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REGULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE mRNA LEVELS BY LPS, INF-γ, TGF-β, AND IL-10 IN MURINE MACROPHAGE CELL LINES AND RAT PERITONEAL MACROPHAGES[1]

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Summary: The molecular mechanisms of LPS, INF-γ, TGF-β, and IL-10 regulation of inducible nitric oxide synthase (iNOS) mRNA expression were evaluated. In murine macrophage cell lines, LPS-induced increases in iNOS mRNA were blocked by either cycloheximide or actinomycin D. Neither TGF-β nor IL-10 alone had any effect on basal expression, and each only slightly reduced LPS induction of iNOS mRNA. However, IL-10 augmented INF-γ induction of iNOS mRNA to very high levels, while TGF-β inhibited INF-γ induction. Human monocytes expressed no detectable iNOS mRNA with any stimuli, though Southern analysis on human genomic DNA revealed a specific human iNOS gene. In human macrophages, the iNOS gene may have become inoperative during evolution.
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Nitric oxide ($NO^{\cdot}$), a free radical gas with crucial immune, cardiovascular and neurological second messenger functions, is synthesized by several isoforms of the enzyme nitric oxide synthase (NOS)(1-4). Inducible NOS (iNOS) (5-8) is regulated at the transcriptional level, whereas expression of the endothelial and neural constitutive isoforms (cNOS) is regulated predominantly at the post-transcriptional level (1-4).

Most studies on the expression of NOS have inferred changes in NOS activity from measurements of nitrites (NO_2^-) and nitrates (NO_3^-). Conclusions about which NOS isoform produced the observed increases have depended largely upon indirect evidence including cell type, time course, stimuli, and calcium/calmodulin dependence or independence (1-4). Recent cloning of the cDNA encoding the transcriptionally regulated macrophage iNOS (5-7)

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Abbreviations : $NO^{\cdot}$,nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase; LPS, bacterial lipopolysaccharide; INF-γ, interferon-γ; IL-10, interleukin-10; TGF-β, transforming growth factor-β; PBMC, peripheral blood monocytes; CGD, chronic granulomatous disease.

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has made possible direct measurements of the regulation of its transcription in various cell types.

In murine macrophages, treatment with bacterial LPS or cytokines such as INF-γ increases iNOS mRNA, levels of NO_2^- and NO_3^- (5-7, 9) and enhances killing of microbes and tumor cells (10, 11). Conversely, treatment of murine macrophages with other cytokines such as IL-10 (12-20), IL-4 (15) and TGF-β (21-24) have been reported to inhibit NO· production and killing activity. There have been no similar reports on human macrophages or peripheral blood monocytes.

Our studies have characterized the induction of iNOS steady state mRNA levels following LPS and cytokine treatment, and the effect of IL-10 and TGF-β on these induced levels. We chose to evaluate mRNA levels rather than NOS activity measurements in order to directly link stimulus-dependent responses to a specific NOS isoform. We examined this induction in both murine cell lines, rat primary cell cultures and human peripheral blood monocytes (PBMC).

MATERIALS AND METHODS

Cells and Reagents

RAW264.7 and J774 cells (American Type Tissue Collection) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma), 10% fetal bovine serum (Flow Labs), 0.58 mg/ml glutamine, and antimicrobial agents (ABAM, Sigma). Rat macrophages were isolated by peritoneal lavage with sterile PBS from male pathogen-free Harlan-Sprague-Dawley rats anesthetized with i.p. sodium pentobarbital (Butler). Cells were placed on ice, spun at 600 g for 10 min and resuspended in complete media. Cells were plated at 10^6 per 60mm dish, allowed to adhere for 3 hr at 37°C, 5% CO_2. Plates were then washed to remove nonadherent cells and 4 ml fresh DMEM added.

Molecular Probes, RNA Isolation and Northern Analysis

The iNOS cDNA probes were derived from a murine cDNA clone kindly provided by Dr. James Cunningham. Nco I digestion of the iNOS plasmid yielded a 1890bp fragment used for primer extension labeling. A rat cathepsin B cDNA probe was used as an internal control (kindly provided by Dr. S. J. Chan). Total RNA was isolated by the acid guanidine thiocyanate-phenol-chloroform extraction method (25). Northern analysis was performed as previously described (26). The membranes were washed at high stringency and subjected to autoradiography and densitometry.

Inflammatory Mediators and Protein and mRNA Synthesis Inhibition

Bacterial LPS (Sigma), murine INF-γ (Genzyme) and the human cytokines TNF-α (Genentech), IL-1β (NCI), IL-10 (R&D Systems), and TGF-β (kindly provided by Dr. Greg Schultz) were used to modulate the induction of iNOS mRNA. Inflammatory mediators were added to the media for 1 to 40 hr at final concentrations of: INF-γ (50-100 U/ml), LPS (20 - 500 ng/ml), IL-1β (5 U/ml), TNF-α, (10 ng/ml), IL-10 (100 U/ml), and TGF-β (10 ng/ml). RAW264.7 cells were treated with actinomycin D, at concentrations of 4 nM to 4 μM or cycloheximide, at 20 nM to 20 μM. Confluent monolayers of cells were treated for 4 h with each inhibitor alone or in combination with LPS (50 ng/ml).

DNA Isolation and Southern Analysis

Genomic DNA was isolated and purified from confluent human pulmonary artery endothelial cells. Restriction enzyme-digested DNA (15 μg/lane) was fractionated on a 1%

agarose gel and Southern analysis performed as previously described (26) using a radiolabeled murine iNOS probe. Blots were then washed at 65°C and subjected to autoradiography.

RESULTS

Characterization of iNOS Response to LPS

Fig. 1 shows the results of the Northern analysis of iNOS mRNA when RAW264.7 cells are exposed to increasing concentrations of LPS for 8 h before RNA isolation. The iNOS mRNA levels increase in response to LPS in a dose-dependent fashion. To compare the time-dependent LPS induction of iNOS mRNA in two different murine macrophage cell lines, we exposed RAW264.7 and J774 cells to LPS and isolated RNA at varying times. Fig. 2 illustrates induction of iNOS mRNA at 4 h, with a peak at 12 hr. Similar kinetics were observed in J774 cells (not shown).

To show whether the effect of LPS on iNOS mRNA levels was limited to mouse macrophage cell lines, we isolated unstimulated and unelicited peritoneal macrophages from pathogen free rats and treated them with LPS for 6 h. Fig. 3 shows that primary unelicited rat peritoneal macrophages have detectable basal expression of iNOS mRNA, unlike murine cell lines. The rat cells demonstrated similar strongly enhanced experession by LPS.

Effect of Actinomycin D and Cycloheximide on LPS Induction of iNOS mRNA

LPS induction of iNOS mRNA steady state levels could result from an increase in transcription, a prolongation of iNOS mRNA half-life, or both. To address these

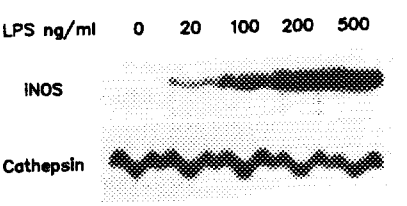


Figure 1. Dose response analysis of induction of iNOS mRNA by LPS. Confluent RAW 264.7 cells were exposed to increasing concentrations of E. coli LPS for 8 h. Northern analysis utilized ^{32}P-labelled murine macrophage iNOS and rat cathepsin B cDNAs. Cathepsin B mRNA signal served as an internal control to document similar RNA loading between lanes.

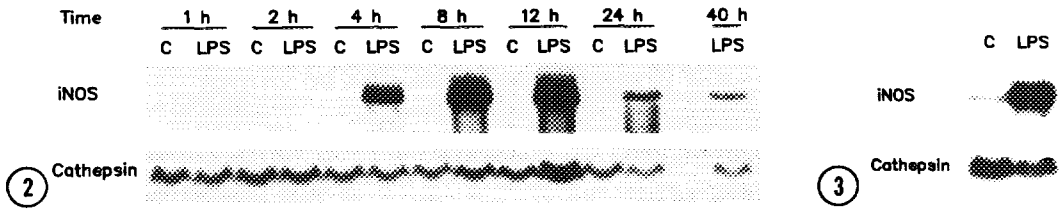


Figure 2. Time course of induction of iNOS mRNA by LPS. Confluent RAW 264.7 cells were treated with medium alone (C) or LPS (200 ng/ml). Total RNA was isolated at the indicated times after addition of LPS and 15 μg was fractionated by size on a 1% agarose/formaldehyde/MOPS buffer gel and electrotransferred to a nylon membrane. Levels of iNOS and cathepsin B mRNA were then determined by Northern analysis. RNA loading was assured by cathepsin B mRNA signal.

Figure 3. Northern analysis of RNA from unelicited resident rat peritoneal macrophages exposed to LPS. Peritoneal macrophages were plated at 10^6 per dish and after 3 h, non-adherent cells were removed by PBS washing. Adherent macrophages were treated with medium alone (C) or LPS (200 ng/ml) for 6 h. Levels of iNOS and cathepsin B mRNA were determined by Northern analysis. RNA loading in each lane was documented by cathepsin B control.

alternatives, RAW264.7 cells were treated for 4 h with LPS alone or co-treated with increasing concentrations of actinomycin D or cycloheximide. Fig. 4A shows the response of iNOS mRNA to LPS and actinomycin D alone and the effects of increasing concentrations of actinomycin D on LPS-induction. The LPS induction was completely inhibited by actinomycin D at 400 nM.

To determine if new protein synthesis plays a role in iNOS induction, cells were exposed for 4 h to LPS alone, increasing concentrations of cycloheximide (20 nM to 20 μM)

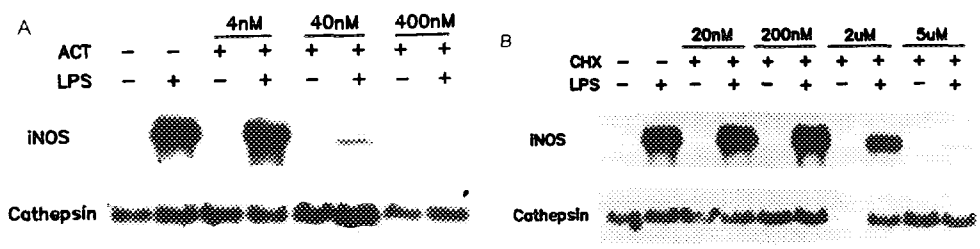
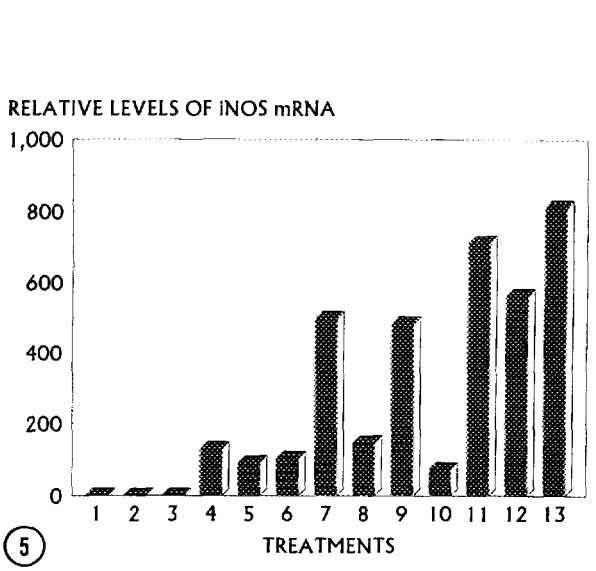


Figure 4. Dose response of actinomycin D and cycloheximide on LPS induction of iNOS mRNA in RAW 264.7 cells. A. Confluent cells were exposed to medium alone, LPS (50 ng/ml), increasing concentrations of actinomycin D alone, or both LPS (50 ng/ml) and increasing concentrations of actinomycin D for 8 h. Total RNA was isolated and evaluated by Northern analysis with ^{32}P-labelled iNOS and cathepsin B cDNA probes. B. Confluent RAW 264.7 cells were exposed to medium alone, LPS (50 ng/ml), increasing concentrations of cycloheximide alone, or both LPS (50 ng/ml) and increasing concentrations of cycloheximide. Total RNA was isolated and evaluated by Northern analysis. Cathepsin B mRNA confirmed RNA loading in each lane. The 2 μM cycloheximide lane is underloaded but does contain detectable RNA on longer exposure.

alone or co-treated with LPS. Fig. 4B illustrates that the inhibition of LPS induced increases in iNOS mRNA is evident at 2 μM cycloheximide and is almost complete at 20 μM.

IL-10 and TGF-β Effects on LPS and INF-γ Induction of iNOS mRNA Levels (Fig. 5)

To further characterize the induction of iNOS mRNA, we next assessed the ability of TGF-β and/or IL-10 to modulate the LPS and INF-γ induced iNOS mRNA expression. Exposure to either IL-10 or TGF-β alone results in no induction after 8 h (Bars 2 and 3). However, IL-10 co-treatment synergistically augmented INF-γ induction of iNOS mRNA



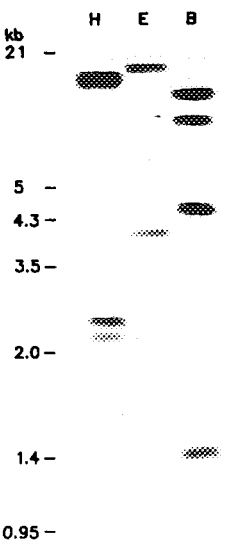


Figure 5. Densitometric analysis of IL-10 and TGF-β modulation of LPS and INF-γ induction of iNOS mRNA in RAW 264.7 Cells. Confluent RAW 264.7 cells were treated for 8 h with medium alone (Bar 1); IL-10 alone (100 U/ml) (Bar 2); TGF-β alone (10 ng/ml) (Bar 3); LPS alone (50 ng/ml) (Bar 4); LPS + IL-10 (Bar 5); LPS + TGF-β (Bar 6); LPS + INF-γ (50 U/ml) (Bar 7); INF-γ alone (Bar 8); INF-γ + IL-10 (Bar 9); INF-γ + TGF-β (Bar 10); LPS + INF-γ + IL-10 (Bar 11); LPS + INF-γ + TGF-β (Bar 12); or LPS + INF-γ + IL-10 + TGF-β (Bar 13). The levels of iNOS and cathepsin B mRNA were determined by Northern analysis as described under "Materials and Methods." The relative level of nitric oxide synthase mRNA expression was determined in each lane after normalization to the respective cathepsin B signal to account for variability in the amounts of RNA that had been loaded. Results of scanning densitometric analysis of the Northern blots are presented. The undetectable iNOS signal in control, IL-10 and TGF-β treated cells were assigned a value of 1.0 (rather than zero) to allow calculation of a relative level of mRNA expression for all other treatments. Comparable results were obtained in 2 independent experiments with duplicate plates of cells.

Figure 6. Southern analysis of human genomic DNA with inducible murine macrophage iNOS cDNA. For each lane 15 μg of human genomic DNA was digested with the restriction enzymes Hind III (H), EcoRI (E), or BamHI (B). After being size fractionated on a 1% agarose gel, electrotransferred and UV cross-linked to a nylon membrane, hybridization was done at 60°C overnight utilizing a radiolabelled 1.89 kb Not I fragment of the murine iNOS cDNA. Blots were washed under high stringency conditions of low salt and 65°C.

(Bar 9) while slightly decreasing the induction of iNOS mRNA by LPS (Bar 5). IL-10 treatment combined with LPS and INF-γ further increased iNOS mRNA expression (Bar 11) with similar results when cells were treated simultaneously with all four mediators (Bar 13). In contrast, TGF-β had minor inhibitory effects on LPS (Bar 6), INF-γ (Bar 10) or their combination (Bar 12),

To determine whether iNOS mRNA could be induced in other rat cells or in human cells, we did Northern analyses looking for iNOS mRNA expression in rat and human pulmonary artery endothelial cells, a rat lung epithelial cell line (L2 cells), and human PBMC from both a healthy volunteer and a patient with chronic granulomatous disease (CGD). In none of these cells was iNOS mRNA basal expression detectable nor was induction observed after 8 h exposure to any of the single agents (LPS, TNF-α, IL-1β, and/or INF-γ) or a mixture of these four cytokines.

Southern Analyses of Human Genomic DNA with Murine iNOS

In order to determine whether the results obtained with our murine cDNA probe in human cells were due to the inability of this probe to cross-hybridize to human iNOS mRNA, we performed Southern blot analysis on human genomic DNA using a radiolabeled probe (1.89kb Not I fragment) from the murine iNOS cDNA. Fig. 6 shows human genomic DNA digested with Hind III, Eco RI or Bam HI, probed with murine iNOS and washed under high stringency conditions. The discrete bands observed indicate the presence of a human iNOS gene in the human genome that cross-hybridizes with the murine cDNA probe.

DISCUSSION

We have used a mouse macrophage-derived iNOS cDNA clone to analyze regulated expression in several cell types including two murine macrophage cell lines, as well as in primary cultures of rat peritoneal macrophages. Northern analyses showed that low concentrations of bacterial LPS dramatically induced iNOS mRNA levels. Cell lines had undetectable basal levels of iNOS mRNA while the unelicited rat peritoneal macrophages demonstrated detectable basal mRNA expression. In two mouse macrophage cell lines the time course of LPS-mediated iNOS induction was similar. It occurred rapidly, detected on long exposures within 2 hours, peaked at about 12 hours, and was declining by 24 hours. Inhibition of iNOS mRNA induction by low concentrations of actinomycin D and cycloheximide is consistent with the requirement for *de novo* transcription and protein synthesis.

Both TGF-β (21-24) and IL-10 (12-20) have been shown to inhibit multiple macrophage functions. Specifically, IL-10 has been reported to decrease INF-γ induced NO·

biosynthesis (14-16), and INF-γ induced parasite killing (12, 15) by mouse peritoneal macrophages elicited by intraperitoneal thioglycolate. In contrast, we observed that IL-10 co-treatment with INF-γ dramatically augmented the iNOS mRNA induction in a murine macrophage cell line. IL-10 also further increased the iNOS mRNA level induced by the synergistic effect of LPS plus INF-γ. These data contradict several previous reports where NOS activity was measured (12,14-16). The previous studies (12, 14, 15) evaluated macrophages from an inflammatory exudate that had been elicited by intraperitoneal periodate or thioglycolate and stimulated by INF-γ, whereas we studied the effects of IL-10 alone or in combination with LPS and INF-γ on a murine cell line. More recently, Corradin et al. reported IL-10 enhanced NO_2^- release from murine bone marrow-derived macrophages stimulated in vitro with INF-γ plus TNF-α, but not INF-γ alone (27). These authors also report that IL-10 inhibited NO_2^- release from these cells treated with LPS in combination with INF-γ. Thus, in the Corradin study as well as the present study, IL-10 has been shown to increase iNOS expression.

Again based on NO_2^- assays, TGF-β has been reported to inhibit $NO^\cdot$ biosynthesis in activated macrophages (15, 21, 22), as well as in cytokine-induced human smooth muscle cells (23) and in rat renal mesangial cells (24). Therefore, we also examined the effects of TGF-β alone on iNOS mRNA expression and on LPS and INF-γ induced expression of iNOS mRNA levels. In contrast to IL-10 effects, and in agreement with published activity data (15, 21-24), co-treatment of cells with TGF-β and 1)LPS, 2)INF-γ or 3)LPS plus INF-γ, slightly decreased iNOS mRNA induced levels.

There have been 2 reports of $NO^\cdot$ production and enhanced microbial killing in infected human macrophages derived from peripheral blood monocytes (28, 29) as well as from human neutrophils and peripheral blood monocytes obtained from both healthy volunteers and patients with CGD (30). However, other efforts to induce $NO^\cdot$ biosynthesis in human mononuclear phagocytes have been largely unsuccessful (1-4, 31). In agreement with these authors, we observed no induction of iNOS mRNA in peripheral blood monocytes isolated from a healthy volunteer or a patient with CGD stimulated with the mixture of inflammatory mediators used successfully by Geller et al. (8) to induce iNOS mRNA in human hepatocytes. Our data, therefore, extend to the mRNA level previous reports that have found no inducible NOS activity in human macrophages.

To address the possibility that the lack of induction in human cells was due to an inability of the murine iNOS radiolabeled probe to cross-hybridize with human iNOS mRNA, we performed Southern analysis on restricted human DNA using our radiolabelled murine cDNA probe. These results demonstrate that the murine probe can cross-hybridize with discrete restriction fragments under high stringency hybridization conditions and therefore,

presumably detect an analogous human iNOS gene. Recently, a cDNA encoding a form of human iNOS has been cloned from a human hepatocyte library. This human hep-iNOS cDNA has 80% homology at the amino acid level with the murine iNOS cloned from RAW264.7 cells. We were unable to compare our Southern data with that published by Geller et al. (8) due to the absence of size markers in their Southern analysis. Therefore, existing Southern data leave open the possibility that a human iNOS isoform exists with Ca^{2+} and calmodulin independent activity analogous to the murine iNOS and distinct from the human hep-iNOS.

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