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Down-regulation of phenobarbital-induced cytochrome P4502B mRNAs and proteins by endotoxin in mice: independence from nitric oxide production by inducible nitric oxide synthase

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Abstract

Multiple hepatic cytochrome P450 enzymes are down-regulated at the mRNA and protein levels during inflammation and infection. A body of evidence suggests that nitric oxide (NO) produced from inducible NO synthase (NOS2) is responsible for some of these effects. The current study was designed to examine the NO dependencies of the down-regulation of phenobarbital-induced CYP2B mRNAs and proteins by bacterial endotoxin (lipopolysaccharide, LPS) treatment *in vivo*, using an *NOS2*-null mouse model. Treatment of C57/BL6 mice with 0.3 mg/kg of LPS maximally suppressed phenobarbital-induced CYP2B9 and 2B10 mRNAs measured 12 hr after injection, whereas 1–10 mg/kg of LPS was required to elevate NO production. Down-regulation of CYP2B mRNAs by 1 mg/kg of LPS was equivalent in wild-type and *NOS2*-null mice. No effect of LPS in the dose range of 0.3 to 10 mg/kg was observed on microsomal CYP2B protein levels measured 12 hr after treatment, whereas 1 mg/kg of LPS suppressed CYP2B proteins 24 hr after treatment in both wild-type and *NOS2*-null mice. We conclude that the main mechanism for the down-regulation of CYP2B proteins in mouse liver following moderate- or high-dose LPS treatment is via NO-independent suppression of CYP2B9 and 2B10 mRNAs. Unlike rat hepatocytes, the contribution of a rapid, NO-dependent mechanism of CYP2B protein suppression in mouse liver appears to be minor or non-existent.

Keywords: Nitric oxide; Cytochrome P450; Bacterial endotoxin; Inflammation; Mouse liver; Phenobarbital

1. Introduction

Cytochrome P450 superfamily monooxygenases are important for the biotransformation of drugs and toxins. Numerous studies indicate that inflammation and infection suppress hepatic CYP levels [1] in various species including human, rat, and mouse. LPS has been used extensively as a model of sepsis to study the inhibitory effects of inflammation and infection on CYP activity and expression [2–5].

During inflammatory responses such as that produced by LPS, cellular levels of nitric oxide increase greatly in many cell types due to the induction of inducible NOS (iNOS, NOS2) expression [6]. Several studies have suggested that the NO produced consequent to hepatic NOS2 induction mediates the suppression of hepatic CYP expression and activity caused by LPS treatment. *In vitro* treatments of rat liver microsomes or purified CYPs with NO, peroxynitrite, or donors of RNS inhibited the catalytic activities of CYP enzymes, a result hypothesized to be caused by reaction of the NO or derivative RNS with heme or amino acid residues on CYPs [7–9]. Inhibitors of NOS, such as *N*-methyl-L-arginine, *N*-nitro-L-arginine methyl ester, or aminoguanidine partially reverse the suppression of CYP mRNAs and proteins in primary rat hepatocytes or whole animals caused by LPS or cytokine treatment [10–13].

Contrary evidence exists that NO is not required for the LPS-induced suppression of some CYPs. We showed that the suppression of several constitutively expressed CYPs caused by LPS treatment is NO-independent [14,15]. Aminoguanidine inhibits the production of NO, but does

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Abbreviations: CYP, cytochrome P450; LPS, bacterial lipopolysaccharide; NO, nitric oxide; NOS, NO synthase; NOS2, inducible NOS; RNS, reactive nitrogen species; PB, phenobarbital; GAP, glyceraldehyde-3-phosphate dehydrogenase; RT–PCR, reverse transcription–polymerase chain reaction; NOx, nitrate + nitrite.

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not prevent the reduced hepatic expression of CYP2C11, 2E1, and 3A2 caused by LPS treatment in rats [15]. Similarly, NOS inhibitors failed to prevent the down-regulation of CYP2C11 mRNA or protein by LPS or interleukin-1β in cultured rat hepatocytes [14]. We also reported that LPS treatment causes equivalent suppression of CYP2C, 3A, and 2E1 mRNA and protein expression in both *NOS2*-null mice and wild-type mice, supporting the hypothesis that NO was not required for the LPS-induced suppression of these CYPs [16].

CYP2B1 and 2B2 are major PB-inducible CYPs in rat liver. Carlson and Billings [17] found that NOS inhibitors block the suppression of CYP2B proteins by a cytokine mixture in cultured rat primary hepatocytes, while an NO donor reduces CYP2B1/2 apoprotein levels, suggesting the involvement of NO in the suppression of CYP2B proteins. Khatsenko *et al*. [18] then reported that LPS treatment of rats inhibits the PB-induced expression of CYP2B1/2 mRNAs and proteins. They also concluded that NO participates in this effect of LPS, because it was attenuated by administration of an NOS inhibitor [18]. Further evidence that NO and RNS may play an important role in the regulation of CYP2B activity was provided by Roberts *et al*. [9], who found that the incubation of purified CYP2B1 protein with peroxynitrite resulted in the nitration of tyrosine residues on CYP2B1, which correlated with the loss of its enzymatic activity.

Recently, we found that the suppression of PB-inducible CYP2B1 expression by LPS treatment in rat hepatocytes occurs by dual mechanisms. Low concentrations of LPS cause an NO-independent suppression of CYP2B1 mRNA that leads to a slow down-regulation of the protein between 24 and 48 hr later [19]. At higher concentrations of LPS (10^{-7} g/mL and above), there is a rapid, NO-dependent suppression of CYP2B proteins that occurs within 6 hr, before there is a significant effect on the CYP2B1 mRNA [19].

The above studies showing that NO production is required for CYP2B mRNA and/or protein down-regulation by inflammatory stimuli relied on the use of NOS inhibitors to block NO production. Due to limitations of specificity inherent in the use of any drug, NOS inhibitors could potentially affect CYP expression in ways other than by inhibiting cellular NO production. The availability of *NOS2*-null mice allows the dissection of NOS2-dependent pathways in the absence of chemical inhibitors, and therefore we used this model to further investigate the role of NO in the *in vivo* regulation of CYP2B mRNAs and proteins by LPS. We report that the down-regulation of PB-induced CYP2B enzymes in mouse liver following LPS treatment occurs by a pretranslational mechanism in the physiologically relevant dose range, with no evidence for a rapid suppression of the CYP2B proteins. The effects were the same in wild-type and null animals, indicating that CYP2B suppression in mouse liver is not dependent upon NO production by NOS2.

2. Materials and methods

2.1. Animals and treatments

Female C57BL/6 and congenic C57BL/6-*NOS2* null mice, 9–12 weeks old, were purchased from Jackson Laboratories. The C57BL/6-*NOS2* mice have a targeted mutation in the *NOS2* gene [20], which is located on chromosome 11. The animals were allowed to acclimate to the animal core facility for at least 1 week before use. *Escherichia coli* LPS, serotype 0127:B8, and PB (Sigma Chemical Co.) were dissolved in sterile 0.9% saline.

To attain stable levels of PB-induced CYP2B mRNA and protein expression, mice were injected i.p. every 8 hr with a previously optimized dose of PB (33 mg/kg body weight) [21] throughout the experiment. LPS was injected at the doses indicated, 24–48 hr after the first dose of PB, simultaneously with the regular dose of PB. Mice continued to receive PB injections every 8 hr thereafter, until they were killed by CO_2 asphyxiation for collection of blood and harvest of the livers. To control for any potential effects caused by LPS-induced hypophagia, mice were either pair-fed or deprived of food after LPS injections, as indicated for each experiment.

Male Fischer 344 rats, 8 weeks old at the beginning of the treatments, were obtained from Charles River Laboratories. Rats were injected with three daily doses of PB (100 mg/kg, i.p.), and then with injections of 50 mg/kg of PB every 12 hr throughout the remainder of the experiment. LPS was injected at a dose of 10 mg/kg, i.p., at the same time as the third 12-hourly injection of PB. Control animals received PB and saline. All animals were deprived of food after the LPS injections. Rats were killed 8 or 14 hr after the LPS or saline injection. Mouse and rat livers were either used immediately for the preparation of RNA or microsomal proteins, or they were snap-frozen in liquid nitrogen and stored at −80° for later analysis.

2.2. Analysis of serum NOx concentration

The stable end-products of NO synthesis, nitrate plus nitrite (NOx), were measured in plasma as an indicator of LPS-dependent NO synthesis. Mouse blood was collected by severing the abdominal aorta during liver harvesting. Blood was allowed to clot at room temperature and centrifuged for 5 min at 500 *g* and 4°. Serum was removed, and stored at −80°. Aliquots of mouse serum were deproteinized by diluting them 5–10 times with water and filtering through Ultrafree-MC filter units (Millipore Corp.) at 10,000 g and 4° for 60 min. Twenty microliters of deproteinized sample was then used for a colorimetric, microplate assay of NOx concentrations, based on the Griess reaction as described previously [15]. In this assay, serum nitrate was reduced to nitrite with nitrate reductase prior to analysis. Nitrite levels were then measured colorimetrically at 550 nm with a Thermomax microplate reader

(Molecular Devices Corp.), using a sodium nitrate standard curve, which was linear within the range of detection.

2.3. Isolation of total RNA and Northern blotting

Total liver RNA was prepared by the acid-phenol extraction method [22], and stored at −80°. The total RNA concentration was determined spectrophotometrically from the absorbances of the samples at 260 nm.

Northern blotting of total mouse liver RNA was performed as described previously [21]. The relative abundances of CYP2B9 and 2B10 mRNAs were measured by probing blots with the respective complementary oligonucleotides [21] labeled with ^{32}P via T4 polynucleotide kinase. Bound ^{32}P-labeled probes were detected and quantified by phosphorimaging. Quantified CYP2B9 and 2B10 mRNA levels were normalized to the GAP mRNA content in the same samples, which was measured by probing blots with a cDNA probe of 780 bp (American Type Culture Collection) [23]. Each of the oligonucleotide probes and the GAP cDNA probe recognized a single band on Northern blots. The assays were conducted under conditions of linearity with respect to the amount of RNA applied to the gel.

2.4. RT–PCR

Total RNA from individual mouse livers was reverse-transcribed using a RETROscript™ kit (Ambion Inc.) following the instructions of the manufacturer. Briefly, 1 μg of total RNA was mixed with 2 μL of random decamers (50 μM), 2 μL of 10× RT buffer, 4 μL of dNTP mix (2.5 mM of each dNTP), 1 μL of placental RNase inhibitor (10 units/μL), 1 μL of reverse transcriptase (100 units/μL), and water to a final volume of 20 μL. The reaction mixture was incubated at 44° for 1 hr, and then at 92° for 10 min to inactivate the enzyme.

Reverse-transcribed NOS2 and cyclophilin cDNAs were PCR amplified with Super*Taq* (Ambion Inc.) using a ''hot-start'' procedure. Three microliters of RT reaction was mixed with 4.5 μL of 10× PCR buffer, 5 μL dNTP mix (2.5 mM of each dNTP), 5 μL of PCR primers (containing a 5 μM concentration of each primer), and water to a total volume of 45 μL. This mixture was heated at 94° for 5 min, then cooled to 65°, and 1 unit Super*Taq* added. NOS2 cDNA was amplified for 30 cycles at 95° for 1 min, 55° for 1 min, and 72° for 1 min, with a final extension of 7 min at 72°. The primer sequences for NOS2 were (5′ to 3′): GAA GTG GGC CGA AGG ATG G (forward) and TTG GTG TTG AAG GCG TAG C (reverse). The NOS2 primer pair produced the expected PCR product of 524 bp using reverse-transcribed hepatic RNA from LPS-treated mice as a template, and gave the predicted products following digestion with *Apa*I and *Alw*NI. This primer pair amplifies only the wild-type NOS2 cDNA, because the reverse primer targets the calmodulin-binding domain, which is deleted in the *NOS2*-null mice [20]. Cyclophilin mRNA in the same samples was used as an internal control, and it was amplified for 25 cycles at 95° for 30 s, 55° for 30 s, and 72° for 40 s, with a final extension of 2 min at 72°. The primers provided by Ambion Inc. were (5′ to 3′): CCA TCG TGT CAT CAA GGA CTT CAT (forward) and CTT GCC ATC CAG CCA GGA GGT CTT (reverse).

2.5. Preparation of liver microsomes, SDS–PAGE, and Western blotting

Pyrophosphate-washed liver microsomes were prepared by differential ultracentrifugation [24], and stored at −80°. Protein concentrations of the samples were measured according to the method of Lowry *et al*. [25], using bovine serum albumin as a standard.

The relative levels of CYP2B proteins in mouse liver microsomes, or of CYP2B1 and 2B2 in rat liver microsomes, were measured by Western blotting as described previously [19,21]. Microsomal proteins were separated via SDS–PAGE (7.5% polyacrylamide), and transferred electrophoretically onto nitrocellulose membranes (Schleicher & Schuell). Blots were probed with anti-rat CYP2B1/2, donated by Dr. James Halpert (University of Texas, Medical Branch at Galveston). Bound antibodies were detected using horseradish peroxidase-coupled goat anti-rabbit IgG and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. Our previous studies led us to conclude that the antibody to rat CYP2B1 recognizes both CYP2B9 and 2B10 in female mouse liver [21]. However, these proteins run together on the gel as a single band with a M_r of 57,000. Thus, they are quantified together as ''CYP2B.'' The intensities of the CYP2B bands were quantified via photodensitometry (Lynx System, Applied Imaging) and were proportional to the amount of antigen loaded on the blots in the range used.

2.6. Statistical analysis

Quantified data from Northern and Western blots were expressed as a percentage ± SEM of the control group (PB-treated, without LPS) in each experiment. A two-tailed independent *t*-test, or one-way analysis of variance, and the Newman–Keuls post-hoc test were used to detect statistically significant differences among the treatment groups. A P value of 0.05 or less was considered to be statistically significant.

3. Results

3.1. Dose-dependencies of CYP2B suppression and NO production in mice

A relatively short treatment duration of 12 hr was chosen for this study because our previous work in rat hepatocytes

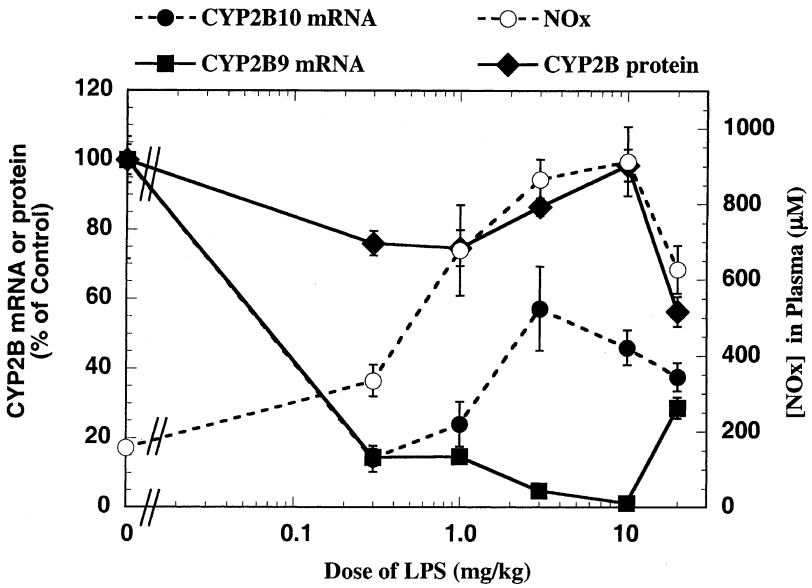

Fig. 1. Dose-dependence of LPS effects in mice. Nine-week-old female C57BL/6 mice were injected i.p. with PB (33 mg/kg) every 8 hr throughout the experiment. Forty-eight hours after the first PB injection, groups of 5 mice were injected i.p. with PB plus 0, 0.3, 1, 3, or 10 mg/kg of LPS. Mice were treated with 20 mg/kg of LPS in a separate experiment with a separate control group. The results from both experiments are combined in the figure. All mice were deprived of food after LPS (or vehicle) treatment, and killed 12 hr later. Plasma NOx concentrations and relative levels of hepatic CYP2B mRNAs and proteins were measured by Northern and Western blotting, respectively, as described in the text. CYP2B mRNA levels were normalized to the GAP mRNA levels in the same samples. Results are expressed as the means ± SEM of each group. In the interests of clarity, significant differences between groups are NOT shown on the figure, but are indicated in the text.

indicated that high concentrations of LPS produced a rapid and NO-dependent suppression of CYP2B1 protein [19]. As shown in Fig. 1, LPS treatment increased the plasma NOx levels dose-dependently. The lowest dose producing a significant increase (4.3-fold) was 1.0 mg/kg, and a maximum increase of 5.8-fold was attained at 10 mg/kg. The mean plasma NOx content in mice treated with 0.3 mg/kg of LPS was not statistically different from the control level ($P = 0.09$).

The dose-dependence of CYP2B mRNA suppression in PB-induced mice was clearly different from that of induction of NOS activity. CYP2B9 and CYP2B10 mRNAs were maximally or near-maximally suppressed to 14 and 14.5% of control levels, respectively, by the lowest dose of LPS used, 0.3 mg/kg (Fig. 1). In contrast, the levels of PB-induced CYP2B proteins were not affected significantly at 12 hr after treatment with 0.3 to 10 mg/kg of LPS (Fig. 1), although the mean values were reduced to 76, 74.6, and 86.5% of control, respectively, by 0.3, 1, and 3 mg/kg of LPS. However, a significant 46% decrease in PB-induced CYP2B protein was found in mice treated with 20 mg/kg of LPS (Fig. 1).

3.2. Effect of a moderate dose of LPS on PB-induced CYP2B expression in NOS2-null mice

The above dose–response study showed that in the 0.3 to 10 mg/kg dose range, LPS caused a significant suppression of PB-induced CYP2B mRNA, but not protein levels within 12 hr of injection. Since the lowest LPS dose that significantly increased the plasma NOx level was 1 mg/kg, we investigated the effects of this dose on PB-induced CYP2B mRNA and protein in *NOS2*-null mice compared with wild-type animals. Two identical experiments were conducted separately, each comprising four mice per group, and they gave very similar results. The results from one experiment are presented in detail: data from the second experiment will only be mentioned to point out where differences between the experiments were seen.

RT–PCR analysis was used to confirm that the *NOS2*-null mice did not express NOS2 in response to LPS treatment. LPS treatment for 8 or 24 hr clearly induced NOS2 mRNA in all of the wild-type mice, but no NOS2 product was detected in the null animals (Fig. 2A). LPS treatment significantly suppressed PB-induced CYP2B9 and CYP2B10 mRNA expression in the wild-type mice to 18 and 42% of control levels, respectively, 8 hr after injection, and to 36 and 53% of control levels, respectively, 24 hr after injection (Figs. 2B and 3A). The effects of LPS treatment on CYP2B mRNAs in *NOS2*-null mice were essentially identical to those in the wild-type animals at both time points (Figs. 2B and 3A).

Treatment with 1 mg/mL of LPS had no effect on PB-induced microsomal CYP2B protein levels in the livers of wild-type or *NOS2*-null mice 8 hr after injection, but significantly decreased CYP2B levels to 58 and 71% of controls in the wild-type and null animals, respectively (Figs. 2C and 3B). In the second experiment, there was likewise no significant effect on CYP2B protein levels in

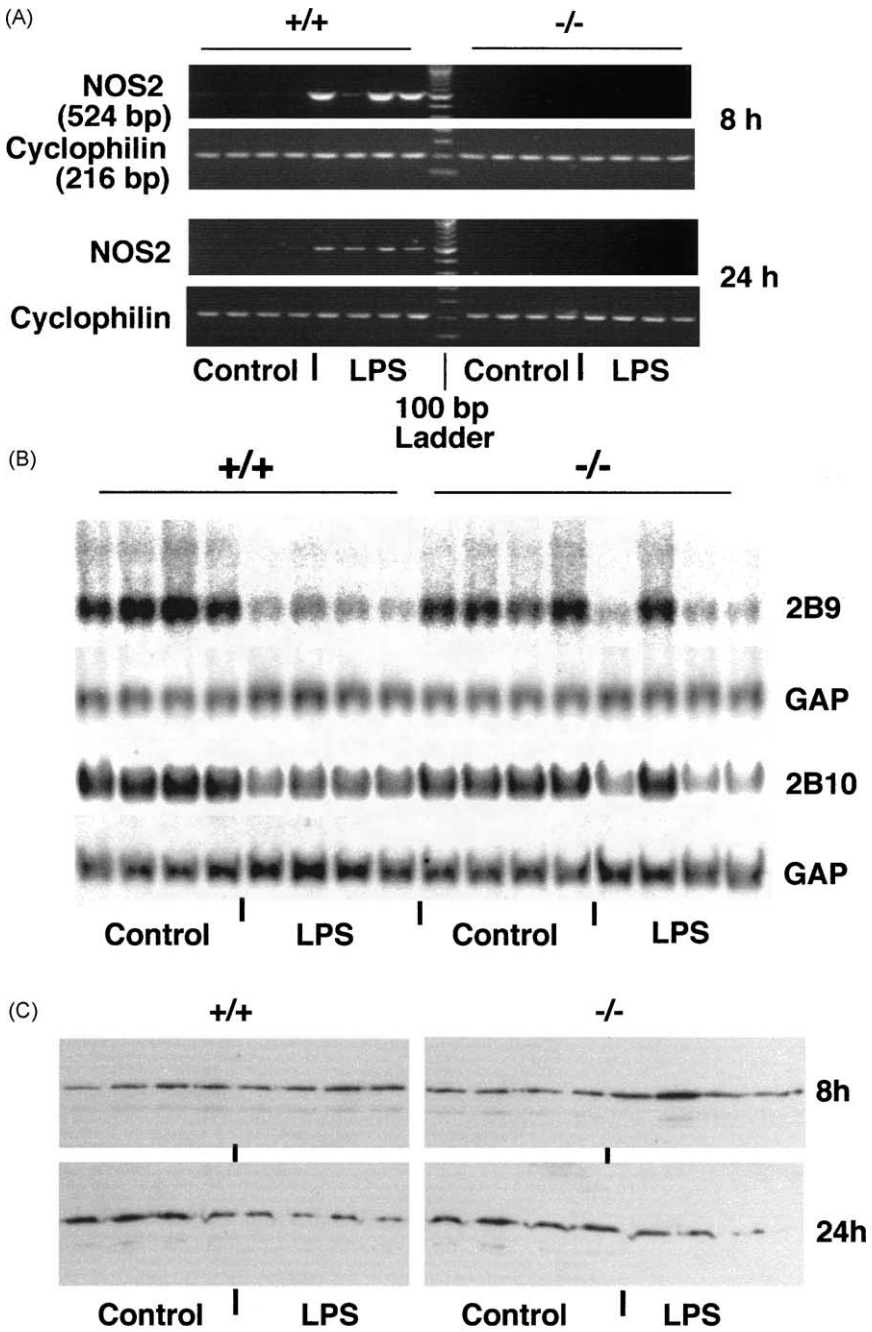

Fig. 2. Effect of 1 mg/kg of LPS on NOS2 and CYP2B expression in wild-type and *NOS2*-null mice. Female C57BL/6 (+/+) or C57BL/6-*NOS2* mice (−/−), 10–12 weeks old, were injected i.p. with PB (33 mg/kg) every 8 hr throughout the experiment. Twenty-four hours after the first dose of PB, groups of 4 mice were injected with a mixture of LPS (1 mg/kg) and PB (33 mg/kg) in sterile saline, or with PB alone (control groups). Control mice were pair-fed with the amount of food that the LPS-treated mice ate. Mice were killed at 8 and 24 hr after LPS or vehicle injection, and their livers were harvested for RNA and microsomal protein preparation. (A) RT–PCR analysis of NOS2 and cyclophilin mRNAs in total hepatic RNA. (B) Twenty micrograms of total hepatic RNA from animals killed 8 hr after injection was subjected to Northern blot analysis of CYP2B mRNA expression. (C) Mouse liver microsomes (0.5 μg) were analyzed for relative CYP2B protein levels by Western blotting.

either genotype, 6 hr after the injection of LPS (data not shown). At 24 hr after injection, the effect of LPS in the second experiment was, if anything, greater in *NOS2*-null mice than in wild-type animals (49 ± 20 and $84 \pm 5\%$ of control, respectively). The results were essentially the same when the data were expressed per gram of liver or per milligram of microsomal protein (not shown).

3.3. Effect of a high dose of LPS on PB-induced CYP2B expression in NOS2-null mice

The experiment shown in Fig. 1 demonstrated that injection of 20 mg/kg of LPS produced a decrease in PB-induced CYP2B protein level measured 12 hr later. Therefore, we investigated whether this decrease was NO-dependent by

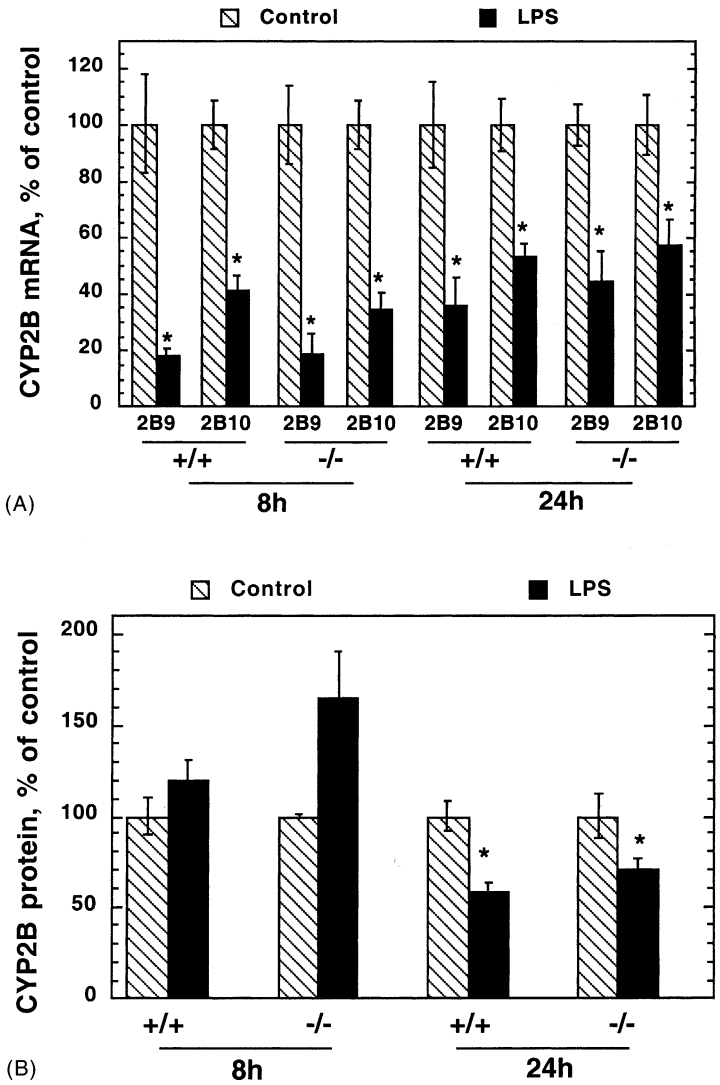

Fig. 3. Effect of LPS (1 mg/kg) on CYP2B expression and activity in wild-type and *NOS2*-null mice. Animals and treatments were as described in the legend of Fig. 2. (A) Relative CYP2B9 and CYP2B10 mRNA levels in total hepatic RNA were determined by Northern blotting and phosphorimaging. Values for the individual P450 mRNAs were normalized to the GAP signals on the blots. (B) Relative levels of CYP2B proteins in mouse liver microsomes were determined by Western blotting and photodensitometry. +/+, wild-type mice; −/−, *NOS2*-null mice. Values shown are the means ± SEM of 4 mice per group and are expressed as percentages of the respective control group means. Key: (∗) significantly different from control, $P < 0.05$.

comparing the responses of wild-type and *NOS2*-null mice to the high dose of LPS.

As observed previously (Fig. 1), the high dose of LPS produced a significant suppression of PB-induced CYP2B9 and 2B10 mRNAs (Fig. 4A). This effect was independent of NOS2 induction, because it was the same in both wild-type and *NOS2*-null mice (Fig. 4A). As expected, serum levels of NOx were elevated significantly by LPS treatment in the wild-type, but not the *NOS2*-null mice (Fig. 4B). However, in this experiment, high-dose LPS treatment failed to produce any effect on CYP2B protein levels in either mouse genotype (Fig. 4A).

3.4. Rapid suppression of CYP2B proteins in rat liver

To determine whether high-dose LPS treatment could produce a rapid suppression of CYP2B proteins in rat liver, male PB-treated rats were injected with 10 mg/kg of LPS and killed 8 and 14 hr later for analysis of microsomal CYP2B1 and 2B2 levels. In contrast to the CYP2B proteins in the mouse, significant suppression of CYP2B1 and 2B2 levels were observed in rat liver 8 and 14 hr after LPS injection (Fig. 5). CYP2B1 was suppressed to 46 and 56% of control levels at these time points, whereas CYP2B2 was slightly more affected.

4. Discussion

The results of these experiments indicate that the dominant mechanism of CYP2B down-regulation by medium to high doses of LPS in PB-induced murine liver *in vivo* is pretranslational, and is independent of NO production by NOS2. The conclusion that the down-regulation of

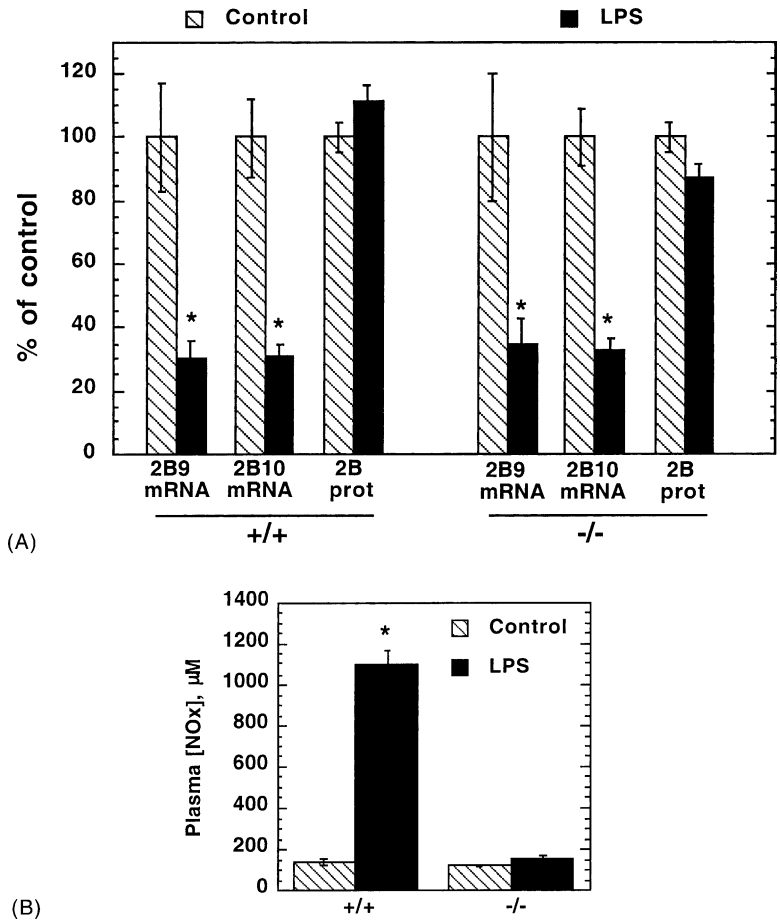

Fig. 4. Effect of 20 mg/kg of LPS on CYP2B expression and activity, and plasma NOx levels in wild-type and *NOS2*-null mice. Female 9-week-old C57BL/6 mice and C57BL/6-*NOS2* mice were injected i.p. with PB (33 mg/kg) every 8 hr throughout the experiment. Forty-eight hours after the first PB injection, groups of 6 mice were treated with LPS (20 mg/kg) and PB (33 mg/kg) in sterile saline, or with PB alone (control groups). All mice were deprived of food after LPS (or vehicle) treatment, and killed 12 hr later. +/+, wild-type mice; −/−, *NOS2*-null mice. (A) Relative levels of CYP2B mRNAs and proteins in the samples were determined as described in the text. The PB-induced CYP2B mRNA and protein levels in the respective control groups were assigned as 100%. (B). The concentrations of NOx were measured in serum obtained when the animals were killed. Values shown are the means ± SEM of 6 mice per group. Key: (∗) significantly different from control, $P < 0.05$.

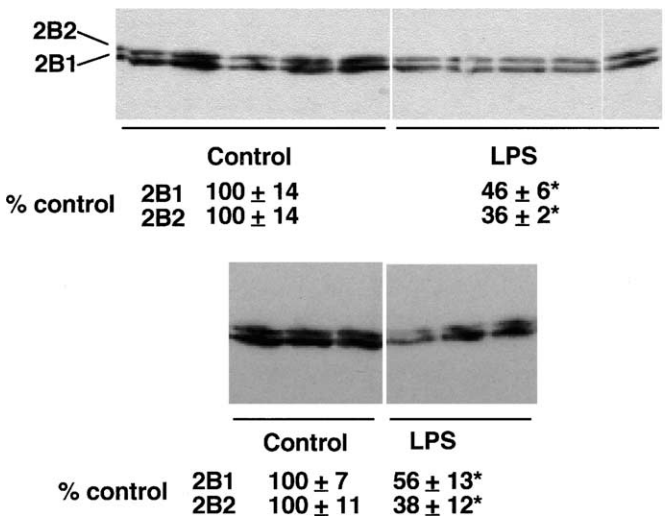

Fig. 5. Effect of 10 mg/kg of LPS on CYP2B protein levels in rat liver microsomes. Male 8-week-old Fischer 344 rats were injected i.p. with three daily doses of PB (100 mg/kg), and then with 12-hourly injections of 50 mg/kg of PB throughout the remainder of the experiment. Twenty-four hours after the first 50 mg/kg dose of PB, rats were treated with LPS (10 mg/kg) and PB (50 mg/kg) in sterile saline, or with PB alone (control groups). All mice were deprived of food after LPS (or vehicle) treatment, and killed 8 and 14 hr later. Relative levels of CYP2B1 and CYP2B2 in hepatic microsomes were determined by Western blotting. Values shown are the means ± SEM of 5 and 3 rats per group at the 8- and 14-hr time points, respectively. Key: (∗) significantly different from control, $P < 0.05$.

PB-induced CYP2B9 and CYP2B10 mRNAs by LPS treatment is not caused by NO derived from NOS2 is supported by the findings that: (a) LPS was more potent in suppression of the CYP2B mRNAs than in elevation of plasma NOx levels; and (b) CYP2B mRNAs were suppressed identically in control and *NOS2*-null mice treated with either a moderate (1 mg/kg) or a high (20 mg/kg) dose of LPS. These findings in a genetic model of NOS2 deficiency are in strong agreement with our previous findings based on pharmacological inhibition of NOS2 in primary cultures of rat hepatocytes [19]. CYP2B1 mRNA suppression by LPS in hepatocytes also occurred at low concentrations of LPS that did not induce NOS2, and was not affected when NO production was blocked by NOS inhibitors.

In rat hepatocytes, high concentrations of LPS evoked a rapid down-regulation of PB-induced CYP2B1 protein (within 6 hr) that was NO-dependent because it was blocked by NOS2 inhibition and mimicked by NO donors [19]. We speculated that this *in vitro* effect of high concentrations of LPS could be relevant *in vivo* during sepsis when LPS concentrations in the plasma are high and NOS2 is maximally activated [19]. However, in the present study, we were unable to detect a rapid decline of CYP2B proteins (8 or 12 hr after injection) at doses of 1–10 mg/kg of LPS, which were sufficient to cause significant increases in NO production in the same animals. At 1 mg/kg of LPS, a significant decrease in CYP2B proteins was only observed 24 hr after LPS injection, consistent with the mechanism of suppression being due primarily to prior down-regulation of the CYP2B mRNAs. Consistent with this conclusion, this slow suppression of the CYP2B protein levels (like the more rapid down-regulation of the mRNAs) was the same in both wild-type and *NOS2*-null mice.

Our first experiment employing the highest dose of LPS studied (20 mg/kg; Fig. 1) indicated that a rapid suppression of PB-induced CYP2B proteins did occur at this very high dose (which is close to the LD_{50} in mice). Therefore, we conducted an experiment using *NOS2*-null mice to determine if this decrease was NO-dependent as was observed previously for CYP2B1 in rat hepatocytes. In this second study, even the high dose of LPS failed to produce a significant effect on CYP2B protein 12 hr after injection (Fig. 4). The reason for the variability in this response is unknown, and it would require studies with much larger numbers of animals to determine whether or not there is a significant effect. Taken together, these discrepant observations suggest that if LPS does suppress PB-induced CYP2B protein at 12 hr after treatment, the decrease is relatively small. Therefore, we conclude that the dominant mechanism of the down-regulation of CYP2B protein in the endotoxemic mouse is pretranslational and independent of NOS2. If there is an NO-dependent mechanism regulating the PB-induced CYP2B protein in mouse liver, its overall contribution to CYP2B down-regulation must be only a minor factor.

The absence of a rapid NO-dependent suppression of CYP2B proteins in mouse liver *in vivo*, in contrast to rat hepatocytes, could mean that the effect observed in hepatocytes is not relevant to *in vivo* suppression of CYP2B enzymes. However, as shown in Fig. 5, rat liver microsomal CYP2B1 and 2B2 proteins were suppressed only 8 hr after the injection of a high dose of LPS, in agreement with studies in rat hepatocytes [19] and in contrast to our observations in mice. These findings are consistent with the interpretation that the lack of a rapid NO-dependent effect in mice is due to a species difference in the mechanisms of regulation of CYP2B proteins by LPS. Work is in progress to determine the NO-dependence of CYP2B expression in rats *in vivo*.

The above presumed species difference in the NO-dependence of CYP2B expression could be related to different overall sensitivities to LPS. Therefore, it is possible that even higher doses of LPS might be required to see the effect in mice. However, it is clear from Fig. 1 that 3–10 mg/kg of LPS is sufficient to maximally induce NOx formation in these mice. Consequently, it would appear unlikely that higher doses would lead to the appearance of an NO-dependent mechanism. Other possibilities to be investigated are that the species difference is due to differential sensitivities of murine and rat P450s to NO, or that the amounts or kinetics of formation of RNS species in the vicinity of the endoplasmic reticulum are different in rat and mouse hepatocytes.

The question of whether or not NO can regulate the expression of P450 enzymes has potential clinical importance because many CYPs are suppressed during inflammation and infections, and if NOS inhibitors are used clinically to treat sepsis or other inflammatory disease, they may block the suppression of CYPs and the related suppressed drug metabolism and clearance. NO donors are also commonly used to treat diseases. Therefore, metabolism and clearance of other drugs that a patient is taking may be reduced if the NO or RNS produced by NO donors causes CYP suppression. CYPs of the 2B subfamily have been investigated for potential gene therapy of cancers, allowing selective toxicity of anti-tumor agents activated by CYP2Bs to cells expressing the transfected gene [26]. Human CYP2B6 metabolizes many clinically used drugs, endogenous compounds, and exogenous toxins [27]. A question of critical importance that we are currently working to address, therefore, is whether the mechanism of regulation of human CYP2B6 during inflammatory stimulation is more similar to that of mouse CYP2B9 and 2B10, or to that of rat CYP2B1.

Acknowledgments

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