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Differential Regulation of Cyclooxygenase-2 (COX-2) mRNA Stability by Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α) in Human *In Vitro* Differentiated Macrophages

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ABSTRACT. Cyclooxygenase-2 (COX-2) is a highly inducible gene in macrophages by pro-inflammatory cytokines. A major mechanism for cytokine-induced COX-2 expression is stabilization of COX-2 mRNA. In this study, we examined the induction of COX-2 expression by interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in human primary *in vitro* differentiated macrophages. IL-1β (5 ng/mL) or TNF-α (1 ng/mL) induced up to an ~40-fold increase of COX-2 mRNA in macrophages during a 2 to 2.5-hr incubation. Run-off experiments demonstrated that cytokine stimulation had only a mild effect on the COX-2 transcription rate (~10–40% increase). The translation blocker cycloheximide (CHM) (10 mg/mL) superinduced COX-2 mRNA during 2 hr of incubation and further stabilized the COX-2 mRNA ($T_{1/2} > 4$ hr). The CHM-superinduced COX-2 mRNA was subject to a rapid degradation after removal of CHM ($T_{1/2} < 1$ hr). Both IL-1β and TNF-α stabilized cytokine-induced COX-2 mRNA ($T_{1/2} \geq 2$ hr). Maximal stabilization of COX-2 mRNA after a short-term stimulation required the continued presence of IL-1β in the medium. Long-term treatment of TNF-α destabilized the induced COX-2 mRNA. Cells simultaneously treated with both IL-1β and TNF-α had a reduced induction of COX-2, IL-1β, and IL-6 mRNA. In transcription-arrested cells, the translation blocker puromycin affected the TNF-α-induced stabilization and destabilization of COX-2 mRNA, but not the IL-1β-induced stabilization. The studies suggest that positive and negative regulation of mRNA stability may play a major role in cytokine-mediated COX-2 induction in human macrophages. TNF-α may play both pro-inflammatory and protective roles during inflammation by regulation of pro-inflammatory gene transcripts. BIOCHEM PHARMACOL **59**;2:187–194, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. cyclooxygenase-2; mRNA stability; tumor necrosis factor-α; interleukin-1β; human primary macrophages

COX-2† is an immediate early response gene in macrophages, and is responsible for elevated prostaglandin secretion during inflammation and immune response [1]. Monocytes macrophages are one of the major sources for elevated prostaglandin output in *in vivo* inflammation. Up-regulation of the COX-2 gene by inflammatory stimuli is found to occur at transcriptional and post-transcriptional levels [2]. In human monocytes macrophages, elevation of COX-2 mRNA can be induced by a variety of pro-inflammatory stimuli, such as LPS and cytokine IL-1β [3–6]. Induction of the COX-2 gene by pro-inflammatory stimuli shares a striking similarity with that of typical pro-inflammatory genes such as IL-1 and TNF-α, since it is a transient and early response [7]. A characteristic AU-rich structure in the 3′-untranslated region (3′-UTR) of COX-2 mRNA has been noted [8]. This non-coding structure is an important feature shared by IL-1β and TNF-α mRNA and is believed to be the determinant for COX-2 mRNA stability [9, 10]. Two types of proteins have been identified in renal mesangial cells that bind to the COX-2 3′-UTR [9]. In the amnion, the increased COX-2 mRNA seen after the onset of labor is entirely in the protein-bound fractions [11]. Protein binding to COX-2 transcripts appears to be a major mechanism for the stabilization and up-regulation of COX-2 mRNA [1, 2, 9, 10]. Moreover, the translation inhibitor CHM can superinduce COX-2 mRNA [12]. This suggests that *de novo* protein synthesis plays an essential role in the regulation of COX-2 mRNA. The COX-2 gene promoter region also has AP-2 and NF-κB binding sites [8].

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† *Abbreviations:* COX-2, cyclooxygenase-2; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; AP-2, activator protein-2; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide; CHM, cycloheximide; IL-6, interleukin-6; PGE_2, prostaglandin E_2; DMEM, Dulbecco's modified Eagle's medium; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; SSC, sodium chloride–sodium citrate; RT–PCR, reverse transcriptase–polymerase chain reaction; and GAPDH, glyceraldehyde-phosphate dehydrogenase.

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Both IL-1β and TNF-α can activate these two transcription factors [13]. Those transcription factors have also been implicated in COX-2 induction by the cytokines [14, 15].

Human macrophages are a major source for IL-1β, TNF-α, and prostaglandin formation. *In vitro* differentiated macrophages derived from circulating monocytes share these important pathophysiological phenotypes [16]. COX-2 is a highly inducible gene in murine macrophages or human macrophage-like cell lines stimulated by pro-inflammatory mediators, such as LPS, IL-1, and TNF-α [3–6]. However, little has been determined about IL-1β and TNF-α induction of COX-2 mRNA in human primary macrophages derived from circulating monocytes. In this study, we examined COX-2 mRNA induction by IL-1β and TNF-α in monocyte-derived macrophages. We found that the stabilization of COX-2 mRNA may be the major mechanism for cytokine-induced elevation of COX-2 mRNA. However, TNF-α and IL-1β regulate COX-2 mRNA stability differently. More importantly, in long-term stimulation, TNF-α enhances COX-2 mRNA degradation and plays a balancing role in the induction of the pro-inflammatory genes COX-2, IL-1β, and IL-6.

MATERIALS AND METHODS

Chemicals

Recombinant human IL-1β and TNF-α were purchased from R & D Systems. The endotoxin level was <0.1 ng/1 μg of the cytokine. [α-^{32}P]dCTP (6000 Ci/mmol) was purchased from Amersham. COX-2 cDNA probe was purchased from Oxford Biomedical Research, Inc. DMEM, human serum type AB, CHM, DRB, and LPS from *E. coli* were purchased from Sigma. Puromycin was purchased from Calbiochem.

Cell Culture

Human monocytes were isolated from the buffy-coat of peripheral blood obtained from the Houston Blood Center, using Plasmagel (Laboratoire Roger Bellon), hypertonic NaCl, and Lymphoprep (NYCOMED PHARMA AS), as previously described [17]. Human monocytes were isolated and suspended in DMEM containing 10% human AB serum, 4 mM L-glutamine, 40 μg/mL of gentamycin and 4 μg/mL of insulin. Insulin was present in the medium only during the first week. The cells become fully differentiated macrophages over a period of 10–15 days [16, 18].

Cell Stimulation and Washout Experiments

Fully differentiated macrophages (~14 days in culture) were incubated with pre-warmed fresh DMEM containing 0.1% low endotoxin BSA prior to exposure to cytokines, LPS, or CHM. The final concentration of endotoxin in the BSA-containing medium was <0.2 ng/mL. To determine the dependence of COX-2 mRNA up-regulation on the continued presence of inducers, the medium containing the stimulating agents was removed. The cells were washed once with pre-warmed PBS and incubated with pre-warmed medium.

Northern Blotting

Total RNA was isolated using RNAzol (Tel-Test, Inc.). Total RNA (10–15 μg) was separated on a 1% agarose gel with 3% formaldehyde and transferred to a GeneScreen Plus nylon membrane (Dupont). The hybridization solution contained $>1 \times 10^6$ cpm of ^{32}P-labeled cDNA probe with a specific activity $>1 \times 10^9$ cpm/μg generated by random prime labeling. Hybridization was carried out at 42° for >14 hr, and the membrane was washed in 0.1 × SSC containing 0.1% SDS and exposed to x-ray film for 6–24 hr. Autoradiograms were quantitated by densitometry.

Nuclei Run-off Assay

Macrophage nuclei ($> 5 \times 10^6$/assay) were collected as previously described [19]. The nuclei were incubated in 25 mM Tris–HCl, pH 8.0; 15% glycerol; 2 mM $MgCl_2$; 150 mM KCl; 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP; 100 μCi [α-^{32}P] UTP; and 2.5 mM dithiothreitol at 30° for 60 min. The protein and DNA were cleared, and RNA was recovered by precipitation. The total radioactivity of each RNA sample was estimated by scintillation counting, and equal amounts of radioactivity were used for the hybridization ($> 5 \times 10^6$ cpm/mL). The hybridization was carried at 55° for 48 hr in the presence of formamide. After hybridization, the target DNA blot was washed in 0.1% SDS and 0.1% SSC at 25° for 1 hr and then at 55° for 1 hr. The blot was exposed to film for 4–6 days. The target DNAs for COX-2 and GAPDH were made from RT–PCR cloning of IL-1β-stimulated macrophages. Human COX-2 primers were 5′-CTCCTCAAGTCCCTGAGCATCTACGGTTT-3′ (sense) and 5′-TTCTTTTAGTAGTACTGTGGGATTGATATC-3′ (antisense). Human GAPDH primers were 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ (sense) and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (antisense). Both PCR products were subcloned into a pCR™II vector (Invitrogen Co.). Products were amplified and sequenced to confirm their identities.

RESULTS

Induction of COX-2 mRNA by TNF-α and IL-1β

To investigate the induction of the COX-2 gene in a primary macrophage model, the basal level and the inducibility of COX-2 expression by IL-1β and TNF-α were determined. As seen in Fig. 1, COX-2 was expressed in fully differentiated macrophages at a very low level. TNF-α (1–10 ng/mL) and IL-1β (1–5 ng/mL) induced COX-2 mRNA up to ~40-fold during a 2-hr incubation. For TNF-α, the maximal level of expression occurred at a concentration of 1–10 ng/mL, depending on the batch of recombinant TNF-α. An interesting phenomenon was that

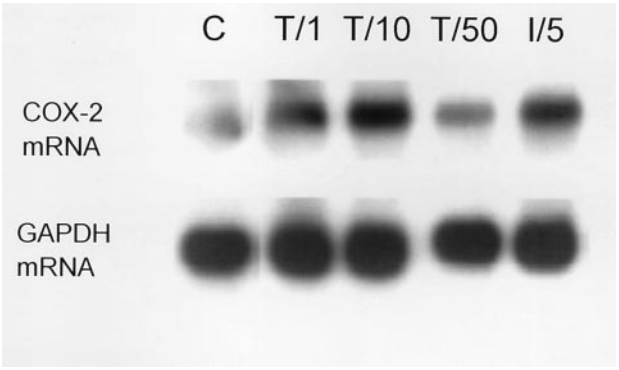

FIG. 1. Northern analysis of COX-2 mRNA in primary macrophages induced by IL-1β and TNF-α. Macrophages (~14 days in culture) were incubated with DMEM containing 0.1% BSA, and exposed to cytokines for 2 hr. Total RNA was isolated and analyzed as described in Materials and Methods. C: control; T/1: TNF-α, 1 ng/mL; T/10: TNF-α, 10 ng/mL; T/50: TNF-α, 50 ng/mL; and I/5: IL-1β, 5 ng/mL. GAPDH mRNA was used as an internal control.

a high concentration of TNF-α (50 ng/mL) could result in reduced induction. As seen in Fig. 1, the relative level of COX-2 mRNA in the cells treated with 10 ng/mL of TNF-α was 100 (COX-2/GAPDH, by densitometry) versus ~65 (COX-2/GAPDH) in the cells treated with 50 ng/mL of TNF-α. We did not see a similar phenomenon in IL-1β stimulation until the concentration of IL-1β was increased to 50 ng/mL (data not shown). For further studies, a concentration of 1 ng/mL for TNF-α or 5 ng/mL for IL-1β was chosen. The fold increase of COX-2 mRNA varied from 2.5- to 40-fold for TNF-α (N = 7) and from 3- to 20-fold for IL-1β (N = 6), depending on the donor. The COX-2 mRNA response to IL-1β and TNF-α usually declined within 6 hr (data not shown). These data suggest that COX-2 mRNA is transiently induced in primary macrophages.

Comparison of COX-2 and IL-1β Induction by LPS

The regulation of IL-1β mRNA stability plays a critical role in its induction during pro-inflammatory stimulation [7, 20]. IL-1β is up-regulated as an early response and has long been thought to have a regulatory effect on induction of the COX-2/prostaglandin pathway during inflammation and immune response [21]. Thus, we examined the temporal relationship in the induction between COX-2 and IL-1β. As seen in Fig. 2, LPS- (100 ng/mL) induced COX-2 mRNA peaked at 2.5 hr in parallel with induced IL-1β mRNA, and both simultaneously declined to the basal level at about 5 hr. This suggests that COX-2 and IL-1β may belong to a family of primary inflammatory response genes, and may be subject to a common regulatory pathway under LPS stimulation.

Effect of IL-1β and TNF-α on the Transcription Rate of COX-2

IL-1β- and TNF-α-induced elevation of COX-2 mRNA could be the result of increased transcription, enhanced

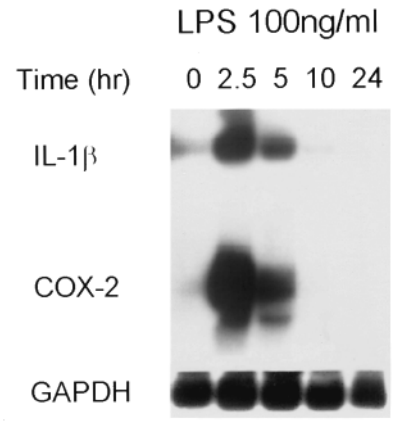

FIG. 2. Northern analysis of COX-2 and IL-1β mRNA in primary macrophages stimulated by LPS. Macrophages (~14 days in culture) were incubated with DMEM containing 10% human serum and exposed to 100 ng/mL of LPS for certain periods of time. Total RNA was isolated and analyzed as described in Materials and Methods. GAPDH was used as an internal control.

stability of mRNA, or a combination of the two mechanisms. We first examined the COX-2 transcription rate under TNF-α or IL-1β stimulation by nuclear run-off experiments. Incubation of cells with TNF-α or IL-1β for 45 min before isolation of cell nuclei did not result in an increased COX-2 transcription rate relative to the control cells (Fig. 3). After a 2.5-hr incubation, TNF-α increased the transcription ratio of COX-2/GAPDH by only ~10% and IL-1β increased this ratio by ~40%. The results demonstrated that in the unstimulated state the COX-2 gene was actively transcribed, and that TNF-α and IL-1β stimulation only led to a small increase in the transcription rate. These increases appear to be insufficient to account for the large rise in the COX-2 mRNA detected.

Regulation of COX-2 mRNA Stability in Macrophages

Due to the low basal levels of COX-2 mRNA expression in untreated macrophages, it was difficult to precisely compare basal and cytokine-regulated changes in COX-2 mRNA turnover rates. We therefore compared the decay rates for COX-2 mRNA after induction by LPS, TNF-α, IL-1β, or superinduction with the translation inhibitor CHM. Macrophages were stimulated first with TNF-α, IL-1β, LPS, or CHM for 2 hr. Then transcription was inhibited by the addition of DRB, a rapid transcription inhibitor [22]. In our studies, the basal level of COX-2 mRNA in macrophages became undetectable within 45 min after DRB treatment. In the cells pretreated with DRB, CHM induced less than 1% of COX-2 mRNA as compared with the control macrophages (data not shown). These results suggested that the inhibition of DBR on COX-2 transcription in macrophages was nearly complete. Thus, the decay in the levels of COX-2 mRNA was measured where the treatment of the cells with DRB was considered as the reference point for

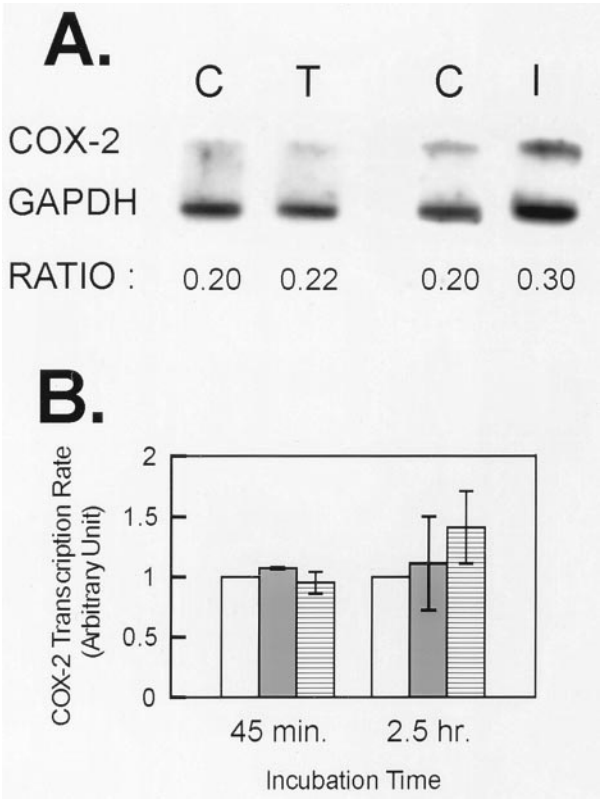

FIG. 3. Transcription rate of COX-2 gene in primary macrophages induced by IL-1β and TNF-α. Macrophages (~14 days in culture) were incubated with DMEM containing 0.1% BSA, and exposed to cytokines for 45 min or 2.5 hr. The nuclei were isolated, and *in vitro* transcription was performed as described in Materials and Methods. Panel A shows a representative experiment at 2.5-hr of incubation. C: control; T: TNF-α; and I: IL-1β. Panel B shows data quantitated by densitometry. The results are the means of three independent experiments ± 1 SD. Open bars: control; gray bars: TNF-α, 1 ng/mL; and line bars: IL-1β, 5 ng/mL.

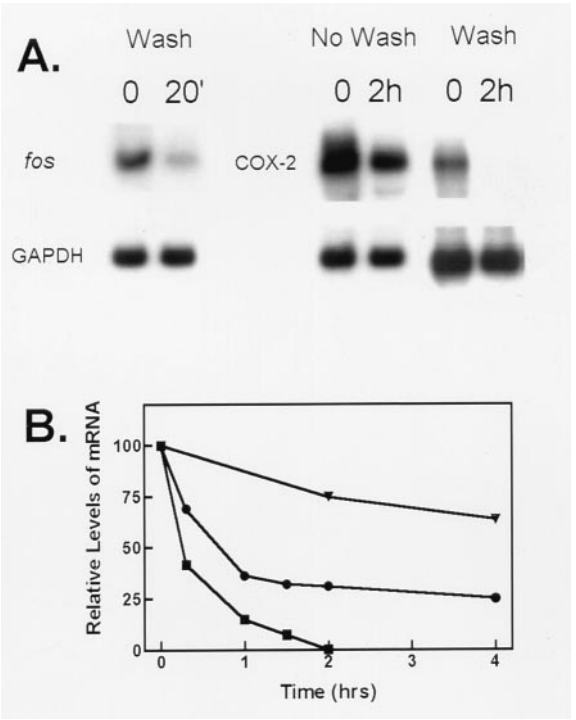

FIG. 4. Effect of CHM on COX-2 mRNA stability in macrophages superinduced by CHM. Macrophages (~14 days in culture) were incubated with DMEM containing 0.1% BSA, and exposed to CHM (10 mg/mL) for 2 hr. The CHM was either removed by washout or remained in the medium. Cell transcription was stopped by adding 25 μg/mL of DRB to the cells. The cells were incubated further for certain periods of time. Total RNA was isolated and analyzed by northern blot as described in Materials and Methods. Panel A shows representative blots. Panel B shows the results of the blots quantitated by densitometry; COX-2 mRNA levels and c-fos mRNA levels were normalized to GAPDH internal controls. The COX-2 or c-fos mRNA levels at transcription stop point were counted as a value of 100. The data were representative of three independent experiments with similar results. Standard deviations were less than 10% in each experiment. Symbols: (●) COX-2 mRNA after washout of CHM; (▼) COX-2 mRNA without washout; and (■) c-fos mRNA after washout.

the maximal level of mRNA expression. As seen in Fig. 4, removal of CHM from the medium following CHM superinduction of COX-2 mRNA resulted in a rapid decay in the mRNA level ($T_{1/2}$ < 1 hr, only ~30% of the COX-2 mRNA remaining at 2 hr). However, the COX-2 mRNA superinduced by CHM was stabilized when CHM was maintained in the medium (~75% of the COX-2 mRNA remaining at 2 hr, Fig. 4). Since, after DRB treatment, CHM only maintains its inhibitory effect on protein synthesis and its superinductory effect on transcription becomes negligible, these results suggest a strong destabilizing effect of resumed protein synthesis on the stability of COX-2 mRNA. The cells also have the capability of maintaining a low level of COX-2 mRNA. As seen in Fig. 4B, in comparison with a typical immediate early response gene, c-*fos*, the COX-2 mRNA decay became obviously slower after the first hour of incubation, and ~25% of the COX-2 mRNA still remained until 4 hr of incubation. However, the decay of COX-2 mRNA, following induction by LPS and removal of LPS from the culture, became slower (45–75% of the COX-2 mRNA remaining at 2 hr) than that seen for COX-2 mRNA following removal of CHM (Fig. 5). For IL-1β, removal of this cytokine had either little effect on COX-2 mRNA decay rate (~57% of the COX-2 mRNA remaining for 2 hr vs ~52% with IL-1β removed for 2 hr, Fig. 5) or decreased COX-2 mRNA stability (Fig. 6). In both cases, the rate of COX-2 mRNA turnover was slower than that superinduced by CHM without further protection of CHM after the stop of transcription. The results indicated that maximal stabilization of COX-2 requires the continued presence of IL-1β in the medium (Fig. 6). In contrast to IL-1β exposure, an accelerated decay for COX-2 mRNA was found when TNF-α remained in the medium (~40% of the COX-2 mRNA remaining at 2 hr) as compared with any of the other COX-2 mRNA inducers used. Interestingly, the decay became slower when TNF-α was removed from the medium (~75% of the COX-2 mRNA remaining at 2 hr). These experiments suggest (a)

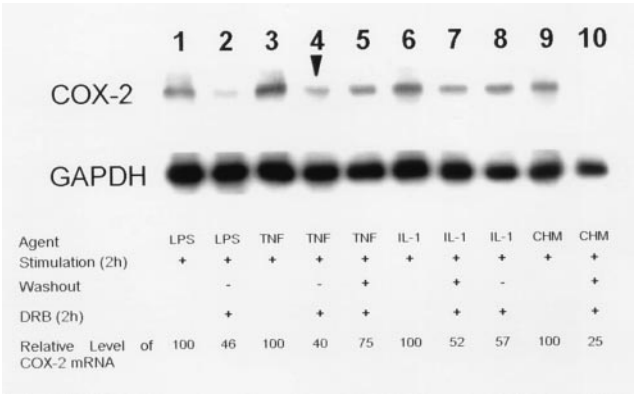

FIG. 5. Effect of LPS, TNF-α, or IL-1β stimulation on COX-2 mRNA stability in macrophages. Macrophages (~14 days in culture) were incubated with DMEM containing 0.1% BSA, and exposed to LPS (10 ng/mL), IL-1β (5 ng/mL), TNF-α (1 ng/mL), or CHM (10 mg/mL) for 2 hr. The total RNA for control levels of COX-2 mRNA (value of 100) under different stimulations was isolated at this point (lanes 1, 3, 6, and 9). The inducers were washed out as described in Materials and Methods or maintained in the medium, and transcription was stopped by 25 μg/mL of DRB. After another 2 hr of incubation, total RNA was isolated for northern analysis. The relative levels of COX-2 mRNA were calculated by normalizing quantitated COX-2 mRNA signals (densitometry) to GAPDH mRNA signals. The blots are representative of three independent experiments with similar results. The arrow (lane 4) indicates that the continued presence of TNF-α in the medium had a negative effect on the COX-2 transcript content in the macrophages (compared with lane 5).

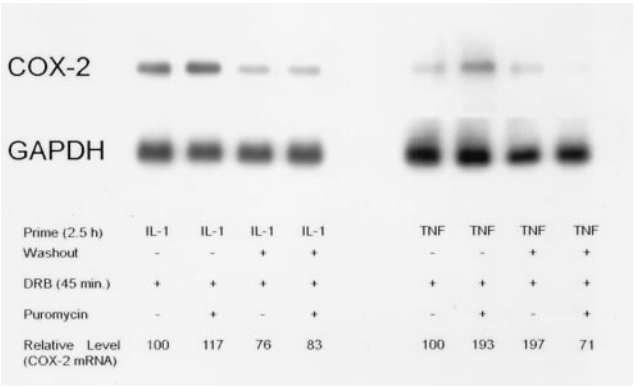

FIG. 6. Role of protein synthesis in COX-2 mRNA stability regulated by IL-1β and TNF-α. Macrophages (~14 days in culture) were incubated with DMEM containing 0.1% BSA, and exposed to IL-1β (5 ng/mL) or TNF-α (1 ng/mL) for 2.5 hr. The inducers were washed out as described in Materials and Methods or maintained in the medium, and transcription was stopped by 25 μg/mL of DRB. The translation inhibitor puromycin (100 μg/mL) was also added to selected cells to block protein synthesis. Total RNA was extracted at the end of a 45-min incubation for northern analysis. The relative value of COX-2 mRNA was calculated as the ratio of COX-2 mRNA/GAPDH mRNA. The relative value of COX-2 mRNA/GAPDH in the cells without washout and addition of puromycin was expressed as a value of 100. The blots are representative of two independent experiments with similar results.

that there is an underlying process of protein synthesis in macrophages, which involves destabilization for COX-2 transcripts, (b) that IL-1β and TNF-α increase the COX-2 mRNA levels by decreasing the rate of mRNA turnover, and (c) that the presence of the two cytokines in the medium during a later stage of stimulation has different effects on mRNA stability. Removal of IL-1β had either little effect or a destabilizing effect on COX-2 mRNA stability. In contrast, the removal of TNF-α markedly increased mRNA stability.

Role of Protein Synthesis in IL-1β- or TNF-α-induced COX-2 mRNA Stabilization

Protein synthesis has been shown to play a crucial role in the regulation of a group of unstable mRNAs, such as histone mRNA, transferrin receptor mRNA, and glucose transporter (GLUT1) mRNA [23–25]. The CHM study, as seen above in Figs. 4 and 5, suggests that basal protein synthesis was destabilizing for COX-2 mRNA in the cells. Those findings raised the possibility that IL-1β- or TNF-α-mediated regulation of COX-2 mRNA may also involve *de novo* protein synthesis. We next examined the effect of protein synthesis inhibition by adding puromycin on IL-1β- and TNF-α-primed macrophages. Puromycin is a potent translation inhibitor, but a weaker superinducer of transcription than CHM [26]. DRB nearly completely stops the transcription so that only < 1% of COX-2 mRNA induced by CHM may still involve some induction of transcription activity. Compared with using CHM, using puromycin in cytokine-induced and DRB-treated cells for the inhibition of translation could further eliminate the effect of transcription on the mRNA level. As seen in Fig. 6, while transcription was blocked by DRB, puromycin only slightly further stabilized COX-2 mRNA during a 45-min incubation (7–17%). This result suggests that IL-1β-induced protein synthesis did not affect COX-2 mRNA stabilization directly. However, in TNF-α-primed cells, puromycin blocked COX-2 mRNA decay by 60% during a 45-min incubation if the cells remained in TNF-α-containing medium. In contrast, TNF-α accelerated the decay by 50% if TNF-α was removed. There appears to be more than one process of protein synthesis in TNF-α-stimulated cells involved in COX-2 mRNA stability, one occurring early and protective, and the other occurring later and destructive.

Role of TNF-α in the Acceleration of COX-2 mRNA During the Later Stage of Stimulation

The next experiment further confirmed the destabilizing role of TNF-α for COX-2 mRNA in the later stage of the stimulation we observed in Figs. 5 and 6. As seen in Fig. 7, COX-2 mRNA was examined after a 2.5-hr incubation with TNF-α. Removal of the medium containing TNF-α resulted in an increase in the level (~40%) of COX-2 mRNA during an additional 30 min of incubation as compared with cells kept in the presence of TNF-α. The addition of fresh medium containing a second bolus of fresh TNF-α resulted in a decrease (~60%) of COX-2 mRNA (Fig. 7). The

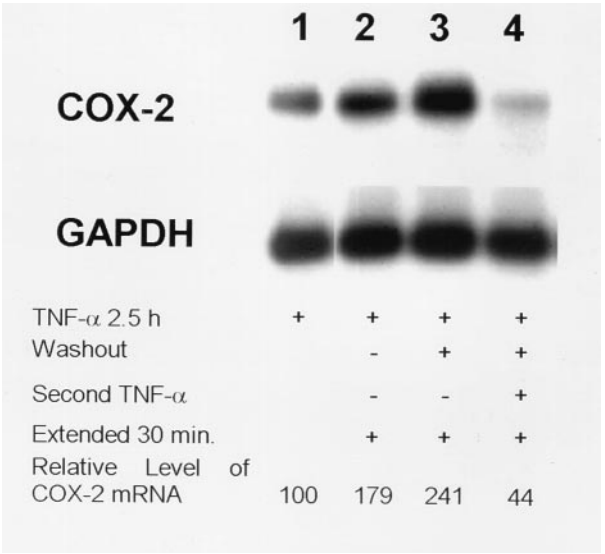

FIG. 7. Negative effect of TNF-α on COX-2 mRNA during the later phase of stimulation. Macrophages (~14 days in culture) were incubated with DMEM containing 0.1% BSA and exposed to TNF-α (1 ng/mL). After a 2.5-hr incubation period, the control cells (TNF-α, 2.5 hr) were harvested (lane 1). The experimental cells were incubated for an additional 30 min in the original medium containing TNF-α, (lane 2) or in fresh medium containing either no cytokine (washout) (lane 3) or a second dose of TNF-α (lane 4). The cells were harvested, and the steady-state levels of COX-2 mRNA were determined by northern analysis. The relative levels of COX-2 mRNA were calculated by normalizing quantitated COX-2 mRNA signals (densitometry) to GAPDH mRNA signals. The blots are representative of three independent experiments with similar results.

data indicate that the continued presence of TNF-α in the medium or the addition of a second bolus of TNF-α adversely affected the maximal induction of COX-2 mRNA. This effect appeared to be mediated directly by TNF-α rather than other autocrine molecules secreted by macrophages during TNF-α priming. These results are consistent with the observation that incubation of high levels of TNF-α (50 ng/mL) with macrophages led to less induction of COX-2 mRNA (Fig. 1), probably due to its more rapid turnover.

Inhibition of the Induction of COX-2, IL-1β, and IL-6 mRNA by Synergistic Use of IL-1β and TNF-α

Other pro-inflammatory gene transcripts such as IL-1β mRNA and IL-6 mRNA also share similar characteristics in mRNA stabilization during pro-inflammatory stimuli [20, 27]. The destabilizing role of TNF-α may also affect IL-1β-induced unstable mRNAs such as COX-2, IL-1β, and IL-6 transcripts. Thus we further examined the synergistic effect of TNF-α with IL-1β on expression of those pro-inflammatory genes. As seen in Fig. 8, both IL-1β and TNF-α induced COX-2, IL-1β, and IL-6 mRNA during a 2-hr incubation. However, the combination of the two cytokines resulted in 20–40% reduction in the induction of those mRNAs compared with those induced by IL-1β alone. These data indicate that the effects of IL-1β and

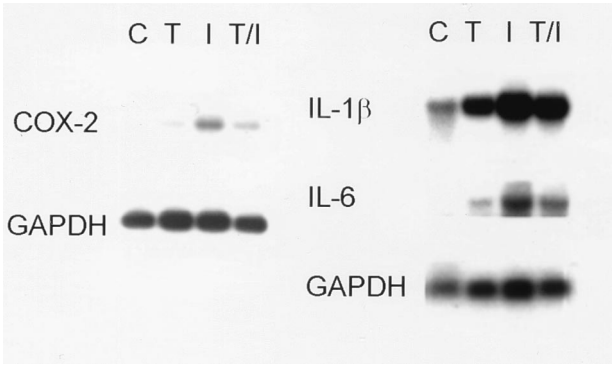

FIG. 8. Reduced induction of pro-inflammatory gene transcripts by the combined use of IL-1β and TNF-α. Macrophages (~14 days in culture) were incubated with DMEM containing 0.1% BSA, and exposed to cytokines for 2.5 hr. Total RNA was isolated and analyzed by northern analysis as described in Materials and Methods. C: control; T: TNF-α; I: IL-1β; and T/I: TNF-α (1 ng/mL) plus IL-1β (5 ng/mL). The blots are representative of two independent experiments with similar results.

TNF-α on those mRNAs are not stimulatory but inhibitory. Since TNF-α has been shown to have a concentration-dependent destabilizing role in COX-2 mRNA stability, a similar mechanism may also be responsible for inhibiting the induction of those transcripts during a combined stimulation with IL-1β and TNF-α.

DISCUSSION

In primary macrophages, a rapid and transient induction of COX-2 mRNA is a noticeable feature for the regulation of this gene expression by LPS, IL-1β, or TNF-α. A transient increase in transcript levels in response to pro-inflammatory stimuli is a common phenomenon seen in pro-inflammatory cytokines, such as interferon-β, IL-1, and TNF-α [7]. These pro-inflammatory gene transcripts, along with proliferation-related c-fos and c-myc mRNAs, share common features in the regulation of expression by the control of mRNA stability. A repeated AUUUA motif in the 3′ untranslated region, which is known to regulate mRNA stability and translatability, is found in all those gene transcripts. High levels of encoded protein products from this group of genes are usually required for only a short period of time and must be expressed in a burst [7]. With respect to its mRNA structure, expression, and biological function, COX-2 mRNA carries all the characteristics of this group of transcripts. In unstimulated macrophages, underlying protein synthesis appears to maintain a low basal level of COX-2 mRNA by accelerating its turnover. An important biological relevance of this finding is that, under non-inflammatory conditions, cells have an autoregulatory mechanism to maintain a low basal level of COX-2. This enzyme carries out catalytic activities converting free arachidonic acid into eicosanoids. Since eicosanoids are highly reactive molecules, and exhibit cytotoxicity if accumulated to a certain level, "housekeeping" protein synthesis allows cells to avoid unwanted COX-2

over-expression. However, macrophages also maintain a low, but stable level of COX-2 expression that is required for the formation of a certain amount of eicosanoids for normal cell functions, such as cell proliferation, differentiation, and apoptosis. This suggests that COX-2 mRNA, after processing, could bind to different protein components in different cellular compartments. This may determine COX-2 mRNA decay rates.

In our nuclear run-off assays, IL-1β mildly, but TNF-α barely, increased the transcription rate of the COX-2 gene. Therefore, increased transcription appeared to play only a minor role in elevating COX-2 message in human macrophages after IL-1β exposure. However, both cytokines as well as LPS did decrease the rate of COX-2 mRNA turnover as compared with CHM-superinduced COX-2 mRNA. Since a basal transcription of the COX-2 gene and basal protein synthesis have provided a condition for the regulation of the COX-2 mRNA level, alteration of protein–COX-2 mRNA interaction or protein–protein interaction could be a rapid and effective means to change COX-2 expression in response to an acute stimulation, such as inflammation. The IL-1β-induced phosphorylation of specific proteins that bind to the 3′-untranslated, AU-rich region in COX-2 transcripts has been implicated in the stabilization of COX-2 mRNA [9] in mesangial cells. These proteins are pre-existing, and do not require *de novo* synthesis. This may explain why we did not observe the correlation between the stabilization of COX-2 mRNA and protein synthesis during IL-1β stimulation. p38 mitogen-activated protein kinase has been implicated in IL-1β-induced COX-2 mRNA stabilization in monocytes [27].

In contrast, TNF-α resulted in a decreased COX-2 mRNA turnover as an early response and an increased COX-2 mRNA turnover as a second-phase reaction. The diverse effects of TNF-α on COX-2 mRNA appear to be associated with a sequential protein synthesis. TNF-α-induced synthesis of multiple proteins has been shown to regulate GLUT1 mRNA by differentially affecting 3′-untranslated AU-rich regions [28]. Similarly, the 3′-UTR of human COX-2 mRNA also has been shown to have two different regulatory regions: proximal and distal regions, the former for COX-2 stability and the latter for its instability [2]. This could provide a structure for multiple protein binding and regulation of COX-2 mRNA stability. Some protein products induced during the later phase of TNF-α stimulation accelerate COX-2 mRNA turnover, and negatively regulate this acute inflammatory reaction. In our studies, the promptness of this negative effect was also associated with a higher concentration of TNF-α or the combined use of IL-1β. Our finding is consistent with a previous report that a higher concentration of TNF-α results in reduced PGE_2 secretion in mouse macrophages [29]. Moreover, this negative role of TNF-α appeared to be able to affect other essential pro-inflammatory genes such as exogenous IL-1β-induced IL-1β and IL-6. TNF-α also can attenuate its own expression [30]. Since these genes share similarities in their transcript structure, pathophysiological function, and regulatory manner in expression during inflammation, TNF-α may have a global regulatory role in attenuating inflammatory reactions during the later stage of inflammation, probably by affecting the stability of these transcripts. This potential has long been noted and proposed: anti-TNF-α antibodies administered to mice with systemic infection were shown to accelerate animal death, confirming that TNF-α also has important protective properties [31]. During inflammatory reactions, the regulation of inflammatory gene expression by protein synthesis or protein modification has advantages over the enhancement of *de novo* transcription. It is fast and economical because it skips a lengthy process of synthesis, activation, and translocation of nuclear factor, mRNA transcription, and the most uneconomical and slowest step: mRNA post-transcriptional processing and translocation out of the nuclei. Under the condition of acute inflammatory reaction, this regulatory manner is probably the optimal one for the cells to be mobilized rapidly. A quick decline of expression of these genes prevents tissues from self-damage by their own reactive products such as prostaglandins. Based on our studies, TNF-α may play an active role in this rapid decline of pro-inflammatory gene expression during the later phase of inflammation.

TNF-α is a pro-inflammatory cytokine that carries obviously opposing effects in apoptosis, cytotoxicity, and cell death [32]. Recent studies indicate that this unique property of TNF-α is mediated by multiple independent but interactive signaling pathways. The induction of protective genes may involve TNF-R1/TRAF2 complex formation and NF-κB-dependent or -independent transcriptional events [33], whereas NF-κB plays a pro-inflammatory, but anti-apoptotic role as does IL-1 [34]. Reactive products of COX-2, such as prostaglandins and thromboxane, are potent mediators for cell apoptosis and death in lymphocytes [35–37]. However, PGE_2 also exhibits protective properties in NO-mediated apoptosis in macrophages [38, 39]. TNF-α may participate in the regulation of those events by meticulously controlling COX-2 mRNA stability, as seen in our studies.

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References

1. Smith WL, Garavito RM and DeWitt DL, Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* **271:** 33157–33160, 1996.
2. Gou Q, Liu CH, Ben-Av P and Hla T, Dissociation of basal turnover and cytokine-induced transcript stabilization of the human cyclooxygenase-2 mRNA by mutagenesis of the 3′-untranslated region. *Biochem Biophys Res Commun* **242:** 508–512, 1998.
3. Endo T, Ogushi F and Sone S, LPS-dependent cyclooxygenase-2 induction in human monocytes is down-regulated by IL-13, but not by IFN-γ. *J Immunol* **156:** 2240–2246, 1996.

4. Arias-Negrete S, Keller K and Chadee K, Proinflammatory cytokines regulate cyclooxygenase-2 mRNA expression in human macrophages. *Biochem Biophys Res Commun* **208:** 582–589, 1995.
5. Reise J, Hoff T, Nordhoff A, DeWitt DL, Resch K and Kaever V, Transient expression of prostaglandin endoperoxide synthase-2 during mouse macrophage activation. *J Leukoc Biol* **55:** 476–482, 1994.
6. Feng L, Xia Y, Garcia GE, Hwang D and Wilson CB, Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-α, and lipopolysaccharide. *J Clin Invest* **95:** 1669–1675, 1995.
7. Shaw G and Kamen R, A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46:** 659–667, 1986.
8. Appleby SB, Ristimaki A, Neilson K, Narko K and Hla T, Structure of the human cyclooxygenase-2 gene. *Biochem J* **302:** 723–727, 1994.
9. Srivastava SK, Tetsuka T, Daphna-Iken D and Morrison AR, IL-1β stabilizes COX II mRNA in renal mesangial cells: Role of 3′-untranslated region. *Am J Physiol* **267:** F504–F508, 1994.
10. Ristimäki A, Garfinkel S, Wessendorf J, Maciag T and Hla T, Induction of cyclooxygenase-2 by interleukin-1α. Evidence for post-transcriptional regulation. *J Biol Chem* **269:** 11769–11775, 1994.
11. Slater D, Berger L, Moore G and Bennett P, Implication of mRNA binding proteins in the regulation of cyclooxygenase in human amnion at term. *Biochem Biophys Res Commun* **203:** 67–71, 1994.
12. Rimarachin JA, Jacobson JA, Szabo P, Maclouf J, Creminon C and Weksler BB, Regulation of cyclooxygenase-2 expression in aortic smooth muscle cells. *Arterioscler Thromb* **14:** 1021–1031, 1994.
13. Barnes PJ, Nuclear factor-κB. *Int J Biochem Cell Biol* **29:** 867–870, 1997.
14. Jobin C, Morteau O, Han DS and Sartor RB, Specific NF-κB blockade selectively inhibits tumour necrosis factor-α-induced COX-2 but not constitutive COX-1 gene expression in HT-29 cells. *Immunology* **95:** 537–543, 1998.
15. Newton R, Stevens DA, Hart LA, Lindsay M, Adcock IM and Barnes PJ, Superinduction of COX-2 mRNA by cycloheximide and interleukin-1β involves increased transcription and correlates with increased NF-κB and JNK activation. *FEBS Lett* **418:** 135–138, 1997.
16. Dougherty GJ and McBride WH, Monocyte differentiation *in vitro*. In: *Human Monocytes* (Eds. Zembala M and Asherson GI), pp. 49–58. Academic Press, San Diego, 1989.
17. Fogelman AM, Elahi F, Sykes K, Van Lenten BJ, Territo MC and Berliner JA, Modification of the Recalde method for the isolation of human monocytes. *J Lipid Res* **29:** 1243–1247, 1988.
18. Fogelman AM, Haberland ME, Seager J, Hokom M and Edwards PA, Factors regulating the activities of the low density lipoprotein receptor and the scavenger receptor on human monocyte-macrophages. *J Lipid Res* **22:** 1131–1141, 1981.
19. Farrell RE Jr, *RNA Methodologies: A Laboratory Guide for Isolation and Characterization*, pp. 235–263. Academic Press, San Diego, 1993.
20. Fenton MJ, Review: Transcriptional and post-transcriptional regulation of interleukin 1 gene expression. *Int J Immunopharmacol* **14:** 401–411, 1992.
21. Varesio L, Espinoza-Delgado I, Gusella L, Cox GW, Melillo G, Musso T and Bosco MC, Role of cytokines in the activation of monocytes. In: *Human Cytokines: Their Role in Disease and Therapy* (Eds. Aggarwal BB and Puri R), pp. 55–69. Blackwell Science, Ann Arbor, MI, 1994.
22. Tamm I and Sehgal P, Halobenzimidazole ribosides and RNA synthesis of cells and viruses. *Adv Virus Res* **22:** 187–258, 1978.
23. Altman M and Trachsel M, Regulation of translation initiation and modulation of cellular physiology. *Trends Biochem Sci* **18:** 429–432, 1993.
24. Melefors O and Hentze MW, Translational regulation by mRNA/protein interactions in eukaryotic cells: ferritin and beyond. *Bioessays* **15:** 85–90, 1993.
25. McGowan KM, Police S, Winslow JW and Pekala PH, Tumor necrosis factor-α regulation of glucose transporter (GLUT1) mRNA turnover. *J Biol Chem* **272:** 1331–1337, 1997.
26. Edwards DR and Mahadevan LC, Protein synthesis inhibitors differentially superinduce c-*fos* and c-*jun* by three distinct mechanisms: Lack of evidence for labile repressors. *EMBO J* **11:** 2415–2424, 1992.
27. Ridley SH, Dean JL, Sarsfield SJ, Brook M, Clark AR and Saklatvala J, A p38 MAP kinase inhibitor regulates stability of interleukin-1-induced cyclooxygenase-2 mRNA. *FEBS Lett* **439:** 75–80, 1998.
28. Amano Y, Lee SW and Allison AC, Inhibition by glucocorticoids of the formation of interleukin-1α, interleukin-1β, and interleukin-6: Mediation by decreased mRNA stability. *Mol Pharmacol* **43:** 176–182, 1993.
29. Bachwich PR, Chensue SW, Larrick JW and Kunkel SL, Tumor necrosis factor stimulated interleukin-1 and prostaglandin E_2 production in resting macrophages. *Biochem Biophys Res Commun* **136:** 94–101, 1986.
30. Grewe M, Gausling R, Gyufko K, Hoffmann R and Decker K, Regulation of the mRNA expression for tumor necrosis factor-α in rat liver macrophages. *J Hepatol* **20:** 811–818, 1994.
31. Smith JG, Magee DM, Williams DM and Graybill JR, Tumor necrosis factor-α plays a role in host defense against *Histoplasma capsulatum*. *J Infect Dis* **162:** 1349–1353, 1990.
32. Malek NP, Pluempe J, Kubicka S, Manns MP and Trautwein C, Molecular mechanisms of TNF receptor-mediated signaling. *Recent Results Cancer Res* **147:** 97–106, 1998.
33. Natoli G, Costanzo A, Guido F, Moretti F and Levrero M, Apoptotic, non-apoptotic, and anti-apoptotic pathways of tumor necrosis factor signaling. *Biochem Pharmacol* **56:** 915–920, 1998.
34. William R, Watson G, Rotstein OD, Parodo J, Bitar R and Marshall JC, The IL-1β-converting enzyme (caspase-1) inhibits apoptosis of inflammatory neutrophils through activation of IL-1β. *J Immunol* **161:** 957–962, 1998.
35. Perez R, Johnson J, Winkler JD, Rudich S, Carter L, Katznelson S and German JB, Kupffer cell-mediated lymphocyte apoptosis: A PGE_2-dependent mechanism of portal venous transfusion-induced immunosuppression? *J Surg Res* **78:** 37–41, 1998.
36. Brown DM, Warner GL, Ales-Martinez JE, Scott DW and Phipps RP, Prostaglandin E_2 induces apoptosis in immature normal and malignant B lymphocytes. *Clin Immunol Immunopathol* **63:** 221–229, 1992.
37. Pica F, Franzese O, D'Onofrio C, Paganini L, Favalli C, Bonmassar E and Garaci E, Effect of PGE_2 on c-Myc and Bcl-2 production and programmed cell death in human lymphocytes. *Adv Prostaglandin Thromboxane Leukot Res* **23:** 457–459, 1995.
38. von Knethen A and Brune B, Cyclooxygenase-2: An essential regulator of NO-mediated apoptosis. *FASEB J* **11:** 887–895, 1997.
39. Zamora R, Bult H and Herman AG, The role of prostaglandin E_2 and nitric oxide in cell death in J774 murine macrophages. *Eur J Pharmacol* **349:** 307–315, 1998.