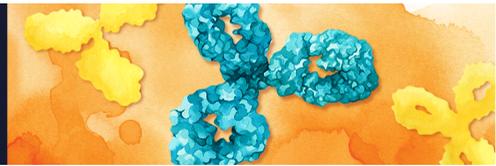


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# Inhibition of MAP Kinase Kinase Prevents Cytokine and Prostaglandin E<sub>2</sub> Production in Lipopolysaccharide-Stimulated Monocytes

Peggy A. Scherle,<sup>1</sup> Elizabeth A. Jones, Margaret F. Favata, Andrea J. Daulerio, Maryanne B. Covington, Sherrill A. Nurnberg, Ronald L. Magolda, and James M. Trzaskos

Activation of the extracellular signal-regulated kinase (ERK) pathway has been shown to occur in monocytes following stimulation with LPS. However, the importance of this event for monocyte function is not clear. To address this issue, we used the novel MAP/ERK kinase (MEK) inhibitor, U0126. Stimulation of monocytes with LPS resulted in activation of the mitogen-activated protein kinase (MAPK) family members ERK, Jun NH<sub>2</sub>-terminal kinase (JNK), and p38. Treatment of monocytes with LPS in the presence of U0126 blocked the activation of ERK1 and ERK2. However, the activation of Jun NH<sub>2</sub>-terminal kinase and p38 family members was not affected by the compound, confirming the selectivity of U0126. To examine the effects of MEK inhibition on monocyte function, we measured production of the cytokines IL-1, IL-8, and TNF, as well as PGE<sub>2</sub>. Monocytes treated with LPS in the presence of U0126 failed to release IL-1, IL-8, TNF, or PGE<sub>2</sub>. The failure to secrete IL-1 and TNF was due to decreased levels of mRNA. These results demonstrate that activation of MEK/ERK is critical for cytokine and PGE<sub>2</sub> production by monocytes in response to LPS. *The Journal of Immunology*, 1998, 161: 5681–5686.

Monocytes play a central role in immune regulation and inflammation. When activated, monocytes produce and release a number of inflammatory mediators, such as IL-1, IL-6, IL-8, TNF- $\alpha$ , and arachidonic acid metabolites. This leads to the recruitment and activation of other immune cells into sites of injury and infection.

One of the most potent activators of monocytes is LPS, a component of the outer membrane of Gram-negative bacteria. At physiologic concentrations, LPS binds to the serum protein, LPS-binding protein, and this complex then interacts with the glycosyl phosphatidylinositol-linked cell surface glycoprotein, CD14, which is present on monocytes, macrophages, and neutrophils (1, 2). Because CD14 lacks both transmembrane and cytoplasmic domains, the mechanism by which CD14 transmits a stimulatory response is still unclear. It has been shown that a number of src family tyrosine kinases, such as lyn, hck, and fgr, become activated in LPS-stimulated macrophages (3–5) and that inhibitors of tyrosine phosphorylation block the secretion of cytokines (6). Activation of protein kinase C and phosphatidylinositol (PI)3-kinase has also been shown to occur (7–9).

Among the most prominently tyrosine phosphorylated proteins in LPS-activated macrophage cell lines are the p42 (extracellular signal-regulated kinase (ERK) 2)<sup>2</sup> and p44 (ERK1) isoforms of the MAP kinase family of serine/threonine kinases (10). MAP kinases have been implicated in a number of signaling events that are potentially important in the inflammatory response. ERKs have

been shown to phosphorylate and activate the transcription factors elk1 and NF-IL6 (11, 12). Elk1 has been shown to be required for c-fos up-regulation, which, as a component of the AP-1 transcription factor, plays an important role in the up-regulation of cytokines and metalloproteases (13) whereas NF-IL6 promotes the up-regulation of granulocyte (G)-CSF, IL-6, IL-8, IL-1, TNF, and the inducible form of nitric oxide synthase (14–17). ERKs can also phosphorylate cytoplasmic phospholipase A<sub>2</sub> (PLA<sub>2</sub>), which catalyzes the release of arachidonic acid (18).

There are additional MAPK family members, JNKs and p38s, whose activity has been shown to be up-regulated in response to LPS in fibroblast and macrophage cell lines (19–22). Recent experiments with a small molecule inhibitor of p38 kinases show that blocking p38 prevents IL-1 and TNF production in LPS-stimulated monocytes at the level of translation (23). It has also been shown that dexamethasone inhibits TNF production at least in part by blocking induction of JNKs (22). These results suggest that JNKs and p38 kinases are also important in the regulation of cytokines in LPS-stimulated monocytes.

The ERK pathway is activated through the stimulation of Ras, which in turn activates the serine/threonine kinase Raf-1 (24). Raf-1 phosphorylates and activates the dual-specificity MAP kinase kinases, MEK1 and MEK2, which phosphorylate ERKs on two critical tyrosine and threonine residues. This event leads to an increase in ERK activity. In this report, we studied the role of the ERK pathway in the regulation of cytokines and PGs in LPS-stimulated monocytes by using a novel inhibitor of MEK, the kinase upstream of ERK in the signaling cascade. We show that treatment of monocytes with the selective MEK inhibitor U0126 blocks production of IL-1, IL-8, TNF, and PGE<sub>2</sub> in response to LPS. These results demonstrate that, although multiple pathways are activated in response to LPS, activation of MEK/ERK is critical for cytokine and PG release.

## Materials and Methods

### Reagents

RPMI 1640 medium and DMEM were obtained from Life Technologies (Gaithersburg, MD). FCS was obtained from Hyclone (Logan, UT). LPS from *Salmonella typhimurium* was purchased from Calbiochem (San Diego, CA). All LPS samples were reconstituted to 10 mg/ml solutions in

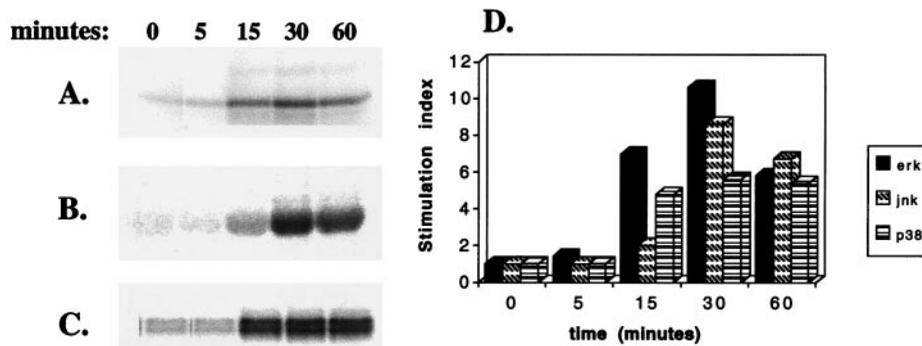
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<sup>2</sup> Abbreviations used in this paper: ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; JNK, Jun NH<sub>2</sub>-terminal kinase; AP-1, activator protein-1; MEK, MAP/ERK kinase; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PGHS-2, PG H synthase 2; GAPDH, glyceraldehyde phosphate dehydrogenase; MKK, MAP kinase kinase.



**FIGURE 1.** ERK, JNK, and p38 are activated in LPS-stimulated human monocytes. Cellular extracts from  $1 \times 10^7$  human monocytes stimulated for various lengths of time with  $1 \mu\text{g/ml}$  LPS were assayed by immunoprecipitation/kinase assay for ERK2 phosphorylation of myelin basic protein (A), JNK phosphorylation of a recombinant glutathione *S*-transferase (GST)-c-Jun protein (B), or p38 phosphorylation of a recombinant GST-activating transcription factor (ATF)2 protein (C). The band represents  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  incorporation into the substrate. Quantitation of the kinase activity was performed using the Image Quant program (Molecular Dynamics, Sunnyvale, CA) (D). The data shown are representative of two independent experiments.

pyrogen-free water containing 0.5% triethylamine or in PBS. Abs to ERK2 and p38 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Ab to ERK2 has been shown to cross-react with ERK1. Phosphospecific Abs were obtained from New England Biolabs (Beverly, MA). Compounds used in this study were prepared by the DuPont Pharmaceuticals Chemistry department and dissolved in 100% DMSO.

#### Monocyte purification

Human monocytes were isolated by a combination of Ficoll-Hypaque gradient centrifugation, with elutriation as previously described (25). This procedure resulted in populations of monocytes of greater than 90% purity as determined by Wright-Giemsa staining.

#### Cell culture

Cells were plated at  $2 \times 10^6$  cells per ml in DMEM or RPMI +10% FCS media (Life Technologies). Cells were plated in Costar (Cambridge, MA) 12-well culture dishes with 1 ml cell suspension per well. Cells were stimulated with  $1 \mu\text{g/ml}$  LPS and incubated at  $37^\circ\text{C}$  for specific lengths of time (4 h, TNF; 16–18 h, IL-1, IL-8, and  $\text{PGE}_2$ ). In experiments to determine the effects of MEK inhibitors, compound was added at various concentrations immediately before addition of LPS. At the end of the incubation, supernatants were removed and assayed for cytokines or  $\text{PGE}_2$ .

#### Cytokine and $\text{PGE}_2$ assays

The quantitative analyses of TNF- $\alpha$  and IL-1- $\beta$  were performed by ELISA as previously described (26, 27).  $\text{PGE}_2$  levels were determined by ELISA (PerSeptive Diagnostics, Cambridge, MA), as were levels of IL-8 (R&D Systems, Minneapolis, MN), according to manufacturer's instructions.

#### Kinase assays

Cellular extracts were prepared from monocytes stimulated for various lengths of time with  $1 \mu\text{g/ml}$  LPS in the presence or absence of U0126 as previously described (28). ERK, JNK, and p38 activities were measured by immunoprecipitation kinase assays as described (28, 29). Determination of phospho-ERK, JNK, and p38 levels was done by Western analysis using phosphospecific Abs (New England Biolabs) as previously described (29).

#### Northern analysis

The human monocytic cell line, THP-1, was stimulated with  $10 \mu\text{g/ml}$  LPS in the presence or absence of compound for 2 h. Total RNA was isolated using RNA-Zol<sup>®</sup> (Tel-Test, Friendswood, TX), and Northern blot analyses were performed as described (30) using  $10 \mu\text{g}$  of RNA/lane. The blots were probed with digoxigenin-labeled cDNA probes for full-length human IL-1 $\beta$ , TNF- $\alpha$ , and PGHS-2 according to the manufacturer (Boehringer Mannheim, Indianapolis, IN).

## Results

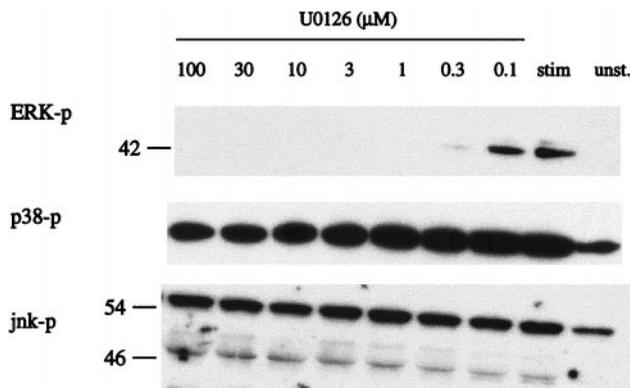
### LPS stimulation leads to the activation of ERK, JNK, and p38 in monocytes

Although it has previously been shown that ERKs, JNKs, and p38 kinases can be activated by LPS in fibroblasts and macrophage cell

lines (7, 10, 19–22), we performed immune complex kinase assays on extracts from LPS-stimulated human monocytes to determine the extent and time course of activation of all three MAPK pathways in our system. As shown in Fig. 1, ERK, JNK, and p38 are activated to a similar extent (5- to 10-fold) in human monocytes. Maximal kinase activity is observed at 30 min for all of the pathways. This response is somewhat delayed relative to other activators, such as TPA, where kinase activity peaks after 5 min, but is in agreement with previous studies on LPS signaling performed in macrophage cell lines (10, 20–22). Although the reagents used in this experiment cannot discriminate among the various ERK, JNK, and p38 family members, the results discussed below suggest that specific family members are preferentially used in this response.

### U0126 selectively blocks ERK phosphorylation in LPS-stimulated monocytes

U0126 was initially identified as an inhibitor of AP-1-driven gene transcription and was later shown to directly block ERK phosphorylation by its upstream kinases, MEK1 and MEK2 (31). As reported previously, U0126 inhibits MEK1 and MEK2 catalytic activity in a direct enzyme assay but does not significantly inhibit the related MAP kinase family members MKK3, MKK4, MKK6, ERK2, JNK1, or p38. We have also shown that U0126 is a non-competitive, reversible inhibitor of MEK and does not inhibit unrelated kinases such as protein kinase C (PKC), abl, S6 kinase, or cdk2 (31). Since these studies were done *in vitro*, we wanted to determine whether MEK1/2 was also selectively inhibited in cells. Monocytes were stimulated with LPS in the presence or absence of various concentrations of U0126 for 15 min. Extracts were then prepared and analyzed by Western blot for phosphorylated forms of ERK1/2, JNK1/2, and p38 or by immune complex kinase assay for MAPK activity. As shown in Fig. 2, ERK2 appears to be preferentially phosphorylated in the LPS-stimulated monocytes, compared with ERK1. The p54 isoform of JNK is also phosphorylated to a greater extent than the p46 form. U0126 blocks ERK2 phosphorylation with an  $\text{IC}_{50}$  value of  $0.2 \mu\text{M}$  and does not inhibit p38 or JNK phosphorylation by greater than 30% at concentrations as high as  $100 \mu\text{M}$ . Further, U0126 blocks ERK activity >90% but not JNK or p38 activities (<20% at  $10 \mu\text{M}$ ) (Fig. 3). The effect of U0126 on ERK phosphorylation vs p38 and jnk phosphorylation is observed at all time points examined (not shown). These data confirm the results of the direct enzyme assays and demonstrate that U0126 is a selective inhibitor of MEK in cells.

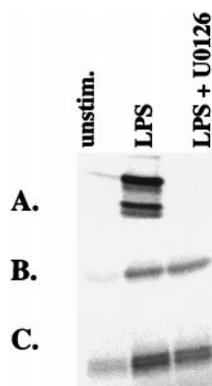


**FIGURE 2.** Effects of U0126 on ERK, JNK, and p38 phosphorylation. Extracts from LPS-stimulated monocytes were analyzed by Western blot using an Ab that recognizes the phosphorylated form of ERK1/2, p38, or JNK1/2. Cells were stimulated for 15 min with 1  $\mu\text{g}/\text{ml}$  LPS in the presence or absence of various concentrations of U0126. The data shown are representative of two independent experiments.

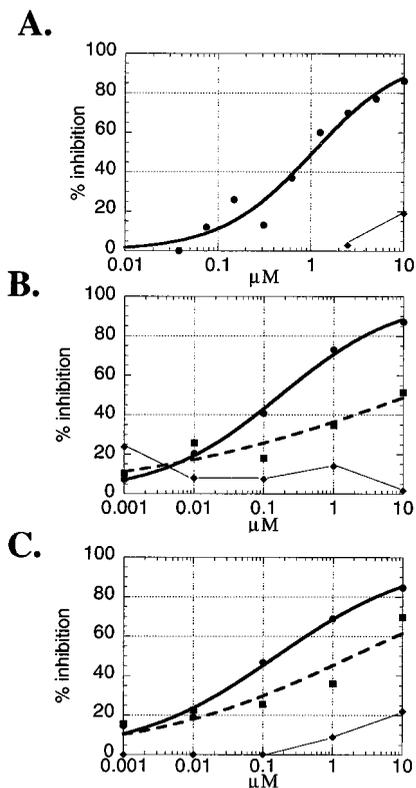
#### U0126 blocks cytokine and PGE<sub>2</sub> production

Although the MAPK pathways are activated by LPS, the role of MEK, specifically, in LPS-mediated effects in monocytes is unclear. To address this issue, we treated monocytes with LPS in the presence or absence of various concentrations of U0126. The levels of IL-1, TNF, and PGE<sub>2</sub> produced were determined by ELISA. As shown in Fig. 4, U0126 blocks IL-1 and TNF production by LPS-stimulated monocytes, having IC<sub>50</sub> values of 1 and 0.3  $\mu\text{M}$ , respectively. U0126 also inhibits the release of PGE<sub>2</sub>, with an IC<sub>50</sub> of 0.2  $\mu\text{M}$ . In addition, U0126 was shown to inhibit release of IL-6 and IL-8 in this system (Fig. 5, and not shown). The IC<sub>50</sub> values for the inhibition of cytokine and PGE<sub>2</sub> release show a good correlation with the effects of U0126 on ERK phosphorylation (IC<sub>50</sub> = 0.2  $\mu\text{M}$ ), suggesting that MEK inhibition is responsible for the effects observed.

In addition, the effects of U0126 in this system are specific since the inactive analogue, U0124, shows minimal to no inhibition. The Parke-Davis MEK inhibitor, PD98059, (32) was also examined for its effects on cytokine and PGE<sub>2</sub> production. As shown in Fig. 4, PD98059 also blocked TNF and PGE<sub>2</sub> release but was 10–30 times less potent (TNF IC<sub>50</sub> = 10  $\mu\text{M}$ ; PGE<sub>2</sub> IC<sub>50</sub> = 2  $\mu\text{M}$ ). These results are in agreement with the difference in potency between the



**FIGURE 3.** U0126 selectively blocks ERK activation in LPS-stimulated monocytes. Cellular extracts were prepared from human monocytes stimulated with 1  $\mu\text{g}/\text{ml}$  LPS for 30 min in the presence or absence of 10  $\mu\text{M}$  U0126. Kinase activity of ERK (A), JNK (B), and p38 (C) was measured as described in Fig. 1. The data are representative of four individual experiments.



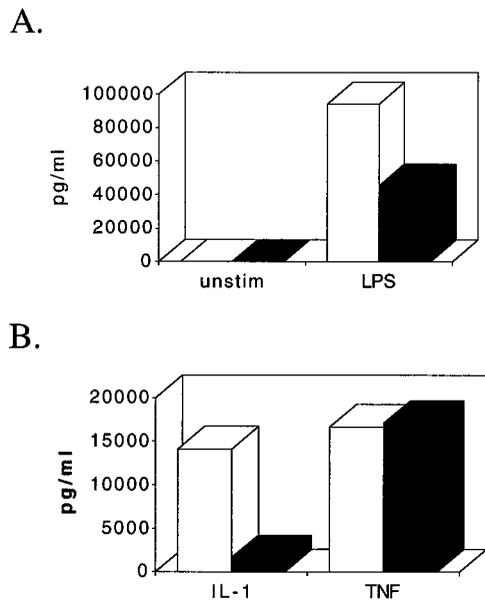
**FIGURE 4.** U0126 inhibits cytokine and PGE<sub>2</sub> release in monocytes. Monocytes were stimulated with LPS in the presence or absence of various concentrations of U0126 (circles), PD98059 (squares), or the inactive analogue U0124 (diamonds). Supernatants were harvested after 4 h and assayed by ELISA for TNF (B) or after 16 h and assayed by ELISA for IL-1 (A) and PGE<sub>2</sub> (C). The data represent the mean of three to four experiments.

two compounds in the direct enzyme assay. U0126 blocks MEK activity with an IC<sub>50</sub> of 70 nM whereas PD98059 has an IC<sub>50</sub> of 5  $\mu\text{M}$  (data not shown).

There were no effects on cell viability or on total RNA and protein levels at concentrations of U0126 as high as 20  $\mu\text{M}$ , suggesting that the cytokine inhibition observed was not due to toxicity (not shown). However, to study this further, we examined the effects of U0126 on cytokine production in response to different stimuli. As shown in Fig. 5, treatment of cells with 1  $\mu\text{M}$  U0126 inhibited IL-8 production in response to LPS or IL-1 but not in response to TNF. At higher concentrations of U0126 (>10  $\mu\text{M}$ ), some inhibition of IL-8 secretion in TNF-treated cells was observed. IL-1 and IL-6 levels were not significantly increased in response to TNF in this system; therefore, the effects of U0126 could not be examined. These results suggest that the involvement of the ERK pathway in the regulation of cytokine production is stimulus dependent and that the effects of U0126 are not due to general toxicity.

#### U0126 blocks cytokine production at the level of transcription

It is known that ERK activation leads to the phosphorylation and increased activity of several transcription factors such as AP-1 and NF-IL6, which have been shown to be involved in the transcriptional up-regulation of cytokines. We therefore performed Northern analyses to determine whether the effects of U0126 on cytokine release were due to inhibition of transcription. We could demonstrate that U0126 blocks IL-1 up-regulation of mRNA levels in the human monocytic cell line, THP-1, in response to LPS stimulation



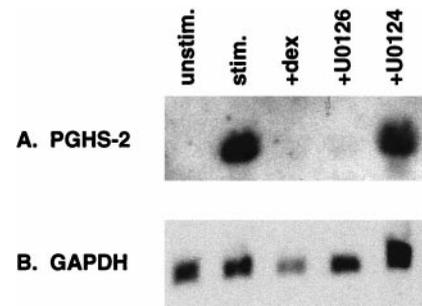
**FIGURE 5.** U0126 blocks IL-8 production in response to LPS and IL-1 but not TNF- $\alpha$ . PBMC were stimulated with LPS (1  $\mu$ g/ml) (A), or TNF (100 ng/ml) and IL-1 (100 ng/ml) (B) in the presence (black bars) or absence (white bars) of 1  $\mu$ M U0126 for 18 h. Supernatants were harvested and assayed for IL-8 levels by ELISA. The data are representative of two experiments.

(Fig. 6). U0126 has an  $IC_{50}$  value of 0.2  $\mu$ M in this system. U0126 also decreases the levels of TNF mRNA (not shown). In both cases, the inactive analogue, U0124, had no effect. The effects of the MEK inhibitor at the mRNA level are in contrast to those observed with the p38 inhibitor SB203580, which has been shown to prevent cytokine release at the level of translation (23).

*U0126 effects on PGE<sub>2</sub> release may be due in part to inhibition of PGHS-2 transcriptional up-regulation*

The enzyme PG H synthase (PGHS) has been shown to be essential for PG release by catalyzing the conversion of arachidonic acid to PG endoperoxide (PGH<sub>2</sub>), the precursor to various PGs, including PGE<sub>2</sub>. (33–35). Two isoforms of PGHS-2 have been identified, a constitutive form (PGHS-1) and an inducible form (PGHS-2). The inducible PGHS-2 has previously been shown to be selectively increased in LPS-stimulated macrophages (36). Since U0126 was shown to block the up-regulation of IL-1 and TNF mRNA, it was possible that U0126 might exert its effects on PGE<sub>2</sub> release by regulating the level of the inducible PGHS-2 enzyme. We therefore examined PGHS-2 mRNA levels in the presence or absence of U0126 by Northern analysis (Fig. 7). We could show

**FIGURE 6.** U0126 prevents LPS-induced up-regulation of IL-1 mRNA. Total RNA was isolated from  $1 \times 10^7$  THP-1 cells stimulated for 2 h with 10  $\mu$ g/ml LPS in the presence or absence of various concentrations of U0126 or 10  $\mu$ M U0124. Northern blots were performed using 10  $\mu$ g/lane of mRNA. The blots were hybridized with probes to IL-1 (A) or GAPDH (B). The data were quantitated using the Image Quant program for  $IC_{50}$  determinations and are representative of two independent experiments.



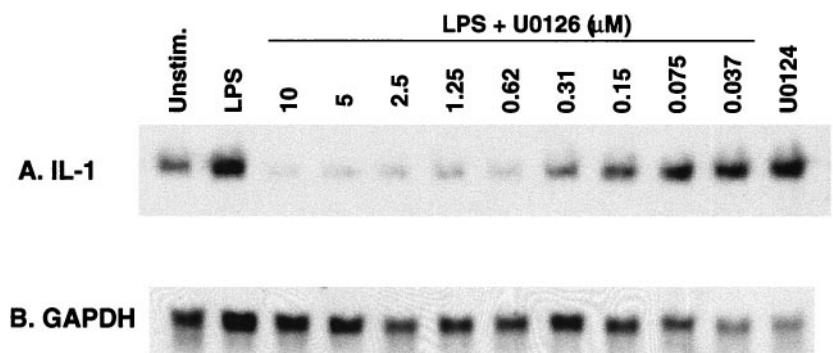
**FIGURE 7.** U0126 prevents the up-regulation of PGHS-2 mRNA. Total RNA was isolated from THP-1 cells after 2 h as described in Fig. 4. U0126 and U0124 were tested at 10  $\mu$ M, and dexamethasone was tested at 1  $\mu$ M. Northern blots were probed with PGHS-2 (A) or GAPDH (B). The data are representative of two experiments.

that U0126 blocks the up-regulation of PGHS-2 at the mRNA level and therefore demonstrate at least one mechanism whereby MEK inhibition can affect PGE<sub>2</sub> release.

## Discussion

In this report, we demonstrate that, in LPS-stimulated monocytes, inhibition of the ERK pathway by the selective MEK inhibitor U0126 blocks cytokine production similar to the effects observed with p38 inhibitors. In contrast to p38 inhibitors, however, the effects of the MEK inhibitor U0126 are mediated, at least in part, at the level of mRNA. Although our experiments do not distinguish between effects of U0126 on mRNA transcription vs mRNA stability, it has recently been reported that the LPS-mediated increase in TNF levels is due primarily to an increase in transcription with no change in mRNA stability (37). Therefore, U0126 most likely prevents gene transcription, possibly by inhibiting ERK phosphorylation and activation of transcription factors such as NF-IL6 and elk1, which have been shown to be important in the up-regulation of a number of inflammatory modulators.

It had previously been shown that overexpression of a Raf1: estrogen receptor (Raf1:ER) chimeric protein in the murine macrophage cell line RAW 264.7 resulted in the rapid and prolonged activation of ERK1 and ERK2 upon treatment with estradiol (38). When the levels of TNF protein and mRNA were measured, however, it was found that they were induced only weakly by Raf1: estrogen receptor. This suggested that other signaling events, such as NF- $\kappa$ B activation, might be required to achieve the maximum response observed with LPS. Although this may be the case in some systems, our data clearly demonstrate that blocking only the ERK pathway is sufficient to inhibit cytokine production in freshly isolated monocytes. These results suggest that, although environmental stimuli may require multiple signaling events to elicit a cellular response, blocking any one of them is adequate for inhibition.



Our results are in agreement with a recent study showing that PD98059 partially inhibits monocyte production of IL-1 and TNF in response to LPS stimulation (39). In this study, they also demonstrated that, in contrast to TNF and IL-1, neither IL-10 secretion nor IL-10 mediated responses are affected, demonstrating that not all LPS-mediated effects will be ERK dependent. Our data show that, similar to IL-1 and TNF, monocyte production of IL-6, IL-8, and PGE<sub>2</sub> in response to LPS are dependent on activation of the MEK, further confirming that proinflammatory events will be suppressed by blocking the ERK pathway. Although these conclusions are based on results with small molecule inhibitors and one can never absolutely exclude the possibility of a second target other than MEK, the finding that two structurally distinct inhibitors give similar results strongly implicates the importance of MEK in LPS-stimulated cytokine production.

The role of the ERK pathway in cytokine production appears to be stimulus dependent since U0126 blocks IL-8 secretion in response to LPS and IL-1 but not TNF. Although we and others have shown that TNF can up-regulate the ERK pathway in monocytes (40), the response is weaker and more transient (not shown), suggesting that other pathways may be more important. In support of this, we have shown that the p38 inhibitor SB203580 does block IL-8 production in response to TNF as well as LPS (not shown). How MEK inhibition results in decreased IL-8 production in response to LPS is under study. IL-8 up-regulation has been shown previously to be dependent on NF- $\kappa$ B sites in its promoter. The role of MEK in regulating NF- $\kappa$ B activity is unclear, but recent studies demonstrating that PD98059 can reduce NF- $\kappa$ B transactivational activity (41) suggest that there is some interaction.

We have shown that U0126 inhibition of PGE<sub>2</sub> release may be due in part to its effects on PGHS-2 since U0126 was shown to block the up-regulation of mRNA levels of this enzyme. This result supports a previous study that demonstrated that overexpression of dominant negative mutants of Raf-1 and ERK blocked induction of PGHS-2 in 3T3 cells stimulated with platelet-derived growth factor (PDGF) or serum (42). Our results are also in agreement with a recent study demonstrating that the Parke-Davis MEK inhibitor, PD98059, blocks PGHS-2 transcription in the RAW 264.7 murine macrophage cell line (43). There is an NF-IL6 site in the PGHS-2 promoter that has been shown to be important for PGHS-2 up-regulation by LPS in vascular endothelial cells (44). Since ERK has been shown to phosphorylate NF-IL6, this may be how the Ras/Raf/MEK/ERK pathway mediates its effects on PGHS-2 transcription. In addition, it is known that the rate-limiting step in PG biosynthesis is the liberation of arachidonic acid and that the release of arachidonic acid requires PLA<sub>2</sub> activation (45). Since ERK has been shown to phosphorylate and activate PLA<sub>2</sub> in vitro (18), it is possible that U0126 blocks PGE<sub>2</sub> release in LPS-stimulated monocytes at this step as well.

Previous studies with inhibitors of the p38 MAP kinase pathway have demonstrated that these molecules have antiinflammatory properties in vivo (46). Our results showing that U0126 inhibits cytokine and PGE<sub>2</sub> production by LPS-stimulated monocytes in vitro suggest that MEK inhibitors may also prevent inflammation in vivo. Based on these results, we have tested U0126 in several animal models of inflammation such as the carrageenan paw and TPA ear edema models and have demonstrated that the compound is efficacious (our unpublished data). Our data therefore demonstrate that MEK is another potential target for the design of therapeutic strategies in the treatment of inflammatory diseases.

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