Primary granule release from human neutrophils is potentiated by soluble fibrinogen through a mechanism depending on multiple intracellular signaling pathways

Florin Tuluc, Analia Garcia, Ovidiu Bredetean, John Meshki, and Satya P. Kunapuli

Department of Physiology, Temple University Medical School, Philadelphia, Pennsylvania 19140

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Tuluc, Florin, Analia Garcia, Ovidiu Bredetean, John Meshki, and Satya P. Kunapuli. Primary granule release from human neutrophils is potentiated by soluble fibrinogen through a mechanism depending on multiple intracellular signaling pathways. Am J Physiol Cell Physiol 287: C1264–C1272, 2004. First published June 30, 2004; doi:10.1152/ajpcell.00177.2004.—N-Formyl-methionyl-leucyl-phenylalanine (fMLP) is a potent activator of neutrophil degranulation. The intracellular signaling mechanisms involved in the potentiating effect of fibrinogen on fMLP-induced primary granule release from human neutrophils were investigated. Fibrinogen caused a significant leftward shift of the concentration-response curve of fMLP-induced elastase release. An antibody against Mac-1 (CD11b/CD18) prevented the potentiating effect of fibrinogen, suggesting that soluble fibrinogen potentiates fMLP-induced degranulating effect by a mechanism mediated by the integrin Mac-1. Fibrinogen enhanced fMLP-induced tyrosine phosphorylation in human neutrophils and markedly enhanced the phosphorylation of mitogen-activated protein kinases (MAPK) caused by fMLP. However, U0126, an inhibitor of p44/42 MAPK activation, or SB-203580, an inhibitor of p38 MAPK, did not alter the effect of fibrinogen on fMLP-induced elastase release. Wortmannin, a phosphatidylinositol 3-kinase (PI3K) kinase inhibitor, and genistein, a nonspecific tyrosine kinase inhibitor, strongly inhibited fMLP-induced elastase release both in the presence and in the absence of fibrinogen. An Akt/ PKB inhibitor failed to alter the potentiating effect of fibrinogen, suggesting that the effect of fibrinogen is mediated by Akt-independent pathways. Gö6976, an inhibitor of classical PKC isofoms, caused a significant inhibition of fMLP-induced elastase release in the presence or absence of fibrinogen, while nonselective inhibitors of PKC, Ro 31-8220, GPS-109203X, and staurosporine, caused potentiation of fMLP-induced elastase release. We conclude that fibrinogen potentiating of primary granule release induced by fMLP is mediated by the integrin CD11b/CD18 through pathways dependent on PI3K and tyrosine kinases, but other regulatory mechanisms may be also involved.

N-formyl-methionyl-leucyl-phenylalanine; elastase; CD11b

POLYMORPHONUCLEAR LEUKOCYTES and monocyte/macrophages play a central role in innate immunity, and they are the major cellular effectors in inflammatory reactions and in responses related to tissue injury. In the past decade, it has become evident that integrin-mediated interactions with extracellular matrix and plasma proteins, as well as with proteins expressed on endothelial cells, are essential in regulating the functions of the inflammatory cells. It was previously shown that fibrinogen is one of the plasma proteins that are able to interact with neutrophils (38) or with macrophages (33) through the β2 integrin CD11b/CD18, also known as Mac-1. Fibrinogen is a dimeric protein composed of identical subunits, each comprising three nonidentical polypeptide chains (α, β, and γ). The binding of fibrinogen to CD11b/CD18 on human neutrophils occurs at a single saturable binding site with an apparent Kd of 0.17 mM and 140,000 sites/cell (38). This recognition site is unique compared with fibrinogen binding to other integrins and is contained within the fragment D of fibrinogen that lacks the Arg-Gly-Asp (RGD) sequences (1). Furthermore, there is experimental evidence suggesting that the fibrinogen γ-chain region Gly190–Val202 functions as a minimal recognition sequence for the leukocyte integrin CD11b/CD18 (2). Given the participation of fibrinogen-leukocyte interaction in inflammation and atherogenesis, it has been postulated that antagonists based on this unique structural motif would effectively interfere with aberrant leukocyte adhesion mechanisms without affecting RGD-directed vascular integrins (2).

The acute phase of the inflammatory response involves an increase in the concentrations of different plasma proteins that include fibrinogen and multiple proinflammatory mediators. The modulatory effects of soluble fibrinogen on the process of sequential neutrophil activation have been poorly investigated (28). Neutrophil recruitment is closely related to neutrophil activation and cell survival (37). In this context, it was recently established that integrin engagement is able to trigger intracellular signaling events that lead to cytokine production (36) and exert regulatory effects in platelet-neutrophil interaction (8), phagocytosis (6, 28, 29), chemotaxis (18), production of reactive oxygen species (ROS) (22), and apoptosis (28, 29). Moreover, it has been suggested that elevated fibrinogen levels may alter vascular remodeling when blood flow is compromised and thus may lead to the development of atherosclerotic lesions (13, 19). Fibrinogen can activate intracellular signaling mechanisms in neutrophils such as intracellular Ca2+ increase and phosphorylation of focal adhesion kinases (29). In addition, enhanced exudation of fibrinogen into the perivascular space was associated with neutrophil migration at the inflammatory site in an in vivo model of acute inflammation in rats (30), strongly suggesting that fibrinogen might have well-defined roles in modulating the neutrophil responses. Passively released or actively secreted elastase from neutrophils has been linked to the pathological processes of a variety of inflammatory diseases, including idiopathic pulmonary fibrosis, rheumatoid arthritis, adult respiratory distress syndrome, and cystic fibrosis (7b). Investigators at our laboratory recently showed that fibrinogen is required for the release of primary granule release from neutrophils trig-
nerged by extracellular nucleotides, such as ATP or UTP (20).

In the present study, we investigated the effects of soluble fibrinogen on isolated human polymorphonuclear leukocytes, with particular emphasis on determining the intracellular signaling mechanisms involved in the potentiating effect of fibrinogen on the release of primary granule release from isolated human neutrophils. We also have shown that soluble fibrinogen has a potentiating effect on other neutrophil responses, such as intracellular Ca\textsuperscript{2+} increase and ROS production. We have found evidence that fibrinogen potentiates of primary granule release induced by N-formyl-methionyl-leucyl-phenylalanine (fMLP) is mediated by the integrin CD11b/CD18 through phosphatidylinositol 3-kinase (PI3K) and tyrosine kinase-dependent pathways. In addition, we show that p44/42 mitogen-activated protein kinase (p44/42 MAPK) and p38 MAPK make no contribution to this mechanism, but it is likely that other regulatory intracellular signaling mechanisms also may be involved in the enhancing effect of fibrinogen on neutrophil degranulation.

**MATERIALS AND METHODS**

Reagents. N-(methoxysuccinyl)-Ala-Ala-Pro-Val p-nitroanilide, SB-203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole], U0126 [1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenyl-mercapto)butadiene], and bovine serum albumin (BSA; fraction V) were obtained from Sigma (St. Louis, MO). Dextran T500, Ficoll-Paque, and enhanced chemiluminescence (ECL) reagents were obtained from Amersham Biosciences (Piscataway, NJ). Fura 2-AM was purchased from Molecular Probes (Eugene, OR). IL-6-Hydroxymethyl-chiro-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarboxonate] (HIMO) was obtained from Alexis Biochemicals (San Diego, CA). Polyclonal anti-phospho-p44/42 MAPK (Thr\textsuperscript{202}/Tyr\textsuperscript{204}), anti-p44/42 MAPK, anti-phospho-p38 MAPK (Thr\textsuperscript{180}/Tyr\textsuperscript{182}) antibodies, and anti-p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA). The monoclonal 4G10 antibody anti-phosphotyrosine was obtained from Upstate Biotechnology (Waltham, MA).

Neutrophil isolation. Venous blood was collected in polypropylene tubes containing anticoagulant (1.5% citric acid, 2.5% sodium citrate, 2% dextrose) upon receipt of written consent from healthy volunteers. Tubes containing anticoagulant (1.5% citric acid, 2.5% sodium citrate, 2% dextrose) upon receipt of written consent from healthy volunteers.

**Measurement of ROS production.** The release of ROS was measured as described elsewhere (9). Briefly, horseradish peroxidase (1 U/ml) and isoluminol (5 µM) were added to neutrophil suspensions (3 million/ml) in a glass cuvette under stirring conditions at 37°C in a luminometer model 560-CA (Chronolog, Havertown, PA). The cells were allowed to equilibrate, reagents were added to the cell suspension, and the luminescence signal was recorded on a chart recorder. A standard solution of hydrogen peroxide was used to determine the sensitivity of the peroxidase-isoluminol system and for normalization of data.

Western blotting. Neutrophils were isolated and stimulated with fMLP (100 nM) in the presence of thrombin concentration (2 µg/ml) of fibrinogen or BSA at 37°C for different intervals. The stimulation was terminated by adding to each sample an equal volume of 2× lysis buffer containing a standard mixture of protease and phosphatase inhibitors. The final concentrations of reagents in the lysis medium were 25 mM Tris·HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, and 10 mM β-glycerophosphate. Lysis was performed on ice for 20 min, followed by centrifugation of the samples at 13,000 rpm for 5 min. A volume of 200 µl of the cytosolic fraction (the supernatant) was collected and mixed with 100 µl of sample buffer containing 6% sodium dodecyl sulfate, 30% glycerol, 3 mM EDTA, and 0.05% phenol red. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and incubated for 30 min in Tris-buffered saline (TBST; 20 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) containing 2% BSA (blocking buffer). Membranes were incubated overnight at 4°C in the presence of the indicated primary antibody, which was diluted 1:1,000 in blocking buffer. Next, membranes were washed four times at room temperature in TBST. The appropriate secondary antibody conjugated with alkaline phosphatase was diluted 1:5,000 in blocking buffer. The membranes were incubated with the secondary antibody for 1 h at room temperature followed by five consecutive washes. CDP-Star with Nitro-Block II (Tropix, Bedford, MA) was used for chemiluminescence detection of the reactive proteins, and a Fujifilm LAS-1000 charge-coupled device system was used for imaging.

**RESULTS**

Soluble fibrinogen causes a transient increase of cytosolic free Ca\textsuperscript{2+} in isolated human neutrophils. Soluble fibrinogen is normally present in blood, and its interactions with the blood cells have well-established roles in various physiological processes, such as platelet aggregation and thrombus formation. We have determined the effect of fibrinogen on intracellular Ca\textsuperscript{2+} mobilization in isolated human neutrophils. When added
to neutrophil suspensions, fibrinogen caused a concentration-dependent increase in intracellular Ca\(^{2+}\) (Fig. 1, A–D). To demonstrate that the effect of fibrinogen was not due to the alteration of the colloid osmotic pressure of the medium in which neutrophils were suspended, we administered equal concentrations of BSA to control samples. Unlike fibrinogen, BSA (≤4 mg/ml) did not cause any detectable change in the intracellular Ca\(^{2+}\) levels (data not shown). Fibrinogen concentrations of 0.5 mg/ml or lower did not cause a significant cytosolic Ca\(^{2+}\) increase, but at concentrations as low as 1 mg/ml, fibrinogen induced a measurable intracellular Ca\(^{2+}\) increase that lasted for ~1 min. Fibrinogen concentrations of 2 mg/ml caused nearly maximal peaks of intracellular Ca\(^{2+}\) concentrations (Fig. 1, A and B).

The ability of fibrinogen to modulate formyl peptide receptor-induced Ca\(^{2+}\) mobilization was also determined. Incubation of neutrophils with 2 mg/ml fibrinogen for 10 min slightly but significantly enhanced the amplitude of the Ca\(^{2+}\) increase caused by fMLP (\(P < 0.05\)) compared with the BSA-treated controls (Fig. 1, C and D).

**Fibrinogen has a potentiating effect on fMLP- or PMA-induced ROS production in isolated human neutrophils.** The production of ROS has a major role in bacterial killing by neutrophils. Therefore we investigated the effect of soluble fibrinogen on the ability of isolated neutrophils to generate ROS. The effect of fibrinogen (2 mg/ml) on the release of ROS was determined on neutrophil suspensions in the presence of isoluminol and horseradish peroxidase as described in MATERIALS AND METHODS. When added alone to neutrophil suspension, fibrinogen did not cause any luminescent signal (data not shown), demonstrating that the intracellular signal provided by fibrinogen alone is not sufficient to cause release of ROS. However, fibrinogen addition altered the responses induced by fMLP or PMA. In control experiments, equal concentrations of BSA were used instead of fibrinogen. An increase with 54 ± 10.7% (\(n = 3\)) of the amplitude of ROS released by fMLP (10 nM) was measured in the presence of fibrinogen and compared with BSA-treated samples (Fig. 2A). To determine whether the duration of preincubation with fibrinogen was critical for its effect, we performed experiments in which fibrinogen was added at different time points before the addition of the agonist. No significant difference was observed between the samples incubated with fibrinogen for 1 min and those incubated for longer times (≤10 min), providing evidence that the...

**Fig. 1.** Effect of soluble fibrinogen on intracellular Ca\(^{2+}\) concentration in isolated human neutrophils. Human neutrophils were loaded with fura 2, and intracellular Ca\(^{2+}\) concentrations were recorded as described in MATERIALS AND METHODS. A and B: resting levels of Ca\(^{2+}\) were recorded for 30 s, then fibrinogen was added to the cuvette. Final concentrations of fibrinogen were 0.5, 1, 2, or 4 mg/ml (A, bottom to top, respectively). Averages ± SE of peak levels of intracellular Ca\(^{2+}\) concentrations are shown in B (\(n = 3–5\)). C and D: resting levels of intracellular Ca\(^{2+}\) were recorded for 30 s, and 2 mg/ml (final concentration) of either bovine serum albumin (BSA; gray tracing) or fibrinogen (black tracing) were added to the cuvette. After 10 min, cells were challenged with N-formyl-methionyl-leucyl-phenylalanine (fMLP). Representative tracings are shown in C. Averages ± SE of the peak levels of intracellular Ca\(^{2+}\) concentrations measured after addition of fMLP are shown in D (\(n = 3\)).

**Fig. 2.** Fibrinogen potentiates fMLP- or PMA-induced reactive oxygen species (ROS) production. Human neutrophil suspensions were placed in a glass cuvette under stirring conditions at 37°C in a lumigregrometer. Horseradish peroxidase (1 U/ml) and isoluminol (5 μM) were added, and cells were incubated with either BSA or fibrinogen (2 mg/ml) for 10 min; then the luminescence was recorded during the addition of fMLP (10 nM) or PMA (10 nM) as indicated in each image. Representative tracings from 3 experiments are shown. Vertical bar represents average luminescence intensity recorded after H₂O₂ (0.3 mM final concentration) was added to medium in the presence of horseradish peroxidase and isoluminol.
potentiating effect of fibrinogen occurs fast and is relatively stable in time (data not shown).

PMA is widely used as a nonspecific activator of PKC in various cell types, and it is well known as a very powerful activator of ROS production in neutrophils. ROS production induced by PMA is not associated with any detectable intracellular Ca\(^{2+}\) increase in neutrophils. To determine whether the effect of fibrinogen depends on the activation of the G\(_i\) protein coupled to fMLP receptor, we also tested the effect of fibrinogen on PMA-induced ROS production (Fig. 2B). The amplitude of the response elicited by PMA was enhanced with 112 ± 10.7% (n = 3) when soluble fibrinogen was added to the medium (P < 0.05).

The potentiating effect of soluble fibrinogen on fMLP-induced primary granule release is dependent on the binding to CD11b. Elastase release from the azurophilic or primary granules is an important physiological response of neutrophils to chemoattractants and is commonly used as a marker for primary granule release. Hence, we measured the elastase release from azurophilic granules using a chromogenic assay as described in MATERIALS AND METHODS. It has been shown that fMLP can cause primary granule release from human neutrophils pretreated with cytochalasin B; in the absence of cytochalasin B, a negligible amount of elastase is released from neutrophils (4). When added to neutrophil suspensions, fibrinogen alone did not cause measurable primary granule release in the presence or absence of cytochalasin B. However, when added 10 min before the addition of fMLP, fibrinogen significantly enhanced the degranulating effect of the formyl peptide.

It has been shown that fibrinogen acts as a ligand for CD11b/CD18, and this integrin is able to cause outside-in signaling in neutrophils. The possibility that the potentiating effect of fibrinogen is due to binding of fibrinogen to Mac-1 was explored. We found that the effect of fibrinogen is completely inhibited (Fig. 3) in the presence of an antibody raised against CD11b (Beckman Coulter, Miami, FL). Hence, we confirmed that the effect of fibrinogen on primary granule release depends on the binding to the integrin Mac-1, in agreement with similar findings of other effects induced by fibrinogen in neutrophils, such as antibody-dependent cytotoxicity and apoptosis (28). The anti-CD11b antibody did not have any effect on degranulation in the absence of fMLP.

Fibrinogen enhances fMLP-induced protein tyrosine phosphorylations in neutrophils. MAPK have been shown to have important roles in intracellular mechanisms responsible for neutrophil activation induced by a variety of stimuli, such as inflammatory cytokines, lipopolysaccharide (LPS), and osmotic shock. Activation of p44/42 MAPK occurs through phosphorylation of threonine and tyrosine residues 202 and 204 by upstream MAPK kinases (MEK1 and MEK2) (25, 34). Both kinases are known to weakly autophosphorylate on tyrosine (34). The activation of p38 MAPK requires phosphorylation at Thr\(^{180}\) and Tyr\(^{182}\) (26). In addition, tyrosine residues are phosphorylated in a variety of proteins during neutrophil activation. Therefore we investigated the effect of fibrinogen on tyrosine phosphorylations using the monoclonal 4G10 antibody (Fig. 4).

Fibrinogen alone had negligible effect on tyrosine phosphorylations as well as on the phosphorylation of MAPK (see below) compared with fMLP. However, fibrinogen enhanced the phosphorylation of several proteins as revealed by the 4G10 monoclonal antibody. Because some of the tyrosine-phosphorylated proteins had apparent molecular mass between 35 and 45 kDa, which corresponded to the molecular mass of the MAPK, we further studied the effect of fibrinogen and fMLP on the phosphorylation of p38 MAPK and p44/42 MAPK.

Phosphorylation of MAPK does not contribute to the effect of fibrinogen on primary granule release. We studied the phosphorylation of p44/42 MAPK and p38 MAPK in neutrophils stimulated with fMLP (Fig. 5, A and B). In parallel, we

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![Fig. 3. Fibrinogen potentiation of fMLP-induced primary granule release is mediated by Mac-1 (CD11b/CD18). Human neutrophils were incubated with an anti-CD11b antibody (shaded bars) or solvent for 30 min, and then fibrinogen (closed bars and shaded bars) or BSA (open bars) was added and an additional incubation of 10 min was performed. Finally, fMLP (100 nM) or solvent was added to cell suspensions as indicated, and after an incubation of 20 min, the activity of elastase released in the medium was determined. Averages ± SE of at least 4 experiments are shown. *P < 0.05 vs. samples stimulated with fMLP in the presence of BSA.](http://ajpcell.physiology.org/)

![Fig. 4. Effect of fibrinogen on tyrosine phosphorylation of cytosolic proteins in isolated human neutrophils. Neutrophils were isolated and incubated for 10 min at 37°C with 2 mg/ml of either fibrinogen or BSA, and then cells were stimulated with fMLP (100 nM) for the indicated time periods. Cells were lysed in a lysis buffer containing 1% Triton X-100 and protease and phosphatase inhibitors, and cytosolic fractions were collected. SDS-PAGE was performed, and proteins were transferred on PVDF membrane, which was probed with an antibody against phosphotyrosine (4G10). Chemiluminescence imaging was performed for detection.](http://ajpcell.physiology.org/)
evaluated the impact of MAPK inhibition on the effect of fibrinogen on elastase release (Fig. 5C). The effect of fMLP (100 nM) on p44/42 MAPK phosphorylation was rapid and lasted longer than 5 min. The phosphorylation of p38 MAPK induced by fMLP was more transient, reaching a maximum at 1 min. Fibrinogen (2 mg/ml) enhanced the phosphorylation of both p44/42 MAPK and p38 MAPK, suggesting that MAPK might be involved in mediating the potentiating effect of fibrinogen. To investigate this hypothesis, we used U0126, a MEK1/2 inhibitor, and SB-203580, a p38 MAPK inhibitor. Both reagents were tested at concentrations that were previously shown to block MAPK activity effectively (21). U0126 (3 μM) did not cause any inhibition of fMLP-induced degranulation. Although SB-203580 (10 μM) slightly inhibited the fMLP-induced degranulation, when fibrinogen was added to the medium, SB-203580 failed to cause any inhibition to primary granule release (Fig. 5C).

Multiple intracellular signaling pathways are involved in the modulatory effect of fibrinogen on primary granule release from human neutrophils. We constructed concentration-response curves for fMLP in the absence and presence of a physiological concentration of fibrinogen (2 mg/ml). In addition, we investigated the role played by several major intracellular signaling pathways in modulating the fMLP-triggered degranulation and the potentiating effect of fibrinogen. When fMLP was added to cytochalasin B-pretreated cells, elastase was released in a concentration-dependent manner (Figs. 5B and 6). In the presence of soluble fibrinogen (2 mg/ml), the degranulating effect of fMLP was significantly enhanced, especially at concentrations of fMLP ranging from 10 to 100 nM. Higher concentrations of fMLP caused a nearly maximal effect on elastase release and fibrinogen had only a slight enhancing effect, most likely because the maximal degranulating ability of neutrophils was reached. Altogether, fibrinogen caused an obvious leftward shift of the concentration-response curve to fMLP.

PI3K has been shown to have a very important role in mediating primary granule release (5, 21). In the presence of wortmannin (100 nM), a PI3K inhibitor, the concentration-response curve to fMLP was shifted rightward to a similar extent in the presence or absence of soluble fibrinogen (Fig. 6A). It was previously shown that Akt can be activated downstream of PI3K in human neutrophils (39). HIMO (10 μM), a recently developed Akt inhibitor (11), only slightly inhibited fMLP-induced degranulation and had no effect on the potentiating effect of fibrinogen (Fig. 6B). Neutrophil activation is

Fig. 5. Mitogen-activated kinases (MAPK) are not involved in the potentiating effect of fibrinogen on fMLP-induced primary granule release. A: human neutrophils were isolated and incubated for 10 min at 37°C with 2 mg/ml of either fibrinogen or BSA, then fMLP (100 nM) was added to cell suspensions. After the time periods indicated, cells were lysed in a lysis buffer containing 1% Triton X-100 and protease and phosphatase inhibitors, and cytosolic fractions were collected. SDS-PAGE was performed, and proteins were transferred on polyvinylidene difluoride membranes, which were probed with antibodies against phospho-p44/42 or phospho-p38 MAPK as indicated. Chemiluminescence imaging was performed for detection. B: image shown in A was subjected to densitometric analysis, and corresponding values are shown. C: neutrophils were isolated from human blood and incubated with the indicated inhibitors or solvent for 30 min at 37°C. Fibrinogen was added to cell suspensions, and an additional incubation of 10 min was performed. Finally, different concentrations of fMLP were added to the medium, and an additional incubation of 20 min was performed. The activity of elastase released in the medium was measured in each sample using a chromogenic assay. Data were normalized for the maximal elastase release caused by fMLP alone. Averages ± SE of at least 4 experiments are shown.
associated with tyrosine phosphorylation of a number of cytosolic proteins. To assess the importance of tyrosine phosphorylation in the degranulating effect of fibrinogen, we used genistein, a nonspecific tyrosine kinase inhibitor. Genistein (100 μM) caused an effect very similar to that of wortmannin, causing a strong rightward shift of the concentration-response curve to fMLP in the absence or presence of fibrinogen (Fig. 6C). PKC has important roles in neutrophil activation, particularly in the production of ROS (7a, 12). Inhibition of classical and novel isoforms of PKC by Ro 31-8220 (10 μM) abolished O₂⁻ production induced by fMLP (data not shown). The same concentration of Ro 31-8220 paradoxically potentiated fMLP-induced elastase release (Fig. 6D). However, when fibrinogen was added to Ro 31-8220-treated cells, the elastase release was not further enhanced.

We further investigated the effect of several other PKC inhibitors on primary granule release from human neutrophils. We found that staurosporine (100 nM) had an effect similar to that observed with Ro 31-8220 (10 μM), causing a potentiating effect on elastase release but a marked inhibitory effect on ROS production induced by fMLP, consistent with the findings previously reported by Krause et al. (15). We also used GF-109203X (1 μM), a nonselective PKC inhibitor, which caused effects similar to those of Ro 31-8220 and staurosporine (Fig. 7). Finally, Gö6976 (1 μM), a selective inhibitor of classical PKC isoform, significantly inhibited fMLP-induced elastase release and abolished ROS production.

**DISCUSSION**

Fibrinogen is a protein present in the plasma in concentrations ranging from 2 to 4 mg/ml and has a crucial role in hemostasis, participating in thrombus formation. However, the role of fibrinogen is not limited to coagulation but extends to other physiological and pathological conditions. In patients with inflammatory diseases, it is very common for fibrinogen levels in the plasma to be elevated; hence fibrinogenemia is routinely determined to detect the presence of acute inflammatory conditions. It was previously demonstrated that CD11b/CD18 is able to bind fibrinogen, and there are indications that fibrinogen can trigger outside-in signaling in neutrophils (3). In addition, soluble fibrinogen has been found to cause enhancement of phagocytosis and antibody-dependent cellular cytotox-

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**Fig. 6.** Fibrinogen potentiates fMLP-induced primary granule release through a mechanism dependent on multiple intracellular signaling pathways. Neutrophils were isolated from human blood and incubated with the indicated inhibitors or solvent for 30 min at 37°C. Fibrinogen was added to cell suspensions, and an incubation of 10 min was performed. Finally, different concentrations of fMLP were added to the medium, and an additional incubation of 20 min was performed. The activity of elastase released in the medium was measured in each sample using a chromogenic assay. Data were normalized for the maximal elastase release caused by fMLP alone. Averages ± SE of at least 4 experiments are shown.

**Fig. 7.** The effect of PKC inhibitors on fMLP-induced primary granule release. Neutrophils were isolated from human blood and incubated with the indicated inhibitors or solvent for 30 min at 37°C. Fibrinogen was added to cell suspensions, and an incubation of 10 min was performed. Finally, fMLP (100 nM) was added to the medium, and an additional 20-min incubation was performed. The activity of elastase released in the medium was measured in each sample using a chromogenic assay. Averages ± SE of at least 3 experiments are shown.
Fibrinogen promotes granule release from neutrophils.

Mechanisms. The activation of MAPK has been suggested to play an important role in the modulating effect of fibrinogen in secondary granule release, antibody-dependent phagocytosis, and apoptosis (28, 29).

Soluble fibrinogen increased the amplitude of the intracellular \( \text{Ca}^{2+} \) increase caused by fMLP, suggesting that fibrinogen might enhance other chemoattractant-induced effects in human neutrophils. Therefore we determined the effect of fibrinogen on ROS production and degranulation triggered by fMLP. We found that fibrinogen has potentiating effects on chemoattractant-induced production of ROS and on primary granule release.

Production of ROS is one of the major mechanisms used by polymorphonuclear leukocytes to kill bacteria, and it is expected that multiple extra- and intracellular mechanisms contribute to the modulation of ROS production. Our data provide evidence that soluble fibrinogen is one of the plasma factors that enhance ROS production induced by chemoattractants. However, this modulating effect appears to have a much smaller impact than other priming agents, such as the extracellular nucleotides (ATP, UTP), which are able to amplify the effect of fMLP three- to eightfold (16, 31). Moreover, because the effect of PMA is enhanced when soluble fibrinogen is added to the medium, it is likely that the potentiating effect of fibrinogen does not depend on the signaling events taking place upstream of PKC, but rather is more directly related to the activation of NADPH oxidase system.

The release of primary granule from isolated human neutrophils was studied by determining the concentration of elastase, a commonly used marker for primary granules, in the extracellular medium. In the presence of soluble fibrinogen, the concentration-response curve to fMLP was shifted to the left, demonstrating that fibrinogen potentiates not only the ROS production but also other microbicidal mechanisms relying on neutrophil degranulation. The intracellular signaling mechanisms responsible for the potentiating effect of fibrinogen were investigated using specific inhibitors for intracellular signaling molecules.

Wortmannin, a PI3K inhibitor, showed a strong inhibitory effect on the degranulating effect of fMLP, causing a rightward shift of the concentration-response curve to fMLP that was similar in the presence or in the absence of fibrinogen. A similar effect was caused by genistein, a nonspecific tyrosine kinase inhibitor. Thus our data strongly support the view that both the PI3K and tyrosine kinases are essential for primary granule release (21). Moreover, it appears that PI3K and tyrosine kinases may be involved in the potentiating effect of fibrinogen on fMLP-induced elastase release because the fibrinogen-induced leftward shift of the concentration-response curve was not significant in the presence of wortmannin or genistein.

Akt has been shown to be activated downstream of PI3K in various cell types. On the basis of the observations that HIMO, a specific inhibitor of Akt, caused no significant effect and PI3K inhibition caused a dramatic rightward shift of the concentration-response curve elicited by fMLP, we suggest that the effect of PI3K in mediating primary granule release from neutrophils is independent of Akt activation.

Because protein kinase C isotypes can be controlled by PI3K through the protein kinase PDK1 (17), we investigated the role of PKC on the effect of fibrinogen. We used Ro 31-8220, a widely used PKC inhibitor, to block the activation of classic and novel PKC isosforms, which were previously shown to have the most significant roles in mediating functional responses in neutrophils (14, 23, 27, 32). In the presence of Ro 31-8220, the concentration-response curve to fMLP was significantly shifted to the left, suggesting that certain classic and/or novel PKC isosforms that are inhibited by Ro 31-8220 have an inhibitory effect on primary granule release. When fibrinogen was added to cells treated with Ro 31-8220, the elastase release elicited by fMLP was not further enhanced (Fig. 6D). To further confirm the effect of PKC inhibition on primary granule release, we used staurosporine and GF-109203X, two other nonselective PKC inhibitors, as well as Gö6976, a selective inhibitor of classic PKC isosforms. While staurosporine and GF-109203X caused potentiation of elastase release in a manner similar to that of Ro 31-8220, Gö6976 significantly inhibited fMLP-induced degranulation in the presence or absence of fibrinogen (Fig. 7).

The data obtained with the PKC inhibitors suggest that the classic PKC isofrom is required for primary granule release and ROS production in human neutrophils. It is possible that one of the novel or atypical PKC isosforms might have a constitutive inhibitory effect on neutrophil degranulation. The nonselective PKC inhibitors block both the potentiating and inhibitory PKC isosforms, and the effect on the inhibitory isosforms appears to be dominant; hence the elastase release is potentiated. On the other hand, Gö6976 may block only the classic PKC isosforms that participate in elastase release, leaving the activity of the inhibitory isosforms unaffected. It is possible that fibrinogen might trigger certain intracellular signaling events that are mimicked by the nonselective PKC inhibitors; however, further investigation is required to confirm this hypothesis.

It has been established that integrin activation is able to initiate intracellular signaling events in neutrophils, leading to changes in gene expression (36), increased phagocytosis, spreading, and chemotaxis (8, 22, 28). In the present study, we have shown that fibrinogen causes a dose-dependent increase in intracellular \( \text{Ca}^{2+} \) concentrations. Fibrinogen potentiates fMLP- and PMA-induced ROS production as well as fMLP-induced primary granule release from isolated human neutrophils. In the presence of the anti-CD11b antibody, the potentiating effect of fibrinogen was completely inhibited. Hence it appears that fibrinogen modulates formyl peptide-induced primary granule release from neutrophils through mechanisms dependent mostly on CD11b/CD18 activation.

PI3K and tyrosine kinase-dependent mechanisms are essential for primary granule release and for the potentiating effect induced by fibrinogen. Although fibrinogen enhances the chemoattractant-induced phosphorylation of p44/42 MAPK and p38 MAPK, the lack of effect of the MAPK inhibitors (U0126 and SB-203580) on the effect of fibrinogen strongly suggests that the activation of MAPK is not responsible for the effect of fibrinogen in degranulation.
noticed that SB-203580 (10 μM) inhibited neutrophil degranulation caused by concentrations of fMLP as high as 1 μM but not by lower concentrations of fMLP, in agreement with previous reports (21, 24). We conclude that the effect of p38 MAPK inhibition on fMLP-induced primary granule release from human neutrophils depends on the concentration of fMLP used as an agonist. Concentrations of fMLP <1 μM cause an effect that is not markedly affected by p38 MAPK blockade, while at 1 μM fMLP causes a degranulating effect sensitive to p38 MAPK blockade. We suggest that the low-affinity receptor for fMLP (fMLP receptor-like 1), which is activated by concentrations of fMLP in the micromolar range and has been shown to be present in neutrophils, might have a significant role in primary granule release induced by high concentrations of fMLP. It is possible that this receptor might cause degranulation using a different pathway than the classic high-affinity fMLP receptor and that this pathway might rely on activation of p38 MAPK. Further investigations using cell lines expressing these receptors are required to confirm this hypothesis.

We conclude that fibrinogen potentiation of primary granule release induced by fMLP is mediated by the integrin CD11b/CD18 through PI3K- and tyrosine kinase-dependent pathways, but other regulatory mechanisms also might be involved.

GRANTS
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REFERENCES


