Endotoxin-Stimulated Nitric Oxide Production Inhibits Expression of Cytochrome c Oxidase in ANA-1 Murine Macrophages

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Endotoxin-Stimulated Nitric Oxide Production Inhibits Expression of Cytochrome c Oxidase in ANA-1 Murine Macrophages

Junping Wei, Hongtao Guo, and Paul C. Kuo

In endotoxin (LPS)-mediated states of sepsis, inducible NO synthase expression and NO production are associated with molecular regulatory functions that determine the host inflammatory response. NO inhibits cellular respiration and mitochondrial electron transport by inhibition of cytochrome c oxidase (CcO) activity. CcO is the terminal complex of the mitochondrial respiratory chain, responsible for 90% of cellular oxygen consumption and essential for cellular energy production. Subunit 1 (CcO I) is considered to be the most critical of the 13 CcO component subunits. In this regard little is known of the effect of NO on the transcriptional program for CcO expression. In ANA-1 murine macrophages, LPS-mediated NO synthesis decreases CcO enzyme activity, CcO I protein expression, and CcO I steady mRNA levels. Mitochondrial run-on analysis demonstrates unaltered CcO I mitochondrial gene transcription. Half-life analysis indicates that CcO I mRNA stability is significantly decreased in the presence of LPS-mediated NO synthesis. In this study using LPS-stimulated ANA-1 murine macrophages, we demonstrate that expression of the mitochondrial gene product, CcO I, is significantly decreased as the result of a unique and previously uncharacterized, NO-dependent post-transcriptional regulatory mechanism. The Journal of Immunology, 2002, 168: 4721–4727.

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y identifying patterns of gene expression associated with varying physiological states, it is possible to determine regulatory mechanisms that are specific to individual physiologic parameters. Suppression subtractive hybridization (SSH) is one potential molecular approach to determine the patterns of gene expression (1). It has recently been used to compare patterns of gene expression in breast cancer cell lines discordant for estrogen receptor expression (2, 3). In a system of ANA-1 murine macrophages, we hypothesized that endotoxin (LPS)-mediated NO production induces a specific set of genetic programs. To identify genes differentially expressed in LPS-stimulated cells producing NO, mRNA from LPS-treated cells was used as the tester and mRNA from LPS- and Nω-nitro-L-arginine methyl ester (L-NAME)-treated cells was used as the driver. In driver cells L-NAME was added to LPS-stimulated cells as a competitive substrate inhibitor of NO production. The resulting subtracted cDNA library was used to generate probes for use in Northern blot analysis to confirm the identities of the differentially expressed genes. A number of mitochondrial genes were significantly decreased in the setting of LPS-mediated NO synthesis, including cytochrome c oxidase (CcO) subunits 1 and 2 (CcO I and II), cytochrome b, and NADH dehydrogenase subunit 1.

In LPS-mediated states of sepsis, inducible NO synthase (iNOS) expression and NO production regulate molecular functions that determine the host inflammatory response. NO inhibits mitochondrial respiration by nitrosylation of the iron-sulfur centers of aconitase, complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), and complex IV (cytochrome c oxidase) (4, 5). Short term exposure to low concentrations of NO specifically and reversibly inhibits CcO activity (4). A mitochondrial heavy strand gene product, CcO, is the terminal complex of the mitochondrial respiratory chain, responsible for 90% of cellular oxygen consumption and essential for cellular energy production (6–9). CcO I is considered to be the most critical of the various CcO component subunits. In this regard although the effect of NO on mitochondrial respiratory physiology has been extensively characterized, the role of NO has not been examined with respect to the transcriptional program for mitochondrial expression of proteins critical to the cellular respiration. In this study using LPS-stimulated ANA-1 murine macrophages, we demonstrate that the expression and activity of the mitochondrial protein, CcO I, are significantly decreased as the result of an NO-dependent post-transcriptional regulatory mechanism.

Materials and Methods

Cell culture and induction of NO synthesis in ANA-1 macrophages

ANA-1 macrophages (gift from Dr. G. Cox, U.S. Uniformed Health Services, Bethesda, MD) were maintained in DMEM with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. LPS (0–10,000 ng/ml) was added in the absence of FCS (10%) to induce NO synthesis. In selected instances the competitive substrate inhibitor of NO synthase, L-NAME (250 ng/ml); the selective inhibitor of iNOS, L-N(1-iminoethyl)lysine hydrochloride (L-NIL; 100 μM); the NO donor, S-nitroso-N-acetyl-penicillamine (SNAP; 100 μM); or a combination of these compounds was added. After incubation for 12 h at 37°C in 5% CO2, the supernatants and cells were harvested for assays.
Suppression subtractive hybridization

SSH was performed using the Clontech PCR-Select cDNA Subtraction and PCR-Select Differential Screening kits. Total RNA was isolated from confluent cells treated with LPS or LPS plus t-NAME for a period of 12 h. Poly(A)~+~ RNA was prepared from total RNA using a Dynabeads mRNA purification kit (Dynal Biotech, Oslo, Norway). A reverse transcripted reaction was performed with 2 μg poly(A)~+~ RNA, 50 mM Tris-HCl (pH 8.5), 8 mM MgCl2, 30 mM KCl, 1 mM DTT, 10 μM dNTP, 10 μM primer (5'TTTTTGTACAGCGTCGAGN3), and 2 U AMV reverse transcriptase for 1.5 h at 37°C. Second-strand cDNA synthesis was performed using DNA polymerase I and T7 DNA polymerase. Double-strand cDNA was treated with a restriction enzyme, RsaI, to generate a blunt-ended, double-stranded cDNA fragment. It was then phenol-extracted and ethanol-precipitated. Adaptors (adaptor 1 or adaptor 2R; Clontech Laboratories, Palo Alto, CA) were attached in 1× ligation buffer containing I U/μl T4 DNA ligase (Clontech Laboratories) and incubated at 16°C for 1 h. Subtraction hybridization of cDNA was conducted by hybridizing adaptor 1-ligated cDNA and the adaptor 2R-ligated cDNA at 68°C for 8 h after denaturation at 98°C for 1.5 min. A second round of hybridization was performed at 68°C overnight after mixing the two primary hybridization samples and adding an excess of freshly denatured adaptor-ligated cDNA. Two hundred microliters of 20 mM HEPES-HCl (pH 8.3), 50 mM NaCl, and 0.2 mM EDTA (pH 8.0) were then added, and the resulting solution was incubated at 68°C for 7 min. Differentially expressed sequences in subtracted cDNA were subjected to PCR to amplify only cDNA with different adaptors at both ends, which were further enriched by a second round of PCR amplification with the nested primer. The desired differentially expressed sequences amplified by PCR were inserted into pT-Adv cloning vector (Clontech Laboratories). After a simple blue/white visual assay, PCR was used to rapidly amplify the cDNA insert using the following PCR primers: forward, AAA CAG CTA TGA CCA TGA; and reverse, TAA TAC GAC TCA CTA TAG GG. Each PCR product was blotted on a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Piscataway, NJ). 32P-labeled cDNA probes were synthesized as first-strand cDNA from tester and driver. Clones corresponding to differentially expressed mRNAs hybridize only with the probe. Following hybridization and washing, the membrane was exposed to x-ray film. Positive clones were sequenced with the sequencing primer AAA AGG CTA TGA CCA TGA, using the ABI PRISM 3737 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). Resulting sequences were compared with the GenBank database using the National Center for Biotechnology Information BLAST server.

Isolation of mitochondria

Mitochondria were isolated using the ApoAlert Cell Fractionation Kit (Clontech Laboratories). The purity of mitochondrial yield was determined by immunoblot analysis for the COX IV mitochondrial protein marker.

Cell culture medium was removed, and plates were rinsed with PBS at room temperature. All the following steps were performed using ice-cold buffers. RIPA buffer (0.6 M; 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, and 60 μg/ml aprotinin) was added to a 65-mm cell culture plate. Plates were scraped, and the cells were lysed. Ten microliters of 10 mg/ml PMSF stock was added, followed by incubation for 30–60 min on ice. Whole cell lysate was preclarified by adding 0.25 μg normal mouse control IgG together with protein A-agarose conjugate, and incubation was performed at 4°C for 30 min. The beads were pelleted, and the supernatant was incubated with primary Ab (monoclonal mouse COX I, COX II, or NADH I-Ab; Molecular Probes, Eugene, OR). Resuspended protein A-agarose was added, and the tubes were incubated at 4°C on a rocker platform overnight. The pellet was collected by centrifugation at 1000 × g for 5 min at 4°C, and the supernatant was discarded. The pellet was washed with RIPA buffer multiple times and resuspended in electrophoretic sample buffer. COX activity was determined by absorbance at 650 nm using protein assay reagent (Bio-Rad, Hercules, CA). Cell lysate (50 μg/lane) was separated by SDS-12% PAGE, and the products were electrotransferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). The membrane was blocked with 5% skim milk PBS, 0.05% Tween for 1 h at room temperature. After being washed three times, blocked membranes were incubated with primary mouse COX I, COX II, or NADH I-Ab (Molecular Probes) for 1 h at room temperature, washed three times in PBS/0.05% Tween, and incubated with HRP-conjugated secondary Ab for 1 h at room temperature. After an additional three washes, bound peroxidase activity was detected by the ECL detection system (Amersham Pharmacia Biotech). Quantification was performed using densitometric analysis.

Assay of NO production

NO released from cells in culture was quantified by measurement of the NO metabolite, nitrite. Cell culture medium (50 μl) was removed from culture dish and centrifuged; the supernatants were mixed with 50 μl sulphanilamide (1%) in 0.5% H NCl. After a 5-min incubation at room temperature, an equal volume of 0.02% N-1-(naphthyl)ethylenediamine was added. Following incubation, filters were washed twice and subjected to autoradiography using Fuji film (Fuji, Tokyo, Japan) for a period of 14 h. The mitochondrial H-strand gene for 16S rRNA was used as the housekeeping gene. Quantification was performed using a PhosphorImager (Storm 840; Molecular Dynamics, Sunnyvale, CA).

Mitochondria run-on assays

Mitochondria run-on assays were performed as described previously (11, 12). Macrophage mitochondria were preincubated in lysis buffer (10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, and 0.5% Nonidet P-40) and pelleted at 500 × g. The mitochondria (2 × 107) were resuspended in 100 μl glycerol buffer, then 150 μCi [32P]UTP (800 Ci/mmol) in 100 μl 10 mM Tris-Cl (pH 8.0), 5 mM DTT, 5 mM MgCl2, 300 mM KCl, and 1 mM each of ATP, CTP, and GTP for 30 min at 30°C were added. Labeled RNA was then treated with 10 μl RNAase-free DNase I (Life Technologies) for 5 min at 30°C, extracted with phenol/chloroform (24/1) and chloroform alone. Following hybridization, the membranes were washed twice and subjected to autoradiography for a period of 14 h. The mitochondrial H-strand gene for 16S rRNA was used as the housekeeping gene. Quantification was performed using a PhosphorImager (Storm 840).
and the absence of the competitive substrate inhibitor, L-NAME (250 ng/ml), or the specific inhibitor of iNOS, L-NIL (100 μM). The nitrite level in unstimulated control cells was 23.1 ± 6.6 nmol/mg. There was a significant concentration-dependent increase in medium levels of nitrite, the NO metabolite, in response to LPS stimulation (by ANOVA, \( p = 0.0001 \)). In the presence of an LPS concentration of 100 ng/ml, nitrite production was 74 ± 11.2 nmol/mg. LPS- plus L-NAME-treated and LPS- plus L-NIL-treated cells exhibited levels of NO production that were not significantly different from controls for all concentrations of LPS used. Nitrite levels of cells treated with L-NAME alone or L-NIL alone did not differ from those of unstimulated control cells (19.3 ± 5.8 and 18.4 ± 4.4 vs 23.1 ± 6.6 nmol/mg). In subsequent assays an LPS concentration of 50 ng/ml was used unless otherwise stated.

**Ceullar CcO activity**

Cellular CcO enzyme activities were normalized to succinate dehydrogenase activity and total cell protein and determined after 1, 6, and 12 h of treatment (Table I). One hour following LPS stimulation, CcO activity was decreased by approximately 50% compared with that in unstimulated control cells. Ablation of NO synthesis in LPS- plus L-NAME-treated cells resulted in restoration of CcO activity. Repletion of NO by addition of SNAP to LPS- plus L-NAME-treated cells again significantly decreased CcO activity. L-NAME or SNAP alone did not alter CcO activity. Following 6 and 12 h of stimulation there was a serial decline in CcO activity in LPS-treated and LPS-, L-NAME-, and SNAP-treated cells. At 12 h of stimulation CcO activity was 20 and 25% of that noted in controls in LPS-treated and LPS- and LPS-, L-NAME-, plus SNAP-treated cells, respectively. Cell viability, as measured by trypan blue exclusion, was not significantly different among various treatment groups following 1, 6, and 12 h of incubation. Subsequently, 1- and 12-h treatment groups of LPS-stimulated cells were washed, medium containing L-NAME was added to inhibit NO production, and CcO activity was measured after an additional 6 h. In the 1-h treatment group CcO activity (1.6 ± 0.2/mg protein) was restored to levels noted in unstimulated control cells. In contrast, in the 12-h treatment group CcO activity (0.4 ± 0.1/mg protein) remained significantly depressed and was not significantly different from that noted before L-NAME addition. The data suggest that CcO enzyme activity is significantly decreased by LPS-mediated NO synthesis. In addition, depression of CcO activity is irreversible following 12-h incubation in the setting of LPS-mediated NO synthesis.

**CcO I protein expression**

Cellular CcO I protein expression was determined using immunoblot analysis (Fig. 1). CcO IV protein, encoded by the nuclear genome, was used as a marker to normalize for levels of total mitochondria. Under all treatment conditions, CcO IV expression was unaltered, indicating that total mitochondrial mass and subunits of CcO encoded by the nuclear genome did not change with the various experimental conditions. LPS treatment decreased normalized CcO I protein by >12-fold compared with control cells (\( p < 0.01 \) vs control). Inhibition of NO synthesis by addition of L-NAME with LPS restored CcO I protein to levels not significantly different from those in control cells. Repletion of NO in the form of SNAP to LPS- plus L-NAME-treated cells again significantly decreased CcO I protein. In this instance, CcO I protein was >10-fold less than the control value (\( p < 0.01 \) vs control). Treatment of ANA-I cells with L-NAME or SNAP alone did not significantly alter CcO I expression compared with that in controls. These results indicate that LPS-mediated NO production inhibits CcO I protein expression. NO alone is necessary, but insufficient.

**CcO I Northern blot analysis**

Steady state mRNA levels of cellular CcO I were determined by Northern blot analysis (Fig. 2). β-Actin and 16S rRNA mRNA

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**Table I. Normalized cellular CcO activity**

<table>
<thead>
<tr>
<th></th>
<th>1 h</th>
<th>6 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>LPS (50 ng/ml)</td>
<td>0.9 ± 0.2*</td>
<td>0.6 ± 0.1*</td>
<td>0.4 ± 0.1*</td>
</tr>
<tr>
<td>LPS + L-NAME</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>LPS + L-NAME (100 μM)</td>
<td>1.1 ± 0.2*</td>
<td>0.7 ± 0.2*</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>LPS + L-NAME + SNAP (100 μM)</td>
<td>1.6 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>L-NAME</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>1.9 ± 0.3</td>
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</table>

*Data, normalized to succinate dehydrogenase activity and total cell protein, are expressed as mg protein. Values are presented as mean ± SEM of eight experiments. *\( p < 0.05 \) vs control, LPS + L-NAME, L-NAME, and SNAP.

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**FIGURE 1.** Immunoblot analysis of CcO I and CcO IV protein expression. CcO I and CcO IV protein expressions were determined as measures of mitochondrial and nuclear-encoded CcO protein subunits, respectively. Mitochondria were isolated using the ApoAlert Cell Fractionation Kit (Clontech). The purity and normalization of mitochondrial yield were confirmed by immunoblot analysis for the CcO IV mitochondrial protein marker. Immunoprecipitation and immunoblot analysis were performed as described in Materials and Methods. LPS, 50 ng/ml; L-NAME, 100 μM; SNAP, 100 μM. The blot is representative of four experiments.

**FIGURE 2.** Northern blot analysis of CcO I steady state mRNA expression. Steady state mRNA levels of cellular CcO I were determined by Northern blot analysis. β-Actin and 16S rRNA mRNA were used as measures of constitutive nuclear and H-strand mitochondrial gene expression. Northern blot analysis was performed as described in Materials and Methods. A 52P[32P]dATP-labeled 600-bp probe was constructed based upon the murine CcO I mitochondrial cDNA sequence (GenBank number AF259518; nt +616 to +1216). LPS, 50 ng/ml; L-NAME, 100 μM; SNAP, 100 μM. The blot is representative of four experiments.
were used as measures of constitutive nuclear and H-strand mitochondrial gene expression. Of note, both β-actin and 16S rRNA mRNA were unaltered by treatment conditions. LPS treatment decreased CcO I mRNA by >20-fold compared with control cells (p < 0.01 vs control). Inhibition of NO synthesis by addition of L-NAME with LPS restored CcO I mRNA to levels not significantly different from those in control cells. Repletion of NO in the form of SNAP to LPS- plus L-NAME-treated cells again significantly decreased CcO I mRNA. In this instance CcO I mRNA was largely undetectable (p < 0.01 vs control). Treatment of ANA-1 cells with L-NAME or SNAP alone did not significantly alter CcO I mRNA expression relative to the control value. These results indicate that LPS-mediated NO production inhibits steady state CcO I mRNA expression. Again, NO alone is necessary, but insufficient.

**CcO I mRNA half-life**

Mitochondrial RNA polymerase is resistant to normal inhibitors of nuclear RNA polymerase (12, 13). Eads and Hand (12) have demonstrated that actinomycin D (100 μg/ml) inhibits mitochondrial gene transcription by 85%, while 0°C incubation during the assay is associated with 96% inhibition. Therefore, CcO I mRNA half-life was determined with both actinomycin D (100 μg/ml) and incubation at 0°C to completely inhibit mitochondrial RNA polymerase (Fig. 3). One hour following stimulation, actinomycin D was added. Expression of mRNA was normalized to that of the housekeeping gene, 28S rRNA, and that of CcO I at time zero. A Northern blot analysis following actinomycin D (100 μg/ml) and incubation at 0°C was performed as described in Materials and Methods. Expression of mRNA was normalized to that of the mitochondrial H-strand gene, 16S rRNA and that of CcO I at time zero. A [32P]dATP-labeled 600-bp probe was constructed based upon the murine CcO I mitochondrial cDNA sequence (GenBank number AF259518; nt +616 to +1216). LPS, 50 ng/ml; L-NAME, 100 μM; SNAP, 100 μM. The blot is representative of four experiments.

Expression of mitochondrial H strand proteins

The effect of NO on other proteins encoded by the mitochondrial H strand genes is largely unknown. Certainly this study indicates that 16S rRNA expression is not changed. In the setting of LPS-mediated NO synthesis in ANA-1 macrophages, we have previously demonstrated that cytochrome b protein expression is inhibited in the presence of unaltered transcription (14). Our SSH data suggest that CcO II and NADH I expression may also be down-regulated in the presence of NO. Therefore, immunoblot and Northern blot analyses were performed (Fig. 6). CcO II and NADH I protein and steady state mRNA levels were not altered under the various treatment conditions. Additional mitochondrial run-on experiments examining CcO II and NADH dehydrogenase
I transcription demonstrated no significant changes in transcription under the various treatment conditions (data not shown). These data indicate that LPS-induced NO synthesis does not uniformly decrease levels of all transcripts encoded by the mitochondrial H-strand.

Discussion

In this study using LPS-mediated NO synthesis in a model of ANA-1 murine macrophages we have demonstrated that NO significantly decreases both CcO activity and CcO I protein expression by increasing CcO I mRNA degradation. In states of sepsis or inflammation NO is known to inhibit mitochondrial respiration by nitrosylation of the iron-sulfur centers of aconitase, complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), and complex IV (CcO) (4, 5). Short term exposure to low concentrations of NO specifically and reversibly inhibits CcO activity (4). However, the effect of NO on the mitochondrial genetic programs that regulate critical components of oxidative phosphorylation and cellular respiration has not been previously examined.
A mitochondrial heavy strand gene product, CcO, is the terminal complex of the mitochondrial respiratory chain, responsible for 90% of cellular oxygen consumption and essential for cellular energy production. The primary three subunits, CcO I, II, and III, are encoded by mitochondrial DNA and perform the catalytic functions of the holoenzyme. CcO I binds three of the enzyme’s redox centers (heme a, heme a₃, and Cu₄) and is the most highly conserved member of the entire CcO complex. As such, it is considered to be the most critical of the 13 CcO subunits (4, 6, 7, 9). In theory, inhibition of CcO I protein expression by NO should dramatically decrease CcO holoenzyme function and inhibit mitochondrial respiration. In this regard it is known that exposure to NO for prolonged time periods or at higher concentrations results in irreversible inhibition of mitochondrial respiration. Induction of iNOS in our studies fulfills these criteria. Previous studies have found that NO damages the mitochondrial iron-sulfur centers and nitrosylates essential thiols in complex I. In addition, the NO metabolite, peroxynitrite, may damage mitochondrial complexes I and II. ATP synthase, creatine kinase, and mitochondrial DNA and induce mitochondrial swelling and uncoupling (4, 5).

The effect of NO on expression of essential protein components of the electron transport chain has not been extensively characterized in this regard. Our results suggest that iNOS-mediated irreversible inhibition of mitochondrial respiration may be the result of NO-dependent post-transcriptional augmentation of CcO I mRNA.

NO-dependent post-transcriptional acceleration of CcO I mRNA degradation has not been previously described in the setting of LPS stimulation. In a model of RAW 264.7 macrophages Lehrer-Graiwer and colleagues (15) found that exposure to an exogenous source of NO for <1 h significantly increased steady state CcO I mRNA and protein expression. Consistent with our observations after administration of SNAP alone, these authors found that an NO donor alone did not significantly alter CcO I protein expression. However, there was no change found in CcO enzyme activity, and addition of the NO donor to the enzyme assay mixture resulted in complete inhibition of CcO activity (15). The difference in results between our two groups is explained by the difference in experimental models. We used a model of LPS-induced NO production with an emphasis on prolonged exposure, and as a consequence, our data indicate that NO is necessary, but insufficient, by itself to inhibit CcO I protein expression. However, in settings using only the NO donor SNAP we found no change in CcO I mRNA or protein expression after an incubation period of 12 h. Janssen et al. (16) exposed rat epithelial cells to spermine 2-[(N,N-diethylamino)-diazene-2-oxide for 4 h and found that mRNA for the mitochondrial genes NADH dehydrogenase subunits 5 and 6 was significantly decreased. Corresponding protein levels and enzyme activity were not measured. Again, the role of LPS-induced NO production is not addressed in this study. Finally, in LPS- and IFN-γ-stimulated astrogial and mixed cortical cell cultures, Nicoletti et al. (17) found that NO induced increased CcO I mRNA levels and transcription following an incubation period of 18 h. However, cytochrome oxidase enzyme activity was decreased. These data contrast with ours and may simply result from differences in cell models. These results notwithstanding, our findings suggest a unique mechanism by which LPS-mediated NO synthesis inhibits CcO activity by enhancing mRNA degradation of CcO I, a mitochondrial gene product critical for electron transport.

The mitochondrial genome is a closed circular dsDNA molecule of approximately 16.6 kb that is highly conserved among mammals (9). The H-strand encodes two rRNAs, 14 tRNAs, and 12 polypeptides, including CcO I. The L strand codes for eight tRNAs and a single polypeptide. All 13 polypeptide products are constituents of enzyme complexes of the oxidative phosphorylation system. Transcription and replication depend upon trans-acting nuclear-encoded factors. An H-strand promoter with two potential initiation sites controls transcription of the H strand. Once initiated, the strands are transcribed as single polycistronic precursor RNAs. Increased mitochondrial transcription has been found in rapidly dividing immortal cells requiring elevated respiratory function and anoxia-induced quiescence in Artemia franciscana embryos and in early Xenopus embryogenesis (11–13, 18). However, differential rates of transcription have not been identified among individual mitochondrial polypeptide mRNAs, indicating that differential mRNA stability accounts for varying levels of mitochondrial mRNA levels. Our run-on results with the various mitochondrial genes corroborate this finding. The mechanisms that control mitochondrial mRNA degradation are less well defined. Polyadenylation of mRNAs is thought to create functional translation stop codons that are encoded in the DNA while also enhancing degradation (9, 18). In Trypanosoma brucei, Miliello and Read (18) found that mitochondrial mRNA is degraded by two biochemically distinct turnover pathways. The first pathway is dependent upon an mRNA poly(A) tail and requires exogenous UTP, while the second requires neither a poly(A) tail nor UTP. The first pathway results in rapid turnover with a mRNA t1/2 of approximately 10–20 min, while the second results in slower turnover with a t1/2 of approximately 3 h. Although the applicability of these observations to our system is unknown, the second slower pathway may be responsible. The details of the interplay of NO with this second mitochondrial mRNA degradation pathway are as yet unknown. Alternatively, exclusive of these pathways, NO may interact with the mitochondrial enzymes, poly(A) polymerase or RNase P, to enhance activity, resulting in increased mitochondrial mRNA degradation (9).

The source of the NO in our studies requires consideration. The two potential sources of NO in our system are inducible NOS and mitochondrial NOS (mtNOS) (19, 20). Characterization of the mtNOS isoform indicates that it is similar to iNOS, but is constitutively expressed and localized to the mitochondrial membrane. It is unknown whether mtNOS expression or activity is enhanced in the presence of LPS or proinflammatory cytokines. Our attempts to induce NO production and demonstrate decreased CcO I protein expression or activity in isolated mitochondria stimulated with LPS were unsuccessful. However, these preliminary results do not rule out involvement of mtNOS in the inhibition of CcO I activity.

In summary, in a system of LPS-stimulated ANA-1 murine macrophages, CcO I protein and mRNA levels, CcO activity, and CcO I mRNA half-life were significantly decreased in the presence of an NOS inhibitor. NO is necessary, but insufficient, to alter CcO I expression. LPS-dependent signal transduction pathways are also required. This effect does not uniformly affect all mitochondrial H-strand genes. The role of NO in mitochondrial respiratory physiology has been extensively characterized in states of inflammation and sepsis. However, the role of NO has not been previously examined with respect to the mitochondrial transcriptional programs that regulate proteins critical for cellular respiration and oxidative phosphorylation. In this regard our observations suggest a novel mechanism by which NO may inhibit mitochondrial function and cellular respiration by enhancing degradation of CcO I mRNA, a critical electron transport protein.

References


