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Down-Regulation of Cytochrome P450 mRNAs and Proteins in Mice Lacking a Functional NOS2 Gene

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ABSTRACT

Endotoxemia results in both the down-regulation of multiple cytochrome P450 genes and the induction of inducible nitric oxide synthase (NOS2). The nitric oxide (NO) released during inflammation has been implicated as the mediator of the decreased catalytic activity and expression of several cytochrome P450 isozymes. We examined the role of NO in the decreases in both gene expression and activity of three P450s in endotoxemic parental and NOS2 knockout mice. Twenty-four hours of endotoxin (LPS) treatment significantly suppressed CYP2C29 and CYP3A11 mRNA expression in both the parental and NOS2 knockout strains. Microsomal CYP2E1, CYP2C-like, and CYP3A-like protein levels were also decreased in both strains of mouse. Similar results were obtained in parental strain endotoxemic mice co-administered the NOS inhibitor aminoguanidine. Six hours after LPS treatment, there was an NO-dependent decrease in testosterone 6β-hydroxylase activity, because no decreases in activity were observed in the NOS2 knockout mice or in mice co-administered aminoguanidine. LPS also evoked decreases in testosterone 15α- and 16β-hydroxylase activity after 24 hr that were observed in the parental strain and not in NOS2 knockout mice. Our results demonstrate that the down-regulation of CYP2C-like, CYP3A-like and CYP2E1 proteins and mRNAs, in the endotoxemic mouse can occur independently of NO production. We do, however, show that the NO released during endotoxemia is capable of causing decreases in some cytochrome P450 catalytic activities.

Administration of LPS to animals decreases the total hepatic P450 content, impairs P450 catalytic activity, and down-regulates the expression of several P450 subfamilies (Gorodischer *et al.*, 1976; Morgan, 1997). In male rats injected with LPS, the levels of constitutively expressed P450 2C11, 2E1, and 3A2 mRNA and protein are suppressed (Morgan, 1989; Sewer *et al.*, 1996). Both *in vitro* and *in vivo* evidence indicated that cytokines such as interleukin (IL)- 1 and IL-6 (Chen *et al.*, 1992; Morgan *et al.*, 1994; Chen *et al.*, 1995) as well as interferons (Morgan and Norman, 1990; Cribb *et al.*, 1994; Chen *et al.*, 1995) can act directly on the hepatocyte to mimic some of the changes in P450 gene expression. The effects of LPS on P450 expression have also been shown in mice where similar decreases are seen in both P450 expression and activity (Stanley *et al.*, 1988). Injecting mice with cytokines (Cantoni *et al.*, 1995), and vaccines (Ansher and Thompson, 1994) which elicit an inflammatory response also suppress P450 expression.

LPS administration induces the expression of NOS2 and the subsequent release of NO both in animals (Curran *et al.*, 1990; Geller *et al.*, 1995; Sewer *et al.*, 1997) and in cultured primary hepatocytes (Geller *et al.*, 1993, 1995; Sewer and Morgan, 1997). NO is capable of binding to the heme moiety and inhibiting P450 catalytic activity (Khatsenko *et al.*, 1993; Wink *et al.*, 1993; Stadler *et al.*, 1994; Osawa *et al.*, 1995).

Several laboratories have proposed that NO is also the mediator of the decreases seen in P450 expression in cytokine and LPS models of inflammation based on the ability of exogenously administered NO to down-regulate P450 gene expression and on attenuation of the down-regulation by NO inhibitors (Stadler *et al.*, 1994; Carlson and Billings, 1996; Khatsenko and Kikkawa, 1997). Khatsenko and Kikkawa have reported that NOS inhibitors are capable of partially reversing the decreases in P450 2C11, 3A2, 1A2, and 2B1/2 activities, protein, and mRNA expression in rats treated with LPS (Khatsenko and Kikkawa, 1997). *In vitro* studies have also reported that NO is the mediator of the decreases seen in P450 2C11, 3A2, 2B1/2, and 1A2 proteins after administration of cytokines in primary hepatocytes (Carlson and Billings, 1996). Our laboratory, however, has provided both *in vivo* (Sewer *et al.*, 1997; Sewer and Morgan, 1998) and *in vitro* (Sewer and Morgan, 1997) evidence for NO-independent down-regulation of several constitutively expressed P450

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ABBREVIATIONS: LPS, bacterial endotoxin; NOS2, inducible nitric oxide synthase; NO, nitric oxide; P450, cytochrome P450; GAP, glyceraldehyde-3-phosphate dehydrogenase; AG, aminoguanidine; IL, interleukin; TLC, thin layer chromatography.

isozymes. Monshouwer *et al.* similarly reported no effect of NOS inhibition on cytokine-evoked decreases in P450-catalyzed steroid hydroxylase activities in cultured pig hepatocytes (Monshouwer *et al.*, 1996). Using interferon inducers, Hodgson and Renton reported no involvement of NO in the down-regulation of P450 content and activity in the mouse (Hodgson and Renton, 1995).

To attempt to resolve the discrepancies between the above cited studies and to confirm findings of previous studies where the use of NOS inhibitors *in vivo* confounded interpretation of the data, we studied the effects of LPS in mice with selective inactivation of the NOS2 gene. Our findings demonstrate that the down-regulation of P450 2C29, 2E1, and 3A11 mRNA expression occurs independently of NO involvement.

Materials and Methods

Animals and treatments. Male B6,129 (parental strain) and B6,129-NOS2 (NOS2 knockout) (Jackson Laboratories, Bar Harbor, ME) mice, 8 weeks old, were used. The animals were allowed free access to food and water at all times and were allowed to acclimatize in the facilities for 7 days before use. Chromatographically purified *Escherichia coli* LPS, serotype 0127:B8 (Sigma Chemical, St. Louis, MO) was dissolved in sterile 0.9% saline and intraperitoneally injected into both parental and NOS2 knockout strains at a dose of 1 mg/kg body weight. Control animals received an equivalent volume of sterile saline. In another experiment, aminoguanidine [AG (133 mg/kg, intraperitoneally)] was administered to B6,129 mice beginning 30 min after a single injection of LPS or saline and every 4 hr thereafter for 6 or 24 hr. Animals that did not receive AG were injected with saline every 4 hr. At 6 and 24 hr after injection of LPS, animals were killed by CO_2 asphyxiation. These procedures were approved by the Institutional Animal Care and Use Committee of Emory University.

Analysis of plasma nitrite and nitrate concentration. The stable end products of L-arginine-dependent NO synthesis, nitrate and nitrite, were measured in the plasma using a colorimetric method based on the Griess reaction (Tracey *et al.*, 1995; Grisham *et al.*, 1996). Briefly, aliquots of plasma were added to 35% sulfosalicylic acid and vortexed every 5 min for 30 min to deproteinize samples. The samples were then centrifuged at 10,000 $\times$ *g* at 4° for 15 min. An aliquot of the supernatant was taken for nitrite and nitrate analysis. Twenty microliters of plasma sample were mixed with 20 μl of 0.31 M phosphate buffer, pH 7.5, 10 μl of 0.1 mM FAD, 10 μl of 1 mM NADPH, 10 ml of nitrate reductase (10 units/ml), and 30 μl of water in a 96-well plate. The reaction was allowed to proceed for 1 hr in the dark. The percent conversion of nitrate to nitrite was 98%. To each sample, 1 μl of lactate dehydrogenase (1500 units/ml) and 10 μl of 100 mM pyruvic acid were added and incubated for 15 min at 37°. The samples were then mixed with an equivalent volume of Griess reagent [1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine and incubated for an additional 10 min at room temperature. Nitrite levels were determined colorimetrically at 550 nm with a Thermomax microplate reader (Molecular Devices, Menlo Park, CA) and a sodium nitrite standard curve.

Preparation of microsomes and total RNA. Livers were excised and perfused with cold 1.15% KCl. Pyrophosphate-washed microsomes were prepared as described by Haugen and Coon (1976). Total RNA was prepared according to the method of Chomczynski and Sacchi (1987). Purified microsomes and total RNA were stored at −80°.

RNA Northern blots. Total RNA concentration was determined spectrophotometrically at 260 nm. Northern blotting was performed as described by Sambrook *et al.* (Sambrook *et al.*, 1989). In short, formaldehyde-containing agarose gels (1.5%) were used to subject denatured RNA to electrophoresis at 70 V for 4 hr. The RNA was blotted onto MagnaGraph nylon transfer membrane filters (Micron Separations, Westbrook, MA) overnight and was fixed by both UV irradiation and baking at 80°. The blots were hybridized to cDNA or oligonucleotide probes, washed, and subjected to autoradiography. A cDNA probe for GAP was used to control for loading and transfer artifacts.

cDNA and oligonucleotide probes. Relative levels of CYP2E1 mRNA were quantified by Northern blot assay using a full-length cDNA for rat CYP2E1, as described previously (18). Relative abundances of CYP2C29 mRNA was measured using an oligonucleotide (5′-ggc cag gcc ctc tcc agc aca aat ccg ttt-3′) complementary to nucleotides 1301–1330 of the published sequence in Genebank (Accession number D17674). The CYP3A11 oligonucleotide probe used (5′-tgt ccg atg ttc tta gac act gcc ttt ctg-3′) corresponds to nucleotides 1631–1660 (Genebank Accession number X60452). The Megaprime labeling kit (Amersham, Arlington Heights, IL) and [α-^{32}P]dCTP was used to radiolabel the cDNA probe. T4 polynucleotide kinase and [γ-^{32}P]ATP were used to 5′-end radiolabel oligonucleotide probes. Blots probed with the CYP2E1 cDNA probe were hybridized at 42° and washed at 62°. Blots probed with oligonucleotides to CYP2C29 and CYP3A11 were hybridized and washed at 45° as described previously for P450 4A rat oligonucleotides (Sewer *et al.,* 1996). Bound ^{32}P-labeled probes were detected by autoradiography and quantified by analysis on either a Personal laser densitometer (Molecular Dynamics, Sunnyvale, CA) or via PhosphorImager scanning (Molecular Dynamics). All assays were performed under previously established conditions of linearity between the amount of the target mRNA on the filter and the densitometric response.

Assays of hepatic microsomes. Total microsomal protein was determined by the method of Lowry *et al.* (1951). P450 concentrations were determined from the CO difference spectrum of the reduced protein at 450 nm (Omura and Sato, 1964).

Western blot immunoassays. The relative levels of various P450 isozymes in the microsomes were measured by Western blotting. Proteins were separated by polyacrylamide gel electrophoresis (7.5% polyacrylamide) in the presence of sodium dodecyl sulfate and were blotted electrophoretically onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The antibodies to rat P450s 2E1 and 3A2 were generous gifts from Dr. Magnus Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden) and Dr. James Halpert (University of Arizona, Tucson, AZ), respectively. A nonimmunoabsorbed antibody to rat P450 2C11 was used to detect any P450 2C-like expression (Morgan *et al.*, 1985). The binding of all antibodies were detected using the electrochemiluminescence detection system (Amersham Life Sciences) according to the manufacturer's instructions. The intensities of the stained bands were measured by laser densitometry, and were determined to be proportional to the amount of antigen loaded on the blot within the experimental range used.

Microsomal P450 activities. Control assays were performed to ensure linearity of both time and protein concentration. Testosterone 6β-, 7α-, 15α-, 16α- and 16β- hydroxylase activities were determined via TLC (Ciaccio and Halpert, 1989; Waxman, 1991). Briefly, 50 μg of microsomal protein was preincubated for 5 min at 37° in buffered solutions containing 250 μM [4-^{14}C]testosterone. The reaction was started by the addition of 1 mM NADPH and the assay was allowed to proceed for 10 min. The reaction was stopped by the addition of 50 μl of tetrahydrofuran and aliquots were spotted on the preabsorbent loading zone of a silica gel TLC plate [250 μM, Si250F (19C); J.T. Baker, Phillipsburg, NJ]. The plates were developed twice in dichloromethane/acetone (4:1, v/v) and the radioactive areas on the plates were scraped and quantified by liquid scintillation counting. Metabolites were localized by autoradiography and identified by comparison with unlabeled standards for 6β-, 7α-, 15α-, 16α-, and 16β-hydroxytestosterone (Steraloids, Wilton, NH). The *p*-nitrophenol hydroxylation activity of P450 2E1 was assayed spectrophotometri-

cally (Koop, 1986). Microsomal protein (300 μg) was preincubated in a phosphate buffer containing 0.2 mM *p*-nitrophenol and ascorbic acid. NADPH (10 mM) was added to initiate the reaction. The reaction was allowed to proceed for 10 min and was then stopped by the addition of 1.5 N perchloric acid and placed on ice for 10 min. After a 10-min spin at 4000 rpm at 4°, the supernatant was mixed with 10 N NaOH and the absorbance read at 510 nm.

Statistical analysis. Data from Northern and Western blot assays and activity assays were expressed as the percentage of the mean of the control group in each experiment. One-way analysis of variance and the Neumann-Keuls test were used to determine differences among treatment groups.

Results

Plasma NO in NOS2 knockout mice. The oxidation products of NO were elevated in the plasma of the parental strain but not in the NOS2 knockout strain treated for 6 hr with LPS (Table 1). No significant elevation of plasma NOx concentrations were seen in either strain 24 hr after LPS treatment.

LPS-evoked suppression of P450 mRNAs in NOS2 knockout mice. Northern blot analysis was carried out on the total RNA prepared from both strains of mice treated for 6 and 24 hr with LPS. Fig. 1A shows representative samples of RNA isolated after 24 hr of LPS treatment from Northern blots probed for CYP2C29, CYP2E1, and CYP3A11. The expression of CYP2C29 and CYP3A11 were suppressed by LPS in both the parent and the NOS2 knockout strain (Fig. 1A). LPS suppressed CYP2C29 to 53% of saline treated mice in both the parent and the NOS2 knockout. CYP3A11 expression was down-regulated to 38% and 45% in the parent and the knockout, respectively, after 24 hr of LPS treatment. No significant effects of LPS were seen on CYP2E1 in either strain of mouse after 24 hr. A trend toward decreased mRNA expression was seen in both parent and NOS2 knockout strains after 6 hr of LPS treatment, but because of variability in the data (not shown), no significant effect was observed.

LPS down-regulates P450 protein expression in NOS2 knockout mice. A polyclonal antibody to rat CYP2C11 that cross-reacts with other P450 2C enzymes (Morgan *et al.*, 1985) was used to detect P450 2C-like protein expression in the microsomes of parent and NOS2 knockout mice treated for 6 and 24 hr with LPS. Two main bands were detected, both of which were decreased by about 40% in both strains of mice after 24 hr of LPS treatment (Fig. 2). Twenty-four hours after LPS injection, P450 2E1 expression was decreased to 50% and 62% of control in the parent and the NOS2 knockout, respectively. Similarly, P450 3A-like immunoreactivity was down-regulated to 49% and 55% of saline treated mice in endotoxemic parent and knockout mice, respectively (Fig. 2). Six hr of LPS exposure did not significantly affect the protein levels of any of the isoforms examined (data not shown). Total P450 content was significantly decreased in both the parent and knockout strains after 24 hr of exposure to LPS (Fig. 3). No significant effect of LPS on total P450 content was seen after 6 hr in either mouse strain.

Microsomal hydroxylase activities in endotoxemic NOS2 knockout mice. TLC was used to measure the microsomal rates of formation of 6β-, 7α-, 15α-, 16α-, and 16β-hydroxy-testosterone (Table 2). Six hr after injecting LPS, 6β- and 16β-testosterone hydroxylase activities were decreased in the B6,129 parental strain but not in the B6,129-NOS2 knockout strain (Table 2). Interestingly, the basal rate of formation of 6β-hydroxytestosterone was significantly less in the knockout strain (Table 2) in the 6-hr but not the 24-hr study. Treating the parental strain with LPS for 6 hr resulted in a 1.4-fold increase in 7α-testosterone hydroxylase activity (Table 2), whereas the activity was decreased in the NOS2 knockout strain. Testosterone 16α- and 15α-hydroxylase activities were unaffected at 6 hr in the parental strain, but 16α-hydroxytestosterone activity was decreased in the knockout animals. Twenty-four hours after LPS treatment,

TABLE 1

Effect of LPS on the plasma NO concentrations in parental and NOS2 knockout mice

B6,129 (parental strain) and B6,129-NOS2 (NOS2 knockout strain) mice were injected with LPS and killed 6 or 24 hr later. Plasma was collected at time of death and analyzed for concentrations of nitrate and nitrite via the Griess reaction. The data presented in the table represent the means ± standard error of five mice per treatment group.

Group	NO	
	6 hr	24 hr
	μM	
Parental saline	49.5 ± 18.4	35.7 ± 23.2
Parental LPS	207.8 ± 33.6*	54.8 ± 30.1
Knockout saline	32.6 ± 21.5	28.4 ± 26.7
Knockout LPS	41.8 ± 17.3	36.3 ± 27.4

* Significantly different from saline-treated control group, $p < 0.05$.

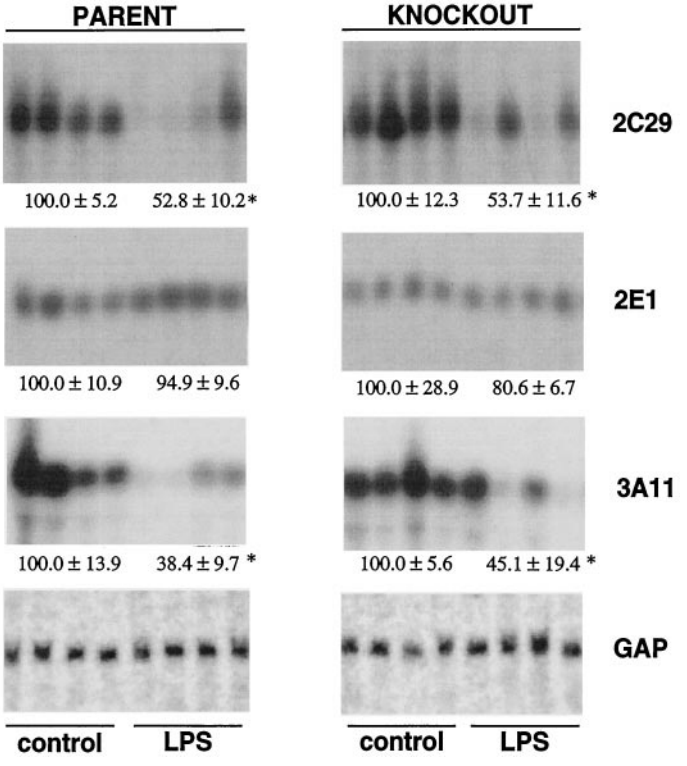

Fig. 1. Effect of LPS on P450 mRNA expression in parental and NOS2 knockout mice. Total RNA was isolated from mice treated for 6 and 24 hr with LPS and subjected to Northern blot analysis as described in Materials and Methods. The autoradiograms presented are four representative samples from each treatment group isolated 24 hr after LPS or saline injection. Nominally, 10 μg of total RNA was loaded in each lane. The blot was probed sequentially for CYP2C29, CYP2E1, and CYP3A11 mRNA expression and for GAP to ensure equal loading. The blots were normalized to GAP mRNA expression. The data presented under the blots represent the mean ± standard error of five mice per treatment group; results are expressed as a percentage of the saline-treated group mean. *, significantly different from saline treated mice, $p < 0.05$.

the hydroxylation of testosterone at the 15α- and 16β-positions was decreased in the parental strain but not in the NOS2 knockout mouse (Table 2). 6β-testosterone hydroxylase activity declined to 75% of control in both the parental and the knockout strains 24 hr after LPS injection (Table 2). No significant changes in testosterone hydroxylation were observed after 24 hr of LPS exposure at the other positions.

As shown in Table 2, 24 hr of exposure to LPS resulted in decreased formation of 4-nitrocatechol from *p*-nitrophenol in both parent and knockout strains to 77% and 64% of control levels, respectively. There were no significant changes in *p*-nitrophenol hydroxylase activity 6 hr after LPS injection (Table 2).

Effect of NOS2 inhibition on the LPS-evoked down-regulation of P450s in the parental strain. B6,129 mice were treated with 1 mg/ml LPS followed by repeated injections of 133 mg/ml AG (a relatively specific inhibitor of NOS2) every 4 hrs for 6- or 24 hr. LPS treatment suppressed CYP2C29 to 37% of the levels seen in saline-treated control rats after 24 hr (Table 3). CYP3A11 mRNA expression was significantly suppressed after 6 and 24 hr to 54% and 16% of the levels seen in control animals (Table 3). Similarly, LPS significantly decreased both P450 2C- and 3A-like protein levels. AG did not have any effect on the LPS-evoked decreases in P450 mRNA and protein expression despite its ability to inhibit NO synthesis (Table 3). As seen in our previous studies in the rat (Sewer and Morgan, 1998). AG alone did decrease P450 3A-like protein expression and total hepatic P450 content (Table 3).

At both 6 and 24 hr after LPS treatment, microsomal testosterone 6β-hydroxylase activity was significantly decreased; however, co-administration of AG was only effective in reversing the LPS-evoked decline in activity at the 6 hr time point (Table 4). The rates of formation of 7α-, 15α-, 16β-, and 16α-hydroxytestosterone were only decreased by LPS at the 24-hr time point in the endotoxemic mouse. AG did not have any effect on 7α-, 15α-, and 16β-hydroxylase activities, but was able to reverse the LPS-stimulated decrease in 16α-hydroxylase activity. AG, when administered alone for 24 hr, significantly decreased 6β-, 15α-, and 16β-hydroxylase activities (Table 4).

p-Nitrophenol hydroxylation was measured as an indicator of P450 2E1 activity. The rate of formation of 4-nitrocatechol was significantly inhibited in microsomes isolated from mice treated with LPS for 6 and 24 hr (Table 4). Co-administering AG for 24 hr partially reversed the LPS-stimulated attenuation of *p*-nitrophenol hydroxylase activity. Injecting mice with AG alone for 6 hr had an inhibitory effect on P450 2E1-mediated hydroxylase activity (Table 4).

Discussion

The results presented herein demonstrate that suppression of P450 gene expression and protein levels by LPS does not require NO generation by hepatic NOS2, and are in agreement with our previous studies in endotoxemic rats (Sewer and Morgan, 1998) and in cultured primary rat hepatocytes (Sewer and Morgan, 1997). They are in contrast with a considerable body of data from other laboratories (Stadler *et al.*, 1994; Carlson and Billings, 1996; Khatsenko and

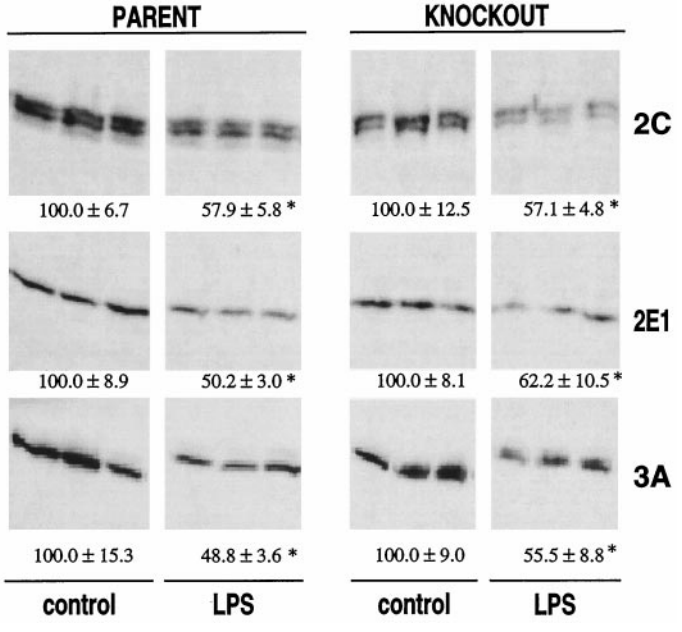

Fig. 2. Effect of LPS on hepatic microsomal protein levels in parental and NOS2 knockout mice. Microsomal protein was isolated from the livers of mice treated for 24 hr as described in Materials and Methods. Equal amounts (2 μg) of protein were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and assayed for P450 2C-like, 2E1, and 3A-like apoproteins using antibodies to rat P450 2C11, 2E1, and 3A2, respectively. Three samples from each treatment group are presented. Densitometric analysis was performed on Western blots. *Numerical values*, mean ± standard error of five mice per treatment group; results are expressed as a percentage of the saline-treated group mean. *, significantly different from saline treated mice, $p < 0.05$.

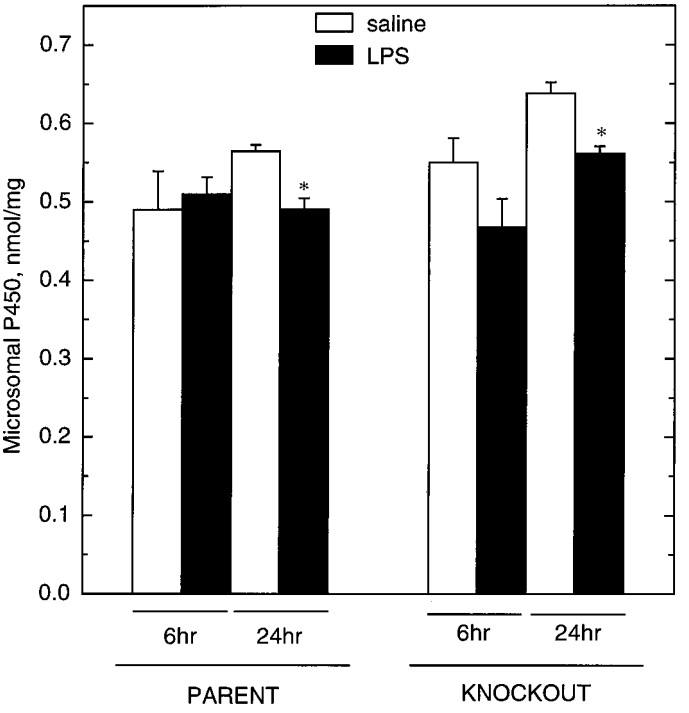

Fig. 3. Effect of LPS on total hepatic microsomal P450 content in parental and NOS2 knockout mice. Microsomal protein was isolated from the livers and total P450 content was assayed from the CO difference spectrum of the reduced protein as described in Materials and Methods. The data presented represent the mean ± standard error of five mice per treatment group; results are expressed as nanomoles of P450 per milligram of protein. *, significantly different from saline treated mice, $p < 0.05$.

Kikkawa, 1997), who found that NOS inhibitors attenuate the down-regulation of various P450 proteins and RNAs; for example, Khatsenko and Kikkawa found that the down-regulation of P450 2C11, 2B1/2, 3A2, and 1A2 mRNA and protein in endotoxemic rats was blocked by co-administration of the NOS inhibitor L-nitro-arginine methyl ester (Khatsenko and Kikkawa, 1997). Similarly, in an *in vitro* study it was reported that *N*-methyl arginine (NOS inhibitor) could reverse the decreased protein levels of P450 2C11, 2B1/2, 3A2, and 1A2 seen after treating cultured primary hepatocytes with cytokines and LPS (Carlson and Billings, 1996).

In the present study, we detected no elevation of NOx in

TABLE 2

Effect of LPS on the microsomal P450 hydroxylase activities in parental and NOS2 knockout mice

B6,129 (parental strain) and B6,129-NOS2 (NOS2 knockout strain) mice were injected with LPS and killed 6 or 24 hr later. Hepatic microsomal protein was isolated and analyzed as described in Materials and Methods. The data presented in the table represent the mean ± standard error of five mice per treatment group.

	Time	Parent		NOS2 Knockout	
		Saline	LPS	Saline	LPS
	hr	*nmol/mg/min*			
Testosterone hydroxylation					
6β	6	1.48 ± 0.02	1.22 ± 0.01*	0.59 ± 0.07	0.48 ± 0.06
	24	1.04 ± 0.04	0.81 ± 0.05*	1.25 ± 0.08	0.83 ± 0.08*
7α	6	0.59 ± 0.03	0.89 ± 0.03*	1.08 ± 0.21	0.53 ± 0.04*
	24	0.62 ± 0.05	0.58 ± 0.10	0.76 ± 0.08	0.66 ± 0.05
15α	6	0.25 ± 0.01	0.35 ± 0.03	0.60 ± 0.06	0.37 ± 0.05
	24	0.52 ± 0.04	0.28 ± 0.03*	0.43 ± 0.11	0.43 ± 0.03
16α	6	0.48 ± 0.06	0.47 ± 0.06	0.79 ± 0.06	0.44 ± 0.06*
	24	0.28 ± 0.04	0.40 ± 0.07	0.45 ± 0.03	0.45 ± 0.08
16β	6	1.27 ± 0.06	0.90 ± 0.05*	1.88 ± 0.31	1.36 ± 0.13
	24	0.88 ± 0.04	0.52 ± 0.08*	0.85 ± 0.09	0.96 ± 0.15
p-Nitrophenol hydroxylation					
	6	2.15 ± 0.32	2.39 ± 0.41	1.32 ± 0.14	1.90 ± 0.24
	24	3.61 ± 0.19	2.86 ± 0.13*	3.93 ± 0.29	2.54 ± 0.14*

* Significantly different from control, $p < 0.05$.

TABLE 3

The effect of AG treatment on LPS-stimulated decreases in total P450 content and P450 mRNAs and protein expression

Mice were injected with LPS followed by repeated injections of AG every 4 hr for 6 and 24 hr. Animals were killed at the indicated times after LPS injection; plasma, hepatic microsomal protein, and total RNA were isolated and analyzed. The data presented in the table represent the mean ± standard error of five mice per treatment group and are expressed as percentages of the saline-treated control group mean.

	Time	LPS	LPS + AG	AG
	hr			
NO	6	241.5 ± 23.6*	112.0 ± 18.6	98.2 ± 24.6
	24	122.8 ± 11.2	127 ± 23.2	108.7 ± 17.2
Cyp2c29	6	73.0 ± 13.4	61.9 ± 11.7	123.8 ± 18.9
mRNA	24	36.8 ± 8.7*	52.4 ± 12.6*	132.2 ± 22.4
Cyp3a11	6	54.1 ± 6.3*	67.2 ± 13.3*	139.9 ± 34.9
mRNA	24	16.2 ± 5.4	25.7 ± 10.9	160.2 ± 43.0
2C-like protein	24	64.4 ± 3.1*	42.0 ± 11.9*	98.9 ± 16.6
3A-like protein	24	27.7 ± 7.4*	18.1 ± 3.0*	57.0 ± 10.9*
Total P450	6	75.8 ± 6.9*	72.6 ± 6.7*	78.9 ± 6.2*
	24	57.5 ± 5.2*	46.2 ± 3.1*	74.8 ± 8.9*

* Significantly different from control, $p < 0.05$.

TABLE 4

The effect of AG treatment on LPS-stimulated decrease in microsomal P450 hydroxylase activities

Mice were injected with LPS followed by repeated injections of AG every 4 hr for 6 and 24 hr. Animals were killed at the appropriate times and hepatic microsomal protein isolated and analyzed as described in Materials and Methods. The data presented in the table represent the mean ± standard error of five mice per treatment group.

	Time	Saline	LPS	LPS + AG	AG
	hr	*nmol/mg/min*			
Testosterone hydroxylation					
6β	6	1.15 ± 0.03	0.85 ± 0.04*	1.23 ± 0.06	1.49 ± 0.26
	24	1.52 ± 0.17	0.76 ± 0.13*	0.29 ± 0.12*	0.71 ± 0.09*
7α	6	0.65 ± 0.01	0.58 ± 0.06	0.54 ± 0.15	0.48 ± 0.10
	24	0.55 ± 0.09	0.13 ± 0.03*	0.22 ± 0.03*	0.40 ± 0.06
15α	6	0.40 ± 0.05	0.22 ± 0.07	0.28 ± 0.08	0.36 ± 0.09
	24	0.36 ± 0.08	0.22 ± 0.01*	0.12 ± 0.03*	0.19 ± 0.02*
16α	6	0.60 ± 0.03	0.44 ± 0.07	0.47 ± 0.07	0.52 ± 0.12
	24	0.19 ± 0.06	0.04 ± 0.01*	0.19 ± 0.05	0.18 ± 0.07
16β	6	0.89 ± 0.09	0.84 ± 0.06	0.59 ± 0.06	0.40 ± 0.12*
	24	0.82 ± 0.06	0.49 ± 0.02*	0.14 ± 0.02*	0.12 ± 0.01*
p-Nitrophenol hydroxylation					
	6	1.51 ± 0.05	1.16 ± 0.12*	0.75 ± 0.11*	0.91 ± 0.04*
	24	1.36 ± 0.24	0.65 ± 0.07*	0.98 ± 0.13	1.21 ± 0.12

* Significantly different from control, $p < 0.05$.

plasma 24 hr after LPS injection, whereas Khatsenko and Kikkawa (1997) found that LPS-treated rats had high levels of NOx in plasma at this time. The persistence of NO in their model could have caused effects on P450 expression that are not present in ours, but because they only measured plasma NOx levels at 24 hr, it is not clear whether the production of NO in their rats is more persistent with respect to our mice, or is delayed. They also did not demonstrate that their treatments with NOS inhibitors were effective in preventing NO production at earlier time points. In rats, we found a time course of plasma NOx levels that was consistent with the present data in mice (Sewer and Morgan, 1998). Therefore, the reason for these differences in time course of NO production observed in the two laboratories is not simply a species difference. It is also unlikely to be related to the doses of LPS used [1 mg/kg here and in our rat study (Sewer and Morgan, 1998), and 1.2 mg/kg by Khatsenko and Kikkawa (1997)], which were very similar.

A critical question in trying to resolve the discrepant reports from different laboratories regarding the role of NOS-derived NO in P450 down-regulation lies in the specificities and efficacies of the NOS inhibitor treatments. Thus, nonspecific effects of NOS inhibitors (present study, Khatsenko and Kikkawa, 1997) could lead to an erroneous interpretation that NOS is involved in a given effect. For instance, in our previous work in rats (Sewer and Morgan, 1998) and in the present study, the repeated administration of AG alone decreased the levels of P450 3A subfamily proteins. On the other hand, incomplete inhibition of NOS2 by drug treatment could lead one to erroneously conclude that NO is not involved. The use in the present study of mice lacking a functional NOS2 gene provided a means to address the role of NO in P450 down-regulation without these potentially confounding problems. Clearly, the down-regulation by LPS of hepatic expression of the P450 mRNAs and proteins examined in the present study was the same in both the parent and knockout mice. The results in the NOS2-knockout mice were also in good agreement with our experiments in mice treated with AG to inhibit NOS2. Therefore, we conclude that NO generated from hepatic NOS2 is not required for down-regulation of these P450s. The possibility remains that other P450s could be down-regulated in an NO-dependent manner.

The lack of widely available specific antibodies to murine P450s necessitates the use of antibodies prepared to rat enzymes to probe for cross-reactive mouse proteins. Because there is only one member of the 2E subfamily in either species, there is some confidence that this form was measured specifically in murine microsomes. In contrast, we do not know the identities of the proteins recognized by the antibodies to rat CYP2C11 and rat CYP3A2. Thus, it is not possible to conclude that the decreases in these proteins are caused by decreases in their specific mRNAs. Despite the fact that CYP2E1 mRNA was not affected 6 or 24 hr after LPS treatment in the mouse, 2E1 protein was decreased at the 24 hr time point. However, we have found in the rat that CYP2E1 mRNA is transiently down-regulated, and returns to control levels within 24 hr (Sewer *et al.*, 1996). Therefore, in the absence of a measurement of CYP2E1 mRNA at an intermediate time point, it is not possible to determine whether the down-regulation of this protein in the mouse is caused by pre- or post-translational mechanisms.

The decrease in total P450 content of hepatic murine microsomes was less than that of the individual forms measured by Western blot. As we noted previously (Morgan, 1989, 1997), this is probably caused by a selective suppression of some P450s, whereas others may be unaffected or induced (Sewer *et al.*, 1997).

It has been shown that exogenously applied NO is capable of both reversible and irreversible inhibition of P450 catalytic activities (Wink *et al.*, 1993, Minamiyama *et al.*, 1997). This occurs by binding to the P450 heme moiety (Wink *et al.*, 1993) and by nitrosylation, respectively (Quaroni, 1996). Because P450-dependent activities are reduced in microsomes of LPS-treated rats after only 6 hr (at which point there are no detectable changes in levels of P450 proteins), we have speculated that NO may be involved in inhibition of P450 catalytic activities at early time points. Therefore, we examined the effects of LPS on P450-dependent activities in wild type mice, NOS2 knockout mice, and mice treated with AG. As discussed in detail below, these results indicated that the involvement of NO in this phenomenon depends on the activity in question, and presumably on the P450 enzyme catalyzing the individual reaction. However, because of a lack of well-characterized antibodies and specific inhibitors for murine P450, it is not possible to assign all of these murine activities to individual enzymes, as can be done in rat. Of the activities measured, only testosterone 15α-hydroxylation and *p*-nitrophenol hydroxylation can be tentatively assigned to CYP2D9 and CYP2E1, respectively.

The LPS-evoked decrease in testosterone 6β-hydroxylase activity at 6 hr seems to be dependent on NO generation, because no decreases in activity were observed in the NOS2 knockout mice (Table 2) or in mice co-administered AG (Table 4). After 24 hr, however, the decline in 6β-testosterone hydroxylase activity is probably caused by suppressed gene expression, because LPS decreases 6β- testosterone hydroxylase activity in the NOS2 knockout mice (Table 2). Data from the knockout animals indicated a role of NO in the reduction of testosterone15α- and 16β-hydroxylase activity at 24 hr, but not at the 6 hr time point. This was not seen in the AG-treated animals, probably because AG alone depressed the activity at 24 hr (an effect that was also seen with the other testosterone hydroxylase activities except 16α-hydroxylase). The results from the knockout animals provided no evidence for involvement of NO in the decreases in activities of testosterone 16α- or 7α-hydroxylases, or of *p*-nitrophenol hydroxylase.

Some unexpected findings may affect clear interpretation of the catalytic activity data. It is evident that the testosterone 7α-, 15α-, 16α- and 16β-, but not 6β-, hydroxylase activities were higher in saline-treated knockout mice than in saline-treated parental strain mice in the 6 hr experiment (Table 2). This might suggest a role of very low constitutively produced levels of NO in the regulation of these activities, but the effect was not seen in the saline-treated mice in the 24 hr experiment. It is unclear why these differences occurred because the same B6,129 parental strain was used in all three experiments. The difference between the 6 hr and 24 hr experiment could be related to seasonal variation (the experiments were conducted 9 months apart), or to diurnal variation (in both experiments mice were injected between 8 and 9 a.m; therefore, the 6 hr animals were killed in the afternoon, whereas the 24 hr mice were killed in the morning). Another inconsistency was that, in the NOS2 knockout

mice experiments, no significant change was seen in testosterone 16α- or 7α-hydroxylase activities in the parental strain after 24 hr, whereas in the AG experiments, both hydroxylase activities were significantly inhibited after 24h. Again, we can only speculate that because these experiments were performed 9 months apart, seasonal variations may be responsible for the differences observed. This is supported by the observation that no discrepancies were seen in the 6 hr NOS2 knockout experiment and AG studies, which were conducted 1 month apart.

Given that our results suggest that NO is involved in the inhibition of some P450-catalyzed reactions in the early stages of endotoxemia, and the fact that exogenous NO can inhibit P450 catalytic activity (Wink *et al.*, 1993; Khatsenko *et al.*, 1993; Stadler *et al.*, 1994; Osawa *et al.*, 1995), the most parsimonious explanation is that NO is directly responsible for inhibiting P450 catalytic activity *in vivo*. However, the ability of endogenously generated NO to bind to and inhibit P450s has yet to be demonstrated directly. It is possible that the involvement of NO in this phenomenon is indirect (e.g., NO could stimulate the release of another inhibitor from either hepatocytes or Kupffer cells).

In summary, the present work clearly demonstrates that NO is not involved in the down-regulation of P450 mRNA and protein expression using both the NOS inhibitor AG and the NOS2 knockout mouse. We do, however, show a possible role for NO in the decreases in P450 catalytic activities. The present findings concur with previous in *vitro* and *in vivo* work by ourselves and others demonstrating NO-independent decreases in P450 gene expression elicited by agents which evoke an inflammatory response.

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References

Ansher SS and Thompson W (1994) Modulation of hepatic mRNA levels after administration of lipopolysaccharide and diphtheria and tetanus toxoids and pertussis vaccine adsorbed (DTP vaccine) to mice. *Hepatology* **20:**984–991.

Cantoni L, Carelli M, Ghezzi P, Delgado R, Faffioni R, and Rizzardini M (1995) Mechanisms of interleukin-2-induced depression of hepatic cytochrome P-450 in mice. *Eur J Pharmacol* **292:**257–263.

Carlson TJ and Billings RE (1996) Role of nitric oxide in the cytokine-mediated regulation of cytochrome P-450. *Mol Pharmacol* **49**:796–801.

Chen YL, Florentin I, Batt AM, Ferrari L, Giroud JP, and Chauvelot-Moachon L (1992) Effects of interleukin-6 on cytochrome P450-dependent mixed function oxidases in the rat. *Biochem Pharmacol* **44:**137–148.

Chen J-Q, Ström A, Gustafsson J-Å, and Morgan ET (1995) Suppression of the constitutive expression of cytochrome P-450 2C11 by cytokines and interferons in primary cultures of rat hepatocytes: comparison with induction of acute-phase genes and demonstration that *CYP2C11* promoter sequences are involved in the suppressive response to interleukins 1 and 6. *Mol Pharmacol* **47:**940–947.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:**156–159.

Ciaccio PJ and Halpert JR (1989) Characterization of a phenobarbital-inducible dog liver cytochrome P450 structurally related to rat and human enzymes of the P450IIIA (steroid-inducible) gene subfamily. *Arch Biochem Biophys* **271:**284–299.

Cribb AE, Delaporte E, Kim SG, Novak RF, and Renton KW (1994) Regulation of cytochrome P-450 1A and cytochrome P-450 2E induction in the rat during the production of interferon α/β. *J Pharmacol Exp Ther* **268:**487–494.

Curran, RD, Billiar TR, and Stuehr DJ (1990) Multiple cytokines are required to induce hepatocyte nitric oxide production and inhibit total protein synthesis. *Ann Surg* **212:**462–471.

Geller DA, deVera ME, Russell DA, Shapiro RA, Nussler AK, Simmons RL, and Billiar TR (1995) A central role for IL-1β in the in vitro and in vivo regulation of hepatic inducible nitric oxide synthase. *J Immunol* **155:**4890–4898.

Geller D, Nussler AK, DiSilvio M, Lowenstein CJ, Shapiro RA, Wang SC, Simmons RL, and Billiar TR (1993) Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthases in hepatocytes. *Proc Natl Acad Sci USA* **90:**522–526.

Gorodischer R, Larsner J, McDevitt JJ, Nolan JP, and Yaffe SJ (1976) Hepatic microsomal drug metabolism after administration of endotoxin in rats. *Biochem Pharmacol* **25:**351–353.

Grisham MB, Johnson GG, and Lancaster JR Jr (1996) Quantitation of nitrate and nitrite in extracellular fluids. *Methods Enzymol* **268:**237–246.

Haugen DA and Coon MJ (1976) Properties of electrophoretically homogeneous phenobarbital-inducible forms of liver microsomal cytochrome P-450. *J Biol Chem* **251:**7929–7939.

Hodgson PD and Renton KW (1995) The role of nitric oxide generation in interferon-evoked cytochrome P450 down-regulation. *Int J Immunopharmacol* **17:**995–1000.

Khatsenko OG, Gross SS, Rifkind AB, and Vane JR (1993) Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants. *Proc Natl Acad Sci USA* **90:**11147–11151.

Khatsenko O and Kikkawa Y (1997) Nitric oxide affects constitutive cytochrome P450 isoforms in rat liver. *J Pharmacol Exp Ther* **280:**1463–1470.

Koop DR (1986) Hydroxylation of p-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isozyme 3a. *Mol Pharmacol* **29:**399–404.

Lowry OH, Rosebrough NJ, Farr AF, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Minamiyama Y, Takemura S, Imaoka S, Funae Y, Tanimoto, and Inoue M (1997) Irreversible inhibition of cytochrome P450 by nitric oxide. *J Pharmacol Exp Ther* **283:**1479–1485.

Monshouwer M, Witkamp RF, Nijmeijer SM, Van Amsterdam JG, and Van Miert AS (1996) Suppression of cytochrome P450- and UDP glucoronosyl transferase-dependent enzyme activities by proinflammatory cytokines and possible role of nitric oxide in primary cultures of pig hepatocytes. *Toxicol Appl Pharmacol* **137:**237–244.

Morgan ET (1989) Suppression of constitutive cytochrome P-450 gene expression in livers of rats undergoing an acute phase response to endotoxin. *Mol Pharmacol* **36:**699–707.

Morgan ET (1997) Regulation of cytochrome P450 during inflammation and infection. *Drug Metab Rev* **29:**1129–1188.

Morgan ET, MacGeoch C, and Gustafsson J-Å (1985) Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16α-hydroxylase cytochrome P-450 apoprotein in the rat. *J Biol Chem* **260:**11895–11898.

Morgan ET and Norman CA (1990) Pretranslational suppression of cytochrome P-450h (IIC11) gene expression in rat liver after administration of interferon inducers. *Drug Metab Dispos* **18:**649–653.

Morgan ET, Thomas KB, Swanson R, Vales T, Hwang J, and Wright K (1994) Selective suppression of cytochrome P450 gene expression by interleukins 1 and 6 in rat liver. *Biochem Biophys Acta* **1219:**475–483.

Omura T and Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. 1. evidence for its hemoprotein nature. *J Biol Chem* **239:**2370–2378.

Osawa Y, Davilla JS, Nakasuka M, Meyer CA, and Darbyshire JF (1995) Inhibition of P450 cytochromes by reactive intermediates. *Drug Metab Rev* **27:**61–72.

Quaroni L, Reglinski J, Wolf R, and Smith WE (1996) Interaction of nitrogen monoxide with cytochrome P-450 monitored by surface-enhanced resonance Raman scattering. *Biochim Biophys Acta* **1296:**5–8.

Sambrook J, Fritsch EF, and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sewer MB, Koop DR, and Morgan ET (1996) Endotoxemia in rats is associated with induction of the P4504A subfamily and suppression of several other forms of cytochrome P450. *Drug Metab Dispos* **24:**401–407.

Sewer MB, Koop DR, and Morgan ET (1997) Differential inductive and suppressive effects of endotoxin and particulate irritants on hepatic and renal cytochrome P-450 expression. *J Pharmacol Exp Ther* **280:**1445–1454.

Sewer MB and Morgan ET (1997) Nitric oxide independent suppression of P-450 2C11 expression by interleukin-1b and endotoxin in primary rat hepatocytes. *Biochem Pharmacol* **54:**729–737.

Sewer MB and Morgan ET (1998) Down-regulation of the expression of three major rat liver cytochrome P450s by endotoxin in vivo occurs independently of NO production. *J Pharmacol Exp Ther*, in press.

Stadler J, Trockfeld J, Schmalix WA, Brill T, Siewert JR, Greim H, and Doehmer J (1994) Inhibition of cytochromes P4501A by nitric oxide. *Proc Natl Acad Sci USA* **91:**3559–3563.

Stanley LA, Adams DJ, Lindsay R, Meehan RR, Liao W, and Wolf CR (1988) Potentiation and suppression of mouse liver cytochrome P-450 isozymes during the acute-phase response induced by bacterial endotoxin. *Eur J Biochem* **174:**31–36.

Tracey WR, Tse J, and Carter G (1995) Lipopolysaccharide-induced changes in plasma nitrite and nitrate concentrations in rats and mice: pharmacological evaluation of nitric oxide synthase inhibitors. *J Pharmacol Exp Ther* **272:**1011–1015.

Waxman DJ (1991) P450-catalyzed steroid hydroxylation: assay and product identification by thin-layer chromatography. *Methods Enzymol* **206:**462–476.

Wink DA, Osawa Y, Darbyshire JF, Jones CR, Eshenaur SC, and Nims RW (1993) Inhibition of cytochromes P450 by nitric oxide and a nitric oxide-releasing agent. *Arch Biochem Biophys* **300:**115–123.

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