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fMet-Leu-Phe Stimulates Proinflammatory Cytokine Gene Expression in Human Peripheral Blood Monocytes: The Role of Phosphatidylinositol 3-Kinase

Zhixing K. Pan, Ling-Yu Chen, Charles G. Cochrane, and Bruce L. Zuraw

The fMLP-stimulated release of proinflammatory cytokines such as IL-1 by human peripheral blood monocytes is an important component of the inflammatory process. The signaling mechanisms used by fMLP to stimulate the release of cytokines are still incompletely understood. We previously demonstrated that fMLP-stimulated NF-κB activation in PBMC and now we present evidence that the lipid products of phosphatidylinositol 3-kinase (PI 3-kinase) are required for fMLP-stimulated activation of NF-κB. Pretreatment with the PI 3-kinase inhibitors, wortmannin and LY294002, effectively blocked fMLP-induced IL-1β gene expression as well as NF-κB activation. Transient transfection of THP1 cells with a dominant-negative mutant of the PI 3-kinase p85 subunit also abrogated fMLP-induced κB activity. These results suggest a potential role of fMLP in the transcription of proinflammatory cytokines and provide the first evidence that such regulation may occur through PI 3-kinase activity. The Journal of Immunology, 2000, 164: 404–411.

Through their unique combination of cellular functions (including chemotaxis, phagocytosis, and generation of lipid mediators and highly reactive superoxide radicals), leukocytes represent an important first line defense of the host against invading micro-organisms (1, 2). It has also become apparent that the leukocyte response to bacteria or bacterial components (such as formylated peptides) involves activation of transcription with de novo gene expression (3).

The presence of viable bacteria in infected tissues has long been observed to attract leukocytes, and fMLP was classically described as a bacterial product that was a leukocyte chemoattractant. Subsequently, fMLP was shown to be able to activate all the classic functional activities of leukocytes (4). More recently, fMLP-stimulated PBMC have been shown to express a defined set of gene products, including IL-1α, IL-1β, and IL-6 (3). The N-formyl peptide receptor has been cloned and shown to belong to the G protein-coupled receptor family (5). Pretreatment of PBMC with pertussis toxin has been shown to abolish fMLP-stimulated cytokine synthesis, suggesting that a Gα-containing heterotrimeric G protein may mediate the process.

Several recent studies have demonstrated activation of the transcription factor NF-κB following binding of cognate ligands to their specific G protein-coupled receptors (6–10). The lipid-derived chemoattractants platelet-activating factor (PAF) and leukotriene B4 were shown to activate NF-κB in both monocytes (9, 11, 12) and transfected cell lines expressing the PAF receptor (8). Furthermore, this activation of NF-κB in monocytes resulted in the transcription of genes encoding both cytokines and growth factors (9, 12). We recently showed that fMLP activates NF-κB in leukocytes, and that this response is cell type and developmental stage specific (13). Regulation of NF-κB activation is of paramount importance to immune cell function due to its ability to activate the transcription of many proinflammatory immediate early genes (14, 15). NF-κB is a multiprotein transcription activator originally found to bind a decameric enhancer sequence in the gene for the Ig κ light chain (14). In leukocytes, NF-κB activation results in the transcription of immediate early genes that encode IL-2, IL-6, IL-8, TNF-α, monocyte chemoattractant protein-1, GM-CSF, as well as several adhesion molecules (15). Numerous stimuli can activate NF-κB, including the bacterial component LPS and other proinflammatory factors, including IL-1β (16) and TNF-α (17).

The DNA binding activity of NF-κB is suppressed by IκBα and IκBβ, cytoplasmic inhibitory proteins that bind the NF-κB dimer (18). Various signals can lead to phosphorylation of IκB (19), which is then ubiquinated and degraded (20). The released NF-κB dimers subsequently translocate to the nucleus and bind specific DNA sequences. Degradation of IκBα and IκBβ results from phosphorylation of two serines by a multicomponent IκB kinase that is activated by cytokines and other signals known to activate NF-κB (21–23).

Although the activation of NF-κB has been extensively studied in cultured cell lines of hemopoietic lineage, the signal transduction pathways that lead to leukocyte transcription activation are still not clear. Recent studies from our laboratory have shown that phosphatidylinositol 3-kinase (PI 3-kinase) inhibitors (wortmannin and LY294002) abolished the NF-κB activation stimulated by bradykinin in human epithelial A549 cells (24). PI 3-kinase is a ubiquitous lipid kinase that phosphorylates the 3-position of the inositol ring of inositol phospholipids to generate such lipid messengers as phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate. The exact roles of these lipid products have not yet been determined; however, increasing evidence suggests that they may serve as intracellular second messengers (25–27). Several distinct PI 3-kinase
forms have recently been described. The classical form of PI 3-kinase consists of a p110 catalytic subunit and a p85 regulatory subunit (28, 29). Recently, myeloid cells have been shown to contain a novel form of PI 3-kinase whose activity is directly regulated by heterotrimeric G protein α and βγ subunits. This form of PI 3-kinase has now been cloned and shown to consist of a unique stranded oligonucleotide (5 pmol) was 32P labeled with T4 polynucleotide kinase from Promega (Madison, WI) and Santa Cruz Biotechnology. The 9-citation of secreted IL-1β has been shown to represent a consensus sequence (33). Double-stranded salmon sperm DNA, and 10% glycerol) for 10 min at room temperature. Then ~20–50 fmol of 32P-labeled oligonucleotide probe (30,000–50,000 cpm) was added, and the reaction mixture was incubated for 10 min at room temperature. The samples were analyzed on 6% acrylamide gels, which were made in 50 mM Tris-borate buffer containing 0.1 mM PMSF. After 30 min at 4°C, lysates were separated by centrifugation (13,000 g, 30 s), and supernatant containing nuclear proteins were transferred to new vials. The protein concentration of extracts was measured using a protein dye (Bio-Rad, Hercules, CA) with BSA as a standard, and samples were diluted to equal concentration in buffer B for use directly or for storage at −80°C.

EMSA

EMSA were performed by incubating 2.5 μg of the nuclear extract in 12 μl of binding buffer (5 mM HEPES (pH 7.8), 5 mM MgCl2, 50 mM KCl, 0.5 mM DTT, 0.4 mg/ml poly(dI-dC) (Pharmacia), 0.1 mg/ml sonicated double-stranded salmon sperm DNA, and 10% glycerol) for 10 min at room temperature. Then ~20–50 fmol of 32P-labeled oligonucleotide probe (30,000–50,000 cpm) was added, and the reaction mixture was incubated for 10 min at room temperature. The samples were analyzed on 6% acrylamide gels, which were made in 50 mM Tris-borate buffer containing 1 mM EDTA or 50 mM Tris380 μM glycine/2 mM EDTA and were pre-electrophoresed for 2 h at 12 V/cm. Electrophoresis was conducted at the same voltage for 2–2.5 h. Gel contents were transferred to Whatman DE-81 paper, dried, and exposed for 3.5 h at −80°C with an intensifying screen. Using this method, a nonspecific DNA-protein complex of unknown origin is sometimes seen in the autoradiograph.

Immunoprecipitation and immunoblotting

Approximately 10 μg of cytoplasmic extracts, collected after the Nonidet P-40 lysis and centrifugation steps (see Preparation of nuclear extracts above), were incubated with the appropriate amount of Ab for 3 h and then precipitated following absorption onto protein A-Sepharose. Precipitates were washed three times, separated by SDS-PAGE, and transferred to Hybond-ECL nitrocellulose (Amersham). Filter strips were incubated with primary Ab for 30 min at room temperature, followed by addition of peroxidase-conjugated goat anti-rabbit IgG at 1/10,000 for 30 min and analysis with enhanced chemiluminescence reagents (DuPont-NEN, Wilmington, DE).

The PI 3-kinase assay

Aliquots of cell lysates normalized for protein content were incubated for 3 h with anti-PI 3-kinase Abs directed against the 85-kDa regulatory subunit (Upstate Biotechnology, Lake Placid, NY). The immune complexes were washed onto protein A-Sepharose and washed as previously described (36). PI 3-kinase assays were performed directly on beads. Briefly, the reaction was conducted for 10 min in a buffer containing 40 mM HEPES (pH 7.2), 6 mM MgCl2, 1 mM EDTA, 10 μg of PI (Avanti Polar Lipids, Alabaster, AL), 10 μM ATP, and 10 μCi [γ-32P]ATP (600 Ci/mmol; DuPont/NEN). Adenosine (0.2 mM) was added to the reaction mixture to inhibit residual PI 4-kinase activity. After the incubation, the reaction was stopped with methanol plus 2.4 N HCl (1:1, v/v), and lipids were extracted and analyzed by TLC.
After development, the extent of CAT activity was measured using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Isolation of transfected cells

Transfected THP1 cells were specifically isolated using the Capture-Tec pHook-2 system (Invitrogen, San Diego, CA) according to the manufacturer’s protocol. Briefly, 4 × 10⁶ THP1 cells were transfected with the pHook-2 plasmid that directs the synthesis of a fusion protein containing the platelet-derived growth factor receptor transmembrane domain fused to a single-stranded cell surface Ab recognizing the hepten 4-ethoxymethylene-2-phenyl-2-oxazzolin-5-one. Transfected cells were then recovered by incubating the cells in suspension for 30 min at 37°C with 2 × 10⁷ magnetic beads coupled to 4-ethoxymethylene-2-phenyl-2-oxazzolin-5-one followed by magnetic separation.

Results

fMLP stimulates de novo production of IL-1β in monocytes

To assess the relationship between fMLP stimulation and synthesis of cytokines, we examined the effects of fMLP on IL-1β synthesis in monocytes. Interleukin-1β, a prototypic proinflammatory cytokine, induces the expression of a variety of genes whose products are involved in acute and chronic inflammatory conditions. Unstimulated human peripheral blood monocytes produced little IL-1β. Addition of fMLP (100 nM) resulted in a time-dependent production of IL-1β as measured by ELISA (Fig. 1). A notable increase in secreted IL-1β was detected 1 h after fMLP stimulation and continued for at least 8 h. To determine whether fMLP-induced up-regulation of IL-1β levels represented new synthesis, monocytes were treated with the transcription inhibitor actinomycin D (ActD; 1 µg/ml) or the protein synthesis inhibitor cycloheximide (CHX; 100 µg/ml) before fMLP stimulation. Pretreatment with either ActD or CHX completely inhibited fMLP-induced IL-1β protein synthesis (Fig. 2). These results indicate that fMLP stimulates de novo IL-1β protein synthesis in monocytes.

fMLP-stimulated production of IL-1β involves in transcription activation

We previously demonstrated that fMLP stimulated NF-κB activity in monocytes, suggesting that activation of NF-κB may be involved in fMLP-stimulated IL-1β gene expression. To test this hypothesis, we used EMSA and promoter-reporter gene constructs to further explore the relationship between NF-κB activation and IL-1β gene expression. Previous studies have indicated a role of NF-κB in regulating IL-1β gene expression (10). EMSA demonstrated a dose- and time-dependent elevation of the DNA κB binding activity following stimulation of monocytes with fMLP (Fig. 3A). The fMLP-stimulated NF-κB activation was first detectable 20 min after agonist stimulation, preceding the later (60 min) appearance of IL-1β protein. Because activation of NF-κB is directly linked to phosphorylation and degradation of its inhibitor, IκB, we assessed cytoplasmic IκB levels by immunoblotting. Fig. 3B demonstrates that fMLP-induced IκB binding activity (lower panel) in monocytes was preceded by IκB degradation, followed by resynthesis of IκB (upper panel).

The results presented above showed a parallel up-regulation of both IL-1β gene expression and NF-κB activation by fMLP. To
investigate whether the IL-1β gene expression was the result of fMLP-induced NF-κB activation, we examined the effect of pyrrolidine dithiocarbamate (PDTC) on both IL-1β gene expression and NF-κB activation; PDTC is an antioxidant that blocks the dissociation of IkB from the cytoplasmic NF-κB, thus preventing

![FIGURE 2.](http://www.jimmunol.org/) The fMLP-induced secretion of IL-1β by monocytes involves de novo synthesis. Monocytes were preincubated with ActD (1 μg/ml) or CHX (100 μg/ml) for 1 h. Cells were then stimulated with 100 nM fMLP for 2 h, and secreted IL-1β was measured by ELISA. Data shown are the mean of two separate experiments.

![FIGURE 4.](http://www.jimmunol.org/) The NF-κB regulated IL-1β gene expression in fMLP-stimulated cells. A, Monocytes were pretreated with PDTC (1 mM) for 1 h (lane 3) followed by fMLP (100 nM) stimulation (lanes 2 and 3) for 2 h, and secreted IL-1β was measured by ELISA. B, Effect of PDTC on fMLP-induced IL-1β mRNA (upper panel) and κB binding activity (lower panel). The monocytes were incubated for 1 h with PDTC as indicated, followed by fMLP stimulation (100 nM, 1 h). Total RNA (20 μg) were subjected to Northern blot analysis with a 32P-labeled human IL-1β probe and IL-1β mRNA, indicated by an arrow (upper panel). The EMSA autoradiograph is shown with the DNA-protein complex marked with a bracket, and the unbound probe is indicated by an arrow (lower panel). C, THP1 cells were cotransfected with 2.5 μg of pSVL-CAT (lane 1, positive control), wt-IL-1β-CAT (lanes 2–4), or the mu-IL-1β-CAT (lanes 5–7) plasmids together with 0.5 μg of the pCMVβ-galactosidase. The transfected cells were stimulated with fMLP (100 nM) or LPS (100 ng/ml) for 60 min, and CAT activity was measured using [14C]chloramphenicol as a substrate, separated by TLC. All results were normalized for transfection efficiency using the expression of β-galactosidase.

The activation and nuclear translocation of NF-κB (39). As shown in Fig. 4A, PDTC treatment of monocytes significantly inhibited fMLP-induced expression of IL-1β. The same treatment almost completely blocked both IL-1β mRNA (Fig. 4B, upper panel) and activation of NF-κB (lower panel) by fMLP, suggesting that NF-κB activation is required for fMLP-stimulated IL-1β gene expression. To further confirm this hypothesis, we transfected the monocyte-like cell line THP1 with a plasmid containing the promoter region of the IL-1β gene fused to the CAT reporter gene to assess the effect of fMLP-induced NF-κB activation on IL-1β transcription (Fig. 4C). The plasmid pIL-1(−4000)CAT (wt-IL-1β-CAT) contains four κB sites from the promoter region of the IL-1β gene and a separate plasmid pIL-1(−133)CAT (mu-IL-1β-CAT) has nonfunctional mutant κB sites. When transfected with the wt-IL-1β-CAT, fMLP (100 nM) and LPS (100 ng/ml) each stimulated increased CAT activity (Fig. 4C, lanes 3 and 4) compared with the unstimulated control (lane 2). When the THP-1 cells were transfected with mu-IL-1β-CAT (from which the four κB sites were deleted), neither fMLP nor LPS stimulated an increase in CAT activity (lanes 6 and 7 compared with lane 5). Taken together, these results demonstrate that fMLP stimulates IL-1β gene expression, and that this is a consequence at least in part of NF-κB activation.
The fMLP stimulates a rapid increase in PI 3-kinase activity

The above experiments indicated that fMLP activates NF-κB and thereby induces IL-1β gene expression in monocytes. The mechanisms of fMLP-induced IL-1β gene expression are not fully understood. Because PI 3-kinase plays a key role in intracellular signal processes linked to diverse receptor types, we examined whether fMLP induced increased PI 3-kinase activity in monocytes. The PI 3-kinase activity was measured by an in vitro kinase assay as described in Materials and Methods. The products of the kinase assay were separated by TLC as described in Materials and Methods. Activity of PI 3-kinase is presented as production of PIP, indicated by an arrow. Experiments were repeated twice with essentially identical results.

Activation of the p85/p110 form of PI 3-kinase involves tyrosine phosphorylation of the p85 subunit (40). To determine whether fMLP stimulated tyrosine phosphorylation of PI 3-kinase, cell lysates from control and fMLP-stimulated monocytes were immunoprecipitated with Ab against the p85 subunit of PI 3-kinase, then precipitated using protein A-Sepharose beads. The PI 3-kinase activity in the immunoprecipitated fraction was determined using an in vitro kinase assay as described in Materials and Methods. Activity of PI 3-kinase is presented as production of PIP, indicated by an arrow. Experiments were repeated twice with essentially identical results.

**FIGURE 5.** The fMLP-induced PI 3-kinase activity in monocytes. Monocytes were stimulated with fMLP (100 nM), PAF (100 nM), C3a (100 nM), or C5a (10 nM) as indicated (A) or with 100 nM fMLP for different times (B). The whole cell lysates were incubated with a rabbit polyclonal Ab against the p85α subunit of PI 3-kinase, then precipitated using protein A-Sepharose beads. The PI 3-kinase activity in the immunoprecipitated fraction was determined using an in vitro kinase assay as described in Materials and Methods. The products of the kinase assay were separated by TLC as described in Materials and Methods. Activity of PI 3-kinase is presented as production of PIP, indicated by an arrow. Experiments were repeated twice with essentially identical results.

The fMLP-induced increase in PI 3-kinase activity was seen within 5 min of stimulation and peaked at 5–10 min. The kinetics of fMLP-induced increase in PI 3-kinase activity was measured by an in vitro kinase assay using phosphatidylinositol as the substrate (36). Monocytes were lysed following stimulation, and the cellular extracts were collected for analysis for PI 3-kinase activity. Both fMLP and other tested leukocyte chemoattractants (PAF, C3a, and C5a) stimulated increased PI 3-kinase activity (Fig. 5A). The fMLP stimulated a time-dependent increase in PI 3-kinase activity (Fig. 5B).

**FIGURE 6.** The fMLP-activated PI 3-kinase contains a tyrosine-phosphorylated p85 subunit. A. Cell lysates from control or fMLP-treated monocytes were immunoprecipitated with anti-p85 of PI 3-kinase and resolved by SDS-PAGE, and then detected using either anti-phosphotyrosine Ab (Fig. 6A, upper panel) or anti-p85 Ab (Fig. 6A, lower panel). As shown in Fig. 6A, fMLP stimulated tyrosine phosphorylation of the p85 subunit in a time-dependent manner. To further confirm the effect of tyrosine phosphorylation, we pretreated monocytes with herbimycin A (1 μM) for 40 min, significantly inhibited fMLP-induced PI 3-kinase activity (Fig. 6B, lane 5), whereas herbimycin A did not reduce the TNF-stimulated PI 3-kinase activity (lane 6). When DMSO (vehicle for herbimycin A) was added, the PI 3-kinase activity level was not significantly changed (lane 4) compared with that using medium alone (lane 1).

The PI 3-kinase activity is required for fMLP-induced NF-κB activation and cytokine gene expression

To assess the role of PI 3-kinase in fMLP-activated NF-κB and IL-1β gene expression, we examined the consequences of preincubating monocytes with PI 3-kinase inhibitors. Wortmannin and LY294002 have both been shown to inhibit PI 3-kinase in multiple cell types with distinct and different modes of action (41, 42). Following pretreatment with wortmannin, LY294002, or medium control, monocytes were stimulated with fMLP, and NF-κB activation was assessed by EMSA. The fMLP-induced NF-κB activation was completely inhibited in monocytes pretreated with wortmannin (Fig. 7, lane 5) or LY294002 (Fig. 7, lane 7). Neither of the inhibitors blocked TNF-α-induced NF-κB activation in the same cells (Fig. 7, lanes 6 and 8). These results suggest that PI 3-kinase activity is required for fMLP-induced NF-κB activation and fMLP-induced intracellular signaling events different from those induced by TNF-α.

Further demonstration of the necessity for PI 3-kinase activity in fMLP-induced NF-κB activation was obtained by overexpressing a dominant negative PI 3-kinase in THP1 cells. The deletion of codons 478–514 from the regulatory p85 component of PI 3-kinase has been shown to confer PI 3-kinase dominant negative activity (34). THP1 cells were transfected with p85ΔN-CΔ478–514, a dominant negative mutant form of PI 3-kinase p85 regulatory
NF-kB and thereby induce IL-1β gene expression by fMLP in monocytes. These results indicate, for the first time, that fMLP stimulates increased PI 3-kinase activity in monocytes, and this PI 3-kinase activity is essential for fMLP-induced activation of NF-kB and IL-1β gene expression.

Using purified peripheral blood cells, we showed that fMLP stimulated IL-1β synthesis as well as NF-kB activation in human monocytes. The fMLP stimulated IL-1β production was blocked by either transcriptional (ActD) or translational (CHX) inhibitors, showing that fMLP stimulated de novo IL-1β synthesis. Furthermore, PDTC, an antioxidant inhibitor of NF-kB, reduced fMLP-induced both NF-kB activation and IL-1β protein secretion (Fig. 4, A and B), suggesting that NF-kB participated in fMLP-stimulated IL-1β gene transcription. Because activation of NF-kB is directly linked to phosphorylation and degradation of its inhibitor, IκB, we demonstrated that fMLP-induced κB binding activity in monocytes was preceded by IκB degradation, followed by resynthesis of IκB (Fig. 3B).

The above experiments indicated that fMLP induced both NF-kB activation and IL-1β gene expression in monocytes. We also showed that there was a time-course correlation between the fMLP-induced NF-kB activation and IL-1β protein secretion (Figs. 1 and 3), and that PDTC not only inhibited NF-kB activation but also abolished IL-1β protein expression (Fig. 4). These observations suggested that fMLP-stimulated IL-1β gene expression could be a consequence of NF-kB activation. To further confirm this hypothesis, we used IL-1β gene promoter:reporter constructs to assess the effect of fMLP-induced NF-kB activation on transcription of the IL-1β gene in THP-1 monocyte-like cells. The fMLP stimulated CAT activity when the promoter region contained functional κB sites, but not when the κB sites were mutated and nonfunctional (Fig. 4C). Taken together with the previous data, these results demonstrate that fMLP-stimulated IL-1β gene expression is a consequence at least in part of NF-kB activation.

Phosphatidylinositol 3-kinase plays a key role in intracellular signal processes linked directly or indirectly to diverse receptor types and is associated with a number of leukocyte responses, such as the generation of superoxide anions and degranulation (44–46). In human peripheral blood monocytes, no evidence has yet been provided for the role of PI 3-kinase in cytokine gene expression. We therefore examined the role of PI 3-kinase in fMLP-induced IL-1β gene expression. Using an in vitro kinase assay with phosphatidylinositol as the substrate, we showed that fMLP-induced PI 3-kinase activity peaked at 5–10 min, while fMLP stimulated NF-kB activation was first detectable after 15–20 min and peaked at 40 min (Fig. 3).

Activation of PI 3-kinase by fMLP suggested that PI 3-kinase could play a role in downstream signaling. We therefore examined whether PI 3-kinase activation is necessary for fMLP-stimulated activation of NF-kB and subsequent IL-1β synthesis. We assessed the effect of inhibiting PI 3-kinase activity on subsequent fMLP-induced NF-kB binding activity and IL-1β gene expression. Wortmannin and LY294002 have been shown to be PI 3-kinase inhibitors. Wortmannin irreversibly inactivates PI 3-kinase by binding to its p110 catalytic subunit (41); LY294002 is a competitive inhibitor, binding to the ATP binding site of the PI 3-kinase (42). Preincubation of monocytes with either wortmannin or LY294002 completely abrogated fMLP-induced NF-kB activation. Neither of the inhibitors blocked TNF-α-induced NF-kB activation in the same cells (Fig. 7, lanes 6 and 8). These results suggest that PI 3-kinase activity is required for fMLP-induced NF-kB activation and fMLP-induced intracellular signaling events different from those used by other NF-kB-activating agents such as TNF-α (Figs.

**FIGURE 7.** The PI 3-kinase inhibitors, wortmannin and LY294002, abolished fMLP-induced NF-kB activation. Monocytes were preincubated with medium (lanes 1 –3), DMSO (lane 4), 100 nM wortmannin (lanes 5 and 6), or 50 μM LY294002 (lanes 7 and 8) for 15 min, then stimulated with 100 nM fMLP (lanes 2, 5, and 7) or 40 ng/ml TNF-α (lanes 3, 6, and 8) for 40 min. Nuclear extracts were prepared and NF-κB activation measured by EMSA as described in Materials and Methods. The EMSA autoradiograph is shown with the DNA-protein complex marked with a bracket and the unbound probe indicated by an arrow. These results are representative of three separate experiments.
6B and 7) and LPS (Fig. 8B). Additional proof that PI 3-kinase was required for fMLP-induced NF-κB activation was provided by the ability of a dominant negative mutant form of the p85 subunit of PI 3-kinase to block fMLP-induced NF-κB activation and IL-1β gene expression (Fig. 8).

Multiple distinct forms of PI 3-kinase have been described in mammalian cells, including two that have been linked to G protein-coupled receptors. The classic PI 3-kinase is linked to receptors with intrinsic or associated tyrosine kinase activity and is a heterodimer consisting of a p110 catalytic subunit and a p85 regulatory subunit containing one SH3 and two SH2 domains (28, 29). A novel p110γ PI 3-kinase that does not bind to p85 has also been described (30, 31). Activation of the p110γ PI 3-kinase in myeloid cells has been reported to be directly regulated by G protein α and βγ subunits (47, 48). Previous studies have shown that fMLP-induced monocyte activation is transduced through a pertussis toxin-sensitive heterotrimERIC G protein and will therefore release GTP-bound Gα and G βγ subunits. Thus, both types of PI 3-kinase forms could potentially be activated as a consequence of signaling through the fMLP receptor. Kular et al. showed that fMLP-sensitive PIP3 formation in human neutrophils involved PI 3-kinase p110γ (49). Studies from other laboratories, however, indicated that fMLP-stimulated leukocytes generate PIP3 through classic p85/p110 PI 3-kinase (47, 48). Our results indicate that PI 3-kinase involved in fMLP-induced NF-κB activation and IL-1β gene expression in human monocytes is the p110/p85 heterodimer. This conclusion is based on 1) its sensitivity to low concentrations of wortmannin, 2) the capacity of an anti-p85 Ab to immunoprecipitate fMLP-induced PI 3-kinase and 3) the ability of a dominant negative p85 mutant to inhibit the response.

In summary, we have shown that fMLP rapidly activates the p85/p110 heterodimeric PI 3-kinase in monocytes. Using both specific inhibitors as well as transient expression of a dominant negative p85 PI 3-kinase mutant, we further showed that fMLP-induced NF-κB activation required PI 3-kinase activity. These findings provide evidence that fMLP-induced both NF-κB activation and IL-1β gene expression uses a signaling pathway that requires activity of PI 3-kinase.

**FIGURE 8.** The PI 3-kinase is necessary for fMLP-stimulated IL-1β gene transcription. A, THP1 cells were cotransfected with 2.0 μg of either p85αN-CA478–514 (dominant negative mutant of the p85 subunit of PI 3-kinase; lanes 3, 5, and 7) or empty vector (lanes 1, 2, 4, and 6) using electroporation. After a 48-h incubation in normal culture medium, the transfected cells were selected by the capture-TecTM pHookTM-2 system, then stimulated with medium alone (lane 1), 100 nM fMLP (lanes 2–7) for 10 min, and harvested. The whole cell lysates were subjected to immunoprecipitation with a rabbit polyclonal Ab against p85, a subunit of PI 3-kinase. The PI 3-kinase activity in the immunoprecipitated fraction was determined using an in vitro kinase assay as described in Materials and Methods. B, THP1 cells were cotransfected with 2.0 μg of either p85αN-CA478–514 (lanes 4–6) or empty vector (lanes 1–3) using electroporation. After a 48-h incubation in normal culture medium, the transfected cells were selected by the capture-TecTM pHookTM-2 system, then stimulated with medium alone (lanes 1 and 4), 100 nM fMLP (lanes 2 and 5), and 100 ng/ml LPS (lanes 3 and 6) for 1 h. Nuclear extracts were prepared for NF-κB activity determined by EMSA (upper panel); culture supernatants were prepared for IL-1β protein detected by ELISA (lower panel). C, THP1 cells were cotransfected with 2.5 μg of the wt-IL-1β-CAT plasmid (lanes 2 and 3), 0.5 μg of pCMVβ (lanes 1–3), and 2.0 μg of either p85αN-CA478–514 (dominant negative mutant of the p85 subunit of PI 3-kinase; lane 3) or empty vector (lanes 1 and 2) using electroporation. After a 48-h incubation in normal culture medium, the transfected cells were stimulated with medium alone (lane 1), 100 nM fMLP (lanes 2 and 3) for 1 h and then harvested. CAT activity was measured in the crude cell lysates using [14C]chloramphenicol as a substrate, separated by TLC as described in Materials and Methods (upper panel). All results were normalized for transfection efficiency using the expression of β-galactosidase. The relative CAT activity of the samples shown in the upper panel is expressed as the percentage of acetylated [14C]chloramphenicol in each lane (lower panel). These results are representative of three separate experiments.
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