

A Labile Transcriptional Repressor Modulates Endotoxin Tolerance

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Summary

Tolerance to bacterial lipopolysaccharide (LPS, endotoxin) is an adaptive cellular process whereby exposure to endotoxin induces a subsequent hyporesponsive state characterized by decreased levels of LPS-induced cytokine mRNA and protein. We demonstrate, in a human promonocytic cell line, THP-1, that endotoxin tolerance is manifested by decreased LPS-induced interleukin 1 β (IL-1 β) transcription. Inhibition of protein synthesis reverses the tolerant phenotype by inducing transcription of IL-1 β in the absence of a second stimulus. These results indicate that a labile protein contributes to the endotoxin-tolerant phenotype, and that this factor acts in a dominant repressive manner to inhibit the activity of existing transcription factors. We provide further data that cellular expression of κ B- α correlates with downregulated IL-1 β gene expression during endotoxin tolerance, implicating κ B- α as a potential candidate for the labile repressor identified herein.

Cells exposed to endotoxin become refractory to further challenge with this agent. This phenomenon of endotoxin tolerance was first demonstrated in animal models of endotoxemia (1), and can be induced in a variety of primary monocyte/macrophage cells and cell lines (2–6). In humans, this condition is observed in the PBMC isolated from subjects given a single intravenous dose of *Escherichia coli* endotoxin (7), and in the neutrophils and monocytes isolated from patients in septic shock (8, 9). The endotoxin-tolerant state is characterized by decreased cytokine production after a challenge dose of LPS, as compared with those levels induced by an initial dose. Adaptation to stimulation by bacterial endotoxin perhaps evolved as a mechanism to curb the infected hosts' continuous response to the invading organism, and to reduce the potentially lethal autotoxic effects precipitated by overproduction of inflammatory mediators such as IL-1 and TNF- α .

The molecular events that regulate endotoxin tolerance are unclear but result in repressed expression of cytokine genes (2–5), and altered cytokine processing and secretion (6). We previously reported that neutrophils isolated from patients in septic shock are consistently tolerant to LPS-induced IL-1 β mRNA and protein synthesis (8). To further study the molecular basis of endotoxin tolerance in human leukocytes, we developed an in vitro model of tolerance in the human promonocytic cell line, THP-1. Here we report that, as with the human neutrophil, endotoxin-tolerant THP-1 cells exhibit reduced levels of LPS-induced steady state IL-1 β mRNA. Downregulation of gene expression is at the level of IL-1 β

gene transcription, and appears to be mediated by the synthesis of a labile dominant transcriptional repressor.

Materials and Methods

Cell Culture and Induction of Endotoxin Tolerance. THP-1 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained at 37°C, 5% CO₂ in RPMI-1640 media supplemented with 10 U/ml penicillin G, 10 μ g/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (FBS)¹ (Hyclone Laboratories, Logan, UT). Cells were cultured to a density of 5.0×10^5 cells/ml, and passaged with a 1:3 ratio split. Unless otherwise indicated in the figure legend, endotoxin tolerance was induced by suspending the cells in RPMI-1640 supplemented with 10 U/ml penicillin G, 10 μ g/ml streptomycin, 2 mM L-glutamine, and 2% FBS at a density of 10^6 cells/ml and treating them with 1 μ g/ml LPS (*E. coli* 0111:B4; Sigma Chemical Co., St. Louis, MO) for 16 h. The cells were then pelleted, washed once in PBS, resuspended in media at 10^6 cells/ml, and stimulated as described in the figure legends. For all assays, control cells were treated similarly, but were not given the primary LPS dose.

Monocyte Isolation and Induction of Endotoxin Tolerance. Human PBMC were isolated from heparinized venous blood obtained from normal adult volunteers by plasma gel (Cellular Products, Inc., Buffalo, NY) sedimentation and Isolymp (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY) centrifugation. The cells

¹ Abbreviations used in this paper: CHX, cycloheximide; FBS, fetal bovine serum; GAPDH, glyceraldehyde phosphate dehydrogenase; NF- κ B, nuclear factor κ B.

were suspended in RPMI-1640 media supplemented with 10 U/ml penicillin G, 10 $\mu\text{g/ml}$ streptomycin, and 2 mM L-glutamine at a density of 10^6 cells/ml and enriched for monocytes by a 2-h adherence step. Fresh media, supplemented with 10% FBS, was added, and the cells were incubated for 24 h in the presence or absence of 1 $\mu\text{g/ml}$ *E. coli* LPS. Media was again removed, the cells washed once with PBS, and fresh media added. The cells were then treated as described in the figure legend.

RNA Extraction and Northern Analysis. Total RNA was isolated using the RNazol B method (Tel-Test, Inc., Friendswood, TX). Routinely, 5.0×10^6 THP-1 cells/condition and 1.0×10^7 monocytes/condition were used. Total RNA (10 $\mu\text{g/lane}$) was fractionated on a 1% agarose, 6.6% formaldehyde gel in 1X MOPS buffer (0.02 M 3-(*N*-morpholino) propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA), and capillary blotted onto nylon membrane filters (Gene Screen Plus; DuPont-NEN, Boston, MA). UV cross-linking was performed using a Stratilinker UV cross-linker (Stratagene, La Jolla, CA).

The plasmid containing a human IL-1 β cDNA insert, pHuIL1/pGEM1 was linearized with EcoRI and labeled using a NEN Nick Translation System kit. Filters were prehybridized in 5–10 ml QuikHyb hybridization buffer (Stratagene) for 15 min at 68°C, in an Autoblot Micro Hybridization Oven (Bellco Glass Inc., Vineland, NJ). 10^6 cpm probe/ml QuikHyb and 1 mg herring sperm DNA were added and hybridization was carried out for 1 h at 68°C. The filters were washed twice for 15 min at room temperature in 2X SSC/0.1% SDS, and once for 30 min at 60°C in 0.1X SSC/0.1% SDS. The filters were exposed for 6–24 h to Kodak XAR film with two intensifying screens at -70°C .

The filters were stripped by boiling for 30 min in 0.1X SSC/0.1% SDS and reprobed, under the same conditions, with EcoRI linearized, nick-translated plasmid containing cDNA of human glyceraldehyde phosphate dehydrogenase (GAPDH) (pHcGAP, ATCC).

IL-1 β mRNA Decay Analysis. Control and endotoxin-tolerant cells were treated with 1 $\mu\text{g/ml}$ LPS, 10 μM cycloheximide (CHX), and 10 μM CHX plus 1 $\mu\text{g/ml}$ LPS for 2.5 h. Actinomycin D (10 μM) was added to inhibit further transcription. Beginning 0.5 h after addition of actinomycin D, aliquots of cells were removed at 1-h intervals over a 10-h time course. Total RNA was isolated, denatured in 50% formaldehyde/10X SSC, and serial dilutions were dot-blotted onto Gene Screen Plus membranes. The filters were probed as described for Northern analysis, and exposed to film for various lengths of time to insure linearity of signal. The signals at each time point were quantitated by densitometry and expressed as a percentage of the signal at the first time point.

Nuclear Run-on Analysis. Nuclei isolation and transcription assays were performed as described (10). Routinely, 5.0×10^7 cells/condition were used. Equal counts of nascent transcripts were hybridized for 48 h to Nytran filters containing 10 $\mu\text{g/slot}$ of linearized, denatured pHuIL1/pGEM1 and pGEM-7 (Promega Corp., Madison, WI). The filters were washed twice for 15 min at room temperature in 2X SSC/0.1% SDS, and once for 30 min at 42°C in 0.1X SSC/0.1% SDS. The filters were exposed for 24 h to Kodak XAR film with two intensifying screens at -70°C . Radioactivity was quantitated by a Radioanalytic Imaging System (AMBIS Inc., San Diego, CA) and specific hybridization of IL-1 β mRNA to pHuIL-1 is expressed relative to nonspecific binding to pGEM-7 (it was not possible to normalize to transcription of housekeeping genes as that of GAPDH was undetectable, and that of β -actin was found to vary with the experimental conditions).

Western Analysis. Control and endotoxin-tolerant cells were treated with 1 $\mu\text{g/ml}$ LPS, 10 μM CHX, and 1 $\mu\text{g/ml}$ LPS plus 10 μM CHX for the times indicated in the figure legend. The cells

were pelleted and lysed in NP-40 lysis buffer (100 mM Tris, 100 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM Na_3VO_4 , 50 mM NaF, 0.1 mM Quercetin, 1 mM PMSF, 1 $\mu\text{g/ml}$ aprotinin, and 1 $\mu\text{g/ml}$ leupeptin). Protein concentrations were determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). 100 μg protein/lane was separated by SDS-PAGE (10% acrylamide) according to the Laemmli method, along with low-range molecular weight markers (Bio-Rad Laboratories, Hercules, CA) and transferred to Hybond-enhanced chemiluminescence (ECL) nitrocellulose (Amersham Corp., Arlington Heights, IL) at 100 mA for 16 h, at room temperature. The nitrocellulose membrane was blocked for 1 h with 5% nonfat dry milk, in TBS-Tween (20 mM Tris base, 137 mM NaCl, and 0.1% Tween-20). The membrane was washed four times in TBS-Tween and incubated for 1 h with rabbit anti-human I κ B- α antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), diluted in 5% BSA in TBS-Tween. The membrane was again washed four times in TBS-Tween and incubated for 1 h with goat anti-rabbit IgG, conjugated to horseradish peroxidase (Organon Teknika Corp., Durham, NC). The membrane was washed four times in TBS-Tween and I κ B- α protein was visualized using Renaissance Western Blot Chemiluminescence Reagent (DuPont-NEN).

Results and Discussion

Endotoxin tolerance was induced by stimulating THP-1 cells with two consecutive doses of LPS and the conditions optimized by varying the concentration and the duration of the primary dose. Northern analysis indicated that endotoxin tolerance was induced by all primary dose concentrations examined, and that the degree of tolerance became more pronounced when the primary dose was increased (Fig. 1 A). To assess the effects of altering the duration of the primary dose, THP-1 cells were incubated with the primary dose of LPS for increasing lengths of time before stimulation with the challenge dose. Fig. 1 B illustrates that cells exposed to the primary dose for 10–19 h become progressively less responsive to the challenge dose. Not shown are data indicating that endotoxin tolerance is observed as early as 6 h and as late as 44 h after the primary stimulus. To compare the kinetics of LPS-induced IL-1 β mRNA accumulation in endotoxin-tolerant cells relative to control cells, we treated control and endotoxin-tolerant THP-1 cells with LPS and measured IL-1 β mRNA levels over a 5-h time course (Fig. 1 C). We found that endotoxin-tolerant cells respond more rapidly to LPS than do control cells, but that the overall response is diminished.

In several models of endotoxin tolerance, downregulated cytokine production is specific for LPS as the challenge stimulus (4, 8, 11), whereas in other models, the endotoxin-tolerant cells are also refractory to challenge by other agents (3, 7). To determine whether endotoxin-tolerant THP-1 cells could respond normally to other stimuli, control and endotoxin-tolerant cells were treated with increasing concentrations of PMA (Fig. 2). We observed that tolerance to stimulation by endotoxin is coincident with an enhanced response to PMA. This observation suggests that the primary LPS dose induces enduring changes in the intracellular pathways leading to IL-1 β gene expression. Thus, in our model, the LPS-induced anergy may be due to synthesis or activation of a negative

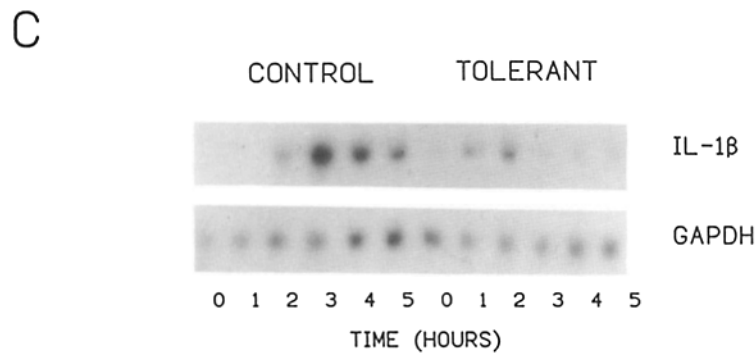
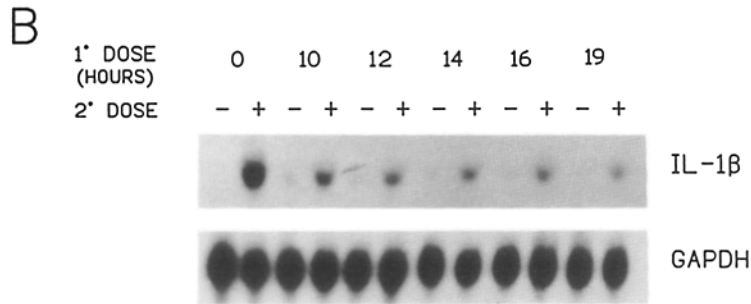
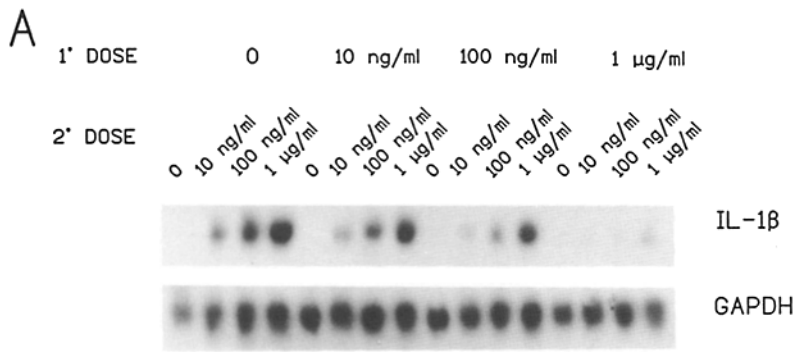


Figure 1. THP-1 cells exposed to endotoxin become refractory to further stimulation with this agent. (A) THP-1 cells were treated with 0, 10 ng/ml, 100 ng/ml, or 1 μ g/ml LPS and incubated for 16 h. The cells were washed once in PBS and challenged with 0, 10 ng/ml, 100 ng/ml, and 1 μ g/ml LPS for 3 h. (B) THP-1 cells were stimulated with 1 μ g/ml LPS for 10, 12, 14, 16, and 19 h before being washed and restimulated with 1 μ g/ml LPS for 3 h. (C) Control and endotoxin-tolerant THP-1 cells were treated with 1 μ g/ml LPS and RNA was isolated at 1-h time points over a 5-h time course. Expression of IL-1 β and GAPDH mRNA was determined by Northern analysis.

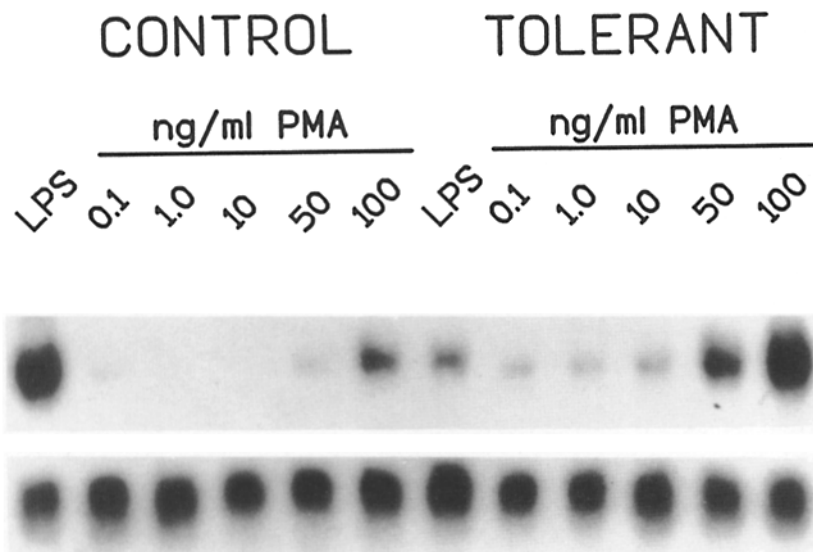


Figure 2. Endotoxin-tolerant THP-1 cells display an enhanced response to PMA. Control and endotoxin-tolerant cells were treated with 1 μ g/ml LPS, and 0.1, 1.0, 10, 50, and 100 ng/ml PMA for 3 h. Expression of IL-1 β and GAPDH mRNA was determined by Northern analysis.

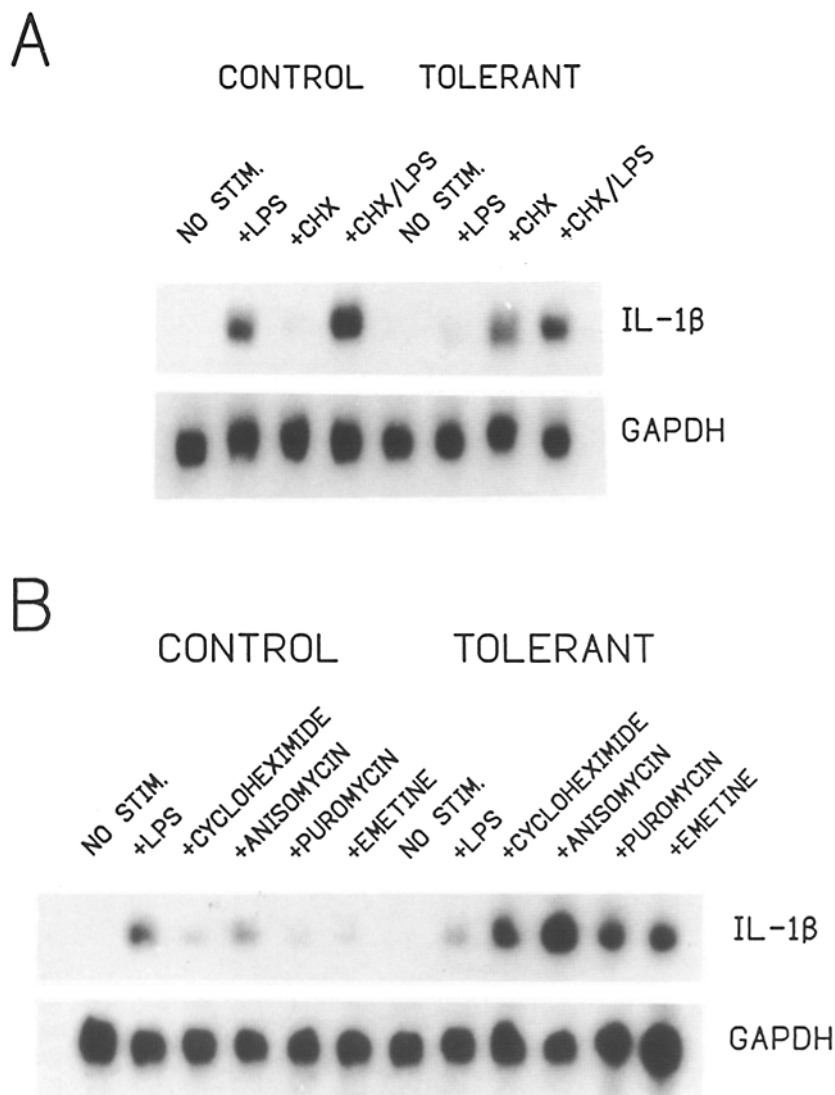


Figure 3. Inhibition of protein synthesis induces IL-1 β mRNA accumulation in endotoxin-tolerant THP-1 cells. (A) Control and endotoxin-tolerant cells were left unstimulated, or treated with 1 μ g/ml LPS, 10 μ M CHX, and 1 μ g/ml LPS plus 10 μ M CHX for 3 h. (B) Control and endotoxin-tolerant cells were left unstimulated, or treated with 1 μ g/ml LPS, 10 μ M CHX, 100 ng/ml anisomycin, 10 μ g/ml puromycin, and 10 μ M emetine for 3 h. Expression of IL-1 β and GAPDH mRNA was determined by Northern analysis.

factor(s) which selectively represses further LPS-induced signaling, whereas the LPS-induced priming effects are due to synthesis or activation of a positive factor(s) in that portion of the pathway shared by LPS and PMA. In support of this hypothesis, a similar phenomenon has been reported by Zhang and Morrison (12), who observed that pretreating mouse peritoneal macrophages with LPS differentially modulated their TNF- α and nitric oxide production in response to challenge with other stimuli.

Regulation of IL-1 β gene expression occurs transcriptionally and posttranscriptionally (13). As labile proteins have been implicated in the regulation of both of these processes (14, 15), we examined whether ongoing protein synthesis was required to maintain the endotoxin-tolerant state. Whereas we had anticipated that the endotoxin-tolerant cells might regain their LPS responsiveness in the absence of de novo protein synthesis, we observed that treating these cells with CHX alone results in a significant accumulation of IL-1 β mRNA, while having minimal effects on the control cells

(Fig. 3 A). To establish that the induction of IL-1 β gene expression by CHX results from inhibition of protein synthesis, and is not due to the activation, by CHX, of alternate signal transduction pathways, we treated control and endotoxin-tolerant cells with a panel of agents known to inhibit translation by different mechanisms (Fig. 3 B). We found that control cells respond only slightly to stimulation with CHX, anisomycin, puromycin, and emetine, whereas the endotoxin-tolerant cells increase IL-1 β mRNA in response to all four protein synthesis inhibitors. This effect is not observed at concentrations of these agents that are insufficient to inhibit protein synthesis (data not shown), but which may activate alternate signal transduction pathways in the cell (16). These data indicate that inhibition of de novo protein synthesis overcomes or reverses downregulation of IL-1 β gene expression in endotoxin-tolerant THP-1 cells, and that the continuous synthesis of a labile protein is required for repression of LPS-induced IL-1 β gene expression during endotoxin tolerance.

The reversal of downregulated IL-1 β mRNA synthesis by

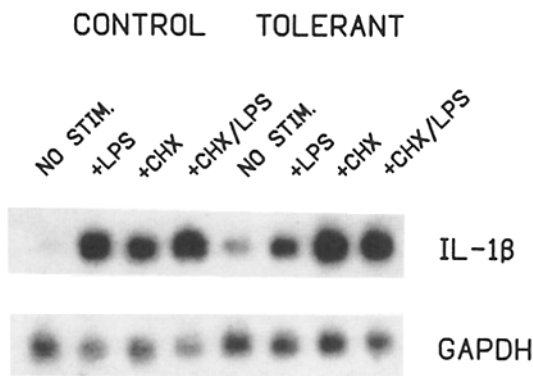


Figure 4. Inhibition of protein synthesis induces IL-1 β mRNA accumulation in endotoxin-tolerant human peripheral blood monocytes. Control and endotoxin-tolerant cells were left unstimulated, or treated with 1 μ g/ml LPS, 10 μ M CHX, and 1 μ g/ml LPS plus 10 μ M CHX for 3 h. Expression of IL-1 β and GAPDH mRNA was determined by Northern analysis.

inhibition of protein synthesis is not unique to the THP-1 cell line, but also extends to human peripheral blood monocytes rendered endotoxin-tolerant *in vitro*. Monocytes that have been preincubated with LPS for 24 h have a decreased

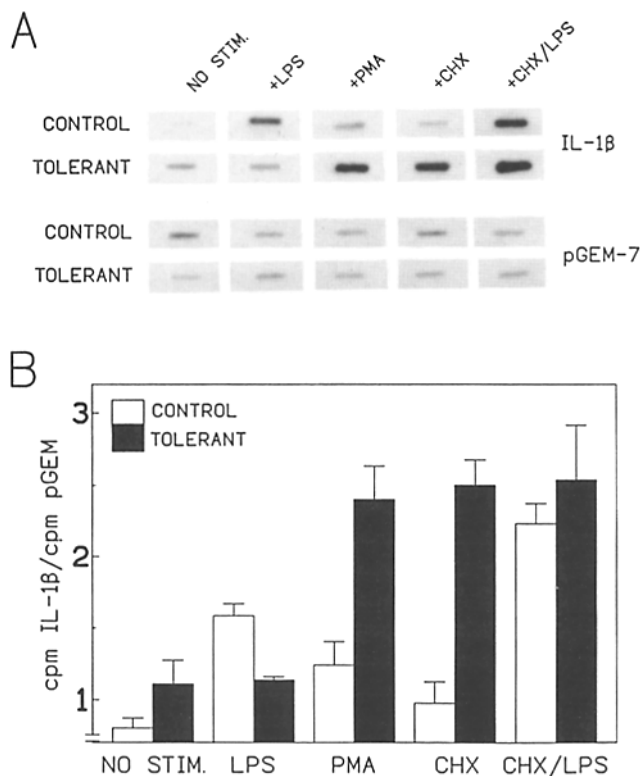


Figure 5. Inhibition of protein synthesis induces IL-1 β transcription in endotoxin-tolerant THP-1 cells. (A) Nuclear run-on analysis was performed on nuclei isolated from control and endotoxin-tolerant cells that were unstimulated, or treated for 2 h with 1 μ g/ml LPS, 100 ng/ml PMA, 10 μ M CHX, and 1 μ g/ml LPS plus 10 μ M CHX. (B) Shown is the average \pm SEM of three separate experiments. The differences observed between control and endotoxin-tolerant cells are statistically significant ($p < 0.05$ by nonpaired *t* test) in all conditions except +CHX/LPS.

IL-1 β mRNA response to a second dose of LPS, but display an enhanced response to treatment with CHX alone, or CHX plus LPS (Fig. 4). We did note that nonendotoxin-tolerant monocytes treated with CHX synthesize greater levels of IL-1 β mRNA than do CHX-treated control THP-1 cells, perhaps as a result of their more advanced state of differentiation.

IL-1 β belongs to a class of rapid response genes containing single or multiple repeats of the consensus sequence ATTTA within their 3'-untranslated region. Since this AU-rich element (ARE) is involved in regulation of transcript stability (17), we examined the contribution of altered IL-1 β mRNA stability to endotoxin tolerance. IL-1 β mRNA decay analysis revealed that the half-life of LPS-induced IL-1 β mRNA is \sim 2.5 h in both control and endotoxin-tolerant cells, indicating that the low levels of LPS-induced IL-1 β transcripts in endotoxin-tolerant cells are not due to enhanced transcript decay. Treating the cells with CHX, and CHX plus LPS, was found to increase the half-life of the message, but did so to equivalent degrees in both control and endotoxin-tolerant cells (data not shown). Whereas this stabilization may be due to elimination of a labile regulatory protein(s), it has been demonstrated that mRNA containing an ARE must be translated to be rapidly degraded (18). In any event, we observed no significant differences in the regulation of IL-1 β transcript stability in control versus endotoxin-tolerant THP-1 cells.

The mRNA decay analysis indicates that the disparate levels of steady state IL-1 β mRNA observed in control and endotoxin-tolerant cells must be regulated predominantly at the level of transcript synthesis. To assess differences in IL-1 β transcription, nuclear run-on analysis was performed on control and endotoxin-tolerant THP-1 cells treated with LPS, PMA, CHX, and LPS plus CHX (Fig. 5). This analysis revealed that LPS induces greater levels of IL-1 β transcription in control cells than in endotoxin-tolerant cells, confirming that the decreased levels of LPS-induced steady state IL-1 β mRNA in these cells is a consequence of decreased transcription. Conversely, PMA induces significantly greater levels of IL-1 β transcription in endotoxin-tolerant cells than in control cells, indicating that the primed response observed previously (Fig. 2) was at least partially due to enhanced transcription. Whereas CHX induces negligible IL-1 β transcription in the control cells, treating endotoxin-tolerant cells with this agent alone, or in concert with LPS, reverses the transcriptional block. This observation supports our concept wherein both positive and negative regulatory factors exist in endotoxin-tolerant THP-1 cells. The primary LPS dose activates the transcription factors involved in upregulation of IL-1 β gene expression, as well as induces the synthesis of a transcriptional repressor, whose activity then produces the endotoxin-tolerant phenotype. Treating the tolerant cells with CHX eliminates this dominant repressor activity by inhibiting synthesis of either the factor itself or, possibly, of another protein that serves to maintain its activity as a repressor (e.g., a kinase or phosphatase). This allows the existing positive factors to reinitiate transcription, even in the absence of a second stimulus.

The transcription factors that regulate LPS-induced IL-1 β gene expression have not yet been fully elucidated, although

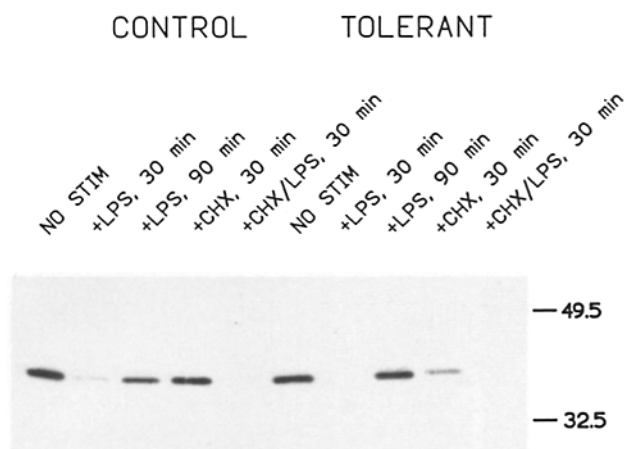


Figure 6. Inhibition of protein synthesis results in a decrease in steady state IκB-α in endotoxin-tolerant THP-1 cells. Western analysis was performed on cell lysates from control and endotoxin-tolerant THP-1 cells that were untreated, or stimulated with 1 μg/ml LPS for 30 and 90 min, or with 10 μM CHX and 10 μM CHX plus 1 μg/ml LPS for 30 min.

recent evidence suggests that nuclear factor κB (NF-κB), NF-IL6 (CCAAT/enhancer binding protein [C-EBPβ]), cAMP response element binding protein (CREB), and other factors may be key participants in the signaling pathway (19–21). In unstimulated cells, the transcription factor NF-κB, typically a heterodimer composed of a 50- and 65-kD protein subunit, resides in the cytoplasm associated with an inhibitory protein subunit, IκB (22). After stimulation with a variety of agents, including LPS, IκB is phosphorylated (23), dissociates from NF-κB, and is rapidly degraded, allowing the NF-κB complex to translocate to the nucleus (24). Among the many genes induced by LPS is that of IκB itself (25), thus providing an efficient negative feedback mechanism for LPS-induced/NF-κB-mediated gene expression. In view of its inducibility by LPS and its labile nature when dissociated from NF-κB (26), IκB is a potential candidate for the dominant negative activity identified in this study. To examine this possibility, we assayed expression of IκB-α (MAD-3) by Western analysis and found that the presence of IκB-α protein does indeed correlate inversely with IL-1β transcription in our system (Fig. 6). Unstimulated control and endotoxin-tolerant THP-1 cells contain equivalent steady state levels of IκB-α, and treating both populations of cells with LPS induces IκB-α decay. However, IκB-α is more rapidly regenerated in the endotoxin-tolerant cells, returning to normal levels

by 90 min after stimulation, while remaining low in the control cells. As IκBα can dissociate NF-κB from its cognate DNA (27), this observation suggests that there may be only a transient pulse of NF-κB activity in the endotoxin-tolerant cells, compared with a more sustained NF-κB-mediated signal in the control cells, thus accounting for the disparate levels of LPS-induced IL-1β mRNA observed in these cells.

Due to the stable nature of IκB-α when associated with NF-κB, inhibition of protein synthesis has little effect on IκB-α levels in unstimulated control cells. However, we found that IκB-α disappears in endotoxin-tolerant cells treated with CHX alone, which suggests that during endotoxin tolerance there is a continuous dissociation of IκB-α from the NF-κB subunits and a constant rate of de novo synthesis to replace the degraded protein. These observations indicate that the pathway leading to IκB-α phosphorylation and dissociation is intact in the endotoxin-tolerant THP-1 cells, and that the endotoxin-tolerant phenotype may be partially due to enhanced rate of synthesis of IκB-α in these cells.

To date, no labile regulatory proteins have been identified for the other transcription factors that regulate IL-1β gene expression. However, it is becoming increasingly apparent that transcription factors from distinct families demonstrate physical and functional interactions that result in subunit and DNA binding site-specific enhancement or repression of transcription (28–30), thus providing yet another level of complexity to the regulation of eukaryotic gene expression.

The ability of cells to adapt to continuous or repetitive stimulation is a general principle of cell biology (31). Adaptation may directly involve control of receptor function, or altered intracellular processes (32). Transcriptional downregulation has been implicated in various adaptive processes, including IFN-α-induced desensitization of fibroblasts (33), antigen-dependent energy to T cell IL-2 expression (34), and IL-4-induced downregulation of IL-2 expression (35). Although labile regulatory factors were not investigated in the latter two systems, the involvement of a protein synthesis-dependent dominant repressor was reported in the former case. Our finding regarding regulation of endotoxin tolerance extends the emerging importance of the role of dominant transcriptional repressors from the control of neoplasia and cell development to that of regulation of inflammation. This encourages the speculation that synthesis of proteins that repress transcription may prove to be a generalized mechanism by which many cell types regulate immunologic and inflammatory anergic states.

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