Nitric Oxide Mediates Hepatic Cytochrome P450 Dysfunction Induced by Endotoxin

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Background: Animals subjected to immunostimulatory conditions (sepsis) exhibit decreased total cytochrome P450 content and decreased P450-dependent drug metabolism. Cytochrome P450 function is of clinical significance because it mediates the metabolism of some opioid and hypnotic drugs. The authors tested the hypothesis that reduced P450 function and decreased drug metabolism in sepsis are mediated by endotoxin-enhanced synthesis of nitric oxide.

Methods: Hepatic microsomes were prepared from male Sprague-Dawley rats in nontreated rats, rats pretreated with phenobarbital and rats receiving aminoguanidine or N\(^\text{\textregistered}\)L-monomethylarginine alone. Nitric oxide synthesis was augmented for 12 h with a single injection of bacterial lipopolysaccharides. Nitric oxide synthase was inhibited with aminoguanidine or N\(^\text{\textregistered}\)L-monomethyl-arginine during the 12 h of endotoxemia in some animals. Plasma nitrite and nitrate concentrations were measured in vivo, and total microsomal P450 content, and metabolism of ethylmorphine and midazolam in vitro.

Results: Administration of endotoxin increased plasma nitrite and nitrate concentrations, decreased total cytochrome P450 content, and decreased metabolism of ethylmorphine and midazolam. Inhibition of nitric oxide formation by aminoguanidine or N\(^\text{\textregistered}\)L-monomethyl-arginine partially prevented the endotoxin-induced effects in the nontreated and phenobarbital-treated groups. Aminoguanidine or N\(^\text{\textregistered}\)L-monomethyl-arginine alone did not have an effect on either total cytochrome P450 content or P450-dependent drug metabolism. Plasma nitrite and nitrate concentrations correlated significantly negatively with P450 content (nontreated r = -0.88, phenobarbital r = -0.91), concentrations of formed formaldehyde (nontreated r = -0.87, phenobarbital r = -0.95), and concentrations of midazolam metabolites (4-OH midazolam nontreated r = -0.88, phenobarbital r = -0.93, and 1-OH midazolam nontreated r = -0.88, phenobarbital r = -0.97).

Conclusions: Altered hepatic microsomal ethylmorphine and midazolam metabolism during sepsis is mediated in large part by nitric oxide. (Key words: Biotransformation; hydroxylation; induction; microsomal; N-demethylation. Enzymes, cytochrome P450: CYP2B1; CYP2C6/11; CYP3A1/2. Interactions, drug: nitric oxide; sepsis. Metabolism, drug: ethylmorphine; midazolam.)

HEPATIC metabolism of drugs is reduced in sepsis. Although this phenomenon was described 40 years ago, the mechanism is still not completely understood. Immunostimulation by endotoxin during sepsis produces substantial quantities of nitric oxide, a free radical that interferes with a number of key metabolic enzymes in the body by oxidizing heme or nonheme iron and iron-sulfur complexes. 3, 7 Nitric oxide transformation of heme to methemoglobin is a well-known example of this reaction. The cytochrome P450 isoenzymes in the liver that metabolize drugs also contain iron, raising the possibility that nitric oxide is a factor in decreased drug metabolism seen during sepsis. 8 This phenomenon

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has been previously demonstrated, but the specificity of nitric oxide for various isoforms has not been examined.

We measured plasma nitrite and nitrate (NOx) as markers for nitric oxide production and correlated the concentrations of these compounds with total hepatic microsomal cytochrome P450 content in a rat sepsis model. We also correlated plasma NOx with the metabolic function of P450 isozymes responsible for the biotransformation of an opioid (ethylmorphine) and a hypnotic drug (midazolam). We used microsomal enzyme induction with phenobarbital and nitric oxide synthesis inhibition to modulate the system in intact animals.

Materials and Methods

The rat isoforms CYP 2B1, CYP 2C6/11, and CYP 3A2 have high catalytic activity for N-demethylation of ethylmorphine (CYP 3A3 in humans), and CYP 3A1 has mainly metabolizing activity for midazolam (CYP 3A5/4 in humans).

Chemicals

Midazolam, 4-OH and 1'-OH midazolam (8-Chloro-6-(2-fluorophenyl)-4H-imidazol[1,5-a][1,4]benzodiazepine-1-methanol) were donated by Hoffman LaRoche (Nutley, NJ). Unless specified, reagents were obtained from Sigma Chemical (St. Louis, MO).

Preparation of Hepatic Microsomes

The protocol was approved by the Institutional Animal Care and Use Committee. Microsomal cytochrome P450 was induced in adult male Sprague Dawley rats (weighing 250–300 g) by intraperitoneal injection of phenobarbital in saline (80 mg/kg, every 24 h for 4 consecutive days). Rats had free access to water and food. General anesthesia was initiated with pentobarbital (70 mg/kg, intraperitoneal), and oxygen by mask. The portal vein was cannulated and blood samples were drawn for subsequent plasma nitric oxide analysis. Livers were perfused with 200 ml iced saline to remove most of the blood and then were quickly excised and stored at −80°C. Microsomes were prepared by homogenizing the liver tissue, thawed in ice water in seven volumes of phosphate-buffered solution (PBS) containing NaCl 136.9 mm, KCl 2.2 mm, NaH2PO4 8.1 mm, KH2PO4 1.5 mm, pH 7.4, in a glass homogenizer with a polytetrafluoroethylene pestle for 3 min on ice. Homogenates were centrifuged at 10,000 × g 4°C for 30 min to remove unbroken cells, lysosomes, and mitochondria. Microsomal pellets were obtained by further centrifugation of the supernatant at 105,000 × g for 1 h at 4°C. The pellets were washed in cold PBS, centrifuged for 40 min at 105,000 × g 4°C to obtain washed microsomes, which were resuspended in PBS and used for further assays as described later. Microsomal protein concentrations were determined by the method of Lowry et al. using bovine serum albumin as the protein standard. Total microsomal cytochrome P450 content was quantified spectrophotometrically (Hitachi Double Beam Spectrophotometer, Danbury, CT) from the carbon monoxide-reduced difference spectrum. 

Stimulation and Inhibition of Nitric Oxide

Table 1 outlines the experimental design used to compare the affects of aminoguanidine and Nω-L-monomethyl-arginine (L-NMMA), phenobarbital treatment, lipopolysaccharide (LPS) treatment, and nitric oxide synthase (NOS) inhibition on nitric oxide formation, cytochrome P450 content, and drug metabolism. To test the effect of nitric oxide production in vivo on hepatic cytochrome P450 activity, rats were divided into two groups: either nontreated receiving saline only or phenobarbital-treated (as described earlier). Rats were given purified endotoxin (LPS, E. coli: 0111:B4, Sigma) as a single dose (10 mg/kg, intraperitoneal). To inhibit nitric oxide formation, rats receiving LPS were treated with either aminoguanidine (80 μmoles/kg in PBS, intraperitoneal) or L-NMMA (80 μmol/kg in PBS, intraperitoneal) beginning 90 min after LPS treatment and repeated at 3-h intervals thereafter, for a total dosage of 320 μmoles/kg. To examine the effects of aminoguanidine and L-NMMA alone, the same regimen was given to untreated animals. Rats were killed 12 h after LPS injection by cardiac arrest during the perfusion with iced saline.

Assays
Plasma Nitrate. Total NOx (nitrite plus nitrate, NO2− plus NO3−) in plasma was determined by conversion to the corresponding 1-nitro-2,4,6-trimethoxybenzene (NTMB) derivatives in the presence of an internal standard (15N)NaNO2 and subsequently analyzed by gas chromatography/mass spectrometry, monitoring m/z NTMB = 213 and m/z 15NTMB = 214. This assay proved to be reproducible in our laboratory, with an r² = 0.999 ± 0.00027 and a coefficient of vari-
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<table>
<thead>
<tr>
<th>Phenacetin treated (PB)</th>
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<th>Ammonioprione</th>
<th>U-NAMMA</th>
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Table 1: Hepatic Micromosomal Cytochrome P450 Content and Activity

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Data represent the mean ± SEM. n = 6 for all groups. See table 2 for ANOVA results.

Ation of 7.2%, and linear over a wide range of standard concentrations (2.5–500 μg/ml NTMB). Practically, the lower limit of sensitivity of this assay in plasma was 200 ng NTMB.

**Ethylmorphine.** N-demethylation of ethylmorphine was assayed by determining the amount of formaldehyde formed using the method of Nash. The microsomal preparation (50–100 μg protein) was preincubated in PBS at 37°C for 10 min, and the reaction was started with the addition of substrate, ethylmorphine (1.5 μmoles), in a final volume of 600 μl, pH 7.4, containing 0.6 mm reduced form of nicotinamide-adenine dinucleotide phosphate, 5 mm MgCl₂, and 15 mm neutralized hydrazinecarboxamide. The reaction mixture was placed in polycarbonate vials in a shaking water bath for 10 min at 37°C under conditions of minimal light and air environment. The reactions were terminated by the addition of 200 μl 15% zinc sulfate heptahydrate, and the mixture was shaken another 5 min, then 200 μl saturated barium hydroxide octahydrate was added. The resultant mixture was centrifuged for 8 min at 6,000 × g; 355 μl Nash reagent (30.0 g ammonium acetate, 0.4 ml acetyl acetone in a total of 100 ml distilled water) was added to 840 μl of the clear reaction solution, mixed thoroughly and heated at 60°C for 30 min. Formaldehyde formation was measured spectrophotometrically at 415 nm with a tissue blank set to 0 absorbance, and quantified using a standard formaldehyde (0.25–3 μg/ml) curve. The standard curve had an r² = 0.998 ± 0.0008 and a coefficient of variation of 5.6%.

**Midazolam.** CYP 3A1 specific activity was assessed measurement of the 4-OH and 1-OH metabolites of midazolam. Microsomal preparation (50–100 μg protein) was incubated in polycarbonate tubes in PBS, pH 7.4, at 37°C for 10 min, in a final volume of 100 μl, in the presence of an NADPH-generating system (5 mm glucose 6-phosphate, 0.15 units of glucose 6-phosphate dehydrogenase, 1 mm nicotinamide-adenine dinucleotide phosphate, and 5 mm MgCl₂). The reaction was started by the addition of substrate, midazolam, as a tenfold concentrate in 20% acetone (60 μM, final concentration), and was conducted in a shaking water bath for 10 min at 37°C under conditions of minimal light and air environment. The reaction was stopped by adding 100 μl cold methanol. Protein was precipitated at 10,000 × g for 6 min, and an aliquot of the supernatant was injected for high-performance liquid chromatographic analysis. The high-performance liquid chromatography system consisted of a pump (Model
510. Millipore Waters, Milford, MA), a programmable absorbance ultraviolet detector (Model 785, Applied Biosystems, Foster City, CA) adjusted to a wavelength of 213 nm; the column (4.6 × 125 mm) was packed with Nucleosil 5-C18 (Phenomenex, Torrance, CA). The eluent solution consisted of 10 mm potassium phosphate, pH 7.4/acetonitrile/methanol (36:280: 200, v/v/v) and was delivered at a constant rate of 1.2 ml/min. Chromatograms were quantitated using a Hewlett-Packard 3396 A integrator (Hewlett-Packard, Avondale, PA). Least-squares linear regression analysis was used to determine the concentration range for 4-OH and T-OH midazolam. The peak-area ratios of 4-OH and T-OH midazolam were compared to an internal standard (diazepam). The calibration curves prepared with chemical standards were linear over a range of 30–800 ng/ml for both 4-OH and T-OH midazolam. The standard curve had an $r^2 = 0.998 ± 0.00082$ with a coefficient of variation of 6.2%. The lower limit of sensitivity of this assay was 15 ng/ml for both metabolites.

**Statistical Analysis**

Two-way analysis of variance was used to determine if phenobarbital induction or LPS administration affected plasma NOx levels or cytochrome P450 content and activity. Transformed (logarithm—base 10) data were used in the analysis to homogenize the variance (the max-min variance ratios of the transformed data were less than 5). An interaction term was included to test the hypothesis that a supraadditive or infraadditive effect of phenobarbital induction and endotoxemia was present. The effect of NOS inhibition with aminoguanidine and L-NMMA was assessed by unpaired $t$-test. Regression analysis using the least-squares method was performed with NOx levels as the independent variable and P450 content and metabolism as the dependent variables. Data are presented as mean ± standard error of mean (SEM) from six rats in each group. Duplicate measurements in each animal were averaged before means were calculated. $P < 0.05$ was taken as the level of significance.

**Results**

*P*50 content, a 2.3-fold increase in formaldehyde formation from ethylmorphine, and a 3.2-fold increase in the rate of 4-OH and T-OH midazolam formation from midazolam compared with that in nontreated animals (table 1). Lipopolysaccharide induced a septic state that reduced cytochrome P450 content and activity in both nontreated and phenobarbital-treated animals. Total microsomal cytochrome P450 content decreased 36.6 ± 2.8% in nontreated animals and 45.7 ± 1.5% in the phenobarbital-treated group. N-demethylation of ethylmorphine decreased 55.8 ± 1.2% in nontreated and 51.3 ± 1.6% in phenobarbital-treated rats after 12 h of endotoxemia. Similar results were obtained for hydroxylation of midazolam. 4-OH midazolam formation decreased 67 ± 0.9% in microsomes from nontreated and 63.4 ± 1.3% in phenobarbital-induced animals; T-OH midazolam formation decreased 65.7 ± 1.3% in nontreated rats and 59.8 ± 2.7% in phenobarbital-treated rats. No interaction between phenobarbital induction and endotoxemia was found for either the content ($P < 0.084$) or the drug metabolizing activity of ethylmorphine ($P < 0.429$) and midazolam ($P < 0.537$; table 2). Plasma NOx concentrations increased approximately 30-fold after 12 h of LPS administration in both nontreated and phenobarbital-treated rats (table 1).

**Nitric Oxide Synthase Inhibition with Aminoguanidine or N°-L-monomethyl arginine Prevents Loss of Cytochrome P450 Content and Activity In Vivo**

Coadministration with aminoguanidine or L-NMMA decreased, but did not completely suppress nitric oxide synthesis. Plasma NOx concentrations were still increased tenfold compared with control rats (table 1). Nevertheless, this degree of inhibition of nitric oxide synthesis was associated with a significant reduction in the effect of LPS on cytochrome P450 content. Inhib-

**Table 2. Analysis of Variance Results**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment (P value)</th>
<th>Interaction (P value)</th>
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<tbody>
<tr>
<td>NOX formation</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P450 content</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Formaldehyde formed</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>4-OH midazolam</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>1-OH midazolam</td>
<td>&lt;0.001</td>
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bition of nitric oxide synthesis also blunted the negative effect of LPS on P450 activity as reflected in the metabolism of ethylmorphine and midazolam (P < 0.002; table 1). The regression analysis supports the concept that nitric oxide plays an important role in the inhibition of cytochrome P450 content and activity during sepsis. Total cytochrome P450 content (fig. 1) and cytochrome P450-dependent metabolism of ethylmorphine (fig. 2) and midazolam (fig. 3) correlated with plasma NOx concentration in a highly significant inverse relationship (P < 0.001). P450 content and activity were greater with nitric oxide synthesis inhibition. Aminoguanidine or L-NMMA given to nontreated rats did not have an effect on P450 content or drug metabolism of ethylmorphine and midazolam (table 1).

Discussion

The results demonstrate an inverse relationship between nitric oxide synthesis (as reflected by NOx concentrations) and hepatic cytochrome P450 enzyme content after the administration of endotoxin to rats. This correlation was observed in nontreated animals as well as animals with phenobarbital-induced microsomal P450 activity. Partial inhibition of inducible nitric oxide synthase with aminoguanidine or L-NMMA produced intermediate plasma NOx concentrations and P450 content. Parallel changes occurred in the P450-dependent metabolism of ethylmorphine and midazolam, suggesting a relationship between nitric oxide release and the diminished hepatic drug metabolism seen in sepsis, possibly through a direct effect on cytochrome P450. The results of this study compare with those obtained by Khatsenko et al.\textsuperscript{3} who also demonstrated an inverse correlation between P450 activity and plasma nitrite concentrations in septic rats. In their study, these authors measured the activity of a different P450 isoform probe (CYP 2B1), and used a different nitric oxide synthase inhibitor, N\textsuperscript{\textdegree}-nitro-L-arginine methyl ester. Our results confirm those of Khatsenko et al.\textsuperscript{3} and extend the concept to include two different NOs inhibitors and two probes for different P450 specific isoforms responsible for metabolism of two major anesthetic drug categories (CYP 2C6/11 and CYP 3A1/2).

Genetic factors, hormones, exogenous and endogenous inducers, inhibitors, and allosteric activators of P450 regulate the expression and function of P450 isoymes in normal and disease states.\textsuperscript{20,21} A reduction during conditions of sepsis of the activity of the cytochrome P450 mixed-function oxidase system has been
observed after treatment of animals or hepatocytes in culture with immunomodulators, including cytokines, and cytokine-releasing agents.  As early as the 1960s, it was demonstrated that cytochrome P450 bind nitric oxide, an observation used in numerous studies to examine the heme ligand environment using electron paramagnetic resonance spectroscopy. The questions in this study were whether binding of nitric oxide to cytochrome P450 would affect the functional process of drug metabolism and whether its effects would be selective for different P450 isofoms.

Induction of hepatic microsomal drug metabolism was introduced in these experiments for two reasons. The first reason was to amplify the quantity of P450 present in the cell system used. The second reason was to consider possible differences between native and induced P450 isofoms in susceptibility to the effects of nitric oxide. Phenobarbital was selected as the inducing drug because of its clinical relevance, its ability to induce all examined subfamilies of P450, and lack of inherent toxicity. Phenobarbital administration to rats induces cytochrome P450 isofoms CYP 2B1, CYP 2C6/11, and CYP 3A1/2. These isofoms are known to metabolize hormones, drugs, and various chemicals. P450 isofoms CYP 2B1, CYP 2C6/11, and CYP 3A2 catalyze ethylmorphine N-demethylation to form formaldehyde. Endotoxin activates a series of inflammatory cells and their mediators, including macrophages, leukocytes, eicosaanoids (prostaglandins and leukotrienes), cytokines, and tumor necrosis factor-α, stimulating formation of inducible NOS in a variety of cells, including macrophages, endothelial cells, hepatocytes, and Kupffer cells. Many of the metabolic, hemodynamic, and inflammatory changes associated with Gram-negative septicemia are attributable to endotoxin. Nitric oxide synthase in the liver is regulated in part by hormones such as glucocorticoids, glucagon, and insulin. These regulatory mechanisms are preserved in an intact animal model.

This study demonstrates that nitric oxide can be produced in sufficient quantities in a living animal to inhibit cytochrome P450 function. Aminoguanidine and L-NMMA substantially suppressed LPS-induced nitric oxide production and associated decreases in cytochrome P450 content and enzymatic function. These concurrent effects suggest a causal linkage. Aminoguanidine is a NOS inhibitor reported to be more selective for the inducible NOS than the constitutive isofom of NOS. Both compounds inhibit by binding to the arginine site. Although aminoguanidine and L-NMMA did not fully prevent the LPS-induced decrease, we assume that the incomplete prevention of loss of microosomal content and activity in vitro is likely caused by incomplete blockade of nitric oxide formation (see plasma NOx concentrations; table 1). We also considered the possibility that L-NMMA might competitively inhibit ethylmorphine metabolism, independent of nitric oxide formation. A similar effect has been demonstrated for the NOS inhibitor, N\textsuperscript{\textregistered}-nitro-
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L-arginine methyl ester.9 Indeed, in vitro assays in our laboratory indicate that L-NMMA in the millimolar concentration reduces formaldehyde formation from ethylmorphine. This did not occur in the current study, probably because of the relatively small amounts of L-NMMA administered to animals (μmoles/kg) compared with the approximately 1,000-fold greater concentrations required to produce direct effects on P450 enzyme function in vitro.

Direct exposure of rat hepatic microsomes to nitric oxide inhibits P450 CYP 1A1,7,10 CYP 2B1/2,7,9 and CYP 3A1.29 The results of our study in intact animals confirm the results from previous studies in isolated tissue culture.7,9,10,29 Inhibition of cytochrome P450 activity by nitric oxide in livers of animals not treated or pretreated with phenobarbital29 suggests that nitric oxide inhibits the activity of cytochrome P450 derived from more than one molecular family. Our results confirm that this is true for at least two more probes for metabolism of P450 isofoms. This supports the view that nitric oxide inhibition is not selective for specific P450 isofoms.

Bertini et al.30 provide evidence of a dual mechanism for the depression of liver cytochrome P450 by LPS and tumor necrosis factor-α. One pathway is mediated by interleukin-1, a potent regulator of immunologic response and inflammatory reactions that inhibits P440; a second, interleukin-1-independent pathway is involved in the acute phase response.30 Our data suggest that nitric oxide may participate directly in the interleukin-1-independent pathway.

Nitric oxide interacts with P450 by binding to the intact hemoprotein. Introduction of nitric oxide to P450 proteins has previously been shown to produce strong iron-nitrosyl complexes with both the ferric and ferrous forms of P450.23 Nitric oxide reacts with hemoprotein at near diffusion-controlled rates,31 and inhibits cytochrome P450 activity by both reversible and irreversible mechanisms.7 Therefore, the inhibition may be explained by binding of nitric oxide to the heme moiety of the P450s, subsequently preventing the binding of oxygen that normally occurs in the catalytic cycle.

Another interpretation of the results in this study might be the suggestion that nitric oxide could impair P450 function by nitration, thus promoting degradation of either heme or apoprotein moieties of P450.32 It has been reported that the transcription of cytochrome P450 genes is decreased by immunostimulants and cytokines32 and has been demonstrated at the mRNA level for CYP 1A1, at least under acute conditions in vitro.10 Under the relatively acute conditions of our experimental protocols, however, we believe the LPS effect on drug metabolism is more likely mediated by functional inactivation of preexisting cytochromes than by inhibiting the process of cytochrome synthesis de novo. Lipopolysaccharide was administered only during the last 12 h after a 96-h microsomal induction period, when P450 content and activity already approached maximum values.

Our findings complements previously published observations5,7,9,10,25,26,27,29,31,32 that all support the following mechanism for LPS inhibition of hepatic cytochrome P450-dependent metabolism: (1) injection of LPS in vitro under conditions of unchanged hormonal and mediator regulation induces the release of a diverse array of cytokines and immunostimulants, which, in turn, induce NOS activity in Kupffer cells and hepatocytes, thereby producing nitric oxide; (2) nitric oxide binds to heme iron in hepatic cytochrome P450 and prevents oxygen binding, thereby blocking enzyme activity; (3) nitric oxide also may promote degradation of cytochrome P450 by nitrosylation of heme or nitratation of thiols present in P450 apoprotein, or it may impair transcription of heme or thiols present in P450 apoprotein; and (4) in vitro inhibition of NOS substantially prevents the loss of total cytochrome P450 content and the reduced activity of isofoms CYP 2B1, CYP 2C6/11, and CYP 3A1/2, as reflected in the metabolism of ethylmorphine and midazolam.

Clinical Implications

Infection in patients commonly impairs drug metabolism as a result of compromised metabolism of the cytochrome P450. Additionally, drugs such as cyclosporine, cimetidine, nifedipine, and quinidine compete with midazolam for CYP 3A in humans and thus prolong the effect of the hypnotic drug.33 The results of this investigation suggest that administration of inhibitors of nitric oxide production in septic patients, in addition to treating the vascular changes associated with this condition, also may offer novel, improved strategies for the choices of drugs and doses used in the treatment of patients with severe sepsis based on the relative role of cytochrome P450 in the disposition of these drugs.

In summary, we have demonstrated a relationship between nitric oxide production during sepsis and reduced cytochrome P450 content and activity in intact rats. This relationship may underlie altered drug metabolism in septic humans. Viewed more broadly, the
results from this study raise further questions about possible roles for nitric oxide in the regulation of normal drug metabolism.

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References


