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Sphingosine Kinase: A Point of Convergence in the Action of Diverse Neutrophil Priming Agent 1

Alison C. MacKinnon,* Avril Buckley,* Edwin R. Chilvers,† Adriano G. Rossi,* Christopher Haslett,* and Tariq Sethi*

Neutrophils are a vital component of the early acute inflammatory response, but can cause profound tissue damage when activated to excess or prevented from undergoing apoptosis. However, much remains unknown about the intracellular signaling pathways regulating neutrophil activity. The structurally diverse neutrophil-priming agents platelet-activating factor, TNF-α, and the substance P analog [d-Arg6, d-Trp7,9,NmePhø8]-substance P (6–11) (SP-G) stimulated a rapid rise in sphingosine kinase activity in freshly isolated human neutrophils. This activity was blocked by preincubation with the sphingosine kinase inhibitor N,N-dimethylsphingosine (DMS). DMS also inhibited the increase in intracellular calcium concentration stimulated by platelet-activating factor, fMLP, and SP-G. This suggests that the increase in intracellular calcium concentration by these agents is dependent on sphingosine kinase activation and the generation of sphingosine-1-phosphate. Changes in cell polarization and the augmentation of the fMLP-induced superoxide anion generation, by all priming agents were also inhibited by DMS, while only the superoxide anion release was blocked by the phosphatidylinositol 3-kinase inhibitor LY294002. Moreover, SP-G and GM-CSF inhibited constitutive neutrophil apoptosis which was completely blocked by DMS. These results suggest a novel role for sphingosine kinase in the regulation of neutrophil priming. The Journal of Immunology, 2002, 169: 6394–6400.

Neutrophils are a vital component for the early nonspecific immune response. Patients who are neutropenic or who have defective leukocyte function are highly susceptible to bacterial infection. However, neutrophils may also cause tissue damage when activated prematurely or to excess and have been implicated in the pathogenesis of a wide variety of inflammatory diseases including adult respiratory distress syndrome. Activation of neutrophils results in superoxide anion generation and degranulation responses that if uncontrolled can lead to local tissue injury (1). The likelihood of neutrophil-mediated tissue damage is determined largely by the activation status of neutrophils that can range from quiescent through primed to fully activated.

Incubation of unprimed neutrophils with a secretagogue agonist (e.g., fMLP) produces only limited cell activation. Exposure to a priming agent, which on its own does not activate neutrophil secretory pathways, causes the initial shape change and polarization necessary for migration to the site of inflammation and greatly amplifies secretagogue-induced respiratory burst activity and cytotoxic degranulation (2). Agents such as GM-CSF, which prime neutrophils to subsequent fMLP-stimulated superoxide anion release, also delay neutrophil apoptosis and prolong neutrophil function at the inflamed site (3, 4). Apoptosis leads to the resolution of inflammation by facilitating the removal of neutrophils from the inflammatory site by phagocytic cells without rupture of the neutrophil membrane or disorgement of the cytotoxic granule contents (5–8). Despite the importance of neutrophil function in host defense and inflammatory disease states much remains unknown about the intracellular signaling pathways regulating neutrophil activity. Priming plays a central role in neutrophil activation (2) and apoptosis and investigating the mechanisms regulating neutrophil priming has important consequences for the development of therapeutic strategies for the treatment of inflammatory disease (9, 10).

The wide variety of priming/chemotactic agents identified so far e.g., TNF-α, GM-CSF, platelet-activating factor (PAF),3 and substance P and its analogs has made it difficult to dissect out the common or shared signal transduction mechanisms underlying priming. At least three different classes of receptor are involved: 1) G protein-coupled receptors (GPCR) (e.g., fMLP and PAF); 2) transmembrane domain receptors such as integrins and FcRs; and 3) single transmembrane domain receptors for growth-regulating cytokines (e.g., TNF-α and GM-CSF). A clue to the mechanism of priming may be provided by searching for a common intracellular signal transduction mechanism that is activated both by low concentrations of the activating agent and by all receptor classes of dedicated primers. A number of signaling mechanisms have been implicated in priming neutrophils, such as changes in receptor number/affinity, changes in intracellular calcium concentration ([Ca2+]i), phospholipase D (PLD), and phosphatidylinositol 3-kinase (PI3K) activity (3, 11, 12), but a direct role for these second
messengers in priming, as opposed to activation, has not been confirmed. To date, no single mechanism has been described that is triggered by all classes of neutrophil priming agent. Furthermore, evidence is emerging that several check points exist during the transition from the inactive state to the primed, activated state and eventually to apoptosis and that more than one type of pharmacological intervention may be possible to modulate neutrophil activity (13).

The lipid second messenger sphingosine 1-phosphate (SIP) has been implicated in the regulation of a variety of cell processes including cell proliferation, differentiation and apoptosis. More specifically, increases in SIP levels have been shown to be involved in the activation of the transcription factor AP-1, the stimulation of the mitogen-activated protein kinase (MAPK) pathways, activation of PLD, and the increased expression of adhesion molecules (14, 15). In differentiated HL-60 cells (a neutrophil-like cell line), FMLPRs can mobilize [Ca\(^{2+}\)], via the stimulation of sphingosine kinase and the subsequent production of SIP (16). This pathway is thought to mobilize [Ca\(^{2+}\)], directly via a novel sphingolipid-gated calcium channel present on the endoplasmic reticulum (17–19). Sphingosine kinase activity has also been shown to be stimulated by other GPCRs (20, 21), growth factor receptors such as platelet-derived growth factor and nerve growth factor receptors (22, 23), FcRs (24), and TNF-α receptors (25). The object of this study was to examine the role of sphingosine kinase in human neutrophil priming and apoptosis using four structurally distinct priming agents PAF, GM-CSF, TNF-α, and the substance P analog [d-Arg\(^6\), d-Trp\(^7,9\), NMePhe\(^5\)]-substance P (6–11) (SP-G).

Materials and Methods

Materials

PBS, HBSS, Dextran 500, Percoll, dihydrodorphamine (DHR), glutaraldehyde, FMLP, PAF, GM-CSF, TNF-α, N,N-dimethylsphingosine (DMS), and SIP were purchased from Sigma-Aldrich (Poole, U.K.); fura 2 tetraacetoxymethyl ester (fura 2-AM) and LY294002 were obtained from Calbiochem (Nottingham, U.K.); annexin V was obtained from Bender Medsystems (Vienna, Austria); [\(^{3}H\)]sphingosine (23 Ci/mmol) was obtained from New England Nuclear (Zaventem, Belgium); SP-G was a kind gift from the Imperial Cancer Research Fund (London, U.K.). All other reagents were of the purest grade available.

Neutrophil preparation

Peripheral venous blood was taken from healthy adult volunteers, anticoagulated with 4 ml of 3.8% sodium citrate/40 ml of blood and centrifuged (300 × g) for 20 min. Neutrophils were isolated as previously detailed (8, 9) using dextran sedimentation and discontinuous plasma-Percoll gradients. The purified neutrophils were washed three times in PBS without CaCl\(_2\) and MgCl\(_2\). Cell purity and viability were routinely >95%.

Measurement of [\(^{3}H\)]SIP formation

SIP was measured essentially as described (20). Neutrophils (1 × 10\(^{6}\) cells in 250 μl) were suspended in HBSS containing 1 mg/ml fatty acid-free BSA and incubated in the presence or absence of 10 μM DMS for 10 min at 37°C. Cells were sedimented and [\(^{3}H\)]sphingosine (15 nM) was added to the cells for 1 min at 37°C before addition of agonist. Following incubation at 37°C for the indicated times, 1 ml of methanol, followed by 0.5 ml of chloroform, was added and the samples were vortexed vigorously. The extracts were cleared by centrifugation and the supernatants were evaporated to dryness in a Speedvac centrifuge. Samples were reconstituted in 25 μl of ethanol and unlabeled SIP was added before spotting onto silica gel 60 TLC plates. The samples were separated in 1-butanol-acetic acid-water (3:1:1). Bands were visualized with iodine and the SIP spots were scraped and radioactivity was measured by scintillation counting. For [\(^{32}P\)]ATP re- 
lease, after stimulation with agonist, cells were rapidly sedimented at 4°C and the supernatant (200 μl) was removed. [\(^{3}H\)]SIP in the cellular and extracellular fraction was extracted and quantitated as described above. Alternatively, mass SIP release was measured by alkaline solvent extraction, dephosphorylation to sphingosine and rephosphorylation by recombinant sphingosine kinase in the presence of [\(^{32}P\)]ATP (26).

Determination of [Ca\(^{2+}\)]\(_i\) by spectrofluorometry

Freshly isolated neutrophils were incubated at 10\(^7\) cells/ml in CaCl\(_2\) and MgCl\(_2\) free HBSS with fura-2-AM (2 μM) for 30 min at 37°C (27). Cells were washed twice and resuspended at 5 × 10\(^6\) cells/ml in HBSS containing CaCl\(_2\) and MgCl\(_2\). The cells were transferred to a cuvette and maintained at 37°C. Agonists were added as described in the figure legends. Ratiometric fluorescence was monitored in a PerkinElmer fluorometric spectrophotometer (Wellesley, MA) with dual excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm. [Ca\(^{2+}\)]\(_i\) was calculated according to the equation [Ca\(^{2+}\)]\(_i\) = K(F − F\(_{\text{min}}\))/(F\(_{\text{max}}\) − F), where F is the ratio of the unknown sample, F\(_{\text{max}}\) is the ratio after the addition of 0.2% Triton X-100 and F\(_{\text{min}}\) is the ratio after [Ca\(^{2+}\)]\(_i\) chelation with 10 mM EGTA. K is the dissociation constant for fura 2-AM which is 224 nM.

Shape change assay

Neutrophils (0.5 × 10\(^6\)/ml) were incubated in PBS with CaCl\(_2\) and MgCl\(_2\) for 30 min at 37°C. Priming agents were added (10 μM) and the cells were incubated at 37°C with gentle shaking, as described in the figure legends. An equal volume of 2.5% glutaraldehyde was added and the cells were analyzed for changes in cell polarity by flow cytometry (Coulter EPICS Profile II; Coulter Electronics, Luton, U.K.). Shape change was calculated as a percentage from the mean forward light scatter of each sample by gating on the nonshape-changed population (28).

Measurement of reactive oxygen species release

Superoxide anion release was estimated as an increase in the generation of reactive oxygen species as determined by DHR fluorescence. Neutrophils were preincubated with inhibitor (DMS, 10 μM) for 10 min at 37°C before the addition of priming agent (SP-G or PAF). Cells were then stimulated by the addition of fMLP (100 nM) for 5 min in the presence of DHR (1 μM). Samples were placed on ice and fluorescence was measured by flow cytometry (Coulter EPICS Profile II; Coulter Electronics, Luton, U.K.).

PtdIns(3,4,5)P\(_3\) mass assay determination

PtdIns(3,4,5)P\(_3\), levels were measured as described (29). In brief, neutrophils (8 × 10\(^6\)) were subjected to standard Folch extraction and lipid extracts containing PtdIns(3,4,5)P\(_3\) were then subjected to alkaline hydrolysis resulting in the release of the polar head group Ins(1,3,4,5)P\(_4\). The mass of Ins(1,3,4,5)P\(_4\) was measured by radioligand displacement of [\(^{3}H\)]Ins(1,3,4,5)P\(_4\) to a recombinant binding protein (GST-GAP\(_{\text{rasp}}\) expressed in Escherichia coli and purified on glutathione-agarose beads) using unlabeled Ins(1,3,4,5)P\(_4\) as standard.

Measurement of apoptosis by morphology

Neutrophils (0.5 × 10\(^6\)) cells/ml were incubated for 20 h in Iscove’s medium containing 10% autologous serum at 37°C. Cells were cytotoxic-fixed in methanol, stained with Diff-Quik, and counted using oil immersion microscopy (×100 objective) to determine the proportion of cells displaying highly distinctive apoptotic morphology (9, 30).

Measurement of apoptosis by annexin V binding

A separate assessment of apoptosis was performed by flow cytometry using FITC-labeled human annexin V which binds to phosphatidylserine exposed on the surface of apoptotic cells. Stock annexin V was diluted 1/200 with binding buffer and then added (25 μl) to 75 μl of the recovered cell samples. Following a 10-min incubation at 4°C, samples were fixed by the addition of 3% paraformaldehyde (100 μl) in PBS before analysis using an EPICS Profile II (Coulter Electronics, Luton, U.K.).

Results

Sphingosine kinase activation

The secretagogue agonist fMLP has been shown to increase sphingosine kinase activity in neutrophil-like HL-60 cells (16). In this study, sphingosine kinase activity was measured in freshly isolated human neutrophils by the formation of [\(^{3}H\)]SIP from [\(^{3}H\)]sphingosine. Basal activity was routinely 450 ± 52 dpm (mean ± SEM) [\(^{3}H\)]SIP/10\(^6\) cells. fMLP, PAF, and SP-G consistently produced a rapid and transient increase in sphingosine kinase activity in human neutrophils (Fig. 1). A maximal stimulation of sphingosine kinase activity was observed at 20–30 s giving a mean increase (±SEM) relative to control of 81 ± 15% (n = 5), 151 ± 17% (n = 3), and 64 ± 35% (n = 3), for SP-G, fMLP, and PAF, respectively.
observed increase in [3 H]S1P was derived mainly from the cellular gosine kinase activity down to or below basal levels (Fig. 1). The tal systems (16, 20, 31). Pretreatment with DMS inhibited sphin-
avate the role of sphingosine kinase in a number of experimen-
a sphingosine kinase inhibitor which has been widely used to in-

\[ \text{from unstimulated cells (}\text{H11021}\text{)} \]

f extracellular (f)

\[ \text{cellular (}\text{H18554}\text{)} \]

\[ \text{fraction as the amount of [ 3 H]S1P detected in the supernatant in} \]

\[ \text{lower limit of detection using this assay. We also measured total} \]

\[ \text{response to PAF or fMLP was} \]

\[ \text{in the supernatants from fMLP-stimulated neutrophils (data not} \]

\[ \text{mobilization} \]

\[ \text{within 20 s of addition to} \]

\[ \text{entry. As expected, neither TNF-} \]

\[ \text{nor GM-CSF} \]

\[ \text{effect of DMS on PAF-} \]

\[ \text{on SP-G-induced} \]

\[ \text{in isolated human neutrophils} \]

PAF, fMLP, and SP-G all increased [Ca\(^{2+}\)]\(\text{i}\) within 20 s of addition to intact fura 2-AM-loaded isolated neutrophil suspensions. S1P (1 nM-1 \(\mu\)M) had no effect on [Ca\(^{2+}\)]\(\text{i}\) (not shown). The response to PAF (33.9 \pm 22.3 to 315.4 \pm 66.1 nM with 3 nM PAF; n = 3; Fig. 2, top panel) and fMLP (31.4 \pm 10.1 to 285 \pm 31 nM with 10 nM fMLP; n = 4; Fig. 2, middle panel) was primarily from mobilization of intracellular stores as the peak response to PAF and fMLP were both abolished by the intracellular Ca\(^{2+}\) antagonist 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB)-8 (Fig. 2) and only marginally affected by the store-operated channel blocker SKF 96365. However, the response to SP-G was significantly reduced by SKF 96365 (62 \pm 12\% inhibition, mean \pm SEM, n = 3; Fig. 2). This suggests that SP-G increases [Ca\(^{2+}\)]\(\text{i}\), both by intracellular Ca\(^{2+}\) mobilization and from external Ca\(^{2+}\) entry. As expected, neither TNF-\(\alpha\) nor GM-CSF produced any change in [Ca\(^{2+}\)]\(\text{i}\) in neutrophils (data not shown) which is in agreement with previous studies (see Ref. 11 and references therein).

**Effects on [Ca\(^{2+}\)]\(\text{i}\) in isolated human neutrophils**

Preincubation for 2 min with 10 \(\mu\)M DMS significantly reduced the PAF- and fMLP-induced rise in [Ca\(^{2+}\)]\(\text{i}\), and completely abolished the response to SP-G (Fig. 3). A similar degree of block was observed if total [Ca\(^{2+}\)]\(\text{i}\), was measured as area under the curve (data not shown). DMS did not inhibit the mean [Ca\(^{2+}\)]\(\text{i}\) observed if total Ca\(^{2+}\) stores. The effect of DMS on SP-G-induced [Ca\(^{2+}\)]\(\text{i}\) was concentration dependent with an IC\(_{50}\) of 6.47 \pm 0.32 \(\mu\)M (Fig. 3).

**Neutrophil shape change**

Changes in neutrophil polarization provide a good indication of neutrophil priming. All priming agents induced morphological changes characteristic of neutrophil shape change. Preincubation for 5 min with DMS (10 \(\mu\)M) significantly inhibited PAF-, GM-CSF-, TNF-\(\alpha\), and SP-G-induced shape change (Fig. 4) at all concentrations of priming agents tested. In contrast, the PI3K inhibitor LY294002 at 10 \(\mu\)M had no effect on neutrophil shape change induced by any priming agent (Fig. 4), suggesting that PI3K, although critical for respiratory burst activation and degranulation responses, is not essential for neutrophil polarization responses. Addition of S1P at concentrations up to 10 \(\mu\)M had no effect on neutrophil polarization (data not shown).
Priming the fMLP-induced respiratory burst

Previous studies have shown that priming agents strongly potentiate the fMLP-mediated increase in neutrophil superoxide anion release. In this study, PAF, GM-CSF, TNF-α/H9251, and SP-G did not induce spontaneous neutrophil superoxide anion release but caused a rapid and concentration-dependent increase in fMLP-stimulated superoxide anion release as measured by an increase in DHR fluorescence (Fig. 5). Addition of exogenous S1P (1 nM to 10 μM for

10 min) had no effect on fMLP-induced superoxide anion release (data not shown). The fMLP-stimulated increase in DHR fluorescence in primed cells was fully inhibited both by DMS (10 μM) and LY294002 (10 μM). Together this suggests a role for sphingosine kinase in neutrophil priming, and for PI3K in fMLP-induced neutrophil activation. There is some evidence that sphingosine analogs such as DMS and dihydrosphingosine also inhibit protein kinase C (PKC) which could explain the effect of DMS on neutrophil priming. However, Fig. 5 shows that the selective PKC

FIGURE 3. DMS inhibits Ca\textsuperscript{2+} mobilization in human neutrophils. Fura 2-AM-loaded human neutrophils were pretreated for 2 min with 10 μM DMS (○) or diluent (□) as indicated and stimulated with 10 nM PAF, 10 nM fMLP, 30 μM SP-G, or ionomycin as indicated by the arrows. The results are representative of three independent experiments. Bottom panel, Concentration response curve for DMS-mediated inhibition of 10 μM SP-G-induced calcium mobilization. Data is expressed as the mean peak response and represents the mean ± SEM of four independent experiments.

FIGURE 4. Effect of DMS and LY294002 on neutrophil shape change. Isolated human neutrophils were incubated for 5 min with vehicle (○), 10 μM DMS (■), or 10 μM LY294002 (■) before stimulation with 1) PAF (5 min), 2) GM-CSF (90 min), 3) TNF-α (30 min), or 4) SP-G (5 min) as indicated. Shape change was calculated from the mean forward light scatter of each sample measured by flow cytometry. The data represents the mean ± SEM of four independent experiments.

FIGURE 5. DMS and LY294002 inhibit neutrophil priming. Isolated human neutrophils were loaded with the cell permeable oxidant-sensitive dye DHR and preincubated for 5 min with vehicle (○), 10 μM DMS (■), or 10 μM LY294002 (■). Cells were primed with 1) PAF (5 min), 2) GM-CSF (90 min), 3) TNF-α (30 min), or 4) SP-G (5 min) as indicated followed by 5-min exposure to 100 nM fMLP. Bottom panel, Effect of GF109203X. Cells were preincubated with GF109203X at the indicated concentrations for 15 min before the addition of 100 nM PMA (○) or by the addition of TNF-α (10 nM) followed by 100 nM fMLP (■) as in 3. Fluorescence was measured by FACS analysis. The data represent the mean ± SEM of four independent experiments.

FIGURE 6. Measurement of PIP3 mass in human neutrophils. Freshly isolated neutrophils were treated with DMS (10 μM; ○), LY294004 (10 μM; □), or vehicle (△) for 5 min at 37°C before addition of TNF-α (10 ng/ml) for 30 min. Cells were stimulated with 100 nM fMLP or vehicle for 5 s at 37°C. Lipid extracts containing PIP3 were assayed as described in Materials and Methods. The results represent the mean ± SEM of four independent experiments.
Inhibitor GF109203X, although inhibiting PMA-induced superoxide release (IC_{50} = 60 nM) had no effect on primed fMLP-induced PKC activation, suggesting that inhibition of PKC could not explain the inhibition of primed superoxide release observed with DMS. We also looked further at the involvement of PI3K in neutrophil activation by measuring phosphatidylinositol (3,4,5) triphosphate (PIP_3) levels following TNF-α-induced priming of the fMLP response, which has been shown to be increased during neutrophil activation (32, 33). Fig. 6 shows that TNF-α primed the fMLP-induced increase in PIP_3 levels in human neutrophils. This was effectively inhibited by pretreatment with LY294002 (10 μM) but not DMS (10 μM).

Effect of DMS on neutrophil apoptosis

Neutrophils aged in culture for 20 h undergo constitutive apoptosis, which can be measured morphologically and by annexin V binding and flow cytometry. GM-CSF and SP-G protected neutrophils from undergoing constitutive apoptosis (Fig. 7). SP-G inhibited apoptosis from 60.5 ± 4.1% in control cultures to 30.8 ± 4.1% in treated cells. The EC_{50} for SP-G-induced inhibition of apoptosis was 10.1 ± 0.6 μM. GM-CSF- and SP-G-induced protection from apoptosis was completely abolished by DMS (10 μM) and LY294002 (10 μM; Fig. 7) supporting a role for sphingosine kinase and PI3K in neutrophil apoptosis.

Discussion

The novel findings of this study are that 1) neutrophil priming agents acting via diverse receptor types (fMLP, PAF, TNF-α, and SP-G) all activate sphingosine kinase; 2) inhibition of sphingosine kinase by DMS abolishes two cardinal markers of neutrophil priming, namely shape change and augmentation of the fMLP-stimulated superoxide anion release; 3) GPCR-induced Ca^{2+} release induced by PAF, fMLP, and SP-G in neutrophils is partially mediated by agonist-stimulated increases in sphingosine kinase activity; and 4) inhibition of sphingosine kinase abolishes the survival effect of SP-G and GM-CSF. The activation of sphingosine kinase is the first demonstration of a common signal activated by structurally diverse priming agents. The stimulation of intracellular sphingosine kinases to generate S1P may therefore be a proximal and central mechanism responsible for the priming of human neutrophils.

S1P is produced from sphingosine by sphingosine kinase. It has become clear that sphingolipids, in addition to being structural components of cell membranes, are sources of important signaling molecules. S1P, as well as acting as an intracellular second messenger, is also a high affinity agonist for the endothelial differentiation gene (EDG) family of GPCRs, EDG1, EDG3, EDG5, EDG6, and EDG8 which couple via different heterotrimeric G proteins to activate multiple effectors (15, 34). This makes S1P unique in eliciting both intra- and extracellular actions. Therefore, it is possible that stimulation of intracellular sphingosine kinase activity could lead to an increase in intracellular S1P which is released from cells to activate cell surface EDG receptors. In this study, we show that S1P released from neutrophils after stimulation with fMLP or PAF was well below the K_d concentration for S1P at any of the EDG receptor subtypes (K_d = 8 nM for EDG1 and ~20–30 nM for EDG3 and EDG5; Refs. 15, 31, and 35). Mass S1P detected in the supernatant following fMLP stimulation was <1 pmol (<2 nM in the 500-μl supernatant). In addition, exogenous S1P at concentrations from 1 nM-10 μM had no effect on [Ca^{2+}], cell polarization, or superoxide release. Furthermore, it has been reported that neutrophils lack cell surface receptors for S1P (35). Therefore, even if low concentrations of S1P are released following stimulation with priming agents, the results from this study suggest that it is unlikely to be responsible (via EDG receptor stimulation) for the effects described in this study. Previous work has shown that TNF-α-induced priming of fMLP-induced superoxide production involves sphingomyelin metabolites (36). Moreover, recent work has shown that FcyR1 aggregation activates NADPH oxidase in primed U937 cells via a pathway involving PLD1 and sphingosine kinase (37). The mechanism whereby an increase in S1P in response to priming agents leads to an increase in superoxide release remains to be elucidated.

We have shown that PAF, fMLP, and SP-G induce a rapid and transient Ca^{2+} signal in isolated human neutrophils that is at least partially dependent on sphingosine kinase activation and the generation of the putative second messenger S1P. This suggests that receptors for PAF, fMLP, and SP-G can use dual pathways leading to calcium release: the conventional phospholipase C-inositol 1,4,5-triphosphate pathway and the sphingosine kinase pathway. The results are similar to those described for lysophosphatidic acid and muscarinic acetyl choline receptors in SH-SY5Y cells (21). The effect of DMS is not due to an emptying of the intracellular Ca^{2+} store, as there was no significant reduction in the ionomycin-sensitive pool. We also show that SP-G, fMLP, and PAF could stimulate sphingosine kinase activity in human neutrophils with similar kinetics to that observed for Ca^{2+} release.

The role of PI3K in neutrophil activation/apoptosis is unclear. Studies have shown that IL-8- and GM-CSF-mediated apoptosis delay is inhibited by LY294002 and the mitogen-activated protein kinase kinase inhibitor PD98059, suggesting a role for PI3K and extracellular signal-regulated kinase (ERK) in neutrophil apoptosis (4). PI3K, but not the MAPKs (ERK, c-Jun-N-terminal kinase, p38) has been shown to be involved in IL-8-induced neutrophil migration (38). Other studies have shown that different chemoattractants (e.g., fMLP and PAF) use different signal transduction pathways to stimulate neutrophils, where PAF and fMLP strongly
activate p38 MAPK but only fMLP activates ERK (13). The ability of fMLP to activate more than one member of the MAPK family as opposed to PAF is reflected by the wider range of functional responses evoked by fMLP. Recently, fMLP and IL-8 have been shown to activate the G protein-dependent PI3K, p110α, and SP-G. Both of these responses could be inhibited by DMS, while only the superoxide anion release was blocked by LY294002. Although LY294002 inhibited PAF-, GM-CSF-, TNF-α, and SP-G-induced superoxide release, it did not inhibit changes in neutrophil polarity suggesting that PI3K is not involved in the initial reorganization of cytoskeletal actin. This suggests a role for sphingosine kinase in neutrophil priming and a role for PI3K primarily in neutrophil activation. This finding is in agreement with recent in vitro studies indicating the pivotal role of activated fMLP receptors in neutrophil activation (15). Moreover, GF109203X did not amenable to genetic manipulation, we are at present characterizing responses in neutrophilic differentiated HL-60 cells with the view to using antisense or a dominant-negative approach to studying further the role of sphingosine kinase in priming. Future work will be important for the design of new therapeutic strategies to modulate the sphingosine kinase/S1P pathway, with the prediction that such agents could have a profound influence on the adverse effects of neutrophil priming. This would be of value in the regulation of chronic inflammatory conditions where the neutrophilic granulocyte plays such a prominent role.

The present study has highlighted a novel role for sphingosine kinase as a regulator of neutrophil priming by a variety of structurally distinct neutrophil priming agents. Although neutrophils are not amenable to genetic manipulation, we are at present characterizing responses in neutrophils using antisense or a dominant-negative approach to modulating the sphingosine kinase/S1P pathway, with the prediction that such agents could have a profound influence on the adverse effects of neutrophil priming. This would be of value in the regulation of chronic inflammatory conditions where the neutrophilic granulocyte plays such a prominent role.

**References**


