mRNA and Protein Stability Regulate the Differential Expression of Pro- and Anti-inflammatory Genes in Endotoxin-tolerant THP-1 Cells*

(Received for publication, November 27, 1999, and in revised form, January 18, 2000)

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The products of proinflammatory genes such as interleukin-1β (IL-1β) and cyclooxygenase-2 (COX-2) initiate many of the events associated with sepsis. Transcription of these genes is subsequently down-regulated, whereas expression of anti-inflammatory genes such as secretory interleukin-1 receptor antagonist (sIL-1 RA) is maintained. Differential expression is associated with endotoxin tolerance, a cellular phenomenon common to sepsis and characterized by reduced proinflammatory gene expression after repeated exposure to lipopolysaccharide. As a model for endotoxin tolerance, we examined the expression of COX-2 and sIL-1 RA in a human promonocyte cell line, THP-1. We observed a 5-fold decrease in COX-2 protein in endotoxin-tolerant cells relative to control cells. In contrast, sIL-1 RA protein increased 5-fold in control and tolerant cells and remained elevated. Decreased COX-2 production is due to repressed transcription and not enhanced mRNA degradation. In addition, COX-2 protein is turned over rapidly. Transcription of sIL-1 RA is also repressed during tolerance. However, sIL-1 RA mRNA is degraded more slowly than COX-2 mRNA, allowing continued synthesis of sIL-1 RA protein that is very stable. These results indicate that differential expression during endotoxin tolerance occurs by transcriptional repression of COX-2 and by protein and mRNA stabilization of sIL-1 RA.

Severe sepsis with septic shock is the major cause of death in critical care units in the United States, killing over 100,000 people each year (1). It is a disease characterized by acute disseminated intravascular inflammation with multiple organ failure and hypotension due to infection by various bacteria, viruses, and fungi. Gram-negative bacterial infections are the most common causative agent of septic shock (2). Bacterial endotoxin lipopolysaccharide (LPS), 1 a component of Gram-negative bacterial cell walls, is a potent inducer of monocyte, macrophage and polymorphonuclear leukocyte inflammatory gene expression during infection and a common inducer of septic shock (3).

COX-2 is a 70-kDa dimeric indolizine enzyme that has a vital role in modulating inflammation through production of prostaglandin H2, a precursor of several potent eicosanoid mediators (4, 5). COX-2 expression is clearly associated with proinflammatory activity in inflammatory diseases and the COX-2 gene responds like other proinflammatory genes during sepsis (6, 7). The induction of proinflammatory genes such as IL-1β, tumor necrosis factor-α (TNF-α), and COX-2 by bacterial products such as LPS is essential and necessary to initiate the septic response (2). However, these genes are rapidly and continuously down-regulated soon after the sepsis syndrome is initiated (2, 8, 9). In contrast to COX-2, regulatory factors, such as secretory interleukin-1 receptor antagonist (sIL-1 RA) (10), serve to neutralize the activity and overproduction of inflammatory defenses (11, 12). Anti-inflammatory genes such as interleukin-1 type II receptor (IL-1 R2), IL-10, and sIL-1 RA are also induced early in sepsis. However, the products encoded by anti-inflammatory genes are persistently elevated during the course of sepsis (13).

In 1947, Bessin first documented that cells exposed to LPS become refractory to further challenge with this stimulus, a process termed “endotoxin tolerance” (14). This adaptive response to LPS is a cellular phenomenon that is associated with reduced levels of inflammatory mediators after a second exposure to LPS when compared with the levels induced by an initial exposure. Adaptation to stimulation by LPS is thought to have evolved as a mechanism to down-regulate the continuous and often injurious inflammatory response the immune system sustains during severe sepsis, presumably to reduce the potentially lethal autotoxic effects brought about by overproduction of inflammatory mediators such as IL-1β, TNF-α, and COX-2 (1). This differential expression of pro- and anti-inflammatory genes leads to a state of immunosuppression. Although adaptation to LPS is protective against the potentially autotoxic effects of LPS when it exists prior to initiation, its presence during sepsis can compromise innate immunity (15). sIL-1 RA protein is expressed at persistently elevated levels in patients suffering from sepsis, while COX-2, IL-1β, and TNF-α protein levels are decreased, thus prolonging an immunosuppressed state (13, 16, 17). Currently, it is unclear how the differential regulation of pro- and anti-inflammatory genes occurs. THP-1 cells respond to repeated exposure to LPS in a fashion similar to leukocytes obtained from patients with sepsis, exhibiting repressed IL-1β and TNF-α expression (3). Suppression of these genes is under the control of a labile protein(s) (3). In the present study, THP-1 cells were used to determine the molecular mechanisms responsible for the differential expression of COX-2 and sIL-1 RA in endotoxin tolerance.

* This work was supported in part by National Institutes of Health Grants HL-29293, HL-50395, and AI-09169 and by National Institutes of Health NCI Training Grant CA-09422. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: LPS, lipopolysaccharide; sIL-1 RA, secretory interleukin-1 receptor antagonist; COX-2, cyclooxygenase-2; IL-1 R2, interleukin-1 type II receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; IL, interleukin; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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EXPERIMENTAL PROCEDURES

Cell Culture and Induction of Endotoxin Tolerance—THP-1 cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10 units/ml penicillin G, 10 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (HyClone Laboratories, Logan, UT) at 37 °C and 5% CO₂ in a humidified incubator as described previously (3). Low passage number and log-phase cells were used for all experiments. THP-1 cells were rendered endotoxin-tolerant by treating with LPS (1 μg/ml, Escherichia coli LPS 0111:B4, Sigma) for 16 h. The cells were centrifuged, washed once in 1× phosphate-buffered saline, resuspended in media at 1×10⁶ cells/ml, and stimulated as described in the figure legends. For all assays, control cells were treated similarly, but were not exposed to LPS during the initial incubation period.

Western Blot Analysis—LPS-responsive control and LPS-tolerant cells were treated with 1 μg/ml LPS for the times indicated in the figure legends. Cells (1×10⁶ cells/ml) were centrifuged and lysed in 100 μl of Nonidet P-40 lysis buffer (100 mM Tris pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM PMSF). Protein concentrations were determined using BCA protein assay reagent (Pierce). Proteins (100 μg protein/lane) were separated by SDS-PAGE (10% acrylamide) according to the Laemmli method (12), along with low range molecular weight markers.
markers (Bio-Rad), COX-2 and COX-1 standards (kindly provided by Dr. S. Prescott, University of Utah) and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose (Amersham Pharmacia Biotech) at 100 mA for 16 h at room temperature. The nitrocellulose membranes were blocked for 1 h with 5% nonfat milk in 1× Tris-buffered saline (TBS), 0.1% Tween 20 and was then probed with COX-2 or COX-1 (kindly provided by Dr. Prescott) or β-actin (Sigma) antibodies, diluted in 5% nonfat milk in 1× TBS, 0.1% Tween 20. The membranes were washed four times in 1× TBS, 0.1% Tween 20 and incubated for 1 h with goat anti-mouse IgG, conjugated to horseradish peroxidase (Organon Teknika Corp., Durham, NC). The membranes were washed four times in 1× TBS, 0.1% Tween 20, and COX-2, COX-1, or β-actin protein was visualized using the Renaissance Western blot chemiluminescence reagent (NEN Life Science Products). In order to compare results between lanes, densitometry data were normalized to an approximately 45-kDa band that was nonspecifically labeled to ELISA. Culture supernatants were determined by ELISA. p = 0.618 at 24 h for control versus tolerant cells. Results are an average (± S.E.) of three experiments.

**FIG. 2.** Effects of LPS stimulation on sIL-1 RA protein expression in control and endotoxin-tolerant THP-1 cells. sIL-1 RA protein was determined by stimulating control (○) and tolerant (■) THP-1 cells with LPS (1 μg/ml) for 0.5–24 h. sIL-1 RA protein expression in the absence of LPS stimulation in control (●) and endotoxin-tolerant (▲) THP-1 cells was assayed, as well. Concentrations of sIL-1 RA proteins in the culture supernatants were determined by ELISA. p = 0.618 at 24 h for control versus tolerant cells. Results are an average (± S.E.) of three experiments.

**CoX-2 and sIL-1 RA mRNA Decay Analysis**—For CoX-2 mRNA decay analysis, control cells (1 × 10⁶ cells/conidion) were stimulated with 1 μg/ml LPS for 2 h to reach the peak mRNA level. LPS-tolerant cells were stimulated for 1 h. Actinomycin D (5 μg/ml) was then added to inhibit further transcription in control and tolerant cells. Beginning 0.5 h after addition of actinomycin D, aliquots of cells were removed at 1-h intervals over the next 6 h for COX-2 and at 2-h intervals over the next 12 h for sIL-1 RA. Total RNA was isolated, and 10 μg of total RNA per condition was analyzed on Northern blots as described previously (3). After hybridization with radiolabeled COX-2, sIL-1 RA, or GAPDH cDNAs, the filters were quantitated by phosphorimaging and mRNA levels were expressed as a percentage of the maximal mRNA level. sIL-1 RA mRNA decay was assayed under the same conditions, with maximal LPS-induced mRNA levels at 6 h for control cells and 2 h for tolerant cells.

**CoX-2 and sIL-1 RA Protein Decay Analysis**—For CoX-2 protein decay analysis, control cells (1 × 10⁶ cells/conidion) were stimulated with 1 μg/ml LPS for 3 h to reach the midpoint between peak mRNA and protein levels. LPS-tolerant cells were stimulated for 1 h. Cycloheximide (10 μM) was then added to inhibit further protein synthesis in control and tolerant cells. Beginning 0.5 h after addition of cycloheximide, aliquots of cells were removed at 1-h intervals over the next 3 h for CoX-2. Proteins (100 μg of protein/lane) were separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose, probed by Western blots, and quantitated as described above. For sIL-1 RA protein decay analysis, control cells (1 × 10⁶ cells/conidion) were stimulated with 1 μg/ml LPS for 5 h to reach the midpoint between peak mRNA and protein levels. LPS-tolerant cells were stimulated for 1 h. Cycloheximide (10 μM) was then added to control and tolerant cells. Beginning 2 h after addition of cycloheximide, aliquots of cells were removed at 2–4 h intervals over the next 24 h for sIL-1 RA. Concentrations of sIL-1 RA in culture supernatants were determined using an IL-1 RA enzyme immunoassay, as described above.

**Statistical Analysis and Data Expression**—A mean constitutive activity or fold induction was determined for each experiment. Data are presented as the mean ± S.E. Statistics were performed using either two-tailed paired or non-paired t tests to determine significant changes.
RESULTS

LPS-induced COX-2 Protein Levels Are Decreased in Endotoxin Tolerance, whereas sIL-1 RA Protein Levels Are Unchanged—In view of the observed difference in the expression of pro- and anti-inflammatory genes in endotoxin tolerance, a condition that is commonly seen during clinical sepsis, we examined the expression of proinflammatory COX-2 and anti-inflammatory sIL-1 RA proteins in control and tolerant THP-1 cells following LPS stimulation. Western blot analysis (Fig. 1A) revealed that the amount of COX-2 protein in control cells increased over 6–8 h following LPS stimulation and then gradually declined over the next 16 h. In contrast, COX-2 protein levels in endotoxin-tolerant cells were much lower (Fig. 1B). At 6 h after stimulation, LPS-induced COX-2 protein expression was nearly 5-fold lower in tolerant cells than in control cells. Protein levels from the constitutively expressed genes COX-1 and β-actin were unchanged between LPS-stimulated control and tolerant cells. The decrease in LPS-induced COX-2 protein expression in tolerant cells was associated with an average 3-fold decrease in release of prostaglandin E₂ in response to LPS (Fig. 1C), a commonly used indicator of COX-2 enzymatic activity (21), as determined by ELISA. Collectively, these data indicate that COX-2 and PGE₂ levels are decreased in endotoxin-tolerant THP-1 cells, similar to results obtained with IL-1β (3).

To determine whether expression of anti-inflammatory genes differs from that of proinflammatory genes in endotoxin-tolerant THP-1 cells, the expression of sIL-1 RA protein in control and tolerant cells following LPS stimulation was assessed by ELISA. sIL-1 RA protein levels increased quickly in control cells, reaching a maximum at 6 h following LPS-stimulation and remained at high levels over the next 16 h (Fig. 2). In the absence of LPS, low levels of sIL-1 RA protein were produced by control cells, consistent with the idea that this gene is constitutively expressed and can be increased approximately 5-fold following LPS treatment in THP-1 cells.

In marked contrast to the decreased amount of COX-2 protein in tolerant cells, sIL-1 RA protein was produced by tolerant cells in response to LPS at levels comparable to those in LPS-stimulated control cells, with peak production occurring at 12 h (Fig. 2). However, the rate of increase of sIL-1 RA from tolerant cells was somewhat slower than from LPS-stimulated control cells. Similarly to unstimulated control cells, sIL-1 RA protein was constitutively expressed at a low level in the absence of LPS in tolerant cells. On average, sIL-1 RA protein levels were increased 4–5-fold above unstimulated levels in both control and tolerant cells. These findings support the idea that the behavior of the anti-inflammatory sIL-1 RA gene is significantly different from that of the proinflammatory genes COX-2 and IL-1β (1).

COX-2 and sIL-1 RA mRNA Levels Are Decreased in Endotoxin-tolerant THP-1 Cells—To determine if the differential expression of COX-2 versus sIL-1 RA proteins in tolerant cells was due to differences in mRNA levels, we analyzed the kinetics of mRNA expression in control and tolerant THP-1 cells.
stimulated with LPS. A 12-h time course was examined because expression in THP-1 cells peaks during the first 6 h of LPS-stimulation (22). In control cells stimulated with LPS, COX-2 mRNA levels are increased rapidly and peak at 2 h (Fig. 3A), exhibiting a 7-fold induction above base-line levels (Fig. 3B). These levels quickly decrease over the next 8 h. sIL-1 RA mRNA levels increased more slowly, reaching a peak at 4 h (Fig. 3A) with a 3.5-fold induction above base-line levels (Fig. 3B). However, sIL-1 RA mRNA levels decline more slowly than COX-2 mRNA between 6 and 12 h after stimulation. mRNA levels for the constitutively expressed gene GAPDH were unchanged in stimulated control cells. These data support the idea that COX-2 behaves like an inducible immediate early gene whose mRNA is rapidly expressed upon stimulation with LPS and rapidly declines, whereas sIL-1 RA is more characteristic of prototypical anti-inflammatory genes that exhibit a slower rate of expression and prolonged mRNA expression (23).

Levels of COX-2 mRNA were repressed to base-line levels in LPS-stimulated tolerant cells (Fig. 3, A and B), exhibiting a 5-fold decrease compared to LPS-stimulated control cells at 2 h after stimulation. This is similar to the results obtained with COX-2 protein and is also similar to those with IL-1β mRNA and protein (3). Reverse transcription-polymerase chain reaction analysis showed that there was a low yet detectable level of COX-2 mRNA in tolerant cells (data not shown), suggesting that the gene may be induced at a low level during endotoxin tolerance. However, the expression of the gene appears to be quickly repressed shortly thereafter. Similarly, sIL-1 RA mRNA was repressed to base-line levels (3.5-fold decrease) in tolerant cells upon stimulation with LPS (Fig. 3, A and B). Reverse transcription-polymerase chain reaction analysis showed that there was also a low yet detectable level of sIL-1 RA mRNA in endotoxin-tolerant cells (data not shown). In contrast, mRNA levels for the constitutively expressed gene GAPDH were unchanged in LPS-stimulated tolerant cells. These data indicate that, whereas COX-2 and sIL-1 RA mRNA are induced in control cells upon stimulation with LPS, expression of these genes is much less responsive to LPS in tolerant cells.

**COX-2 and sIL-1 RA Transcription Are Repressed in Endotoxin-tolerant THP-1 Cells**—The low levels of COX-2 and sIL-1 RA mRNA in LPS-stimulated tolerant cells could be due to a
lower level of transcription or a higher rate of mRNA turnover. The transcriptional activity of the COX-2 promoter in control and tolerant THP-1 cells was determined using a series of COX-2 promoter fragments linked to a chloramphenicol acetyltransferase (CAT) reporter gene in transient transfection experiments. LPS-induced CAT activity in control cells was greatest with the construct containing 2494 bp to 1101 bp of the COX-2 gene (Fig. 4A). LPS stimulation of cells transfected with this promoter construct resulted in an approximately 6-fold induction above base-line. Plasmids containing larger amounts of upstream sequence (2833 and 2582) resulted in lower amounts of LPS-induced CAT activity. The enhanced activity associated with the 2494 bp fragment is consistent with the conclusion that an endogenous repressor binding site may exist in the region of 2833 to 2494 bp. LPS-induced CAT expression also decreased with constructs containing promoter fragments with less than 2494 bp upstream of the promoter (2265 bp and 292 bp), although even the shortest promoter fragment tested retained LPS inducibility in control THP-1 cells. However, when tolerant cells transfected with any of the CAT promoter plasmids were incubated with LPS, stimulation of CAT expression was minimal from all constructs. These data are consistent with the notion that the COX-2 promoter is transcriptionally active in control cells stimulated with LPS; however, its promoter is repressed in stimulated tolerant cells. In contrast, there was no significant difference in CAT fold induction in control and tolerant cells that had been transfected with the constitutively active CMV-CAT construct (Fig. 4A). The promoterless pCAT.Basic construct, serving as a negative control, yielded no CAT expression in either control or tolerant cells (data not shown). Thus, reduced expression of the COX-2 promoter fragments in tolerant cells is not due to a general effect on transcription. Results from nuclear run-on analyses of the endogenous COX-2 gene are consistent with the lower transcriptional activity in tolerant cells versus control cells, as well (data not shown).

The transcriptional activity of the sIL-1 RA promoter was analyzed similarly using a series of promoter fragments linked to a luciferase reporter gene in control and tolerant THP-1 cells. RA-294.luc, which contains a 294-bp region directly upstream of the sIL-1 RA transcription start site (2267 bp to +27 bp), was the most LPS-responsive. In control cells stimulated with LPS, there was a 6.5-fold induction of luciferase activity in comparison to unstimulated control cells (Fig. 4B). Plasmids containing larger amounts of upstream sequence (~1.68 kilobase pairs and ~7.0 kilobase pairs) were less responsive. However, in tolerant cells, there was no LPS-induced increase in luciferase activity from any of the promoter constructs, indicating a loss of LPS responsiveness in tolerant cells. In contrast, there was no significant difference in luciferase fold induction in control and tolerant cells that had been transfected with the constitutively active pGL3-CMV construct (Fig. 5).
4B). The promoterless pGL3-Basic construct, serving as a negative control, yielded no luciferase expression in either control or tolerant cells (data not shown). These results indicate that the sIL-1 RA promoter is transcriptionally active in LPS-stimulated control cells, yet is repressed in stimulated tolerant cells, a conclusion that is consistent with the observation that there is little increase in sIL-1 RA mRNA in LPS-stimulated tolerant cells (Fig. 3).

**COX-2 and sIL-1 RA mRNAs Are Stabilized in Endotoxin-tolerant THP-1 Cells**—The repression of transcription of the COX-2 and sIL-1 RA genes in endotoxin-tolerant cells clearly contributes to the lower levels of the corresponding mRNAs. However, increased mRNA decay might also contribute to the regulation of COX-2 and sIL-1 RA genes in tolerant versus control THP-1 cells. To assay mRNA decay, control and tolerant THP-1 cells were stimulated with LPS to achieve peak COX-2 and sIL-1 RA mRNA levels. Transcription was halted with the addition of actinomycin D, and the levels of COX-2 and sIL-1 RA mRNA were analyzed on Northern blots.

In LPS-stimulated control cells, the decay of COX-2 mRNA was biphasic, exhibiting a t_{1/2} of approximately 1 h (Fig. 5A). However, in LPS-stimulated tolerant cells, there was no rapid phase of decay and the t_{1/2} for COX-2 mRNA was 6 h. These data indicate that reduced levels of COX-2 mRNA in tolerant cells are not due to enhanced degradation, since COX-2 mRNA was actually more stable in tolerant than control cells. In contrast to COX-2 mRNA, the decay of sIL-1 RA mRNA was not biphasic and was significantly slower. The t_{1/2} for sIL-1 RA mRNA was 6 h in LPS-stimulated control cells and increased to 12 h in LPS-tolerant cells (Fig. 5B). As with COX-2 mRNA, sIL-1 RA mRNA turnover is slower in endotoxin-tolerant cells than in control cells and cannot account for the lower levels of sIL-1 RA mRNA in tolerant cells. Collectively, these data support the idea that COX-2 and sIL-1 RA mRNAs are stabilized in endotoxin-tolerant THP-1 cells and that sIL-1 RA protein expression may continue in tolerant cells, due in part to the long half-life of sIL-1 RA mRNA.

**COX-2 Protein Is Turned Over Rapidly, whereas sIL-1 RA Protein Is Stable**—mRNA stability appears to be one mechanism for maintaining sIL-1 RA levels during endotoxin tolerance in the absence of continued transcription. However, the observed increase in sIL-1 RA protein levels following LPS stimulation required an additional explanation since the levels of sIL-1 RA mRNA in tolerant cells. In view of the apparent discrepancy between the observed transcriptional repression of COX-2 and sIL-1 RA mRNA and the elevated levels of sIL-1 RA protein in endotoxin-tolerant cells, we examined the decay rates of COX-2 and sIL-1 RA proteins. Control and tolerant THP-1 cells were stimulated with LPS to reach the midpoint between peak mRNA and protein decay in LPS-stimulated control and endotoxin-tolerant THP-1 cells. A, COX-2 protein decay analysis in LPS-stimulated control (○) and endotoxin-tolerant (□) THP-1 cells. Control and tolerant THP-1 cells were stimulated with LPS (1 μg/ml). At the midpoint between peak mRNA and protein levels, protein synthesis in control and tolerant cells was halted with the administration of cycloheximide (10 μM). The cells were cultured for 0–3 h, protein was isolated, and 100 μg of protein/sample was separated by SDS-PAGE and analyzed on Western blots. p = 0.183 at 2 h for control versus tolerant cells. Results are an average (± S.E.) of four experiments. B, sIL-1 RA protein decay analysis in LPS-stimulated control (○) and endotoxin-tolerant (□) THP-1 cells. Control and endotoxin-tolerant THP-1 cells were stimulated with LPS (1 μg/ml). At the midpoint between peak mRNA and protein levels, protein synthesis in control and tolerant cells was halted with the administration of cycloheximide (10 μM). The cells were cultured for 0–24 h, and concentrations of sIL-1 RA in culture supernatants were determined by ELISA. p = 0.075 at 24 h for control versus tolerant cells. Results are an average (± S.E.) of three experiments.

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**FIG. 6.** COX-2 and sIL-1 RA protein decay in LPS-stimulated control and endotoxin-tolerant THP-1 cells. A, COX-2 protein decay analysis in LPS-stimulated control (○) and endotoxin-tolerant (□) THP-1 cells. Control and tolerant THP-1 cells were stimulated with LPS (1 μg/ml). At the midpoint between peak mRNA and protein levels, protein synthesis in control and tolerant cells was halted with the administration of cycloheximide (10 μM). The cells were cultured for 0–3 h, protein was isolated, and 100 μg of protein/sample was separated by SDS-PAGE and analyzed on Western blots. p = 0.183 at 2 h for control versus tolerant cells. Results are an average (± S.E.) of four experiments. B, sIL-1 RA protein decay analysis in LPS-stimulated control (○) and endotoxin-tolerant (□) THP-1 cells. Control and tolerant THP-1 cells were stimulated with LPS (1 μg/ml). At the midpoint between peak mRNA and protein levels, protein synthesis in control and tolerant cells was halted with the administration of cycloheximide (10 μM). The cells were cultured for 0–24 h, and concentrations of sIL-1 RA in culture supernatants were determined by ELISA. p = 0.075 at 24 h for control versus tolerant cells. Results are an average (± S.E.) of three experiments.
protein levels for COX-2 and sIL-1 RA. Protein synthesis was altered with the addition of cycloheximide and cell extracts were then analyzed on Western blots for COX-2 proteins and culture media were analyzed by ELISA for sIL-1 RA proteins.

In both LPS-stimulated control and tolerant cells, there was no significant difference in COX-2 protein stability ($t_{1/2} = 2$ h, Fig. 6A). Similarly, there was no significant difference in the stability of sIL-1 RA in LPS-stimulated control versus tolerant cells (Fig. 6B). However, in contrast to COX-2, sIL-1 RA was very stable over a 24-h period following cycloheximide addition. In additional experiments, assaying as long as 96 h after cycloheximide addition showed that this protein remained stable in both control and tolerant cells (data not shown). Collectively, these data support the idea that differences in the stability of COX-2 and sIL-1 RA proteins contribute to their differential expression in endotoxin-tolerant THP-1 cells.

**DISCUSSION**

In endotoxin-tolerant THP-1 cells, the level of COX-2 protein is decreased, whereas the level of sIL-1 RA protein is not. This differential expression results from repressed transcription of both COX-2 and sIL-1 RA genes combined with stabilization of sIL-1 RA protein and mRNA. The mechanism(s) that decrease transcription and stabilize protein and mRNA in tolerant cells are not yet known, but may involve a labile repressor(s) of transcription as well as protein and mRNA stabilizing/destabilizing elements (3, 24). The resulting steady state mRNA levels are amplified at the level of protein with dramatic differences in COX-2 and sIL-1 RA expression in endotoxin-tolerant THP-1 cells.

Given the observation that COX-2 and sIL-1 RA were differentially expressed at the protein level, we examined their transcriptional regulation in THP-1 cells. COX-2 promoter fragments exhibit 3–5.5-fold decreases in reporter gene activity in transient transfection assays (Fig. 4A). Similarly, transcription of the endogenous COX-2 gene exhibits an equivalent decrease in tolerant cells upon stimulation with LPS in comparison to control cells (data not shown). Based on these data and mRNA levels (Fig. 3B), we find that COX-2 transcription is induced early and then quickly repressed. In addition, PGE2 lipid levels are decreased approximately 3-fold in tolerant cells when compared to control cells stimulated with LPS, possibly due to lower levels of COX-2 protein or available substrate present in tolerant cells. Similar to COX-2, sIL-1 RA transcription is also markedly reduced in tolerant cells (Fig. 4B). This transcriptional repression may involve negative regulatory element(s) similar to those required for silenced activity in interferon-A gene promoters (25). These findings are consistent with repressed transcription of the two genes, possibly by the same type of labile repressor that is thought to act on IL-1β (3). The large increase in sIL-1 RA protein in the presence of repressed transcriptional activity and mRNA levels (Fig. 3A) indicates that other regulatory mechanisms must contribute to the elevated protein levels in endotoxin-tolerant THP-1 cells. We propose two potential mechanisms. First, differences in mRNA stability may account for the increase in sIL-1 RA protein levels in tolerant cells. Second, enhanced translational efficiency of declining pools of mRNA may potentiate sIL-1 RA expression in tolerance. We believe that it most likely is a combination of these two potential mechanisms. In support of this notion, Cassatella (26) has shown that anti-inflammatory IL-10 up-regulates IL-1RA production in LPS-stimulated human polymorphonuclear leukocytes by delaying mRNA degradation. We have also shown that delaying mRNA degradation in THP-1 cells tolerant to IL-1β overcomes repression of IL-1β synthesis (27). These studies and others (28) suggest that increased anti-inflammatory expression may result from positive feedback mechanisms that are in place to help counterbalance proinflammatory expression. In addition, Beutler (29) has shown that cis-acting elements from the 3′-untranslated region of TNF-α are required for efficient translational activation of the gene. A similar form of translational regulation by cis-acting elements within the IL-1 RA 3′-untranslated region is therefore plausible.

Post-transcriptional mechanisms of gene regulation have important roles in the expression of pro- and anti-inflammatory genes (26, 29). It was unknown whether similar mechanisms regulated COX-2 and sIL-1 RA expression. We investigated this issue by mRNA decay analysis. Our findings demonstrated that the turnover rate for the biphasic decay of COX-2 mRNA (Fig. 6A) in tolerant cells ($t_{1/2} = 6$ h) is 6 times as long as in control cells ($t_{1/2} = 1$ h). In addition, we believe that even at lower levels in tolerant cells, COX-2 mRNA is efficiently translated (Fig. 1A). The biphasic decay of COX-2 in control cells may be important in that elevated levels of proinflammatory mRNAs are rapidly turned over, whereas lower levels are subject to different turnover mechanisms and kinetics. Similarly, mRNA half-life analysis (Fig. 6B) demonstrated that the turnover rate for sIL-1 RA in tolerant cells ($t_{1/2} = 12$ h) is doubled in comparison to control cells ($t_{1/2} = 6$ h), indicating that sIL-1 RA mRNA is relatively stable in tolerant cells. This mRNA stability may permit continued translation of sIL-1 RA. Additionally, mRNA stabilization may serve as a general mechanism of regulating expression for certain pro- and anti-inflammatory genes, potentially as a negative feedback loop (i.e. COX-2) or as means for continued expression (i.e. sIL-1 RA). Brooks et al. have shown that IL-1-induced JunB mRNA levels are not directly correlated with the level of JunB protein synthesis (30). Rather, JunB protein levels remain elevated as a result of enhanced translational efficiency. Our findings with sIL-1 RA in endotoxin-tolerant THP-1 cells are consistent with this mechanism. In addition, Cassatella and others have shown that the sIL-1 RA 3′-untranslated region does not contain AU-rich destabilizing sequences that are characteristic of rapidly turned over proinflammatory mRNA, such as COX-2 and IL-1 (26, 31).

With the data supporting the notion that mRNA stability may in part explain differential expression of COX-2 and sIL-1 RA, we investigated protein stability by half-life analysis. Protein levels for COX-2 in both control and tolerant cells reached 50% of their maximal level within 2 h of cycloheximide addition (Fig. 5A). This relatively short turnover time for COX-2 is consistent with what is known for the rapid decay of proinflammatory proteins in vitro (3) and their relative absence clinically in sepsis (17). In marked contrast, sIL-1 RA protein is very stable (Fig. 5B). In both control and tolerant cells, sIL-1 RA protein is stable over 24 h. Interestingly, this stability is still maintained at 96 h (data not shown). These findings are supportive of clinical studies in sepsis in which anti-inflammatory protein levels remain elevated for several days (18). Although elevated levels of anti-inflammatory proteins, such as sIL-1 RA, often help to decrease the potentially lethal inflammatory response in sepsis, the stability of these proteins may in part promote the severe immunosuppression commonly seen in sepsis patients.

Taken collectively, these data are consistent with the notion that there is no significant difference in protein stability between control and endotoxin-tolerant cells for COX-2 or sIL-1 RA. However, the corresponding mRNAs are more stable in tolerant cells than in control cells, delaying degradation. In addition, due to undefined differences between pro- and anti-inflammatory genes, sIL-1 RA mRNA and protein are significantly more stable than those of COX-2, resulting
in a pronounced difference at the level of protein in endotoxin tolerance.

In summary, the results of this study demonstrate differential regulation and expression of COX-2 and sIL-1 RA in endotoxin-tolerant THP-1 cells. COX-2 protein levels are decreased, yet sIL-1 RA protein levels remain elevated. Differential expression is consistent with repressed transcription and protein turnover for COX-2, whereas sIL-1 RA mRNA and protein are stabilized. We believe that increased stability of sIL-1 RA mRNA may be coupled with enhanced translational efficiency of sIL-1 RA protein in tolerant cells. These data demonstrate that COX-2 and sIL-1 RA proteins are differentially expressed in endotoxin tolerant THP-1 cells, similar to differential expression observed during sepsis (13, 17). In addition, the time courses for the THP-1 expression of COX-2 (rapid and transient) and sIL-1 RA (slow and constitutive) proteins are similar to the pattern of expression of pro- and anti-inflammatory genes observed during clinical sepsis (23). These results indicate that the THP-1 cell line is as a useful system to dissect endotoxin responsiveness and the regulation of pro- and anti-inflammatory genes. Our results not only establish mechanisms for the differential regulation of pro- and anti-inflammatory genes and their protein products, but also serve as a foundation for future studies on the factors that are responsible for repressing and destabilizing COX-2 mRNA and protein and that promote sIL-1 RA expression.

Acknowledgments—We thank Jean Hu, Sue Cousart, and Jon Wells for excellent technical support. We are especially grateful to Dr. Michael F. Smith, Dr. Steve Prescott, Dr. Eugene O’Neill, and Dr. Timothy Hla, who supplied us with reagents as identified in the text; Dr. Barbara K. Yoza for insightful discussion; and Dr. Douglas Lyles for critical reading of the manuscript. Oligonucleotide synthesis was performed in the DNA Synthesis Core Laboratory of the Cancer Center of Wake Forest University.

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J. Biol. Chem. 2000, 275:12185-12193.
doi: 10.1074/jbc.275.16.12185

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