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Suppression of Flavin-Containing Monooxygenase by Overproduced Nitric Oxide in Rat Liver

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ABSTRACT

Effects of excessive nitric oxide (NO) produced in vivo by an i.p. injection of bacterial lipopolysaccharide (LPS) on hepatic microsomal drug oxidation catalyzed by flavin-containing monooxygenase (FMO) were determined. At 6 and 24 h after the LPS injection, liver microsomes were isolated and FMO activities were determined by using FMO substrates like thiobenzamide, trimethylamine, *N,N*-dimethylaniline, and imipramine. Liver microsomal FMO activities of LPS-treated rats were decreased significantly for all these substrates. Microsomal content of FMO1 (the major form in rat liver) in LPS-treated rats as determined by immunoblotting, was severely decreased as well. In support of this, hepatic content of FMO1 mRNA was decreased by 43.6 to 67.3%. However, the hepatic content of inducible NO synthase (iNOS) mRNA was increased by 2.6- to 5.4-fold and the plasma nitrite/nitrate concentration was increased by about 30-fold in the LPS-treated rats. When this overproduction of NO in the LPS-treated rats was inhibited in vivo by a single or repeat doses of either a general NOS inhibitor N^G-nitro-L-arginine or a specific iNOS inhibitor aminoguanidine, the FMO1 mRNA levels were not severely depressed (70−85% of the control level). Attendant with the reduction of plasma nitrite/nitrate concentration by single and repeated doses of NOS inhibitors, activity and content of FMO1 in liver microsomes isolated from these NOS inhibitor cotreated rats were restored partially (in single-dose inhibitors) or completely (in repeat doses). In contrast to these NO-mediated in vivo suppressive effects on the mRNA and enzyme contents of FMO1 as well as the FMO activity, the NO generated in vitro from sodium nitroprusside did not inhibit the FMO activities present in microsomes of rat and rabbit liver as well as those present in rabbit kidney and lung. Combined, the excessive NO produced in vivo (caused by the LPS-dependent induction of iNOS) suppresses the FMO1 mRNA and enzyme contents as well as the FMO activities without any direct in vitro effect on the activities of premade FMO enzyme. These findings suggest that NO is an important mediator involved in the suppression of FMO1 activity in vivo. Thus, together with the previously reported suppression on the cytochrome P-450 activities, the overproduced NO in the liver caused by induction of iNOS under conditions of endotoxemia or sepsis suppresses FMO and appears to be responsible for the decreased drug oxidation function observed generally under conditions of systemic bacterial or viral infections.

In patients with sepsis or endotoxemia caused by bacterial or viral infections, hepatic drug metabolism function is known to be depressed (Chang et al., 1978; Kraemer et al., 1982). In the septic condition, large quantities of cytokines like interferon-γ and tumor necrosis factor-α are known to be produced and the hepatic drug metabolism function in rats treated with these cytokines has been demonstrated to be suppressed (Ghezzi et al., 1986; Mannering and Deloria, 1986).

Experimental animals treated with endotoxin or/and cytokines to mimic the endotoxemia or septic conditions have been demonstrated to have increased inducible nitric oxide synthase (iNOS) activity in liver and to produce excessive amounts of nitric oxide (NO) (Billiar et al., 1989; Curran et al., 1989). Although the excessive NO produced in vivo is known to act mainly as an immunological messenger molecule mediating the antibacterial or antitumoral activities (Nathan and Hibbs, 1991; Nathan, 1992; Nussler and Billiar, 1993), excessive NO produced in septic condition has been speculated to interact with the heme iron of cytochrome P-450 (CYP), the major enzyme involved in hepatic drug oxidation, and to be responsible for the depressed hepatic drug metabolism function (NO-dependent pathway). Wink et al. (1993) and Minamiyama et al. (1997) have demonstrated that NO binds reversibly to the heme moiety of CYP in vitro and irreversibly to the protein of CYP, such as those occurring under in vivo condition, and inhibits the CYP activity.

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ABBREVIATIONS: NO, nitric oxide; FMO, flavin-containing monooxygenase; CYP, cytochrome P-450; LPS, lipopolysaccharide; SNP, sodium nitroprusside; NNA, N^G-nitro-L-arginine; AG, aminoguanidine; TB, thiobenzamide; TMA, trimethylamine; DMA, *N,N*-dimethylaniline; IMP, imipramine.

Furthermore, Khatsenko et al. (1993) reported that the decrease in microsomal CYP content observed in vivo caused by injection of endotoxin in rats is reversed by coadministration of an inhibitor of NO synthase (NOS; EC 1.14.13.39) and has demonstrated that the decrease in drug metabolism function under inflammatory conditions is the result of excessive NO production. In further studies, other investigators have demonstrated that NO decreases not only the hepatic microsomal CYP content and activity but also suppresses the expression of CYP mRNAs in rat liver (Stadler et al., 1994; Nadin et al., 1995; Khatsenko and Kikkawa, 1997).

In addition to these effects of endotoxin on the activity and expression of CYP, injection of endotoxin has also been demonstrated to increase the heme oxygenase activity accelerating the degradation of heme moiety in CYP and also to decrease the δ-aminolevulinate synthetase activity inhibiting the synthesis of heme required for incorporation into CYP (Bissell and Hammaker, 1976a,b). Based on these results, Kim et al. (1995) suggested that overproduction of NO enhances the degradation of heme, causes liberation of heme iron from CYP, and also inhibits heme biosynthesis in a concerted manner, all leading to suppress the CYP catalyzed hepatic drug oxidation function.

In addition to the above-mentioned NO-dependent pathway, several studies have demonstrated the existence of NO-independent mechanisms by direct injection of interleukins or cytokines and also explained the down-regulation of CYP gene expression, which leads to suppression of CYP contents and activities (Hodgson and Renton, 1995; Monshouwer et al., 1996). These studies have been performed both in vivo and in vitro (cultured primary hepatocytes). More recently, Sewer and Morgan (1997, 1998) and Sewer et al. (1998) clearly demonstrated the NO-independent suppression of some CYP expression by using the iNOS knock-out mice and rat hepatocytes and suggested that it is mediated directly by LPS or cytokines. In particular, this cytokine-dependent pathway suppressing the CYP gene expression was demonstrated to be caused by tumor necrosis factor-α (Nadin et al., 1995) and interleukin-1β (Sewer and Morgan, 1997).

Together with the heme-containing CYP monooxygenases, the flavin-containing monooxygenases (FMOs; EC 1.14.13.8), also contained in liver microsomes, catalyze the oxidation of many clinically important medicines and dietary plant alkaloids and play an important role in the overall oxidative hepatic drug metabolism function. However, unlike the CYP, which contains Fe-heme as the prosthetic center and binds NO directly, the FMOs are known to contain flavin adenine dinucleotide (FAD) as the prosthetic group, which does not bind NO directly. As for the in vivo suppressive effect of NO on FMO activities, although not implicated directly, in liver microsomes isolated from rats with acute hepatitis and cirrhosis induced by chemicals, conditions in which the in vivo production of NO is generally known to be increased, the FMO activity has been shown to be depressed (Nakajima et al., 1998). In a recent metabolic study using isolated livers obtained from rats pretreated with LPS, we observed a dramatic reduction in their ability to produce theobromine or ranitidine *N*-oxide from the infused caffeine or ranitidine, respectively (C-S.P., H-M.B., W-G.C., K-H.L., and Y-N.C., unpublished observation). In previous studies, the production of theobromine from caffeine has been demonstrated to be catalyzed primarily by the FMO present in rat and human liver microsomes (Chung and Cha, 1997; Chung et al., 1998).

Thus, in the present study, we determined the effect of NO, overproduced in vivo by the elevated iNOS in the liver of rats pretreated with LPS, on hepatic microsomal FMO activity and FMO1 (primary form in rat liver) gene expression.

Materials and Methods

Animals and Treatments. In an effort to enhance the NO synthase activity (iNOS) in vivo in the liver, male Sprague-Dawley rats weighing about 250 g obtained from the Animal Breeding Laboratory of Inha University were given an i.p. injection of a single dose of bacterial lipopolysaccharide (LPS) (1 mg/kg in autoclaved saline, *Escherichia coli* 0111:B4; Sigma Chemical Co., St. Louis, MO). For some experimental rats, N^G- nitro-L-arginine (NNA) (20 mg/kg/treatment; Tocris Cookson Inc.), a competitive inhibitor of the L-arginine-dependent NO biosynthesis, or aminoguanidine (AG) (20 mg/kg/treatment; Sigma), prototypical selective inhibitor of iNOS, was injected together with LPS to inhibit the excessive NO production by the elevated iNOS. These inhibitors were given to rats with LPS either by an i.p. single dose simultaneously or by repeated doses at every 4 h for 6 and 24 conseuctive hours. Control rats were given a single or repeated i.p. injection of autoclaved saline alone or with each of the above NOS inhibitors. At 6 and 24 h after the injection of LPS or NOS inhibitors, in separate or in combination, rats were sacrificed by cervical dislocation and liver tissues were removed and frozen immediately by immersing in liquid nitrogen. The liver samples were stored at −80°C for isolation of hepatic microsomes and total liver RNAs at a later time.

Determination of NO Production. The concentration of stable NO metabolites (nitrite/nitrate; NOx) present in plasma at the time of sacrifice was determined. After converting the plasma nitrate (NO_3^-) to nitrite (NO_2^-) using nitrate reductase (10 U/ml in 100 μM Tris buffer, pH 7.6), the total concentration of nitrite was determined spectrophotometrically at 560 nm using a method based on the Griess reaction (Green et al., 1982; Khatsenko and Kikkawa, 1997).

Determination of Liver Microsomal FMO Activities. Liver microsomes were prepared from the stored frozen liver tissues using a procedure developed by Chung and Buhler (1994) and the protein contents were measured by the method of Lowry et al. (1951) using BSA as the standard. Hepatic microsomal FMO activity was measured by using the thiobenzamide (TB) *S*-oxidation assay developed by Cashman and Hanzlik (1981) and as modified by Itoh et al. (1993). Briefly, 0.5 mg of rat liver microsomes was added to the reaction mixture (1.0 ml) containing 50 mM Tris-HCl (pH 8.4), 0.25 mM $NADP^+$, 2.0 mM glucose 6-phosphate, 0.5 U of glucose 6-phosphate dehydrogenase, and 7.0 mM $MgCl_2$. After a 10-min preincubation at 37°C, the reaction was started by adding 1 mM TB, and the rate of TB *S*-oxide formation was determined by following the 370 nm absorption for 5 min. In addition to the rate of TB *S*-oxide formation, FMO activities also were measured also by determining the rates of NADPH consumption on oxidation of other FMO substrates like trimethylamine (TMA; 1 mM), *N,N*-dimethylaniline (DMA; 0.1 mM), and imipramine (IMP; 0.1 mM), which are all known to be oxidized by FMOs (Ziegler, 1988; Lemoine et al., 1990). After a preincubation for 3 min at 37°C in the absence of these FMO substrates, the rates of NADPH oxidation (starting with 0.15 mM) caused by addition of these FMO substrates were monitored at 340 nm for 5 min. The microsomal FMO activities oxidizing these compounds were measured in the absence and presence of prior CO-bubbling, which was done to remove the participation of CYPs in the microsomal oxidation of these four FMO substrates.

In Vitro Attempts to Inhibit FMO Activities with Exogenous NO Donor. To examine the direct in vitro effect of NO on microsomal FMO activities, a well known NO donor sodium nitroprusside (SNP) dissolved in distilled water was added to the micro-

somal incubation mixture at concentrations of 1, 5, and 10 mM. After 30 min of incubations both at 0° and 37°C or in absence and presence of SNP, differential FMO activities were determined by TB *S*-oxidation assay.

Extraction of Total Liver RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total liver RNAs were isolated from the stored frozen liver tissues using the protocol designated in RNeasy kit (Qiagen, Hilden, Germany). Frozen liver tissues stored at −80°C were ground to a fine powder with mortar and pestle under liquid nitrogen and then homogenized in the presence of a highly denaturing guanidinium isothiocyanate buffer by repeated suction through a 20-gauge needle using a syringe. Ethanol was added to provide an appropriate binding condition for the hepatic RNA to an RNA binding column in which the total liver RNAs would bind favorably to the silica-gel based membrane. After the contaminants have been washed away, the RNA was then eluted from the column with distilled water containing diethylpyrocarbonate to inhibit the RNase. The purity and concentration of isolated liver RNAs were determined spectrophotometrically.

Total liver RNAs (1.5 μg) were then reverse transcribed for 30 min at 42°C in the presence of viral reverse transcriptase of avian meyloblastosis (AMV Rtase) and oligo-dT adapt primer (Takara, Shiga, Japan). For the amplification of rat the FMO1 cDNA fragment, the employed sense primer sequence from 1033 to 1052 nt was 5′-GTTGAGGATGGCCAGGCATC-3′ and the antisense primer sequence from 1358 to 1377 nt was 5′-CAGGCGTGGGTCAGTCAGGA-3′ (Cashman and Hanzlik, 1981). Identity of iNOS mRNA induced by the LPS treatment was confirmed with the sense primer covering from 3241 to 3263 nt (5′-GGAGGACCACCTCTATCAGGAAG-3′) and the antisense primer from 3601 to 3624 nt (5′-GTGCCTTTGGGCTCCTCCAAGGTG-3′). To normalize the PCR products in a quantitative manner, β-actin cDNA fragment was used as the control and was amplified with primers (sense: 5′-AGAAGAGCTATGAGCTGCCTGACG-3′, antisense: 5′-CTTCTGCATCCTGTCAGCGATGC-3′). The PCR amplifications were carried out in the reaction mixture containing 4 μl of the single strand cDNAs. Denaturation, annealing, and elongation were performed for 30 cycles at 94°, 55° and 72°C, and for the durations of 40, 40, and 90 s, respectively. The PCR products were electrophoresed on a 2% agarose gel (Nusieve 3:1; FMC, Rockland, ME) containing 1 μg/ml ethidium bromide and then observed under a UV-transilluminator. The relative amounts of PCR products formed were compared by using the image analysis software Bio-1D (Ver.97; Vilber Lourmat, France).

DNA Sequencing. To confirm the DNA sequences of the RT-PCR products shown in Fig. 4, direct sequencing of DNA was carried out by the dideoxy chain-termination method using the Sequenase version 2.0 T7 DNA polymerase (U.S. Biochemicals, Cleveland, OH) and [α-^{35}S]-labeled dATP (Amersham, Buckinghamshire, UK).

Electrophoresis and Immunoblotting. Liver microsomal protein (10 μg) was subjected to the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 6 to 18% Tris-glycine polyacrylamide gradient gels, transferred to polyvinylidene difluoride membrane (Bio-Rad Labs., Hercules, CA), and blocked with a buffer containing 5% nonfat dry milk. The first polyclonal pig antibody (anti-FMO1; a gift from Dr. Ziegler at Texas A&M University) and another antibody (antinitrotyrosine; a gift from Dr. Y.M. Kim of Kangwon University in Korea) were incubated at 1:1000 dilutions and the anti-rabbit or anti-mouse IgG/horseradish peroxidase conjugate diluted at 1:2000 was added, respectively. The enhanced chemiluminescence method (Pierce, Rockford, IL) was used to visualize the protein bands, and the quantification was performed by using the image analysis software Bio-1D.

Statistical Analysis. Data are presented as the mean ± S.D. obtained from triplicate samples for each of four to five individual rats and are analyzed by using the multiple comparison of Kruskal-Wallis test for three group comparisons. Comparison between two groups was analyzed by using unpaired *t* test. The *p* values <0.05 (95% confidence interval) and 0.01 (99%) are noted as significant.

Results

Plasma NOx Concentration. The plasma concentration of NOx in LPS-pretreated rats was increased markedly and the NOx concentration in the NOS inhibitor-treated rats was decreased significantly (Fig. 1). The suppressive effect of NNA or AG on NOx production was the strongest at the early time point (6 h). The small increases of plasma NOx concentration in the NNA- or AG-treated groups did not appear to be significant (p = .21 for NNA, p = .09 for AG; Fig. 1).

Effects on Hepatic Microsomal FMO Activities. FMO activities of liver microsomes isolated from rats pretreated were measured using TB, TMA, DMA, and IMP with and without the prior CO-bubbling (Table 1 and Fig. 2). The TB *S*-oxidase activities obtained with liver microsomes isolated at 24 h (LPS alone), 6 h (2 doses of saline), and 24 h (6 doses of saline) after the LPS treatment were decreased by 41.4, 61.9, and 70.1%, respectively (p <.01 for all) before the CO-bubbling and those obtained after the CO-bubbling were decreased by 54.0, 60.9, and 68.5%, respectively (p <.01; Fig. 2). When the NOS inhibitor NNA or AG was injected (once only) with LPS to rats, the isolated liver microsomal TB *S*-oxidase activity was partially recovered to about 80 to 87% (with and without the CO bubbling, p <.01) of the control levels (Fig. 2). However, when these inhibitors were given repeatedly every 4 h for 6 to 24 h after the LPS treatment, the FMO activities were completely restored (93.2−125.0% of the control level). And the FMO activities obtained from liver microsomes of rats pretreated only with single or repeated doses of NNA or AG were not significantly different from those of saline-treated controls. Furthermore, the TB *S*-oxidase activities obtained in the absence or presence of CO-bubbling were not significantly different and this indicated that the *S*-oxidation of TB is catalyzed primarily by FMO.

Microsomal FMO activities were determined also by measuring the rate of NADPH consumed for oxidation of other *N*- or *S*- containing FMO substrates like TMA, DMA, and IMP, with or without the CO-bubbling (Table 1). The rates of NADPH oxidation (FMO activities) obtained with LPS-microsomes metabolizing TMA, DMA, and IMP after a prior

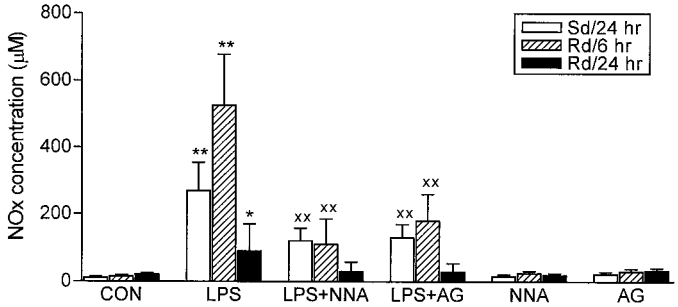

Fig. 1. Plasma NOx levels in rats pretreated with LPS and LPS plus NOS inhibitors. Plasma collected from 18 experimental groups was used for determination of NO production (nitrite and nitrate = NOx concentration; in μM) by using Griess reaction. Data represent means ± S.D. obtained from four or five rats per experimental group. *p < .05 and **p < .01 indicate significant increase of NOx concentration when compared with those obtained from the respective saline-treated control groups, and ^{xx}p < .01 indicates significant restoration toward the normal level when compared with those of the respective LPS-pretreated groups. Abbreviations: Sd/24 h, single dose saline or inhibitors and sacrificed at 24 h; Rd/6 h, 0 and 4 h repeat saline or inhibitor treatments and sacrificed at 6 h (early time point); Rd/24 h, every 4 h repeat doses of saline or inhibitors and sacrificed at 24 h.

CO-bubbling (to block the CYP activity) were decreased to 73.8, 67.8, and 51.6%, respectively, of the values obtained with control rat liver microsomes ($p < .01$). As shown in Table 1, the FMO activities of LPS microsomes treated repeatedly with saline (6 and 24 h) were higher than those treated with LPS alone. Even without the CO-bubbling, the rates of NADPH consumption obtained for the LPS microsomes (CYP and FMO activities) were decreased to about the same extent as that obtained with the CO-bubbling (FMO activity only). In the liver microsomes isolated from rats pretreated with LPS and LPS together with a single dose of NNA or AG, the FMO activities obtained for TMA and DMA oxidation were restored completely (>110% of control); whereas those observed for IMP oxidation were recovered partially up to 72.2% (in the absence of CO bubbling) and ~80.5% (in the presence of CO bubbling) of that obtained in the control rat liver microsomes (Table 1). However, the FMO activities of LPS microsomes obtained after repeated doses of NOS inhibitors (every 4 h for 6- and 24-h time point liver microsomes) were restored completely (~95–150% of control). As expected, the FMO activities obtained for DMA and IMP measured before and after the CO bubbling were significantly different. This difference is caused by the contribution of CYP, which is inhibited by the CO bubbling. However, the FMO activities obtained with the microsomes of either single or repeated dose NNA (or AG) were not significantly different from those of saline-treated controls except for IMP at 24 h (repeat dose) which was inhibited significantly ($p < .05$).

In Vitro Effect of Exogenous NO on Microsomal FMO Activity. In an effort to determine the direct effect of NO on premade FMOs present in various tissue sources, SNP, a well known NO donor, was added to the microsomal incubation mixture undergoing TB oxidation. In addition to the rat liver microsomes (with high FMO1), rabbit liver (FMO1), kidney (FMO4), and lung (FMO2) tissue microsomes (Cashman, 1995) were used as well to determine the direct effect of NO on other isoforms of FMO which also metabolize the TB.

Exposure to SNP at 1, 5, and 10 mM concentrations at both 0° and 37°C did not produce any significant changes ($p = .923$) in the FMO activities of these tissue microsomes (Fig. 3). Results shown in the figure represent the effect obtained only with 1 mM concentration of SNP.

Hepatic Content of FMO1 mRNA in LPS-Treated Rats. Effects of administering LPS alone (induction of iNOS) or LPS together with NNA or AG (inhibition of NO production) on hepatic contents of FMO1 mRNA as well as the iNOS mRNA were determined by RT-PCR (Fig. 4). The relative differences in the levels of FMO1 and iNOS mRNAs present in the total liver RNAs isolated from the 18 experimental groups [single (24 h) and repeat doses (6 h and 24 h) of NOS inhibitors or saline for each of the control, LPS, LPS plus NNA, LPS plus AG, NNA and AG] were compared. As the results show in Fig. 4, the relative hepatic content of FMO1 mRNA in LPS-treated rats was severely decreased to 33.0% of the control level (the ratio of FMO1 mRNA versus β-actin mRNA: control; 0.85 ± 0.15, LPS; 0.28 ± 0.09, $p < .01$), but those in repeat dose saline (6 and 24 h) were decreased to 56.4 and 32.0%, respectively. By contrast, the relative iNOS mRNA content in the liver was increased by about 2.7-fold (control, 0.18 ± 0.08; LPS, 0.48 ± 0.15, $p < .01$), 5.4-fold (6 h LPS + repeat saline), and 2.7-fold (24 h LPS + repeat saline; Fig. 4). In all the livers of rats treated together with LPS plus single or repeated doses of NNA or AG, although the relative iNOS mRNA contents were still increased by 2.5- to 4.5-fold, the FMO1 mRNA levels were restored up to 70, 75, and 83%, respectively, of the control level. Unexpected inhibitory effects on iNOS mRNA expression in LPS-treated rats were observed in the 6- and 24-h time points with repeat dose NNA and AG ($p = .067$), but not in the single dose-treated rats. In the livers of rats treated only with the inhibitors of NO production (NNA or AG individually), the level of FMO1 mRNA appeared to be decreased, but without statistical significance ($p = .62$ for the NNA and $p = .33$ for the AG-treated rats, unpaired t test).

TABLE 1

Liver microsomal FMO activities of rats pretreated with LPS and LPS plus NOS inhibitors

Before and after bubbling with CO gas, the liver microsomal FMO activities were measured by determining rate of NADPH oxidation at 340 nm on addition of various FMO substrates as described in *Materials and Methods*. Values represent mean ± S.D. obtained from triplicate measurements with liver microsomes obtained from four or five individual rats in each treatment group.

Substrates	Groups	CON	LPS	LPS + NNA	LPS + AG	NNA	AG
TMA	Sd/24 h	1.41 ± 0.15	1.04 ± 0.11[a]	1.61 ± 0.17[c]	1.64 ± 0.29[c]	1.56 ± 0.42	1.59 ± 0.26
		(1.34 ± 0.41)	(0.94 ± 0.18)[b]	(1.53 ± 0.32)[c]	(1.52 ± 0.44)[d]	(1.49 ± 0.33)	(1.47 ± 0.61)
	Rd/6 h	1.59 ± 0.22	1.42 ± 0.39	2.54 ± 0.41[c]	2.28 ± 0.21[c]	1.41 ± 0.22	1.59 ± 0.36
		(1.52 ± 0.29)	(1.33 ± 0.31)	(2.50 ± 0.34)[c]	(2.19 ± 0.25)[c]	(1.37 ± 0.15)	(1.55 ± 0.31)
	Rd/24 h	1.74 ± 0.31	1.29 ± 0.16[a]	2.52 ± 0.31[c]	2.68 ± 0.20[c]	1.39 ± 0.49	1.72 ± 0.29
		(1.75 ± 0.24)	(1.28 ± 0.24)[a]	(2.45 ± 0.29)[c]	(2.61 ± 0.19)[c]	(1.36 ± 0.31)	(1.74 ± 0.35)
DMA	Sd/24 h	2.39 ± 0.59	1.62 ± 0.15[a]	2.62 ± 0.27[c]	2.91 ± 0.33[c]	2.88 ± 0.76	3.16 ± 0.82
		(1.64 ± 0.23)	(1.19 ± 0.32)[b]	(1.84 ± 0.22)[c]	(1.91 ± 0.39)[c]	(1.96 ± 0.55)	(1.89 ± 0.63)
	Rd/6 h	2.91 ± 0.49	2.85 ± 0.31	4.40 ± 0.71[c]	4.01 ± 0.33[c]	3.05 ± 0.29	3.21 ± 0.40
		(2.11 ± 0.34)	(2.12 ± 0.26)	(2.89 ± 0.31)[c]	(2.74 ± 0.19)[c]	(2.13 ± 0.33)	(1.99 ± 0.27)
	Rd/24 h	3.09 ± 0.35	2.20 ± 0.29[b]	4.21 ± 0.31[c]	3.84 ± 0.25[c]	2.71 ± 0.21	2.84 ± 0.39
		(2.27 ± 0.28)	(1.45 ± 0.11)[b]	(3.29 ± 0.39)[c]	(2.81 ± 0.39)[c]	(1.88 ± 0.21)	(2.11 ± 0.33)
IMP	Sd/24 h	3.45 ± 0.46	1.78 ± 0.29[a]	2.49 ± 0.34[c]	2.60 ± 0.42[c]	3.32 ± 0.49	3.13 ± 0.81
		(1.59 ± 0.26)	(0.78 ± 0.32)[a]	(1.28 ± 0.17)[d]	(1.44 ± 0.38)[c]	(1.71 ± 0.55)	(2.22 ± 0.41)
	Rd/6 h	2.89 ± 0.38	1.84 ± 0.31[b]	3.04 ± 0.51[c]	2.72 ± 0.64[d]	2.89 ± 0.61	2.71 ± 0.39
		(1.62 ± 0.34)	(1.11 ± 0.19)[b]	(2.49 ± 0.35)[c]	(2.14 ± 0.29)[d]	(1.79 ± 0.44)	(1.61 ± 0.51)
	Rd/24 h	2.91 ± 0.62	1.99 ± 0.29[b]	2.83 ± 0.39[d]	2.68 ± 0.46[d]	2.32 ± 0.61	2.14 ± 0.66[b]
		(1.76 ± 0.31)	(1.38 ± 0.26)[b]	(2.17 ± 0.35)[c]	(1.92 ± 0.40)[d]	(1.61 ± 0.21)	(1.49 ± 0.32)

Abbreviations: Sd/24 h, single dose saline or inhibitors and sacrificed at 24 h; Rd/6 h, 0 and 4 h repeat doses of saline or inhibitors and sacrificed at 6 h (early time point); Rd/24 h, every 4 h repeat doses of saline or inhibitors and sacrificed at 24 h.

[a] $p < .01$ and [b] $p < .05$ indicate significant suppression of FMO activities when compared with those obtained from control rat liver microsomes, and [c] $p < .01$ and [d] $p < .05$ indicate significant restoration toward normal level when compared with those of the respective LPS-treated groups. Values in parentheses indicate FMO activities determined after CO-bubbling.

DNA Sequence Analysis. Nucleotide sequence analysis of the RT-PCR products (FMO1 band; 345 bp in Fig. 4) revealed a 99.8% identity with that of the previously published rat liver FMO1 cDNA (Itoh et al., 1993). In the original gel electrophoresis result, there were two nonspecific RT-PCR products appearing above the FMO1 band and these RT-PCR products had no similarity with the cDNA sequences of FMO isoforms, but showed some homology with glucagon receptor or α-internexin genes (data not shown). At present, we cannot offer any explanations for these extra bands on top of the FMO1 band. Thus, for the reversed picture shown in Fig. 4, we deleted these potentially unrelated bands to make only the FMO1 band would appear.

FMO1 Content in Hepatic Microsomes of LPS-Treated Rats. Content of FMO1 protein present in the liver microsomes isolated from rats pretreated with LPS was decreased down to about 20% of the control level (Fig. 5). The content in microsomes of repeat dose saline (6 and 24 h) were decreased to 86 and to 58%, respectively. However, the contents in the liver microsomes of LPS plus single (24 h) and repeated doses of NNA or AG (6 and 24 h)-pretreated rats were restored partially and completely (up to ~80% and to 110−150% of the control level, respectively). Both the single or repeated NNA and AG treatments without the LPS do not appear to suppress the FMO1 enzyme protein content in the isolated liver microsomes (Fig. 5).

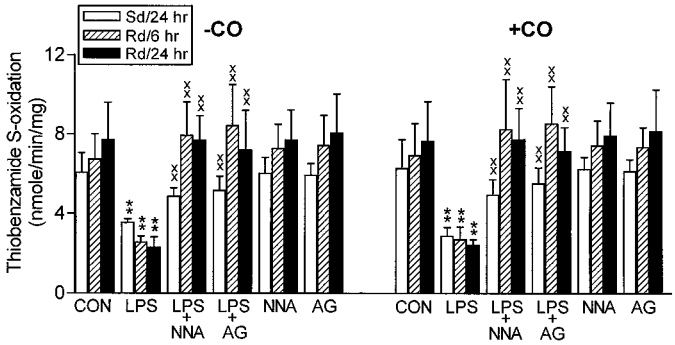

Fig. 2. Hepatic microsomal FMO activity of rats pretreated with LPS and LPS plus NOS inhibitors. FMO activity (TB *S*-oxidation) was determined before and after bubbling with CO gas as described in *Materials and Methods*. Data represent means ± S.D. obtained from triplicate measurements with liver microsomes of four or five individual rats for each treatment group. $^{**}p < .01$ indicates significant suppression of FMO activities when compared with those obtained from respective saline-treated control groups, and $^{\times\times}p < .01$ indicates significant restoration toward the normal level when compared with the FMO activities of respective LPS-pretreated groups. Abbreviations: Sd/24 h, single dose saline or inhibitors and sacrificed at 24 h; Rd/6 h, 0 and 4 h repeat doses of saline or inhibitors and sacrificed at 6 h (early time point); Rd/24 h, every 4 h repeat doses of saline or inhibitors and sacrificed at 24 h.

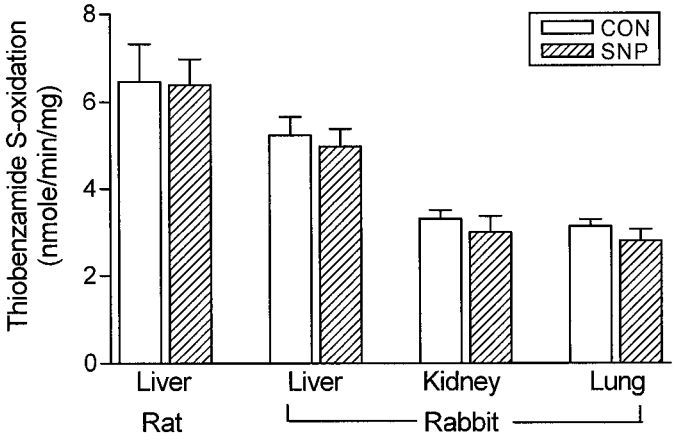

Fig. 3. Effect of adding SNP to the in vitro microsomal FMO assay system. FMO activity (thiobenzamide *S*-oxidation) was measured using microsomes isolated from rat liver (primarily FMO1) and rabbit liver (FMO1), kidney (FMO4), and lung (FMO2) in the presence of 1 mM SNP as described in *Materials and Methods*.

Discussion

As mentioned above, hepatic drug metabolism function is known to be depressed in patients with endotoxemia or sepsis caused by bacterial or viral infection (Chang et al., 1978; Kraemer et al., 1982). Studies conducted with experimental rats treated with bacterial endotoxin or cytokines like interferon-γ and tumor necrosis factor-α mimicking the endotoxemic or septic condition have demonstrated that hepatic microsomal CYP activities were depressed as well (Ghezzi et al., 1986; Mannering and Deloria, 1986). In the liver of these rats treated with bacterial endotoxin or cytokines, the NO-producing enzyme (iNOS) was found to be elevated. The increased iNOS activity was found in hepatic parenchymal cells, Kupffer cells, and even in hepatic endothelial cells (Billiar et al., 1990; Pittner and Spitzer, 1992; Geller et al.,

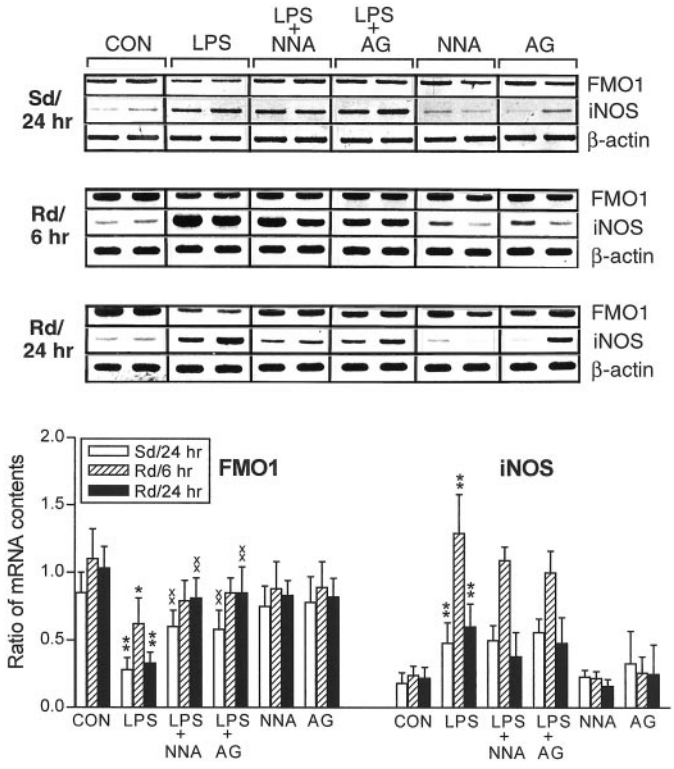

Fig. 4. Effects of LPS and LPS plus NNA or AG pretreatment on FMO1 and iNOS mRNA contents in rat liver. FMO1, iNOS, and β-actin mRNA contents were determined by RT-PCR (reversed picture) using specific primers designed from rat FMO1, iNOS, and β-actin cDNAs. FMO1 and iNOS mRNA contents were compared with respective β-actin mRNA contents and their ratios are shown in the bottom as bar graphs. $^{*}p < .05$ and $^{**}p < .01$ indicate significant suppression or increase when compared with those of respective saline-treated control groups and $^{\times\times}p < .01$ indicates significant restoration toward the normal level when compared with those of the respective LPS-pretreated groups. Abbreviations: Sd/24 h, single dose saline or inhibitors and sacrificed at 24 h; Rd/6 h, 0 and 4 h repeat doses of saline or inhibitors and sacrificed at 6 h (early time point); Rd/24 h, every 4 h repeat doses of saline or inhibitors and sacrificed at 24 h.

1993). Under such a condition, an excessive amount of NO was produced, and this overproduced NO has been demonstrated to bind directly with the prosthetic heme moiety of CYP, forming iron-nitrosyl complexes and leading to inhibition of oxygen binding and CYP activities (reversible inhibition). Also, irreversible inhibition of CYP enzymes caused by nitrogen oxides (in vivo condition) have been suggested (Wink et al., 1993). Conversely, inhibition of NO synthesis under such experimental conditions performed by administering inhibitors of NOS activity has been shown to prevent the degradation of heme and reductions of CYP content and activity (Kim et al., 1995). Thus, the depression of CYP catalyzed hepatic drug metabolism function observed in such a septic condition appears to be caused by the overproduced NO, at the least.

Together with the heme-containing CYP monooxygenases, the FAD containing FMO also plays a major role in hepatic drug oxidation. In spite of the fact that FAD contained in FMO does not bind CO or NO directly, in rats pretreated with LPS in which the NO was overproduced, the FMO activity was found to be severely decreased. As shown in Table 1 and Fig. 2, LPS-treated rat liver microsomal FMO activities determined with some of the well known FMO substrates such as TB, TMA, DMA, and IMP were found to be markedly decreased. Even after the contribution of CYP activities contained in liver microsomes was eliminated by prior bubbling with CO gas, the FMO activities of LPS microsomes were severely depressed.

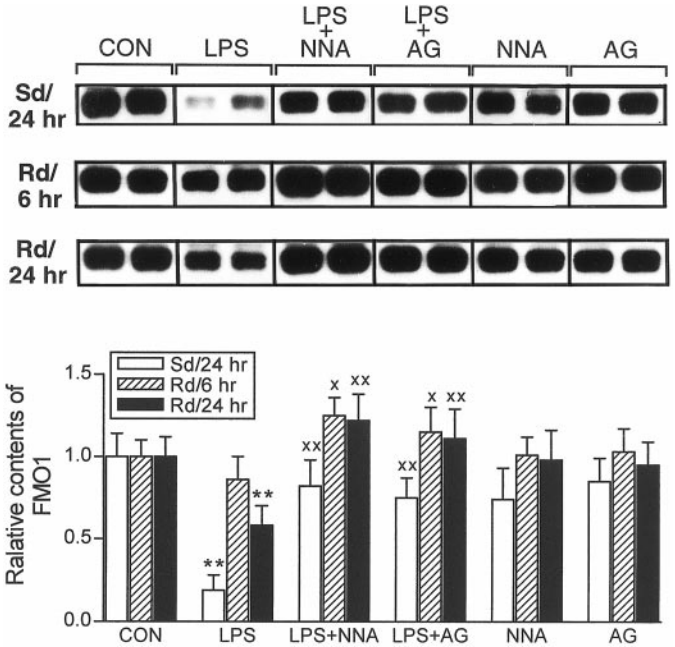

Fig. 5. Liver microsomal FMO1 contents of rats pretreated with LPS and LPS plus NOS inhibitors. FMO1 contents were determined by immunoblot analysis. FMO1 contents of LPS and LPS plus NOS inhibitors were compared with those of respective control groups and shown in the lower part of the figure as a bar graph. Data represent means ± S.D. obtained from duplicate measurements with liver microsomes of four or five individual rats in each treatment group. $^{**}p < .01$ indicates significant reduction of FMO1 content when compared with that obtained in the respective control group liver microsomes and $^{\times\times}p < .01$ indicates significant restoration toward normal level when compared with those obtained in the respective LPS- pretreated groups. Abbreviations: Sd/24 h, single dose saline or inhibitors and sacrificed at 24 h; Rd/6 h, 0 and 4 h repeat doses of saline or inhibitors and sacrificed at 6 h (early time point); Rd/24 h, every 4 h repeat doses of saline or inhibitors and sacrificed at 24 h.

The contents of FMO1 mRNA (Fig. 4) and enzyme protein (Fig. 5) were decreased most severely at 6 h after the LPS administration. In support of this, the iNOS mRNA content (Fig. 4) and plasma NOx concentration (Fig. 1) were increased most markedly at 6 h and suggested that the overproduced NO resulting from the elevated iNOS was responsible for the observed decrease of FMO1 activity. Conversely, when the production of NO was blocked by coadministering NOS inhibitors like NNA (general) or AG (iNOS specific) with LPS, even though the level of iNOS mRNA was still elevated (Fig. 4), the contents of FMO1 mRNA (Fig. 4) and enzyme protein (Fig. 5) as well as the FMO activities (Fig. 2) were not suppressed or restored (partially by single dose inhibitors and completely by repeat dose inhibitors) to the control level. This suggested that the reduction of FMO-catalyzed drug oxidation in LPS-treated rats was caused by the overproduced NO.

In addition to CO, NO is also known to bind to the heme center of CYP directly and to inhibit the hepatic microsomal CYP activity (Khatsenko et al., 1993; Stadler et al., 1994; Kim et al., 1995). However, Wink et al. (1993) and Minamiyama et al. (1997) demonstrated that there are both reversible (nitrosylation of heme moiety) and irreversible (nitrogen oxides affecting the protein structure) inhibitions in the NO-derived suppression of CYP activities in vivo. As for the FMO, because it contains FAD, which does not bind CO or NO directly, the FMO activity would not be inhibited either by CO (Stefek et al., 1989) or NO added in vitro from exogenous sources (Fig. 3). In support of this, the microsomal FMO activities of rat and rabbit liver (primarily FMO1) or rabbit kidney (primarily FMO4) and lung (primarily FMO2) tissues measured in vitro in the presence of SNP, an NO donor, or bubbling with NO gas (data not shown) were not inhibited at all (Fig. 3). This excludes inhibition of FMO by the reversible or irreversible nitrosylation mechanism. However, the FMO activities of liver microsomes isolated from LPS-treated rats (exposed to excessive NO in vivo) were severely decreased. This suggested that the decreased FMO activity in LPS microsomes was due either to the NO-mediated transcriptional down-regulation and/or post-translational modification (nitration) of FMO in the LPS-treated rat liver.

In an effort to determine whether this in vivo suppression of FMO activities was caused by the post-translational nitration of FMO, we have attempted a Western blot analysis of the LPS microsomes using an antinitrotyrosine monoclonal antibody. Unfortunately, we could not identify any bands equivalent to FMO1 enzyme size (~59 kDa; data not shown). This result indicated that the observed reduction in the FMO mRNA contents in the liver as well as the FMO contents and activities in LPS microsomes may be due to the NO-derived suppression of FMO mRNA expression (transcriptional down-regulation) or enhanced unstability of the mRNA rather than post-translational protein modifications by nitration. Because this was an in vivo study, however, we could not employ actinomycin D (a blocker of mRNA synthesis) to make certain about the transcriptional down-regulation hypothesis.

Upon inhibition of NO production in vivo by coadministration of either single or repeated doses of NNA or AG, even

though the hepatic content of iNOS mRNA was still elevated, the plasma levels of NOx were decreased and the microsomal contents of FMO1 protein as well as the FMO activities were restored (partially by single dose and completely by repeated dose treatments of inhibitors). However, the FMO mRNA levels were restored only partially (about 80% of control) both in single and repeated NOS inhibitor-treated rats. This suggested that factors other than NO (perhaps directly by LPS or indirectly by cytokines generated by the LPS pretreatment) may also be responsible for the down-regulation of FMO transcription.

Saline injections (both single and repeated) did not significantly affect the plasma NOx (control in Fig. 1) and FMO activities (control in Fig. 2). However, when saline injections (single and repeated) were given to the LPS-treated rats, even though the NOx level at 6 h post-LPS was elevated further, it was decreased markedly at 24 h (LPS in Fig. 1). As for the suppression of FMO activity produced by pretreatment of LPS, however, there was no significant differences between the two repeated saline-treated groups (6 and 24 h post-LPS). Thus, it appears that some unknown early stress factors arising from the saline treatment was responsible for the extra elevation of NOx at 6 h without affecting the FMO activity. We cannot offer any reasonable explanation for this 6-h saline effect at this time.

Also, both the single and repeated injections of NOS inhibitors alone (NNA and AG) did not significantly affect the plasma NOx levels (Fig. 1) or FMO activities (Fig. 2). However, when these NOS inhibitors were given to the LPS-treated (iNOS-induced) rats either by single or repeated dose regimen, plasma NOx level returned to the control level only by the 24-h repeated dose treatments (LPS + NNA and LPS + AG; Fig. 1). Even with these differential plasma NOx levels at the 6- and 24-h time points, the restoring effects on FMO activities were not significantly different (Fig. 2). Interestingly, however, the complete restorative effect on FMO activity was demonstrable only by giving repeated doses of NOS inhibitors. This indicated that single treatment of competitive inhibitors of NOS did not provide prolonged blockade on NO production.

In conclusion, in rats pretreated with LPS, the overproduced NO resulting from the enhanced iNOS activity appeared to be responsible for the down-regulation of FMO1 gene expression, eventually leading to the reduced FMO1 content and FMO activity in liver microsomes. These results strongly suggest that the depression of hepatic drug metabolism function observed in sepsis patients with bacterial endotoxemia may be caused both by the cytokine and NO-mediated suppression on both FMO and CYP gene expression as well as by reversible and irreversible inhibition of CYP activities in the liver. Although both reversible and irreversible nitrosylation together with some nitration (post-translational modification) appeared to be involved in the suppression of CYP activities, we could not detect their involvement in the suppression of FMO activities. In the present study, we were unable to determine the role of cytokines (NO-independent pathway) and thus, we can only suggest that at least the excessive activation of L-arginine/NO pathway (NO-dependent pathway) is responsible for the suppression of FMO activities, perhaps by transcriptional down-regulation in the LPS-treated rats.

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