Combinations of ERK and p38 MAPK Inhibitors Ablate Tumor Necrosis Factor-α (TNF-α) mRNA Induction

EVIDENCE FOR SELECTIVE DESTABILIZATION OF TNF-α TRANSCRIPTS*

Karine Rutault‡, Catherine A. Hazzalin§, and Louis C. Mahadevan¶

From the Nuclear Signalling Laboratory, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom

Tumor necrosis factor-α (TNF-α) is a potent proinflammatory cytokine whose synthesis and secretion are implicated in diverse pathologies. Hence, inhibition of TNF-α transcription or translation and neutralization of its protein product represent major pharmaceutical strategies to control inflammation. We have studied the role of ERK and p38 mitogen-activated protein (MAP) kinase in controlling TNF-α mRNA levels in differentiated THP-1 cells and in freshly purified human monocytes. We show here that it is possible to produce virtually complete inhibition of lipopolysaccharide-stimulated TNF-α mRNA accumulation by using a combination of ERK and p38 MAP kinase inhibitors. Furthermore, substantial inhibition is achievable using combinations of 1 μM of each inhibitor, whereas inhibitors used individually are incapable of producing complete inhibition even at high concentrations. Finally, addressing mechanisms involved, we show that inhibition of p38 MAP kinase selectively destabilizes TNF-α transcripts but does not affect degradation of c-jun transcripts. These results impinge on the controversy in the literature surrounding the mode of action of MAP kinase inhibitors on TNF-α mRNA and suggest the use of combinations of MAP kinase inhibitors as an effective anti-inflammatory strategy.

Monocytes and macrophages play a pivotal role in inflammation and immune regulation. Upon activation, monocytes produce and release many inflammatory mediators such as IL-1,1 IL-6, IL-8, TNF-α, or arachidonic acid metabolites, as well as the anti-inflammatory mediators IL-10, soluble TNF receptor, and IL-1 receptor antagonist. Among the different products released, TNF-α is thought to be one of the most important mediators of inflammatory disease (1), and therefore the understanding of molecular mechanisms of TNF-α gene induction is of considerable medical interest.

Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is a very potent inducer of TNF-α production and release in human monocytes. Two proteins, CD14 and LPS-binding protein (LBP), are implicated in mediating the cellular response to LPS (2–5). CD14 is a 55-kDa glycoprotein found as a glycosyl phosphatidylinositol-anchored membrane protein (mCD14) and as a soluble form (sCD14) in plasma. LBP is a 60-kDa plasma protein that binds to LPS and functions primarily to accelerate the binding of LPS to CD14 (5). However, because CD14 lacks transmembrane and intracellular domains, the mechanism by which the LPS signal is transduced across the plasma membrane is not fully understood.

Recent work indicates that Toll-like receptors, a family of human receptors related to Drosophila Toll, mediate LPS-induced signal transduction and that this response is dependent on LBP and enhanced by CD14 (6–9).

The involvement of all three mitogen-activated protein (MAP) kinase subtypes, c-Jun N-terminal kinase (10, 11), p38 (12–14), and the extracellular signal-regulated kinase (ERK) p42/44 (13, 15), in LPS-induced cytokine production and release has been documented extensively. However, the precise role of these MAP kinase subtypes in the processes of transcriptional induction and/or translation of cytokine transcripts remains in contention. In particular, although the production of human TNF-α in response to LPS is clearly regulated at both transcriptional and post-transcriptional levels (16, 17), the role of the three MAP kinase subtypes, especially of p38 MAP kinase, within these processes has been quite controversial. The major advance in this area was the discovery of pyridinyl imidazole compounds, such as SB203580, which was identified by a high-throughput screen for compounds that would inhibit the release of proinflammatory cytokines including TNF-α. These compounds function by binding specifically to and inhibiting p38 MAP kinase (12). However, in the study of Lee et al., it was claimed that inhibition of p38 primarily affected the translation of the TNF-α transcripts but not the level of TNF-α mRNA in these cells. By contrast, we have shown that induction of several immediate-early genes is clearly inhibited at the transcriptional level by these compounds (18, 19). In addition, pyridinyl imidazoles have now been reported to affect the LPS-stimulated transcription of several cytokine genes including IL-1β (20, 21). Manthey et al. (20) reproduced earlier findings (12, 22, 23) that p38 inhibitors appeared to suppress TNF-α protein levels more than TNF-α mRNA. By contrast, Dean et al. (14) using these inhibitors to study LPS-stimulated cyclooxygenase-2 and TNF-α production, came to the very opposite conclusion and found no discrepancy between the suppression of TNF-α protein and mRNA levels, suggesting that inhibition of mRNA accumulation might account completely for the effect of this compound on TNF-α secretion in human blood mono-

* 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.
‡ Supported by Aventis.
§ Supported by the Cancer Research Campaign.
¶ To whom correspondence should be addressed. Tel.: 44-1865-285-345; Fax: 44-1865-275-259; E-mail: louiscm@bioch.ox.ac.uk.
1 The abbreviations used are: IL, interleukin; TNF-α, tumor necrosis factor α; LPS, lipopolysaccharide; LBP, LPS-binding protein; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein ERK kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; DRB, 5,6-dichlorobenzimidazole riboside; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay.
cytes. A further complication is the very recent indication of a novel mode of control of TNF-α transcript levels, namely through modulation of their sensitivity to degradation (24–26).

The differences outlined above may arise from the use of different cell types or different experimental methods to measure gene induction. The low numbers of primary monocytes purified from human blood, and the use of THP-1 cell line in place of primary monocytes, most likely account for the differences in gene induction levels. The human monocytic cell line THP-1 corresponds to an immaturity stage of monocyte differentiation and may not accurately reflect the situation in mature monocytes. Here, we show first that although THP-1 cells are a poor model for studying LPS-stimulated TNF-α mRNA induction, they become more sensitive to LPS stimulation after differentiation with TPA and compare well with mature human monocytes purified from human blood. Moreover, we show in TPA-differentiated THP-1 cells and in purified blood monocytes that individual blockade of p38 or ERK pathways does certainly affect TNF-α transcript levels, although neither inhibitor is capable of completely blocking TNF-α mRNA induction. Most importantly however, using a combination of these inhibitors it is possible to ablate TNF-α induction totally at the mRNA level. Finally, we show that the inhibition of p38 MAP kinase selectively destabilizes TNF-α transcripts without affecting other inducible transcripts such as c-jun. These results are discussed in relation to the controversy in the literature outlined above and to the possible use of combinations of MAP kinase inhibitors at reduced concentrations as a therapeutic strategy.

EXPERIMENTAL PROCEDURES

Materials—Lymphoprep was from Nycomed Pharma (Oslo, Norway). RPMI 1640 medium and Hanks’ balanced salt solution were purchased from Life Technologies, Inc. FCS was obtained from Globepharm (Guildford, UK). LPS (Escherichia coli serotype 055:B5) and TPA were from Sigma. SB203580 was a gift from J. S. Sweeney (Aventis Pharma Ltd., Dagenham, UK). D98059 was obtained from Alexis (Nottingham, UK). U0126 was purchased from Promega (Southampton, UK). The following monoclonal antibodies were used: fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins and CD54 were from Dako (Glostrup, Denmark); CD14 (Hb246) and CD3 (UCHT-1) were from ATCC; CD19 (BU12) was a gift of D. Hardie (University of Birmingham, UK), CD58 was from Roche Molecular Biochemicals. Rabbit anti-phospho-p38 antibody was purchased from New England Biolabs (Hitchin, UK). Dynabeads M-450 sheep anti-mouse IgG were from Dynal (Oslo, Norway). [32P]CTP was purchased from ICN Pharmaceuticals (Irvine, CA). The random primed DNA labeling kit was obtained from Roche Molecular Biochemicals.

Cell Culture—THP-1 cells were grown in RPMI 1640 medium with 10% fetal calf serum and 20 μM β-mercaptoethanol. Differentiated THP-1 cells were obtained by treatment with 5 nM TPA for 2 days and then starved overnight in 0.5% FCS-RPMI in the presence of 5 nM TPA before stimulation. Human peripheral blood monocytes were freshly prepared from the buffy coat fraction of 1 unit of donor blood. Mononuclear cells were separated on Ficoll Lymphoprep (400 g, 30 min) and the remaining red blood cells were lysed for 15 min in 0.1% Tri-HCl containing 8 g/liter ammonium chloride. Mononuclear cells were then allowed to adhere (5 × 10^6 cells/ml) in 10% FCS-RPMI. After 90 min at 37 °C, 6% CO2, nonadherent cells were removed, and adherent cells were recovered by a 30-min treatment in 0.02% EDTA. Monocytes were then depleted of contaminating T and B cells using CD3 and CD19 monoclonal antibodies followed by sheep anti-mouse IgG-coated Dynabeads.

Monocyte purity was assessed by fluorescence-activated cell sorting in a Becton Dickinson FACScan. Cells were <3% CD3 or CD19 and >90% CD14. Cells were rendered quiescent by an overnight incubation in 0.5% FCS-RPMI before stimulation for MAPK analysis or Northern blots.

p38 Activation by Western Blot—THP-1 or TPA-differentiated THP-1 cells were lysed in ice-cold lysis buffer consisting of 20 mM Heps, pH 8, 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 10% glycerol, 1 mM dithiothreitol, 400 mM KCl, 0.4% Triton X-100, 20 mM sodium β-glycerophosphate, 1 μM microcystin, 0.2 μM okadacid acid, 0.1 mM sodium orthovanadate, and proteasome inhibitors. Lysates were incubated on ice for 10 min and cleared by centrifugation at 13,000 × g for 10 min. Protein concentrations were measured in the supernatants by the Bradford method (27), and equal amounts of proteins were loaded onto electrophoresis gels.

p38 and ERK Activation by Western Blot—Expression of p38 and ERK activation was measured by Western blot analysis in specific phospho-p38 antibodies. Cell extracts were loaded on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore). Membranes were blocked overnight at 4 °C in 5% skimmed milk in TBST (20 mM Tris-HCl, pH 8, 137 mM NaCl containing 0.1% Tween 20). After several washes, membranes were probed with phospho-specific antibodies and peroxidase-conjugated secondary antibody for 1 h at room temperature. After washes, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h, and blots were then developed using a chemiluminescent detection.

Northern Blot Analysis—Total cellular RNA in THP-1 cells was isolated according to the method of Chomczynski and Sacchi (28). RNA from human blood monocytes was purified using the Purescript RNA isolation kit (Genta system, Minneapolis, MN). Aliquots containing 5 μg of RNA were resolved on 1% agarose gels containing 0.41 mM formaldehyde. RNA was transferred onto nylon membranes (Hybond N+, Amersham Pharmacia Biotech) by capillary transfer in 50 mM NaOH. cDNA probes for TNF-α, c-jun, and GAPDH were labeled with 50 μCi of [α-32P]CTP by random priming. GAPDH mRNA was detected using a 738-base pair EcoRI fragment of human TNF-α cDNA clone in pBluescript SKⅠ’, kindly provided by D. R. Edwards (University of East Anglia, Norwich, UK). TNF-α mRNA was detected using a 738-base pair EcoRI fragment of human TNF-α cDNA clone in pbluescript SKⅠ’, kindly provided by D. R. Katz (University College London). c-jun mRNA was detected using a 749-base pair EcoRI/SalI fragment derived from the mouse c-jun PHA1119 plasmid (generously provided by R. Bravo, European Molecular Biology Laboratory, Heidelberg, Germany). TNF-α and c-jun mRNA were visualized by autoradiography and quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and corrected by reference to the corresponding GAPDH reading to compensate for slight variation in loading.

Flow Cytometry Analysis—Cells (10^6 cells/ml) in RPMI containing 10% rabbit serum and 0.1% NaN3 were incubated for 30 min at 4 °C with murine monoclonal antibodies followed by a 30-min incubation with fluorescein isothiocyanate rabbit anti-mouse IgG. After washing, cells were fixed in 3.7% formaldehyde in Hanks’ balanced salt solution and analyzed using a FACScan flow cytometer (Becton Dickinson).

RESULTS

Differentiated THP-1 Cells Show Stronger Responsiveness to LPS Stimulation: Analysis of TNF-α mRNA and Protein Levels—The human monocytic cell line THP-1 corresponds to an immature stage of monocyte differentiation and thus does not represent an ideal model of human monocytes for studying cytokine regulation. Several studies have shown that TPA treatment of THP-1 cells resulted in a more differentiated phenotype in terms of adherence, loss of proliferation, or expression of surface markers (29, 30). To study the levels at which TNF-α production is regulated, we first compared LPS-induced TNF-α mRNA and protein in TPA-differentiated and nondifferentiated THP-1 cells. Normal THP-1 cells or THP-1 cells differentiated for 2 days with 5 nM TPA were incubated with 10 ng/ml of LPS, and TNF-α gene induction was evaluated by Northern blot analysis (Fig. 1A). In THP-1 cells, LPS only weakly induces TNF-α transcripts, whereas TPA differentiation with 10 ng/ml of TPA resulted in higher levels of TNF-α mRNA, which was detectable in response to LPS (Fig. 1A). In the differentiated cells, TNF-α mRNA was detectable 30 min after LPS treatment. Maximal induction was observed between 1 and 2 h, and transcript levels declined thereafter, with barely detectable levels remaining after 6 h. We have shown further that this response is detectable in the presence of translational...
inhibitors (data not shown), and this finding, together with the transient response, characterizes the TNF-α gene as an immediate-early gene in response to LPS stimulation.

We next analyzed levels of TNF-α protein released in the culture medium of these two cell systems in response to LPS. Dose-response analysis showed that normal THP-1 cells secreted only low levels of TNF-α protein when stimulated with up to 1 μg/ml of LPS, and we could only detect significant amounts of TNF-α in these supernatants using 5 μg/ml of LPS (Fig. 1B). By contrast, the TPA-differentiated cells secreted large quantities of TNF-α protein following treatment with concentrations of LPS as low as 1 ng/ml, the lowest concentration tested (Fig. 1B); this was approaching maximal and showed no significant further increase at higher concentrations.

Time-course analysis showed that normal THP-1 cells produce no detectable TNF-α protein over a 48-h incubation period using 100 ng/ml LPS (Fig. 1C), the same stimulus as used for analysis of transcript levels shown above. By contrast, after TPA differentiation, these cells release detectable quantities of TNF-α cytokine as early as 2 h after LPS-stimulation. The level of secreted protein peaks at around 6 h and diminishes to a quite stable but submaximal level over 48 h. Dose-response and time course analyses were also performed in freshly isolated monocytes from human blood (discussed further below). TNF-α mRNA and protein levels compared well with those seen in TPA-differentiated THP-1 cells (data not shown). These studies revealed a very marked enhancement of TNF-α response in the TPA-differentiated cells, especially when stimulated with lower concentrations of LPS. Thus, TPA-differentiated THP-1 cells are a better model for events in purified human monocytes than the nondifferentiated THP-1 cells, in particular for the study of TNF-α mRNA and protein.

**TPA-induced Differentiation of THP-1 Cells Increases Expression of CD14 and CD54 Cell Surface Markers**—To confirm that differentiated THP-1 cells mimic human monocytes more closely than undifferentiated cells, we next determined the levels of expression of two monocyte surface markers, CD14 and CD54, in undifferentiated and TPA-differentiated cells by FACSan and compared it with expression levels in purified human blood monocytes. This showed that TPA treatment enhanced the expression of monocyte markers in THP-1 cells (Fig. 2). Undifferentiated THP-1 cells very weakly express the LPS receptor CD14; only 13.1% of the cells are positive. By contrast, 42.2% of differentiated THP-1 cells express the CD14 marker compared with 94.7% in purified blood monocytes. The same observation was made in analyses of the adhesion molecule CD54 (ICAM-1). CD54 is not detectably expressed in normal THP-1 cells but is expressed in 53.9% of the TPA-differentiated cells compared with 61.3% in purified blood monocytes. This confirms indications from the TNF-α expression studies above that TPA-differentiated THP-1 cells are a better model for human monocytes than the undifferentiated cells. Because CD14 is part of the LPS receptor, the increased levels of CD14 seen here may explain why the differentiated cells mount a stronger response to LPS than undifferentiated cells.

**LPS Induces p38 Activation in TPA-differentiated THP-1 Cells and Blood Monocytes**—Because TPA differentiation increases the expression of the LPS receptor CD14, we then compared the ability of LPS to activate p38 MAP kinase in THP-1 and TPA-differentiated THP-1 cells to that in purified human blood monocytes. For positive controls, stimuli such as anisomycin, UV radiation, and hyperosmotic stress, which are known to strongly activate p38, were used. As expected, anisomycin, UV radiation, or osmotic stress using sodium chloride or sorbitol strongly induced p38 activation in normal THP-1 cells (Fig. 3) showing that the p38 cascade is present and activated normally in these cells. However, LPS stimulation only produced very weak and barely detectable activation of p38 in these cells. By contrast, in TPA-differentiated THP-1 cells, as in purified blood monocytes, all of the five stimuli tested, including LPS, proved to be very efficient in activating the p38 pathway. This finding reiterates at the signal transduction level all previous indications that the differentiated cells more closely resemble mature monocytes than the undifferentiated cells.

**Inhibition of ERK or p38 MAP Kinase Cascades Differentially Affects TNF-α Protein Released by Human Monocytes**—Lee et al. (12) showed that pyridinium imidazole compounds that inhibit p38 MAPK are very potent inhibitors of LPS-induced TNF-α secretion in human monocytes. Further, PD98059, a MEK inhibitor that blocks ERK activation, also causes a marked inhibition of LPS-stimulated TNF-α release in human blood monocytes (13). To investigate the mechanism of action of
these two inhibitors, we first carried out dose-response analyses to assess the level of inhibition of TNF-α protein released by purified blood monocytes.

Human monocytes were purified from buffy coat preparations as described (see “Experimental Procedures”) and pretreated for 30 min with the indicated concentrations of the p38 inhibitor SB203580 (Fig. 4A) or the MEK1/2 inhibitor PD98059 (Fig. 4B), followed by a 16-h incubation with 10 ng/ml LPS. Cell supernatants were then harvested and assayed for the presence of TNF-α protein. These data confirmed that both inhibitors inhibit LPS-induced TNF-α release from human monocytes in a dose-dependent manner. PD98059 only partially blocked TNF-α production (47% inhibition using 50 μM PD98059). In agreement with previous data, the p38 inhibitor SB203580 was shown to be a very powerful inhibitor of TNF-α release, since it blocked more than 77% of the TNF-α production at a concentration of 120 nM (Fig. 4A). No obvious loss of viability of human monocytes cells was detected at the concentrations of inhibitors used.

Inhibition of ERK or p38 MAP Kinase Pathways Reduces LPS-stimulated TNF-α mRNA in Purified Human Monocytes—The inhibition seen with these compounds, particularly the p38 inhibitor, has previously been ascribed principally to translational inhibition of TNF-α transcripts, with minimal effect on the levels of transcripts (12, 22, 23). We next asked whether the presence of SB203580 or PD98059 would have any effect on the level of TNF-α mRNA in purified human monocytes. These experiments were done in triplicate, but because of the difficulty of obtaining purified monocytes in sufficient numbers for mRNA analysis, only a subset of data points corresponding to the protein data from Fig. 4, A and B, was chosen for mRNA analysis (Fig. 5, A and B). This showed that over the concentration range selected, SB203580 produced increasing and pronounced inhibition of TNF-α transcript levels, contradicting the proposal that SB203580 acts primarily at the level of translational regulation. It is important to note that a reduction in secreted TNF-α protein (Fig. 4A) was always more substantial than its effects on transcript levels (Fig. 5A). This finding is in agree-
mental with published data indicating TNF-α translation as another point of action of this inhibitor. However, contrary to the prevailing view, the data showed that SB203580 also has a clear concentration-dependent effect on TNF-α transcript levels, and this must also contribute to the overall loss of TNF-α production. By contrast, the milder reduction of TNF-α protein seen with varying concentrations of PD98059 (Fig. 4B) corresponds well with a reduction in transcripts (Fig. 5B) and conceivably could account for most of the reduced protein levels seen under these conditions. Although strict comparisons of protein and transcripts levels are not safe, the data suggest that as opposed to SB203580, which affects TNF-α production at both mRNA and translational levels, the less marked effects of PD98059 may occur principally at the mRNA level.

Combination of SB203580 and PD98059 Produce Virtual Ablation of TNF-α Induction at the mRNA Level—We next investigated whether combinations of the two inhibitors described above might produce more pronounced inhibition of TNF-α transcript levels. Because of the difficulty of obtaining sufficient quantities of purified monocytes for these studies, we first analyzed these phenomena in TPA-differentiated THP-1 cells, shown above to be a good model of human monocytes. Cells were pretreated with different concentrations of SB203580, PD98059, or a combination of both compounds as indicated (Fig. 6A) for 60 min before LPS stimulation (100 ng/ml, 60 min). Used on its own, each inhibitor at the highest concentration tested blocked slightly more than 50% of LPS-stimulated TNF-α mRNA accumulation. Note that SB203580 was less efficient in inhibiting TNF-α mRNA expression in differentiated THP-1 cells than in purified human monocytes, (50% inhibition compared with >75% inhibition using 10 μM SB203580). More interestingly, we observed that a combination of SB203580 (10 μM) and PD98059 (50 μM) almost completely ablated TNF-α mRNA accumulation in response to LPS, suggesting that p38 and ERK pathways are both necessary for maximal TNF-α gene induction.

This observation was then confirmed using purified human blood monocytes (Fig. 6B). Again, more than 90% inhibition of TNF-α gene induction was obtained using a combination of the two inhibitors. We then carried out repeated analyses of the effect of these inhibitors on LPS-stimulated TNF-α mRNA levels in several different preparations of human monocytes and TPA-differentiated THP-1 cells, subjecting the data to quantitative and statistical analyses (Table I). Over the course of several experiments that were in close agreement, LPS-induced TNF-α mRNA levels were inhibited on average by 53.7% in TPA-differentiated THP-1 cells and by more than 79% in TPA-differentiated THP-1 cells.

![Figure 4](http://www.jbc.org/)

**Fig. 4. Effect of SB203580 and PD98059 on TNF-α release in purified human blood monocytes.** A, freshly purified blood monocytes were pretreated for 30 min with Me2SO (□) or various concentrations of SB203580 (■) before a 16-h incubation with 10 ng/ml LPS. TNF-α levels were then measured by ELISA. Data are the means of quadruplicate culture supernatants ± S.E. and are representative of at least two separate experiments. B, the same conditions as in A, using different concentrations of PD98059 (■).

![Figure 5](http://www.jbc.org/)

**Fig. 5. Effect of SB203580 and PD98059 on TNF-α gene induction, analyzed by Northern blot, in purified human blood monocytes.** A, freshly purified blood monocytes were pretreated for 30 min with Me2SO (□) or various concentrations of SB203580 (■) followed by stimulation with 100 ng/ml LPS for 1 h. Total cellular RNA was then isolated using the Purescript RNA isolation kit and analyzed by Northern blot using cDNA probes for TNF-α and GAPDH. Quantification of radioactivity was performed by phosphorimaging, corrected for loading against the corresponding GAPDH signals, and expressed as a percent ± S.E. of the signal measured with LPS only. Quantification of the data represents the average of three independent experiments. B, the same conditions as in A, using different concentrations of PD98059 (■).
Regulation of Tumor Necrosis Factor-α at the mRNA Level

Inhibition of p38 MAPK Destabilizes TNF-α mRNA but Not c-jun mRNA—Contrary to previous work, these data show that TNF-α mRNA levels are considerably reduced by MAP kinase inhibitors. However, Baldassare et al. (21) using nuclear run-on analyses, provided evidence that SB203580 does not significantly affect LPS-stimulated TNF-α gene transcription, raising the possibility that the compound could have an effect on TNF-α transcript stability. To address this possibility, we then analyzed the effect of SB203580 and PD98059 on TNF-α mRNA stability. Differentiated THP-1 cells were stimulated for 1 h with 100 ng/ml of LPS, following which the transcriptional inhibitor DRB (25 μg/ml) was added to the cells to block any further transcription of the TNF-α gene. Five minutes later, MAP kinase inhibitors were added to the cells and the levels of TNF-α, c-jun, and GAPDH mRNA over the next 90 min were measured by Northern blotting analyses (Fig. 8A).

In the absence of inhibitors, TNF-α transcripts decayed gradually over 90 min (TNF-α mRNA half-life = 52 ± 7.5 min). This decay is slightly quicker in the presence of PD98059 (t1/2 = 39.2 ± 9.4 min) but when cells are treated with SB203580, pronounced acceleration of TNF-α transcript degradation was obtained (t1/2 = 26.4 ± 4.8 min) and no TNF-α mRNA could be detected after 60 min of LPS treatment. To assess whether this effect on mRNA stability was specific to TNF-α, we compared this with the degradation of c-jun mRNA, also induced by LPS, by re-probing this blot for c-jun transcripts (Fig. 8A). This showed that in TPA-differentiated THP-1 cells c-jun transcripts decayed more rapidly than TNF-α (c-jun mRNA t1/2 = 33.4 ± 4.1 min without an inhibitor). The addition of either p38 or ERK pathway inhibitors had no effect on c-jun mRNA stability (t1/2 = 30.2 ± 4.9 min in the presence of SB203580; t1/2 = 29.9 ± 5.7 min in the presence of PD98059). No change was observed in GAPDH transcripts, which are extremely stable. Finally, we asked whether a combination of SB203580 and PD98059 would have additive or synergistic effects on TNF-α mRNA stability (Fig. 8B). Over five separate experiments, we found that the majority of the destabilizing effect could be accounted for by the presence of SB203580 and that the additional presence of PD98059 only very slightly accelerated this decay (Fig. 8B). We conclude that p38 MAP kinase appears to have the major role in stabilizing TNF-α transcripts, although a slight effect of PD98059 was reproducibly observed. These findings are discussed further below in the context of recent observations of novel stability determinants, potentially regulated via MAP kinase cascades, in the 3’ untranslated regions of this gene.

**DISCUSSION**

In their seminal paper, Lee et al. (12) identify p38 MAP kinase as the primary target of anti-inflammatory pyridinyl imidazole drugs, previously shown to inhibit LPS-stimulated production of pro-inflammatory cytokines such as IL-1 and TNF-α. Several pieces of data have been advanced to support the idea that the inhibitory effect of these drugs on the production of cytokines, and in particular TNF-α, is mediated primarily at the translational level (12, 22, 23), a finding that is much cited in the literature (10, 13, 15, 21). In contrast, a recent study carried out by Dean et al. (14) reported that pyridinyl imidazole compounds inhibit cyclooxygenase-2 and TNF-α in response to LPS at the mRNA level, suggesting that the decrease in mRNA could fully account for the reduced protein levels.

In this report we have re-examined, using pyridinyl imidazole compounds, the role of p38 MAPK in LPS-induced TNF-α release in TPA-differentiated THP-1 cells as well as in purified

---

**Blood samples**

Blood monocytes (75–86%) are obtained from healthy volunteers by density gradient separation. They are cultured for 3 days in the presence of phorbol 12-myristate 13-acetate (TPA) and then stimulated with LPS or SB203580 (a p38 inhibitor) or PD98059 (a MEK inhibitor) for 1 h, followed by Northern blot analysis using cDNA probes for TNF-α and GAPDH. The quantification of radioactivity shown on these blots was performed by phosphorimaging, corrected for loading against the corresponding GAPDH signals, and expressed as fold-stimulation over the signal in control unstimulated cells. Data are representative of three independent experiments.

**Fig. 6. Effect of combinations of SB203580 and PD98059 on TNF-α gene induction, analyzed by Northern blot.** A, TPA-differentiated THP-1 cells were pretreated for 30 min with MeSO4 ( ), or with SB203580 (SB, /), PD98059 (PD, ) or a combination of both compounds (SB + PD, ) at the concentrations indicated followed by stimulation with 100 ng/ml LPS for 1 h. Total cellular RNA (5 μg) was then isolated and analyzed by Northern blot using cDNA probes for TNF-α and GAPDH. The quantification of radioactivity shown on these blots was performed by phosphorimaging, corrected for loading against the corresponding GAPDH signals, and expressed as fold-stimulation over the signal in control unstimulated cells. Data are representative of three independent experiments. B, Blood monocytes were freshly purified and pretreated for 30 min with MeSO4 ( ), 10 μM SB203580 (SB), 50 μM PD98059 (PD), or a combination of both compounds at these concentrations (SB + PD) followed by stimulation with 100 ng/ml LPS for 1 h. Cellular RNA was isolated and analyzed as detailed in A. Data are representative of three independent experiments.
TPA-differentiated THP-1 cells or purified blood monocytes were pretreated for 30 min with SB203580 (10 μM), PD98059 (50 μM), or both compounds followed by stimulation with 100 ng/ml LPS for 1 h. Total RNA was then extracted, and Northern blots were performed. The numbers show the percent of inhibition ± S.E. of LPS-induced TNF-α gene induction compared with cells treated with LPS only and correspond to the average of at least three independent experiments.

| Pretreatment | TPA-differentiated THP-1 cells | Purified blood monocytes |
|--------------|-------------------------------|--------------------------|
| SB203580     | 53.7 ± 4.3% (n = 5)           | 79.9 ± 3.7% (n = 8)      |
| PD98059      | 50.2 ± 1.9% (n = 4)           | 46.5 ± 19.9% (n = 8)     |
| SB203580 + PD98059 | 91.8 ± 2.6% (n = 4) | 93.3 ± 6.6% (n = 3)      |

The p38 inhibitor SB203580 and the ERK pathway inhibitor PD98059 have been reported to act individually and in combination to reduce LPS-stimulated secretion of TNF-α protein in human monocytes (13), but that study did not distinguish between transcriptional, post-transcriptional, or translational modes of action. We have now extended these findings and show here that a combination of ERK and p38 inhibitors is able to ablate LPS-stimulated TNF-α induction at the mRNA level. Thus, both p38 and ERK pathways are necessary for normal LPS-induced TNF-α mRNA accumulation in these cells. Furthermore, using a new MEK inhibitor, U0126, which is effective at lower concentrations, together with SB203580, we observed virtually complete ablation of TNF-α transcripts by using a combination of inhibitors at a concentration of 1 μM each.

The effect of p38 inhibition on TNF-α mRNA levels could be due to decreased transcription and/or reduced mRNA stability. Baldassare et al. (21) performing nuclear run-on analysis showed SB203580 did not inhibit TNF-α expression at the transcriptional level, in contrast with the effect of this inhibitor on IL-1β transcription. Since we observed SB203580 greatly decreased TNF-α mRNA level, we assessed the effect of this compound on TNF-α mRNA stability and showed that this compound clearly destabilizes TNF-α mRNA in response to LPS, providing evidence for a role of p38 in TNF-α mRNA stabilization. We reproducibly observed a slight effect of PD98059 on TNF-α mRNA stability but to a much lesser extent than with SB203580. The combination of both inhibitors produced only slightly accelerated destabilization compared with the use of SB203580 alone. These data suggest that p38 MAP kinase influences both the levels of TNF-α transcripts and their translation, whereas the effect of ERK inhibition on TNF-α transcript levels alone could be sufficient to account completely for its effect on TNF-α production. Most importantly, however, this study suggests the use of combinations of MAP kinase inhibitors at reduced concentrations as a most efficient means of inhibiting TNF-α production.

Wang et al. (31), studying the effect of another p38 inhibitor SB202190 on cytokine production by reverse transcriptase-polymerase chain reaction, also suggested that specific mRNA destabilization represents an important site of action for the cytokine suppressive effect of p38 inhibitors. However, there are crucial differences between our work and that of Wang et al. (31). First, they used the p38 inhibitor either 2 h before or instead of the transcriptional inhibitor actinomycin D, and thus, their studies do not totally exclude an indirect effect of SB202190 on cytokine mRNA levels that involves transcription. In our experiments, transcription was halted with 5,6-
absence of any further transcription. A second important difference is that Wang et al. (31) suggest that this effect might be mediated through AU-rich elements and could therefore affect all transcripts that carry these motifs within their 3′ untranslated regions, including c-fos and c-jun, in which induction is known to be inhibited by SB203580 (18). However, this assertion did not accommodate the earlier demonstration that SB203580 clearly did not affect the stability of these two transcripts nor that of three other transiently induced messages (19) in mouse fibroblasts. To eliminate cell-specific variability, we have shown directly that under identical conditions c-jun transcripts in the cells used here are not affected by blockade of p38 MAP kinase, whereas TNF-α mRNA is destabilized. Thus, our data do not agree with the assertion that SB203580 destabilizes all transcripts with AU-rich elements in their 3′ untranslated regions and argue instead for destabilization that is selectively targeted at a subset of AU-rich-element-containing labile transcripts.

Recent data suggest a role for the p38 pathway in the posttranscriptional regulation of several cytokines (26, 32, 33). A key factor of TNF-α regulation is the presence of AU-rich elements in the 3′ untranslated region of TNF-α mRNA (34, 35); several proteins could regulate cytokine mRNA stability by binding to the regulatory AU-rich elements such as AUF1 (36), TIAR (35), or tristetraprolin (24, 25). Tristetraprolin is a member of a family of zinc finger proteins, which has been shown to interfere with cytokine production by binding to AU-rich elements and destabilizing TNF-α mRNA (24, 25, 37) as well as granulocyte/macrophage colony-stimulating factor (38). However, little is known about the signaling pathways involved in the binding of this protein on the AU-rich elements. In NIH-3T3 cells, tristetraprolin has been shown to be phosphorylated rapidly on Ser residues after stimulation with different mitogens, and p42 MAPK could in vitro phosphorylate tristetraprolin on Ser-220 (39). Since we show here that p38 is clearly involved in controlling TNF-α mRNA stability, it would be interesting to investigate whether tristetraprolin can be directly or indirectly phosphorylated by p38 MAPK in human monocytes following LPS stimulation, thereby explaining how MAP kinases may selectively regulate TNF-α mRNA stability. Note that since the submission of this paper, Brook et al. (40) have also reported that inhibition of p38 MAP kinase destabilizes TNF-α transcripts.

Acknowledgments—We are grateful to Dr. John Souness and Brenda Burton (Aventis, Dagenham, UK) for many helpful discussions and for assisting with the measurements of TNF-α protein levels and to Prof. Benjamin Chain (University College, UK, London) for providing access to FACScan facilities.

REFERENCES
1. Beutler, B., Krochin, N., Milisark, I. W., Luedike, C., and Cerami, A. (1986) Science 232, 977–980
2. Wright, S. D. (1995) J. Immunol. 155, 6–8
3. Hozier, A., Ferrero, E., Koslgen, F., Hijmans, N., Yamamoto, S., Silver, J., Stewart, C. L., and Goyert, S. M. (1996) Immunity 4, 407–414
4. Wurfel, M. M., Kunitake, S. T., Lichenstein, H., Kane, J. P., and Wright, S. D. (1994) J. Exp. Med. 180, 1025–1035
5. Haiman, E., Lichenstein, H. S., Wurfel, M. M., Miller, D. S., Johnson, D. A., Kelley, M., Busse, I. A., Zakowski, M. M., and Wright, S. D. (1994) J. Exp. Med. 179, 269–277
6. Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard, A., Wood, W. I., Gurney, A. L., and Godowski, P. J. (1998) Nature 395, 284–288
7. Kirschning, C. J., Wesche, H., Merrill Ayres, T., and Roth, M. (1998) J. Exp. Med. 188, 2091–2097
8. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejandro, E., Silva, M., Galanias, C., Freundenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) Science 282, 2085–2088
9. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. (1999) J. Biol. Chem. 274, 10689–10692
10. Swantek, J. L., Cobb, M. H., and Geppert, T. D. (1997) Mol. Cell. Biol. 17, 6274–6282
11. Hambleton, J., Weinstein, S. L., Lem, L., and DeFranco, A. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2774–2778
Regulation of Tumor Necrosis Factor-α at the mRNA Level

12. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., White, J. R., Adams, J. R., and Young, P. R. (1994) Nature 372, 739–746

13. Foey, A. D., Parry, S. L., Williams, L. M., Feldmann, M., Foxwell, B. M. J., and Brennan, F. M. (1998) J. Immunol. 160, 920–928

14. Dean, J. L. E., Brook, M., Clark, A. R., and Saklatvala, J. (1999) J. Biol. Chem. 274, 264–269

15. Scherle, P. A., Jones, E. A., Favata, M. F., Daulerio, A. J., Covington, M. B., Nurnberg, S. A., Magolda, R. L., and Trzaskos, J. M. (1998) J. Immunol. 161, 5681–5686

16. Raabe, T., Bukrinsky, M., and Currie, R. A. (1998) J. Biol. Chem. 273, 974–980

17. Myokai, F., Takahashi, S., Lebe, R., and Amar, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4518–4523

18. Hazzalin, C. A., Cano, E., Cuenda, A., Barratt, M. J., Cohen, P., and Mahadevan, L. C. (1999) Curr. Biol. 6, 1028–1031

19. Hazzalin, C. A., Cano, E., Cohen, P., and Mahadevan, L. C. (1997) Oncogene 15, 2321–2331

20. Manthey, C. L., Wang, S.-W., Kinney, S. D., and Yao, Z. (1998) J. Leukocyte Biol. 64, 409–417

21. Baldassare, J. J., Bi, Y., and Bellone, C. J. (1999) J. Immunol. 162, 5367–5373

22. Pritchett, W., Hand, A., Shelds, J., and Dunnington, D. (1995) J. Inflamm. 45, 97–105

23. Young, P., McDonnell, P., Dunnington, D., Hand, A., Laydon, J., and Lee, J. (1995) Agents & Actions 39, C67-C69

24. Lai, W. S., Carballo, E., Strum, J. R., Kennington, E. A., Phillips, R. S., and Blackshear, P. J. (1999) Mol. Cell. Biol. 19, 4311–4323

25. Carballo, E., Lai, W. S., and Blackshear, P. J. (1998) Science 281, 1001–1005

26. Kontoyiannis, D., Pasparakis, M., Pizarro, T. T., Cominelli, F., and Kollias, G. (1999) Immunity 10, 387–398

27. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254

28. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159

29. Hoff, T., Spenceker, T., Emmedoerffer, A., and Goppelt-Strukebe, M. (1992) J. Leukocyte Biol. 52, 173–182

30. Shwede, H., Fitzke, E., Amb, P., and Dieter, P. (1996) J. Leukocyte Biol. 58, 555–561

31. Wang, S.-W., Pawlowski, J., Wathen, S. T., Kinney, S. D., Lichenstein, H. S., and Manthey, C. L. (1999) Inflamm. Res. 48, 533–538

32. Kotlyarov, A., Neininger, A., Schubert, C., Eckert, R., Birchmeier, C., Volk, H.D., and Gaestel, M. (1999) Nat. Cell Biol. 1, 94–97

33. Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C.Y.A., Shyu, A.-B., Muller, M., Gaestel, M., Resch, K., and Holtmann, H. (1999) EMBO J. 18, 4989–4996

34. Lewis, T., Gueydan, C., Huez, G., Toulme, J. J., and Kruys, V. (1998) J. Biol. Chem. 273, 13781–13786

35. Gueydan, C., Droogmans, L., Chalon, P., Huez, G., Caput, D., and Kruys, V. (1999) J. Biol. Chem. 274, 2322–2326

36. Sirenko, O. I., Lofquist, A. K., DeMaria, C. T., Morris, J. S., Brewer, G., and Haskell, J. S. (1997) Mol. Cell. Biol. 17, 3988–3996

37. Taylor, G. A., Carballo, E., Lee, D. M., Lai, W. S., Thompson, M. J., Patel, D. D., Schenkmak, D. I., Gilkeson, G. S., Brunnmeyer, H. E., Haynes, B. F., and Blackshear, P. J. (1996) Immunity 4, 445–454

38. Carballo, E., Lai, W. S., and Blackshear, P. J. (2000) Blood 95, 1891–1899

39. Taylor, G. A., Thompson, M. J., Lai, W. S., and Blackshear, P. J. (1995) J. Biol. Chem. 270, 13541–13547

40. Brook, M., Sully, G., Clark, A. R., and Saklatvala, J. (2000) FEBS Lett. 483, 57–61
Combinations of ERK and p38 MAPK Inhibitors Ablate Tumor Necrosis Factor-α (TNF-α) mRNA Induction: EVIDENCE FOR SELECTIVE DESTABILIZATION OF TNF-α TRANSCRIPTS

Karine Rutault, Catherine A. Hazzalin and Louis C. Mahadevan

J. Biol. Chem. 2001, 276:6666-6674.
doi: 10.1074/jbc.M005486200 originally published online November 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005486200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 22 of which can be accessed free at http://www.jbc.org/content/276/9/6666.full.html#ref-list-1