further studies with inhibitors of other proteolytic pathways suggest a possible role for calpains, especially when the proteasome is inhibited. NO donors also down-regulated CYP51A1 mRNA in Huh7 cells, but to a lesser degree than the down-regulation of the protein.

Keywords

nitric oxide; cytochrome P450; inflammation

INTRODUCTION

Nitric Oxide (NO) is a free radical gas that is used in the body for a number of biological processes, including vasodilation [1], neuronal signaling [2] and promoting immune function [3]. NO reacts with oxygen and reactive oxygen species to form reactive nitrogen species such as peroxynitrite and dinitrogen trioxide [4], which can affect protein function or expression. Reversible modification of proteins by NO via nitrosylation of cysteine residues has been likened to phosphorylation as a mode of cellular regulation. Nitrosylation and denitrosylation of proteins occur physiologically and regulate the activities, trafficking,

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DECLARATIONS OF INTEREST

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTION STATEMENT

Ji Won Park, Aria Byrd and Choon Lee designed and conducted the experiments, analyzed the data and contributed to writing the manuscript. Edward Morgan designed experiments, analyzed data and contributed to writing the manuscript

stability and redox sensing properties of a wide diversity of cellular proteins of diverse function [5].

One family of proteins that are affected by NO are the cytochrome P450 (P450) proteins. P450s are a superfamily of enzymes responsible for a number of metabolic activities including metabolism of xenobiotics [6] as well as biosynthesis of endogenous compounds. There are three known mechanisms by which NO and P450s interact; i) All P450s contain a heme-iron center which is nitrosylated by NO in a coordination complex [7, 8]; ii) Cysteine nitrosylation [9] and iii) Tyrosine nitration, in which peroxynitrite reacts with the phenolic oxygen of tyrosines. Peroxynitrite, a product of nitric oxide and superoxide anion interaction, is capable of nitrating tyrosine residues of rat CYP2B1 [10] and human CYP2B6, 2E1 and 3A4 [11, 12] *in vitro*. Inactivation of CYP2B1 by peroxynitrite can be abrogated by mutation of tyrosine residue 190 [13]. CYP3A4 and 2E1 are inactivated via both heme modification and tyrosine nitration [11].

In addition to inhibition of P450 enzymes, NO can also stimulate their degradation. We demonstrated that induction of nitric oxide synthase 2 (NOS2) by bacterial lipopolysaccharide or interleukin (IL)-1 causes the NO-dependent proteasomal degradation of the drug metabolizing P450s, CYP2B1 [9, 14] and CYP3A1 [15] in rat hepatocytes, and that proteasomal degradation of CYP2B1 is ubiquitin-dependent [9]. CYP2B1 was shown to undergo S-nitrosylation by GSNO *in vitro* [9], but it has not yet been determined whether this modification, or indeed tyrosine nitration or heme nitrosylation is responsible for the degradation of the protein. Human CYP2B6 also undergoes NO-dependent down-regulation in human hepatocytes [16]. Sensitivity to NO-dependent down-regulation is enzyme-selective: for example rat CYP2C11 [17] and human CYP3A4 are not affected [16].

The biosynthesis of cholesterol is reliant on CYP51A1, which is the only enzyme that converts lanosterol to 4, 4-dimethylcholesta-8(9), 14, 24-trien-3 β -ol via 14 α -demethylation. CYP51A1 is believed to be the oldest recognizable P450, as it is the only P450 with a conserved function across animal, fungal and plant kingdoms [18]. In keeping with its vital function, the enzyme is ubiquitously expressed in the endoplasmic reticulum of mammalian cells, and maintaining homeostasis of this protein is important in cellular function. The enzyme also plays a vital role in production of sterols regulating meiosis in testis [19]. Transcriptional regulation of CYP51A1 enzymes is highly conserved in mammals [19]. The enzyme is regulated via the sterol regulatory element binding protein pathway in a cholesterol feedback loop and by a cAMP/cAMP element response modulator mechanism in germ cells [20]. Here, we sought to investigate a novel regulatory pathway of CYP51A1 via NO-dependent degradation.

EXPERIMENTAL

Materials and Reagents

Dipropyleneetriamine NONOate (DPTA) was purchased from Cayman Chemicals, Ann Arbor, MI. N^ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), cycloheximide, doxycycline and chloroquine (CQ) were from Sigma-Aldrich, St. Louis, MO. IL-1 β , tumor necrosis factor- α (TNF α) and interferon- γ (IFN γ) were obtained from R&D Systems,

Minneapolis, MN. Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) was from Boston Biochemicals (Cambridge, MA) and bortezomib was from LC laboratories (Woburn, MA). 3-methyladenine (3-MA) was from ARCOS Organics, Geel, Belgium. N-acetyl-L-leucyl-N-[(1S)-1-formylpentyl]-L-leucinamide (ALLN), Calpeptin (Cal P) and ((2S,3S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (EST, also known as E-64d) were obtained from Calbiochem, San Diego, CA. TRIzol reagent was from Zymo Research Corp., Irvine, CA.

Mouse monoclonal antibodies to the V5-peptide (catalog # V8012) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, catalog # MAB374) were purchased from Sigma-Aldrich (St. Louis, MO) and Millipore (Billerica, MA), respectively. Affinity purified anti-actin antibody (catalog # A2066) was purchased from Sigma-Aldrich. Mouse monoclonal anti-hemagglutinin (HA) antibody was from Santa Cruz Biotechnology, Dallas, TX (catalog # 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.

Lentiviral construction

Plasmids pLX304-CYP51A1V5 and pLX304-CYP2B6V5 were obtained from the Arizona State/DNASU plasmid repository, <https://dnasu.org/DNASU/Home.do>. Viruses containing pLX304-CYP51A1V5 or pLX304-CYP2B6V5 were produced in human embryonic kidney HEK293T cells using a second generation lentiviral packing system consisting of pMD2.G and psPAX2 (gifts from Didier Trono; Addgene (Cambridge, MA) plasmids # 12259 and # 12260, respectively) and a virus production protocol from Addgene (<https://www.addgene.org/tools/protocols/plko/>). Virus-containing supernatants were collected at 48h and 72h post-transfection, combined, passed through a 0.45 µm filter and stored at -80°C. We described previously the construction of the pLIX-hNOS2 virus expressing human NOS2 under control of tetracycline [21].

Cell Culture

HeLa and Huh7 hepatocarcinoma cell lines were obtained from the American Type Culture collection and the laboratory of Dr. Arash Grakoui of Emory University, respectively. Short tandem repeat analysis by the Emory Integrated Genomics Core facility was used to verify the cell identities. Huh7 and HeLa cells were cultured in 10% FBS/1% penicillin/streptomycin-Dulbecco's Modified Eagle Medium (DMEM) and treated as described in the individual figure legends.

Cells were cultured in 10% FBS in DMEM at 5% CO₂ and 37°C. For viral transduction, the frozen lentivirus was thawed at room temperature. Two µl of 4 mg/mL polybrene was added to 1mL of viral media and this was added to cells (HeLa or Huh7) at 60 to 80% confluency in 6-well plates. The plates were 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.

Human hepatocytes were obtained from the University of Pittsburgh via the NIDDK-s Liver Tissue Cell distribution system. Information about the donors is given in Supplemental Table

1. These experiments were carried out in accordance with the Declaration of Helsinki, and were designated exempt from review by the Emory University Institutional Review Board. Cells were cultured for 24 h prior to delivery in hepatocyte maintenance medium (Cambrex Bioscience, Walkersville MD). Upon receipt, cells were placed at 37°C in 5% CO₂ and medium was changed 1–2 h later to Williams E cell culture media supplemented with 10 nM insulin, 25 nM dexamethazone, and 1% penicillin/streptomycin. Media were changed every other day. After 4 days of culture in the Williams E medium, cells were treated with a mixture of cytokines (ILmix; IL-1 β (2.5 ng/ml), TNF α (2.5 ng/ml) and IFN γ (5 ng/ml)) and/or L-NAME (200 μ M) for 12 or 24 h to simulate an inflammatory event. At the end of the treatments period, media samples were removed and reserved for assay of the stable end products of NO production, nitrate + nitrite (NOx) using the Griess reaction. Total cell lysates were used for immunoblotting.

NO assay

NO production from human hepatocytes or cell lines containing the pLIX-hNOS2 virus was assessed by measuring the stable NO oxidation products nitrite+nitrate (NOx) in the culture media via the Griess reaction [22]. 50 μ L of cell culture media were incubated with 1 mM NADPH in 0.4M potassium phosphate buffer pH 7.4, 0.11 mM FAD and 1 unit of nitrate reductase for 1 hour at room temperature. Lactate dehydrogenase (Sigma-Aldrich, Catalog # L2625-50K):pyruvic acid (1:10) was added into the solution and incubated at 37°C for 30 minutes). Finally, Griess reagent (1% sulfanilamide in 5% H₃PO₄, 0.1% N-(1-naphthyl) ethylenediamine: NaNO₂ (1:1) was added to the solution and absorbance was measured at 550nm.

Real time PCR

Experiments for RNA determination were performed in twelve-well plates. Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions. Two micrograms of total RNA was used for cDNA synthesis with the High-Capacity cDNA Archive Kit (Applied Biosystems, ThermoFisher, Grand Island, NY), and real-time PCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems) using an ABI 7300 real-time PCR system. GAPDH mRNA was used as the normalization control. The primers described by Sato et al [23] were used for CYP51A1 isoform measurements by real-time PCR; the glyceraldehyde-3-phosphate dehydrogenase primers used were ATCTTCCAGGAGCGAGATCC and AGGAGGCATTGCTGATGATC. Analysis of real-time PCR was carried out by the Ct method [24].

Immunoblotting

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 P83400). Cell lysates were centrifuged at 15,000 $\times$ g for 10 min and the supernatants were collected. Protein concentration was determined with a bicinchoninic acid assay kit (ThermoFisher Scientific, Grand Island, NY). Equal amounts of protein were loaded and subjected to SDS-PAGE and Western blotting on a nitrocellulose membrane (Bio-Rad, Hercules, CA). Relative levels of native CYP51A1 in total cell lysates were measured with a siRNA knockdown-validated rabbit anti-CYP51A1 antibody (catalog #

13431-1-AP) from Proteintech Group Inc, Rosemont, IL. CYP51A1 specific monoclonal antibody (1:3,000) which was incubated with the membrane at 4°C overnight. V5-tagged CYP51A1 was detected with the anti-V5 antibody described above, at a dilution of 1:10,000. Actin (1:5,000) and/or GAPDH (1:10,000) antibodies were included in the incubations as loading controls. Membranes were incubated in IRDye-labeled anti-rabbit or anti-mouse polyclonal antibodies (1:10,000 Li-COR) at room temperature for 1 hour. For IR fluorescence detection, blots were incubated with IRDye® 680RD Goat anti-Rabbit IgG and/or 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, Lincoln, NE). Fluorescence intensity was measured using Image Studio™ software (LI-COR Biosciences). The native red/green fluorescent signals were converted to grayscale for the figures. In most experiments, both GAPDH and actin antibodies were present, and the relative CYP51A1 contents of the samples were normalized to both loading controls. In some experiments only a single loading control antibody was used, and this is noted in the figure or legend.

Ubiquitination assay

Huh7 cells expressing CYP2B6V5 were cultured in DMEM containing 10% FBS in a 5% CO₂ incubator. Plasmid pCDMA3.1-HA-Ub [25] wild type, expressing hemagglutinin (HA)-tagged ubiquitin was transfected into the cells using Lipofectamine 2000 (Invitrogen) using 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% confluent cell cultures on 6-well plates. After 24 h, cells were treated with DPTA and/or bortezomib for the indicated times. After harvesting the cells, total cell lysates were subjected to immunoprecipitation of CYP2B6V5 or CYP51A1 proteins. For CYP2B6V5 immunoprecipitation, 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. To pull down CYP51A1, 20 µl of anti-CYP51A1 antibody was added to half of the cell lysate, incubated overnight under the same conditions as CYP2B6V5, and then 75 µl of Protein A/G UltraLink Resin (Pierce, Grand Island, NY) was added to the antigen-antibody complex. The complex was incubated for 1 h at room temperature. After extensive washing of the magnetic bead mixture or resin complex, P450 proteins were 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), anti-V5 or anti-CYP51A1 at 4 °C overnight, and then with IRDye secondary antibodies as described above.

Statistical Analysis

Unless otherwise stated, data are presented as the means $\pm$ SD of several independent cell culture experiments, where n is noted in the figure legends. Statistical analyses were performed using Prism 6 (GraphPad Software Inc, La Jolla, CA) and the null hypothesis was rejected at $p < 0.05$. The post-hoc tests recommended by Prism for each experiment were used, and these are stated in each figure legend. To combine data from multiple experiments, we first calculated the ratio of the CYP51A1 signal to that of the control genes (GAPDH and/or actin) for each sample. This was then divided by the mean of the normalized values for all the samples on the blot.

For sample x , blot normalized value $v = \frac{CYP51A1x}{GAPDHx} * \frac{n}{\sum \left(\frac{CYP51A1n}{GAPDHn} \right)}$ 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. Experiments for which no statistical comparisons were performed are deemed exploratory, and in this case the error bars, if shown, are replicates from different cell culture wells within a single experiment.

RESULTS

Down-regulation of CYP51A1 by NO donors

To determine if nitric oxide could regulate CYP51A1 protein expression, we treated Huh7 cells with the nitric oxide donor DPTA, which has a NO-releasing half-life of 1–4 h in solution. DPTA treatment reduced CYP51A1 protein levels to 54% of control within 2h. The effect was essentially maximal at 38% of control after 6 h. The concentration dependence of the response to DPTA was examined at the 4h time point (Fig. 1B). Down-regulation of CYP51A1 protein by DPTA was concentration-dependent within the range 10–500 μ M, with an apparent EC50 <250 μ M. At 1000 μ M DPTA, two out of three higher mean expression at 1000 μ M than at 250 μ M.

DPTA treatment also down-regulated CYP51A1 mRNA (Fig. 2). CYP51A1 mRNA levels were 70, 65 and 57% of control at 2, 4 and 6h of treatment, respectively. This down-regulation was slower and of smaller magnitude than the changes in CYP51A1 protein at the same time points (Fig. 1A data shown in Fig. 2 for comparison). Similar results were obtained using diethylenetriamine NONOate, an NO donor with a longer half-life (data not shown).

Protein degradation of CYP51A1

To determine the contribution of protein degradation, versus reduced rate of synthesis, to CYP51A1 protein down-regulation, experiments were carried out in the presence of the protein synthesis inhibitor cycloheximide. Fig. 3 shows that DPTA rapidly down-regulated CYP51A1 protein in the absence of protein synthesis. The rate and extent of down-regulation were very similar to those seen in the absence of cycloheximide (Fig. 1A), indicating that protein degradation is the main mechanism of down-regulation. The protein levels of CYP51A1 declined only about 8% after 6h of incubation in the absence of DPTA suggesting that in the basal state CYP51A1 turns over relatively slowly.

Regulation of CYP51A1 by cellular NO

Since Huh7 cells do not produce significant amounts of nitric oxide in response to inflammatory stimuli, we engineered a HeLa cell line expressing human NOS2 under control of the tetracycline regulator (TetR) protein. As seen in Fig. 4A, treatment of the cells with doxycycline resulted in the production of NO and down-regulation of CYP51A1 protein, and both of these effects were blocked by the NOS inhibitor L-NAME. Furthermore, CYP51A1 exhibited NO-dependent down-regulation in primary human hepatocytes when stimulated by a cytokine mixture (ILm, interleukin mixture) that we

previously showed was optimum for NOS2 induction [16] (Fig. 4B). The effect of ILm was greater after 12h of treatment than at 24h, which is reflective of the modest amount of NO formed after 12 (Fig. 4B), and was reversed when production of NO was inhibited by L-NAME.

Pathways of CYP51A1 degradation

Our previous studies on CYP2B and 3A enzymes in rat hepatocytes had shown that these enzymes undergo proteasomal degradation in response to NO [9, 15]. We next studied the effect of the proteasome inhibitor bortezomib, as well as the autophagy inhibitor 3-MA, on DPTA-elicited CYP51A1 degradation. Results indicated a small but non-significant difference ($p=0.52$), between cells treated with DPTA versus cells treated with DPTA and bortezomib (Supplemental Fig. 1 A, C). There was no effect of 3-MA. Exploratory experiments conducted in parallel also showed no effect of the lysosomotropic drug chloroquine, whereas like bortezomib the proteasome inhibitor MG132 tended to partially inhibit the down-regulation (Supplemental Fig. 1 A, C). Increasing the gain on the IR fluorescence signal revealed no evidence of the accumulation of higher molecular mass (HMM) species in the presence of proteasome inhibition, which would be indicative of ubiquitinated CYP51A1 species (Supplemental Fig. 1B).

Since the effect of bortezomib in the previous experiment was not statistically significant, we designed another series of experiments with adequate statistical power to detect the effect observed, if indeed it was an effect. This experiment detected a small but significant reversal of CYP51A1 down-regulation by bortezomib (Fig. 5A). Greatly increasing the gain on the IR fluorescence signal revealed higher molecular mass (HMM) species, which might be indicative of ubiquitinated CYP51A1, but only one of these was slightly increased in cells treated with DPTA and bortezomib (Fig. 5B).

To facilitate experiments on the mechanisms of the observed regulation, we expressed CYP51A1 tagged with the bacterial V5 peptide fused to the C-terminus of the P450 protein, and characterized the responses of the resulting Huh7-CYP51A1V5 cell lines to NO donors. The labeling of second antibodies with different IR-fluorescent dyes allows simultaneous visualization of the native enzyme in the red channel and V5-tagged enzyme in the green channel, without affecting the accuracy of quantitation of either. As seen in Fig. 6A, CYP51A1V5 was down-regulated by DPTA in the presence or absence of cycloheximide, with similar time dependence to the native enzyme. If anything, both forms of CYP51A1 tended to be more downregulated in the presence of cycloheximide than in its absence. The calpain inhibitors Cal III, and to a lesser extent ALLN, tended to antagonize the down-regulation of both CYP51A1-V5 and native enzyme (Fig. 6B), whereas the native enzyme was more sensitive to bortezomib inhibition than the V5-tagged enzyme. Notably, when the gain was increased in the green channel, we observed V5-reactive CYP51A1 fragments of approximately 35, 25 and 15 kDa in the samples treated with bortezomib, which were enhanced in the cells treated with bortezomib plus DPTA (Fig. 6C). We did not observe these fragments with the native enzyme, perhaps due to the much lower specificity of the antibody.

To confirm whether or not CYP51A1 is ubiquitinated in response to NO, we transiently transfected the Huh7 cells expressing V5-tagged human CYP2B6 with hemagglutinin-tagged Ubiquitin (HA-Ub). After 24 h to allow for HA-Ub expression, cells were treated with DPTA for 2 h in the presence or absence of bortezomib. CYP51A1 was immunoprecipitated from the cell lysates using the V5 antibody, and the precipitates were subjected to Western blotting with the HA-Ub antibody. Under conditions where Ub complexes of CYP2B6-V5 (as a positive control) were clearly observed, no substantial ubiquitination of CYP51A1 was found, whereas Ub complexes of CYP2B6-V5 (as a positive control) were clearly observed (Fig. 7C). When the gain was greatly increased, faint bands corresponding to ubiquitinated CYP51A1V5 could be detected (Fig 7D).

The detection of discrete, lower molecular weight V5 immunoreactive bands in the presence of bortezomib (Fig. 6C) suggested the action of a non-processive protease. Therefore, we studied the effects of three cell-permeable calpain inhibitors Cal P, Cal III and EST on down-regulation of native CYP51A1 by DPTA. As seen in Fig 8A, each of the calpain inhibitors tended to diminish the down-regulation of CYP51A1 by DPTA. None of these effects attained the criterion of significance ($P=0.065$ for Cal III; 0.189 for Cal P; 0.91 for EST), although Cal III was very close to significance. To further investigate the roles of calpain and proteasomal degradation, we subjected cells to a combination of bortezomib and the three calpain inhibitors (calpain cocktail, CC). Results show that, consistent with previous results, bortezomib or CC alone each produced a small but nonsignificant effect on DPTA-evoked down-regulation. However, the combination of the calpain cocktail and bortezomib effectively restored CYP51A1 levels to 75% of control, a value that was significantly different from both DPTA treatment alone and control cells (Fig 8B).

DISCUSSION

Like several rat P450 enzymes [9, 15, 26] and human CYP2B6 [21], CYP51A1 protein is targeted for degradation when exposed to nitric oxide generated under inflammatory conditions by NOS2 or released from NO donor compounds. Three pieces of evidence support this conclusion: 1) the magnitude of down-regulation of the protein in response to DPTA is greater than that of the mRNA; 2) down-regulation of the protein has similar magnitude in the presence or absence of the protein synthesis inhibitor cycloheximide; 3) down-regulation of V5-tagged CYP51A1 expressed from a lentivirus controlled by a cytomegalovirus CMV promoter is almost identical to that of the native protein expressed in the same cells. Moreover, this regulation of CYP51A1 by NO was shown in three different cell types: Huh7 hepatoma cells, HeLa cervical carcinoma cells, and cultured primary human hepatocytes.

We observed a partial inhibition of the down-regulation of CYP51A1 protein by the proteasome inhibitor bortezomib in two different series of experiments, and in the second series which was sufficiently powered to detect this small effect, it was shown to be statistically significant (Fig. 5). This leads us to conclude that CYP51A1 undergoes proteasome-dependent degradation in response to NO. In studying the effect of NO on V5-tagged CYP2B6 in Huh7 cells, we have also routinely observed this partial effect of bortezomib [21]. However, CYP51A1 degradation appears to be largely independent of

ubiquitination, because increases in HMM complexes indicative of CYP51A1 ubiquitination were not observed in the presence of DPTA plus proteasome inhibition, and HA-Ub-tagged CYP51A1 was only detected at very low levels in cells transfected with HA-Ub. Ub-independent proteasomal degradation has been demonstrated before for rabbit CYP2E1 expressed in HeLa cells [27].

Since bortezomib could only partially inhibit CYP51A1 degradation, we reasoned that other proteolytic pathways must take over when the proteasome is inhibited. Therefore, we tested the autophagic inhibitor 3-MA, the lysosomotropic agent chloroquine and several calpain inhibitors for their abilities to inhibit NO-evoked CYP51A1 down-regulation. Of these, the calpain inhibitors tended to have a partial effect (Fig. 8A). Therefore, we tested these inhibitors in combination (CC) and found that the combination treatment significantly attenuated the down-regulation. Moreover the effects of CC and bortezomib were additive (Fig. 8B), suggesting that both calpain and proteasome pathways are active in CYP51A1 degradation, perhaps in concert. These results must be interpreted with caution however, because experiments with other P450s in our lab have found that inhibitors or substrates of certain P450s can inhibit their down-regulation by NO by an unknown mechanism (unpublished data). Although CYP51A1 is not a drug-metabolizing P450, it remains possible that the effect of calpain inhibitors that we observed could be due to binding to the enzyme and not calpain inhibition. However, the observation in Fig. 6C of discrete cleavage products of CYP51A1 in the presence of proteasome inhibitors and DPTA supports the idea that calpain is involved.

CYP51A1 mRNA was also found to be down-regulated after treatment of Huh7 cells with DPTA. CYP2D6 mRNA was reported to be down-regulated by NO donors in HepG2 cells [28], and CYP1A1 mRNA was down-regulated in an NO-dependent manner by a combination of cytokines in cultured rat hepatocytes [29]. The down-regulation of CYP2D6 was shown to be due to hepatocyte nuclear factor 4 by NO [28]. Although protein degradation is clearly the dominant mechanism for the initial decline in CYP51A1 activity in our experiments, the pretranslational effect may be an important contributor to decreased activity in vivo, and further studies are needed to elucidate this.

The majority of the experiments herein were performed using the NO donor DPTA. Some variability in the magnitudes of the response to the donor can be seen in different experiments. The concentrations of DPTA we found to be effective (250–500 μ M) are comparable to concentrations of NO donors necessary to evoke down-regulation of other NO-sensitive proteins [30–34]. The relevance of these results to regulation of CYP51A1 by cellular NO is further supported by our findings that inflammatory cytokines down-regulated CYP51A1 in primary cultured human hepatocytes in a NO-dependent manner, and that generation of NO in HeLa cells via a doxycycline-induced NOS2 gene also recapitulated the effect.

In conclusion, the targeted degradation of CYP51A1 by NO represents a novel mechanism of regulation of this important, highly conserved and ubiquitous enzyme. Further investigation is needed to determine the importance of this phenomenon in human inflammatory disease and its mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

ALLN	N-acetyl-L-leucyl-N-[(1S)-1-formylpentyl]-L-leucinamide
N-Cal III	[N-[(Phenylmethoxy)carbonyl]-L-valyl]-phenylalaninal
Cal P	calpeptin
CQ	chloroquine
DMEM	Dulbecco's modified Eagle Medium
DPTA	Dipropylenetriamine NONOate
EST	((2S,3S)- <i>trans</i> -Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HA	hemagglutinin
IFNγ	interferon- γ
IL	interleukin
3-MA	3-methyladenine
L-NAME	N ^ω -Nitro-L-arginine methyl ester hydrochloride
MG132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
NO	nitric oxide
NOS2	inducible nitric oxide synthase
NOx	nitrate + nitrite
P450	cytochrome P450
TNFα	tumor necrosis factor- α

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Figure 1.

Down-regulation of CYP51A1 protein by NO donors in Huh7 cells. A. Time course. Cells were treated with 500 μ M DPTA for the indicated times. Relative CYP51A1 protein levels were measured by Western blotting. The panel shows the fluoroimage from a sample experiment B. Analysis of three independent identical time course experiments. C. Concentration dependence. Cells were treated for 4 h with the indicated concentrations of DPTA. Values represent the mean $\pm$ SD of values from three independent experiments. Time course and concentration response experiments were often performed simultaneously, thus their control groups were shared. Significantly different from untreated cells ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA and Dunnet's test, $n = 3$

Figure 2. Regulation of CYP51A1 mRNA expression by an NO donor in Huh7 cells. Cells were treated with 500 μ M DPTA for the indicated times, and relative levels of cellular CYP51A1 mRNA were measured by RT-qPCR. Values represent the mean $\pm$ SD of values from three independent experiments. ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA and Dunnett's test. The protein data from Fig 1A are shown for comparison.

Figure 3.

CYP51A1 protein degradation. Huh 7 cells were treated with 20 μ g/ml of the protein synthesis inhibitor cycloheximide (CHX) for 30 minutes prior to addition of 500 μ M DPTA or vehicle. Cells were harvested after 2, 4 or 6 h of treatment and subjected to Western blot analysis. A. Western blot from a single experiment. B. Results of Western blot quantitation. Values represent the means $\pm$ SD of three independent experiments. ** $P < 0.01$; *** $P < 0.001$ versus control cells at same time point; unpaired two-tailed t-test with Holm-Sidak correction.

Figure 4.

CYP51A1 regulation by cellular NO. A. HeLa-hNOS2 cells were treated with 10 μM doxycycline with or without 300 μM L-NAME for 18 h. Media and cells were harvested for measurement of NO production (NOx) and Western blotting. The upper panel shows the Western blot from a single experiment. The lower panels are data from three independent experiments. B. Cultured primary human hepatocytes were incubated for 12 h or 24 h with a mixture of cytokines that we had previously characterized to be optimal for hNOS2 induction in these cells: ILm containing 5 ng/mL IL-1β, 10 ng/mL TNFα, and 10 ng/mL IFNγ. The upper panel shows a Western blot including both time points. The lower panels show results from 3 independent experiments at the 12 h time point only. Both experiments were analyzed by one-way ANOVA and Tukey's test. *P<0.05, **P<0.01, ***P<0.001 compared to control and #P<0.05, ##P<0.01, ###P<0.001 compared to ILm-treated group.

Figure 5.

Partial inhibition of CYP51A1 with proteasome inhibition. Huh7 cells were treated with 500 μ M DPTA for 4 h, with or without bortezomib (Bort, 10 μ M). The upper panel is from a sample experiment, the lower panel is the analysis of 4 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to DPTA treated group; ^^ $P < 0.001$ compared to bortezomib alone, one-way ANOVA and Tukey's test.

Figure 6.

Regulation of V5-tagged CYP51A1 by NO. These are exploratory experiments in which the SD presented is that of three different wells in a single cell culture experiment. Although identical experiments were not performed to allow statistical analyses, the essential findings with respect to cycloheximide and calpain and proteasomal inhibition have been replicated in at least two other experiments. A. Huh7-CYP51A1 cells were treated with 500 μ M DPTA in the presence or absence of cycloheximide (CHX) for 2 or 4 h. CHX was added to the cells 30 min before the addition of DPTA. Cell lysates were analyzed by Western blotting for expression of CYP51A1-V5 (green channel) or native CYP51A1 (red channel). B. Huh7-CYP51A1 cells were treated with DPTA in the presence or absence of bortezomib (Bort, 10 μ M), N-Acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN, 10 μ M), or Cal III (100 μ M) for 4 h. Lysates were analyzed as described in panel A. C. Same Western blot as in panel B showing only the green channel, with the gain increased to detect degradation products of CYP51A1-V5 (indicated by the *arrows*).

Figure 7.

Low levels of CYP51A1 ubiquitination in response to NO. Huh7 cells expressing CYP2B6V5 and native CYP51A1 were transfected with HA-tagged ubiquitin. 24 h later, cells were incubated for 2 h with 500 μ M DPTA and/or 10 μ M bortezomib. **A.** Western blot of three different wells in the same experiment. The panels are images of the red and green channels from the same blot. **B.** Western blot of the pooled samples for each group. **C.** Western blots of samples immunoprecipitated with anti-V5 or anti-CYP51A1 as described in the Experimental section. The immunoprecipitated samples were subjected to Western blot analysis with anti-HA antibody to detect ubiquitinated P450s, and with anti-CYP51A1 or anti-CYP2B1 to detect unmodified P450s. **D.** same blot as in C with the gain increased. C, control; D, DPTA; B, bortezomib.

Figure 8.

Role of calpains. A. Huh7 cells were treated individual calpain inhibitors Cal III (100 μ M), Cal P (50 μ M) or EST (10 μ M) for 1 hour prior to addition of 500 μ M DPTA or medium. Cells were harvested after further incubation for 4 h. Data are the mean $\pm$ SD of four independent experiments. Differences between means were determined by one way ANOVA for non-repeated measures and Sidak's multiple comparison test. *** P <0.001, compared to control group; ###, P <0.001 compared to DPTA group. B. Huh 7 cells were treated with a calpain inhibitor cocktail (CC) consisting of 100 μ M Cal III, 50 μ M of CalP, and 10 μ M EST and/or bortezomib (Bort, 10 μ M), with or without DPTA and harvested 4 h later. Differences between means were determined by one way ANOVA for non-repeated measures and Sidak's multiple comparison test. * P <0.0001 and *** P <0.0001, compared to control group; ## P <0.01 compared to DPTA group, ^^ P <0.01 compared to inhibitors alone.