Rapid Induction of Messenger RNA for Nitric Oxide Synthase II in Rat Neutrophils *In Vivo* by Endotoxin and its Suppression by Prednisolone (43700)

JAY KOLLS,* JIANMING XIE,* RICHARD LEBLANC,* TADEUS MALINSKI,† STEVE NELSON,* WARREN SUMMER,* AND STAN S. GREENBERG*,[1]

Section of Pulmonary and Critical Care Medicine, Department of Medicine, Physiology and Pharmacology, Louisiana State University Medical Center, New Orleans, Louisiana 70112; and Department of Chemistry,† Oakland University, Rochester, Michigan, 48309-4401*

Abstract. **Nitric oxide is believed to participate in nonspecific cellular immunity. Gram negative bacterial endotoxins increase the production of reactive nitrogen intermediates (RNI) in phagocytic cells by inducing the enzyme nitric oxide synthase II (NOS II). Anti-inflammatory glucocorticoids attenuate endotoxin-induced increases in RNI. This study evaluated the effect of *in vivo* administration of prednisolone on *Escherichia coli* lipopolysaccharide endotoxin (LPS)–induced increases in plasma RNI and neutrophil mRNA for NOS II and production of RNI in the rat. We show that LPS rapidly induces mRNA for NOS II and production of RNI (NO_2^- and NO_3^- anion) in rat neutrophils within 2 hr after *in vivo* administration of a sublethal dose of 0.5 mg/kg, iv. A pharmacologic dose of prednisolone (50 μg/kg, im) given 15 min before LPS-attenuated production of NO_2^- and NO_3^- by neutrophils and suppressed LPS-stimulated mRNA for NOS II. 3-Amino, 1,2,4-triazine inhibited NO_2^- and NO_3^- production without affecting gene expression for NOS II. These data demonstrate that LPS rapidly induces functional gene expression for NOS II and prednisolone prevents induction of NOS II activity by inhibiting transcription of its mRNA.** [P.S.E.B.M. 1994, Vol 205]

Nitric oxide synthase (NOS) enzymatically converts L-arginine into L-citrulline and NO. NOS exists as several isozymes including Ca^{2+}-calmodulin activated, constitutive NOS I and III in neurons and endothelial cells, and cytokine and *Escherichia coli* lipopolysaccharide endotoxin (LPS)–inducible Ca^{2+}-independent NOS II in neutrophils (PMN), macrophages, and other cells (1, 2). Glucocorticoids such as dexamethasone and cortisol (3) attenuate LPS-inducible increases in NO in the lung and liver without affecting the activity of the constitutive Ca^{2+}- and calmodulin-dependent NO synthases in the brain and aortic endothelium (3, 4). It was proposed that inhibition of induction of the Ca^{2+}-independent NO synthase *in vivo* may underlie some of the physiological and pharmacological effects of the anti-inflammatory glucocorticoids (3, 4).

Glucocorticoid-induced suppression of NOS II activity may result from inhibition of NOS II gene expression, post-transcriptional inhibition of translation of NOS II mRNA, or inhibition of NOS II activity, since glucocorticoids inhibit reactive nitrogen intermediates (RNI) production, an index of NOS II activity, when given either before or shortly after LPS administration (3–6). This study evaluated the effect of *in vivo* administration of prednisolone on LPS-induced increases in PMN NOS II mRNA and production of RNI in the rat.

Methods and Materials

All experiments were performed with an approved LSU Institutional Animal Care and Use Committee

[1] To whom requests for reprints should be addressed at Department of Medicine, Section of Pulmonary and Critical Care Medicine, Suite 3205, 1901 Perdido Street, New Orleans, LA 70112.

Received May 25, 1993. [P.S.E.B.M. 1994, Vol 205]
Accepted November 16, 1993.

0037-9727/94/2053-0220$10.50/0

Protocol (LSU-NO-859). Male, Sprague-Dawley rats (325–350 g) were anesthetized with ketamine-xylazine (25 mg/kg, ip), and catheters were inserted into the carotid artery and jugular vein for measurement of blood pressure and drug administration, respectively (7, 8). After a 30-min equilibration period, they were given either PBS (0.01 ml/100 g, im), prednisolone hemisuccinate (50 μ/kg, im) or 3-ATINE (75 mg/kg, iv) (9, 10) followed 15 min later by either LPS (0.5 mg/kg, iv) (GIBCO Inc., Long Island, NY) or PBS (0.01 ml/100 g, iv). Blood pressures were monitored for the next 2 hr, after which a thoracotomy was performed and blood (8–10 ml) was obtained by cardiac puncture in heparin-treated tubes (1000 U/ml) for isolation of PMN and assay for RNI as described previously in detail (11).

Neutrophil Preparation. Rat circulating PMN were isolated from heparin-treated (1000 U/ml) blood obtained by cardiac puncture and prepared to a purity of greater than 90%–95% with PolymorphPrep (Nycomed, Gibco/BRL, Gaithersburg, MD) following the manufacturer's protocol (12). The percentage of viable cells determined by trypan blue exclusion was greater than 98%.

RNI Production by Rat Neutrophils. PMN (10^6/0.5 ml) obtained from PBS- and LPS-treated rats were incubated in HEPES buffered salt solution containing (m*M*): NaCl (128), KCl (4.9), $MgCl_2$ (1.2), $CaCl_2$ (1.6), dextrose (10), NaHEPES/HEPES buffer (18.7), and NaH_2PO_4 (1.18). Following a 30 min incubation at 37°C the cellular response was stopped by placing the reaction tubes in ice water. The cells were removed by centrifugation and the incubate assayed for RNI (11, 13). Briefly, the incubate (50 μl) was added to 100 ml of a reducing solution (2.3% vanadium chloride in 2 *N* HCl at 100°C) under a stream of nitrogen gas. Quantification of the NO formed from RNI was determined from the specific chemiluminescence resulting from the reaction of NO with ozone using a Dasibi Model 821 Nitric Oxide–NO_X Analyzer (Dasibi Environmental Inc., Glendale, CA). Conversion of standard nitrate and nitrite solutions to NO was 94%–96% when compared with calibrated standards of NO gas.

Assay of mRNA for NOS II. Transcripts for NOS II were measured in isolated PMN by competitive cDNA equalized reverse transcription-polymerase chain reaction (cERT-PCR) (14). Briefly, RNA was isolated by lysing the PMN in RNAzol (Biotecx, Friendsville, TX) following the manufacturer's protocol. Reverse transcription was carried out in 40 μl reactions with a final concentration of 500 p*M* random hexamer as primer, dATP-dGTP-dTTP (1 m*M*) and dCTP (0.03 m*M*) (Pharmacia Inc., Gaithersburg, MD), PCR buffer (50 m*M* KCl, 25 m*M* $MgCl_2$, and 10 m*M* Tris, pH 8.3), RNasin (40 units), dithiothreitol (1 m*M*) and Moloney Murine Leukemia Virus–Reverse Transcriptase (400 units) (Gibco/BRL, Gaithersburg, MD). The resulting cDNA was labeled by the addition of 1 μCi of α-[^{32}P]dCTP and quantitated by electrophoresing an aliquot of the reaction mixture on a denaturing polyacrylamide gel and scanning the gel on a Phosphor Imager (Molecular Dynamics, Mountain View, CA) as described previously (14). Varying amount of cDNA were then analyzed and amplified using PCR with primers specific for murine-inducible NOS II (15). The primer sequences were as follows: NOS II A, 5′-AATGGCAACATCAGGTCGGCCATCACT-3′ and NOS II B, 5′-GCTGTGTGTCACAGAAGTCTC-GAACTC-3′. The PCR product was quantified by cERT-PCR (16) by co-amplifying known amounts of cDNA and competitor template (15) which was made by creating a small deletion in the NOS II PCR product. Preliminary experiments demonstrated that this competitor competes linearly with NOS II over a 4-fold logarithmic range of NOS II mRNA concentrations from 10^{-14} to 10^{-10} g/ml (10, 17). Data are expressed as picograms of NOS II mRNA per nanogram of cDNA as previously described (14).

Data Analyses. Data were analyzed with ANOVA using Bonferroni's correction for time series measurements (18). A *P* value of 0.05 or less was accepted for statistical significance of differences between and among means.

Results

Animal Experiments. Mean arterial blood pressure was stable throughout the 2-hr observation period in rats treated only with PBS, prednisolone (50 μg/kg, im), or 3-ATINE (Fig. 1). Administration of LPS to rats pretreated with PBS initially decreased mean arterial pressure which slowly returned towards pre-LPS values over the 2-hr period of evaluation. However,

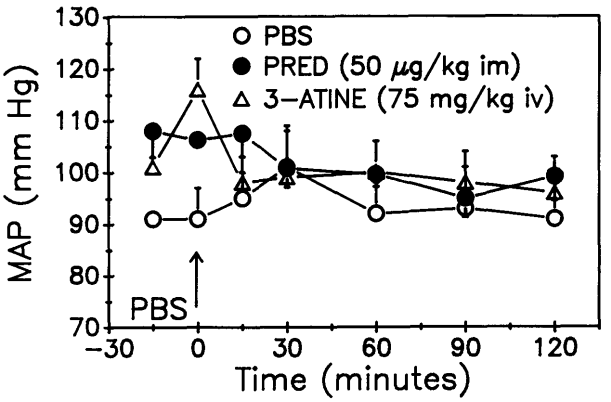

Figure 1. Pretreatment of rats with phosphate buffered saline (PBS, 0.01 ml/100 g, iv), prednisolone (PRED; 50 μg/kg, im), or 3-ATINE (75 mg/kg, iv) 15 min before administration of vehicle (PBS) does not affect mean arterial pressure of the ketamine-anesthetized rat. The ordinate is the mean arterial pressure (mm Hg) ± SEM. The abscissa is the time of the experiment. PBS was administered at the arrow. Vertical lines are the SEM from four to six experiments.

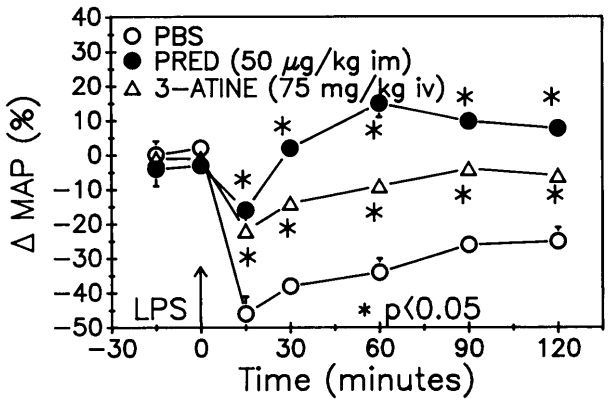

Figure 2. Pretreatment of rats with prednisolone (PRED; 50 μg/kg, im) or 3-ATINE (75 mg/kg, iv) 15 min before administration of LPS (0.5 mg/kg iv) attenuates LPS-induced hypotension observed in ketamine-anesthetized rats pretreated with vehicle (PBS). The ordinate is the percent change of mean arterial pressure from control values obtained prior to pretreatment with PBS, PRED, or 3-ATINE. Vertical lines are the SEM. The abscissa is the time of the experiment. LPS was administered at the arrow. Vertical lines are the SEM from four to six experiments. An asterisk indicates the responses to LPS in PRED and 3-ATINE pretreated animals differ ($P < 0.05$) from the LPS response in PBS pretreated animals. The absolute mean arterial pressures in each of the experimental groups are summarized in Table I.

mean arterial pressure remained 25 ± 3% below control values 2 hr after administration of LPS (Fig. 2). Rats pretreated with prednisolone exhibited a transient hypotension to iv LPS. However, within 30 min after administration of LPS, mean arterial pressure returned to control values and subsequently increased by 10%–15% above control values over the remainder of the experiment (Fig. 2). In contrast, mean arterial pressure transiently declined and then returned to pre-LPS values within 15 to 30 min in rats pretreated with 3-ATINE (Fig. 2). In each of the prednisolone and 3-ATINE pretreated animals, mean arterial pressure and the change in mean arterial pressure were significantly different from the LPS-treated rats at 15 to 120 min after administration of LPS (Table I, Fig. 2).

RNI in Plasma and Rat Neutrophils. Plasma obtained from rats treated with PBS contained low concentrations of RNI, which were unaffected by prednisolone or 3-ATINE but were significantly increased 2 hr after iv administration of LPS. LPS-induced increases in plasma RNI were attenuated when obtained from rats pretreated with prednisolone or 3-ATINE (Fig. 3). We used the PMN as a model for LPS-inducible NOS II. PMN obtained from PBS- or prednisolone-treated rats exhibited a small and variable production of RNI, which did not differ from that measured in buffer incubated without PMN (Fig. 4). LPS administration *in vivo* increased the *in vitro* production of RNI in the supernatant obtained from the 30-min incubation of PMN (Fig. 4). When obtained from rats pretreated with either 3-ATINE or prednisolone prior to *in vivo* administration of LPS, the capability of PMN to generate RNI *in vitro* was attenuated (Fig. 4).

Assay of mRNA for NOS II. No detectable mRNA for NOS II was evident in PMN preparations obtained from rats treated with saline, prednisolone, or 3-ATINE. However, a significant induction of NOS II transcripts occurred in PMN obtained from rats treated *in vivo* with LPS (Fig. 5). This induction was associated with the spontaneous release of RNI by PMN *in vitro* as measured by chemiluminescence (Fig. 4). Pretreatment of rats with prednisolone decreased LPS-induced NOS II transcripts by approximately 40% with a concomitant 73 ± 11% decrease of RNI by PMN *in vitro* (Fig. 4). In contrast, pretreatment of rats with 3-ATINE (75 mg/kg, iv) had no effect on NOS II mRNA transcripts in PMN obtained from PBS- or LPS-treated rats (Fig. 5) despite its ability to suppress RNI production by PMN (Fig. 4).

Discussion

Within 2 hr after *in vivo* administration of LPS to rats, mRNA for NOS II is increased in PMN. Hypotension, increased plasma levels of RNI, and spontaneous *in vitro* production of RNI by PMN accompanied the increased transcript. Prednisolone prevented LPS-induced hypotension and suppressed LPS-

Table I. Effect of Prednisolone and 3-ATINE on LPS-Induced Hypotension[a]

Time (min)	PBS	LPS	P	P + LPS	3-ATINE	3-ATINE + LPS
	Mean arterial pressure (mm Hg ± SEM)					
0	91 ± 2	95 ± 7	108 ± 5	96 ± 8	103 ± 9	98 ± 7
15 TREATED	85 ± 2	105 ± 10	108 ± 5	90 ± 9	101 ± 8	97 ± 5
30 LPS OR PBS	91 ± 6	105 ± 9	106 ± 2	89 ± 8	116 ± 6*	97 ± 3
45	95 ± 5**	63 ± 8*	108 ± 5**	75 ± 3*	98 ± 9**	75 ± 6*
60	101 ± 7**	74 ± 7*	101 ± 4**	90 ± 5**	99 ± 10**	85 ± 5**
90	92 ± 6**	71 ± 7*	100 ± 5**	105 ± 7**	100 ± 6**	88 ± 7**
120	93 ± 8**	80 ± 7*	95 ± 4**	101 ± 6**	98 ± 6**	95 ± 6**
150	91 ± 7**	80 ± 7*	99 ± 5**	99 ± 5**	96 ± 7**	89 ± 3**

[a] Ketamine anesthetized rats were administered PBS (0.01 ml/100g, iv), prednisolone (50 μg/kg, im) or 3-ATINE (75 mg/kg, iv) at 15 min and either PBS (0.01 ml/100 g, iv) or LPS (0.5 mg/kg, iv) at 30 min and mean arterial pressure monitored for additional 2 hr. *Differs from time 0 ($P < 0.05$). **Differs from LPS ($P < 0.05$) (n = 4–6). For details see Methods and Materials.

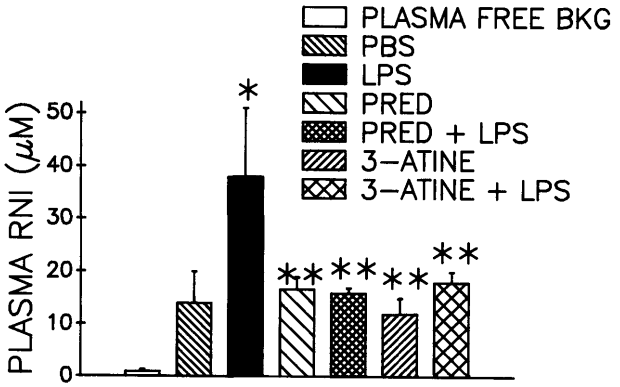

Figure 3. Pretreatment with PBS (0.01 ml/100 g, iv), prednisolone (PRED; 50 μg/kg, im), or 3-ATINE (75 mg/kg, iv) decreases LPS-induced (0.5 mg/kg, iv) increases in plasma RNI. Vertical lines are the SEM from four to six experiments. *Differs from PBS; **differs from LPS.

induced increases in plasma levels of RNI and release of RNI from PMN. This was associated with prednisolone-mediated suppression of LPS-induced increases in mRNA for NOS II in PMN. However, 3-ATINE, the mixed catalase/peroxidase/NOS II inhibitor (9, 10) attenuated LPS-induced hypotension, plasma levels of RNI, and *in vitro* production of RNI by PMN, without any effect on LPS-induced increases in mRNA for NOS II. These data demonstrate that the LPS-inducible NOS II is functional in rat PMN both *in vivo* and *in vitro*. The data also support the conclusions that (i) prednisolone suppresses LPS-induced increases of NOS II and RNI production by suppressing gene expression for NOS II and (ii) 3-ATINE acts at a post-transcriptional level to inhibit the activity of NOS II in PMN (9).

NOS exists as at least four isoforms: two different constitutive Ca^{2+}-activated calmodulin-dependent constitutive enzymes in vascular endothelium and neurons, a cytokine-inducible Ca^{2+}-independent, cal-

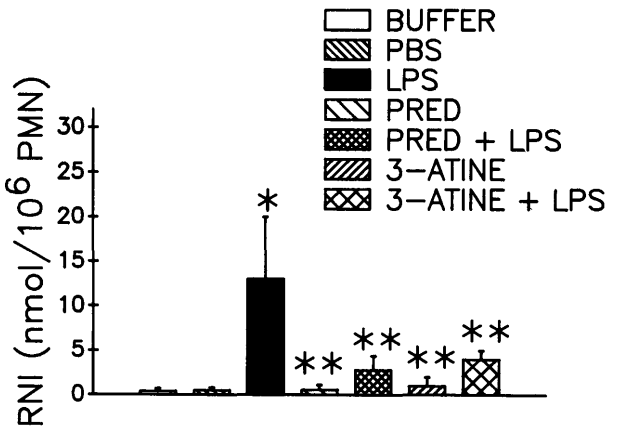

Figure 4. Pretreatment with PBS (0.01 ml/100 g, iv), prednisolone (PRED; 50 μg/kg, im), or 3-ATINE (75 mg/kg, iv) decreases LPS-induced (0.5 mg/kg, iv) increases in PMN production of RNI. Vertical lines are the SEM from four to six experiments. *Differs from PBS; **differs from LPS.

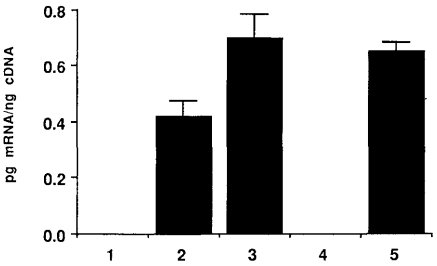

Figure 5. Upper panel: a representative polyacrylamide gel of competitive PCR of rat neutrophil mRNA for NOS II obtained from rats pretreated with PBS (0.01 ml/100 g, iv), prednisolone (PRED; 50 μg/kg, im), or 3-ATINE (75 mg/kg, iv) 15 min before administration of PBS or LPS (0.5 mg/kg, iv). PMN were obtained 2 hr after LPS administration. Lane 1, kilobase marker; Lane 2, negative PCR control; Lane 3, PRED + LPS; Lane 4, LPS; Lane 5, 3-ATINE + LPS; Lane 6, 3-ATINE. The bottom band is competitor template, the top band is NOS II mRNA. The bottom panel is the mean ± SEM of the mRNA for NOS II expressed per ng of cDNA for three experiments.

modulin-bound enzyme (1, 2), and an interleukin-1_β-inducible, Ca^{2+}-dependent isozyme in rabbit chondrocytes (19). Anti-inflammatory glucocorticoids (e.g., dexamethasone and hydrocortisone) have been shown to inhibit LPS-induced stimulation of RNI in rat aortic rings (20), porcine aortic endothelial cells (3, 6), rat peritoneal neutrophils (21), EMT6 adenocarcinoma cells (22), and smooth muscle cells (23). However, they do not affect the constitutive enzyme nor the rabbit chondrocyte inducible NOS (3–6, 20, 23). This has been taken as evidence that glucocorticoids suppress induction of the cytokine-inducible NOS II, rather than inhibiting enzyme activity. However, RNI production alone may not always distinguish between effects on induction and enzyme activity. 3-ATINE acted in a manner similar to glucocorticoids in that it inhibited NOS II without any affect on NOS activity in vascular endothelium (10, 17). Moreover, measurement of RNI alone cannot distinguish between glucocorticoid effects on transcription or translation. Yet direct evidence supporting an effect of glucocorticoids on transcription or post-transcriptional processes involving NOS II has been lacking.

Recently, using Northern blot hybridization techniques, Geller *et al.* (24) showed that cytokines, LPS, and glucocorticoids regulate gene expression of LPS-inducible NOS II in hepatocytes. This study extends this observation to PMN and shows with cERT PCR techniques that (i) LPS rapidly induces mRNA for NOS II in PMN, (ii) the increased mRNA for NOS II is associated with increased RNI in plasma and PMN,

(iii) LPS-induced increases in mRNA are attenuated by prednisolone and (iv) prednisolone-induced attenuation of mRNA for NOS II is associated with suppression of spontaneous RNI in both plasma and PMN. It has been suggested that a constitutive form of NOS, which requires calcium ion but not calmodulin for maximal activation, exists in PMN (25). This has lead some investigators to classify the enzyme as a constitutive NOS I_c (2). However, this enzymatic activity was not found by McCall *et al.* (21) using the same oyster-glycogen-elicited peritoneal neutrophils as those used by Yui *et al.* (25). Furthermore, McCall *et al.* (21) demonstrated that LPS induced NOS II activity in the elicited PMN. In addition, experiments conducted in our laboratories with bioassay and chemiluminescence measurement of RNI failed to elicit RNI or relaxation of endothelial denuded blood vessels with perfused, unstimulated, or A23187 stimulated PMN (unpublished observations). Thus, our data support the observations of Moncada *et al.* (21) that LPS induces NOS II in PMN. Moreover, our data extend their observations to show that (i) LPS rapidly induces gene expression for NOS II in the PMN, (ii) the mRNA is rapidly translated into NOS II enzymatic activity as reflected by the RNI production, (iii) the PMN enzyme is inhibited by glucocorticoids and (iv) there exists significant homology between the macrophage and the PMN NOS mRNA at the molecular level.

One may challenge the latter two conclusions. Reverse PCR, while an extremely sensitive assay, is very nonspecific in that it is difficult to determine whether the assay is measuring mRNA for NOS II in PMN or macrophages. While this is true for reverse PCR, it is not true for competitive reverse PCR which coamplifies the message and the cDNA (14). We initially used reverse PCR to test the concept that LPS-induced mRNA for NOS II and prednisolone suppressed this induction in the PMN from LPS-treated rats. However, once this was confirmed, the competitive technique was applied and the data expressed per unit of DNA. Since our preparations were between 90%–95% PMN, the maximum contribution of macrophage NOS II to total NOS II, if all the mRNA for NOS II inhibited by prednisolone was derived from macrophages, could not exceed 5%–10% of the NOS II/ng DNA. Since prednisolone suppressed mRNA for NOS II by more than 43%, it is unlikely that prednisolone suppressed mRNA for NOS II solely within macrophages. Thus, while a fraction of the prednisolone-induced suppression of gene expression for NOS II may have reflected macrophage and monocyte mRNA for NOS II, the majority of the prednisolone effect—similar to that observed by McCall *et al.* on NOS II enzyme activity (21)—must have primarily reflected corticosteroid-induced suppression of gene expression for NOS II in the PMN.

Our data can also be challenged since we show an increase in mRNA for NOS II at 2 hr after administration of LPS *in vivo*, whereas most investigators show a slow induction of NOS II activity *in vitro* (3–5, 21). Using Northern blot techniques, Geller *et al.* (24) found increases of mRNA for NOS II in hepatocytes within 1 hr after administration of LPS. Using the more sensitive technique of cERT PCR, increased gene expression for NOS II and associated increases in plasma and PMN RNI were evident at 15 min after LPS administration to rats *in vivo*, and increased in time-dependent manner at 1 and 2 hr after LPS administration (26). Moreover, similar results were found in mouse lung using an RNase protection assay (R. Lyons, personal communication) and in rat Kuppfer's and endothelial cells (27). One explanation for the discrepancy between our findings using cERT PCR in combination with chemiluminescence sensitive to 1 ppb of NO and those which only measure the formation of RNI using the Greiss reaction is the relative difference in the sensitivities of the methodologies. The Greiss reaction is sensitive to micromolar concentrations of RNI. Interferon gamma sensitized macrophages and PMN maximally generate approximately 340 n*M* free NO within 5 hr after exposure to LPS in culture (17). Several hours would be required for sufficient RNI to accumulate in the cell culture medium to produce sufficient RNI to be detected by the Greiss reaction (17). Thus, LPS-induced gene expression for NOS II appears to be more rapid than previously assumed (3–5, 21) and is not an anomaly of the rat PMN.

The present data do not allow us to distinguish between a direct effect of prednisolone on induction of NOS II and an indirect effect resulting from glucocorticoid-mediated suppression of cytokine synthesis which would also result in down-regulation of gene expression for NOS II. Nevertheless, the data clearly show that prednisolone, whether directly or indirectly through suppression of gene expression for cytokines, attenuates expression of mRNA for NOS II and that this effect is associated with decreased RNI production by PMN, decreased RNI levels in plasma and attenuation of LPS-induced hypotension. Since LPS and TNF_α actually inhibit vascular endothelial cell production of RNI (13, 28–30), in part by suppression of mRNA for NOS I (31) or by enhancement of the degradation of mRNA for NOS I (32), our data also support the conclusion (33) that selective inhibition of NOS II may prevent the early hypotension associated with bacterial sepsis and septic shock. Further studies are in progress to test this postulate.

Dr. Xie was a Transfer Postdoctoral Fellow of the New Jersey Affiliate of the American Heart Association 91–002 during these studies. Dr. Kolls is supported by a Parker B. Francis Pulmonary

Research Fellowship Grant and Maternal Child Health Grant MCJ-229163.

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