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# Nitric Oxide-Independent Suppression of P450 2C11 Expression by Interleukin-1β and Endotoxin in Primary Rat Hepatocytes*

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**ABSTRACT.** Hepatic expression of multiple cytochrome P450 genes is suppressed in the livers of rats undergoing an inflammatory response. Nitric oxide (NO) released during inflammation has been implicated in the decreased activities and expression of several cytochrome P450 isozymes. We examined the role of cytokine-mediated NO release on cytochrome P450 2C11 expression in rat hepatocytes cultured on Matrigel. Lipopolysaccharide (LPS), interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), but not interferon-γ (IFN-γ), suppressed the expression of P450 2C11 mRNA. Neither IL-6 nor IFN-γ caused NO release into the medium or induction of inducible nitric oxide synthase (iNOS) mRNA. IL-1β and LPS were the most effective in causing NO release and iNOS induction, and in down-regulating P450 2C11 mRNA expression. Combinations of the cytokines, IFN-γ, and LPS produced an additive release of NO but did not synergize to further suppress P450 2C11 mRNA. To investigate the role of NO in the IL-1β- or LPS-mediated suppression of P450 2C11, *N*-monomethyl-L-arginine (NMA) was administered at concentrations ranging from 30 to 300 μM. Three hundred micromolar NMA returned NO release back to control levels, but did not affect the IL-1β- or LPS-mediated down-regulation of P450 2C11 mRNA or protein expression. Our results suggest that NO is not required for IL-1β- or LPS-mediated down-regulation of P450 2C11 expression in cultured hepatocytes. BIOCHEM PHARMACOL **54**;6:729–737, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** cytochrome P450 2C11; nitric oxide; interleukin-1; lipopolysaccharide; inflammation; hepatocytes

It is well established that the ability of the liver to carry out cytochrome P450-dependent drug biotransformation is compromised during inflammation or bacterial infection. The decreases seen in cytochrome P450 content, expression, and catalytic activity can be mimicked in primary cultured hepatocytes by treatment with cytokines and interferons [1, 2]. Many studies have examined the suppressive effects of cytokines on the expression of inducible P450 isozymes *in vitro*. P450 2C11 is an isozyme that is highly and constitutively expressed in male rat liver, and can be stably expressed in hepatocytes cultured on Matrigel in the absence of added growth hormone [3]. We have demonstrated the down-regulation of P450 2C11 by IL-1β‡, IL-6, IFN-α, and TNF-α in primary rat hepatocytes [4]. Further, we showed that the cytokines that suppress P450 2C11 also induce the expression of the acute phase genes AGP and β-fibrinogen. P450 2C11 expression has also been found to be suppressed by growth hormone [3, 5] and epidermal growth factor [6] *in vitro*.

In the hepatocyte and Kupffer cells, IFNs, cytokines, and LPS evoke the release of NO via an induction of iNOS [7, 8]. It has also been shown that the NO released following cytokine administration can inhibit the catalytic activity of P450 isozymes [9, 10]. Khatsenko *et al.* [10] demonstrated *in vivo* that NOS inhibitors were able to restore the LPS-stimulated decreases in hepatic microsomal P450 activity. They also found that treating microsomes with NO-generators *in vitro* results in decreased P450-dependent oxygenation reactions. Other studies have demonstrated in primary rat hepatocytes that the NOS inhibitor NMA can reverse the decreases in P450 1A1 activity, mRNA, and protein levels evoked by treating with a mixture of cytokines and LPS [11]. In these studies, the chemicals phenobarbital [10] and β-naphthoflavone [11] were used to induce the expression of P450 2B1/2 and 1A1, respectively.

A role for NO in the cytokine-mediated down-regulation of several constitutively expressed isozymes has also been proposed [12]. In short-duration primary rat hepatocyte cultures, NOS inhibitors were able to restore the decreases in P450 2B, 2C11, and 3A2 proteins elicited by cytokine administration [12]. In the present study, we investigated

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‡ *Abbreviations:* AGP, α-acid glycoprotein; GAP, glyceraldehyde-3-phosphate dehydrogenase; GSNO, *S*-nitrosoglutathione; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide; LPS, lipopolysaccharide; NMA, *N*-monoethyl-L-arginine; NO, nitric oxide; and TNF-α, tumor necrosis factor-α.

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the effects of NOS inhibition on the cytokine- and LPS-mediated down-regulation of P450 2C11 mRNA and protein in 5- to 7-day cultures of hepatocytes on Matrigel, in which P450 2C11 expression has been shown to be stable [3, 4]. Our studies showed that NO is not required for the LPS- or cytokine-mediated down-regulation of P450 2C11 in cultures of primary rat hepatocytes.

## MATERIALS AND METHODS

### *Materials*

Male Fischer 344 (F344) rats (150–200 g) from Harlan Sprague Dawley (Indianapolis, IN) were used for hepatocyte isolation. Cell culture medium (Waymouth's MB 752/1), insulin, antibiotics, murine recombinant IL-1β, IL-6, and TNF-α were purchased from GIBCO-BRL Life Technologies (Bethesda, MD). Murine recombinant IFN-γ was purchased from Boehringer Mannheim (Indianapolis, IN). Chromatographically purified *Escherichia coli* LPS, serotype 0127:B8, NMA, sulfanilamide, *N*-(1-naphthyl)-ethylenediamine, NADPH:nitrate reductase, and Type IV collagenase were purchased from the Sigma Chemical Co. (St. Louis, MO). The P450 2C11 cDNA is described in Ström *et al.* [13] and polyclonal antibody was purified as described by Morgan *et al.* [14]. The iNOS cDNA probe was donated by Dr. D. L. Feinstein, New York Hospital-Cornell Medical Center. Matrigel was prepared by us, according to the method of Schuetz *et al.* [15], from murine Engelbreth-Holm-Swarm sarcoma provided by Dr. H. Kleinman, National Institutes of Health.

### *Hepatocyte Isolation and Culture*

Matrigel was diluted to 7.7 mg/mL with Waymouth's medium and applied to 60-mm plastic culture dishes (Nunc; Fisher Scientific, Pittsburgh, PA) and allowed to gel at 37° for 1–2 hr. Isolation of rat hepatocytes was performed by *in situ* collagenase perfusion [3]. The viability of hepatocytes was 70–85% (as determined by trypan blue exclusion) and the yield was 200–400 × $10^6$ viable cells/liver. Cells (3.0 × $10^6$/plate) were plated in 3 mL of Waymouth's medium containing 0.15 μM insulin [3]. Cultures were maintained for 5–7 days at 37° in 5% $CO_2$ atmosphere. The medium was replaced every 48 hr, commencing 4 hr after plating. Diaminobenzidine staining [16] of three representative cell preparations revealed Kupffer cell contamination to be less than 0.5%.

### *Treatments*

Cells were cultured for 5 days before beginning treatment to allow recovery of stable expression of P450 2C11 [3, 4]. The cells were treated on day 5 by changing to medium containing 6 ng/mL IL-1β, 6 ng/mL IL-6, 5 ng/mL IFN-γ, 100 ng/mL TNF-α, or 10 μg/mL LPS. Twenty-four hours after treatment, cell viability assessed by trypan blue exclusion was 96% for control cells, 92% for IL-1β-treated cells, and 87% for cells treated with either LPS alone or LPS, IL-1β, IFNγ, and TNFα. There was no statistically significant effect ($P < 0.05$) of any of the treatments. None of the treatments resulted in any significant increases in the number of dead or living cells recovered in the culture medium after 24 hr. NMA was co-administered with IL-1β or LPS at concentrations ranging from 30 to 450 μM. For analysis of P450 2C11 mRNA cells were treated for 24 hr and for microsomal P450 2C11 protein isolation cells were treated every 24 hr for 24 or 48 hr.

### *Isolation of Total RNA and Northern and Slot-Blot Assays*

Total hepatocyte RNA was prepared by the acid phenol extraction method [17]. Northern blotting was performed as described by Sambrook *et al.* [18]. Briefly, hepatocyte RNA was denatured and subjected to agarose gel electrophoresis (1.5% agarose) in the presence of formaldehyde at 60 V for 4 hr. The RNA was blotted onto MagnaGraph nylon transfer membrane filters (Micron Separations Inc., Westborough, MA) overnight and was fixed by both UV irradiation and baking at 80°.

Slot blots were prepared as described previously [19]. Total RNA was denatured using formaldehyde and loaded onto Nytran maximum strength filters (Schleicher & Schuell, Keene, NH) in the slot-blot manifold. The RNA was immobilized by both UV irradiation and baking at 80°.

Relative levels of CYP2C11 and iNOS mRNAs were assayed by northern and slot-blot assay using full-length cDNAs for CYP2C11 [13] and iNOS. A cDNA for GAP was used to control for loading and transfer artifacts on northern blots, as described previously [1]. All slot-blot results were normalized to the content of poly($A^+$) RNA, measured by probing slot blots with an oligo$(dT)_{30}$ probe [20, 21]. The Megaprime labeling kit (Amersham, Arlington Heights, IL) and [α-$^{32}$P]dCTP were used to radiolabel cDNA probes. These probes hybridized to single mRNA bands of appropriate size in northern blots of RNA from 6-day hepatocyte cultures. Bound $^{32}$P-labeled probes were detected by autoradiography and quantified by analysis on a Lynx video densitometer (Applied Imaging, Santa Clara, CA). The amounts of total RNA were determined previously to be in the range giving a linear response for the assays.

### *Microsomal Protein Isolation and Western Blotting*

Microsomes were isolated by differential ultracentrifugation, using a Beckman TLK tabletop ultracentrifuge, as described previously [3]. Microsomal protein concentration was determined by the method of Lowry *et al.* [22] using bovine serum albumin as a standard. The amount of P450 2C11 protein in the microsomes was assayed under conditions of linearity, using an immunoabsorbed polyclonal antibody to P450 2C11, as described previously [19]. The bound second antibody-horseradish peroxidase complex

was visualized by chemiluminescent detection (ECL; Amersham), followed by autoradiography and video densitometric quantitation.

### *Analysis of Nitrite and Nitrate Concentration*

The stable end-products of L-arginine-dependent NO synthesis, nitrate and nitrite, were measured in the culture supernatant using a colorimetric method based on the Griess reaction [23, 24]. Briefly, total nitrite and nitrates were measured by obtaining an aliquot of medium and reducing the nitrate to nitrite with NADPH:nitrate reductase. The percent conversion of nitrate to nitrite was 98%. The reduced aliquot of medium was 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 540 nm with a Thermo max microplate reader (Molecular Devices, Sunnyvale, CA) and converted to $NO_x$ concentrations by using a sodium nitrite standard curve.

### *Statistical Analysis*

Data from slot-blot and western blot 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

### *Suppression of P450 2C11 mRNA and Induction of iNOS mRNA in Hepatocytes Treated with Cytokines and LPS*

The objectives of our study were 2-fold: first, to determine which cytokines could induce iNOS expression and NO release in our culture system; and second, to examine whether the NO released after treatment with these cytokines is required for the suppression of P450 2C11 mRNA and protein seen after cytokine and LPS administration *in vitro*.

As shown in Figs. 1, 2A and 2B, IL-1β, TNF-α, and LPS treatment all resulted in significant decreases in P450 2C11 mRNA expression. These agents also induced iNOS mRNA expression to 607, 851, and 888% of control levels, respectively (Fig. 2C). Although both northern and slot blots were assayed for the mRNAs examined in this study, we found the slot blots to be a more accurate way of quantitating changes seen in mRNA expression. Densitometric scanning and quantitation of northern blots yielded similar results. IL-6 did not reduce P450 2C11 mRNA significantly in this experiment (although previous studies have shown a consistent decrease [4]) and did not induce iNOS mRNA expression (Fig. 2). As we have shown previously [4], IFN-γ had no significant effect on P450

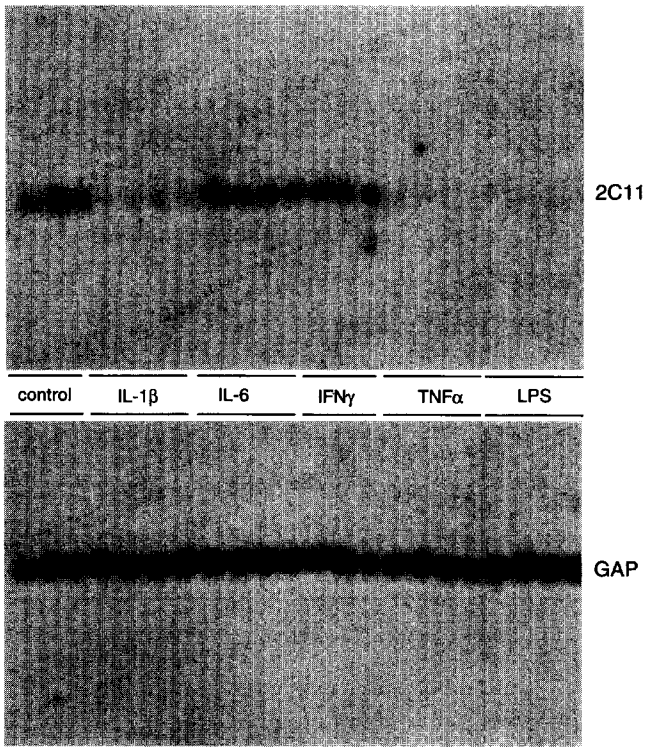


**FIG. 1. Suppression of P450 2C11 mRNA following cytokine and LPS administration. Hepatocytes were cultured on Matrigel for 5 days and then treated for 24 hr with 6 ng/mL IL-1β, 6 ng/mL IL-6, 5 ng/mL IFN-γ, 100 ng/mL TNF-α, or 10 μg/mL LPS. Cells were harvested and total RNA was isolated and subjected to northern blot analysis as described in Materials and Methods. Three samples from each treatment group are presented.**

2C11 mRNA; it also did not induce iNOS mRNA expression (Fig. 2). Treating the hepatocytes with a combination of cytokines resulted in a larger increase in iNOS mRNA expression than was produced by the individual agents (Fig. 2C), but the combination did not suppress P450 2C11 expression more than did IL-1β, TNF-α, or LPS alone (Fig. 2B).

### *Effect of NMA on the IL-1β- and LPS-Mediated Suppression of P450 2C11 mRNA*

To determine whether NO released after IL-1β or LPS treatment was mediating the suppression of P450 2C11 mRNA observed following treatment with these agents, hepatocytes were incubated with the competitive NOS inhibitor NMA over a range of concentrations in the presence and absence of either IL-1β or LPS. After 24 hr, IL-1β treatment decreased P450 2C11 mRNA levels to 4% of control expression (Fig. 3A). Administration of NMA at 30, 90, and 300 μM had no effect on the suppression of P450 2C11 elicited by IL-1β, whereas it produced a concentration-dependent inhibition of NO release that was complete at 300 μM (Fig. 3B). The 300 μM concentration of NMA did not have any significant effect on P450 2C11

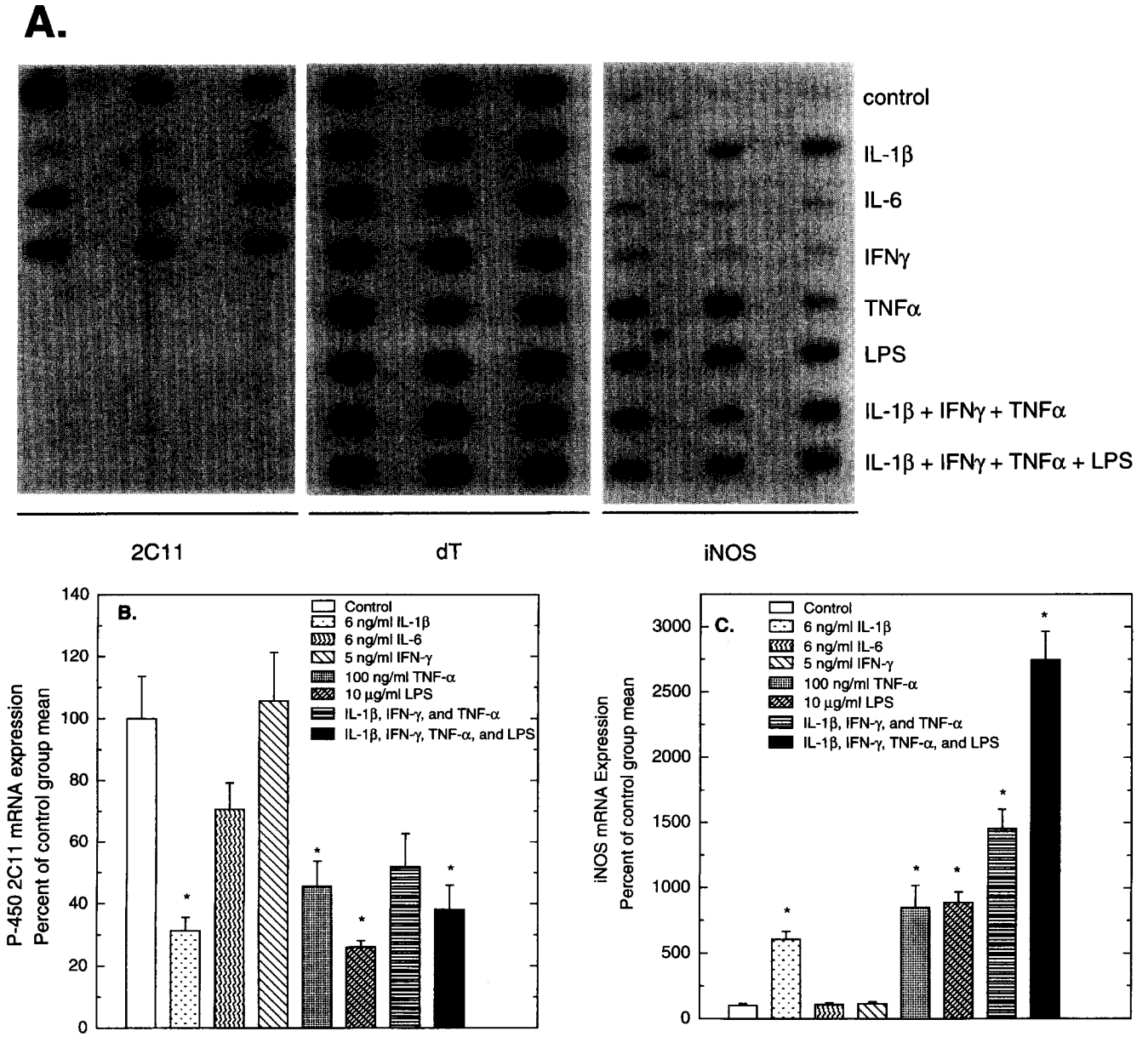


FIG. 2. Effects of cytokines, IFN-γ, and LPS on P450 2C11 and iNOS mRNA expression. Hepatocytes were cultured on Matrigel for 5 days and then treated for 24 hr with 6 ng/mL IL-1β, 6 ng/mL IL-6, 5 ng/mL IFN-γ, 100 ng/mL TNF-α, 10 μg/mL LPS, or combinations of the stimuli. Hepatocytes were harvested and total RNA was isolated from each sample and subjected to RNA slot-blot analysis as described in Materials and Methods. A slot blot of three representative samples from each group is presented, showing the effects of cytokines, IFN-γ, and LPS on P450 2C11 and iNOS mRNA (A). A blot probed with oligo-dT is shown to illustrate similar poly($A^+$) content of the samples. Panels B (2C11 mRNA) and C (iNOS mRNA) present the quantitative data obtained from the P450 2C11 and iNOS mRNA assays, normalized to the poly($A^+$) RNA contents of the samples. The data represent the means ± SEM of six culture plates for each treatment group and are expressed as percentages of the mean value for control cells. Key: (*) significantly different from untreated cells, $P < 0.05$.

mRNA expression when administered in the absence of IL-1β (Fig. 3A).

LPS treatment also significantly suppressed P450 2C11 mRNA expression (Fig. 4A). As seen with IL-1β, despite the ability of 300 μM NMA to block NO release (Fig. 4B), the suppression of P450 2C11 evoked by LPS was unaffected. In cultures stimulated with a combination of IL-1β, IFN-γ, and LPS, NMA also was unable to restore the suppression of P450 mRNA evoked by the cytokine/LPS mixture (data not shown).

### *Time-course of P450 2C11 Expression*

To exclude the possibility that we were missing a contribution of NO at other time points, we examined the effects of NMA on the time–course of P450 2C11 mRNA down-

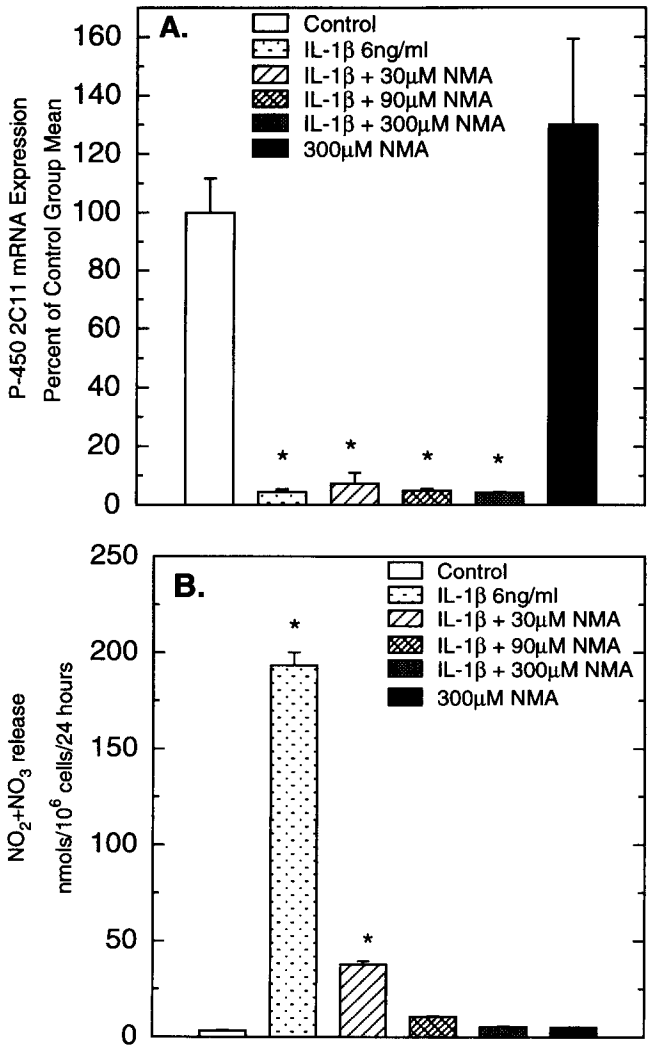


FIG. 3. Effect of NMA on the IL-1β-evoked suppression of P450 2C11 mRNA expression. Hepatocytes were cultured as described for Fig. 1 and were exposed for 24 hr to IL-1β (6 ng/mL) in the presence and absence of 30, 90, and 300 μM NMA. The amount of $NO_2$ + $NO_3$ released into the medium was determined by obtaining an aliquot of medium after the 24-hr incubation and analyzing via the Griess reaction. The data represent the means ± SEM of fifteen culture plates for each treatment group (N = 5 from three individual experiments) and are expressed as percentages of the mean value for control cells. The effect of NMA on P450 2C11 mRNA expression (A) and $NO_2$ + $NO_3$ released into the medium (B) is shown. Key: (*) significantly different from untreated cells, $P < 0.05$.

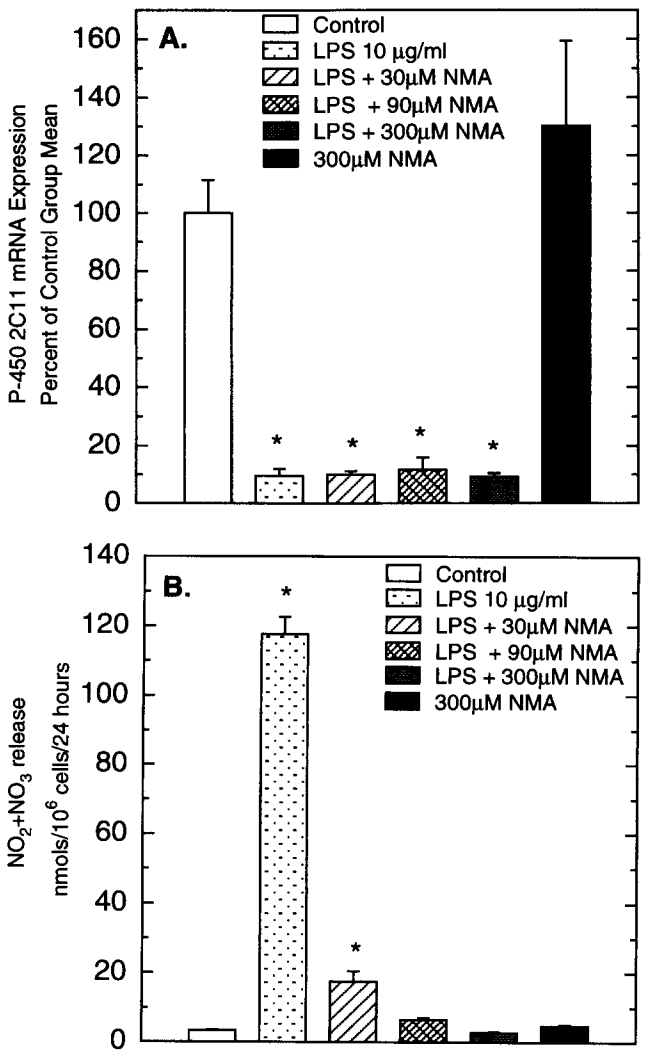


FIG. 4. Effect of NMA on the LPS-evoked suppression of P450 2C11 mRNA expression. Hepatocytes were cultured as described for Fig. 1 and were exposed for 24 hr to LPS (10 μg/mL) in the presence and absence of 30, 90, and 300 μM NMA. The amount of $NO_2$ + $NO_3$ released into the medium was determined by obtaining an aliquot of medium after the 24-hr incubation and analyzing via the Griess reaction. The data represent the means ± SEM of fifteen culture plates for each treatment group (N = 5 from three individual experiments) and are expressed as percentages of the mean value for control cells. The effect of NMA on P450 2C11 mRNA expression (A) and $NO_2$ + $NO_3$ released into the media (B) is shown. Key: (*) significantly different from untreated cells, $P < 0.05$.

regulation. As shown in Fig. 5, the basal level of P450 2C11 mRNA expression remained unaltered by NMA for 48 hr past the day 5 treatment point. IL-1β elicited maximal suppression of P450 2C11 mRNA, 4% of control, at the 24-hr time point and the effect lasted for at least 48 hr. Treatment with 300 μM NMA was unable to attenuate the down-regulation of P450 2C11 mRNA at any time point. Similar results were obtained when LPS was used to evoke P450 2C11 suppression (Fig. 5). NMA treatment did block NO release into the medium, as measured by nitrite and nitrate formation, at all time points assayed (data not shown).

### *Effect of NMA on IL-1β- and LPS-Mediated P450 2C11 Microsomal Protein Expression*

P450 2C11 microsomal protein expression was also examined to determine if NO could be participating in the declines seen in P450 2C11 protein expression following cytokine and LPS treatment in our system (Fig. 6). Hepatocytes were treated with IL-1β or LPS for 24 or 48 hr both

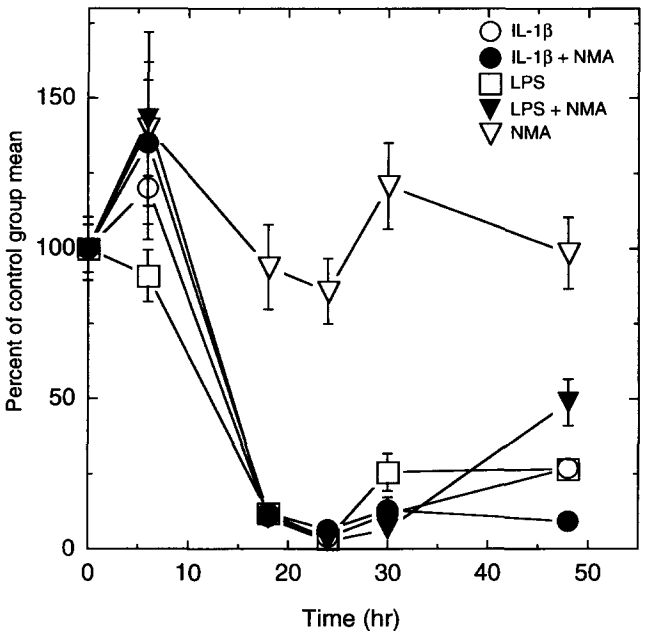


**FIG. 5. Time–course of the response of P450 2C11 to IL-1β and LPS and the effect of NMA on this response. Hepatocytes were cultured as described for Fig. 1. IL-1β at 6 ng/mL and LPS at 10 μg/mL were then added with the medium change either alone or in combination with 300 μM NMA. The cells were harvested at the indicated times, and total RNA isolated from each sample was subjected to slot-blot analysis as described in Materials and Methods. Each data point represents the mean ± SEM of six independent samples.**

in the absence and the presence of a range of NMA concentrations. NMA at 300 μM blocked IL-1β or LPS-evoked synthesis of NO (Table 1). In agreement with the results obtained for P450 2C11 mRNA, NMA administered alone (300 μM) had no significant effect on P450 2C11 microsomal protein levels, and failed to attenuate the decreases in P450 2C11 protein caused by IL-1β or LPS at either time point (Table 1).

**TABLE 1. Effect of NMA on IL-1β-mediated down-regulation of P-450 2C11 protein expression**

| | P450 2C11 protein (% of control mean) | $NO_2 + NO_3$ (nmol/$10^6$ cells) |
|---|---|---|
| | 24 hr | |
| Control | 100 ± 19.8 | 7.0 ± 0.9 |
| IL-1β | 24.3 ± 8.7* | 142 ± 19.7* |
| LPS | 34.0 ± 14.9* | 120.8 ± 18.6* |
| IL-1β + NMA | 32.9 ± 12.0* | 10.1 ± 2.3 |
| LPS + NMA | 38.5 ± 11.4* | 7.4 ± 1.9 |
| NMA | 184.9 ± 72.7 | 8.8 ± 3.3 |
| | 48 hr | |
| Control | 100 ± 17.6 | 4.1 ± 0.3 |
| IL-1β | 11.5 ± 3.8* | 163.4 ± 15.0* |
| LPS | 41.0 ± 7.0* | 132.4 ± 6.9* |
| IL-1β + NMA | 10.4 ± 4.3* | 6.3 ± 0.9 |
| LPS + NMA | 27.8 ± 14.9* | 6.6 ± 0.1 |
| NMA | 74.3 ± 31.9 | 3.5 ± 0.3 |

Hepatocytes were cultured for 5 days as described in Materials and Methods and then treated for 24 or 48 hr with 6 ng/mL IL-1β or 10 μg/mL LPS alone or in combination with 300 μM NMA. Microsomal proteins isolated from each sample were subjected to 7.5% SDS–PAGE, transferred to nitrocellulose paper, and assayed for P450 2C11 apoprotein. NO levels were determined by assaying the $NO_2 + NO_3$ accumulation in the medium for the duration of the treatments. For each determination, N = 15 (five samples from 3 independent experiments) with results given as means ± SD.
*Significantly different from untreated cells (100%), $P < 0.05$.

### *Lack of Effect of the NO Donor GSNO on P450 2C11 mRNA Expression*

To determine whether chemically produced NO could cause a decrease in CYP2C11 expression in our system, primary hepatocytes were treated with the NO donor GSNO at concentrations ranging from 5 to 500 μM for 24 hr. None of these concentrations of GSNO affected P450 2C11 mRNA expression measured 24 hr later, whereas IL-1β again caused a significant suppression (Fig. 7). These findings are in agreement with our inhibitor studies showing that IL-1β and LPS are able to evoke an NO-independent suppression of P450 2C11 mRNA expression in cultured hepatocytes.

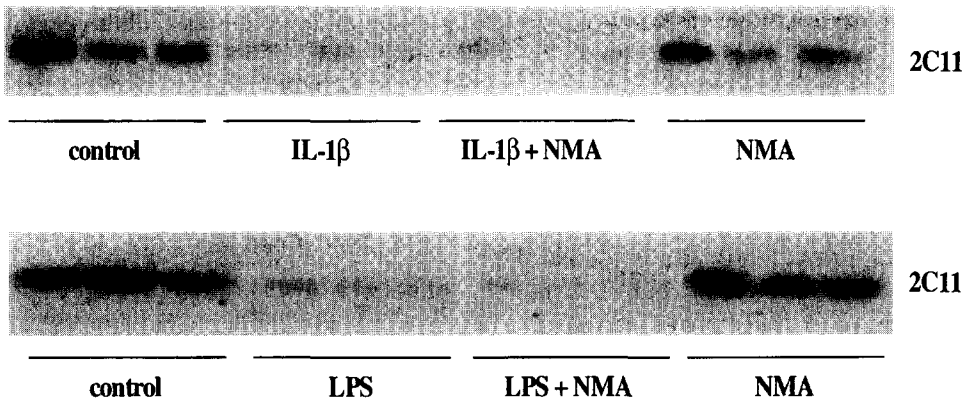


**FIG. 6. Western blot of P450 2C11 protein levels after incubation with IL-1β or LPS ± NMA. Microsomal samples from cells treated every 24 hr for 48 hr with IL-1β, IL-1β + NMA, LPS, LPS + NMA, or NMA alone were subjected to western blot analysis as described in the text. Three samples from each treatment group of the experiment summarized in Table 1 are shown.**

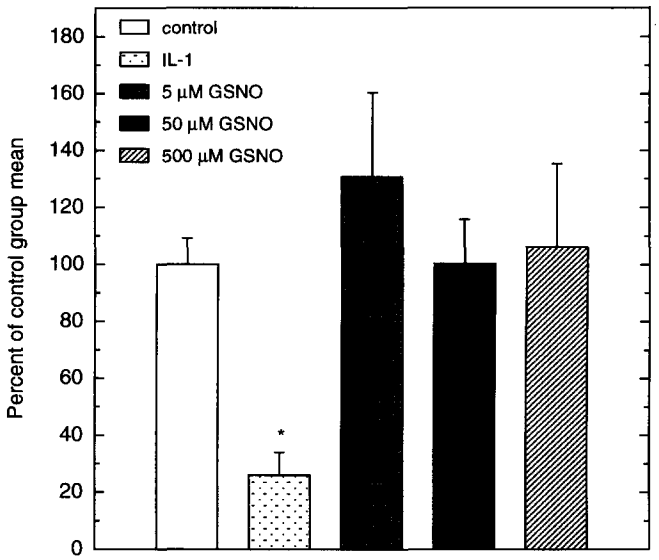


**FIG. 7. Effect of the NO donor GSNO on P450 2C11 mRNA expression. Hepatocytes were cultured as described in the legend of Fig. 1. IL-1β (6 ng/mL) or GSNO (5, 50, or 500 μM) was added to the cultures with the medium change. Cells were harvested 24 hr after treatment, and total RNA isolated from each sample was subjected to slot-blot analysis as described in Materials and Methods. Each data point represents the mean ± SEM of ten independent samples. Key: (*) significantly different from untreated cells, $P < 0.05$.**

## DISCUSSION

Previous *in vitro* and *in vivo* work has suggested a role for NO in the decreased activity [9–11] and expression [11, 12] of several cytochrome P450 isozymes following treatment with various cytokines, interferons, and LPS. In the present study, IL-1β, TNF-α, and LPS each induced the expression of iNOS and suppressed the expression of P450 2C11 in primary rat hepatocytes cultured on Matrigel. However, while the competitive NOS inhibitor NMA was able to block IL-1- and LPS-evoked NO release into the culture medium, the inhibitor was unable to reverse the down-regulation of P450 2C11 mRNA or protein elicited by either IL-1β or LPS. Our findings indicate that NO is not required for the IL-1β- or LPS-evoked decreases seen in P450 2C11 mRNA and protein expression levels.

Although we found that NMA treatment reduced NO production to levels equivalent to those in control cultures, it might be argued that small residual concentrations of NO could be responsible for the effects of cytokines on P450 2C11 mRNA and protein. Also, NMA metabolism results in the production of small amounts of NO [25], and this could conceivably offset the inhibition of NO production by iNOS. These scenarios appear to be unlikely, since neither NMA nor the NO donor GSNO had an effect on P450 2C11 expression in the absence of cytokines or LPS.

The expression of iNOS in hepatocytes has been shown to be induced by various agents, including cytokines, LPS, and interferon. The combination of LPS, TNF-α, IL-1β, and IFN-γ results in a synergistic induction of iNOS mRNA [7, 8, 26]. The present results showing that LPS, TNF-α, or IL-1β, but not IL-6, can individually induce this enzyme are similar to those reported recently by Duval *et al.* [27], and are in contrast to several older studies which indicated that multiple cytokines and LPS were needed to obtain iNOS mRNA induction in hepatocytes [7, 8].

Carlson and Billings [12] found that the addition of inhibitors of NOS significantly prevented the cytokine-mediated decreases in P450 2C11, 3A2, 1A2, and 2B1/2 protein, and further postulated a role for NO in the down-regulation of cytochrome P450 protein expression. It is possible, and perhaps even likely, that different mechanisms will pertain in the down-regulation of different forms of P450 by inflammatory factors, and so our findings cannot be extrapolated to P450s other than P450 2C11. This point is emphasized by our recent *in vivo* findings in rats administered particulate irritants. P450 2C11 was down-regulated by administration of kaolin, Celite, and barium sulfate, although these treatments did not induce hepatic iNOS expression [28]. Thus, NO is not required for down-regulation of P450 2C11 by inflammatory agents *in vivo*. Interestingly, LPS, which does evoke hepatic iNOS induction when injected into rats, down-regulates hepatic expression of P450 2C11, 2E1, and 3A2 [29], whereas particulate irritants only suppress P450 2C11 and do not affect significantly the expression of P450 3A2 or 2E1 [28]. These findings and those of Carlson and Billings [12] are consistent with a role for NO in the suppression of P450 2E1 and/or 3A2 by LPS and cytokines.

The differences between our results and those of Carlson and Billings [12] with regard to P450 2C11 expression may reflect the different culture systems used. There is considerable evidence to indicate that the culture system used in the present study is relevant to the study of the physiological regulation of P450 2C11. In animals treated with LPS, the down-regulation of P450 2C11 mRNA clearly precedes the decrease in the protein [19, 29]. There is little evidence for stimulation of protein degradation; if anything, protein degradation may be inhibited [19]. IL-1 or IL-6 suppresses P450 2C11 pretranslationally both *in vivo* [1] and in the Matrigel culture system [4], and again the primary mechanism is pretranslational. Also, the relative efficacies of IL-1 and IL-6 in producing these changes are the same in cultures and in whole animals [1, 4]. Carlson and Billings did not report any data on P450 mRNAs in their published study using short-term cultures [12], but unpublished preliminary data supported the conclusion that the NO-dependent effects they observed were at the posttranslational level.* Preliminary studies conducted in our laboratory revealed findings similar to those contained in Carlson and Billings [12] when their culture conditions were used: i.e. that NMA treatment did attenuate the (smaller) decreases in P450 2C11 protein (Sewer MB and Morgan ET, unpublished results). The decreases in P450 2C11 protein expression that were observed in the short-term

* Billings R, personal communication. Cited with permission.

culture system were small when compared with the NO-dependent decreases seen in P450 2B and 3A proteins in the same system [12], or to the decreases in P450 2C11 protein that we observed in the present study. This may be due to the relatively minor importance of changes in protein degradation rates in the suppression of P450 2C11. However, it does not explain why the NO dependence of P450 2C11 protein suppression is different in the two culture systems.

Another study suggested that NO can inhibit inducible P450 gene expression in hepatocytes. Using β-naphthoflavone to induce P450 1A expression and a mixture of cytokines and LPS to induce iNOS, Stadler *et al.* [11] have shown an NO-dependent decrease in both mRNA and protein content of P450 1A1 that was reversed upon addition of NMA. Based on these results and the findings presented in this paper, one can postulate that NO may play a role in inhibiting the inductive mechanism of P450 1A1 but does not mediate the suppression of constitutive expression of P450 2C11 in response to cytokines or endotoxin.

Although the role of NO in the suppression of P450 gene expression by cytokines may be controversial and may depend on the P450 isoform, the ability of NO to inhibit the activities of P450 isozymes is well established. Several studies have attributed the decreases in P450 activities observed during LPS-mediated inflammation to a direct effect of NO [9, 10, 30]. Khatsenko *et al.* [10] demonstrated that incubating hepatic microsomes with NO produces an attenuation of cytochrome P450-dependent oxygenation reactions. Moreover, they showed that *in vivo* administration of an NOS inhibitor with LPS reverses the LPS-mediated attenuation of P450 2B activity in phenobarbital-treated rats. Other studies have also demonstrated that NO-releasing agents cause reversible and irreversible inhibition of P450 isozyme activity *in vitro* [9] due to a direct binding of NO to the heme moiety. Further, LPS and a mixture of cytokines decreased *in situ* testosterone 2α-, 16α-, and 6β-hydroxylase activities in short-term hepatocyte cultures, decreases that were reversed only partially by NMA when nearly complete inhibition of nitrite production was observed [30]. Testosterone 2α- and 16α-hydroxylation is catalyzed by P450 2C11, whereas 6β-hydroxylation is due mostly to P450 3A2 [31]. The findings of Osawa *et al.* [30] led them to postulate that either very low levels of NO were still having an inhibitory effect on P450 activity or, more likely, that other NO-independent mechanisms are also involved.

There is evidence for at least one other mechanism by which the expression of P450 2C11 can be regulated during inflammation. IL-1β has been found to induce the hydrolysis of sphingomyelin and the accumulation of ceramide in hepatocytes [32] and a number of other nonhepatic cell types [33–35]. Ceramide appears to play a role as a second messenger and mimic the effects of IL-1β [36] and TNF-α [37]. We have found that both exogenous and endogenous ceramides mimic the effects of IL-1β on the expression of P450 2C11 and the acute phase protein AGP, suggesting a role for sphingolipids in the regulation of hepatic P450 mRNA by cytokines [32]. In summary, the present work illustrates NO-independent down-regulation of P450 2C11 mRNA and protein by cytokines and LPS in primary cultures of rat hepatocytes. Whether NO is involved in the regulation of other P450s remains an important focus of investigation.

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