Endotoxin-mediated nitric oxide synthesis inhibits IL-1β gene transcription in ANA-1 murine macrophages

REBECCA A. SCHROEDER, CHARLES CAI, AND PAUL C. KUO
Department of Surgery, Georgetown University Medical Center, Washington, District of Columbia 20007

Schroeder, Rebecca A., Charles Cai, and Paul C. Kuo. Endotoxin-mediated nitric oxide synthesis inhibits IL-1β gene transcription in ANA-1 murine macrophages. Am. J. Physiol. 277 (Cell Physiol. 46): C523–C530, 1999.—On the basis of previous work demonstrating nitric oxide (NO)-mediated inhibition of nuclear factor-kB (NF-κB) DNA binding, we hypothesized that NO downregulates NF-κB-dependent interleukin-1β (IL-1β) production in an ANA-1 macrophage model of lipopolysaccharide (LPS) stimulation. In the presence of LPS (100 ng/ml), levels of IL-1β protein and mRNA were significantly upregulated with NO synthase inhibition. Using nuclear run-on analysis and transient transfection studies, IL-1β gene transcription and IL-1β promoter activity were also found to be increased with inhibition of NO production. Parallel transfection studies using an NF-κB long terminal repeat-reporter plasmid exhibited similar findings, suggesting an NO-mediated effect on NF-κB activity. Gel shift studies showed that LPS-associated NF-κB DNA binding was increased, both in the setting of NO synthase inhibition and in a reducing environment. Repletion of NO by addition of an S-nitrosothiol restored IL-1β protein synthesis, mRNA levels, gene transcription, promoter activity, and NF-κB DNA binding to levels noted in the presence of LPS alone. Our studies indicate that NO may regulate NO synthase-associated inflammation by downregulating IL-1β gene transcription through S-nitrosation of NF-κB.

S-nitrosation; inducible nitric oxide synthase; cytokine; nuclear factor-κB; lipopolysaccharide; interleukin-1β

In pathophysiological states that are characterized by the elaboration of proinflammatory cytokines, inducible nitric oxide synthase (iNOS) is expressed and nitric oxide (NO) is produced (20). This multifunctional free radical alters numerous biochemical functions, such as mitochondrial electron transport, activation of guanylyl cyclase, and expression of adhesion molecules. However, although it is apparent that NO production frequently accompanies inflammatory states, it is unclear whether NO is indifferent, promotes, or inhibits the inflammatory process. In this regard, we have demonstrated that NO S-nitrosylates a key thiol group in the DNA binding domain of nuclear factor-κB (NF-κB), a key transcription factor for the elaboration of multiple proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (1, 2, 6, 7). S-nitrosation of NF-κB at the redox-sensitive C62 residue of p50 is associated with inhibition of NF-κB DNA binding. Given the central role of NF-κB in proinflammatory gene transcription, S-nitrosation-associated inhibition of NF-κB-dependent promoter activity suggests a potential role for NO as a feedback inhibitor of the inflammatory process.

Excessive macrophage elaboration of IL-1β plays a fundamental role in the pathogenesis of the sepsis syndrome (8). Transcriptional regulation is a major determinant of IL-1β protein synthesis, and maximal transcription of the IL-1β gene is NF-κB dependent (1, 2, 5, 8, 10). NF-κB therefore plays a central role in regulating IL-1β production and subsequent IL-1β-dependent inflammatory processes. Using a murine macrophage model of endotoxin (lipopolysaccharide (LPS))-mediated NO production, we sought to test the hypothesis that endogenous synthesis of NO inhibits the synthesis of IL-1β, an NF-κB-dependent proinflammatory protein, by S-nitrosation of NF-κB. Our results suggest a novel mechanism in which NO production feedback inhibits IL-1β gene transcription in the context of LPS stimulation.

MATERIALS AND METHODS

Materials. ANA-1 macrophages were a gift from Dr. George Cox (National Cancer Institute, Frederick, MD). The murine IL-1β promoter-chloramphenicol acetyltransferase (CAT) reporter plasmid construct and NF-κB-TNF-CAT reporter plasmid were gifts from Dr. Clifford J. Bellone (St. Louis University School of Medicine, St. Louis, MO). Human recombinant NF-κB p50 and the Gel Shift assay system, containing the NF-κB consensus oligonucleotide 5′-GCGTGTGATGCAGCCCGAAA-3′, were obtained from Promega (Madison, WI). The rabbit anti-human NF-κB p50 monoclonal antibody (MAb) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The DIG gel shift kit was purchased from Roche Diagnostics (Indianapolis, IN). LPS (Escherichia coli serotype 0111:B4), L-arginine, sodium nitrite, and nitrate reductase were obtained from Sigma (St. Louis, MO). DMEM, glutamine, penicillin-streptomycin, trypsin-EDTA, and endotoxin-free FCS were purchased from Gibco BRL (Grand Island, NY). All other chemicals were of reagent grade.

Induction of NO synthesis in ANA-1 macrophages. ANA-1 macrophages were maintained in DMEM with 0.4 mM L-arginine. Sodium nitrite (200 µM) was added. After incubation for 6 h at 37°C in 5% CO2, the supernatant was aspirated, and the cells washed twice with Dulbecco's PBS. After treatment with trypsin-EDTA, the macrophages were harvested for biochemical assays.

Measurement of NO. NO release from cells in culture was quantified by measurement of the NO metabolite nitrite by a Griess reaction (18). The concentration of nitrite in the culture supernatant was measured by a colorimetric method that utilizes the Griess reagent (24) to generate a purple formazan product (at 550 nm) that is proportional to the nitrite concentration. The nitrite concentration was determined by comparing the absorbance of the sample to a standard curve of nitrite standards. The nitrite concentration in the culture supernatant was expressed as nanomolars per milliliter of culture supernatant. The results are expressed as the mean ± SD of duplicate determinations.

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the technique of Snell and Snell (27). To reduce nitrate to nitrite, conditioned medium (200 µl) was incubated in the presence of 1.0 unit of nitrate reductase, 50 µM NADPH, and 5 µM FAD for 15 min at 37°C in the dark. Sulfanilamide (1%, 50 µl) in 0.5 N HCl (50% vol/vol) was then added to 50 µl of the treated medium. After a 5-min incubation at room temperature, an equal volume of 0.02% (N-1-naphthyl)ethylenediamine was added; after incubation at room temperature for 10 min, the absorbance at 570 nm was compared with that of an NaNO₂ standard.

Determination of IL-1β and TNF-α concentration. Cell culture medium levels of IL-1β and tumor necrosis factor-α (TNF-α) were determined by the University of Maryland Cytokine Core Laboratory with an ELISA technique utilizing murine mAbs.

Immunoblot analysis of IL-1β and iNOS proteins. Isolated murine macrophages were washed three times in PBS and incubated with boiling 2X nonreducing electrophoresis sample buffer for 2 min. Separation was performed by SDS-12% PAGE, and then the products were electrotransferred to a polyester-supported nitrocellulose membrane for 90 min at 150 mA. The membrane was blocked overnight at 4°C in Tris-buffered saline (TBS) containing 3% BSA. Blocked membranes were incubated with the anti-mouse IL-1β MAb or anti-rat iNOS MAb (Transduction Laboratories, Lexington, KY), washed three times in TBS-0.1% Tween, and incubated with biotinylated sheep anti-mouse IgG (Amersham, Arlington Heights, IL) for 1 h. After being washed three additional times, membranes were incubated with strepavidin-horseradish peroxidase conjugate. After an additional washing, bound antibodies were detected by the ECL detection system (Amersham). Blots were scanned with a computerized laser densitometer (Hoeffer Scientific Instruments, San Francisco, CA), and the area under the curve was normalized to the human NF-κB p50 standard.

Synthesis of SNAC. SNAC was synthesized by combining equimolar NaNO₂ and N-acetylcysteine in 0.5 N HCl for 30 min at room temperature as previously described. Before use, the S-nitrosoprotein solution was neutralized to pH 7.0 with 0.1 N NaOH. Previous work has confirmed the presence of S-nitrosothiol bonds in the above species by 1H-NMR spectroscopy (28).

Transient transfection with murine IL-1β promoter and reporter gene constructs. Transient transfection of ANA-1 macrophages was performed by electroporation with the Bio-Rad Gene Pulser (9). After cells were washed twice with media, 20 µg of plasmid DNA containing the IL-1β promoter construct were added with 20 µg of β-galactosidase reference plasmid to 10³ cells in 1 ml of medium and the medium was transferred to two 0.4-cm cuvettes. After a single 250-V, 960-µF pulse, the cells were combined into a 60-mm dish containing 5 ml of complete medium. At least 24 h later, the medium was changed, and LPS, LPS-NAME, or LPS-NAME-SNAC was added. Approximately 24 h later, the cells were washed with ice-cold PBS, resuspended in 0.25 mM Tris (pH 7.8), and subjected to three cycles of freezing and thawing. Lysates were centrifuged (11,700 g for 10 min at 4°C); the supernatant was heated at 65°C for 10 min to inactivate CAT inhibitors and then centrifuged as described above. The supernatant was used as the sample for CAT activity by a CAT ELISA technique (Boehringer Mannheim). Transfection efficiency was normalized by cotransfection of a β-galactosidase reporter gene with a constitutively active simian virus 40 promoter. All values are expressed as picograms of CAT per milliliter.

Nuclear run-on assays. Nuclear run-on analysis was performed as previously described (14). Briefly, 100 µl of hepatocyte nuclei were incubated for 5 min at 30°C with 150 µCi of [α-32P]UTP (800 Ci/mmol) in 100 µl of 10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 300 mM KCl, and 5.0 mM (each) ATP, CTP, and GTP. Labeled RNA was isolated by the acid-guanidinium thiocyanate method. Before ethanol precipitation, labeled RNA was treated with 0.2 M NaOH for 10 min on ice. The solution was neutralized by the addition of HEPES (acid free) to a final concentration of 0.24 M. After ethanol precipitation, the RNA pellet was resuspended in 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.4), 0.2% SDS, and 10 mM EDTA. Target RNA was spotted onto nylon membranes with a slot blot apparatus. Target DNA for murine IL-1β was amplified by PCR based on the published sequence (1,800 bp; –1021 to –2821; GenBank X04964). β-Actin and λ-bacteriophage DNA served as positive and negative controls, respectively. Hybridization was performed at 42°C for 48 h with 5 x 10⁶ cpm of labeled RNA in hybridization buffer [50% formamide, 4 x sodium chloride-
sodium citrate (SSC), 0.1% SDS, 5× Denhardt's solution, 0.1 M sodium phosphate (pH 7.2), and 10 μg/ml salmon sperm DNA. After hybridization, the membranes were washed twice at room temperature in 2× SSC and 0.1% SDS, and three times at 56°C in 0.1× SSC and 0.1% SDS. The membranes were then exposed to X-ray film and scanned on a laser densitometer.

Statistical analysis. All values are presented as means ± SD of three or four experiments. Data were analyzed by one-way ANOVA, Student's t-test, or the Wilcoxon rank sum test, as appropriate. P values < 0.05 were considered significant.

RESULTS

The NO dose-response relationship with concentrations of LPS ranging from 1 to 1,000 ng/ml is depicted as a semilogarithmic plot in Fig. 1A. Increasing concentrations of LPS were associated with significantly increased NO production (P < 0.0001 by ANOVA). When L-NAME (100 μM) was added with LPS, NO production at all concentrations of LPS was ablated to a level that was no different from that noted in the absence of LPS, 22.3 ± 2.1 nmol/mg protein (P < 0.01 for LPS vs. LPS-L-NAME). The addition of SOD did not alter NO synthesis in a significant fashion. The concentration of IL-1β in the cell culture medium was then determined (Fig. 1B). In the absence of LPS, the IL-1β concentration was 1.5 ± 3.2 pg/mg protein. Logarithmically increasing concentrations of LPS (1–1,000 ng/ml) were associated with significant increases in IL-1β production (P < 0.0001 by ANOVA). In the presence of L-NAME (100 μM), LPS-induced IL-1β synthesis was increased two- to threefold (P < 0.01). NO was then made replete by the addition of the S-nitrosothiol SNAC (100 μM). In LPS-L-NAME-treated cells, the addition of SNAC restored IL-1β production to levels that were not different from that noted with LPS alone. Similarly, the addition of SNAC to LPS-treated cells did not alter the extent of IL-1β synthesis. IL-1β production in the LPS-L-NAME-SOD-treated cells was not altered. These data indicate that inhibition of NO production increases IL-1β levels in cell culture media after LPS stimulation. The effect of LPS-mediated NO synthesis on TNF-α was then analyzed (Fig. 1C). In the absence of LPS, TNF-α levels in the culture medium were 6.7 ± 10.1 pg/mg protein. The addition of LPS (1 ng/ml) resulted in a dramatic increase in TNF-α production. Subsequent concentrations of LPS (10, 100, and 1,000 ng/ml) were associated with incremental but significant increases in TNF-α levels (P = 0.01 by ANOVA). The addition of L-NAME (100 μM), SOD (100 μM), and SOD (100 μM) decreased TNF-α levels in the culture medium.
U/ml, and/or SNAC (100 µM) with LPS did not alter the TNF-α production profile, indicating that NO synthesis does not alter the extent of TNF-α synthesis in ANA-1 murine macrophages. Subsequent studies were performed at an LPS concentration of 100 ng/ml.

Immunoblot analysis was performed on cellular protein extracts (Fig. 2). TNF-α protein expression in cellular extracts reflected that seen in the cell culture medium (data not shown). In the absence of LPS, no immunoreactive protein was detected. In contrast, in LPS-, LPS-L-NAME-, and LPS-L-NAME-SNAC-treated macrophages, immunoreactive TNF-α protein was readily detected. However, the presence or absence of NO, whether endogenous or exogenous in origin, did not alter the amount of TNF-α detected. In contrast, IL-1β protein expression was noted to vary significantly with NO production. In the absence of LPS (and NO), no IL-1β immunoreactive protein was detected. In LPS-treated cells, IL-1β was detected. In the presence of both LPS and L-NAME (100 µM), the amount of IL-1β synthesized was increased by approximately three- to fourfold over that found in the presence of LPS alone (P < 0.01). When NO was made replete in LPS-L-NAME-treated cells by the addition of SNAC (100 µM), IL-1β production decreased to a level that was equivalent to that of LPS-treated cells. When SNAC (100 µM) was added to LPS-treated cells, the immunoreactive IL-1β protein level did not change, reflecting that noted in the cell culture medium (data not shown). These data corroborate the cell culture medium findings regarding the influence of NO on IL-1β protein production. An immunoblot analysis of iNOS protein expression was performed under these conditions. iNOS protein was detected in the LPS cells. Inhibition of iNOS by the addition of L-NAME resulted in a 35% increase in iNOS protein expression (P < 0.01 vs. LPS or LPS-L-NAME-SNAC). Again, the addition of SNAC to LPS-treated cells returned iNOS protein expression to a level equivalent to that for LPS treatment alone.

Steady-state levels of IL-1β mRNA were then analyzed semiquantitatively by RT-PCR (Fig. 3). In control cells, no iNOS or IL-1β mRNA was detected. In the presence of LPS, iNOS and IL-1β mRNA was expressed. Inhibition of NO synthesis by the addition of L-NAME (100 µM) was associated with a four- to fivefold increase in the normalized density of the IL-1β mRNA band (P < 0.01 vs. LPS alone) and a two- to threefold increase in the iNOS density (P < 0.01 vs. LPS alone). When NO was made replete in LPS-L-NAME-treated cells by the addition of SNAC (100 µM), steady-state levels of IL-1β mRNA production decreased to a level that was equivalent to that for LPS-treated cells. To determine the effect of NO on IL-1β gene transcription, nuclear run-on studies were performed (Fig. 4). Inhibition of NO synthesis by the addition of L-NAME (100 µM) with LPS resulted in a 2.5-fold increase in IL-1β gene transcription (P < 0.01). Again, repletion of NO by the addition of SNAC (100 µM) to LPS-L-NAME-treated cells was associated with IL-1β gene transcription that was not different from that for cells exposed to LPS alone. The addition of SNAC to LPS-treated cells did not alter the extent of IL-1β gene transcription as determined by the nuclear run-on assay (data not shown). These data indicate that NO synthesis inhibits IL-1β gene transcription in LPS-treated macrophages.

Transient transfection studies were performed with the murine IL-1β promoter to determine whether the effect of NO was dependent on the IL-1β promoter region or a noncontiguous enhancer/repressor region (Fig. 5). The IL-1β murine promoter plasmid contains 4,093 bp of the 5’-flanking sequence containing the entire first exon, first intron, and untranslated portion of the second exon. This has been previously shown to have strong inducibility by LPS (over 30-fold) (9). As a

**Fig. 2. IL-1β and inducible nitric oxide synthase (iNOS) protein expression in ANA-1 macrophages.** A: immunoblot analysis of IL-1β and iNOS protein in whole cell lysates, as described in MATERIALS AND METHODS. Blot is representative of four experiments. B: histogram representation of densitometric analysis of IL-1β and iNOS immunoreactive protein relative to standard. Values are means ± SD of 3 or 4 experiments.
positive control for LPS induction of NF-κB-dependent transcriptional activity, parallel experiments were performed by transfection of a CAT plasmid containing three multimerized NF-κB sites upstream from a TNF-α minimal promoter (9). After exposure to LPS (100 ng/ml), LPS-L-NAME (100 µM), LPS-SNAC (100 µM), or LPS-L-NAME-SNAC, levels of IL-1β promoter and NF-κB inducibility were determined by CAT expression. Transfection of a mock plasmid containing only a CAT reporter revealed no detectable CAT protein in the presence of LPS stimulation. Full-length IL-1β promoter transfection was associated with minimal CAT expression under unstimulated control conditions. Stimulation with LPS (100 ng/ml) resulted in significantly increased CAT expression, over sixfold greater than that of the control (P < 0.01 vs. control). Compared with that resulting from LPS treatment, CAT expression was increased an additional fivefold in LPS-L-NAME-treated cells (P < 0.01 vs. LPS alone). Repletion of NO by exogenous administration of the S-nitrosothiol SNAC decreased IL-1β promoter activity to a level that was not different from that for LPS-treated cells. Last, IL-1β promoter activity in LPS-SNAC-treated cells was not different from that in ANA-1 macrophages treated with LPS alone. Results from the parallel experiments with the NF-κB long terminal repeat (LTR) CAT reporter plasmid mirrored those for the IL-1β promoter. Inhibition of LPS-mediated NO synthesis with L-NAME significantly increased NF-κB LTR activity. This activity was decreased to baseline levels with repletion of NO in LPS-L-NAME-SNAC-treated cells. These data suggest that inhibition of NO synthesis increases LPS-induced IL-1β promoter activation and NF-κB LTR activation.

Fig. 3. Effect of NO on steady-state IL-1β mRNA levels in ANA-1 macrophages. A: RT-PCR analysis of iNOS, IL-1β, and β-actin mRNA levels. Gel is representative of four experiments. B: histogram representation of relative IL-1β and iNOS cDNA expression levels normalized to β-actin, as described in MATERIALS AND METHODS. P < 0.01 for control vs. LPS, LPS-L-NAME, and LPS-L-NAME-SNAC for IL-1β and iNOS and P < 0.01 for LPS-L-NAME vs. LPS and LPS-L-NAME-SNAC for IL-1β and iNOS by Student's t-test. Values are means ± SD for 4 experiments.

Fig. 4. Effect of NO on IL-1β gene transcription in ANA-1 macrophages. A: nuclear run-on blot of IL-1β gene transcription. Target DNA for murine IL-1β was amplified by PCR on the basis of published sequence (1,800 bp; 1,021 to 2,821; GenBank X04964). β-Actin and λ-bacteriophage DNA served as positive and negative controls, respectively. Gel is representative of 3 experiments. B: histogram representation of LPS-induced IL-1β gene transcription normalized to β-actin. P < 0.01 for control vs. LPS, LPS-L-NAME, and LPS-L-NAME-SNAC and P < 0.01 for LPS-L-NAME vs. LPS and LPS-L-NAME-SNAC by Student's t-test. Values are means ± SD of 3 experiments.
production. Gel is representative of 3 experiments. The addition of LPS (100 ng/ml) resulted in NF-κB DNA binding was significantly increased in the presence of the reducing agent DTT. This would suggest that NF-κB DNA binding may be the result of S-nitrosation of NF-κB, as we and others have previously demonstrated.(6, 7, 16)

**DISCUSSION**

In this study, we hypothesized that LPS-mediated NO production inhibits production of the NF-κB-dependent protein IL-1β. Our results indicate that inhibition of NO production in the setting of LPS stimulation 1) increases IL-1β protein expression in the cell culture supernatant and cell lysates, 2) increases steady-state levels of IL-1β mRNA, 3) augments IL-1β gene transcription, 4) increases IL-1β promoter activity, and 5) promotes NF-κB DNA binding. The addition of the S-nitrosothiol SNAC as an exogenous NO donor restores IL-1β protein expression, gene transcription, and NF-κB DNA binding to levels noted with LPS alone. Finally, gel shift analysis showed that NF-κB DNA binding in LPS-treated cells is significantly increased in the presence of the reducing agent DTT. This would suggest that S-nitrosation of NF-κB with resulting inhibition of DNA binding is a plausible mechanism underlying decreased expression of the NF-κB-dependent protein IL-1β. Given the central position of NF-κB in proinflammatory gene transcription, S-nitrosation-associated inhibition of NF-κB-dependent promoter activity suggests a potential role for NO as a feedback inhibitor of specific components in the inflammatory process, such as IL-1β.

We and others have previously demonstrated that ex vivo biochemical modification of NF-κB p50 inhibits DNA binding as determined by the gel shift assay. In particular, Matthews and co-workers (16) showed that NO donors inhibit the DNA binding activity of p50 and that a p50 mutant with a serine substitution (C62S) was significantly resistant to DNA binding inhibition; they also showed, by electron spray ionization mass spectrometry, that the C62 residue in p50 was S-nitrosylated by NO. We characterized the biochemical kinetic effects of p50 S-nitrosation and found that NO decreased the dissociation constant by fourfold, from $1.0 \times 10^{-10} \text{ M}^{-1}$ to 0.29 $\times 10^{-10} \text{ M}^{-1}$ (6). Using the ANA-1 macrophage model, we have subsequently demonstrated that inhibition of the endotoxin-mediated synthesis of NO enhances NF-κB p50 DNA binding without altering NF-κB activation and nuclear translocation (7). Immunoprecipitation studies confirm the presence of S-NO bonds in p50 isolated from endotoxin-stimulated cells and, conversely, the absence of S-NO bonds in the presence of the INOS inhibitor L-NAME. The p50...
isolated in this fashion retains functional DNA binding properties, mirroring that found in our ex vivo kinetic studies. Transcription of the NF-κB-dependent genes for iNOS and macrophage colony-stimulating factor (M-CSF) was enhanced in the setting of NO inhibition. In total, these results strongly suggest a role for S-nitrosation as a specific modulator of NF-κB DNA binding, with its associated effects on NF-κB-dependent gene transcription. In this study, using ANA-1 macrophages we demonstrate that NO alters IL-1β gene transcription and, ultimately, IL-1β protein expression.

These observations correlate well with others relating NO synthesis and the functional results of NF-κB inhibition. NF-κB activation is a prerequisite for the transcription of a variety of proinflammatory mediators and markers, including IL-1β, iNOS, M-CSF, TNF-α, and vascular cell adhesion molecule (VCAM) (21, 25, 26). In particular, a number of previous studies have documented the regulatory interplay between NO and IL-1β synthesis in a variety of experimental models (4, 12, 13, 17, 18, 24, 29). Inhibition of NO synthesis is associated with increased IL-1β protein expression. Until recently, an underlying mechanism has not been elucidated. In models of macrophage synthesis of NO, Kim and colleagues (12) have demonstrated that NO suppresses IL-1β protein processing by inhibition of caspase-1, a key enzyme required for IL-1β maturation and release. The inhibition of caspase-1 activity is reversed in the presence of DTT. This, in combination with previous work by the same group, indicates S-nitrosation of caspase proteases as a potential mechanism (11). These results notwithstanding, Kim and colleagues also demonstrate that inhibition of NO synthesis is associated with 1) increased IL-1β and TNF-α mRNA expression and 2) increased IL-1β protein expression but unchanged TNF-α protein expression. Our findings certainly corroborate the results of Kim and coworkers and indicate that S-nitrosation may regulate the activity of key regulatory proteins involved at multiple levels of protein synthesis, including transcription and posttranslational processing.

Previous work has demonstrated that S-nitrosation of NF-κB p50 is associated with decreased gene transcription and synthesis of the NF-κB-dependent proteins iNOS and M-CSF (7). However, the effects of S-nitrosation are not generalized to all NF-κB-dependent proteins, as evidenced by our observation that LPS-stimulated TNF-α elaboration is not altered by inhibition of NO synthesis. Posttranscriptional regulatory mechanisms may obviate the effects of transcriptional inhibition.

Although TNF-α mRNA levels are increased in the presence of NO inhibition, as shown by Kim et al. (12), additional posttranscriptional mechanisms in the macrophage may obviate these effects. In contrast, other investigators have demonstrated that inhibition of NO synthesis is associated with increased expression of both IL-1β and TNF-α proteins (17, 18, 29). Although these differences in the effect of NO on TNF-α biology may be the result of differences in the experimental models, inhibition of IL-1β synthesis by NO is a recurring theme in the work of many groups. The results from the present study suggest that NF-κB DNA binding properties, IL-1β gene transcription, and IL-1β protein expression are significantly decreased in the presence of NO. The underlying mechanism is consistent with our previous observations concerning S-nitrosation of NF-κB and its effects on DNA binding and gene transcription (7).

As a result of its participation in redox chemistry, NO is a pluripotent regulator of multiple cellular functions (19). The formation of S-nitrosothiols exemplifies these pathways of NO oxidation, which lead to surrogate NO-like bioactivity and result in allosteric receptor modification, inhibition of sulfhydryl enzyme activities, and downregulation of transcriptional activators. With respect to NF-κB activity, studies utilizing exogenous NO donors have shown that NF-κB DNA binding is inhibited (3, 21, 22). In a system of human vascular endothelial cells, Peng and colleagues (21, 22) showed that exogenous NO stabilized NF-κB by inhibiting the dissociation of the IκB inhibitor while simultaneously increasing IκB mRNA levels. These investigators also found that NO minimally altered activation of nuclear binding proteins AP-1, GATA-2, and GATA-3 (21, 22). Recently, Camaels and colleagues (3) also demonstrated NO-mediated inhibition of p53 DNA binding resulting from NO-induced conformational and functional modifications, suggesting S-nitrosation, given the absence of an IκB correlate for p53. Of added interest is the relative specificity of S-nitrosation for NF-κB- and, perhaps, p53-associated activity. Nuclear proteins, such as IRF, GATA, AP-1, oct-2, and STAT, do not exhibit NO-induced alteration in activity (6, 21, 22). Future identification of a pool of NO-modulated nuclear protein transcription factors would allow elucidation of the pathways of NO-mediated transcriptional regulation.

These results indicate that NO may play a direct regulatory role in the inflammatory response. Our study suggests a novel mechanism in which NO production feedback inhibits IL-1β gene transcription in the context of LPS stimulation. Although initial studies in the field suggested that iNOS expression and NO production were associated with deleterious biological effects, more recent studies have elucidated a feedback inhibitory role for iNOS and NO in the elaboration of IL-1β, IFN-γ inducing factor, IL-6, and VCAM-1 (12, 23, 24). Under certain inflammatory states, we propose that NO may function to inhibit feedback inhibition of specific NF-κB-dependent proteins in an autoregulatory inducible fashion.

Address for reprint requests and other correspondence: P. C. Kuo, Dept. of Surgery, Georgetown Univ. Medical Center, 4 PHC; 3800 Reservoir Rd. NW, Washington, DC 20007 (E-mail: kuop@gunet.georgetown.edu).

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