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Regulation of Cytochrome P450 enzyme activity and expression by Nitric Oxide in the context of inflammatory disease

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Abstract

Many hepatic cytochrome P450 enzymes and their associated drug metabolizing activities are down-regulated in disease states, and much of this has been associated with inflammatory cytokines and their signaling pathways. One such pathway is the induction of inducible nitric oxide synthase (NOS2) and generation of nitric oxide (NO) in many tissues and cells including the liver and hepatocytes. Experiments in the 1990s demonstrated that NO could bind to and inhibit P450 enzymes, and suggested that inhibition of NOS could attenuate, and NO generation could mimic, the down-regulation by inflammatory stimuli of not only P450 catalytic activities but also of mRNA expression and protein levels of certain P450 enzymes. This review will summarize and examine the evidence that NO functionally inhibits and down-regulates P450 enzymes *in vivo* and *in vitro*, with a particular focus on the mechanisms by which these effects are achieved.

Keywords

Cytochrome P450; Nitric Oxide; Inflammation; Protein degradation; Enzyme inhibition; Gene transcription

Introduction

Regulation of P450 enzyme activity and expression in inflammation and infection.

Cytochrome P450s are responsible for the inactivation and elimination of a majority of small molecule drugs. They also detoxify, but sometimes bioactivate, environmental toxicants. In various animal models of inflammation and infection, many hepatic P450 proteins, mRNAs and activities are down-regulated (Aitken et al. 2006). Viral, bacterial and parasitic infections depress P450-dependent drug clearance in humans (Renton 2001; Morgan 2017). The clearance of antipyrine was dramatically reduced, and its half-life prolonged up to five-fold, in children with bacterial sepsis (Carcillo et al. 2003). Bacterial lipopolysaccharide

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(LPS) administered to humans in low doses caused impairment of hexobarbital, aminopyrine and theophylline clearances (Shedlofsky et al. 1994). Other clinical conditions with an inflammatory response have been associated with reduced P450-dependent drug clearance: e.g. elective surgery, congestive heart failure and cancer (Aitken et al. 2006), rheumatoid arthritis and Crohn's disease (Coutant and Hall 2018). Growth of a sarcoma in mice caused down-regulation of mouse liver CYP3A11, as well as reduced hepatic transcription of a human CYP3A4 reporter transgene (Charles et al. 2006). This was associated with secretion of interleukin-6 (IL-6) from the tumor. Inflammatory cytokines, including IL-6, IL-1 and tumor necrosis factor- α (TNF α), mimic the down-regulation of P450s in cultured rodent (Aitken et al. 2006) and human (Aitken and Morgan 2007) hepatocytes. Studies in knockout mice have suggested roles for IL-6 (Siewert et al. 2000) and IL-1 (Ashino et al. 2004) in hepatic down-regulation of P450s in inflammation caused by turpentine and tuberculosis vaccine.

Since many inflammatory stimuli that cause down-regulation of P450s in the liver also result in the production of NO in both hepatocytes and Kupffer cells (Billiar et al. 1992), the role of NO in the suppression of hepatic CYP activity and expression has been studied in many laboratories. There is evidence that NO contributes to decreases in enzyme activity, protein and/or mRNA levels of distinct P450 enzymes during inflammation.

Nitric oxide and nitric oxide synthases.

NO is a short-lived cellular messenger with important roles in the cardiovascular and nervous systems that is synthesized from arginine through the actions of three forms of nitric oxide synthase (NOS) (Bredt and Snyder 1994). NO forms an iron-nitrosyl complex with hemoproteins, and nitrosylation of soluble guanylyl cyclase (sGC) is the mechanism by which NO stimulates sGC to elevate cellular cGMP levels resulting in vascular smooth muscle relaxation (Bredt and Snyder 1994).

Inducible NOS (iNOS, NOS2) is induced in endothelial cells, stellate cells, Kupffer cells and hepatocytes in response to inflammatory stimuli (Curran et al. 1990; Marletta 1993; Bredt and Snyder 1994; Morris and Billiar 1994). Thus the hepatocyte is exposed to NO produced both internally and externally. In hepatocytes, NOS2 is induced by a combination of cytokines and LPS (Geller et al. 1993). NO causes decreased hepatocyte protein synthesis and an inhibition of mitochondrial enzymes which is partially reversible (Curran et al. 1990; Stadler et al. 1991).

Protein modifications by NO.

As the multiple functions of NO in biology were being discovered, most of the physiological effects of NO were attributed to its regulation of the activity of guanylate cyclase. However, modification of critical amino acids by reactive nitrogen species (RNS) formed from NO is now recognized as an important mode of NO signaling.

Tyrosine nitration.—Peroxynitrite, the product of the reaction between NO and superoxide anion, can nitrate tyrosine residues on proteins, resulting in the formation of 3-nitrotyrosine. It is now thought that peroxynitrite is not the major cellular nitrating species

(Radi 2004; Lancaster 2006). Rather, nitration occurs via oxidation of tyrosine to the tyrosyl radical which then combines with the nitrating radical species $\cdot\text{NO}_2$. $\cdot\text{NO}_2$ can be formed by the homolysis of peroxynitrite, oxidation of nitrite in the presence of H_2O_2 and metal ions, and by myeloperoxidase (Radi 2004; Lancaster 2006). Regardless of the mechanism, high concentrations of NO in cells result in protein nitration, although there is no consensus sequence for nitration (Abello et al. 2009). Nitration is a relatively stable modification (cf. nitrosation, below), although denitration mechanisms have been identified (Abello et al. 2009).

Using 2D-gel electrophoresis/MS, Aulak and co-workers identified more than 30 proteins nitrated in rat livers after treatment with LPS in a reversible and dynamic process (Aulak et al. 2001). Many other nitrated proteins have been identified under physiological and pathophysiological conditions by mass spectrometry (MS) (Zhan et al. 2015). The activity of Manganese superoxide dismutase is inhibited by nitration (Radi 2004; Yamakura and Kawasaki 2010); conversely, nitration has been shown to stimulate peroxidase activity of cytochrome c, enhance the clotting properties of fibrinogen, activate the ϵ isoform of protein kinase C (Radi 2004) and activate the small GTPase RhoA (Rafikov et al. 2014). Exposure of PC12 cells to peroxynitrite caused a proteasome-dependent reduction in steady state levels of tyrosine hydroxylase that correlated with tyrosine nitration of the enzyme (Souza et al. 2000).

Cysteine nitrosation.—Reversible S-nitrosation of protein thiols is an important mode of NO signal transduction (Stamler et al. 2001; Gaston et al. 2003). Nitrosation and denitrosation regulate the activities, trafficking, stability or redox sensing properties of various cellular proteins (Gould et al. 2013). Protein nitrosation by RNS can occur directly or via transnitrosation from low-molecular weight thiols such as S-nitrosoglutathione (GSNO) or even from other proteins (Smith et al. 2012; Gould et al. 2013). Denitrosation can occur via thioredoxin 1 (Trx1) catalysis, or indirectly via reduction of GSNO by glutathione oxidoreductase or carbonyl reductase 1 (Smith et al. 2012).

There are no well-defined linear sequence motifs that direct site-specific nitrosation (Doulias et al. 2010; Smith et al. 2012). pKa of the cysteine and hydrophobicity are not predictive either (Doulias et al. 2010; Smith et al. 2012). There may be a slight tendency for nitrosated residues to be localized near flexible regions of the protein (Doulias et al. 2010). Nitrosated residues tended to be on large surface accessible areas (Doulias et al. 2010; Smith et al. 2012), yet 71% of residues in the rat liver nitrosoproteome were not accessible to solvent (Doulias et al. 2010; Gould et al. 2013).

Hundreds of S-nitrosated or potentially nitrosated cellular proteins have been identified via proteomic studies (Jaffrey et al. 2001; Miersch and Mutus 2005; Lee YI et al. 2014; Ibanez-Vea et al. 2018). S-nitrosylation of proteins can regulate their functions in diverse ways; e.g. activation of thioredoxin and the Ras GTPase, inhibition of caspases and glyceraldehyde 3-phosphate dehydrogenase, inhibition of DNA-binding activity of the transcription factors NF- κ B and AP-1 (Gaston et al. 2003; Miersch and Mutus 2005).

Reversible and irreversible inhibition of P450 enzymes by NO

In vitro studies—Incubation of microsomal P450s with NO results in both reversible and irreversible inhibition (Wink et al. 1993; Kim YM et al. 1995). Reversible inhibition is ascribed to formation of the ferric heme-nitrosyl complex, whereas the irreversible component was blocked by albumin, suggesting that RNS formed from NO are involved (Wink et al., 1993). CYP2B1-associated activities were more susceptible to both modes of inhibition than were CYP1A-associated activities (Wink et al. 1993). NO derived from the NO-generating compound 3-morpholinosydnonimine (SIN-1), or from added NO, also caused a concentration-dependent inhibition of microsomal CYP2B1/2 activity *in vitro*, and this was correlated with the formation of a heme-NO adduct (Khatsenko OG et al. 1993). Gaseous NO or NO-donating compounds have also been demonstrated to inhibit CYP1A1, 1A2 and 2E1 activities (Stadler et al. 1994; Nakano et al. 1996; Gergel et al. 1997). Electron spin resonance (ESR) spectroscopy has demonstrated ferric heme-nitrosyl complexes in microsomal P450s (Gergel et al. 1997; Minamiyama et al. 1997).

Several mechanisms have been proposed to explain the irreversible inhibition. Incubation of rat liver microsomes with the NO donors NOC7 or peroxyxynitrite caused parallel reductions of P450 activities and microsomal free thiols (Minamiyama et al. 1997). It was proposed that thiol oxidation or nitrosation was responsible, but there is no direct evidence to support this mechanism. Reaction of peroxyxynitrite with tyrosine residues is causative for the inactivation of CYP8A1 (prostacyclin synthase) and CYP2B1. CYP8A1 is physiologically nitrated upon stimulation of mesangial cells with IL-1 via a heme-catalyzed reaction in which reaction of peroxyxynitrite with ferric heme generates a ferryl complex and the NO₂ radical. The ferryl species can be reduced by a nearby tyrosine, with the resulting phenoxy radical adding NO₂ to form nitrotyrosine (Zou MH et al. 2000). When incubated with peroxyxynitrite *in vitro*, CYP2B1 undergoes nitration of Y190 and Y203, and this correlates with loss of catalytic activity (Roberts et al. 1998). Mutation of Y190, but not Y203, to arginine abrogates the inactivation by peroxyxynitrite (Lin et al. 2003). Nitration of Y190 inhibits catalytic activity by disrupting a hydrogen bond with Q149, which results in a conformational change that is suboptimal for catalysis (Lin et al. 2005). Treatment of human CYP2B6 or 2E1 with peroxyxynitrite *in vitro* resulted in their nitrations on multiple tyrosine residues (Lin et al. 2007). Nitration of tyrosine 354 as well as adduction of the heme porphyrin ring were both found to contribute to the inactivation on CYP2B6 by peroxyxynitrite. As yet nitration of CYP2B1, 2B6 or 2E1 has not been detected in cells or *in vivo*.

Studies with purified bacterial P450 enzymes CYP102A1 and CYP51A1 found that NO reversibly coordinates to the ferric P450s with micromolar or sub-micromolar affinities (Quaroni et al. 2004; Ouellet et al. 2009). However, there are notable differences between the enzymes. Reduction of the ferric-NO complex of CYP102A1 resulted in the restoration of a native oxidized visible spectrum. Dissociation of NO from ferric CYP102A1 was accompanied by formation of nitrotyrosine as detected by Resonance Raman spectroscopy, and studies with the Y51F mutant suggested that this residue located in the substrate access channel is the major target (Quaroni et al. 2004). Ferrous bacterial CYP51A1 bound NO ‘almost irreversibly’, possibly due to dissociation of the heme-thiolate bond and nitrosylation of the axial thiolate (Ouellet et al. 2009). The CYP102A1 enzyme thiolate bond

was also labilized by NO, although freeze-thawing was needed to fully fracture it (Quaroni et al. 2004).

Heme dissociation from CYP enzymes may also be involved in their irreversible inhibition by NO. In freshly isolated rat hepatocytes, inhibition of NOS blocked the 65% decrease in total P450 content of the microsomes caused by incubation of the cells for 24 h with a cytokine cocktail (Kim YM et al. 1995). Total spectral P450 content was restored by dialysis of the microsomes with heme (Kim YM et al. 1995). One potential confounding factor is that these experiments were performed against a changing background of P450 expression, which declines rapidly in the first 48 h after hepatocyte isolation. The authors noted that different P450s may be differentially affected by NO. In line with that principle, NO forms a stable ferric-NO complex with mammalian CYP1A2 (Nakano et al. 1996) that is resistant to reduction in the absence of chemical reductants. The resistance to reduction was dependent on Glu 318, which may form an ionic bridge with the NO.

NO binding to P450 heme can be either inhibited or enhanced by substrates, and this can vary even in the same P450 (1A2). This may be related to the size of the ligand and its effect on rigidity of the active site (Nakano et al. 1996). In CYP101 (P450cam), substrates that increased enzyme rigidity also inhibited CO binding *via* limiting access to the heme (Jung et al. 2002).

In vivo models of inflammation and infection—Enzyme inhibition by NO (or RNS) is thought to be important for changes in P450-dependent metabolism in the early phases of inflammation. Animal studies have demonstrated that NOS inhibition attenuates or blocks reductions in clearance of P450 substrates (Kitaichi et al. 1999) or in microsomal P450-dependent enzyme activities (Khatsenko OG et al. 1993; Muller et al. 1996; Khatsenko O and Kikkawa 1997; Sewer et al. 1998; Takemura et al. 1999) caused by administration of bacterial LPS. In some cases, these effects were observed in the absence of decreases in the levels of measured P450 mRNAs and proteins. For example, in rats treated with a high dose of LPS (10 mg/kg, iv), midazolam sleeping time was prolonged 6-fold within 8h after injection, concomitant with reduced catalytic activities of CYP2C11 and 3A2. A NOS inhibitor prevented all of these effects (Takemura et al. 1999). By contrast, CYP3A2 and 2C11 protein contents were not reduced (Takemura et al. 1999). Heme-nitrosyl complexes have been demonstrated in the livers of rats treated with LPS, (Takayama et al. 1999), providing further evidence that direct inhibition by NO does occur in vivo. On the other hand, some investigators have found NO-dependent changes in P450 mRNAs and proteins in the LPS model (discussed in detail below), suggesting that at least part of the NO-dependent reductions in some P450 activities might be due to decreased enzyme levels. The importance of enzyme inhibition is likely to be greatest at earlier times before changes in enzyme expression have occurred. Dissecting the relative contributions of enzyme inhibition and enzyme down-regulation to NO-dependent changes in enzyme activity in published literature is difficult because in many cases neither the substrates nor the antibodies used are completely specific for a given mouse, rat or rabbit P450 enzyme.

Hepatic heme-nitrosyl complexes have also been detected in the livers of mice treated with killed *Corynebacterium parvum* bacteria in the presence or absence of LPS (Chamulitrat et

al. 1995). In rats treated with *C. parvum*, the clearance of vecuronium and antipyrine was reduced and this was partially reversed by NOS inhibition (Blobner et al. 1999). However, levels of P450 proteins were not measured. In a model of sterile inflammation caused by turpentine injection in rabbits, a NO-dependent reduction in the clearance of antipyrine was observed in the absence of changes in CYP1A2 (Barakat et al. 2001). Similarly, a NO-dependent reduction of antipyrine clearance in rats treated with type II Shiga-like toxin was not accompanied by changes in CYP2C11 and CYP3A2 proteins (Kitaichi et al. 2004). In a model of Type I allergy in mice, an NO scavenger blocked the observed decreases in Cyp1a2, Cyp2c, Cyp2e1 and Cyp3a activities, whereas there were no changes in Cyp protein levels (Tanino et al. 2016). On the other hand, decreases in P450-dependent activities in the livers of mice treated with polyinosinic. polycytidylic acid (a toll-like receptor 3 agonist) were not affected by NOS inhibition (Hodgson and Renton 1994).

In vivo and ex vivo studies with NO donating compounds—Several therapeutic drugs release NO (e.g. sodium nitroprusside (SNP) or S-nitrosoglutathione (GSNO)), or release NO upon metabolism (e.g. glyceryl trinitrate (GTN)). SNP is a vasodilator used for treatment of acute heart failure and hypertensive crises, whereas GTN is given sublingually for the treatment of angina. GSNO has undergone more than 20 clinical trials, mostly in cardiovascular diseases (Broniowska et al. 2013). Studies in isolated perfused rat livers demonstrated the loss of P450 heme and P450 activities within 30–60 minutes of perfusion with SNP or the NO donor isosorbide dinitrate (ISDN) (Vuppugalla and Mehvar 2004b, 2004a). Effects of NO on drug metabolizing CYPs were seen in less than one hour and varied depending on the isoform involved. CYP2B1/2 activity was one of the most sensitive. Most P450 protein levels were unchanged following these short treatments. The NO donors inhibited P450 activities at 2–4 fold lower concentrations than they decreased total P450, and to a much greater extent (80–85%), suggesting that heme dissociation was not the primary mechanism. Kinetic analyses showed that ISDN or SNP reduced the V_{max} of 2C11, 2B1/2, 3A2, 2E1 and 1A1/2 in the perfused livers. K_m s for 2B1/2 and 2D1 were unchanged, the K_m of 2C11 increased, and the K_m of 1A1/2 decreased, (Vuppugalla and Mehvar 2005). The K_m effects were hypothesized to be due to thiol modification of the enzymes, because treatment of the microsomes with dithiothreitol tended to reverse the declines in activities after 1 hr of SNP treatment, less so after 30 min.

Hodgson and Renton found no decrease in total P450 levels, or in activities associated with CYP1A or CYP2E1 in the livers of mice given four injections of either SNP (20–100 mg/kg, i.p.) or GTN (16–80 mg/kg, i.p.) every 4h (Hodgson and Renton 1994). On the other hand, continuous i.v. infusion of GTN or ISDN, but not SNP, caused a decrease in total P450 and the levels of 1A2, 2C11, 2E1 and 3A2 proteins in rat liver (Minamiyama et al. 2004). Immunohistochemical evidence suggested that CYP2B1 was greatly affected. Tolerance to GTN appears to be due to P450 down-regulation (Minamiyama et al. 2001).

Role of NO in inflammatory down-regulation of P450 mRNAs and proteins

In vivo models of inflammation and infection—Many studies have investigated the role of NO in the down-regulation of P450 mRNAs and proteins during inflammatory responses in vivo, mainly using NOS inhibitors. Similar numbers of them seem to support or

refute a role for NO in these processes, even those that studied the same P450s in the same disease model in the same species. When interpreting the effects of NOS inhibition or NOS gene deletion on the regulation of P450 enzymes in vivo, it's important to recognize that NOS inhibition could indirectly affect P450 regulation via attenuation of other inflammatory pathways. Variability in the impact of NOS inhibition on systemic inflammatory responses may partly contribute to the different results observed in different laboratories. Inflammatory pathways can regulate P450 expression via redundant mechanisms, and nullifying one such pathway (e.g. NO/NOS) might or might not affect a measured endpoint depending on the relative contributions of the other pathways.

Following their initial demonstration that inhibition of NOS attenuated LPS-evoked decreases in total P450 and CYP2B-catalyzed pentoxifyresorufin O-dealkylation in Wistar rat liver (Khatsenko OG et al. 1993), Khatsenko and co-workers reported that NOS inhibition abrogated the down-regulation of CYP2C11 mRNA and protein, as well as that of CYP3A2 protein, but not CYP3A2 mRNA (Khatsenko O and Kikkawa 1997). The decrease in CYP2B protein was partially reversed. Using a lower dose of LPS, our laboratory found no effect of NOS inhibition on the down-regulation of CYP2C11, 2E1, 3A2 mRNAs and proteins in F344 rat liver (Sewer and Morgan 1998). Even with a high dose of LPS, Takemura et al found no effect of NOS inhibition on the down-regulation of CYP2C11 or CYP3A2 mRNAs in Wistar rats (Takemura et al. 1999), suggesting that the different results among laboratories were not entirely due to the strains of rats used. However, in another model of bacterial sepsis, i.e. cecal ligation and puncture Eum et al found that down-regulation of hepatic CYP1A1, 1A2 and 2E1 mRNAs, proteins and activities in Sprague-Dawley rats were attenuated by NOS inhibitors (Eum et al. 2006).

Mice with a deletion of the *Nos2* gene offer a different tool to assess the role of NO in P450 regulation. Our laboratory found no effect of *Nos2* gene deletion on the down-regulation of Cyp2c, 2e1, Cyp3a mRNAs or proteins (Sewer et al. 1998) in the LPS model, and the same result was obtained for Cyp2b mRNAs and proteins (Li-Masters and Morgan 2002). While these results could be explained by NO formation from other NOS isoforms, the negative findings were supported by experiments using the NOS2 inhibitor aminoguanidine (Sewer et al. 1998).

Cultured cells—NO donors are important tools used to study NO-mediated effects in cultured cells. Of course this is not physiological, and the concentrations of NO donors used in most studies (100–500 μM) are well above concentrations of nitrate + nitrite (NO_x) measured in incubations of e.g. human hepatocytes with inflammatory cytokines (20–60 μM) (Nussler et al. 1992; Aitken et al. 2008). However, NO_x concentrations in cell culture media represent a huge dilution of NO_x from its sites of generation in the cells. The volume of a hepatocyte is $\sim 3 \times 10^{-9}$ mL (Duarte et al. 1989). In a well of a 6-well culture plate there are $\sim 1 \times 10^6$ hepatocytes: the total volume of these cells therefore is 3×10^{-3} mL. A measurement of 20–60 μM NO_x in 2.0 mL of cell culture medium therefore represents a 600-fold dilution. One must also consider the stability of the donor (rate of NO release), stability of NO in solution and the site of NO generation. The half-life of DPTA, the NO donor used in most of our experiments, is 1–4 h (Keefer et al. 1996), although the generally accepted value is 3h (Mooradian et al. 1995). Each molecule of DPTA releases two

molecules of NO with different rates, but the released NO is not stable. NO autooxidation kinetics are second order with respect to NO (Ford et al. 1993): at 10–20 μ M its half-life is about 2 minutes, and is even shorter at higher concentrations. Clearly, NO concentrations are much lower than the concentration of the donor at any given time.

In 1994 Stadler and colleagues reported that the down-regulation of CYP1A1 and 1A2 proteins, and of CYP1A1 mRNA by a cytokine mixture were attenuated by the NOS inhibitor N-monomethyl arginine (NMA) in cultured primary rat hepatocytes treated with the CYP1A inducer β -naphthoflavone (BNF) (Stadler et al. 1994). Although the responses were not quantified, this was the first demonstration that NO could regulate P450 mRNA and protein expression in cell cultures. In short-term cultures of primary rat hepatocytes plated on collagen, inhibitors of NOS also attenuated the suppression of CYP1A2 and CYP2C11 proteins by cytokines that induce NOS expression, and completely blocked the suppression of CYP2B and CYP3A2 proteins (Carlson and Billings 1996), suggesting a role for NO in their down-regulation. Our later work supported these findings for CYP2B (Ferrari et al. 2001) and 3A2 enzymes (Lee et al. 2009) in rat hepatocytes. However, we found that in rat hepatocytes overlaid with Matrigel the down-regulation of CYP2C11 mRNA and protein by cytokines was NO-independent (Sewer and Morgan 1997). The disparity in the findings for CYP2C11 could be explained by different culture conditions: in short-term cultures on collagen the basal expression of many P450s falls rapidly, whereas culturing on Matrigel allows the relatively stable expression of CYP2C11 (Liddle et al. 1992).

LPS treatment causes the down-regulation of CYP2B proteins and mRNAs (primarily CYP2B1) in primary rat hepatocytes. The down-regulation of the mRNAs occurs at lower LPS concentrations and is NO-independent, whereas the down-regulation of the proteins is NO-dependent and only occurs at LPS concentrations that induce NOS (Ferrari et al. 2001). Moreover, the protein down-regulation is faster than that of the mRNA (Ferrari et al. 2001), suggesting that NO causes degradation of CYP2B proteins, which was demonstrated in a subsequent publication (Lee et al. 2008). At early time points, NOS inhibition attenuates CYP2B protein down-regulation, but at later time points it does not, because at later time points the NO-independent transcriptional repression results in decreased protein levels regardless of the NO-dependent degradation mechanism (Ferrari et al. 2001). These findings illustrate the importance of choosing the right time point to elucidate mechanisms.

A proteomic analysis of primary rat hepatocytes treated with either IL-1 or IL-1 + NMA identified 229 proteins in the cell lysates, of which 3 were regulated by NO: CYP2B, CYP3A and CYP2C22 (Lee et al. 2009; Lee CM et al. 2014). Western blotting confirmed that CYP3A proteins underwent NO-dependent down-regulation at early time points when CYP3A1 mRNA, the predominant CYP3A transcript in the cells, was unaffected (Lee et al. 2009). Similarly CYP2C22, a retinoic acid hydroxylase, underwent NO-dependent protein degradation in the absence of mRNA down-regulation (Lee CM et al. 2014).

Human P450s also undergo regulation by NO. The NOS inhibitor NMA attenuated the down-regulation of CYP3A4 protein by interferon- γ (IFN γ) in primary human hepatocyte cultures. Results from 3 different human donors showed that IFN γ caused ~30% decreases

in de novo synthesis of CYP1A2 and 3A4 proteins, that were attenuated by NMA (Donato et al. 1997). Measurement of CYP3A4 mRNAs by qualitative RT-PCR suggested a similar decrease in CYP3A4 mRNA that was reversed by NMA in 2 out of three hepatocyte preparations. Together these results suggest that NO inhibits the synthesis of CYP3A4 via down-regulation of its mRNA. In human hepatocytes treated with a cytokine and LPS mixture, we found that the down-regulation of CYP3A4 was not blocked by NOS inhibitors (Aitken et al. 2008), even though the NO donor 3,3-bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC18) caused a down-regulation of CYP3A4 mRNA. These reports from different labs illustrate that the observed NO dependence may depend on the inflammatory stimulus. NO dependence in the presence of pure IFN γ (Donato et al. 1997) may be obscured by an NO-independent mechanism when a cytokine mixture (Aitken et al. 2008) is used as the stimulus. The latter study (Aitken et al. 2008) demonstrated that like rat CYP2B, human CYP2B6 protein was down-regulated (31% of control) by treatment of human hepatocytes with NOC18 and its down-regulation by the cytokine mixture was attenuated by NOS inhibitors. NOS inhibition failed to affect CYP2B6 mRNA down-regulation by the cytokine mixture.

To study the mechanisms of P450 down-regulation by NO, we established human Huh7 hepatoma and HeLa cell lines expressing CYP2B6 via lentiviral transduction. These cells recapitulated the down-regulation of CYP2B6 protein by NO donors (100–500 μ M) observed in primary human hepatocytes, in the absence of any effect on CYP2B6 mRNA (Lee et al. 2017). CYP2B6 with a C-terminal V5 peptide tag (CYP2B6V5) showed the same regulation. This occurred in the presence of cycloheximide, demonstrating that it is due to stimulated protein degradation (Lee et al. 2017). Since neither Huh7 nor HeLa cells mount a robust induction of NOS2 in response to inflammatory mediators, we also co-expressed CYP2B6V5 in HeLa cells with a tetracycline-regulated human NOS2 gene, and demonstrated that NO derived from NOS2 down-regulated the 2B6V5 protein (Lee et al. 2017). Using the same approaches, we demonstrated that native and V5-tagged human CYP51A1 (Park et al. 2017), CYP2J2V5 (Park et al. 2018) and CYP2A6V5 (Cerrone Jr et al. 2020) are each susceptible to NO-mediated degradation. CYP51A1 is a ubiquitous enzyme that catalyzes lanosterol 14 α -demethylation, an obligatory step in the biosynthesis of cholesterol and intermediate bioactive sterols (Stromstedt et al. 1996) (Rozman et al. 2002). Despite being expressed in all cells it shows tissue-dependent regulation, including responses to cAMP signaling in spermatogenesis (Rozman et al. 1999) and in the liver (Halder et al. 2002). CYP2J2 is an arachidonic acid epoxigenase with important roles in inflammation and the cardiovascular system (Karkhanis et al. 2017). It will be important to discover whether their regulation by NO in disease states contributes to disease pathology or mitigation. On the other hand, CYP3A4V5 and CYP3A5V5 were refractory to down-regulation by NO (Lee et al. 2017).

Together, the above observations provide strong evidence that both human and rat P450 enzymes display differential sensitivity to NO-stimulated degradation. Studies with NO donors are supported by data using NO inhibitors to block down-regulation in primary hepatocytes or in cells transduced with tetracycline-regulated hNOS2.

Mechanisms of P450 down-regulation by NO

Transcriptional regulation—NO activates guanylate cyclase to produce cGMP, which has a number of physiological functions. cGMP regulates the expression of genes at both transcriptional and post-transcriptional levels (Pilz and Casteel 2003). An important mode of cGMP action is regulation of gene transcription by phosphorylation. Transcription factors (TF) including cAMP response element-binding protein (CREB), Activator Protein-1 (AP-1) and Early Growth Response Protein-1 (ERG-1) are regulated in this manner. While on one hand cGMP signaling is well studied and well confirmed, S-nitrosation of proteins and not the cGMP pathway seems to be the major mechanism of transcriptional regulation by NO (Sha and Marshall 2012).

The S-nitrosation of target proteins can occur in multiple cellular compartments (Sha and Marshall 2012). NO can modulate gene expression through S-nitrosation of at least 14 TFs, including Nuclear Factor-kappaB (NF- κ B), a TF that regulates expression of genes involved in apoptosis, inflammatory response and cell adhesion, etc. (Diatchenko et al. 2005). Altered NO levels affect the activity of NF- κ B and can act on multiple steps in the signal cascade of NF- κ B by S-nitrosation (Hayden and Ghosh 2008; Sha and Marshall 2012). For example S-nitrosation of its p50 monomer inhibits NF- κ B dependent DNA binding (Matthews et al. 1996; Marshall et al. 2000). S-nitrosation by NO in other signal transduction pathways that cross-talk with NF- κ B pathway can also affect gene expression controlled by NF- κ B (Sha and Marshall 2012).

As discussed above, NO is implicated in down-regulation of multiple cytochrome P450 mRNAs and proteins in the liver. However, the general mechanism of NO transcriptional regulation of P450 genes remains unknown. Data collected in the human hepatoma HepG2 cell line suggest that hepatocyte nuclear factor (HNF) 4 α that regulates liver-specific gene expression may have an important role in suppression of P450 gene transcription by NO. NO released from an NO donor suppressed *CYP2D6* at the transcriptional level (Hara and Adachi 2002) in a guanylate cyclase-independent manner. Exogenous NO inhibited the DNA binding activity of HNF4 α in a concentration dependent manner which was shown to cause the suppression of *CYP2D6* (Hara and Adachi 2002). *CYP2D6*-mediated clearance was reported to be suppressed in HIV patients (Jones et al. 2010), but it is not known if this is a transcriptional effect or whether it is NO-mediated. Inhibition of HNF4 α by NO could have broader implications for many human P450 genes: more than 20 *CYP2* genes have the HNF4 α binding element found in the proximal promotor regions (Chen et al. 1994; Ibeanu and Goldstein 1995). HNF4 α was also identified in regulation of other major drug metabolizing genes, such as *CYP3A4*, *CYP3A5*, *CYP2A6*, *CYP2B6*, *CYP2C9*, and *CYP2D6* (Jover et al. 2001). More research is needed to determine the generality of this mechanism.

The aryl hydrocarbon receptor (AhR), a ligand-activated TF, controls various cytochrome P450 genes including *CYP1A1*, *CYP1A2*, *CYP1B1* and *CYP2S1* (Nebert et al. 1993). In mouse Hepa 1c1c7 cells, induction of Cyp1a1 and NAD(P)H-quinone oxidoreductase-1 (Nqo1) by the AhR agonist BNF was antagonized by the inflammatory agents LPS and TNF α , accompanied by the formation of significant amounts of NO and an elevation in

NOS2 mRNA expression (Gharavi and El-Kadi 2007). The suppression of Cyp1a1 and Nqo1 was partially prevented by the NOS2 inhibitor L-N6-(1-iminoethyl) lysine, consistent with an effect of NO on the AhR-mediated transcriptional regulation of these genes, as well as NO-independent effects of LPS and TNF α . More work is needed to prove that the effect of NO is transcriptional and mediated via the AhR. The suppression of human *CYP1A1* by TNF α is also regulated via redox regulation of the nuclear factor-1 (NF-1) in HepG2 cells (Morel and Barouki 1999).

Another critical NO target may be the vitamin D3 receptor (VDR). CYP3A4 is known to be induced by 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in the colon carcinoma cell line Caco-2. NO donors N-(E)-(4-Ethyl-2-[(Z)-hydroxyimino]- 5-nitro-3-hexene-1-yl)-3-pyridine carboxamide (NOR-4) or S-nitroso-N-acetyl-penicillamine, dose-dependently inhibited the induction of CYP3A4 mRNA by 1,25(OH)₂D₃ in these cells, via a guanylate cyclase-independent mechanism (Hara et al. 2000). Subsequently, Watabe et al reported that down-regulation of CYP3A4 could be antagonized by antisense oligonucleotides to the TF c-myc, suggesting that antagonism of 1,25(OH)₂D₃ induction of CYP3A4 may be due to a suppressive effect of c-myc (Watabe et al. 2003).

The synthesis of bile acids (BA) involves two distinct pathways: the classic and the alternative pathway with cholesterol-7-hydroxylase (CYP7A1) and sterol-27-hydroxylase (CYP27A1) as the first enzymatic steps, respectively. CYP7A1 is induced by dietary cholesterol via the oxysterol-activated nuclear receptor liver X receptor (LXR) and there is evidence that LXR in turn is regulated by NO. Activation of LXR could inhibit NOS2, iNOS2 expression and NO production in LPS-stimulated microglia, while nuclear translocation of NF-kappaB1 p50 was not inhibited (Secor McVoy et al. 2015). The findings in macrophage foam cells show that exposing macrophages to excess NO downregulated the LXR α -ABCA1 pathway, and this impaired the efficacy of cholesterol efflux (Zhao et al. 2013).

Protein degradation

Major proteolytic degradation systems of the cell: The main proteolytic systems in the cell are the ubiquitin (Ub)-proteasome system (UPS), and the autophagic-lysosomal pathway (ALP). About 80% of cellular proteins are degraded by the UPS (Wang J and Maldonado 2006). For P450s, the UPS pathway takes the form of endoplasmic reticulum-associated degradation (ERAD) (Kincaid and Cooper 2007; Correia et al. 2014). Several P450 proteins, including CYP2B1, undergo ubiquitination and proteasomal degradation after inactivation by a suicide inhibitor (Correia et al. 2005). Some native P450s, including rat and human CYP3As are also degraded via ERAD (Faouzi et al. 2007; Kim SM et al. 2010).

In general, proteasomal degradation regulates the turnover of short-lived proteins, while the ALP regulates degradation of organelles and long-lived proteins (Schmid and Munz 2007; Ohsumi 2014). Proteins are delivered to the lysosomes by three mechanisms. In microautophagy, the cytosol is imported into the lysosomes via invagination of the lysosomal membrane. In chaperone-mediated autophagy, targeted proteins carry signal peptide tags, and cytosolic and lysosomal HSC70 chaperones assist the LAMP-2a transporter to import the protein cargo (Schmid and Munz 2007). Macroautophagy is the

most common autophagic pathway, involving the engulfment of cytosolic constituents by a double membrane vesicle, the autophagosome (Ohsumi 2014). This fuses with lysosomes or late endosomes for degradation.

Calpains are cysteine proteases, consisting of 15 members of which the best-characterized are Capn1 and Capn2 (Ono et al. 2016). These are also known as μ and m calpains, respectively activated by micromolar and millimolar concentrations of Ca^{++} (Liu X et al. 2004; Ono et al. 2016). Calpains are found in almost every subcellular compartment including the ER and Golgi (Franco and Huttenlocher 2005). Although calpains can cleave many proteins, their roles in physiology are still not fully appreciated (Liu X et al. 2004; Ono et al. 2016). Calpains are so-called “accessory” or “modulator” proteases that perform limited proteolysis by cleaving at distinct sites on a protein.

NO-stimulated degradation of P450 proteins and the proteases involved: Table 1 summarizes the published cellular studies of NO-evoked P450 degradation, together with the effects of protease inhibitors. NO-stimulated down-regulation of CYP2C22, 2A6, 2B6, 51A1 and 2J2 (Table 1) has been demonstrated in the presence of the translational inhibitor cycloheximide, from which one can conclude that the effect is due to degradation since no protein synthesis is ongoing. Inhibition of down-regulation by an inhibitor of a specific proteolytic enzyme or pathway is also suggestive evidence that degradation is occurring, but it cannot exclude the possibility that NO is affecting enzyme synthesis and the inhibitor is affecting the basal rate of degradation.

We have found no evidence for participation of the autophagy-lysosomal pathway in NO-mediated degradation for any P450 enzymes (Table 1). There is strong evidence that NO stimulates the Ub-dependent proteasomal degradation of rat CYP2B1 (Lee et al. 2008) and CYP2B6 (Lee et al. 2017), in that NO stimulates their ubiquitination and proteasome inhibitors block or substantially inhibit their down-regulations. CYP3A1 can tentatively be assigned to the same category since its down-regulation in primary rat hepatocytes was blocked by proteasome inhibition (Lee et al. 2009). CYP51A1 (Park et al. 2017) and CYP2J2 (Park et al. 2018) degradations are only partially inhibited by proteasome inhibitors. Ubiquitination of CYP51A1 could not be detected (Park et al. 2017) and although CYP2J2 was ubiquitinated under basal conditions, its ubiquitination was not stimulated by NO (Park et al. 2018). This suggests that these two enzymes undergo Ub-independent proteasomal degradation in addition to other pathways. On the other hand, NO-stimulated accumulation of ubiquitinated CYP2A6 was detected in the presence of proteasome inhibitors (Cerrone Jr et al. 2020), clear evidence that ubiquitin-dependent proteasomal degradation of CYP2A6 is stimulated by NO. However, the inhibitors did not attenuate down-regulation of the parent CYP2A6 protein, again suggesting that in the absence of proteasomal activity the enzyme could be degraded by another pathway.

In searching for other proteases that could explain the relatively small effects of proteasome inhibitors on CYP2J2 and CYP51A1 degradation, we found that calpain inhibitors partially blocked their degradation. Degradation of CYP51A1 was inhibited by a combination of the calpain inhibitors Cal III ([N-[(phenylmethoxy) carbonyl]-L-valyl]-phenylalaninal), calpeptin and (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (EST,

also known as E64d) (Park et al. 2017), and their effect was additive with that of the proteasome inhibitor bortezomib. CYP2J2 degradation was also inhibited by the calpain inhibitors calpeptin and by Cal III (Park et al. 2018). Precedent for calpain involvement in P450 degradation was provided by Huan et al. (Huan et al. 2004), who reported that E64d partially inhibited the steady-state turnover of CYP2E1 (but not 2B1) in stably transfected HeLa cells. Perhaps significantly, the proteasomal degradation of CYP2E1 in these cells was also Ub-independent (Huan et al. 2004). However, they found no effect of calpeptin (Huan et al. 2004).

Interpretation of the calpain inhibitor results is complicated by our finding that the CYP2J2, CYP2B6 and CYP2A6 -specific enzyme inhibitors danazol, 4-chlorophenylimidazole and pilocarpine respectively blocked NO-dependent degradation of these enzymes (Park et al. 2018; Cerrone Jr et al. 2020). In the context of protease inhibition, this signifies that protease inhibitors should be tested for their ability to directly inhibit the P450 enzyme under study. Indeed, the prototypic proteasome inhibitors MG132 and epoxomicin inhibit CYP3A4 in cultured human hepatocytes, whereas the therapeutic proteasome inhibitor bortezomib does not (Lee et al. 2010). We found that Cal III, but not calpeptin inhibited catalytic activity of CYP2J2. Thus the inhibition of CYP2J2 degradation by calpeptin may be indeed be due to calpain inhibition. However, we also found that CYP2J2 degradation was not inhibited by the calcium chelator BAPTA-AM as would be expected for a calcium-dependent protease (Park et al. 2018). Therefore the involvement of calpains in CYP2J2 degradation remains to be conclusively established. In the absence of a facile assay for CYP51A1 (which routinely entails 3H labeled substrates in a reconstituted enzyme system(Cotman et al. 2004)) we do not know what effect calpain or proteasome inhibitors might have on its activity.

Unlike proteasomal and lysosomal degradation, which reduce proteins to very small peptides (Raynes et al. 2016), calpains cleave at specific sites on proteins. Additional evidence for participation of calpains in CYP51A1 degradation was provided by the detection of discrete fragments of CYP51A1V5 in cells treated with DPTA in the presence of a proteasome inhibitor (Park et al. 2017). Because we have not detected fragments of CYP51A1 (or any of the other P450s we have studied) in the absence of proteasome inhibitors, our working hypothesis is that the dominant pathway for NO-stimulated degradation of P450s is via the proteasome and that other proteases (such as calpains) become more important when the proteasome is inhibited (Fig. 1). Unlike any of the other P450 enzymes we tested, NO-dependent CYP2C22 degradation in rat hepatocytes and CYP2A6V5 in Huh7 cells was not attenuated by inhibitors of the UPS, the autophagy-lysosomal pathway or calpain inhibitors. The secondary pathways for CYP2C22 and CYP2A6 degradation remain to be determined.

Role of the immunoproteasome.: IFN γ regulates proteasomal activity in antigen-presenting cells, in part by upregulating three proteasomal subunits LMP2, LMP7 and MECL-1 (Fruh and Yang 1999) that replace the homologous catalytic subunits of the constitutive proteasome. LMP2 is thought to be responsible for the increased chymotrypsin-like activity of the ‘immunoproteasome’ seen with IFN γ treatment (Fruh and Yang 1999; Raynes et al. 2016). Since NO induces immunoproteasome subunits in bovine aortic endothelial cells (Kotamraju et al. 2006) we hypothesized that NO might cause P450 suppression via the immunoproteasome. We found that in rat hepatocytes, IL-1 induced the

immunoproteasome subunit LMP2 within 6–12 h of treatment, and suppression of CYP2B proteins by the NO donor NOC-18 was accelerated by pretreatment with IL-1 (Sun et al. 2012). However, contrary to our hypothesis, this effect of IL-1 occurred in the presence of a NO synthase inhibitor, suggesting that an NO-independent action of IL-1 contributes to the lability of CYP2B proteins. CYP2B protein degradation in response to IL-1 was attenuated by the selective LMP2 inhibitor UK-101 ((2*R*,4*S*)-2-tert-butyldimethylsiloxyethyl-4-[(*S*)-*N*-heptanoylalanyl-amino]-6-methyl-1,2-oxiranylheptane) but not by an LMP7 inhibitor (Sun et al. 2012). This work revealed CYP2Bs as novel substrates of LMP2, and suggests that induction of LMP2 may be involved in the potentiation of NO-dependent degradation by IL-1 and in the degradation of CYP2B proteins in response to inflammatory stimuli. The participation of the immunoproteasome in the degradation of other P450 enzymes remains to be determined.

Mechanisms of NO-stimulated P450 degradation

In early studies on the NO-stimulated degradation of rat CYP2B enzymes, the effects were insensitive to inhibition by cGMP-dependent protein kinase (Lee et al. 2008). Therefore, subsequent studies have focused on other mechanisms. NO and RNS derived from reaction of NO with oxygen, superoxide or carbonate can modify cysteine and tyrosine residues on proteins, thereby providing alternative mechanisms for regulating protein functions (Davis et al. 2001; Lancaster 2006). As discussed below, the reaction of peroxynitrite with P450 enzymes can result in the selective nitration of tyrosine residues, which could result in degradation. We and others have demonstrated that P450 enzymes can also undergo S-nitrosation, and there is ample evidence that this can be a trigger for degradation of other proteins. Finally, we have already discussed evidence that NO can cause dissociation of heme from P450s and the resultant conformational changes could also serve as a signal for degradation. In contemplating these mechanisms, it is important to consider that they could differ among individual enzymes, and that indeed multiple mechanisms could be ongoing within the same enzyme.

Tyrosine nitration.—Tyrosine nitration of P450 enzymes is a plausible mechanism for NO-stimulated degradation. Nitration of tyrosine causes a change in its pKa from 10.1 to 7.5 (Gow et al. 1996), which could lead to conformational effects exposing hydrophobic residues and generate a degradation signal. As described above, CYP8A1 (prostacyclin synthase) is inhibited by nanomolar concentrations of peroxynitrite, and nitration can be detected at low micromolar concentrations (Zou M et al. 1997). CYP8A1 is physiologically nitrated upon stimulation of mesangial cells with IL-1 (Zou MH et al. 1998). However, there is no evidence that this nitration causes increased degradation of the enzyme.

As noted above, rat CYP2B1 undergoes stimulated, Ub-dependent proteasomal degradation in response to NO (Lee et al. 2008). Incubation of CYP2B1 with peroxynitrite in vitro resulted in selective nitration of Y190 and 203 and enzyme inhibition (Roberts et al. 1998), later shown to be due to nitration of Y190 (Lin et al. 2003). Several years ago we presented data describing the results of experiments on the NO-dependent degradation of CYP2B1 tyrosine mutants (Lee et al. 2011). One such experiment is shown in Fig. 2, in which the Y190A mutation partially inhibited the down-regulation by the NO donor NOC18. Five such

experiments were conducted, in which three gave the results shown in Fig 1 and the other two showed only a small effect of the mutation. Several other tyrosine mutants (not shown) showed partial effects that were also variable in size, leading us to hypothesize that the degradation signal could be due to cumulative nitration of multiple residues.

Human CYP2B6 is also nitrated when exposed to peroxynitrite in vitro (Lin et al. 2007). Systematic mutation of tyrosine residues in CYP2B6V5 expressed in Huh7 cells revealed that mutations of Y190, Y317 and Y380 to alanine each caused a partial reduction in the down-regulation of the enzyme by DPTA or by NO produced by co-expressed NOS2 (Lee et al. 2020). The partial effects of these mutations led to the hypothesis that cumulative nitration of Y190, 317 and Y380 causes destabilization of the enzyme, ubiquitination and proteasomal degradation. As shown in Fig. 3, these residues are all located on the surface of the enzyme, exposed to solvent. None of them were reported to be nitrated upon treatment of CYP2B6 with peroxynitrite in vitro (Lin et al. 2007). Thus, nitration sites identified by incubation with peroxynitrite might be different from those nitrated physiologically. The effect of Y190A on CYP2B6 degradation is consistent with the observations for CYP2B1 in Fig. 2.

We have been unable to detect NO-stimulated CYP2B nitration in cells. While it is relatively easy to measure protein nitration and assess its impact on protein function in vitro, it is much more difficult to detect nitration under physiological conditions. Nitrotyrosine groups are in very low abundance in the in vivo proteome (Zhan et al. 2015). Nitrated P450 enzymes were not detected in microsomes (Minamiyama et al. 1997) or isolated perfused rat liver treated with NO donors (Vuppugalla and Mehvar 2004b, 2004a).

The residues identified in CYP2B6 as important for down-regulation are not consistently represented in NO-sensitive P450s (Fig. 4). Thus, while Y190 is present in the NO-sensitive enzymes CYP2J2, 2B1, 2B6 and 2C22, it is absent in the sensitive CYP51A1 and present in the insensitive CYP2C11. Y317 in CYP2B6 is present in all the sensitive enzymes except CYP51A1, and absent in the insensitive CYP2C11. Y380 is present in all 6 enzymes. Thus it is likely that the residues involved differ to some extent among the sensitive enzymes.

S-nitrosation.—It has been proposed that CYP inhibition and/or down-regulation by NO may be caused by S-nitrosylation of one or more cysteine residues (Minamiyama et al. 1997; Vuppugalla and Mehvar 2004b). S-nitrosation regulates the degradation of a number of cellular proteins, including iron regulatory protein-2 (C178) (Kim S et al. 2004); liver kinase B1 (C430) (Liu Z et al. 2015); phosphodiesterase-5 (C220) (Wang Y et al. 2015); peroxisome proliferator-activated receptor- α (PPAR α)(C168) (Yin et al. 2015); p35 (C92) (Zhang et al. 2015); and topoisomerase-1 (C7) (Sharma et al. 2015). S-nitrosation of P450 enzymes is therefore an attractive hypothesis to explain NO-mediated degradation. We used the biotin-switch method to detect nitrosation of CYP2B1 (Lee et al. 2008) and CYP2J2 (Park et al. 2018) upon exposure to NO donors in vitro. However, we have yet to demonstrate that NO increases the nitrosation of these enzymes in cells. This could indicate that S-nitrosation is not the trigger for NO-stimulated degradation, or it could be explained by a rapid de-nitrosylation of the enzymes in cells. Experiments with cysteine mutants

should be informative for this question, but mutation of the surface-exposed C180 in CYP2B6 did not attenuate its down-regulation (Lee et al. 2020).

Cysteine oxidation.—Protein thiol oxidation results in the reversible formation of a sulfenic acid (S-OH), and further irreversible oxidation to the sulfinic or sulfonic acids can occur. Biologically relevant cysteine thiol oxidations by RNS include the inactivation of tyrosine hydroxylase (Kuhn et al. 1999), inactivation of glutathione reductase via formation of a sulfenic acid on Cys 63, and the inactivation of cathepsin K due to irreversible oxidation of the active site Cys 25 by GSNO (Claiborne et al. 2001).

Human CYP4A11 undergoes reversible sulfenylation of the heme thiolate after treatment with H₂O₂ in vitro, concomitant with enzyme inhibition (Albertolle et al. 2017). Reduction of the native enzyme stimulated activity 3-fold, suggesting that this redox regulation might have a physiologic role. In a subsequent study, CYP2C8, CYP2D6 and CYP3A4 were each shown to be sensitive to oxidation by H₂O₂, with the oxidation of CYP3A4 being irreversible (Albertolle et al. 2018). At least for CYP2C22, it appears that this is not the mechanism for NO-directed degradation, since treatment of cells with H₂O₂ did not stimulate the degradation of that enzyme (Lee CM et al. 2014).

Heme nitrosylation and dissociation.—Mutation of no single amino acid residue of CYP2B1 or CYP2B6 could abolish the down-regulation of that enzyme by NO. As discussed above, this could be explained if the signal for degradation was accumulation of nitrative and/or nitrosative modification of the enzyme on multiple residues. Another possibility is that heme nitrosylation, or subsequent heme dissociation is also required for degradation. Evidence was presented (above) that NO can stimulate heme dissociation from at least some P450 enzymes in cultured hepatocytes (Kim YM et al. 1995). We have found that reversible inhibitors of CYP2J2 (danazol (Park et al. 2018)), CYP2A6 (pilocarpine (Cerrone Jr et al. 2020)) and CYP2B6 (4-chlorophenylimidazole (Lee et al. 2020)) inhibit their NO-stimulated degradation, suggesting that NO needs access to the heme or at least the active site to trigger degradation. However, interpretation is not straightforward, as formation of the ferric nitrosyl complex can be inhibited or enhanced by substrates, even in the same enzyme (Nakano et al. 1996). Ligand binding is known to cause significant changes in the conformation of P450 enzymes, especially CYP2B enzymes (Scott et al. 2004). Thus, the impact of ligand binding on NO-mediated degradation could limit the access of RNS to crucial tyrosine and/or cysteine residues. This is unlikely to be true for CYP2B6, in which the identified tyrosines are solvent exposed.

Notably, experiments with the mechanism-based inhibitor of CYP2A6, methoxsalen, revealed that preincubation with this inhibitor failed to block NO-sensitive down-regulation of the enzyme (Cerrone Jr et al. 2020). This demonstrates that at least for CYP2A6, the inactivation mechanism does not require a catalytically active enzyme.

Conclusions

Nitric oxide inhibits the catalytic activities of P450 enzymes via heme nitrosylation in an enzyme-specific manner, and both reversible and irreversible inhibition occur. In the context

of an inflammatory response, the main impact of these effects is likely to be early in the response before the changes in gene expression are manifest. For some enzymes, tyrosine nitration may cause or contribute to irreversible inhibition. NO can also reduce P450 expression via down-regulation of specific mRNAs and via stimulated degradation of selected isoforms. NO can directly repress transcription of CYP2D6 via inhibition of HNF4 α , and more work is needed to test for NO regulation of other P450 genes through HNF4 α and other nuclear receptors such as VDR and LXR, as well as via the AhR. NO also selectively stimulates the degradation of multiple P450 enzymes in hepatocytes. Both ubiquitin-dependent and -independent proteasomal degradation have been found for different enzymes. The failure of proteasome inhibitors to block NO-evoked degradation of some enzymes is hypothesized to be due to the use of alternative degradation pathways when the proteasome is inhibited. For CYP2B6, three key solvent-exposed tyrosine residues have been identified that, when mutated, attenuate its degradation. For P450s tested so far, reversible ligands of the enzymes block their NO-dependent degradation. These observations led to a working model in which both heme nitrosylation and cumulative tyrosine nitration are necessary triggers for ubiquitination and proteasomal degradation.

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Figure 1.

Multiple pathways of NO-stimulated protein degradation. Under normal conditions, NO-evoked protein degradation proceeds via the proteasome, via either Ub-dependent or Ub-independent mechanisms. Other pathways may participate but are relatively minor. Under experimental conditions of proteasome inhibition, some P450s enzymes are degraded by other proteases to a greater or lesser extent depending on the enzyme.

Figure 2.

Role of Y190 in down-regulation of CYP2B1 by NO. HeLa cells were transfected with a plasmid encoding WT CYP2B1 or the Y190A mutant using Lipofectamine 2000. After 24h of transfection, cells were treated with or without 500 μ M NOC18 for an additional 24 h and CYP2B1 protein levels were assessed by Western blotting. Each lane corresponds to a triplicate cell culture well within the same experiment. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 3. Positions of the mutated tyrosine residues on CYP2B6. Shown from above the heme (left) and from a side view (right). Reprinted with permission from (Lee et al. 2020).

CYP				
51A1	209	-----C--LHGKEIRS-----Q---		218
2J2	189	SNIICSITFGERFEYQDSWFQQLLK		213
2B1	176	ANIICSIVFGERFDYTDRQFLRLLE		200
2B6	176	ANIICSIVFGKRFHYQDQEFLKMLN		200
2C22	175	CNVISSVIFQHRFDYSDEKFQKFIE		199
2C11	175	RNVICSIIFQNRFDYKDPTFLNLMH		199
51A1	329	RDKTLOKKCYLEQKTVCGENLPPLT		353
2J2	329	LYPEIQEKVQAEIDRVIGQGQQPST		353
2B1	316	KYPHVAEKVQKEIDQVIGSHRLPTL		340
2B6	316	KYPHVAERVYREIEQVIGPHRPPPEL		340
2C22	314	KYPEVTAKIQEEITRVIGRHRRPCM		338
2C11	315	KHVDVTAKVQEEIERVIGRNRSPCM		339
51A1	388	QTVAGYTIPPGHQVCVSPTVNQRLK		412
2J2	388	TTLAGYHLPKGTMITNLTLALHRDP		412
2B1	375	TMFRGYLLPKNTEVYPILSSALHDP		399
2B6	375	TSFRGYIIPKDTEVFLILSTALHDP		434
2C22	373	VEFRGYHIPKGTSMACLTALHDD		432
2C11	374	IKFRNYFIPKGTNVIVSLSSILHDD		433

Figure 4.

Alignment of tyrosine residues in CYP2B6 that were identified to be important in NO-mediated down-regulation (Lee et al. 2020).

Table 1.

Cellular studies of NO-stimulated P450 protein degradation

Enzyme (CYP)	Cells	Down-regulated by NO?	Cycloheximide chase	Effect of protease inhibitors			NO-stimulated ubiquitination?	Reference
				Proteasome	ALP pathway	Calpains		
2B1	PRH	Yes	ND	Partial	Slight	ND	ND	(Lee et al. 2008)
Myc-2B1	HeLa	Yes	ND	Complete	ND	ND	Yes	(Lee et al. 2008)
2C11	PRH	No	ND	NA	NA	NA	NA	(Sewer and Morgan 1997)
2C22	PRH	Yes	Yes	None	None	None	ND	(Lee CM et al. 2014)
2C22 *	Huh7	Yes	ND	None	None	None	ND	(Lee CM et al. 2014)
3A1	PRH	Yes	ND	Complete	ND	ND	ND	(Lee et al. 2009)
2A6V5 *	HuH7	Yes	Yes	None	None	None	Yes	(Cerrone Jr et al. 2020)
2B6	PHH	Yes	ND	ND	ND	ND	ND	(Aitken et al. 2008)
2B6 *	HeLa	Yes	ND	Partial	None	None	ND	(Lee et al. 2017)
2B6V5 *	Huh7	Yes	Yes	Partial	None	None	Yes	(Lee et al. 2017)
3A4V5 *	HeLa	No	ND	NA	NA	NA	NA	(Lee et al. 2017)
3A5V5 *	HeLa	No	ND	NA	NA	NA	NA	(Lee et al. 2017)
51A1 *	Huh7	Yes	Yes	Partial	None	Partial	Trace levels	(Park et al. 2017)
51A1V5 *	Huh7	Yes	Yes	None	ND	Partial	ND	(Park et al. 2017)
2J2 *	Huh7	Yes	ND	ND	ND	ND	ND	(Park et al. 2018)
2J2V5 *	Huh7	Yes	Yes	Partial	None	Partial	No	(Park et al. 2018)

* lentivirally-transduced cells. Abbreviations: PHH, primary human hepatocytes; PRH, primary rat hepatocytes; NA, not applicable; ND, not determined. V5 indicates enzyme tagged on the c-terminus with the bacterial V5 peptide.