0022-3565/04/3102-718–727$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS Vol. 310, No. 2
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65557/1156929
JPET 310:718–727, 2004 *Printed in U.S.A.*

Hepatic Disposition and Effects of Nitric Oxide Donors: Rapid and Concentration-Dependent Reduction in the Cytochrome P450-Mediated Drug Metabolism in Isolated Perfused Rat Livers

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Received January 18, 2004; accepted March 26, 2004

ABSTRACT

Various mechanisms, including high levels of cytokines and nitric oxide (NO), have been proposed as mediators for inflammation-induced cytochrome 450 down-regulation. However, the contribution of each of these mediators to the observed effects is controversial. We used an isolated perfused rat liver (IPRL) model to test the direct effects of NO donors on CYP450 down-regulation in the absence of cytokines or other confounding in vivo factors. Our hypothesis was that NO rapidly and concentration-dependently decreases CYP450 activities in IPRL. Livers were perfused (60 min) with 50 to 500 μM sodium nitroprusside (SNP) or 100 to 500 μM isosorbide dinitrate (ISDN) as NO donors, and the perfusate and biliary disposition of SNP, ISDN, and generated nitrate/nitrite (NO_x) were determined. Additionally, at the end of perfusion, catalytic activities and protein levels of various cytochrome isoenzymes were measured. Both SNP and ISDN exhibited linear hepatic disposition with extraction ratios of ~0.30 and 0.50, respectively. Furthermore, although in small amounts, both NO donors and NO_x were found in the bile. Except for CYP2D1, the catalytic activities of all the studied isoenzymes were substantially (up to 85%) decreased by both NO donors. However, the apoprotein levels of isoenzymes remained largely unchanged. Additionally, the inhibitory effects of NO donors were concentration-dependent, with the concentrations of SNP producing one-half of maximum inhibition being in the order of 2C11 > 2B1/2 > 2E1 = 3A2 > 1A1/2. These studies indicate that the effects of NO on the down-regulation of cytochrome 450 catalytic activity are rapid, concentration-dependent, and isoenzyme-selective.

Cytochrome P450 (P450) microsomal monooxygenases are a major class of heme-containing proteins that catalyze the biotransformation of many endogenous and exogenous compounds. Many studies have shown that infection and inflammation reduce the metabolism of drugs (Morgan, 1997). To date, several proinflammatory mediators, such as cytokines and nitric oxide (NO), have been shown to contribute to this down-regulation. A large body of data shows that the cytokines secreted from the immune cells have the capacity to lower the P450 content of the liver (Morgan, 1997). Also, the use of null mice for certain cytokines or cytokine receptors has resulted in abolishment or modification of P450 response to inflammatory stimuli, supporting an important role for cytokines (Warren et al., 1999).

Inflammation is also known to induce nitric-oxide synthase (iNOS) that results in the production of NO in hepatocytes, macrophages, and endothelial cells (Nathan, 1992). Because NO reacts strongly with hemoproteins, it was proposed that NO would alter the P450 activities as well as expression (Khatsenko et al., 1993; Wink et al., 1993; Khatsenko and Kikkawa, 1997). Indeed, various studies using whole animals (Khatsenko and Kikkawa, 1997), hepatocytes (Stadler et al., 1994), or microsomes (Wink et al., 1993; Minamiyama et al., 1997) have implicated NO in the down-regulation of P450-mediated drug metabolism. Conversely, Morgan and coworkers (Sewer and Morgan, 1997, 1998) reported that NO is not required for the suppression of P450 catalytic activities, mRNAs, or proteins by lipopolysaccharide (LPS) or cytokines. Support for the independence of P450 down-regulation from NO was also provided by others who reported that NOS inhibition had no effect on cytokine-evoked decreases in P450-catalyzed activities (Hodgson and Renton, 1995; Mon-

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.104.065557.

ABBREVIATIONS: P450, cytochrome P450; NO, nitric oxide; iNOS, inducible nitric-oxide synthase; LPS, lipopolysaccharide; AG, aminoguanidine; SNP, sodium nitroprusside; ISDN, isosorbide dinitrate; IPRL, isolated perfused rat liver; NO_x, nitrate/nitrite; HPLC, high-performance liquid chromatography; *E*, hepatic extraction ratio; CL_h, hepatic clearance; CL_{int}, intrinsic clearance; CL_{bile}, biliary clearance; ELISA, enzyme-linked immunosorbent assay; γ, steepness factor; ANOVA, analysis of variance; AUC, area under the curve.

shouwer et al., 1996). Therefore, the role of NO in down-regulation of P450 activity remains controversial.

The above discrepancies might be attributed to different experimental manipulations adopted by various investigators. For example, in vivo studies have mostly induced iNOS expression by LPS, which also triggers cytokine release and several known and unknown pathways that might impact the effects of NO (Stadler et al., 1994). Additionally, the use of NOS inhibitors N(G)-monomethyl-L-arginine or aminoguanidine (AG) have produced conflicting results (Khatsenko and Kikkawa, 1997) due perhaps to nonspecific effects of these agents (Sewer and Morgan, 1998). For example, the injection of AG alone to rats caused a 50% decrease in the expression of CYP2C11 mRNA (Sewer and Morgan, 1998). Similarly, AG alone significantly reduced the total P450 content and CYP3A-like protein levels and formation of 6β-, 15-α, and 16β-hydroxy testosterone by mouse liver microsomes (Sewer et al., 1998). Others have reported that N(G)-monomethyl-L-arginine might competitively inhibit ethylmorphine metabolism, independent of its effect on NO formation (Muller et al., 1996). Therefore, investigating the effects of NO directly, in the absence of any confounding factors, is crucial to an understanding of the role of NO in reduction of drug metabolism in different disease states.

Attempts at delineating the effects of NO directly have resulted in the use of iNOS knockout mice (Sewer et al., 1998) and the microsomes exposed to NO donors directly (Khatsenko et al., 1993; Wink et al., 1993; Minamiyama et al., 1997). Because murine and rat P450 show different sensitivities to NO (Li-Masters and Morgan, 2002), comparison of data from knockout mice with the large body of data available in rats becomes difficult. Furthermore, the experiments that use microsomal fractions are not physiologic because they lack barriers and communications that exist among different cells of an intact organ (Khatsenko and Kikkawa, 1997).

Alternatively, in the present study, we investigated the direct effects of exogenous NO donors SNP and ISDN on the inhibition of P450 using an isolated perfused rat liver (IPRL). This model lacks the confounding factors associated with the in vivo LPS models of inflammation, while preserving the integrity of the intact organ. Because NO reacts with hemoproteins at diffusion-controlled rates (Minamiyama et al., 1997), we hypothesized that NO rapidly and concentration-dependently decreases the activities of various P450 isozymes in IPRL. Therefore, specific P450 activities and apoprotein levels were determined in microsomes of livers perfused with various doses of SNP or ISDN. Given that the kinetics of SNP or ISDN impact the exposure of P450 to these donors, the hepatic disposition, including the biliary excretion, of the NO donors and the generated NO were also investigated. Finally, the quantitative concentration-effect relationships were constructed for the concentration (or amount) of NO donors or generated NO and the P450 inhibitory effects.

Materials and Methods

Chemicals. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): SNP, ISDN, sodium dithionite, cytochrome *c*, NADPH, NADP$^+$, chlorzoxazone, umbelliferone, dextromethorphan, dextrorphan, methoxy morphinan, resorufin, cortexolone, isocitrate dehydrogenase, magnesium chloride, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), horse serum, anti-goat rabbit alkaline phosphate conjugate, and anti-rabbit goat alkaline phosphate conjugates. Testosterone, 6β-hydroxy testosterone, and 16α-hydroxy testosterone were obtained from Steraloids (Wilton, NH). Ethoxyresorufin and benzyloxyresorufin were purchased from Molecular Probes (Eugene, OR). Rabbit antirat primary antibodies for CYP3A2 and CYP2C11 were purchased from Chemicon International (Temecula, CA) and Affinity Bioreagents (Golden, CO), respectively. Goat antirat primary antibody for CYP2E1 was purchased from BD Gentest (Woburn, MA). All other reagents used in this study were of analytical grade and obtained from commercial sources.

Animals. Male Sprague-Dawley rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Animals were housed in a temperature- and humidity-controlled room under a 12-h light/dark cycle, with free access to water and food. All the experiments were conducted with nonfasting male rats weighing 240 to 300 g. Texas Tech Health Sciences Center Animal Care and Use Committee approved the study protocol, and all the animals received humane care in compliance with guidelines set by the National Institutes of Health (publication no. 85-23, revised 1985, Bethesda, MD).

Isolated Perfused Rat Liver Studies. The procedures used for the isolation and perfusion of the livers were similar to those reported by us before (Mehvar and Zhang, 2002). In brief, after the incision of the abdominal wall, bile duct, portal vein, and suprahepatic vena cava were cannulated. The liver was then transferred to a water-jacketed (37°C), all-glass perfusion system (Radnoti Glass Technology Inc., Monrovia, CA) and perfused in a single-pass manner, at a constant flow rate of 30 ml/min. Krebs-Henseleit bicarbonate buffer containing 1.2 g/1 glucose and 75 mg/l sodium taurocholate, oxygenated with 95% oxygen and 5% carbon dioxide mixture, was used as the perfusion buffer. Perfusion pressure was constantly monitored from tubing attached proximally to the inflow cannula using a pressure transducer. All the perfusions were performed for 1 h.

SNP and ISDN were used as NO donors in our study. Because of the instability of SNP in the presence of light (Baaske et al., 1981), all the perfusions were conducted under a safelight (Delta 1/CPM, Inc. Dallas, TX). The study consisted of 32 IPRLs, divided into eight experimental groups (n = 4/group). Livers were perfused either without any NO donor (control) or with 50 (SNP 50), 100 (SNP 100), 200 (SNP 200), or 500 (SNP 500) μM SNP or 100 (ISDN 100), 200 (ISDN 200), or 500 (ISDN 500) μM ISDN for 1 h. Outlet perfusate samples were collected at 10-min intervals, wrapped with aluminum foil, and stored at −80°C for further analysis of SNP, ISDN, and/or nitrate/nitrite (NO_x) levels. Additional samples were collected from the outlet at the beginning and end of the perfusion for the analysis of liver enzymes (alanine aminotransferase and aspartate aminotransferase). Bile samples were also collected in preweighed microcentrifuge tubes at 15-min intervals for the analysis of SNP, ISDN, and/or NO_x levels. At the end of the perfusion, the livers were blotted dry, weighed, and used for the preparation of microsomes.

Analysis of Perfusate and Bile Samples. Perfusate and biliary SNP or ISDN concentrations were estimated using previously reported HPLC methods (Baaske et al., 1981; Pramar et al., 1991). For SNP, samples were prepared under a safelight and transferred to an HPLC autosampler that prevented exposure to light. The perfusate enzyme levels were measured using commercially available spectrophotometric kits from Sigma-Aldrich (procedure no. 505). The concentrations of NO in the last outlet perfusate samples and bile were measured based on NO_x determination using a commercially available kit (Active Motif, Carlsbad, CA) that makes use of Griess reagent.

Pharmacokinetic Analysis of Hepatic Disposition of SNP and ISDN. The area under the outlet perfusate concentration-time curve (AUC) was estimated using linear trapezoidal rule for the

duration of perfusion. Hepatic extraction ratio (E) was calculated according to the following equation:

$$E = \frac{C_{\text{in}} - C_{\text{out}}}{C_{\text{in}}} \tag{1}$$

where C_{in} and C_{out} were the constant inlet and steady-state outlet concentrations of the NO donors (SNP or ISDN), respectively. The latter (C_{out}) was the average concentration of the last six outlet perfusate samples for SNP and last four outlet samples for ISDN. The hepatic (CL_h), intrinsic (CL_{int}), and biliary (CL_{bile}) clearances of SNP or ISDN were then calculated using the following equations:

$$\mathrm{CL_h} = E \cdot Q \tag{2}$$

$$\mathrm{CL_{int}} = \frac{\mathrm{CL_h}}{1-E} \tag{3}$$

$$\mathrm{CL_{bile}} = \frac{A_{\text{bile}}}{\mathrm{AUC_{perfusate}}} \tag{4}$$

where A_{bile} is the amount of unchanged drug eliminated in the bile during 60 min of perfusion, and $AUC_{perfusate}$ is the AUC of the NO donor in the outlet perfusate from 0 to 60 min of perfusion.

Microsomes, Total P450 and Cytochrome b_5 Content, and Cytochrome *c* Reductase Activity. Microsomes were prepared at the end of 1-h perfusion using standard ultracentrifugation methods (Lake, 1987) and stored at −80°C. Cytochrome b_5 and carbon monoxide-bound P450 contents were determined by the method of Omura and Sato (Lake, 1987). Total protein content was measured based on the Bradford assay using bovine serum albumin as protein standard. Cytochrome *c* reductase activity was measured using a kinetic assay as described before (Guengerich, 1994).

Analysis of Heme, Thiols, and Nitrotyrosine. Total heme was measured by subjecting the microsomes to the pyridine-hemochromogen method (Lake, 1987). Briefly, after the addition of 0.5 ml of 0.5 M NaOH and 0.5 ml of pyridine to 1.5 ml of microsomal suspensions (~1.5 mg protein/ml), the test sample was reduced with sodium dithionite and its spectrum analyzed against a fully oxidized (potassium ferricyanide) reference. Free heme was measured by the same method after ultrafiltration of microsomal suspensions using Microcon YM-10 filters with 10-kD molecular mass cutoff (Millipore Corporation, Bedford, MA). For determination of thiols, the Ellman's reagent was used as described in detail previously (Minamiyama et al., 1997). Nitrotyrosine levels in the microsomes (~25 mg protein/ml or ~20 μM P450) were quantitated using a commercially available ELISA kit (OxisResearch, Portland, OR) according to the manufacturer's specifications. The detection limit of the assay is 2 nM, corresponding to 0.1 pmol nitrotyrosine/nmol P450.

Analysis of P450 Isoform Activities and Protein Contents. Activities of CYP3A2 and 2C11 were analyzed using formation of 6β- and 16α-hydroxytestosterone, respectively, from testosterone, based on modifications made to a previously published method (Purdon and Lehman-McKeeman, 1997). Briefly, a reaction mixture of 1 ml containing 0.2 mg of microsomal protein, $MgCl_2$ (3 mM), isocitric acid (0.4 M), isocitrate dehydrogenase (77.7 U/ ml), EDTA (1 mM), and $NADP^+$ (1 mM) in phosphate buffer (pH 7.4) was incubated at 37°C for 10 min. The reaction was initiated by the addition of testosterone (150 μM). The remainder of the procedure was similar to the published method (Purdon and Lehman-McKeeman, 1997).

The activity of CYP2E1 was assessed based on the modifications made to a previously established method (Jayyosi et al., 1995) that uses chlorzoxazone as a substrate. In brief, a 250-μl reaction mixture containing chlorzoxazone (250 μM), NADPH (1 mM), and $MgCl_2$ (5 mM) in Tris-HCl buffer, pH 7.4 was incubated at 37°C for 20 min. The reaction was initiated by the addition of 0.1 mg of microsomal protein. After termination of the reaction with 5 μl of 70% perchloric acid, 100 μl of 10 μg/ml umbelliferone (internal standard) was added. The contents were vortexed and centrifuged before injection onto the HPLC system.

Dextromethorphan hydroxylation was employed to assess the activity of CYP2D1. The method used for the analysis was based on modifications made to a previously reported HPLC method (Yu and Haining, 2001). Briefly, dextromethorphan (10 μM) was added to 100 μl of the reaction mixture containing 50 μg of microsomal protein, $MgCl_2$ (5 mM), isocitric acid (5 mM), isocitrate dehydrogenase (77.7 U/ml), and $NADP^+$ (1 mM), and the mixture was incubated at 37°C for 20 min. After the termination of the reaction with 5 μl of 70% perchloric acid, and the reaction mixture was vortexed, centrifuged, and injected onto the HPLC system.

The activities of CYP2B1/2 and CYP1A1/2 were assessed by formation of resorufin from benzyloxy- and ethoxy-resorufin as substrates, respectively (Burke et al., 1985; Rutten et al., 1992). The concentrations of resorufin were measured fluorometrically.

Protein contents of CYP3A2, 2E1, and 2C11 were measured according to an ELISA method reported earlier (Snawder and Lipscomb, 2000). Microsomal proteins used were 0.15 μg for CYP2E1 and CYP2C11 and 1 μg for CYP3A2 analysis. Isoform protein values were expressed as picomoles per milligram of protein.

Concentration (or Amount)-Effect Relationships. The P450 inhibitory effects of SNP or ISDN (I) and the concentrations (or amounts) of the drugs or the generated NO_x (C) were fitted to the sigmoidal I_{max} model using the following equation:

$$I = \frac{I_{\max} \cdot C^{\gamma}}{\mathrm{IC}_{50}^{\gamma} + C^{\gamma}} \tag{5}$$

where I_{max}, IC_{50}, and γ are the maximum inhibitory effect, the concentration (or amount) producing one-half of I_{max}, and the steepness factor, respectively. The model parameters were estimated using the nonlinear regression program WinNonlin (Pharsight Co., Mountain View, CA).

Statistical Analysis. All the statistical comparisons were conducted using ANOVA with subsequent Fisher's test. The linearity of relationships between two parameters (e.g., NO_x concentrations versus NO donor concentrations) was tested using an F test based on the comparison of sum of squares due to the deviation of the observed values from the regression line and those due to the variability within each treatment group (Bolton, 1984). All tests were conducted using a significance level of 0.05. Data are presented as mean ± S.E.

Results

SNP and ISDN Kinetics and NO Release. The time courses of the concentrations of SNP and ISDN in the outlet perfusate samples obtained upon perfusion of livers with different doses of SNP and ISDN are presented in Fig. 1. The concentrations of SNP in the outlet perfusate reached steady state within the first sampling time (10 min) for all the groups. Also, the concentrations of SNP in the outlet perfusate increased proportional to its inlet concentrations, suggesting linear hepatic disposition (Fig. 1, top). In agreement with this observation, dose-corrected AUC values of SNP (data not shown) were not significantly different from each other ($P > 0.05$). Compared with SNP, the achievement of steady-state outlet concentrations for ISDN (~30 min) was delayed (Fig. 1, bottom). However, similar to SNP, the outlet concentrations of ISDN were proportional to its inlet concentrations ($P > 0.05$ for comparison of dose-corrected AUC values).

The hepatic disposition parameters of SNP and ISDN are presented in Table 1. For both SNP and ISDN, the kinetic parameters E, CL_h, and CL_{int} were independent of the administered dose ($P > 0.05$), confirming the linearity of the

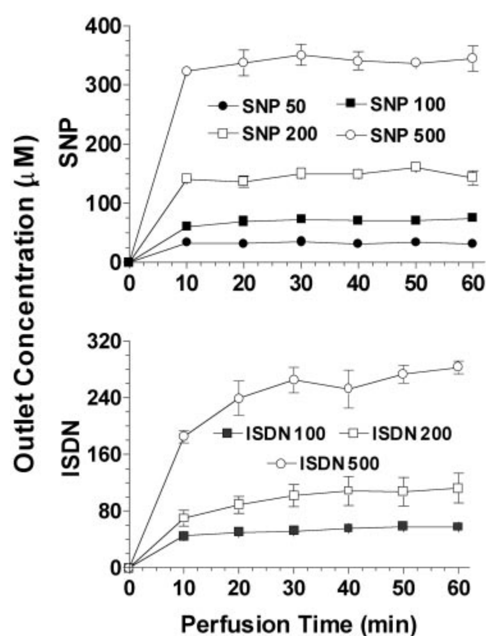

Fig. 1. The concentration-time courses of SNP (top) and ISDN (bottom) in the outlet perfusate samples of isolated rat livers perfused with 50, 100, 200, or 500 μM SNP or 100, 200, or 500 μM ISDN. Symbols and bars represent the average and S.E. values, respectively.

kinetics of these NO donors in IPRLs. However, both E and CL_h values of ISDN were ~60% higher than those of SNP ($P < 0.05$). This was due to ~2-fold higher CL_{int} of ISDN, compared with SNP (Table 1). Nevertheless, the E values show that ~30% and ~50% of SNP and ISDN present in the inlet are extracted during one single pass through the liver, respectively. Additionally, both intact SNP and ISDN were found in the bile. However, the CL_{bile} values (Table 1) were only a small percentage of CL_h (0.47%–1.2% for SNP and 0.06%–0.18% for ISDN), indicating that most of the extracted SNP or ISDN is metabolized in the liver. In contrast to the CL_h values, the CL_{bile} values of ISDN were significantly ($P < 0.01$) lower than those of SNP (Table 1). Furthermore, there was an apparent dose dependence in the CL_{bile} of SNP; CL_{bile} of SNP in livers perfused with 500 μM of the drug was significantly ($P < 0.05$) higher than that in livers perfused with 50, 100, or 200 μM SNP (Table 1). As for bile flow rates, the values in the livers treated with either drug were higher ($P < 0.05$) than those in the untreated livers (Table 1).

The concentrations of NO_x in the outlet perfusate at the end of perfusion (60 min) and the total (0–60 min) amounts of NO_x recovered in the bile are presented in Fig. 2 as a function of SNP or ISDN inlet concentrations. An increase in the inlet concentration of SNP or ISDN resulted in a nonlinear ($P < 0.01$) increase in the NO_x concentrations in the outlet perfusate (Fig. 2, top). For SNP, an inlet concentration of 50 μM was associated with an NO_x level of ~10 μM. However, a 10-fold increase in the inlet concentrations of SNP to 500 μM caused only a 2-fold increase in the NO_x levels in the perfusate (Fig. 2, top). Similarly, a 5-fold increase in the inlet concentrations of ISDN resulted in a less than proportional (3 fold) increase in the NO_x concentrations (Fig. 2, top). Nevertheless, at inlet concentrations of 200 and 500 μM, ISDN produced higher ($P < 0.05$) NO_x levels in the outlet perfusate than did SNP (Fig. 2, top). In contrast to the NO_x concentrations in the outlet perfusate, the amount of NO_x in bile increased linearly with an increase in the inlet concentrations of SNP or ISDN (Fig. 2, bottom). Additionally, significantly ($P < 0.01$) smaller amounts of NO_x were found in the bile after ISDN than SNP (Fig. 2, bottom).

The concentrations of alanine aminotransferase and aspartate aminotransferase in the outlet perfusate samples at the beginning of perfusion were low and remained low (<10 U/l) during the entire period of perfusion (60 min).

General Microsomal Markers. Total P450 and Cytochrome b_5 contents and cytochrome c reductase activities measured after the isolation of microsomes from the livers perfused with different concentrations of SNP or ISDN are presented in Fig. 3. Perfusion of livers with SNP 200 or SNP 500 resulted in a significant decrease in the total P450 levels by ~60% ($P < 0.001$) when compared with the control livers (Fig. 3, top). Similar results were found with ISDN 200 or 500 ($P < 0.001$). However, perfusion of livers with SNP 50, SNP 100, or ISDN 100 did not affect the total P450 levels (Fig. 3, top). For cytochrome b_5 levels, a concentration-dependent decline (12%–50%) was observed when the livers were perfused with SNP or ISDN at 100 to 500 μM concentrations, with SNP 50 showing no effect (Fig. 3, middle). In contrast to the total P450 and cytochrome b_5 levels, the cytochrome c reductase activities were not significantly affected by either SNP or ISDN (Fig. 3, bottom) ($P > 0.05$).

TABLE 1

Kinetic parameters of SNP and ISDN in isolated rat livers perfused with 50, 100, 200, or 500 μM of SNP or 100, 200, or 500 μM of ISDN (n = 4/group)

Values are expressed as mean ± S.E.

Group	Concentration	E	CL_h	CL_{int}	D_{bile}	CL_{bile}	Bile Flow Rate
	μM		*ml/min*	*ml/min*	*nmol*	*μl/min*	*ml/h*
Control							0.567 ± 0.049
SNP	50	0.343 ± 0.021	10.3 ± 0.60	15.8 ± 1.5	86.2 ± 9.6	48.2 ± 7.1	0.742 ± 0.034[a]
SNP	100	0.283 ± 0.024	8.50 ± 0.72	12.0 ± 1.4	293 ± 80	75.6 ± 18.4	0.750 ± 0.068[a]
SNP	200	0.270 ± 0.027	8.00 ± 0.79	11.1 ± 1.4	597 ± 94	74.9 ± 13.3	0.707 ± 0.024[a]
SNP	500	0.310 ± 0.019	9.32 ± 0.57	13.6 ± 1.3	2130 ± 164	114 ± 7.2[b]	0.841 ± 0.039[a]
ISDN	100	0.450 ± 0.037[c]	13.4 ± 1.1[c]	24.9 ± 3.3[c]	23.3 ± 0.8	8.10 ± 0.19[d]	0.598 ± 0.036
ISDN	200	0.470 ± 0.097[d]	14.0 ± 2.9[d]	31.1 ± 9.3[d]	136 ± 21	25.7 ± 1.2[d]	0.829 ± 0.017[a]
ISDN	500	0.470 ± 0.028[d]	14.0 ± 0.9[d]	26.8 ± 3.0[d]	262 ± 31	19.5 ± 2.3[d]	0.774 ± 0.051[a]

[a] Significantly different from control: $P < 0.05$, ANOVA, followed by Fisher's test.
[b] Significantly different from SNP 50, 100, and 200: $P < 0.05$, ANOVA, followed by Fisher's test.
[c] Significantly different from SNP 100 and 200: $P < 0.05$, ANOVA, followed by Fisher's test.
[d] Significantly different from SNP 100, 200, and 500: $P < 0.01$, ANOVA, followed by Fisher's test.

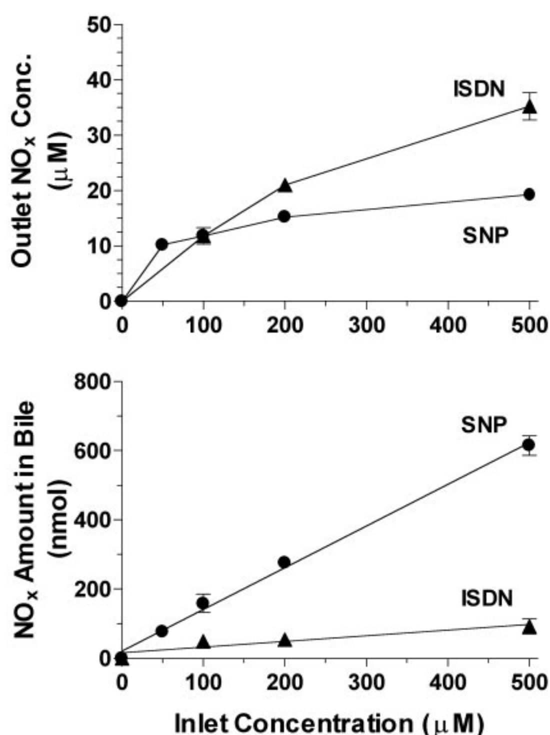

Fig. 2. Top, concentrations of NO_x in the outlet perfusate at the end of perfusion of livers with 50, 100, 200, or 500 μM SNP or 100, 200, or 500 μM ISDN. Bottom, total amount of NO_x recovered in bile as a function of inlet concentrations of SNP or ISDN. Symbols and bars represent the average and S.E. values, respectively.

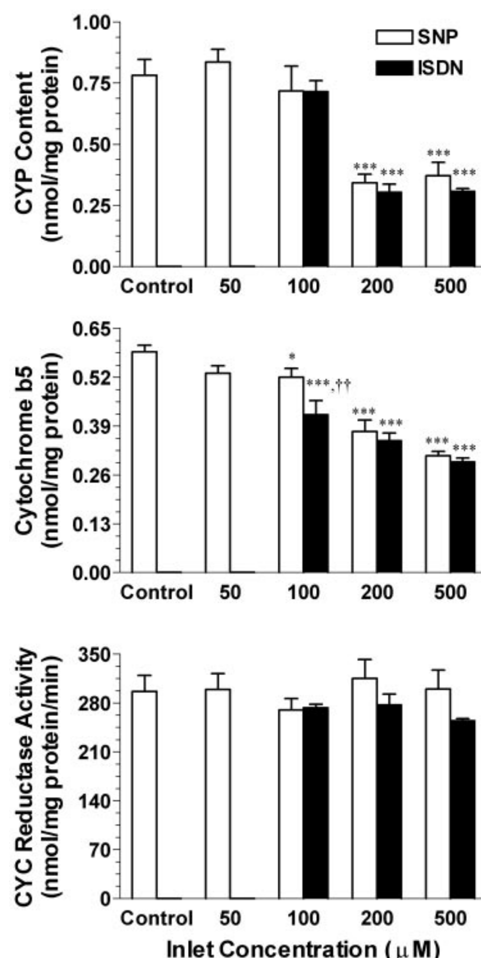

Fig. 3. Cytochrome P450 (CYP) content (top), cytochrome b_5 content (middle), and cytochrome c reductase (CYC) activities (bottom) of rat livers perfused with 50, 100, 200, or 500 μM SNP or 100, 200, or 500 μM ISDN. * and ***, significant difference ($P < 0.05$ and <0.001, ANOVA) between treated and control livers; ††, significant difference ($P < 0.01$) between SNP and ISDN at similar concentrations. Columns and bars represent the average and S.E. values, respectively.

The levels of microsomal heme and thiols are depicted in Fig. 4. At concentrations of 100, 200, and 500 μM, SNP caused, respectively, 18% ($P < 0.01$), 40% ($P < 0.001$), and 51% ($P < 0.001$) reductions in the total heme content of microsomes (Fig. 4, top). For ISDN, the reductions (~50%) were significant ($P < 0.001$) only at 200 and 500 μM concentrations. No free heme was detected in our microsomal preparations obtained from either control or treated livers. As for free (non-nitrosylated) thiols, significant ($P < 0.05$), but moderate (17%–24%), reductions were observed only at high concentrations (200 and/or 500 μM) of SNP or ISDN (Fig. 4, bottom).

The levels of nitrotyrosine in our microsomal preparations were below the detection limit of the ELISA method, which was 2 nM or 0.1 pmol nitrotyrosine/nmol P450.

CYP3A2, 2C11, and 2E1 Activities and Protein Levels. Profiles of CYP3A2 activities and protein levels are presented in Fig. 5. The activity of CYP3A2 in livers perfused with SNP significantly decreased ($P < 0.05$) in a dose-dependent manner (Fig. 5, top). The degrees of reduction in the activity of CYP3A2 were 34%, 47%, 69%, and 75% for 50, 100, 200, and 500 μM concentrations of SNP, respectively. Additionally, perfusion of livers with ISDN 200 or ISDN 500 also resulted in a significant decrease (~47% for both concentrations) in the CYP3A2 activity ($P < 0.01$) (Fig. 5, top). The inhibitory effect of ISDN appeared to be less than that observed after similar concentrations of SNP (Fig. 5, top). A substantial interindividual variability was observed in the protein levels of CYP3A2, measured by the ELISA method (Fig. 5, bottom). However, unlike the activities, protein levels remained unaltered in all the treatment groups and were comparable with the control livers (Fig. 5, bottom).

Profiles of CYP2C11 activities as well as protein levels are presented in Fig. 6. Similar to CYP3A2, the activity of CYP2C11 was inhibited by SNP in a dose-dependent manner. However, the inhibitory effect of SNP 50 was not significant (Fig. 6). The percentages of reduction in the CYP2C11 activities were 40, 82, and 80, for SNP inlet concentrations of 100, 200, and 500 μM, respectively ($P < 0.05$). Additionally, CYP2C11 activity was significantly reduced in ISDN 200 and ISDN 500 groups by 58% and 76%, respectively (Fig. 6, top). As for protein levels, they were not significantly affected by all the treatments except for the 500 μM SNP or ISDN, which resulted in 43% or 66% reductions, respectively (Fig. 6, bottom).

Perfusion of livers with SNP also caused a dose-dependent decrease in the activity of CYP2E1 (Fig. 7, top); percentages of reduction were 27, 53, 76, and 74 for SNP concentrations of 50, 100, 200, and 500 μM, respectively ($P < 0.05$). Although ISDN also significantly reduced the activity of 2E1, its effects were significantly less than that of SNP at similar concentrations (Fig. 7, top). The protein levels of CYP2E1 remained unchanged in five of the six treated groups (Fig. 7, bottom).

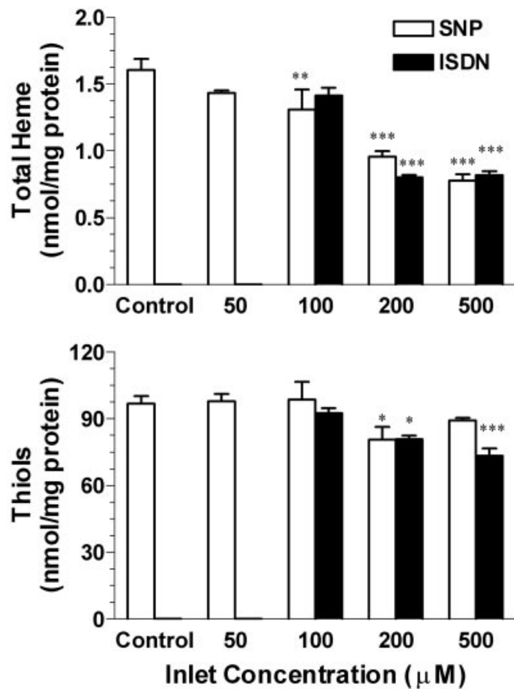

Fig. 4. Microsomal levels of heme (top) and thiol (bottom) in rat livers perfused with 50, 100, 200, or 500 μM SNP or 100, 200, or 500 μM ISDN. *, **, and ***, significant difference ($P < 0.05$, <0.01, and <0.001, respectively; ANOVA) between treated and control livers. Columns and bars represent the average and S.E. values, respectively.

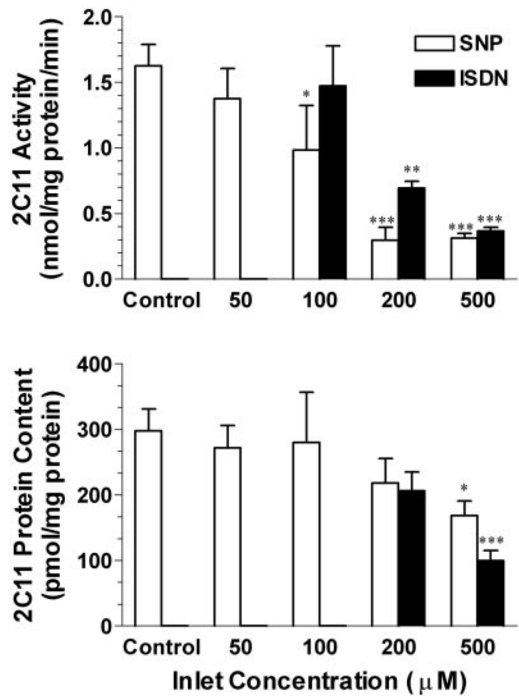

Fig. 6. The activities (top) and protein levels (bottom) of CYP2C11 in rat livers perfused with 50, 100, 200, or 500 μM SNP or 100, 200, or 500 μM ISDN. *, **, and ***, significant difference ($P < 0.05$, <0.01, and <0.001, respectively; ANOVA) between treated and control livers. Columns and bars represent the average and S.E. values, respectively.

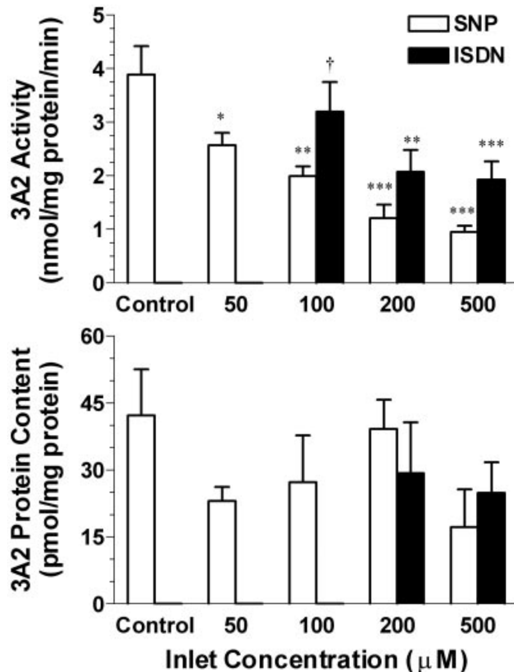

Fig. 5. The activities (top) and protein levels (bottom) of CYP3A2 in rat livers perfused with 50, 100, 200, or 500 μM SNP or 100, 200, or 500 μM ISDN. *, **, and ***, significant difference ($P < 0.05$, <0.01, and <0.001, respectively; ANOVA) between treated and control livers; †, significant difference ($P < 0.05$) between SNP and ISDN at similar concentrations. Columns and bars represent the average and S.E. values, respectively.

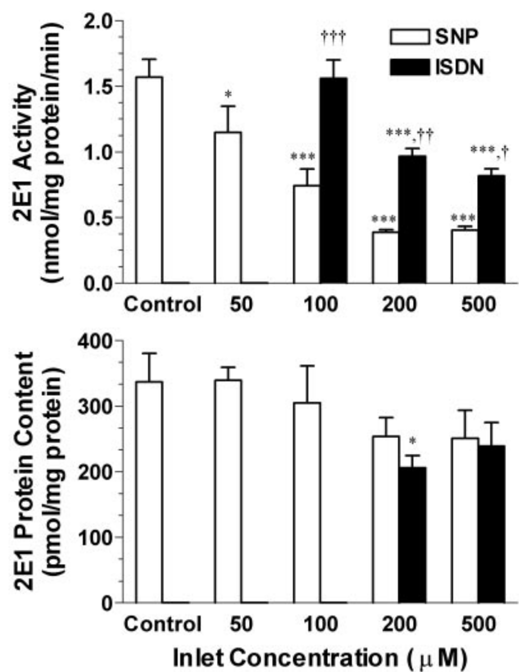

Fig. 7. The activities (top) and protein levels (bottom) of CYP2E1 in rat livers perfused with 50, 100, 200, or 500 μM SNP or 100, 200, or 500 μM ISDN. * and ***, significant difference ($P < 0.05$ and <0.001, respectively; ANOVA) between treated and control livers; †, ††, and †††, significant difference ($P < 0.05$, <0.01, and <0.001, respectively; ANOVA) between SNP and ISDN at similar concentrations. Columns and bars represent the average and S.E. values, respectively.

CYP1A1/2, 2B1/2, and 2D1 Activities. The microsomes obtained from livers perfused with different concentrations of SNP or ISDN were also analyzed for the dealkylation activities of CYP1A1/2, 2B1/2, and 2D1 (Fig. 8). The extent and pattern of inhibitory effects of NO donors on the activity profiles of CYP1A1/2 (Fig. 8, top) and CYP2B1/2 (Fig. 8, middle) were similar to those seen for CYP3A2, 2C11, and

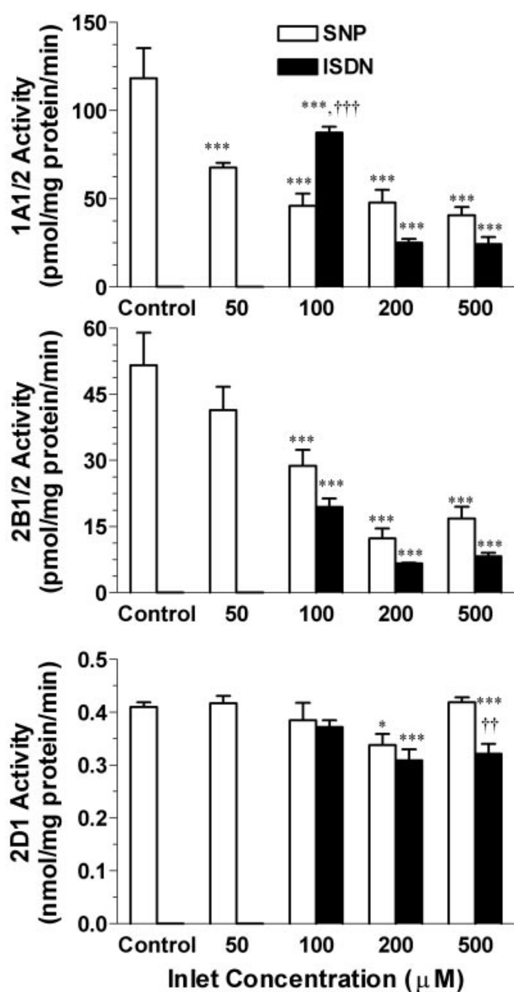

Fig. 8. The activities of CYP1A1/2 (top), CYP2B1/2 (middle), and CYP2D1 (bottom) in rat livers perfused with 50, 100, 200, or 500 μM SNP or 100, 200, or 500 μM ISDN. *, **, and ***, significant difference ($P <$ 0.05, $<$0.01, and $<$0.001, respectively; ANOVA) between treated and control livers; †† and †††, significant difference ($P <$ 0.01, and $<$0.001, respectively; ANOVA) between SNP and ISDN at similar concentrations. Columns and bars represent the average and S.E. values, respectively.

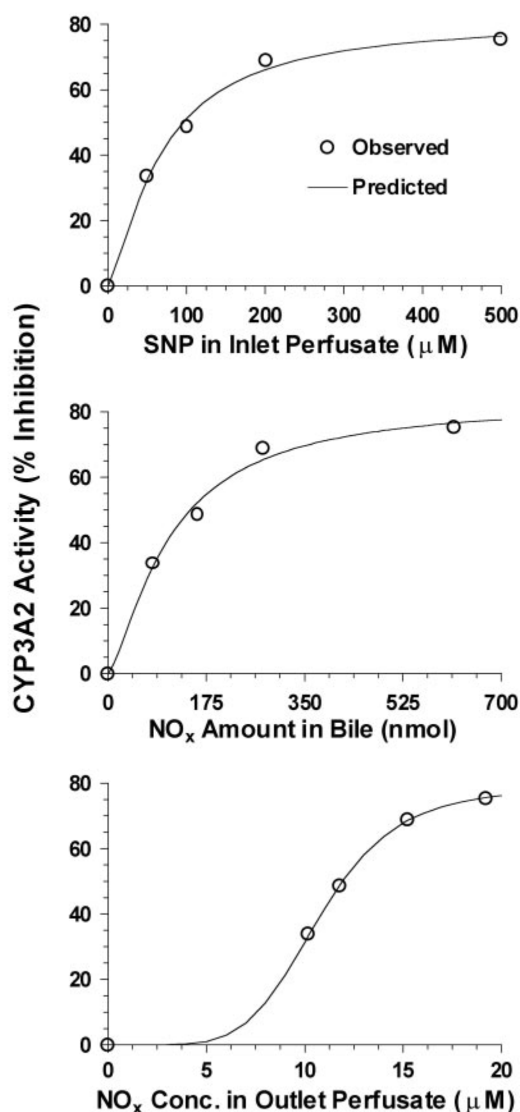

Fig. 9. The relationships between the inhibitory effects of SNP on CYP3A2 activity and the SNP inlet concentration (top), NO_x amounts in bile (middle), or NO_x concentrations in the outlet perfusate (bottom).

2E1. However, unlike the other studied P450s, the influence of NO donors on the activity of CYP2D1 was minimal, if any (Fig. 8, bottom).

Concentration-Inhibitory Effect Relationships. The concentration-inhibitory effect relationships are shown in Fig. 9 for CYP3A2 activity versus the concentrations of SNP in the inlet perfusate, NO_x amounts recovered in bile, and NO_x concentrations in the outlet perfusate. Similar profiles were observed for other isoenzymes affected by NO donors (data not shown). Additionally, the pharmacodynamic parameters estimated from these relationships are presented in Table 2 for all the affected enzymes. For each P450 isozyme, the estimated I_{max} values obtained from three different regression methods were similar (Fig. 9; Table 2). However, as expected, the IC_{50} and γ values were dependent on the type of dependent variable (concentrations of SNP in the perfusate or concentrations or amounts of NO_x in the perfusate or bile) (Table 2). The maximum inhibitory effects of SNP treatment on the enzyme activity were relatively similar for all the studied isozymes and ranged from 63% (1A1/2) to 85% (2C11) (Table 2). However, up to 2.5-fold differences were observed in the IC_{50} values when the inhibitory effect was plotted against the SNP inlet concentrations or NO_x amounts in bile (Table 2); the IC_{50} values for different isozymes were in the order of 2C11 > 2B1/2 > 2E1 = 3A2 > 1A1/2. Although showing similar rank order, the IC_{50} values obtained from the NO_x concentrations in the outlet perfusate were very close for all the isozymes (Table 2).

Because of a small number of data points (three concentrations), the concentration-effect relationships were not constructed for ISDN.

Discussion

Hepatic Disposition of NO Donors. Although the in vivo disposition of SNP and ISDN has been reported previously in both humans (Schulz, 1984; Fung, 1985) and rats (Kreye and Reske, 1982; Morrison and Fung, 1984), our study is the first to show the detailed hepatic disposition, including biliary excretion, of SNP or ISDN in an IPRL model (Fig. 1; Table 1). In addition to metabolism by liver microsomes (Rao

TABLE 2

The pharmacodynamic parameters for the inhibition of the individual P450 isozymes as a function of SNP concentrations in the inlet perfusate, NO_x concentrations in the outlet perfusate, and NO_x amounts in bile

Values in parentheses are percentage of coefficient of variations.

P450 Isozyme	SNP Concentrations in the Inlet Perfusate			NO_x Amounts in Bile			NO_x Concentrations in the Outlet Perfusate		
	I_{max}	IC_{50}	γ	I_{max}	IC_{50}	γ	I_{max}	IC_{50}	γ
	% Control	*μM*		*% Control*	*μM*		*% Control*	*nmol*	
CYP3A2	81.5 (7.5)	67.4 (14.1)	1.35 (23.7)	83.6 (11.6)	110 (20)	1.4 (31.7)	78.7 (1.1)	10.8 (0.5)	5.50 (4.6)
CYP2C11	84.7 (8.6)	97.4 (12.8)	2.95 (34.5)	82.3 (11.0)	153 (14.2)	3.2 (41.6)	83.1 (4.0)	11.8 (1.8)	11.3 (20.5)
CYP2E1	77.6 (4.8)	67.7 (8.6)	2.30 (20.3)	78.2 (6.6)	108 (11)	2.37 (25.2)	76.0 (1.8)	10.8 (0.8)	10.2 (9.9)
CYP1A1/2	62.8 (3.7)	40.9 (14.6)	3.75 (71.0)	63.3 (3.9)	63.4 (14.0)	3.26 (59.8)	62.6 (3.5)	9.83 (3.3)	20.6 (88.6)
CYP2B1/2	73.0 (10.0)	77.9 (17.0)	2.72 (41.0)	73.6 (12.4)	122 (19)	2.82 (45.3)	72.5 (6.5)	11.2 (2.8)	11.4 (33.4)

et al., 1991; Minamiyama et al., 2001), both ISDN and SNP undergo metabolism to generate NO and other metabolites in vascular smooth muscle (Kreye and Reske, 1982; Fung et al., 1984). Therefore, the E and CL_h values presented here (Table 1) represent metabolism of SNP and ISDN by both hepatic vasculature and the hepatocytes.

The linearity in the hepatic disposition of SNP within the concentration range of 50 to 500 μM (Fig. 1; Table 1) is consistent with the drug's main metabolic pathway; in hepatocytes, SNP is reduced by P450 reductase (Rao et al., 1991), a pathway that remained unchanged after treatment with NO donors (Fig. 3, bottom). Similarly, despite a significant NO-dependent reduction in the catalytic activities of microsomal 1A1/2 (Fig. 8, top) and 2E1 (Fig. 7, top), which reportedly (Minamiyama et al., 2001) participate in the in vitro metabolism of ISDN, the E of ISDN was dose independent (Table 1). This apparent discrepancy suggests that the contribution of these two isoenzymes to the overall metabolism of the drug in the IPRL model is relatively small.

The E and Cl_{int} values of ISDN observed here (Table 1) revealed, for the first time, the true ability of the liver to eliminate ISDN. This is because the systemic (i.e., metabolic) clearance of ISDN (118 ml/min/kg) (Morrison and Fung, 1984) far exceeds the hepatic blood flow in rats (Davies and Morris, 1993), indicating extrahepatic metabolism. Therefore, estimation of CL_h, E, and/or CL_{int} from the in vivo data has not been possible. Our CL_{int} values (Table 1) predict an in vivo CL_h that cannot account for more than one-third of the reported systemic clearance of ISDN (Morrison and Fung, 1984). Therefore, approximately two-thirds of the systemic clearance of ISDN in rats is indeed due to extrahepatic metabolic pathways.

Although in small amounts, the presence of intact SNP or ISDN in bile (Table 1) indicates that hepatocytes have indeed been exposed to intact NO donors which could generate NO within the hepatocytes. The presence of NO_x in the bile (Fig. 2) further supports this argument.

Effects of NO Donors on P450 Activity and Protein. Our observations clearly show that NO donors rapidly and concentration-dependently decrease the total P450 content (Fig. 3) and activities (Figs. 5–8) in an IPRL model. However, the protein levels of the affected isoenzymes are largely unaltered (Figs. 5–7). We measured the total P450 content using the standard CO differential spectra analysis, which requires the presence of intact protein-bound heme. Theoretically, the observed decrease in the total P450 content may be due to binding of NO to the heme prosthetic groups (Wink et al., 1993; Minamiyama et al., 1997) and/or loss of enzyme-bound heme (Kim et al., 1995). However, because the microsomal P450-NO complexes dissociate rapidly (Minamiyama et al., 1997) (e.g., during microsomal preparation), the reduction in P450 content, observed in our IPRL studies, is due mostly to the loss and degradation of enzyme-bound heme (Kim et al., 1995). The observed reductions in total heme contents of microsomal preparations in the livers treated with NO donors (Fig. 4, top) are in agreement with this postulate. Although the loss of heme is expected to reduce the P450 catalytic activities, it is unlikely to affect the ELISA-measured isoenzyme protein levels.

The reductions in P450 content alone (Fig. 3, top) cannot fully explain the changes in the activities of various isoforms observed after NO exposure (Figs. 5–8). Therefore, other mechanisms must be in place. One such mechanism is the interaction of NO with sulfhydryl groups of cysteine amino acid residues in various P450s, forming reversible *S*-nitrosothiols that have relatively long half-lives (Minamiyama et al., 1997). If the cysteine residues are critical for the interaction of ligand with the isoenzyme, formation of *S*-nitrosothiols could result in a reduced activity. In fact, the reaction of NO with cysteinyl groups of 3A2 and 2C11 were held responsible for the reductions in the 16α- and 6β-hydroxylation of testosterone by microsomes that were directly exposed to an NO donor (Minamiyama et al., 1997). In our studies, NO donors caused a moderate reduction in the free sulfhydryl groups only at high concentrations (Fig. 4, bottom). However, it should be noted that the reaction of NO with one or more critical cysteine groups of a P450 isoform may have a dramatic effect on its activity without a substantial effect on the overall number of free thiols. Therefore, the percentage reduction in thiols may not be compared with the percentage reduction in the activity of the individual P450 isoforms.

Another potential mechanism for the NO-induced inhibition of P450 activity is an irreversible nitration of tyrosine residues, which are positioned at the active site of the enzyme or are involved in electron transfer, by the NO-derived peroxynitrite (Roberts et al., 1998). For example, peroxynitrite-mediated nitration of tyrosine residues of CYP2B1 significantly reduced its catalytic activity (Roberts et al., 1998), an effect which was abolished by mutation of tyrosine 190 to alanine (Lin et al., 2003). However, in agreement with a microsomal study (Minamiyama et al., 1997), nitrotyrosine was not detected in our model.

The P450 isoenzyme-specific inhibitory responses to NO (Figs. 5–8) may be explained by the reductions in thiol (Fig. 4) and/or cytochrome b_5 (Fig. 3) contents. Various P450 isoenzymes contain four to nine free cysteinyl residues (Minamiyama et al., 1997). Therefore, depending on the number of free cysteinyl groups and their position relative to the

active site, the response of different isoenzymes to the presence of NO may be different. Additionally, because cytochrome b_5 has isoenzyme-specific stimulatory effects on the P450 activities (Yamazaki et al., 2002), the NO-induced reduction in the cytochrome b_5 contents (Fig. 3, middle) may affect the activities of different isoforms to a different extent.

In contrast to significant reductions in catalytic activities observed for all the other studied isoenzymes (Figs. 5–8), the NO-induced changes in the activity of CYP2D1 were minimal (Fig. 8, bottom). This observation suggests that NO does not interact with CYP2D1 at critical sites. Indeed, recent reports (Paine et al., 2003) have clearly shown that negatively charged carboxylate-containing amino acids, such as aspartate-301 and glutamate-216, are critical for the interaction of 2D6 with basic nitrogen-containing ligands. Therefore, the lack of the effect of NO donors on 2D1 may be due to the lack of cysteine and/or tyrosine residues at the critical site(s) of the isozyme. Furthermore, several in vitro studies (Yamazaki et al., 2002) have shown that, in contrast to most other P450 isoforms, the activity of CYP2D6 is not stimulated by cytochrome b_5. Hence, the NO-induced reduction in cytochrome b_5 content (Fig. 3, middle) is not expected to affect the activity of this isoform.

In addition to the alterations in the catalytic activities of P450 isoenzymes, NO could potentially decrease P450 mRNA and apoprotein levels (Stadler et al., 1994; Khatsenko and Kikkawa, 1997). However, the protein levels of CYP3A2 (Fig. 5), 2C11 (Fig. 6), and 2E1 (Fig. 7), which constitute majority of total P450 in male rats, were not substantially altered in our studies. This is most likely due to our short experimental time (1 h).

Concentration (Amount)-Effect Relationships. Because of its linear hepatic disposition (Table 1), the inlet concentrations of SNP are expected to be directly related to the concentrations of SNP and generated NO in hepatocytes. Similarly, the biliary amounts of NO_x reflect the exposure of the hepatocytes to SNP and NO. Therefore, it is not surprising that the inhibitory effect-concentration (or amount) profiles were qualitatively similar when SNP inlet concentrations or biliary NO_x amounts were used as the independent variable (Fig. 9). Conversely, the NO_x concentrations in the outlet perfusate may not be a true representation of hepatocyte exposure to NO because NO may be generated from SNP in the liver vasculature (Kreye and Reske, 1982). Therefore, the inlet concentrations of NO donors or the biliary amounts of NO_x are more appropriate predictors of the P450 inhibitory effects of NO donors than are the perfusate NO_x levels (Wink et al., 1993).

It should be noted that the Griess reagent used in our studies measures NO metabolites (NO_x) as an indirect measure of NO exposure. However, the metabolism of ISDN may produce NO_x independent of the formation of NO (Fung et al., 1984). Therefore, the NO_x levels after ISDN administration may be an overestimation of the liver exposure to NO. Furthermore, NO species other than free radical (e.g., nitroxyl or nitrosonium intermediates), which may be present after SNP administration (Rao et al., 1991), may also react with P450s. Consequently, the effects of exogenously administered SNP on P450s may not be completely similar to those of NO derived in vivo from NO synthase.

Conclusions

In conclusion, our findings indicate that NO decreases the P450-mediated drug metabolism in an isolated perfused rat liver model. The NO effect on drug metabolism is direct, rapid (<1 h), and differential to various P450 isoforms. Different mechanisms such as nitrosylation of heme and/or cysteinyl amino acids may be responsible for this effect. Because NO is released in substantial amounts under various pathophysiologic conditions of the liver, the rapid inhibitory effects of NO on metabolism of drugs used in these conditions are of clinical relevance. Further experiments are currently underway to determine whether the NO-mediated inhibition of P450 in IPRLs is reversible and/or time-dependent.

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