SHORT-TERM INHIBITORY EFFECTS OF NITRIC OXIDE ON CYTOCHROME P450-MEDIATED DRUG METABOLISM: TIME DEPENDENCY AND REVERSIBILITY PROFILES IN ISOLATED PERFUSED RAT LIVERS

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**Running Title:** Reversibility of Nitric Oxide-Mediated CYP450 Inhibition

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**ABBREVIATIONS:** P450, cytochrome P450; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; IPRL, isolated perfused rat liver; SNP, sodium nitroprusside; NO₃, nitrate/nitrite; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DTT, dithiotreitol; V_{max}, maximum velocity of metabolism; K_{m}, Michaelis-Menten constant.
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ABSTRACT

Nitric oxide (NO) is implicated as a mediator in the decreased catalytic activities of P450 enzymes during inflammation or infection. Here, we examined the time course and the reversibility of the NO effect on P450s using isolated perfused rat livers. Livers were perfused at a constant rate with the NO donor sodium nitroprusside (SNP) for 0.5 or 1 h, followed by washout periods of 0-2.5 h. At the end of perfusion, microsomes were prepared and analyzed for P450 activities and other metabolic markers. Whereas 0.5 h of NO exposure caused an irreversible decline (~30%) in total P450 content, a greater decline after 1 h of NO (~55%) was mostly (~30%) reversible, a pattern identical to that observed for the microsomal heme content. NO exposure also caused an enzyme-selective and time-dependent decline in P450 activities. Whereas the pattern of decline and reversibility of activities were qualitatively similar for CYP3A2, 2C11, 2E1, and 1A1/2, they differed for 2B1/2 and 2D1 in that the decline in the activity was delayed (1 h) for 2B1/2 and not observed for 2D1. This may be attributed to the accessibility of heme or cysteine thiolate and/or the presence/reactivity of critical cysteinyl amino acid residues in various P450 enzymes. Additionally, for most enzymes, the activity showed a biphasic decline, one within 1 h of SNP perfusion and another after 2 h of washout. This was associated with an identical biphasic decline in the microsomal free thiols, presumably due to the rapid and slow reaction of NO and peroxynitrite, respectively, with critical P450 thiols. The short-term effects of NO on P450 are time-dependent, enzyme-selective, with both reversible and irreversible mechanisms.
The cytochrome P450 (P450) superfamily is a group of closely related enzymes that catalyze the biotransformation of a variety of xenobiotic and endogenous compounds. The major P450 enzymes that are primarily involved in xenobiotic biotransformation are grouped into the CYP1, CYP2, and CYP3 families (Porter and Coon, 1991). It has been demonstrated repeatedly that the activation of the host-defense mechanisms during immune response results in the inhibition of P450-mediated drug biotransformation in both humans and animals (Morgan, 1997). Furthermore, it is now widely recognized that infection with bacteria or treatment with cytokines or lipopolysaccharides (LPS) attenuates P450 enzymes by a mechanism that is partly mediated by nitric oxide (NO) (Khatsenko et al., 1993), although some controversies still exist (Li-Masters and Morgan, 2002).

Nitric oxide is a short lived, readily diffusible free radical secreted by several cell types that are known to play important bioregulatory roles in diverse physiological processes, like regulation of vascular tone, platelet aggregation, neurotransmission, and host-defense mechanisms (Moncada and Higgs, 1991; Nathan, 1992). These physiological actions of NO are based on its ability to modulate the activities of different cellular enzymes by binding to either the heme or non-heme iron complexes (Henry et al., 1991). The P450 enzymes in the liver also contain heme and, therefore, are one of the targets for NO (Morgan et al., 2001).

Several probable mechanisms have been put forth to explain NO-induced attenuation of P450. These include liberation of heme from P450 proteins (Kim et al., 1995), inhibition of P450 protein synthesis (Stadler et al., 1994; Khatsenko and Kikkawa, 1997), NO reaction with superoxide to form peroxynitrite that effects nitration of critical amino acid residues (Roberts et al., 1998), and/or binding of NO to the heme moiety with subsequent oxidation of free thiols in P450 enzymes (Minamiyama et al., 1997). Thus, all the aforementioned mechanisms are
presumed to operate either independently or in concert and are thought to serve as the basis for the ability of NO to inhibit P450 enzymes.

Earlier studies on the alterations of P450 activities have mainly focused on studying the relatively long-term and indirect effects of NO on P450 by either employing nitric oxide synthase (NOS) inhibitors and/or by inducing the NOS expression with immunostimulants like LPS or cytokines (Khatsenko and Kikkawa, 1997; Takemura et al., 1999). However, less emphasis has been placed on elucidating the short-term (< 3 h), direct effects of NO on P450 and its reversibility kinetics. Although, there are a few reports investigating the reversibility of NO effect on P450, the experiments have either used microsomes or purified enzyme systems instead of an intact liver (Wink et al., 1993; Minamiyama et al., 1997). Use of microsomal or purified enzyme systems impose an inherent limitation, as they lack the structural and functional integrity that exists in an intact cell and the crosstalk that exists among different cells (hepatocytes, Kupffer, and endothelial cells) in an intact liver. Also, it is known that the mechanisms by which NO inhibits P450 enzymes may vary with the experimental conditions and the cellular or subcellular components under study (Kim et al., 1995; Minamiyama et al., 1997; Nakajima et al., 2002). Moreover, NO delivered continuously over the entire period of the study is known to best mimic its biological mode of release (Feelisch, 1998). Therefore, investigating the effects of NO on P450 directly in an intact liver under the steady state condition is necessary for elucidating the reversibility and selectivity of the NO action on P450 enzymes.

Very recently (Vuppugalla and Mehvar, 2004), we showed that the effects of NO on the down-regulation of P450 catalytic activity are rapid, concentration-dependent, and enzyme-selective in an isolated perfused rat liver (IPRL) model. Both NO donors, sodium nitroprusside (SNP) and isosorbide dinitrate decreased the activities of various P450 enzymes in 1 h,
indicating that the decline is due to the generation of NO and not to possible nonspecific effects of these drugs. In the present study, we investigated the time courses and mechanisms of NO-mediated P450 inhibition and its degree of reversibility using one of these NO donors (SNP) in an IPRL model. The results of this work provide evidence indicating that the direct as well as reversible/irreversible effects of NO are dependent on both the length of exposure of the tissue to NO and the type of enzyme studied.

**Materials and Methods**

**Chemicals and Reagents.** The following reagents were purchased from Sigma-Aldrich (St. Louis, MO): Sodium nitroprusside (SNP), sodium dithionite, cytochrome c, NADPH, NADP+, potassium ferricyanide, 5,5’-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent), magnesium chloride, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, chlorzoxazone, 6-hydroxy chlorzoxazone, dextromethorphan, methoxy morphinan, dextrorphan, resorufin, cortexolone, umbelliferone, isocitrate, isocitrate dehydrogenase, horse serum, anti-rabbit goat alkaline phosphate conjugate, and anti-goat rabbit alkaline phosphate conjugates. K-Gold (p-nitrophenol phosphate substrate) was obtained from Neogen (Lexington, KY). Benzyloxyresorufin and ethoxyresorufin were purchased from Molecular Probes (Eugene, OR). Testosterone, 6β-hydroxy testosterone, and 16α-hydroxy testosterone were purchased from Steraloids (Wilton, NH). Rabbit antirat primary antibodies for CYP3A2 and 2C11 were purchased from Chemicon International (Temecula, CA) and Affinity Bioreagents (Golden, CO), respectively. Goat antirat primary antibody for CYP2E1, rabbit antirat primary antibody for CYP1A1/2, and rat supersomes for CYP1A2, 3A2, 2C11, and 2E1 were purchased from BD
Gentest (Woburn, MA). All the other reagents were of analytical grade and obtained from commercial sources.

**Animals.** Male Sprague-Dawley rats weighing 230-320 g were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All animals were maintained on a 12-h light/dark cycle with free access to food and water. The procedures involving animals were approved by the Texas Tech Health Sciences Center Animal Care and Use Committee and were consistent with the guidelines set by the National Institute of Health (publication no. 85-23, revised 1985, Bethesda, MD).

**Isolation and Perfusion of Livers.** Liver isolation and portal vein, hepatic vein, and bile duct cannulation were carried out according to the methods reported by us before (Mehvar and Zhang, 2002). In brief, rats were anesthetized with ketamine:xylazine (80:12 mg/kg body wt), and after incision of the abdominal wall, the bile duct was cannulated with PE-10 tubing. Next, both the suprahepatic vena cava and portal vein were cannulated and the infrahepatic vena cava was ligated. Livers were then immediately mounted on a water-jacketed (37˚C), all-glass perfusion system (Radnoti Glass Technology Inc., Monrovia, CA) and perfused with Krebs-Henseleit Bicarbonate buffer containing 75 mg/l sodium taurocholate and 1.2 g/l glucose. The perfusate was oxygenated with a 95% oxygen and 5% carbon dioxide mixture, and the perfusion pressure was constantly monitored using a pressure transducer. Livers were perfused in a single-pass manner, at a flow rate of 30 ml/min and were allowed to stabilize for ~10 min before the initiation of the experiments.

**Experimental Protocol.** An outline of the study design is depicted in Scheme 1. A total of 40 livers were divided into three broad groups consisting of livers perfused with a constant inlet concentration (200 µM) of NO donor SNP for 0 (Control), 0.5 (SNP0.5), or 1.0 (SNP1.0) h.
The livers in the SNP_{0.5} group were then further subdivided into 3 groups with 0 (SNP_{0.5}^{0.0}), 0.5 (SNP_{0.5}^{0.5}), or 2.5 (SNP_{0.5}^{2.5}) h of washout perfusion in the absence of SNP. Similarly, the SNP_{1.0} group was also divided into 3 subgroups with 0 (SNP_{1.0}^{0.0}), 1.0 (SNP_{1.0}^{1.0}), or 2.0 (SNP_{1.0}^{2.0}) h of washout perfusion. Additionally, control livers with perfusion times of 0.5, 1.0, 2.0, or 3.0 h were used to match the corresponding total (SNP plus washout) perfusion times in the SNP_{0.5} and SNP_{1.0} groups (Scheme 1). This design resulted in a total of 10 groups (Scheme 1) with each group consisting of 4 livers. For each group, periodical samples (5-30 min intervals) were taken from the outlet perfusate. Because SNP degrades on exposure to light (Baaske et al., 1981), the perfusion experiments were carried out under the safelight (Delta 1/CPM, Inc. Dallas, TX). The collected samples were then wrapped in aluminum foil and stored at –80°C for further analysis of nitrate/nitrite (NOx) levels. Extra samples were collected from the outlet at the beginning and end of the perfusion for the analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme levels. Additionally, bile samples were collected in preweighed microcentrifuge tubes at 15-min intervals for assessment of bile flow rates.

The concentration of SNP (200 µM) was selected based on our recent study (Vuppugalla and Mehvar, 2004), indicating that it will result in NOx concentrations of ~10 µM in the outlet perfusate. Nitric oxide is produced in large quantities during the immune response in various diseases, resulting in plasma NOx concentrations as high as 250 µM in liver transplant patients (Ioannidis et al., 1995; Zanaro et al., 2001) or >100 µM in septic patients (Ochoa et al., 1991). Similarly, in endotoxemic animals, plasma NOx concentrations as high as 800 µM have been reported (Sewer and Morgan, 1998; Li-Masters and Morgan, 2002). Therefore, the expected concentrations of NOx in the outlet perfusate in our study (~10 µM) are likely to be easily achieved and exceeded in the presence of pathophysiologic production of NO.
Outlet Sample Analysis. The concentrations of NO\textsubscript{x} in the perfusate were measured using a commercially available kit (Active Motif, Carlsbad, CA) that utilizes Griess reagent. The perfusate transaminase enzyme levels were measured using commercially available spectrophotometric kits from Sigma-Aldrich (procedure no. 505).

Microsomal Preparation, Total Heme, Cytochrome P450 and Cytochrome b\textsubscript{5} Content, and Cytochrome c Reductase Activity. At the end of perfusion, livers were blotted dry and subjected to the preparation of microsomes using well-established differential centrifugation methods (Lake, 1987) and stored at -80°C for further analysis. Total heme, cytochrome b\textsubscript{5}, and cytochrome P450 contents were determined based on the method of Omura and Sato (Omura and Sato, 1964). Bradford assay (Bradford, 1976) was employed for the measurement of total protein content of the microsomes. Activity of cytochrome c reductase was measured spectrophotometrically at 550 nm as described previously (Guengerich, 1994).

Free Thiol and Nitrotyrosine Levels in Microsomes. Levels of free thiols in the microsomes were measured using the method that utilizes Ellman’s reagent (Minamiyama et al., 1997). Nitrotyrosine levels were quantified using a commercially available ELISA kit (OxisResearch, Portland, OR). The detection limit of the assay was 2 nM, equivalent to 0.1 pmol nitrotyrosine/nmol P450.

Spectral Evidence for the Nitrosyl-Heme Complex. NO binding to the heme of 450 was detected using a spectrophotometric method described earlier (Gergel et al., 1997). Briefly, the microsomes (1 mg/ml) obtained from the SNP\textsubscript{0.0}, SNP\textsubscript{0.5} and SNP\textsubscript{1.0} livers were scanned between 400 to 500 nm using a Hitachi dual beam spectrophotometer and the characteristic absorbance maximum at 437 nm, attributed to the NO-heme adduct, was recorded. As a positive control, the
control microsomes (1 mg/ml) were incubated with 100 µM of SNP for 5 min and the spectrum from 400 to 500 was recorded.

**Measurement of P450 Enzyme Activities and Protein Contents.** Activities of CYP3A2 and 2C11 were analyzed by HPLC using the formation of 6β- and 16α-hydroxytestosterone, respectively, from testosterone, based on our modification (Vuppugalla and Mehvar, 2004) of a published method (Purdon and Lehman-McKeeman, 1997).

Chlorzoxazone and dextromethorphan were used as substrates for the measurement of CYP2E1 and 2D1 activities, respectively. The details of the microsomal incubation and HPLC analyses of the drugs and metabolites have been described by us before (Vuppugalla and Mehvar, 2004).

The dealkylations of ethoxyresorufin and benzyloxyresorufin were used as measures of the activities of CYP1A1/2 and 2B1/2, respectively. In both cases the formation of resorufin was monitored fluorometrically (Burke et al., 1985; Rutten et al., 1992).

A previously reported ELISA method (Snawder and Lipscomb, 2000) was used for the determination of the protein contents of CYP2E1, 3A2, 2C11, and 1A1/2. Microsomal proteins used were 0.15 µg for CYP2E1, 2C11, and 1A1/2 and 1 µg for CYP3A2 analysis. The concentrations of enzymes in the samples were determined against calibration standards, which were constructed using their respective supersomes. Enzyme protein values were expressed as pmol per milligram protein.

**Determination of the Role of Thiols in the NO Effects.** To examine the potential role of NO-oxidized thiols on the spectral levels of P450 and cytochrome b5 and on the catalytic activities of CYP3A2, 2C11, 2E1, 1A1/2, and 2B1/2, 10 mM dithiotreitol (DTT) (Minamiyama et al., 1997) was added to the microsomes (4 mg/ml) obtained from the $SNP_{0.5}^{0.0}$ and $SNP_{1.0}^{0.0}$
livers. After incubation for 30 min, the spectral levels of P450 and cytochrome b5 and activities of various enzymes were determined as described above.

**Data Analysis.** All the levels and activities related to P450-mediated metabolism in the SNP$_{0.5}$ and SNP$_{1.0}$ groups were corrected for the corresponding values in the *Control* livers perfused for the similar length of time (Scheme 1) and expressed as %Control values. The statistical comparisons were carried out using ANOVA with subsequent Fisher’s test. A paired *t* test was used for comparing the levels and activities of microsomes treated with and without DTT. The relationships between the enzyme activity and biochemical markers were determined using regression analysis. In all cases, a *p* value of < 0.05 was considered significant. Data are presented as mean ± S.E.M.

**Results**

**NO$_x$ and Hepatic Enzyme Levels and Bile Flow Rates.** The outlet perfusate concentrations of NO$_x$ at the end of SNP perfusion (200 µM) for 0.5 (SNP$_{0.5}$) and 1.0 (SNP$_{1.0}$) h were 9.1 ± 1.1 and 10 ± 1 µM, respectively. In all groups, the concentrations of NO$_x$ in the outlet perfusate reached steady state within the first 10 min of infusion with 200 µM of SNP and remained relatively constant during the entire period of SNP infusion (data not shown). However, the NO$_x$ outlet concentrations during the washout periods were below the level of detection.

The outlet levels of ALT and AST, which were used as markers of liver viability, were low (<30 U/l) during the entire period of perfusion. Additionally, bile flow rates ranged from 0.552 to 0.772 ml/h in different groups.
Effect of NO on Total P450 Content. The profiles of total P450 contents measured after isolation of microsomes from the SNP$_{0.5}$ and SNP$_{1.0}$ groups with various washout periods are presented in Fig. 1. Perfusion of the livers with SNP for 0.5 h without any washout (SNP$_{0.5}$) resulted in a significant decrease in the total P450 content by ~30% ($p < 0.001$) (Fig. 1, top). Moreover, total P450 levels continued to remain significantly ($p < 0.01$) low even after 0.5 (~30%) and 2.5 (~20%) h of washout (Fig. 1, top). The perfusion of the livers with SNP for 1 h (SNP$_{1.0}$) (Fig. 1, bottom) resulted in an even more dramatic decrease of 67% in the total P450 content ($p < 0.001$). However, in contrast to the SNP$_{0.5}$ groups, most of this decline was reversed ($p < 0.001$) in the 1 (19% decline) and 2 (16% decline) h washout groups (Fig. 1, bottom). Nevertheless, similar to the SNP$_{0.5}$ groups, the P450 levels in the 1- and 2-h washout groups were still significantly ($p < 0.05$) lower than their respective controls (Fig. 1).

In addition to the peak at 450 nm, we also observed a minor peak at 420 nm, representing cytochrome P420. However, the significant reductions in the spectral P450 in SNP-treated livers (Fig. 1) were not associated with a significant increase in the P420 peak (data not shown).

Effect of NO on P450 Enzyme Activities. The activities of CYP3A2 measured in the microsomes obtained from livers in the SNP$_{0.5}$ and SNP$_{1.0}$ groups are reported in Fig. 2, top and bottom panels, respectively. The activity of CYP3A2 in the SNP$_{0.5}$ and SNP$_{1.0}$ groups, which are subjected to no washout period, decreased ($p < 0.0001$) by 54% and 60%, respectively. However, the activities were partially reversible when the livers were subjected to varying periods of washout in both groups (Fig. 2). Unexpectedly, the activities observed in the microsomes of the SNP$_{1.0}$ livers were significantly ($p < 0.05$) lower than those for the SNP$_{1.0}$ group (Fig. 2, bottom), despite longer washout time (2 h) for the former.
Profiles of CYP2C11 activities are reported in Fig. 3 for the SNP_{0.5} (top) and SNP_{1.0} (bottom) groups. Similar decreases were observed in the 2C11 microsomal activities obtained from the SNP_{0.5}^{0.0} (55%) and SNP_{1.0}^{0.0} (56%) groups (Fig. 3). In the SNP_{1.0} groups, 1 h of washout caused a partial reversal of the activity ($p < 0.05$). However, similar to CYP3A2 (Fig. 2), the 2-h washout resulted in a further decline in the activity ($p < 0.01$), when compared with the 1-h washout group. Indeed the 2C11 activities of SNP_{1.0}^{0.0} and SNP_{1.0}^{2.0} groups were similar (Fig. 3, bottom). Qualitatively similar patterns were observed for the SNP_{0.5} groups (Fig. 3, top). However, the differences between the washout groups did not reach statistical significance.

Perfusion of the livers with SNP for 0.5 and 1 h also caused significant decreases in the activities of both 2E1 (Fig. 4) and 1A1/2 (Fig. 5); percentages of reduction in the activities of 2E1 and 1A1/2 in the 0.5-h infused groups (SNP_{0.5}^{0.0}) were 66% and 62%, respectively (Figs. 4 and 5, top). Moreover, the percentages of reduction in the 1-h infused groups (SNP_{1.0}^{0.0}) were 75% and 60%, respectively (Figs. 4 and 5, bottom). For both 2E1 (Fig. 4) and 1A1/2 (Fig. 5), the activities remained significantly low in all the washout groups showing little or no sign of recovery.

The patterns of the inhibitory effects of SNP on the activities of 2B1/2 (Fig. 6) and 2D1 (Fig. 7) were quite different from those observed with the other P450 enzymes discussed above (Fig. 2-5). The effect of NO on CYP2B1/2 activity seemed to be delayed, as no inhibition was noticed upon the infusion of SNP for 0.5 h (Fig. 6, top). However, a significant ($p < 0.001$) inhibition of activity (50%) was observed in the 0.5 h (SNP_{0.5}^{0.5}) washout group, which partially reversed back ($p < 0.05$) after 2.5 h (SNP_{0.5}^{2.5}) of washout (Fig. 6, top). On the other hand, perfusion of the livers with SNP for 1 h (SNP_{1.0}^{0.0}) resulted in a significantly decreased activity of CYP2B1/2 by 67% (Fig. 6, bottom). Although the activity reversed back to control values in the
1-h washout group, it significantly ($p < 0.05$) dropped down again (by 30%) in the 2-h washout group (Fig. 6, bottom). As for 2D1, SNP infusion for either 0.5 or 1.0 h did not have any significant impact on its activity (Fig. 7).

Effect of NO on P450 Enzyme Protein Levels. The protein levels of various P450 enzymes are presented in Table 1. Unlike their activities, protein levels of all the studied enzymes, except 2C11, remained unaffected (Table 1). For 2C11, protein levels declined by 32% only in the $\text{SNP}_{1.0}^{2.0}$ group ($p < 0.01$).

Effect of NO on Microsomal Thiol, Total Heme, Nitrotyrosine and Cytochrome b$_5$ Levels and Cytochrome c Reductase Activity. Perfusion of livers with SNP for 0.5 ($\text{SNP}_{0.5}^{0.0}$) and 1 ($\text{SNP}_{1.0}^{0.0}$) h resulted in modest, but significant ($p < 0.05$ and 0.0001), decreases (~10 and 21%, respectively) in the levels of microsomal thiols (Table 2). The levels continued to remain low in all the washout groups except for the $\text{SNP}_{1.0}^{0.0}$ livers (Table 2).

Infusion of SNP also caused significant reductions in the levels of total heme in the $\text{SNP}_{0.5}^{0.0}$ (20%) and $\text{SNP}_{1.0}^{0.0}$ (40%) groups (Table 2). The pattern of reversal (in the $\text{SNP}_{1.0}^{1.0}$ washout groups) of, or lack thereof (in the $\text{SNP}_{0.5}^{1.0}$ washout groups), total heme (Table 2) paralleled those for the total P450 contents (Fig. 1).

Unlike the microsomal thiol and heme levels, the levels of cytochrome b$_5$ declined (by 36%) only in the group of livers infused by SNP for 1 h ($\text{SNP}_{1.0}^{0.0}$). However, the levels recovered in the washout groups (Table 2). Cytochrome c reductase activities remained unchanged in all of our experimental groups (Table 2). Furthermore, nitrotyrosine levels, as determined by ELISA, were below the level of detection of our assay, which was 2 nM.

Effect of Reduction of Thiols on Total P450 and Cytochrome b$_5$ Levels and Enzyme Activities. Dithiotreitol (DTT) has been shown to reduce the oxidized thiols (Minamiyama et al.,...
The effects of thiol reduction on the spectral levels of P450 and activities of various P450 enzymes in the $SNP_{0.5}$ and $SNP_{1.0}$ groups are presented in Fig. 8. In the $SNP_{1.0}$ group, the activities increased significantly ($p < 0.01$) for 3A2 (49%), 2C11 (42%), and 2B1/2 (39%); although showing similar trends, DTT did not cause any significant changes ($p > 0.05$) in the activities of 2E1 and 1A1/2 (Fig. 8, bottom). The increase in activities was associated with a significant ($p < 0.001$) increase (39%) in the total P450 contents in the $SNP_{1.0}$ group (Fig. 8, bottom). Similar observations were made for the $SNP_{0.5}$ group (Fig. 8, top). However, because the activity of 2B1/2 was already close to 100%, DTT did not change the activity of this enzyme in this group (Fig. 8, top). Additionally, the 11% increase in the P450 contents in this group did not reach statistical significance ($p > 0.05$) (Fig. 8, top).

In contrast to enzyme activities and total P450 content, DTT did not have any significant influence on the cytochrome bs levels (data not shown).

Spectral Evidence for the Nitrosyl-Heme Complex. This study was conducted to determine the contribution of ferric iron-NO complexes to the NO-induced reduction in the spectral P450 content, observed in our IPRL studies (Fig. 1). In vitro addition of SNP (100 µM) to the non-reduced microsomes (obtained from control livers) resulted in the appearance of a peak at 437 nm (attributed to the heme-NO adduct), serving as a positive control (Fig. 9). However, no characteristic peak was observed in the microsomal samples obtained from the $SNP_{0.5}$ and $SNP_{1.0}$ livers (Fig. 9), indicating the absence of iron-NO complex.

Relationship Between Enzyme Activities or P450 Contents and Biochemical Markers. There were significant relationships ($p < 0.05$) between the activities of CYP3A2, 2C11, 2E1, or 2B1/2 and the microsomal levels of thiol for the $SNP_{1.0}$ group, with the strongest relationship observed for CYP2C11 and 2E1 (Fig. 10). Although a similar trend was also
observed for CYP1A1/2, the relationship did not reach statistical significance (Fig. 10). As expected, however, there was no relationship between the CYP2D1 activity and thiol levels (Fig. 10). Further, there was a significant ($p < 0.001$) relationship ($r^2: 0.89$) between the P450 contents (Fig. 1) and microsomal heme levels (Table 2) for the SNP1.0 group (data not shown). Because the variations in both thiol and heme levels in the SNP0.5 group were low ($< 9\%$) (Table 2), respective relationships for this group did not reach statistical significance in most cases (data not shown).

**Discussion**

The data presented here demonstrate that the length of exposure of the liver to NO may drastically alter the pattern of decline and/or reversibility of the P450 contents (Fig. 1) and enzyme activities (Fig. 2-6). For instance, although the relatively modest effect of NO on the total P450 content in the SNP0.5 group (~30% decline) was mostly irreversible (Fig. 1, top), the more intense decline in the SNP1.0 group (~55%) consisted of an additional rapidly reversible component (~30%) (Fig. 1, bottom).

Various mechanisms have been proposed for the decline in the P450 content after NO exposure. One of these mechanisms, which is very rapidly reversible (Wink et al., 1993), is the formation of nitrosyl-heme complexes which can prevent binding of CO to heme and hence hinder its spectral detection. However, our inability to detect the ferric-NO complex (Fig. 9) indicates that this mechanism is not responsible for the reductions in the microsomal P450 content observed in our studies (Fig. 1). This observation, however, does not exclude the possibility of formation of such a complex in the liver and its subsequent dissociation during the preparation of microsomes. Indeed, mere washing and resuspending NO-exposed microsomes are reported to reduce the peak at 437 nm (Gergel et al., 1997).
Another mechanism for the NO-induced decrease in P450 is the loss and degradation of the protein-bound heme (Kim et al., 1995). In our study, both the percentages of the P450 content and its pattern of decline (Fig. 1) were very similar to those for the microsomal heme (Table 2); in addition to an irreversible component for both SNP0.5 and SNP1.0 groups, a rapidly reversible component of total heme decline was observed only in the SNP1.0 group (Table 2), a pattern that mimicked the P450 content data (Fig. 1). This data suggest that both the reversible and irreversible declines in spectral P450 observed in our study (Fig. 1) are related to heme loss (Table 2).

The question then arises why the decline in the spectral P450, and heme, is mostly reversible only after 1 h, and not after 0.5 h, of SNP perfusion. The answer to this question is not immediately apparent from our data. However, a mechanism based on the reported (Kim et al., 1995) capability of heme to reconstitute with P450 apoprotein may be involved, at least in part, in this observation. After 0.5 h of SNP perfusion, the relatively small amounts of heme lost (~20%, Table 2) may be degraded by the low, baseline levels of heme oxygenase in the hepatocytes (Immenschuh et al., 2003), resulting in an irreversible loss of spectral P450. On the other hand, perfusion of the liver with SNP for 1 h results in a more substantial loss of heme (~40%, Table 2), causing only a partial degradation of the lost heme by the low, saturable, baseline levels of heme oxygenase. The free heme-induced feedback mechanisms for induction of heme oxygenase take much longer than our ≤ 3 perfusion period (Kim et al., 1995). Consequently, the undegraded heme remains available for reconstitution with the apoprotein during the washout period, thus accounting for the reversible component in the SNP1.0 group (Fig. 1). This postulate, however, remains to be proven.
Alternatively, the irreversible decline in the spectral P450 may be due to the loss/degradation of heme, whereas the reversible component observed in the $SNP_{1.0}^{0.0}$ group may be because of the reversible binding of NO to the heme moiety of P450 via the cysteine thiolate-modification pathway (Nakajima et al., 2002). This is in agreement with the DTT-mediated partial recovery of the spectral P450 contents in the $SNP_{1.0}^{0.0}$, and not $SNP_{0.5}^{0.0}$, groups (Fig. 8). The lack of the reversible pathway in the $SNP_{0.5}^{0.0}$ group may be because the reaction of NO with the cysteine thiolate requires longer than 0.5 h. In agreement with our results, Nakajima et al. (Nakajima et al., 2002) showed that the treatment of NO-exposed microsomes with NaBH₄, a reducing agent, returned the P450 levels to normal.

In addition to its effect on heme (Table 2) and total P450 content (Fig. 1), NO may exert its effects selectively on the individual enzyme activities through modification of the apoprotein. These modifications include nitrosylation of critical amino acids (Minamiyama et al., 1997; Roberts et al., 1998) and/or a decrease in the levels of individual enzyme proteins (Stadler et al., 1994; Khatsenko and Kikkawa, 1997; Ferrari et al., 2001). Each P450 enzyme may be affected selectively by one or more of these pathways, depending on the presence and reactivity of critical cysteinyl or tyrosine amino acid residues or the susceptibility of the protein levels to modifications by NO. Furthermore, a decline in cytochrome b₅ contents may selectively affect the activities of some, but not all, P450 enzymes (Yamazaki et al., 2002).

In addition to the reaction of NO with the heme-bound cysteine thiolate discussed above, NO may react with cysteinyl residues of the apoprotein, resulting in a reduction in the free thiol levels. In our study, NO effects on thiol levels showed different patterns for the $SNP_{0.5}$ and $SNP_{1.0}$ groups (Table 2). In the $SNP_{0.5}$ groups, the maximum reduction in the thiols was observed after 0.5 h of washout in the $SNP_{0.5}^{0.5}$ group, whereas the maximum reduction for the $SNP_{1.0}$
groups was observed at the end of the 1.0 h perfusion of SNP (Table 2), suggesting that the reaction of NO with thiols is relatively slow. The thiol profiles may explain, at least in part, why the significant decline in the activity of 2B1/2 occurs only after 0.5 h of washout in the SNP0.5 group, whereas the maximum decline for the SNP1.0 group is observed at the end of 1-h perfusion (Fig. 6). Therefore, in addition to other factors, nitrosylation of thiols in 2B1/2 enzyme may have a critical impact on its activity. Indeed, thiol levels had a significant effect on the activities of most of the studied enzymes (Fig. 10).

The thiol profiles (Table 2) may also explain, at least in part, the unexpected further decline in the activity of the 2-h washout group (SNP2.0) following partial recovery of the activities in the 1-h washout group (SNP1.0) observed for most enzymes only in the SNP1.0 group (Figs. 2-6). The recovery and further decline of activities in the SNP1.0 and SNP2.0 groups, respectively, were accompanied by a total recovery and a significant decline in the thiol levels (Table 2 and Fig. 10). The rapid (observed at the end of 1-h SNP perfusion) and delayed (observed after 2-h of washout) phases of reduction in free thiol levels (Table 2) may be attributed, respectively, to the interaction of intact NO and its metabolite peroxynitrite with P450 thiols. This postulate is in agreement with the reported (Minamiyama et al., 1997) 1000-fold faster reaction of thiols with NO as opposed to that with peroxynitrite.

The role of thiols in the inhibitory effects of NO is further confirmed by the partial recovery of the activity of the studied P450 enzymes in microsomes incubated in vitro with DTT (Fig. 8). The accompanied partial recovery in the P450 content in the SNP0.0 group (Fig. 8) suggests that the recovery of enzyme activities following incubation with DTT in this group is mostly due to the recovery of the cysteine thiolate.
In contrast to the thiol levels and in agreement with previous reports (Minamiyama et al., 1997; Vuppugalla and Mehvar, 2004), no detectable amounts of nitrotyrosine were found in our relatively short-term study. This observation suggests that this mechanism does not play a significant role in the results obtained in our study.

It has been suggested that NO is partly responsible for the reductions in the mRNA and/or protein levels of various P450s observed after relatively long-term, in vivo studies in which iNOS is stimulated by LPS (Khatsenko and Kikkawa, 1997). Our IPRL studies lasted only for \( \leq 3 \) h, reducing the likelihood of protein changes due to transcriptional events. However, modification of P450s by NO could potentially accelerate their degradation. Indeed, our protein data showed a significant decline (~30%) in the levels of 2C11, which was observed only 3 h after the start of SNP infusion in the \( SNP_{1.0}^{2.0} \) group (Table 1). The decrease in 2C11 protein has most likely contributed to the decline in its activity in the \( SNP_{1.0}^{2.0} \) group, following a partial recovery in the \( SNP_{1.0}^{1.0} \) livers (Fig. 3).

Despite an overall reduction in the spectral P450 content (Fig. 1), the activity of CYP2D1 was not affected by NO in our experiments (Fig. 7). This suggests that the reduction in spectral P450 levels may not be attributed equally to all P450 enzymes. Others (Gergel et al., 1997) have also suggested that because of differences in the accessibility of heme, and possibly cysteine thiolate, the sensitivity to NO differs among different P450 enzymes. Therefore, it is likely that cysteine thiolate and/or heme in CYP2D1 is not easily accessible to NO. Alternatively, the differences between the effects of NO on CYP2D1 and other enzymes may be related to the NO effects on the \( V_{\text{max}} \) and/or \( K_{\text{m}} \) values. If NO simultaneously reduces both the \( V_{\text{max}} \) and \( K_{\text{m}} \) values of 2D1, it is possible that at certain substrate concentrations, the rate of metabolism remains the same in treated and control livers. Nevertheless, more extensive studies, including kinetic
DMD #1487

analysis, are needed to unequivocally determine the reason(s) for the apparent enzyme-selective effects of NO.

Conclusions

In conclusion, the exposure of a physiologically-preserved intact liver to exogenous nitric oxide causes a very rapid decline in the P450 activity within 30 min. Multiple mechanisms with different time courses contribute to this reduction. Although some of these mechanisms are reversible within a short period of time, others are not. Isolated perfused rat liver may be a suitable model to study the short-term effects of nitric oxide on P450 enzymes.
DMD #1487

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DMD #1487


LEGENDS FOR FIGURES

SCHEME 1. The experimental protocol for isolated perfused rat livers.

Bullets indicate groups of livers included in the study.

FIG. 1. Microsomal contents of cytochrome P450.

Microsomes were prepared from livers perfused with 200 µM SNP for 0.5 (top panel) or 1 (bottom panel) h and subjected to various washout periods (n = 4/group). The average microsomal P450 contents of control livers perfused for 0.5, 1, 2, and 3 h were 0.88, 0.78, 0.69, and 0.54 nmol/mg protein, respectively. The cytochrome P450 content in each group is presented as % of its respective control value. *, **, and ***, Significant differences (p < 0.05, < 0.01, and < 0.001, respectively, ANOVA) between treated and respective control livers. †††, Significant differences (p < 0.001, ANOVA) between no washout (0 h) and washout groups. Columns and bars represent the average and S.E.M. values, respectively.

FIG. 2. Microsomal activities of CYP3A2.

Microsomes were prepared from livers perfused with 200 µM SNP for 0.5 (top panel) or 1 (bottom panel) h and subjected to various washout periods (n = 4/group). The average microsomal activities of control livers perfused for 0.5, 1, 2, and 3 h were 6.6, 4.5, 4.4, and 2.9 nmol/mg protein/min, respectively. The activity in each group is presented as % of its respective control value. ** and ***, Significant differences (p < 0.01 and < 0.001, respectively, ANOVA) between treated and respective control livers. † and †††, Significant differences (p < 0.05 and < 0.001, respectively, ANOVA) between no washout (0 h) and washout groups. #, Significant difference (p < 0.05, ANOVA) between the 1- and 2-h washout groups. Columns and bars represent the average and S.E.M. values, respectively.
FIG. 3. Microsomal activities of CYP2C11.

Microsomes were prepared from livers perfused with 200 µM SNP for 0.5 (top panel) or 1 (bottom panel) h and subjected to various washout periods (n = 4/group). The average microsomal activities of control livers perfused for 0.5, 1, 2, and 3 h were 3.2, 2.1, 1.8, and 1.7 nmol/mg protein/min, respectively. The activity in each group is presented as % of its respective control value. ** and ***, Significant differences (p < 0.01 and < 0.001, respectively, ANOVA) between treated and respective control livers. † and #, Significant differences (p < 0.05, ANOVA) between 0 and 1-h and 1- and 2-h washout groups, respectively. Columns and bars represent the average and S.E.M. values, respectively.

FIG. 4. Microsomal activities of CYP2E1.

Microsomes were prepared from livers perfused with 200 µM SNP for 0.5 (top panel) or 1 (bottom panel) h and subjected to various washout periods (n = 4/group). The average microsomal activities of control livers perfused for 0.5, 1, 2, and 3 h were 1.3, 1, 0.7, and 0.6 nmol/mg protein/min, respectively. The activity in each group is presented as % of its respective control value. ** and ***, Significant differences (p < 0.01 and < 0.001, respectively, ANOVA) between treated and respective control livers. Columns and bars represent the average and S.E.M. values, respectively.

FIG. 5. Microsomal activities of CYP1A1/2.

Microsomes were prepared from livers perfused with 200 µM SNP for 0.5 (top panel) or 1 (bottom panel) h and subjected to various washout periods (n = 4/group). The average microsomal activities of control livers perfused for 0.5, 1, 2, and 3 h were 110, 120, 110, and 60 pmol/mg protein/min, respectively. The activity in each group is presented as % of its respective
control value. * and **, Significant differences ($p < 0.05$ and $< 0.01$, respectively, ANOVA) between treated and respective control livers. Columns and bars represent the average and S.E.M. values, respectively.


Microsomes were prepared from livers perfused with 200 µM SNP for 0.5 (top panel) or 1 (bottom panel) h and subjected to various washout periods ($n = 4$/group). The average microsomal activities of control livers perfused with 0.5, 1, 2, and 3 h were 39, 59, 42, and 30 pmol/mg protein/min, respectively. The activity in each group is presented as % of its respective control value. * and ***, Significant differences ($p < 0.05$ and $< 0.001$, respectively, ANOVA) between treated and respective control livers. †, ††, and †††, Significant differences ($p < 0.05$, $< 0.01$, and $< 0.001$, respectively, ANOVA) between no washout (0 h) and washout groups. #, Significant difference ($p < 0.05$, ANOVA) between 0.5 and 2.5 h washout groups. Columns and bars represent the average and S.E.M. values, respectively.

FIG. 7. Microsomal activities of CYP2D1.

Microsomes were prepared from livers perfused with 200 µM SNP for 0.5 (top panel) or 1 (bottom panel) h and subjected to various washout periods ($n = 4$/group). The average microsomal activities of control livers perfused for 0.5, 1, 2, and 3 h were 0.33, 0.35, 0.36, and 0.33 nmol/mg protein/min, respectively. The activity in each group is presented as % of its respective control value. Columns and bars represent the average and S.E.M. values, respectively.
FIG. 8. *Effect of thiol reduction with DTT on the recovery of P450 content and enzyme activities*

Microsomes were prepared from livers perfused with 200 μM SNP for 0.5 (top panel) or 1 (bottom panel) h with no washout period (n = 4/group). P450 content and various enzyme activities were then determined in the presence and absence of DTT. *, ** and ***, Significant differences (p < 0.05, < 0.01, and < 0.001, respectively, paired t-test) in the content or activities between the No-DTT and DTT-treated groups. The activities are expressed as % of their respective controls (without SNP). Columns and bars represent the average and S.E.M. values, respectively.

FIG. 9. *Absorbance spectra of P450-NO complexes.*

A: microsomal P450-NO complex after addition of 100 μM of SNP to non-reduced microsomes obtained from control (untreated) livers; B: P450-NO complex was absent in the microsomes obtained from rat livers perfused for 0.5 or 1 h, with 200 μM of SNP.

FIG. 10. *The relationship between enzyme activities and microsomal levels of thiols.*

Microsomes were prepared from livers perfused with 200 μM SNP for 1 h and subjected to 0.0, 1.0, or 2.0 h of washout (n = 4/group). The data from all the washout groups were used for the regression analysis. Symbols, solid lines, and dashed lines represent experimental points, regression lines, and 95% confidence intervals, respectively.
The microsomal protein levels of P450 isoforms

Microsomes were prepared from livers perfused with 200 µM of sodium nitroprusside (SNP) for 0.5 or 1 h and subjected to various periods of washout. Values are expressed as mean ± S.E.M. (n = 4/group). The protein levels (pmol/mg microsomal protein) for control livers perfused for 0.5, 1.0, 2.0, and 3.0 h were 390, 440, 290, and 280 (2C11); 31, 29, 29, and 18 (3A2); 580, 560, 460, and 370 (2E1); and 260, 250, 230, and 180 (1A1/2), respectively.

<table>
<thead>
<tr>
<th>P450’s</th>
<th>SNP Perfusion Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 h</td>
</tr>
<tr>
<td></td>
<td>Washout Period</td>
</tr>
<tr>
<td>0 h</td>
<td>0.5 h</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>92 ± 15</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>86 ± 8</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>CYP1A1/2</td>
<td>86 ± 10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from its respective control: p < 0.01, ANOVA, followed by Fisher’s test.

<sup>b</sup> Significant difference between 1- and 2-h washout groups: p < 0.01, ANOVA, followed by Fisher’s test.
TABLE 2
Microsomal levels of thiols, total heme, and cytochrome b5 and the activities of cytochrome c reductase

Microsomes were prepared from livers perfused with 200 µM of sodium nitroprusside (SNP) for 0.5 or 1 h and subjected to various periods of washout. Values are expressed as mean ± S.E.M. (n = 4/group). The control values in livers perfused for 0.5, 1.0, 2.0, and 3.0 h were 81, 87, 70, and 66 nmol/mg protein (thiols); 1.5, 1.6, 1.3, and 1.1 nmol/mg protein (total heme); 0.47, 0.59, 0.38, and 0.31 nmol/mg protein (cytochrome b5); and 280, 300, 260, and 240 nmol/mg protein/min (cytochrome c reductase), respectively.

<table>
<thead>
<tr>
<th>Marker</th>
<th>SNP Perfusion Time</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Washout Period</td>
</tr>
<tr>
<td></td>
<td>0.5 h</td>
</tr>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>% Control</td>
<td></td>
</tr>
<tr>
<td>Thiols</td>
<td>90 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total heme</td>
<td>80 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Cytochrome b5</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>CYC Reductase</td>
<td>110 ± 1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from its respective control: p < 0.05, ANOVA, followed by Fisher’s test.

<sup>b</sup> Significantly different from its respective control: p < 0.01, ANOVA, followed by Fisher’s test.

<sup>c</sup> Significantly different from its respective control: p < 0.001, ANOVA, followed by Fisher’s test.

<sup>d</sup> Significant difference between 0 and 1-h washout groups: p < 0.001, ANOVA, followed by Fisher’s test.

<sup>e</sup> Significant difference between 1- and 2-h washout groups: p < 0.001, ANOVA, followed by Fisher’s test.

<sup>f</sup> Significant difference between 0 and 2-h washout groups: p < 0.001, ANOVA, followed by Fisher’s test.

<sup>g</sup> Significant difference between 0 and 2-h washout groups: p < 0.01, ANOVA, followed by Fisher’s test.
Scheme 1
**FIG. 1**

The figure shows the effect of SNP on P450 content (% Control) over different washout periods. Two graphs are presented:

- **0.5-h SNP**
  - The graph depicts P450 content over a 2.5-hour washout period, with control and SNP conditions.
  - The bars for SNP at 0.0, 0.5, and 2.5 hours show significant differences compared to control.

- **1.0-h SNP**
  - The graph shows P450 content over a 2-hour washout period, with significant differences noted.

The asterisks (**, ***, *, †††) indicate statistical significance between control and SNP conditions.
FIG. 2
**FIG. 3**

CYP2C11 Activity (% Control)

**0.5-h SNP**

0.0 0.5 2.5

Control SNP

0 30 60 90 120

**0.5-h SNP**

0.0 0.5 2.5

**1.0-h SNP**

0 1 2

Washout Period (h)

***, **, †

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FIG. 4
FIG. 5

CYP1A1/2 Activity (% Control)

0.5-h SNP

<table>
<thead>
<tr>
<th>Washout Period (h)</th>
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<th>SNP</th>
</tr>
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<tbody>
<tr>
<td>0.0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
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1.0-h SNP

<table>
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<tr>
<th>Washout Period (h)</th>
<th>Control</th>
<th>SNP</th>
</tr>
</thead>
<tbody>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant difference from control
**Highly significant difference from control
FIG. 6

CYP2B1/2 Activity (% Control)

0.5-h SNP

Control

SNP

Washout Period (h)

0.0 0.5 2.5

0 30 60 90 120

***,†††

**

†, #

1.0-h SNP

Washout Period (h)

0 1 2

0 30 60 90 120

***

††

*,†
FIG. 7
FIG. 8
FIG. 9
FIG. 10

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