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Nitric Oxide-Dependent Ribosomal RNA Cleavage Is Associated with Inhibition of Ribosomal Peptidyl Transferase Activity in ANA-1 Murine Macrophages

Charles Q. Cai,* Hongtao Guo,* Rebecca A. Schroeder,† Cecile Punzalan,* Paul C. Kuo²*

NO can regulate specific cellular functions by altering transcriptional programs and protein reactivity. With respect to global cellular processes, NO has also been demonstrated to inhibit total protein synthesis and cell proliferation. The underlying mechanisms are unknown. In a system of ANA-1 murine macrophages, iNOS expression and NO production were induced by exposure to endotoxin (LPS). In selected instances, cells were exposed to an exogenous NO donor, S-nitroso-N-acetylpenicillamine or a substrate inhibitor of NO synthesis. Cellular exposure to NO, from both endogenous and exogenous sources, was associated with a significant time-dependent decrease in total protein synthesis and cell proliferation. Gene transcription was unaltered. In parallel with decreased protein synthesis, cells exhibited a distinctive cleavage pattern of 28S and 18S rRNA that were the result of two distinct cuts in both 28S and 18S rRNA. Total levels of intact 28S rRNA, 18S rRNA, and the composite 60S ribosome were significantly decreased in the setting of cell exposure to NO. Finally, 60S ribosome-associated peptidyl transferase activity, a key enzyme for peptide chain elongation, was also significantly decreased. Our data suggest that NO-mediated cleavage of 28S and 18S rRNA results in decreased 60S ribosome associated peptidyl transferase activity and inhibition of total protein synthesis. The Journal of Immunology, 2000, 165: 3978–3984.

Expression of inducible NO synthase (iNOS)³ protein in settings rich in pro-inflammatory cytokines or endotoxin is associated with multiple alterations in cellular function. These include inhibition of protein synthesis, induction of cytostasis, modification of transcriptional programs, inhibition of mitochondrial respiration, and altered cellular redox state (1). In some instances, the underlying mechanism is related to NO-mediated S-nitrosation of key protein thiols with subsequent alteration in enzymatic function or protein binding properties (2, 3). Alternatively, NO alters the cellular redox balance by virtue of its oxidant properties, and induces downstream counterregulatory events (4, 5). However, the association between high concentrations of NO, as expressed by iNOS, and inhibition of total protein synthesis has not been extensively examined. The effect of NO upon global cellular translation, gene transcription, or cellular architecture critical to the protein synthetic process is unknown. In particular, the effect of NO upon the ribosomal scaffolding, which is critical to initiation and potentiation of protein translation, has not been examined.

In this study, using a model of endotoxin-mediated iNOS expression in ANA-1 murine macrophages, we demonstrate that NO inhibits total protein synthesis and cell proliferation without alteration in global gene transcription. However, induction of iNOS and inhibition of total protein synthesis in this cell line are associated with a specific and reproducible cleavage pattern in 28S and 18S rRNA that is both NO- and time-dependent. We examined enzymatic function of the 60S ribosome, which is largely composed of intact 28S and 18S rRNA. Levels of 60S ribosome and 60S ribosome-associated peptidyl transferase activity, an essential part of peptide chain elongation are both significantly depressed in the settings of both LPS-mediated endogenous NO synthesis and exposure to an exogenous NO donor, S-nitroso-N-acetylpenicillamine (SNAP). We conclude that NO-mediated inhibition of total protein synthesis is the result of decreased 60S ribosome-associated peptidyl transferase activity.

Materials and Methods

Cell culture

ANA-1 murine macrophages, a gift from Dr. George Cox (Uniformed Services University of the Health Sciences, Bethesda, MD), were maintained in DMEM with 5% heat-inactivated FCS. LPS (Escherichia coli serotype 0111:B4; 0–10,000 ng/ml) was added to the medium to induce NO synthesis. In selected instances, the competitive substrate inhibitor of NOS, N⁴-nitro-l-arginine methyl ester (l-NAME; 4 mM), alone or together with LPS and the NO donors, SNAP or S-nitroso-N-acetylcysteine were added. Cells were harvested after incubation for 2–20 h at 37°C in 95% O₂/5% CO₂. To determine cell viability, cells were washed with ice-cold PBS three times, stained with 0.2% trypan blue solution (w/v) for 10 min, and viable cells were counted under the light microscope. Cell viability was routinely >95% under all treatment conditions.

Determination of NO synthesis

NO release from cells in culture was quantified by measurement of the NO metabolite, nitrite, using the technique of Snell and Snell (6). To reduce nitrate to nitrite, 200 μl conditioned media was incubated in the presence of 1.0 U nitrate reductase, 50 μM NADPH, and 5 μM flavin adenine dinucleotide. Sulfanilamide (1%) in 0.5N hydrochloric acid (50% v/v) was then added. After a 5-min incubation at room temperature, an equal volume of 0.02% N-(1-(naphthyl))ethylenediamine was added; following incubation at room temperature for 10 min, absorbance at 570 nm was compared with that of a NaNO₂ standard.

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RNA and DNA extraction and Northern blot analysis

Total RNA was purified from cultured ANA-1 macrophages with the use of TRIzol Reagent (Life Technologies, Rockville, MD). The purified total RNA (5 μg) was subjected to electrophoresis on a 1% (w/v) agarose gel for 3 h at 80 V. The RNA was blotted onto Nytran nylon transfer membrane (Schleicher & Schuell, Keene, NH) with 10× SSC (1.5 M NaCl/0.15 M sodium citrate) and 0.1% SDS, and washed three times with 2× SSC and 0.1% SDS. The membranes were cross-linked to the membrane by UV irradiation. cDNA probes complementary to murine 28S and 18S rRNA were prepared by PCR amplification with primers based on their cdna sequence and randomly labeled with [32P]dCTP. Hybridization of the labeled probe was performed in hybridization solution (50% formamide, 5× SSC, 5 mM EDTA, 20 mM sodium phosphate buffer (pH 7), 1% SDS, 200 μg/ml salmon sperm DNA, and 5× Denhardt’s solution) at 42°C overnight with 3 h of prehybridization at 42°C. The blots were washed briefly with 2× SSC containing 0.1% SDS at room temperature, once for 15 min with 0.5× SSC containing 0.1% SDS at 42°C, and once for 15 min with 0.2× SSC containing 0.1% SDS at 50°C and 65°C. Autoradiographs were exposed at room temperature. Genomic DNA was purified from cultured ANA-1 macrophages with the DNAzol Reagent (Life Technologies). The purified genomic DNA were subjected to electrophoresis on a 0.6% (w/v) agarose gel for 3 h at 100 V.

Determination of protein synthesis

Cells were washed with methionine-free DMEM three times and incubated for 20 min in short-term medium (methionine-free DMEM containing 5% diazoy FCS) in a humidified 37°C, 5% CO2 incubator to deplete intracellular pools of methionine. Medium was then replaced with fresh short-term labeling medium containing 10 μCi/ml [3H]methionine. After 15–30 min, cell pellets were washed twice with 95–100% ethanol. When dry, the filter papers were transferred to a scintillation vial containing 4 ml scintillation fluid. Radioactivity was then measured.

Cell proliferation assays

ANA-1 macrophages were labeled with 5 μCi/ml [3H]thymidine for 20 h in the presence or absence of LPS. The plates were treated with 0.5 ml/well ice-cold 10% trichloroacetic acid at 4°C. After the addition of 0.5 ml lysis solution containing 20 mM Tris-HCl (pH 8.0), 400 mM KCl, 20 mM MgCl2, and 1 mM sodium citrate, the reaction mixture was extracted with 1 ml ethyl acetate. A portion of the extract was mixed with scintillation mixture and counted. Peptidyl transferase activity is expressed as nmol/mg protein/min.

Nuclear run-on analysis

A total of 100 μl ANA-1 macrophage nuclei was incubated for 5 min at 30°C with 150 μCi of [32P]rUTP (800 Ci/mmol) in 100 μl 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 re-suspended in 10 mM N-tris(hydroxy-

Cellular ribosome profile

ANA-1 murine macrophages were isolated in ice-cold PBS. The cells were pelleted at maximum speed in a microcentrifuge and resuspended in hypertonic buffer (40 mM Tris-HCl pH 7.4), 20 mM KCl, 3 mM MgCl2, 20 mM sodium fluoride, and 150 mM sucrose and kept on ice for an additional 10 min. Following cell lysis in 1% Triton X-100 and 1% deoxycholate, the preparation was vortexed and centrifuged for 1 min in a microcentrifuge. The supernatant formed the total ribosomal fraction and a portion was used for peptidyl transferase activity. The remaining supernatant was layered onto a linear 10–45% sucrose gradient containing 25 mM Tris-HCl, 80 mM KCl, 4 mM MgCl2, and 20 mM sodium fluoride. Following centrifugation for 4 h in a Beckman (Fullerton, CA) SW-41 rotor, ribosomal profiles were monitored by continuously measuring A280.

Ribosome-associated peptidyl transferase activity

Peptidyl transferase activity was measured in ANA-1 macrophage total ribosomal fractions using a peptidyl transferase reaction with full-length formyl-[3H]Met RNA as a donor substrate (7). Typically, cell fractions were incubated with 2.5 pmol formyl-[3H]Met RNA in 40 μl buffer containing 20 mM Tris-HCl (pH 8.0), 400 mM KCl, 20 mM MgCl2, and 1 mM puromycin. Reactions were initiated by adding 20 μl cold methanol and incubated for 20 min at 0°C. To terminate the reaction, 10 μl 4 M KOH was added and incubated for an additional 20 min at 37°C. After the addition of 200 μl 1 M KH2PO4, the reaction mixture was extracted with 1 ml ethyl acetate. A portion of the extract was mixed with scintillation mixture and counted. Peptidyl transferase activity is expressed as nmol/mg protein/min.

Statistical analysis

Data are presented as mean ± SEM of three or four experiments each performed in triplicate. Statistical analysis was performed using the Student’s t test or rank sum test, as appropriate.

Results

LPS and NO synthesis in ANA-1 macrophages

Following stimulation with 10–10,000 ng/ml LPS for a period of 20 h, ANA-1 murine macrophage production of NO was determined by measuring levels of nitrite and nitrate in the culture media (Fig. 1). The dose response was plotted in a semilogarithmic fashion. In the absence of LPS, the nitrite level in the media was 9.9 ± 1.0 nmol/mg protein. NO production increased in a significant dose-related fashion at LPS concentrations of 10, 100, 1,000, and 10,000 ng/ml (p < 0.01 vs control for LPS = 100, 1,000, and 10,000 ng/ml). The competitive substrate inhibitor L-NAME (4 mM) was added. In the presence of L-NAME alone, nitrite production was not significantly altered from that of control cells (10.1 ± 1.1 nmol/
In cells treated with LPS + L-NAME, NO production was ablated at all concentrations of LPS (10–10,000 ng/ml) and did not significantly differ from that of control cells. In addition, trypan blue exclusion and media levels of lactate dehydrogenase were determined as measures of cell viability. Under all treatment conditions, cell viability was routinely >95%, and LDH levels did not differ among the various treatment groups. These data indicate that LPS-mediated NO production is dose-dependent and does not alter cell viability. Subsequent studies use 100 ng/ml LPS and 4 mM L-NAME, unless stated otherwise.

Cell proliferation and total protein synthesis

Following a 20-h incubation, total protein synthesis was determined by [35S]methionine incorporation in control, LPS-, LPS + L-NAME-, and SNAP 400 μM-treated macrophages (Fig. 2A). In all instances, there was a significant linear, time-dependent increase in protein synthesis. In LPS-treated cells, total protein synthesis was consistently decreased by 40–70% at all time points when compared with those of control and LPS + L-NAME (p < 0.05 vs control and LPS + L-NAME). The greatest incremental difference in protein synthesis was present following a 15-min pulse. Protein synthesis in control and LPS + L-NAME cells was not significantly different. In SNAP-treated cells, total protein synthesis was significantly decreased at all time points, in comparison to control and LPS + L-NAME and also, when compared with LPS alone (p < 0.05 vs control, LPS + L-NAME, and LPS). When cells were incubated in the presence of LPS + L-NAME + SNAP, the protein synthesis curve was not statistically different from that of SNAP alone (data not shown). These data suggest that NO, whether from an exogenous or endogenous source, is associated with significant depression of total protein synthesis in ANA-1 murine macrophages.

The assay was then repeated by determination of [35S]methionine incorporation following a 240-min pulse while varying the duration of LPS stimulation (100 ng/ml), 0, 8, 12, 16, and 20 h (Fig. 2B). There was no difference in protein synthesis following 8 h of LPS stimulation. However, at LPS incubation times greater than 12 h, protein synthesis progressively decreases in a significant fashion (p < 0.05 vs control and LPS + L-NAME for 16 and 20 h). The time dependence of total NO synthesis was also determined and plotted in Fig. 2B. NO production increases dramatically following 12 h of LPS stimulation and reaches a near maximum after 20 h of LPS stimulation. The time course of this increase in NO is associated with a parallel decline in total protein synthesis. When [35S]methionine incorporation is plotted vs nitrite from LPS stimulated cells, an inverse linear relationship (r² = 0.70) is present (Fig. 2C). This suggests that NO production is inversely associated with decreased protein synthesis in LPS stimulated macrophages.

FIGURE 2. A, Total cellular protein synthesis in ANA-1 murine macrophages. ANA-1 murine macrophages were exposed to 100 ng/ml LPS for a period of 20 h and pulsed with [35S]methionine. Radioactivity was determined following the designated time period; measured values were corrected for total cell protein. In selected instances, 4 mM L-NAME or 400 μM SNAP were added. Results represent data from four separate experiments. *p < 0.05; LPS vs control and LPS + L-NAME. #p < 0.05; SNAP vs LPS, control, and LPS + L-NAME. B, Total cellular protein synthesis and NO production in ANA-1 murine macrophages. ANA-1 murine macrophages were exposed to 100 ng/ml LPS for a designated period of incubation and pulsed with [35S]methionine. Radioactivity was determined following 2 h and corrected for total cell protein. Nitrite production was simultaneously determined in LPS-treated cells. Results represent data from three separate experiments (p < 0.05, LPS vs control and LPS + L-NAME). C, Total cellular protein synthesis and NO production in 100 ng/ml LPS-treated ANA-1 murine macrophages. First order linear regression analysis was performed (r² = 0.70).
ANA-1 cell proliferation in the setting of LPS-induced NO synthesis was determined by the incorporation of [3H]thymidine (Fig. 3). LPS-mediated NO synthesis was associated with a significant 50% decrease in tritiated thymidine incorporation following a 20-h incubation in the presence of LPS (p < 0.05 vs control and LPS + L-NAME).

These data suggest that LPS-mediated NO synthesis inhibits total protein synthesis and cell proliferation in this model of ANA-1 murine macrophages. Total protein synthesis initially declines following 12 h of exposure to NO.

LPS-mediated NO production and gene transcription
To determine the role of NO production in ANA-1 macrophage gene transcription, nuclear run-on analysis was performed using β-actin, GAPDH, and cyclophilin as target “housekeeping” genes (Fig. 4). Following 20 h of LPS incubation, there was no difference in gene transcription for any of the target genes tested. Transcription was not significantly different among control, LPS-, L-NAME-, and LPS + L-NAME-treated cells. These results indicate that inhibition of total protein synthesis is not the result of decreased global gene transcription in LPS-treated ANA-1 murine macrophages.

rRNA expression patterns
The pattern of total RNA expression was then examined in this experimental model (Fig. 5A). In control cells, the typical electrophoretic pattern of 28S and 18S rRNA expression was found. In contrast, in the presence of 50 and 100 ng/ml LPS, a distinctive cleavage pattern of 18S and 28S rRNA was found. This pattern is associated with decreased levels of 18S and 28S rRNA, as determined by intensity of the bands on gel electrophoresis. Inhibition of NO production by the addition of L-NAME resulted in restoration of the normal rRNA pattern. The electrophoretic pattern of genomic DNA expression in LPS- and LPS + L-NAME-treated cells did not differ and specifically did not exhibit an apoptotic pattern (Fig. 5B).

An LPS dose-response experiment was performed using LPS concentrations varying from 50 ng/ml to 10 μg/ml and an incubation period of 20 h (Fig. 6A). Again, a specific cleavage pattern of rRNA was exhibited in all cells treated with LPS. In contrast, the typical rRNA pattern was found in control, L-NAME-, and LPS + L-NAME-treated cells. A transition in the intensity of RNA degradation was noted between LPS concentrations of 50–200 ng/ml. The experiments were then repeated using LPS concentrations of 50, 100, 150, 200, and 250 ng/ml to better characterize the transition (Fig. 6B). No distinct transition corresponding to a specific LPS concentration was evident from these experiments.

When an exogenous source of NO, 400 μM SNAP, or 400 μM S-nitroso-N-acetylcysteine was added with LPS + L-NAME to ANA-1 macrophages, the distinctive NO-mediated cleavage pattern for rRNA seen in LPS-treated cells was noted (data not shown). The time dependence of this rRNA cleavage pattern was examined. Control, LPS-, and LPS + L-NAME-treated cells were incubated for a period of 0, 4, 8, 12, 16, and 20 h before RNA extraction was performed. The RNA cleavage pattern appeared in LPS-treated cells at 12, 16, and 20 h of incubation. The cells incubated for a period of 20 h exhibited a more intense pattern with diminished bands corresponding to both 18S and 28S rRNA when compared with those found in cells treated for only 12 or 16 h. Of interest is the finding that the time course of RNA cleavage parallels that found for inhibition of total protein synthesis and maximal NO production (Fig. 2, B and C).

To determine the identity of these various RNA bands, Northern blot analysis was performed (Fig. 7). A full-length oligonucleotide probe was constructed that was complementary to murine 18S rRNA (1869 bp). Due to the size of 28S rRNA, three separate cDNA probes were made as follows: 28S-1 (nt 0–1500), 28S-2 (nt 1559–3268), and 28S-3 (nt 3275–4712). Based upon the results of the Northern blot analysis, 18S rRNA is cleaved into three fragments of ~1.0, 0.8, and 0.6 kb. In contrast, 28S rRNA is cleaved into four fragments. One binds 28S-1 (size ~3.3 kb); another (size ~2.6 kb) binds 28S-2, and a final two are complementary to 28S-3 (sizes ~1.5 and ~1.0 kb). These data indicate that NO, from both endogenous and exogenous sources, induces a specific time-dependent cleavage pattern in rRNA resulting in decreased levels of 18S and 28S rRNA expression.

ANA-1 ribosomal profiles
ANA-1 macrophages were exposed to 100 ng/ml LPS for 20 h. Unstimulated control and LPS + L-NAME-treated cells served as comparison groups. In cells exposed to LPS, there was a marked 4-fold decrease in 60S ribosomal A280 (0.025 ± 0.012) compared with that of control (0.102 ± 0.14; p < 0.05 vs LPS) and LPS + L-NAME (0.115 ± 0.18; p < 0.05 vs LPS) cells, indicating a...
decrease in quantity of 60S ribosomes. In addition, predictably, the A280 of the 80S ribosomal particle composed of 60S and 40S ribosomes was also decreased in LPS-treated cells (0.012 ± 0.009) compared with that of control (0.265 ± 0.019; p < 0.05 vs LPS) and LPS + l-NAME-treated (0.281 ± 0.023; p < 0.05 vs LPS) cells. These data suggest that LPS-mediated NO synthesis is associated with a decrease in relative quantities of 60S and 80S ribosomal particles, which are requisite for protein synthesis.

Ribosome-associated peptidyl transferase activity

Cleavage of 28S and 18S rRNA may alter 60S ribosomal peptidyl transferase activity and inhibit total protein synthesis. Peptidyl transferase activity from ANA-1 total ribosomal fractions was determined using LPS concentrations of 0, 1, 5, 10, 25, 50, 100, and 1000 ng/ml at time points varying from 0 to 20 h (Fig. 8A). In certain instances, 4 mM l-NAME was also added. In unstimulated control cells, peptidyl transferase activity was maintained throughout the study period. LPS concentrations of 1, 5, 10, and 25 ng/ml were associated with a progressive, concentration- and time-dependent decrease in peptidyl transferase activity beginning at 12 h of incubation. In contrast, LPS treatment at concentrations of 50, 100, and 1000 ng/ml was associated with maximally depressed enzyme activity beginning at 12 h with a subsequent decline thereafter at 16 and 20 h of incubation. At LPS concentrations of 50, 100, and 1000 ng/ml, peptidyl transferase activity was 2-fold less than that of controls at 20 h of incubation. Ablation of NO synthesis by addition of 4 mM l-NAME restored peptidyl transferase activity to control levels at all time points studied. The addition of an exogenous source of NO in the form of SNAP significantly depressed enzyme activity at all time points compared with both control and LPS cells. l-NAME alone did not alter peptidyl transferase activity in comparison to those of control cells (data not shown). To further examine the threshold characteristic of these data, the IC50 of peptidyl transferase activity was plotted as a function of LPS concentration (Fig. 8B). These data suggest that NO,
from either exogenous or endogenous sources, inhibits ribosome fraction-associated peptidyl transferase activity.

**Discussion**

In this model of LPS-mediated iNOS expression in ANA-1 murine macrophages, we demonstrate that NO, both from endogenous and exogenous sources, inhibits total protein synthesis and cell proliferation. This inhibition parallels total NO production beginning after ~12 h of exposure to NO and reaches its maximum following ~20 h of exposure. In addition, 28S and 18S rRNA cleavage occur at all concentrations of LPS examined during a standard 20-h incubation, suggesting that rRNA cleavage is both NO- and time-dependent. Transcription, as determined by nuclear run-on analysis of the standard “housekeeping” genes, β-actin, GAPDH, and cyclophilin, is not altered by LPS-mediated NO production. Northern blot analysis demonstrated the presence of two cleavage sites in both 18S and 28S rRNA. The time course and NO concentration dependence of decreased total protein synthesis correlates with appearance of this distinctive rRNA cleavage pattern. We hypothesized that NO-mediated inhibition of cellular protein synthesis may be the result of fragmentation of the rRNA components essential to 60S ribosome-mediated protein elongation. Subsequent studies found that levels of 60S ribosome and ribosomal peptidyl transferase activity were significantly decreased in ANA-1 murine macrophages exposed to endogenous and exogenous sources of NO. We conclude that NO-mediated inhibition of total protein synthesis is the result of depressed 60S ribosome-associated peptidyl transferase activity.

Eukaryotic protein synthesis is catalyzed by ribosomes, which are large complexes of proteins and rRNA (8). The smaller 40S rRNA subunit binds both mRNA and tRNA, while the larger 60S rRNA subunit catalyzes peptide bond formation. Together the 60S and 40S ribosomes compromise the larger functional unit of the 80S ribosome. The process of protein translation centers upon binding of an aminoacyl-tRNA molecule to the vacant A site on the 40S ribosomal subunit. The carboxyl end of the polypeptide chain is uncoupled from the tRNA molecule linked to the growing end of the polypeptide chain at the adjacent P site; a peptide bond is formed to the amino acid linked to the tRNA molecule in the A site.
in the protein synthetic pathway, including eIF-2α. A 14-h incubation with LPS. They postulated that NO impairs phosphorylation of the active site of peptidyl transferase to oxidative stress suggests another possible mechanism by which NO may modulate peptidyl transferase function and ultimately, total protein synthesis.

In our model, we hypothesize that NO mediates a specific pattern of 18S and 28S rRNA cleavage. As a result of depletion of intact components of the 60S ribosome, peptidyl transferase activity is decreased, total protein synthesis is depressed, and inhibition of cell proliferation ensues. Alternatively, it is possible that these RNA bands are the result of altered maturation or cleavage of rRNA precursors rather than or in addition to that of the mature rRNA forms. NO-mediated inhibition of protein synthesis has been previously observed in multiple experimental models using hepatocytes and macrophages. However, the underlying mechanism has not been extensively studied. Recently, Kim et al. (5) examined NO-mediated inhibition of total protein synthesis in RAW 264.7 murine macrophages. They found that exposure to NO was associated with phosphorylation of the protein synthesis initiation factor, eIF-2α. 80S ribosome formation was inhibited following a 14-h incubation with LPS. They postulated that NO impairs protein translation by phosphorylation of the α subunit of eIF-2α and inhibits binding of the initiator methionyl-tRNA to 40S rRNA. These investigators did not specifically address the role of NO in rRNA processing.

In a similar fashion, our results also indicate diminution in quantities of both 80S and 60S ribosomes, which are essential structural scaffolds upon which translation occurs. In addition, 60S ribosomal peptidyl transferase activity paralleled the time course of 28S and 18S rRNA degradation. We did not specifically address eIF-2α activity. However, NO may certainly act at multiple points in the protein synthetic pathway, including eIF-2α phosphorylation and inhibition of 60S ribosome formation with consequent ablation of peptidyl transferase activity.

In this experimental model, NO mediates a specific cleavage pattern in rRNA that is not paralleled by apoptosis. The effect of NO on rRNA metabolism has not been previously addressed. However, work in the early 1980’s by Varesio and colleagues (11–14) examined murine macrophages activated by individual exposure to IFN-α, IFN-γ, and LPS. Similar to what found in our study, they demonstrated a specific cleavage pattern in 28S rRNA with decreased accumulation of 28S rRNA and associated ribosomal particles. This pattern was associated with a cytotoxic functional profile rather than a suppressive profile. Given that this body of work predates the discovery of NO, the authors did not specifically address the presence or absence of NO. However, the similarities with the results from our study are compelling. Varesio et al. (12) may have been the first to identify a specific rRNA cleavage pattern that is mediated by cytokine- or LPS-mediated NO synthesis in macrophages. The specific sites of rRNA cleavage in 18S and 28S rRNA are the subject of ongoing investigation in our lab. It is unclear whether the cleavage site is specific for a defined nucleotide sequence or a rRNA tertiary structural motif. In addition, the mechanism may reside in enzymatic endonuclease or ribozyme activity. Finally, it will be important to determine the signaling pathway for the induction of this cleavage pattern.

The functional correlate of NO-mediated inhibition of total protein synthesis in the setting of LPS stimulation is unknown. Is it a cytotoxic or cytoprotective response? It has been hypothesized that inhibition of protein synthesis and mitochondrial respiration may serve as a stress response (5). The purpose may be to transiently shut-down nonessential cellular functions and conserve cellular resources in the presence of proinflammatory cytokines and/or endotoxemia. However, when chronically maintained, this process may lead to ultimate loss of cell viability. In our study, there were no discernible differences in cell viability based upon trypan blue exclusion or LDH release.

These considerations notwithstanding, we have demonstrated that LPS-mediated NO production is associated with decreased total protein synthesis without change in global gene transcription programming. This process is paralleled by a specific, reproducible, NO- and time-dependent cleavage pattern in rRNA constituents of the 60S ribosome, 28S and 18S rRNA. This results in decreased expression of both the 60S ribosome and the complete 80S ribosome, both of which are essential for protein synthesis, and significant inhibition of 60S ribosome-associated peptidyl transferase. The time course of decreased peptidyl transferase activity closely parallels that noted with cleavage of 28S and 18S rRNA. In this model of LPS-treated ANA-1 murine macrophages, we conclude that one potential mechanism underlying the inhibition of total protein synthesis is NO-mediated degradation of constituent rRNA components of the 60S ribosome and decreased 60S-associated peptidyl transferase activity, an essential central step in the protein synthetic pathway.

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