Chemoattractant-stimulated NF-κB Activation Is Dependent on

The Low Molecular Weight GTPase RhoA*

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SUMMARY

Chemoattractants bind to seven transmembrane-spanning, G-protein-coupled receptors (GPCR) on monocytes and neutrophils and induce a variety of functional responses, including activation of the transcription factor NF-κB. The signaling mechanisms utilized by chemoattractants to activate NF-κB in human peripheral blood monocytes are poorly defined. We previously demonstrated that fMLP-stimulates NF-κB activation, and this function of fMLP requires phosphatidylinositol 3-kinase (PI3K). Here we present evidence that fMLP activates RhoA, and that fMLP-induced NF-κB activation requires this small GTPase. Stimulation of monocytes with fMLP rapidly activated RhoA as well as NF-κB and their activation was markedly reduced by pertussis toxin treatment. Pre-treatment of monocyte with a RhoA inhibitor, C3 transferase from Clostridium botulinum, effectively blocked fMLP-induced NF-κB activation as well as IL-1β gene expression. A dominant negative form of RhoA (T19N) also inhibited fMLP-stimulated reporter gene expression in a κB dependent manner. Cotransfection of the monocytic THP1 cells with a constitutively active form of RhoA (Q63L) with the promoter reporter plasmid results in a marked increase in NF-κB-mediated reporter gene expression. Furthermore, the PI3K inhibitors wortmannin and LY294002 block RhoA activation induced by fMLP. These results demonstrate that low molecular weight GTPase RhoA is a novel signal transducer for fMLP-induced NF-κB activation and Gαi or Gαo class of heterotrimeric G proteins likely mediate RhoA activation via PI3K in human peripheral blood monocytes.
INTRODUCTION

Leukocytes constitute the first line of host defense against invading microorganisms and are a major cellular component of the inflammatory reaction. When exposed to chemoattractants, such as formyl peptide, leukocytes become rapidly activated. The bacterial tripeptide fMet-Leu-Phe (fMLP)\(^1\) is able to activate all major functions of neutrophils and is a prototypical ligand for the N-formyl peptide receptor (FPR), which contains 7 putative transmembrane domains characteristic of the G protein-coupled receptor (GPCR) of the rhodopsin family (1, 2).

fMLP stimulates peripheral blood mononuclear cells (PBMC) to express a defined set of gene products, including IL-1, IL-6 and IL-8. Pretreatment of the PBMC with pertussis toxin abolishes fMLP-stimulated cytokine synthesis, suggesting that a G\(\alpha\)i containing heterotrimeric G protein may mediate the process (3). Several recent studies have demonstrated activation of the transcription factor NF-\(\kappa\)B by G protein-coupled receptors (4-8). NF-\(\kappa\)B is of paramount importance to immune cell function owing to its ability to activate the transcription of many proinflammatory immediate-early genes (9, 10). NF-\(\kappa\)B is a multiprotein transcription activator originally found to bind a decameric enhancer sequence in the gene for the immunoglobulin \(\kappa\) light chains. In leukocytes, NF-\(\kappa\)B activation results in the transcription of immediate-early genes that encode IL-2, IL-6, IL-8, TNF\(\alpha\), MCP-1, GM-CSF, as well as several adhesion molecules (10). Numerous stimuli can activate NF-\(\kappa\)B, including the bacterial components LPS, as well as other proinflammatory factors including IL-1 and TNF\(\alpha\). We recently reported that chemoattractants (fMLP, PAF, C3a and C5a) can activate the transcription factor NF-\(\kappa\)B (7, 11, 12) and provided the evidence that fMLP-induced NF-\(\kappa\)B activation is essential for proinflammatory cytokine synthesis in human peripheral blood monocytes (13). We
further showed that fMLP-induced NF-κB activation required PI3K activity and this activation in monocytes is mediated by p85/p110 heterdimeric PI3K (13).

GTPases of the Rho family exist in both GDP-bound inactive (GDP-Rho) and GTP-bound active (GTP-Rho) forms. When cells are stimulated with different ligands, GDP-Rho is converted to GTP-Rho, which binds to specific targets and then exerts its biological functions. Low molecular weight G proteins of the Rho family (consisting of Cdc42, Rac, and RhoA) have been shown to regulate actin cytoskeletons, focal adhesion complex formation, cell aggregation and cell motility (14-16). The function of these small G proteins in leukocyte cytokine gene transcription, however, has not been previously addressed. Recent reports indicate that Rho GTPases regulate c-fos transcription activation (17), that constitutively active Rho proteins can activate NF-κB, and that TNFα-induced activation of NF-κB in NIH-3T3 cells is dependent on Cdc42 and RhoA (18). Chang et al demonstrated that Rho activation is also involved in AP-1 mediated transcription in Jurkat cells (19). C. difficile toxin B, which inactivates Rho family proteins including RhoA, Rac, and Cdc42, has been reported to reduce the LPS-induced IL-8 expression in human umbilical vein endothelial cells (20). Despite an apparent role of the Rho GTPases in gene expression, relatively little is known about the molecular mechanisms of Rho signaling in gene transcription.

In this study we therefore investigated the role of Rho GTPase in the signaling events that lead to NF-κB activation in fMLP-stimulated human peripheral blood monocytes. We found that stimulation of monocytes with fMLP rapidly activated RhoA as well as NF-κB in a pertussis toxin-sensitive manner. Furthermore, inhibition of RhoA activity blocked fMLP-induced NF-κB activation. These results indicate that fMLP stimulates RhoA and NF-κB activation and that the RhoA activity is required to mediate this effect in human peripheral blood monocytes.
EXPERIMENTAL PROCEDURES

Reagents FMLP and recombinant human C5α were obtained from Sigma (St. Louis, MO). Platelet-activating factor (PAF) was obtained from Calbiochem (San Diego, CA). Human C3a was obtained from Advanced Research Technologies (San Diego, CA). Pertussis and cholera toxins were purchased from Calbiochem (San Diego, CA). Recombinant murine TNFα was kindly provided by V. Kravchenko (Scripps Research Institute). A monoclonal antibody against RhoA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotides and their complementary strands for electrophoretic mobility shift assays (EMSA) were from Promega (Madison, WI), with the sequence of (underlined), 5'-AGTTGAGGGGACTTTCCCAGGC-3' (NF-κB) (21). The NF-κB oligonucleotide has been shown to represent a consensus κB sequence (22, 23). Double-stranded oligonucleotide (5 pmoles) was 32P-labeled with T4 polynucleotide kinase. [γ-32P]ATP (> 5000 Ci/mmol) was from Amersham (Arlington Heights, IL). The RhoA dominant negative (T19N), constitutively active (Q63L) pCMV plasmids, and recombinant Clostridium botulinum C3 transferase were obtained as previously described (21).

Preparation of monocytes from peripheral blood Heparinized human peripheral blood from health donors was fractionated on Percoll (Pharmacia) density gradients. Mononuclear cells and neutrophils were initially separated by centrifugation through a 55%/74% discontinuous Percoll gradient. Monocytes were further prepared from the mononuclear cell population with gelatin/plasma coated flasks as described (7). The purity of monocytes was greater than 85-90% as determined by staining with an anti-CD14 monoclonal antibody (Coulter Immunology, Miami, FL), and cell viability was greater than 95% as measured by trypan blue exclusion. Monocytes were resuspended in RPMI-1640 medium (Irvine Scienfic, Santa Ana, CA) with 10%(V/V) heat-inactivated fetal
bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM; Irvine Scientific, Santa Ana, CA).

**Detection of cellular GTP-Rho** When activated, Rho undergoes GDP-GTP exchange, and activated Rho can thus be detected by analyzing GTP-bound Rho. RhoA activity was detected by the method recently described by Ren et al (24). This assay utilizes the Rho-binding domain (RBD) from the effector protein Rhotekin as a probe to specifically isolate the active forms of RhoA. Human peripheral blood monocytes (5x10^6) were stimulated with fMLP, or control media and then lysed (lysis buffer: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml each of leupeptin and aprotinin). Equal volumes of lysates were incubated with GST-RBD (20 µg) beads at 4°C for 45 min. The beads were washed three times with a Tris buffer containing 1% Triton X-100, 10 mM MgCl₂, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml each of leupeptin and aprotinin. Bound Rho proteins were detected by Western blotting using a monoclonal antibody against RhoA (Santa Cruz, Inc). The amount of RBD-bound Rho was normalized to the total amount of Rho in cell lysates for comparison of Rho activity in different samples.

**Electrophoretic mobility shift assay (EMSA)** Nuclear extracts were prepared from human peripheral blood monocytes using a modified method of Dignam et al. (25), and EMSA were performed using 2.5 µg of the nuclear extract as described previously (7).

**Chloramphenicol acetyltransferase (CAT) assay** The promoter:reporter plasmid pIkB-CAT (WT-IκB-CAT) contains an κB-like enhancer from the promoter region of the IκB gene. This enhancer was deleted in the plasmid MU-IκB-CAT. Both constructs were previously described in (21, 26). The plasmid pBLCAT2 was the parent vector for the IκB constructs. The plasmid pCMVβ (Clontech) was used as a control for monitoring the
transfection efficiency by the expression of β-galactosidase. The plasmid DNA was transiently transfected into THP1 cells by electroporation. After 6 hours, the DNA-containing media was removed, the cells were washed with PBS and incubated with normal medium for 16 hours. The cells were then stimulated with agonists for the indicated time and harvested by scraping. Crude cell extracts were prepared for the measurement of CAT activity with the use of $[^{14}\text{C}]$chloramphenicol (Amersham) as the substrate and thin layer chromatography (TLC) for the separation of the native from the acetylated forms as described (21, 27). After development, the extent of CAT activity was measured using the ImageQuant software (Molecular Dynamics).
RESULTS

FMLP-induced \( \kappa B \) binding is blocked by a RhoA inhibitor

To assess the role of Rho GTPase in fMLP-induced NF-\( \kappa B \) activation, we examined the consequences of pre-incubating cells with a specific Rho inhibitor. The C3 transferase is an exotoxin produced by \textit{Clostridium botulinum} that specifically inhibits the Rho small GTP binding proteins (Rho A, B, and C) but does not inhibit Rac or Cdc42 (28). Following pretreatment with 10 \( \mu \text{g/ml} \) of recombinant C3 transferase or media control for 16 hours, human monocytes were stimulated with fMLP or TNF\( \alpha \) for 60 min and NF-\( \kappa B \) activation assessed by EMSA. The DNA binding activity of NF-\( \kappa B \) was potently induced by fMLP and TNF\( \alpha \) (Fig. 1A, lanes 2-3), fMLP-induced NF-\( \kappa B \) activation was completely inhibited in monocytes pretreated with C3 transferase (Fig 1A, lane 5), however, the C3 transferase only marginally affected TNF\( \alpha \)-induced NF-\( \kappa B \) activation (Fig. 1A, lanes 6). These results suggest that RhoA is required for fMLP- but not TNF\( \alpha \)-induced NF-\( \kappa B \) activation. We next examined the dose-response of the inhibitory effect of C3 transferase on fMLP-induced NF-\( \kappa B \) activation. As shown in figure 1B, C3 transferase at doses greater than or equal to 5 \( \mu \text{g/ml} \) significantly inhibited fMLP-induced NF-\( \kappa B \) activation. NF-\( \kappa B \) is known to stimulate the transcription of many inflammatory genes, including a large number of cytokines (22). Preincubation with recombinant C3 transferase also inhibited fMLP-stimulated IL-1\( \beta \) synthesis (Fig. 1C), but only marginally affected TNF\( \alpha \)-induced IL-1\( \beta \) synthesis (Fig. 1C, lane 5 versus 3).

fMLP stimulates a rapid but transient increase in RhoA activity

The results presented above demonstrate that inhibition of RhoA activity abrogates fMLP-induced NF-\( \kappa B \) activation. We next examined whether fMLP would induce increased RhoA activity in human peripheral blood monocytes.
At various times following stimulation, monocytes were lysed and total cell lysates incubated with GST or GST-RBD beads. Immunoblotting of the bound proteins was performed as described in Experimental Procedures. Both fMLP and other tested leukocyte chemoattractants (PAF, C3a, and C5a) stimulated increased RhoA activity (Fig. 2A). fMLP stimulated a time-dependent increase in RhoA activity (Fig. 2B). The fMLP induced increase of RhoA activity was seen within 5 minutes of stimulation and peaked at 5-10 minutes. The kinetics of fMLP-induced RhoA activity preceded that of fMLP-induced NF-κB activation (13), consistent with a role for RhoA in the activation of NF-κB.

**fMLP-induced RhoA and NF-κB activation involves fMLP receptors coupled to pertussis sensitive heterotrimeric G proteins**

We previously demonstrated that fMLP-induced NF-κB activation in peripheral blood mononuclear cells (PBMC) is mediated through the fMLP receptor (11), a member of the seven transmembrane G protein coupled receptor (GPCR) superfamily. The identity of the heterotrimeric G proteins coupling the fMLP receptor to RhoA activity and NF-κB activation in monocytes has not been elucidated. We therefore examined the effect of pertussis toxin and cholera toxin on fMLP-induced NF-κB activation and RhoA GTPase. Monocytes were pre-treated with pertussis and cholera toxins separately, then stimulated with fMLP. Pertussis toxin (0.5 µg/ml) markedly reduced fMLP-stimulated NF-κB activation (Fig. 3A, lane 5), while cholera toxin had no such inhibitory effect in monocytes (Fig. 3A, lane 8). Neither of the toxins inhibited TNFα-induced NF-κB activation in the same cells (Fig. 3A, lanes 6 and 9). FMLP-induced RhoA activity was also blocked by pertussis toxin (Fig. 3B, lane 5) but not cholera toxin (Fig. 3B, lane 7); while neither toxin inhibited TNFα-induced RhoA activity (Fig 3B, lanes 6 and 8). Thus, our results indicate that fMLP stimulates both RhoA and NF-κB activation through fMLP receptors that are coupled to pertussis toxin sensitive heterotrimeric G proteins.
RhoA activity is required for fMLP-induced NF-κB activation

Further demonstration of the necessity for RhoA activity in fMLP-induced NF-κB activation was obtained by overexpressing a dominant-negative form of RhoA (RhoA-T19N). THP1 cells were co-transfected with an expression vector encoding a dominant negative form of RhoA (RhoA-T19N) together with an IκB promoter-CAT reporter plasmid prior to fMLP stimulation. Figure 4 shows the effect of the dominant negative RhoA mutant protein on fMLP-stimulated CAT activity in THP1 cells transfected with the chimeric IκB-CAT plasmid. fMLP stimulated increased CAT activity in THP1 cells transfected with the WT-IκB-CAT plasmid (Fig. 4A, lane 2). Co-transfection of the dominant negative RhoA plasmid (2 µg) with the WT-IκB-CAT plasmid completely blocked fMLP-stimulated CAT activity (Figure 4A, lane 4). These results confirm that RhoA activity is required for fMLP-induced NF-κB activation.

The relationship between RhoA activity and subsequent NF-κB activation was further explored using RhoA-Q63L, a constitutively active form of RhoA. Transfection of THP1 cells with RhoA-Q63L resulted in increased CAT activity when the cells were co-transfected with a WT-IκB-CAT reporter plasmid containing a functional κB site (Fig. 4B, lane 2). Thus, we have shown that RhoA activity alone is sufficient to induce NF-κB activity. Taken together with the previous results, this strongly suggests that fMLP-mediated NF-κB activation results at least in part from RhoA activity.

Effects of PI3K inhibitors on fMLP-induced RhoA activation in human monocytes

We have previously demonstrated that fMLP stimulated phosphatidylinositol 3-kinase (PI3K) activity and this activity is required for fMLP-induced NF-κB activity in human monocytes (13). Based on our current results and our previous report, the relationship between PI3K activation and activation of the RhoA appears to be an
important issue. To investigate the role of PI3K in RhoA activity induced by fMLP, we used wortmannin and LY294002, that specifically inhibit PI3K by distinct mechanisms. As shown in Fig. 5, fMLP-induced RhoA activation was dose-dependently inhibited by wortmannin and LY294002, indicating that PI3K activity is required for the Gi-mediated activation of RhoA.
DISCUSSION

Although the activation of NF-κB has been extensively studied in cultured cell lines of hematopoietic lineage, the signal transduction pathways for this activation process are still incompletely understood. The Rho GTPases are known to regulate actin cytoskeletons, focal adhesion complex formation, cell aggregation and cell motility (14-16). Results from recent studies have shown an important role of Rho GTPases in gene transcription and expression (17-20). To explore the mechanism by which fMLP promotes inflammatory disorders, we examined the ability of fMLP to regulate gene transcription and the signal transduction pathways that lead to NF-κB activation in human peripheral blood monocytes.

We demonstrated that fMLP induced a time-dependent increase in RhoA activity, suggesting that RhoA play a role in downstream signaling. These results were supported by the ability of C3 transferase, an inhibitor of Rho, to abolish fMLP-induced NF-κB activation. The role of Rho in fMLP-induced NF-κB activation was then confirmed by co-transfecting THP1 cells with a dominant negative RhoA plasmid as well as the chimeric IκB-CAT reporter plasmid. The dominant negative RhoA plasmid significantly abrogated the fMLP-stimulated CAT activity. These results suggest that fMLP-induced NF-κB activation require activation of RhoA. The relationship between RhoA activity and subsequent NF-κB activation was further explored using a constitutively active form of RhoA (RhoA-Q63L) which increased NF-κB activity in the absence of additional stimulation. Taken together with the previous results, these data suggest that fMLP-induced NF-κB activation results from RhoA activity.

The N-formyl peptide receptor (FPR) is a member of the G protein-coupled receptor family (1, 2). This class of receptors is typified by the presence of seven membrane-spanning domains. The G-protein known to play a major role in FPR signal
transduction is a pertussis toxin substrate, since treatment of neutrophils with the pertussis 
toxin blocks the majority of FPR-mediated responses (29, 30). To assess the type of 
heterotrimeric G protein coupling the FPR to RhoA and NF-κB, we analyzed the effects of 
pertussis and cholera toxins. Pertussis toxin ADP-ribosylates Gαi and Gαo proteins, while 
cholera toxin ADP-ribosylates Gαs proteins (31). FMLP stimulated RhoA activity and 
NF-κB activation were both inhibited by pertussis toxin but not by cholera toxin, 
indicating that both responses are transduced through Gαi or Gαo class of heterotrimeric G 
proteins. The mechanism for the potentiation of fMLP induced RhoA activity by cholera 
toxin is currently unknown. It is possible that Gs and adenylyl cyclase might also function 
in the signaling mechanism governing RhoA activity in these system, but this will require 
进一步的调查。

We previously demonstrated that fMLP induced NF-κB activation required 
PI3K activity in human monocytes (13) and here we present evidence that RhoA activity is 
essential for fMLP-induced NF-κB activation. To test the possibility that PI3K is a 
component of the fMLP stimulated signaling pathway leading to RhoA activation, we 
assessed the effect of inhibiting PI3K activity on subsequent fMLP-induced RhoA 
activation. Wortmannin and LY294002 have been shown to be specific PI3K inhibitors. 
Wortmannin irreversibly inactivates PI3K by binding to its p110 catalytic subunit (32); 
LY294002 is a competitive inhibitor, binding to the ATP-binding site of the PI3K (33). 
Pre-incubation of human monocytes with either wortmannin or LY294002 significantly 
abrogated fMLP-induced RhoA activity. Our results indicate that PI3K likely functions 
upstream of RhoA in the fMLP-induced signaling pathway. The mechanisms linking PI3K 
activation to RhoA activity are unknown. D3-phosphorylated phosphatidylinositols are 
known to play important roles in cell growth and survival, although the exact role and 
immediate downstream molecular targets of PtdIns(3)P, PtdIns(3,4)P2, and 
PtdIns(3,4,5)P3 are only now emerging (34, 35). Several studies have demonstrated that
PI3K may be activated downstream of the small Rho GTPases (36-38). GTP-loaded Rac was shown to directly bind PI3K (39). Furthermore, inactivation of Rho using bacterial toxin C3 inhibited lysophosphatidic acid-induced PI3K activation in Swiss 3T3 cells (40). A more recent study suggests that Toll-like receptor-2 (TLR2)-mediated NF-κB activation requires Rac1 and PI3K is involved as a downstream effector (41). Other studies, however, have suggested that PI3K may activate the small Rho GTPases. Expression of a constitutively active PI3K mutant in Swiss 3T3 cells induced a subset of Rac and Rho-mediated cellular responses (42). The PI3K product PtdIns(3,4,5)P3 has been shown to bind the pleckstrin homology (PH) domain of guanine nucleotide exchange factors (GEF), providing a potential mechanism for PI3K-mediated regulation of Rho activation (42, 43). Additionally, the p85 regulatory subunit of PI3K contains a breakpoint cluster region-homology domain (BH) that has been shown to have GTPase activating protein (GAP) activity (44).

In summary, we have shown that fMLP rapidly activates the RhoA GTPase in human peripheral blood monocytes. FMLP stimulated RhoA activity and NF-κB activation were both inhibited by pertussis toxin but not cholera toxin, suggesting that both FPR-mediated responses are the results of coupling to the G\textsubscript{i}/G\textsubscript{o} class of G\textsubscript{α} proteins. Utilizing both a specific inhibitor and transient expression of a dominant-negative RhoA mutant, we further showed that fMLP-induced NF-κB activation required RhoA activity. We have previously reported that RhoA is also involved in NF-κB activation through the Gq-coupled B2 bradykinin receptor (21), but it was not clear at that time whether bradykinin stimulation could activate RhoA in fibroblasts. Although the mechanism by which Gq and Gi activates RhoA may not be the same, these findings combined suggest that the signaling pathways initiated by Gi and Gq may converge at a point prior to RhoA activation. Although TNF\textsubscript{α} also stimulated RhoA activity, TNF\textsubscript{α} stimulated NF-κB activation was not affected by inhibition of RhoA activity. These findings provide
evidence that fMLP-induced NF-κB activation utilizes a signaling pathway which requires both PI3K and low molecular weight GTPase RhoA and is distinct from the signaling pathway utilized by TNFα. The signaling molecules linking FPR-associated Gi/Go proteins and the PI3K, small GTPase RhoA, however, remain to be determined.
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FOOTNOTES

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1 Abbreviations used in this paper: fMLP, fMet-Leu-Phe; FPR, N-formyl peptide receptor; PAF, platelet-activating factor; PBMC, peripheral blood mononuclear cells; GPCR, G-protein-coupled receptors; PI3K, phosphatidylinositol 3-kinase; RBD, Rho-binding domain; IL-1, interleukin 1; TNF, tumor necrosis factor; rC3, recombination C3 transferase; NF-κB, nuclear factor κB; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; TLC, thin layer chromatography; PH, pleckstrin homology; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein.
FIGURE LEGENDS

**Figure 1.** The RhoA inhibitor, C3 transferase, abolishes fMLP-induced NF-κB activation and IL-1β synthesis. (A) human peripheral blood monocytes were pre-incubated with media alone or recombinant C3 transferase (rC3 inhibitor; 10 µg/ml overnight), stimulated with media alone (M), 100 nM fMLP (F), or 100 ng/ml TNFα (T) for 60 min; and NF-κB activation was monitored by EMSA. A PhosphoImager screen was exposed, and the EMSA autoradiograph is shown. The DNA-protein complex is marked with a bracket, and the unbound probe is indicated by an arrow. (B) rC3 transferase inhibits fMLP stimulated NF-κB activation in a dose dependent manner. Human peripheral blood monocytes were pre-incubated with varying concentrations of rC3 inhibitor for 16 hours, then stimulated with fMLP (100 nM) for 60 min. NF-κB activation was determined as described for Fig. 1A 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. (C) human peripheral blood monocytes were pre-incubated with media alone or recombinant C3 transferase (rC3 inhibitor; 10 µg/ml overnight), stimulated with media alone, fMLP (100 nM), or TNFα (100 ng/ml) for 4 hours, and IL-1β was measured in the culture supernatants by ELISA.

**Figure 2.** FMLP induces RhoA activity in human peripheral blood monocytes. Monocytes were stimulated with fMLP (100 nM), PAF (100 nM), C5a (10 nM), C3a (100 nM), and TNFα (100 ng/ml) for 10 min as indicated (A), or with 100 nM with fMLP for different times (B). The whole cell lysates were incubated with either GST beads (control) or GST-RBD (20 µg) beads at 4°C for 45 min. Rho activity is indicated by the amount of RBD-bound RhoA by Western blotting using a monoclonal antibody against RhoA (Santa Cruz, Inc) as described under “Experimental Procedures”. Experiments were repeated 3 times with essentially identical results.
Figure 3. Pertussis toxin inhibits fMLP-stimulated NF-κB activation and RhoA activity. (A) Human monocytes were incubated for 4 h with either pertussis toxin (0.5 µg/ml) or cholera toxin (5 µg/ml) and then for 60 min with 100 nM fMLP (F) or 100 ng/ml TNFα (T), before NF-κB activation was determined as described for Fig. 1, bracket marks the DNA-protein complex. (B) Activity of RhoA, measured as described for Fig. 2, is indicated by the amount of RBD-bound RhoA assessed by Western blotting. These results are representative of two experiments.

Figure 4. RhoA is necessary for fMLP-stimulated NF-κB activation. (A) THP1 cells were co-transfected with 2.5 µg of the WT-IκB-CAT plasmid (lanes 1-4), 0.5 µg of pCMVβ (lanes 1-4), and 2.0 µg of either RhoA-T19N (dominant negative mutant of the RhoA; lanes 3 and 4) or empty vector (lanes 1 and 2). After 48 hours incubation in normal culture media, the transfected cells were stimulated for 2 hours with media alone (lanes 1 and 3) or 100 nM fMLP (lanes 2 and 4) and then harvested. (B) THP1 cells were co-transfected with 2.5 µg of the WT-IκB-CAT plasmid (lanes 1 and 2), MU-IκB-CAT (lane 3), 0.5 µg of pCMVβ (lanes 1-3), and 2.0 µg of either the RhoA-Q63L plasmid (lanes 2 and 3) or empty vector (pBLCAT2, lane 1). CAT activity was measured in the crude cell lysates using [14C]-chloramphenicol as a substrate, separated by TLC as described in the Methods. All results were normalized for transfection efficiency using the expression of β-galactosidase. A PhosphoImager screen was exposed, and the autoradiograph is shown. These results are representative of two separate experiments.

Figure 5. Effects of PI3K inhibitors on fMLP-stimulated RhoA activation in human monocytes. Human peripheral blood monocytes were preincubated for 15 min with indicated concentrations of wortmannin (Fig. 6A) or LY294002 (Fig. 6B) and then stimulated for 5 min with 100 nM fMLP. The GTP-bound, active RhoA was detected as described above.
Fig. 1

A. rC3 10 µg/ml

B. rC3 + fMLP

C. IL-1β protein (pg/ml)

- Media
- fMLP
- TNFα
- rC3+fMLP
- rC3+TNFα
Fig. 2

A. 

| fMLP (min) | 0  | 1' | 5' | 10' | 20' |
|------------|----|----|----|-----|-----|
| Activated RhoA |    |    |    |     |     |
| Beads      | GST | GST-RBD |

B. 

| fMLP PAF C5a C3a TNFα |    |    |    |     |     |
| Activated RhoA |    |    |    |     |     |
| Beads      | GST | GST-RBD |
Fig. 3

A.  

|   | PT (0.5 μg/ml) | CT (5 μg/ml) |
|---|----------------|--------------|
| M | F | T | M | F | T | M | F | T |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |

NF-κB

B.  

Relative RhoA Activity

| GST | GST-RBD |
|-----|---------|
|     |         |

| 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
|----|----|----|----|----|----|----|----|
| Media | fMLP | TNFα | fMLP | TNFα | fMLP | TNFα |

PT (0.5 μg/ml)  CT (5 μg/ml)
Fig. 4

A. WT-IκB-CAT
   Vector fMLP Media fMLP
   Rho-T19N Media fMLP

B. WT-IκB-CAT MU-
   Vector Q63L IκB-CAT
   Q63L

Fig. 5

A. Wortmannin (nM)
   Wortmannin (nM) 0 0 10 100
   Activated RhoA
   fMLP (nM) 0 100 100 100

B. LY294002 (μM)
   LY294002 (μM) 0 0 5 50
   Activated RhoA
   fMLP (nM) 0 100 100 100
Chemoattractant-stimulated NF-kB activation is dependent on the low molecular weight GTPase RhoA

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