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*J Immunol* 2002; 168:3527-3535; doi: 10.4049/jimmunol.168.7.3527
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Soluble Fibrinogen Modulates Neutrophil Functionality Through the Activation of an Extracellular Signal-Regulated Kinase-Dependent Pathway

Carolina Rubel,* Gabriela C. Fernández,* Fernanda Alves Rosa,* Sonia Gómez,* Macarena Beigier Bompadre,* Omar A. Coso,† Martín A. Isturiz,* and Marina S. Palermo*

The integrin family not only mediates the recruitment of polymorphonuclear leukocytes (PMN) to sites of inflammation but also regulates several effector functions by binding to specific ligands. We have recently demonstrated that soluble fibrinogen (sFbg) is able to trigger an activating signal in PMN through an integrin-dependent mechanism. This activation results in degranulation, phagocytosis enhancement, and apoptosis delay. The aim of the present work was to further elucidate the molecular events that follow sFbg interaction with CD11b in human PMN, and the participation of this signaling pathway in the regulation of neutrophil functionality. We demonstrate that sFbg triggers a cascade of intracellular signals that lead to focal adhesion kinase and extracellular signal-regulated kinase 1/2 tyrosine phosphorylation. The activation of this mitogen-activated protein kinase pathway plays a central role in the sFbg modulation of secondary granule degranulation, Ab-dependent phagocytosis, and apoptosis. However, fibrinogen-induced secretory vesicle degranulation occurs independently of the signaling transduction pathways investigated herein. In the context of an inflammatory process, the intracellular signal pathway activated by sFbg may be an early event influencing the functionality of PMN. The Journal of Immunology, 2002, 168: 3527–3535.

Polyomorphonuclear leukocytes (PMN) circulate within the vasculature in a quiescent state but are capable of responding rapidly to bacterial products or cytokines synthesized by other cells in response to such products, rendering primed PMN. Once primed, neutrophils migrate to inflammatory foci through a sequential process that involves selectin and integrin adhesion molecules. The integrin family not only mediates the recruitment of PMN to sites of inflammation by binding to specific ligands but also regulates several effector functions (1). CD11b/CD18 is a β2 integrin that serves as the receptor for complement factor C3bi, fibrinogen, fibrin, and collagens (2–4). CD11c binds C3bi and fibrinogen (5), but the physiological impact of these interactions seems less important due to the low surface expression on PMN when compared with the high abundance of CD11b (6). Thus, the β2 integrins mediate a variety of different cell and cell-substrate interactions of PMN during the inflammatory response.

We have recently demonstrated that soluble fibrinogen (sFbg) activates human neutrophils, which have been primed by a purification procedure, through a CD11b-dependent mechanism. This activation results in degranulation, phagocytosis enhancement, and apoptosis delay (7).

Much progress has been made in understanding the adhesive functions of the β2 integrins and the intracellular events that follow cytokines and/or inflammatory stimuli upon surface-adherent PMN. However, molecular events directly triggered by soluble ligands to β2 integrins are still incompletely understood. The first evidence for the signaling capacity of the β2 integrins was obtained by the finding that TNF-α-induced superoxide anion production in human PMN depends on β2 integrin adhesion (8). Subsequently, in the same experimental conditions, activation of different signaling components, including tyrosine phosphorylation of Syk and the Src kinases Fgr, Hck, and Lyn, has been reported (9–12). Besides, in many cell types, integrin-dependent adhesion leads to the formation of specialized structures known as focal adhesions in regions in which the cell is in close contact with extracellular matrix proteins (12). Focal adhesion kinase (FAK) is a 125-kDa nonreceptor tyrosine kinase commonly found at these structures (13) that associates with the cytoplasmic domains of integrins and is phosphorylated and activated in response to integrin ligation in cells such as fibroblasts and carcinoma cells (14, 15). Although PMN do not form a true focal adhesion structure, phosphorylated FAK and paxillin have been identified in adherent neutrophils (16).

Furthermore, integrin-mediated cell adhesion has also been shown to strongly activate mitogen-activated protein kinase (MAPK), a key downstream effector of the signaling pathway in many cells. Recently, it has been shown that neutrophils use the MAPK cascade in response to a wide variety of stimuli (17). Three major families of MAPKs cascades have been described in

*División Inmunología, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, and Laboratorio de Fisiología y Biología Molecular, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina
†Laboratorio de Fisiología y Biología Molecular, Facultad de Ciencias Exactas y Naturales, Buenos Aires, Argentina

Received for publication May 10, 2001. Accepted for publication January 18, 2002.

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1 This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Tecnológicas de la República Argentina, Fundación Antorchas and Alianzas Científicas Argentinas. Address correspondence and reprint requests to Dr. Carolina Rubel, División Inmunología, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Pacheco de Melo 3081 (1425), Buenos Aires, Argentina. E-mail address: rubel@sinectis.com.ar
2 Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; Fbg, fibrinogen; sFbg, soluble Fbg; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein/ERK kinase; JNK, c-Jun N-terminal kinase; PI, propidium iodide; PVDF, polyvinylidene difluoride; MFI, mean fluorescence intensity; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine.
mammalian cells: c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs), and p38. ERK1/2 and p38 are activated in human neutrophils by cytokines (18), chemoattractants (19–21), and bacterial LPS (22). Although JNK is present in human neutrophils, proinflammatory stimuli do not increase JNK activity (18, 20). Activation of a MAPK is the final step in a three-part intracellular signal transduction cascade in which a MAPK kinase kinase activates (through phosphorylation) a MAPK kinase, which in turn phosphorylates specific single tyrosine and threonine residues on a MAPK (22, 23). Once activated, MAPKs appear capable of further signal transduction through phosphorylation and induction of diverse transcription factors.

The aim of the present work is to further elucidate the molecular events that follow sFbg interaction with CD11b in human PMN, and the involvement of the triggered signaling pathway in the regulation of neutrophil functionality.

The results reveal that sFbg triggers a cascade of intracellular signals that leads to FAK and ERK1/2 tyrosine phosphorylation. Furthermore, the activation of this MAPK pathway plays a central role in the sFbg modulation of degranulation of secondary granules, AB-dependent phagocytosis, and apoptosis. However, up-regulation of CD11b by sFbg occurs independently of the signaling transduction pathways evaluated herein. Identifying distinct mechanisms of integrin responses to extracellular stimuli and patterns in the classes of responding molecules will be crucial for understanding how integrins function.

Materials and Methods

Reagents and mAbs
Acridine orange, ethidium bromide, propidium iodide (PI), aprotinin, leupeptin, pepstatin A, PMA, diisopropyl fluorophosphate, and PMSF were obtained from Sigma-Aldrich (St. Louis, MO); human fibrinogen (Fbg) was obtained from Baxter Immuno (Buenos Aires, Argentina). The kinase inhibitors herbimycin, genistein, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), PP2, SB203580, and PD98059 were purchased from Calbiochem-Novabiochem (La Jolla, CA). The anti-phosphotyrosine mAb (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). The mAbs against phospho-ERK1/2, phospho-p38, and FAK, as well as polyclonal Abs against p38 and ERK2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), FITC-conjugated mouse mAb Bear-1 (IgG1) against human CD11b/CD18 (Mac-1), FITC-conjugated mouse mAb 80H3 (IgG1) against human CD66b, and FITC-conjugated isotype control mouse IgG1 were obtained from Caltag Laboratories (Burlingame, CA). Azida-free mAbs Bear-1 against CD11b (IgG1) and isotype control of mouse IgG1 were obtained from Immunotech (Marseille, France).

Blood samples
Blood samples were obtained from healthy volunteer donors who had taken no medication for at least 10 days before the day of sampling. Blood was obtained by venipuncture of the forearm vein and was drawn directly into citrated plastic tubes.

Neutrophil isolation
Neutrophils were isolated by Ficoll-Hypaque (Pharmacia Biotech, Uppland, Sweden) and Winthrop Products (Buenos Aires, Argentina), respectively, gradient centrifugation and dextran sedimentation, as previously described (24). Contaminating erythrocytes were removed by hypotonic lysis. After washing, the cells (>96% neutrophils on May Grunwald/Giemsa-stained cyto-preps) were suspended in RPMI 1640 supplemented with 1% heat-inactivated FCS.

Degranulation of neutrophils
The expression of the surface markers CD11b (Mac-1) and CD66b on the neutrophil surface was used as an indicator of degranulation of secretory vesicles and secondary granules, respectively (25). After preincubation at 37 °C during different periods with or without sFbg, neutrophils were washed with cool PBS supplemented with 1% FCS and incubated with mAb against CD11b and CD66b. Control of isotype-matched Ab was assayed in parallel. Cells were then washed with cool PBS supplemented with 1% FCS and suspended in 0.3 ml of ISOFLOW (International Link, Buenos Aires, Argentina). Fluorescence was measured with a FACScan (BD Biosciences, Mountain View, CA). The analysis was conducted on 20,000 events on each sample by using the CellQuest program (BD Biosciences).

Immunoblotting of human cell lysates and kinase assays
For each condition, PMNs at 2 × 10^6 cells/ml were used. At the end of the experimental treatment, the cells were washed with PBS. They were lysed by incubation on ice for 20 min in 0.5 ml 100 mM Tris-HCl (pH 8), 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (RIPA buffer), 1 mM Na3VO4, 50 mM NaF, 0.3 U/ml aprotinin, 2 mM PMSF, and 1 μg/ml each of leupeptin and pepstatin A. Lysates were centrifuged for 15 min at 14,000 × g. Protein concentrations were determined using a micro Bradford assay (Pierce, Rockford, IL). The supernatants were prepared for SDS-PAGE under reducing conditions.

SDS-PAGE was run on 10% minigels using standard Tris-glycine buffers. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) for 1 h at 300 mA and blocked with PBS 3% nonfat dry milk for 1 h. The membrane was probed with primary Ab in PBS 0.4% BSA (1 μg/ml anti-phosphotyrosine mAb 4G10 and 0.4 μg/ml for the other Abs) overnight. After washing three times with PBS 0.2% Tween 20, blots were incubated for 1 h with a HRP-conjugated goat anti-mouse or anti-rabbit IgG (Amersham, Aylesbury, U.K.). Immunoreactivity was detected using the ECL Western blotting detection reagent (Amersham).

The activity of MAPK ERK1/2 was measured by an in vitro kinase enzyme assay system (Amersham). Total cell lysates were incubated with 5 μl of magnesium [γ-32P]ATP buffer (200 μCi/ml) and 10 μl of substrate buffer. After incubation at 30°C for 30 min, the reaction was terminated by adding 10 μl of stop reagent. To separate phosphorylated peptide, 30 μl of terminated reaction mixture were added on the peptide binding paper. After washing twice with 1% acetic acid and twice with distilled water, 10 ml of liquid scintillation mixture were added and each vial was counted in a scintillation counter.

Immunoprecipitation
PMNs were lysed in lysis buffer as described above. Lysates were clarified and protein concentrations were determined as outlined previously. Lysates (100–200 μg protein) were incubated overnight at 4°C with 3 μg of anti-FAK or 2 μg of anti-phosphotyrosine mAbs and protein G-Sepharose with rotation. The beads were washed thrice with lysis buffer and adsorbed proteins were solubilized in 1% SDS and separated on 8% SDS-PAGE minigels. Proteins were transferred to PVDF membranes and subsequently immunoblotted with anti-FAK.

Ab-mediated erythrophagocytosis
Erythrophagocytosis was performed as previously described (26). Briefly, after preincubation with saline or sFbg for 1 h at 37°C, human neutrophils (50 μl, 7 × 10^6/ml) were mixed with sheep erythrocytes (50 μl, 3% v/v) sensitized with subagglutinating amounts (200 μg) of rabbit IgG anti-sheep erythrocytes (Sigma-Aldrich). After incubation for 30 min at 37°C in 5% CO2–95% humidified air, the noningested erythrocytes were lysed by hypotonic shock. The percentage of phagocytic neutrophils was evaluated by microscopic examination. At least 100 cells were scored in each sample. No phagocytosis was detected when neutrophils were incubated with unsensitized erythrocytes.

Quantification of cellular apoptosis and viability by fluorescence microscopy
Quantification was performed as previously described (27), using the fluorescent DNA-binding dye acridine orange (100 μg/ml) to determine the percentage of cells that had undergone apoptosis and ethidium bromide (100 μg/ml) to differentiate between viable and nonviable cells. To assess the percentage of cells showing morphologic features of apoptosis, at least 200 cells were scored in each experiment.

Quantification of neutrophil apoptosis by PI staining and flow cytometry
The proportion of neutrophils that displayed a hypoploid DNA peak, i.e., apoptotic cells, was determined using a modification of Nicoletti’s protocol (28). Briefly, cell pellets containing 2.5 × 10^6 neutrophils were washed in 400 μl of hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100) and incubated for 2 h at 4°C. The red fluorescence of PI of individual nuclei was measured using a FACScan flow cytometer (BD Biosciences). The forward scatter and side scatter of
particles were simultaneously measured. Cell debris were excluded from analysis by appropriately raising the forward-scattered threshold. The red fluorescence peak of neutrophils with normal (diploid) DNA content was set at channel 250 in the logarithmic mode. Apoptotic cell nuclei emitted fluorescence in channels 4–200.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis of the data was performed using a nonparametric paired Mann-Whitney test. Values of \( p < 0.05 \) were considered significant.

Results

Temporal analysis of sFbg effects on granule mobilization

To investigate the underlying intracellular mechanisms that lead to regulation of granule mobilization by sFbg, we first studied the kinetics of degranulation induced by sFbg. PMN were incubated in the presence of sFbg (6 \( \mu \)M) (7) for different times up to 60 min at 37°C before analysis of CD11b and CD66b membrane expression, as degranulation markers of secretory vesicles and secondary granules, respectively. As shown in Fig. 1, significant up-regulation of CD11b was evident 15 min after sFbg incubation, reaching a plateau from 30 to 60 min. However, degranulation of secondary granules was delayed. Enhancement of CD66b membrane expression was significant at 30 min, but maximal response was achieved after 60 min of sFbg incubation.

Modulation of sFbg-induced PMN degranulation by tyrosine kinase inhibitors

Activation of tyrosine kinases is important in mediating many \( \beta_2 \) integrin-dependent functions in PMN, including reactive oxygen intermediate production and spreading (29, 30). Therefore, we sought to examine whether sFbg-dependent effects were mediated by this important downstream stage of CD11b signaling. This possibility was tested by the use of tyrosine kinase inhibitors herbimycin and genistein. These two agents have distinct mechanisms of action; genistein is a competitive inhibitor with respect to ATP (31) while herbimycin attacks critical sulfhydryl groups in tyrosine kinases (32). As shown in Fig. 2, 100 \( \mu \)M genistein and 5 \( \mu \)M herbimycin caused a strong inhibition of sFbg-induced exocytosis of secondary granules (CD66b). In contrast, H7, an inhibitor for the serine-threonine protein kinases A, C, and G (33), did not significantly inhibit sFbg-induced up-regulation of CD66b. On the other hand, the sFbg-induced up-regulation of CD11b, a marker of secretory vesicles, was not significantly reversed by any of these inhibitors. Interestingly, the simultaneous incubation with sFbg and H7 even induced a higher expression of CD11b than sFbg alone.

Among the tyrosine kinases so far implicated in adherent-dependent signaling in PMN there are Src family tyrosine kinases such as Syk and Fgr (34). Moreover, agonists such as TNF-\( \alpha \), PMA, and FMLP enhance the kinase activity of Fgr through a \( \beta_2 \) integrin activation-dependent mechanism (35, 36). These findings prompted us to investigate sFbg effects in the presence of 10 \( \mu \)M PP2, a selective inhibitor of Src family kinases (37). As shown in Fig. 2, PP2 diminished the exocytosis of secondary granules but had no considerable effect on the release of the secretory vesicle marker CD11b. Taken together these data suggest that secondary granule mobilization induced by sFbg involves protein-tyrosine phosphorylation, while the content of secretory vesicles are mobilized rapidly and independently of this signaling pathway. In addition, members of the Src kinase family may also participate in sFbg-induced degranulation of secondary granules, but not in that of secretory vesicles.

sFbg induces protein tyrosine phosphorylation in PMN

Because one of the earliest events of integrin signal transduction is the tyrosine phosphorylation of several intracellular proteins we investigated in PMN the pattern of protein phosphorylation by sFbg. After different times of PMN incubation with 6 \( \mu \)M sFbg, cells were lysed and equal amounts of cell protein were electrophoresed on SDS-PAGE gels and Western blotted with antiphosphotyrosine mAb. Immunoblotting of whole cell lysates from

FIGURE 1. Kinetics of PMN degranulation induced by sFbg. Neutrophils (2.5 \( \times \) 10\(^6\)/ml) were incubated for different times as indicated at 37°C in the presence of sFbg (6 \( \mu \)M). Then the cells were centrifuged, washed, and stained with specific mAb anti-CD66b or mAb anti-CD11b as described in Materials and Methods. Data represent the mean ± SEM of mean fluorescence intensity (MFI) from six donors. *, Statistical significance (\( p < 0.005 \)) compared with control.

FIGURE 2. Effect of tyrosine kinase inhibitors on sFbg-induced PMN degranulation. Neutrophils (2.5 \( \times \) 10\(^6\)/ml) were preincubated 1 h with medium (control), genistein (100 \( \mu \)M), herbimycin (5 \( \mu \)M), PP2 (10 \( \mu \)M), or H-7 (10 \( \mu \)M), followed by stimulation for 1 h with sFbg (6 \( \mu \)M) at 37°C. Then the cells were centrifuged, washed, and stained with specific mAb anti-CD66b or mAb anti-CD11b as described in Materials and Methods. Data represent the mean ± SEM of MFI from eight donors. *, Statistical significance (\( p < 0.005 \)) compared with control; #, statistical significance (\( p < 0.005 \)) compared with sFbg.
sFbg-activated PMN showed phosphorylation of several proteins. Among them, mainly two bands at relative molecular mass around ~120 and ~40 kDa exhibited a rapid and reversible tyrosine phosphorylation (Fig. 3a). Tyrosine phosphorylation of both proteins was evident within 5–15 min of the addition of sFbg. Over the 60-min period both species showed a decrease in the extent of tyrosine phosphorylation.

To determine whether tyrosine phosphorylation in response to sFbg occurs in a concentration-dependent fashion, PMN were exposed to concentrations of sFbg ranging from 1.5 to 6 μM for 5 min. Fig. 3b shows that phosphorylation of both proteins (~120 and ~40 kDa) is a dose-dependent sFbg effect, and the major effect was reached at 6 μM. However, the ~120 kDa protein seems to be phosphorylated even with sFbg 1.5 μM. Protein phosphorylation in response to sFbg was completely blocked by anti-CD11b (Fig. 3b), confirming that sFbg acts through specific interaction with CD11b receptor.

sFbg induces FAK phosphorylation

Considering that in several cell types there is a clear relationship between integrin ligation and the tyrosine phosphorylation of FAK (125 kDa) (38), we tested FAK phosphorylation after neutrophil stimulation with sFbg. Neutrophils were incubated during different times with sFbg (6 μM). The cells were then lysed and the cellular extracts were immunoprecipitated with anti-phosphotyrosine mAb and immunoblotted with anti-FAK mAb, as described in Materials and Methods. The results shown in Fig. 4a demonstrated FAK phosphorylation in response to sFbg, which was maximal at 5–15 min (Fig. 4, a and b). To confirm that the same amount of FAK was present in the lysates that were then immunoprecipitated, total lysates from neutrophils treated for 15 min with medium or sFbg (6 μM) were immunoblotted with the anti-FAK mAb (Fig. 4c). In addition, lysates from sFbg-treated neutrophils immunoprecipitated with the anti-phosphotyrosine mAb or the anti-FAK mAb were run in parallel. Immunoblotting using anti-FAK demonstrated similar concentration of total FAK and its phosphorylated form (Fig. 4c).

FIGURE 3. sFbg-stimulated protein tyrosine phosphorylation in PMN. a, PMN were incubated with sFbg (6 μM) at 37°C for the times indicated. Proteins were extracted with RIPA buffer and, after electrophoresis in SDS-polyacrylamide gels, transferred to PVDF membranes and probed with the anti-phosphotyrosine mAb 4G10 (1 μg/ml) as described in Materials and Methods. In experiments in which incubation with 4G10 was done in the presence of 1 mM phosphotyrosine no reactivity was detected (data not shown). Numbers at the left show migration of molecular mass markers. b, PMN were preincubated with or without anti-CD11b (1.8 mg/ml) for 30 min and/or with different concentrations of sFbg for 5 min. Proteins were extracted and analyzed as described in Fig. 3 and in Materials and Methods.

FIGURE 4. sFbg-induced FAK phosphorylation in PMN. a, Cell lysates from PMN incubated with sFbg (6 μM) for the times indicated were immunoprecipitated with anti-phosphotyrosine mAb 4G10 and subjected to Western blot analysis with anti-FAK mAb. b, The time course of FAK phosphorylation obtained by immunoprecipitation was scanned and the band intensity was quantified by the Image 1.569 program (National Institutes of Health, Bethesda, MD). These data represent the mean ± SEM of three separate experiments, expressed as fold increase with respect to control. *, Statistical significance (p < 0.05) compared with control. c, Total lysates from PMN incubated with medium or sFbg (6 μM) were run in parallel with the product of immunoprecipitation with anti-phosphotyrosine or anti-FAK and subjected to Western blot analysis with anti-FAK mAb.
sFbg induces MAPK activation

Because of its molecular mass, the ~40kDa protein probably represents a MAPK. In neutrophils, activation of p38 and ERK1/2 have been demonstrated after several inflammatory stimuli (18–22). Because phosphorylation of MAPK closely correlates with their activation (39), this parameter was measured in PMN extracts subjected to Western blotting with mAb specific for phosphorylated forms of the respective MAPK. As shown in Fig. 5, a and b, sFbg induced phosphorylation of ERK1/2 but not p38 after 5–15 min. As a positive control of p38 activation, lysates from PMN activated by osmolar stress were run in parallel. Immunoblotting using anti-ERK2 and anti-p38 Abs demonstrated similar concentrations of the respective total proteins but not phosphorylated forms.

The ability of sFbg to activate ERKs in a time-dependent manner was confirmed by an in vitro kinase enzyme assay. As shown in Fig. 5c, ERK activity was clearly increased at 5 min after addition of sFbg. After 60 min it returned to basal levels. Furthermore, pretreatment with mAb to CD11b completely blocked enhancement of ERK activity induced by sFbg.

Modulation of sFbg degranulation by MAPK inhibitors

To determine the involvement of ERK1/2 cascade in the exocytic activity of sFbg we next investigated the effect of pharmacological
inhibition of the ERK and p38 MAPK pathways in sFbg-induced degranulation of PMN. We used PD98059, an inhibitor of mitogen-activated protein/ERK kinase (MEK)1 and MEK2, the kinases responsible for phosphorylation of MAPK ERK1/2. This compound blocks phosphorylation of ERK through an allosteric mechanism that does not involve inhibition of ATP binding (40). In parallel, we tested the role of p38 MAPK in the sFbg-induced degranulation using SB203580, an inhibitor of this kinase (41). As shown in Fig. 6a, 50 μM PD98059 significantly decreased the sFbg degranulation of secondary granules without affecting the up-regulation of CD11b expression. In contrast, 10 μM SB203580 was not able to modify the pattern of degranulation by sFbg. Under identical conditions, we confirmed that PD98059 specifically inhibited sFbg-mediated activation of ERK kinase by immunoblotting using anti-phospho-ERK Ab (Fig. 6b). In Fig. 6b, it is also shown that activation of ERK1/2 protein was blocked by 100 μM genistein and 10 μM PP2, raising the possibility that the inhibition of this MAPK might mediate the effect of tyrosine kinase inhibitors on the degranulation response induced by sFbg. These data were confirmed evaluating ERK activity by an in vitro kinase enzyme assay (Fig. 6c).

Taken together, these data indicate that ERK MAPK pathway is involved in sFbg-induced degranulation of secondary granules but not in that of secretory vesicles.

**Effect of tyrosine kinase inhibitors on sFbg enhancement of erythrophagocytosis**

Although the use of ERK1/2 or p38 pathway depends largely upon the nature of the stimulus, the functional relevance of the activation of such signal transduction cascade could be variable depending on the response analyzed. In this regard, we have previously demonstrated that sFbg induces the enhancement of Fc-dependent phagocytosis (7). We then investigated the participation of MAPK ERK1/2 activation induced by sFbg on erythrophagocytosis. As shown in Fig. 7, pharmacological inhibition of tyrosine kinases completely abrogated the enhancing effect of sFbg on Fc-mediated phagocytosis, while inhibition of Src proteins and ERK1/2 blocked it partially. On the contrary, inhibition of serine kinases or p38 MAPK did not interfere with the sFbg effect on erythrophagocytosis. None of the pharmacological inhibitors used modified the basal levels of erythrophagocytosis (data not shown).

**Effect of tyrosine kinase inhibitors on sFbg delay of apoptosis**

Considering that degranulation and phagocytosis are short-time reactions, we assayed the involvement of sFbg-triggered signaling pathways in the regulation of a long-lasting, gene induction-dependent reaction such as apoptosis. For this purpose, neutrophils were incubated with or without sFbg 6 μM, in the presence of specific inhibitors for tyrosine kinases, serine kinases, ERK1/2 kinase (MEK), and p38. The percentage of apoptotic cells was determined by flow cytometry (Fig. 8) and confirmed by fluorescence microscopy (data not shown). The results shown in Fig. 8 clearly demonstrate that the sFbg inhibitory effect on neutrophil apoptosis is dependent on tyrosine phosphorylation and ERK1/2 activation. Pharmacological inhibition of p38 MAPK did not abrogate sFbg effects on neutrophil apoptosis. In contrast, the serine-kinase inhibitor H7 induced an increase in the percentage of apoptotic cells, both in the absence (data not shown) and in the presence of sFbg, suggesting the involvement of serine kinases in the modulation of spontaneous neutrophil apoptosis.

**Discussion**

The results obtained indicate that human sFbg, through CD11b interaction, rapidly and transiently phosphorylate (activate) two proteins in human neutrophils, FAK and ERK1/2. Furthermore, we demonstrate that neutrophil degranulation of secondary granules, enhancement of Ab-dependent phagocytosis, and inhibition of apoptosis, all induced by sFbg, are functional responses associated to ERK activation.

The following observations indicate that phosphorylation and the consequent activation of ERK1/2 MAPK represent a key element in the signaling pathway triggered by sFbg: 1) the rapid and specific activation of ERK1/2, following sFbg binding to CD11b, precedes the degranulation response; 2) the decrease of degranulation in response to pretreatment with genistein, herbimycin, and PP2 correlates with a similar decrease in the phosphorylation of ERK MAPK; and 3) most important, the direct inhibition of ERK MAPK by PD98059 impairs the degranulation response. Furthermore, the sFbg effects on Ab-dependent phagocytosis and apoptosis are also associated with ERK activation. To our knowledge this is the first study to show that sFbg activates the ERK1/2 MAPK signaling pathways in PMN and to demonstrate its functional relevance in degranulation, phagocytosis, and apoptosis.

Activation of ERKs initiated by serpentine receptors (e.g., the FMLP and C5a receptors), as well as by those that are more closely linked to tyrosine kinase activity (e.g., GM-CSF and FcγRs) (18), appears to require sequential activation of the small molecular mass GTPase p21ras, Raf-1, and MEK (ERK kinase) (42). Herein, we demonstrate that β2 integrin ligation sFbg also activates a signal pathway where MEK-1 and/or MEK-2 are the predominant upstream activators of ERK1/2. In keeping with our results, Downey et al. (43) have demonstrated that PD98059 substantially inhibited the triggering of the oxidative burst by other inflammatory stimuli such as the FMLP, indicating the involvement of ERK1/2 MAPK in this FMLP response. In contrast, we did not obtain any evidence of p38 participation because we found neither
The nonreceptor FAK localizes with integrins (45) and participates in integrin signaling cascade of carcinoma cells, fibroblasts, and platelets (14, 38). Our results indicate that FAK is also involved in the intracellular signal triggered by sFbg in PMN. Because integrins lack catalytic activity, FAK activation may be an important event for integrin-mediated signal transduction processes. The NH2 domain of FAK may interact with integrin cytoplasmic domains, providing a means for FAK activation upon integrin clustering (38). The C-terminal region of FAK interacts with one or more focal adhesion proteins, e.g. paxillin (46), and with the Src family of nonreceptor kinases (14). Conversely, Src interaction with FAK causes the maximal activation of FAK (47). In our experimental conditions, the Src family kinases seem to participate in the signaling of the exocytic process induced by sFbg because the application of the selective inhibitor PP2 significantly decreases the sFbg-induced granule release (Fig. 2), although we did not observe a consistent increase in tyrosine phosphorylation of proteins of the Src family. The observation that PP2 prevented activation of ERK1/2 places the site of action of Src-related kinases between the receptor and ERK1/2 MAPK. Similarly, Mócsai et al. (48) have demonstrated that FMLP exocytosis of primary and secondary granules of PMN proceeds through p38 MAPK activated by a Src family kinase-dependent mechanism. In this regard, Schlaepfer et al. (49) have demonstrated that Src phosphorylation of FAK enable FAK to interact with the Ras/MAPK pathway.

Several reports have analyzed the response of CD11b-adherent PMN upon an additional inflammatory stimulus demonstrating phosphorylation of several proteins, most of which are from Src family kinase (9–12, 23). Interestingly, we have observed a completely different pattern of protein phosphorylation depending on whether PMN are exposed to soluble or immobilized Fbg. While sFbg induces a major phosphorylation of two proteins (FAK and ERK1/2), PMN plated on Fbg-coated plates show phosphorylation of a variety of at least 10 proteins of different molecular mass (data not shown), confirming previous results (9–12). This distinct activation pattern depending on the ligand presentation has been previously reported in platelets (15, 50, 51). One interpretation of these findings is that ligand occupancy and limited aggregation of integrins provide a very rapid trigger for the first step of intracellular response, and that other signals are induced by the subsequent aggregation of these integrins due to immobilized ligand (15).

The degranulation of secretory vesicles and secondary granules in response to sFbg display a different sensitivity to the inhibitors used. In fact, while tyrosine kinase activity and MAPK activity constitute a central event in the signal cascade leading to degranulation of secondary granules, they are not involved in the release of secretory vesicles upon sFbg stimulation. Similarly, Mócsai et al. (48) and Capodici et al. (42) have demonstrated that exocytosis of secretory vesicles in response to FMLP is independent of tyrosine phosphorylation and PI-3K activation, respectively. These results taken together suggest that other early and simultaneous intracellular signals are implicated in the mobilization of secretory vesicles. Future investigations would be necessary to define, for example, the role of calcium influx induced by sFbg on secretory vesicle exocytosis.

Another key component of the microbialic function of neutrophils tightly associated to cytoskeletal assembly is their ability to ingest foreign organisms by phagocytosis. In this regard, we observed that enhancement of FcγR-dependent phagocytosis by sFbg is also dependent of ERK activation. Similarly, Downey et al. (43) have demonstrated that ERK inhibition of PMN significantly inhibited phagocytosis of opsonized zymosan without affecting their binding capacity. In addition, a recent report has shown that ERK activation is necessary for actin polymerization in neutrophils responding to FcγR engagement (52).

Peripheral blood neutrophils are relatively short-lived cells (t1/2 = 6–8 h) and undergo spontaneous apoptosis when maintained in culture (53). However, exposure to sFbg or cytokines such as GM-CSF and IL-6 has been shown to delay apoptosis (7, 54, 55), thereby contributing to host defense or, under other circumstances, tissue injury. It has been previously reported that ERK MAPK pathway is involved in cell survival and preventing or delaying apoptosis (56–58). In this regard, the fact that sFbg activates ERK MAPK pathway and that the specific MEK inhibitor PD98059 reverses the effect of sFbg on apoptosis suggests that, in neutrophils, ERK activation leads to the extension of their half-life. However, as apoptosis is a complex process which involves gene induction, additional regulation steps could participate in sFbg modulation of cell survival. In this regard, preliminary evidence using NF-κB activation inhibitors, suggests that NF-κB would be involved in the protection of spontaneous apoptosis by sFbg (our unpublished observations). These results are in agreement with the role of NF-κB as a protector factor against apoptosis (59, 60) and have important implications for the regulation of inflammatory processes.

In conclusion, integrins are responsible for attachment and migration, but these molecules also contribute to intracellular signaling processes, either by transducing signals themselves or by enabling and/or coordinating signaling via other receptor systems. We hypothesize that during an inflammatory process chemoattractants or cytokines could prime neutrophils in circulation or in extravascular sites, turning them into sensitive targets for fibrinogen. In this context, the intracellular signal triggered in PMN by sFbg may be a central and early event influencing the fate of the inflammatory response.
Acknowledgments

We thank Marta Felipio, Nora Galassi, and Norma Riera for their technical assistance and Fundación de la Hemofilia and Academia Nacional de Medicina for the use of the FACScan flow cytometer. We also thank Sergio Fridman, Viviana Pressiani, and Antonio Fernández from the Department of Hemotherapy of Centro de Educación Médica e Investigación para Clinias for blood samples.

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