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Research Article

Sphingosine 1-phosphate antagonizes human neutrophil apoptosis via p38 mitogen-activated protein kinase

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Abstract. Sphingosine 1-phosphate (SPP) is associated with the regulation of apoptosis, although its role in neutrophil apoptosis remains poorly investigated. Here, we show that exogenous SPP antagonizes spontaneous and anti-Fas-induced apoptosis in neutrophils. Pre-treatment with pertussis toxin clearly reduced the apoptosis-inhibiting capacity of SPP. Consequently, we investigated the involvement of potential modulators of apoptosis that are activated downstream of G_i/G_0-coupled receptors. Neither Akt activity nor change in basal activity of c-Jun N-terminal kinases was detected during apoptosis or after adding SPP. In contrast, there was a transient decrease in phosphorylation of both extracellular-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) during both spontaneous and anti-Fas-induced apoptosis. Although exogenous SPP reversed these reductions in kinase activity, experiments with inhibitors of ERK (PD98059) and p38 MAPK (SB203580) revealed that only SB203580 counteracted the effect of SPP. Thus, SPP counteracts neutrophil apoptosis via a G_i/G_0 protein survival-signalling pathway that includes modulation of p38 MAPK activity.

Key words. Sphingosine 1-phosphate; signal transduction; p38 MAPK; apoptosis; neutrophil.

The inflammatory process is one of the bodily defences against invading microorganisms. Unfortunately, overly extensive inflammation can lead to destruction of host tissue [1]. An important factor in the resolution or progression of this process is the mechanism whereby recruited neutrophils are removed from inflammatory foci [2]. In neutrophils, apoptosis is associated with early loss of cellular functions and entails recognition and uptake of these cells by macrophages [2]. Consequently, induction and control of neutrophil apoptosis is essential for rapid resolution of an inflammatory reaction [2]. Neutrophil apoptosis has been shown to be regulated by factors in the local inflammatory environment, including interleukins and inflammatory mediators such as IL-8, lipopolysaccharide (LPS), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α) and Fas ligand (FasL) [3–5]. However, the intracellular signalling pathways that mediate the actions of these stimuli have not yet been fully elucidated.

The well-recognized mitogen-activated protein kinase (MAPK) superfamily of signalling proteins has been closely linked to regulation of apoptosis in a wide variety of situations and cell types. This family of kinases consists of proteins such as extracellular-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK), each of which has different isoforms [6, 7]. Most types of cell surface receptors, including receptor tyrosine kinases, cytokine and adhesion receptors, and G protein-coupled receptors, can activate or modulate the mentioned MAPKs [7, 8]. A dynamic balance between the ERK and p38 MAPK/JNK signalling pathways is responsible for determining cell fate, and induction of apoptosis occurs through the JNK pathway in some types of cells [8–12],

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whereas ERKs are believed to negatively regulate such cell death [7, 9]. Although p38 MAPK has been clearly implicated in the regulation of apoptosis in different kinds of cells [6, 9, 11, 12], the specific role of this kinase in neutrophil apoptosis is unclear. Frasch et al. [13] did not detect any activation of p38 MAPK during spontaneous or anti-Fas-induced apoptosis in neutrophils, and Aoshiba et al. [14] have suggested that activation of p38 MAPK is involved in spontaneous neutrophil apoptosis. In contrast, recent reports have shown that p38 MAPK signalling contributed to the survival of mice granulocytes [15] and we recently found that p38 MAPK activity reduced apoptosis in human neutrophils [16].

Over the years, studies of many different types of cells have outlined the participation of sphingolipids in a number of important cellular functions, including apoptosis [17]. Initially, sphingomyelinase-induced accumulation of ceramide was suggested to mediate apoptosis caused by ionizing radiation, anti-cancer drugs, and TNF receptor ligation [18–20]. Although the ceramide pathway was earlier suggested to be activated in Fas-induced apoptosis [21], other authors have now shown that ceramide is not a Fas-initiated apoptotic signal [22, 23]. In a more recent study, ceramide was clearly shown to be dispensable for the apoptotic process [24]. In contrast, exposure to sphingosine has been found to induce or potentiate apoptosis in neutrophils [25] and in the leukaemic cell line HL-60 [26]. A phosphorylated derivative of sphingosine, sphingosine 1-phosphate (SPP), has on the other hand been implicated in suppression of apoptosis in other cell types [27–29]. This has also been demonstrated by overexpression of sphingosine kinase and thus accumulation of SPP in Jurkat cells [30]. A role of SPP in regulating neutrophil apoptosis has not yet been investigated despite the fact that this cell type exhibits a high sphingosine kinase activity and that a significant part of the generated SPP is released from the cells [31]. Consequently, the release of SPP from neutrophils might enable it to generate intracellular signals in these cells by an auto- or paracrine mechanism. In other cell types, SPP has been shown to induce intracellular signalling by binding to specific cell surface G protein-coupled receptors of the endothelial differentiation gene (Edg) family [32]. In accordance with these findings, mRNA for SPP-binding Edg receptors has been detected in human neutrophils [33]. In several cell types, SPP has been shown to affect ERK and p38 MAPK activities by a mechanism that is sensitive to pertussis toxin and therefore most probably occurs through a G protein-coupled receptor [32–35]. However, in Edg receptor-transfected CHO cells, the SPP-induced effects on both p38 MAPK and JNK activities were shown to be mediated by a pertussis toxin-insensitive signalling pathway [36].

In the present study, we performed experiments to investigate the potential role of SPP as a regulator of neutrophil apoptosis and, more specifically, the ability of this lipid to activate intracellular signals related to survival in these cells.

Materials and methods

Reagents

SPP and D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (D-MAPP) were obtained from Biomol Research Laboratory (Plymounth, Pa.). Fumonisin B1 (FB1) was purchased from Alexis Biochemicals (Läufelfingen, Switzerland), pertussis toxin from Speywood Pharma (Maidenhead, U. K.), Asp-Glu-Val-Asp-aminomethylcoumarin (DEVD-AMC) from Upstate Biotechnology (Lake Placid, N. Y.) and the fluorescent calcium indicator Fura2/AM from Molecular Probes (Eugene, Ore.). The p38 MAPK inhibitor (SB203580) and the MEK inhibitor (PD98059) were obtained from Calbiochem (La Jolla, Calif.), and SDS-PAGE reagents were from Bio-Rad (Rockford, IU.). Ficoll-Paque and an enhanced chemiluminescence (ECL) kit were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), and cell culture medium and supplements were from Gibco-BRL (Gaithersburg, Md.). D-erythro-sphingosine, acridine orange, ethidium bromide and all other reagents were from Sigma (St. Louis, Mo). The antibodies (Abs) were obtained from several sources: anti-phospho-JNK monoclonal Ab (clone G-7: sc-6254) and anti-JNK1 polyclonal Ab (clone FL: sc-571) were from Santa Cruz Biotechnology (Santa Cruz, Calif.); anti-phospho-ERK1/2, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-Akt and anti-phospho-Akt polyclonal Abs were from New England BioLabs (Hitchin, U. K.); horseradish peroxidase-conjugated Abs were from DAKO (Copenhagen, Denmark); the anti-ERK1/2 Ab was from Calbiochem; the anti-Fas CH-11 monoclonal Ab was from Immunotech (Marseille, France) and the anti-Edg-1 and anti-Edg-6 polyclonal Abs were from Biosource International (Nivelles, Belgium).

Isolation of human neutrophils

Blood samples were collected from healthy volunteers (a general approval to use cells isolated from these samples was given by the local ethics committee) and the neutrophils were isolated as previously described [37]. Briefly, erythrocytes were eliminated by sedimentation for 30 min at room temperature in the presence of 6% dextran, followed by a brief hypotonic lysis. Thereafter, the cell suspension was applied on a Ficoll-Paque density gradient and centrifuged at 400 g for 30 min. The resulting granulocyte fraction (>95% neutrophils) was washed twice and then gently dispersed in RPMI 1640 cell culture medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum.

Preparation of sphingolipid mediators

Synthetic preparation of sphingosine was dissolved in absolute ethanol. Aliquots of the ethanol stock were slowly diluted in a buffer that contained 0.6 mM $CaCl_2$, 1.5 mM KH_2PO_4, 2.6 mM KCl, 0.5 mM $MgCl_2$, 136 mM NaCl and 8 mM Na_2HPO_4 (pH 7.4) and was supplemented with 4 mg/ml fatty acid-free bovine serum albumin [38]. The mediator was subsequently incubated for 2 h at 37 °C and then used immediately. SPP powder was crushed and dissolved in methanol (1 mg/ml) by heating to 65 °C. The solvent was evaporated under nitrogen, and the thin film that remained was dissolved in the buffer described above for 30 min at 37 °C immediately prior to use.

Incubation of neutrophils

The isolated neutrophils (5×10^6/ml) were incubated in multiwell cell culture plates (in RPMI 1640 medium supplemented with 5% fetal bovine serum) at 37 °C in 95% air and 5% CO_2 in the absence or presence of sphingosine (15 µM) or 150 ng/ml anti-Fas Ab for the indicated times. In experiments with SPP, neutrophils were pre-incubated with or without this lipid (15 µM) for 15 min and then incubated in the absence or presence of either 15 µM sphingosine or 150 ng/ml anti-Fas Ab. The alkaline ceramidase inhibitor D-MAPP (30 µM) or the ceramide synthase inhibitor FB1 (30 µM) was added 60 min before beginning the incubations with or without anti-Fas Ab. In the experiments with pertussis toxin (PTX), the cells were pre-incubated for 2 h at 37 °C with 200 ng/ml of the toxin. The MAPK inhibitors PD98059 (50 µM) and SB203580 (20 µM) were added to the medium 15 min before SPP or vehicle and 30 min before anti-Fas Ab or vehicle.

Analysis of apoptosis

Caspase-3 activity was determined after 4 h of initiations of an apoptotic response using the fluorogenic peptide substrate DEVD-AMC. The cells (10^6 per sample) were washed in PBS, re-suspended in 500 µl of lysis buffer [10 mM Tris-HCl, 10 mM NaH_2PO_4 (pH 7.5), 130 mM NaCl, 1% Triton-X-100 and 10 mM $NaPP_i$] and placed on ice for 10 min. An aliquot (50 µl) of each cell lysate was then incubated for 60 min at 37 °C with 200 µl of substrate buffer [20 mM HEPES (pH 7.5), 10% glycerol, and 2 mM dithiothreitol (DTT)] and 4 µl of the DEVD-AMC substrate (1 µg/ml). The fluorescence of the AMC released was measured using an excitation wavelength of 390 nm and an emission wavelength of 460 nm in a BMG plate reader (Offenburg, Germany). As a blank, plain lysis buffer (lacking cells) was incubated with the substrate as described above. The value obtained for the blank was subtracted from all measurements made on the same plate. Due to the variations in caspase-3 activities among the different batches of cells, each isolated from a unique donor, we have chosen to normalize the data on caspase-3 activities. The observed variations can in part be explained by a varying onset of apoptosis in the different cell batches, since variations such as these are often noted for signalling activities in isolated human neutrophils.

Six hours after initiating the indicated incubations, the neutrophils were stained with acridine orange/ethidium bromide to analyse the distinctive morphological features of apoptosis and necrosis. Nuclei of normal neutrophils display diffuse fluorescence and several easily discerned lobes that are indicative of normal chromatin, whereas the nuclei of apoptotic neutrophils exhibit characteristically condensed chromatin and no distinguishable lobes. To analyse chromatin, 1 µl of a dye solution (acridine orange and ethidium bromide, 100 µg/ml each) was added to 25 µl of neutrophil suspension (12.5×10^4 cells), and 10 µl of this mixture was placed on a glass slide, mounted with a cover slip, and examined in a Nikon Eclipse E800 fluorescence microscope. Using a DAPI/FITC filter combination, normal and apoptotic neutrophils from at least four separate experiments were scored, counting a minimum of 100 nuclei per sample.

Measuring cytosolic free calcium

The cytosolic free Ca^{2+} concentration was measured as previously described [39, 40]. Briefly, neutrophils (5×10^6) were incubated for 20 min at 37 °C with 2 µM Fura2/AM in calcium-containing medium (136 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 20 mM Hepes, 1.0 mM $CaCl_2$ and 5.5 mM glucose; pH 7.4). The cells were then washed to eliminate excess Fura2/AM and re-suspended in a cuvette containing 2 ml of the above medium lacking $CaCl_2$. Fluorescence was measured on a SPEX spectrofluorometer equipped with a thermostatted (37 °C) cuvette holder equipped with a continuous stirring device. Excitation wavelengths were 340 and 380 nm, and the emission wavelength was 505 nm. Due to an interference of the added SPP with the calibration procedure, we have chosen to present the data as 'relative fluorescence'.

Western blotting

Neutrophils (10^7 cells/sample) were washed in cold PBS and then pelleted and lysed in a mixture of 200 µl of PBS and 100 µl of pre-warmed and concentrated Laemmli buffer [625 mM Tris (pH 6.8), 10% SDS, 25% glycerol and 0.015% bromophenol blue]. Equal amounts of proteins were loaded in each lane of a 10% SDS-PAGE mini-gel and separated by electrophoresis. Thereafter, the proteins were electrotransferred (100 V, 90 min) to polyvinylidene difluoride (PVDF) membranes in a buffer containing 48 mM Tris (pH 9.2), 39 mM glycine, 20% methanol and 0.037% SDS. Unspecific protein binding was blocked by pre-incubation with 5% (w/v) BSA in PBS supplemented with 0.1% (v/v) Tween-20 (designated PBST) for 1 h. The membranes were incubated overnight at 4 °C with a primary antibody. After washing in PBST,

the membranes were incubated for 1 h with one of the following secondary antibodies: horseradish peroxidase-conjugated anti-mouse IgG (1:3000) to detect phospho-JNK; anti-rabbit IgG (1:5000) for JNK, Edg receptors, phospho-Akt, and active and total p38 MAPK and ERK; or anti-goat IgG (1:5000) for Akt. All immunoblots were thoroughly washed in PBST, and signals from bound and conjugated Abs were detected using the ECL kit.

Results

Effects of SPP on neutrophil apoptosis

We analysed apoptosis in human neutrophils by measuring caspase-3 activity and evaluating morphological characteristics (when experimentally possible). Few (<10%) freshly isolated human neutrophils showed morphological changes characteristic of apoptosis. Furthermore, in accordance with previously published results [16, 41], we observed that approximately 30 and 58% of the neutrophils incubated under normal conditions underwent spontaneous apoptosis after 6 and 24 h, respectively. As shown in figure 1A, exposure to either the agonistic anti-Fas Ab or sphingosine for 6 h significantly increased the number of apoptotic cells. No pronounced necrosis was noticed in controls or in cells treated with anti-Fas Ab or sphingosine for this period of time; however, if longer incubations were used this became a problem (data not shown). Coincubation with SPP reduced the number of apoptotic cells that arose spontaneously or in anti-Fas Ab-treated cells and, as expected, in sphingosine-treated controls (fig. 1A). Similar results were recorded when the activity of caspase-3 was analysed in these different situations (fig. 1B).

To exclude the possibility that anti-Fas-induced apoptosis is mediated via formation of sphingosine, we used an inhibitor of alkaline ceramidase (D-MAPP, 30 µM) that has been shown to block the conversion of ceramide to sphingosine [17]. Despite recently published arguments against a role of ceramide in the apoptotic process [24], we also tested the effect of an inhibitor of ceramide synthesis (FB1, 30 µM) on neutrophil apoptosis. In accordance with the recent results found in different cell types [24], we observed no effects of either of these inhibitors on anti-Fas Ab-induced apoptosis in neutrophils (data not shown).

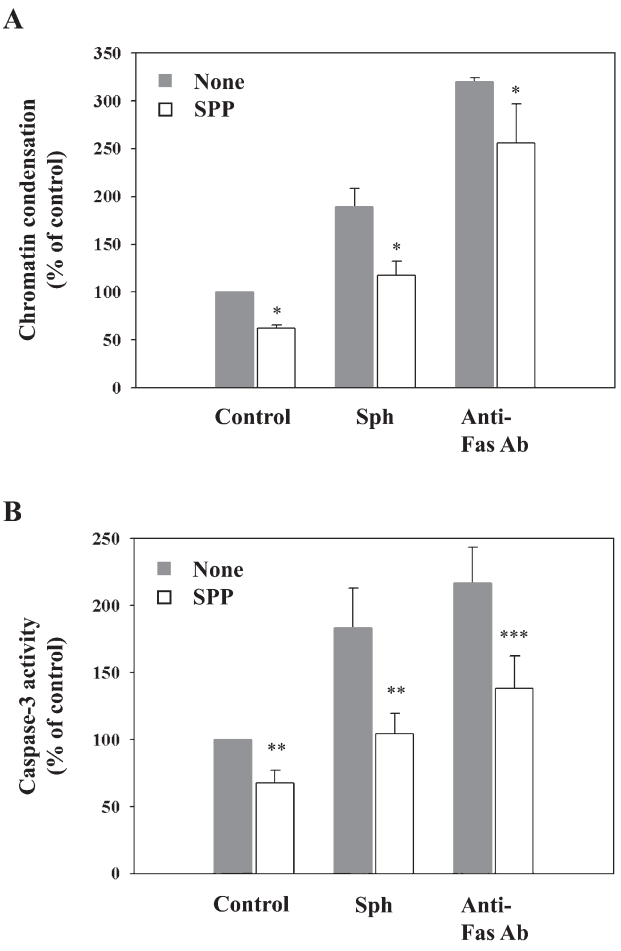

Figure 1. SPP protects neutrophils from spontaneous and sphingosine- and anti-Fas-induced apoptosis. (*A*) Effects of SPP on spontaneous and sphingosine- and anti-Fas-induced chromatin condensation. Neutrophils were pre-incubated for 15 min with or without 15 µM SPP and then incubated in the absence or presence of either 15 µM sphingosine (Sph) or 150 ng/ml of an anti-Fas Ab. Data are given as means ± SE of four experiments. Statistically significant differences were evaluated by paired Student's t test, but only the results representing the effects of SPP are shown; *$p<0.05$. (*B*) Effects of SPP on spontaneous and sphingosine- and anti-Fas-induced caspase-3 activity. Neutrophils were treated with SPP, sphingosine and anti-Fas Ab as above. Data are given as means ± SE of eight experiments. Statistically significant differences were evaluated by paired Student's t test, but only the results indicating the effects of SPP are shown; **$p<0.01$, ***$p<0.001$.

PTX reverses the effects of SPP on cytosolic Ca^{2+} and apoptosis

We first analysed the inhibitory effect of PTX on G protein-mediated signals by recording the effects of SPP and fMet-Leu-Phe (fMLP) on levels of cytosolic Ca^{2+} in the presence or absence of 200 ng/ml of PTX. We found that SPP induced a sustained increase in cytosolic Ca^{2+}, which was prevented by pre-treatment with PTX, and we confirmed that fMLP elicited a transient and much shorter Ca^{2+} signal, which was also blocked by PTX (fig. 2). Furthermore, the results show that the magnitudes of the SPP- and the fMLP-induced Ca^{2+} signals are approximately similar and also in the range detected in other cell types stimulated by a variety of agonists.

We then tested the effect of PTX on neutrophil apoptosis. Although it had no effect of its own, we found that it reversed the SPP-induced inhibition of both spontaneous and Fas-induced apoptosis, as indicated by the caspase-3 activities (fig. 3). These data suggest that SPP mediates its anti-apoptotic effect via a PTX-sensitive G protein sig-

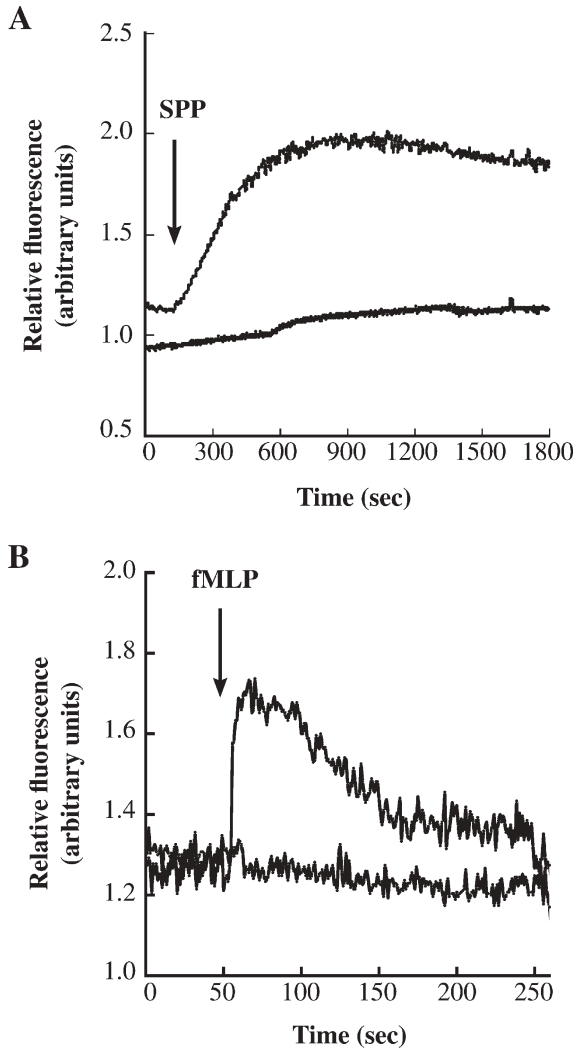

Figure 2. Effects of SPP and fMLP on the level of cytosolic free calcium in human neutrophils. Isolated human neutrophils were loaded with Fura2, as described in Materials and methods. The cells were then washed and subjected to fluorescent analysis at 37 °C under continuous stirring. The arrows indicate stimulation of the cells with 15 µM SPP (*A*) and 10^{-7} M fMLP (*B*). The lower traces represent recordings from cells that were pre-incubated with PTX, whereas the upper traces represent recordings from cells that were pre-incubated with vehicle alone. Each trace is representative of four similar experiments. Note the different time scales of the x-axis in *A* and *B*.

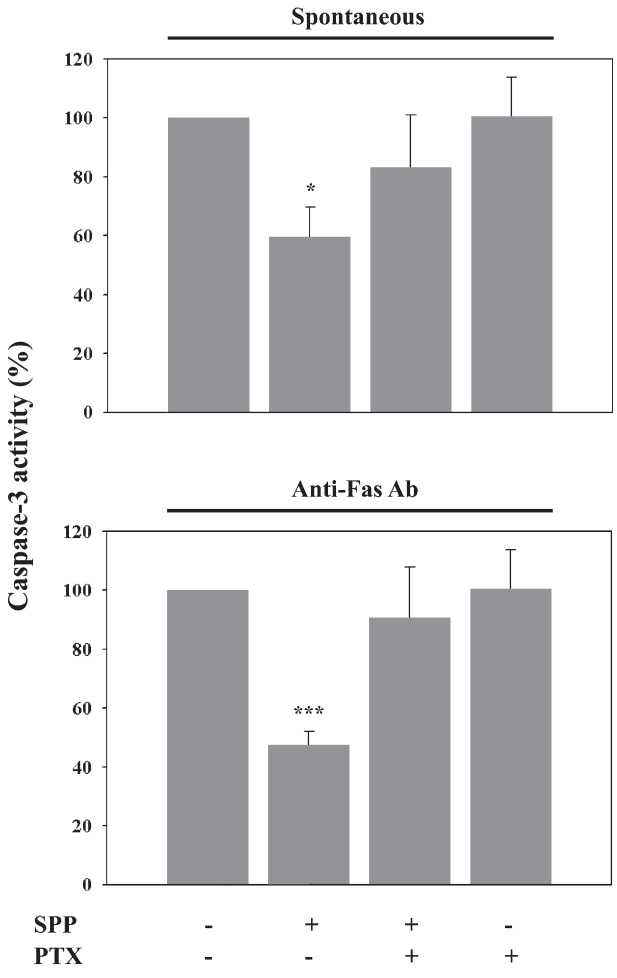

Figure 3. Effect of PTX on SPP-induced inhibition of caspase-3 activity. Neutrophils were pre-incubated for 2 h with or without PTX (200 ng/ml) and then treated with vehicle, SPP or anti-Fas Ab alone or together with SPP. Data are given as means ± SE of five separate experiments and are presented as percent of values representing spontaneous (upper panel) or anti-Fas Ab-induced (lower panel) activity. Statistically significant differences were evaluated by paired Student's t test, and no effects of SPP were noted in the presence of PTX.

nalling pathway. The effect of PTX on chromatin condensation could not be tested since the required conditions for testing this toxin, high cell density and a 2+6 h incubation period, resulted in a significant degree of secondary necrosis.

SPP counteracts the transient decrease in activation/phosphorylation of both p38 MAPK and ERK

In light of our findings that SPP modulates neutrophil apoptosis, and that these effects are impaired by PTX, we performed experiments to determine whether survival signals, for example different MAPKs, exist downstream of G protein activation. Neutrophils were pre-incubated for 15 min in the absence (control) or presence of SPP and then incubated for different amounts of time in the absence (fig. 4A) or presence (fig. 4B) of anti-Fas Ab. Immunoblotting with an anti-phospho-p38 MAPK Ab confirmed previous observations [16] that the constitutive expression of the active form of p38 is rapidly reduced under control conditions (spontaneous apoptosis) and during anti-Fas-induced apoptosis (fig. 4A, B). In neutrophils pre-treated with SPP, the re-phosphorylation of p38 MAPK occurred much more rapidly, which suggests that inhibition of apoptosis by SPP could

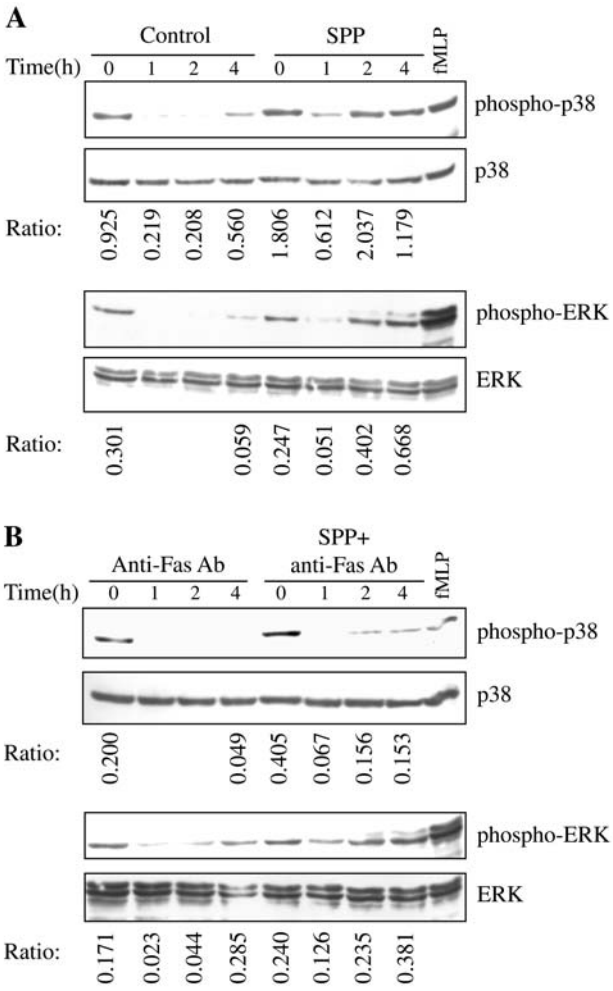

Figure 4. Effects of SPP on phosphorylation of p38 MAPK and ERK in the absence or presence of anti-Fas Ab. (*A*) Neutrophils were pre-incubated for 15 min with or without 15 µM SPP and then incubated at 37 °C for the indicated times in the absence of anti-Fas Ab. Lysates were subjected to immunoblot analysis with a specific Ab against the phosphorylated form of either p38 MAPK (phospho-p38) or ERK (phospho-ERK; p42 and p44). The immunoblots were stripped and re-probed with Abs against total p38 MAPK (p38) or total ERK (ERK; p42 and p44). A lysate of cells stimulated for 2 min with fMLP (10^{-7} M) was included in each blot as a positive control. The blots are representative of five separate experiments. (*B*) Cells were pre-incubated for 15 min with or without 15 µM SPP and then incubated at 37 °C for the indicated times in the presence of anti-Fas Ab. Immunoblot analysis of the lysates was done as above. Each blot is representative of three separate experiments. (*A*, *B*) In the outlined blots, the optical density (OD) of all bands of phosphorylated MAPK and total MAPK were scanned, and the calculated ratios for the two values ($OD_{phospho}/OD_{total}$) are indicated below each respective sample.

be mediated via a less pronounced inactivation of p38 MAPK.

Although not as consistently as p38 MAPK, analysis of ERK phosphorylation (fig. 4A, B) often also revealed a constitutive activity that was rapidly reduced and subsequently regained very slowly during spontaneous and Fas-mediated apoptosis. Similar to its effects on p38 MAPK activity, SPP caused a less pronounced dephosphorylation of ERK in these situations (fig. 4A, B). These results indicated that ERK might also be a regulatory signal involved in mediating the protective effect of SPP on neutrophil apoptosis.

In this context, we also analysed the activities of two other signalling molecules that have been implicated in regulation of apoptosis, namely Akt and JNK. We did not detect any activation of the well-known survival factor Akt, at any time points, during either spontaneous or anti-Fas-induced apoptosis [16], nor did we find that SPP induced any immediate or late activation of this kinase (data not shown). Furthermore, Western blot analysis showed that treatment with SPP did not affect the basal level of phospho-JNK during either spontaneous or anti-Fas-induced apoptosis (data not shown). Considering these findings, we focused our interest on possible involvement of p38 MAPK and ERK in SPP-mediated regulation of neutrophil apoptosis. To address this issue, we investigated the effects of specific inhibitors of these two kinases on the SPP-induced modulation of neutrophil apoptosis.

Effects of the ERK inhibitor PD98059 and the p38 MAPK inhibitor SB203580 on the apoptosis-obstructing effects of SPP

We pre-incubated neutrophils with the ERK inhibitor PD98059 (50 µM) to ascertain whether the ERK pathway plays a role in regulation of SPP-effected survival. The results show that PD98059 did not influence the ability of SPP to promote neutrophil survival during anti-Fas-induced apoptosis (table 1). In the presence of PD98059 alone we found no effects on chromatin condensation (15.7 ± 1.9% of total cells) or on caspase-3 activity (100.1 ± 5.1% of the control). Because of this lack of effect of PD98059, we also analysed the impact of this inhibitor on SPP-induced activation/phosphorylation of ERK and confirmed that PD98059 did indeed inhibit the activity of ERK (fig. 5). The action of SB203580 cannot be tested by a similar approach, since the inhibitor binds to the ATP-binding site of p38 MAPK and inhibits its activity without impairing tyrosine phosphorylation of the p38 MAPK [42]. These findings suggest that the observed modulation of ERK activity is not part of the SPP-induced survival signal in human neutrophils.

In contrast to the above results, pre-treatment of neutrophils with the p38 MAPK inhibitor SB203580 (20 µM) significantly reversed the impeding effect of SPP on anti-Fas-induced apoptosis towards levels seen in the absence of SPP and SB203580 (table 2). The inability of SB203580 to totally reverse the effect of SPP is most likely explained by the presence of the δ isoform of p38 MAPK in human neutrophils [43] and the fact that SB203580 does not inhibit this particular isoform [44]. In the presence of SB203580 alone we observed an increase in chromatin condensation (23.2 ± 2.6% of total cells) and

Table 1. Influence of PD98059 on SPP-induced inhibition of apoptosis.

	Chromatin condensation (% of total cells)	Caspase-3 activity (% of control)
Controls	13.7 ± 0.6	100.0
Anti-Fas Ab	34.9 ± 3.7	228.3 ± 16.5
SPP + anti-Fas Ab	23.6 ± 1.6	145.2 ± 10.1
PD98059 + anti-Fas Ab	32.0 ± 3.3	210.4 ± 23.4
PD98059 + SPP + anti-Fas Ab	25.1 ± 2.9	164.9 ± 3.1

Neutrophils were pre-incubated for 15 min in the absence or presence of 50 µM PD98059 and then with or without 15 µM SPP for 15 min and subsequently exposed to an anti-Fas Ab (150 ng/ml). Levels of apoptosis were analysed as described in Materials and methods. Data are given as means ± SE of four (chromatin condensation) and three (caspase-3 activity) separate experiments. Statistically significant effects were evaluated by paired Student's t test.

Table 2. Influence of SB203580 on SPP-induced inhibition of apoptosis.

	Chromatin condensation (% of total cells)	Caspase-3 activity (% of control)
Controls	14.3 ± 0.7	100.0
Anti-Fas Ab	35.5 ± 3.0	234.5 ± 19.5
SPP + anti-Fas Ab	22.6 ± 1.6	114.9 ± 21.2
SB203580 + anti-Fas Ab	38.3 ± 4.9	244.7 ± 31.5
SB203580 + SPP + anti-Fas Ab	29.7 ± 2.6*	180.0 ± 27.3*

Neutrophils were pre-incubated for 15 min in the absence or presence of 20 µM SB203580 and then with or without 15 µM SPP for 15 min and subsequently exposed to an anti-Fas Ab (150 ng/ml). Levels of apoptosis were analysed as described in Materials and methods. Data are given as means ± SE of five (chromatin condensation) and three (caspase-3 activity) separate experiments. Statistically significant differences were evaluated by paired Student's t test. The statistical significances for the effects of SB203580 are indicated; *$p < 0.05$.

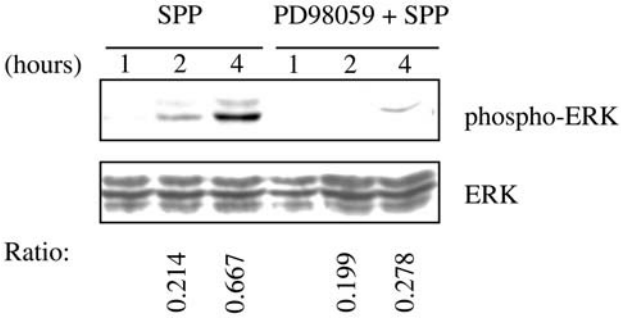

Figure 5. Effects of PD98059 on SPP-induced phosphorylation of ERK**.** Neutrophils were pre-incubated for 15 min in the absence or presence of 50 µM PD98059 and subsequently exposed to 15 µM SPP for 15 min. Thereafter, the cells were incubated for up to 4 h with anti-Fas Ab. Immunoblot analysis of lysates was done using a specific Ab against the phosphorylated form of ERK (phospho-ERK; p42 and p44). The immunoblots were stripped and re-probed with an Ab against total ERK (ERK; the upper two stronger bands represents p42 and p44). Each blot is representative of three separate experiments. In these blots, the OD of all bands of phosphorylated ERK and total ERK were scanned, and the calculated ratios for the two values ($OD_{phospho}/OD_{total}$) are indicated below each respective sample.

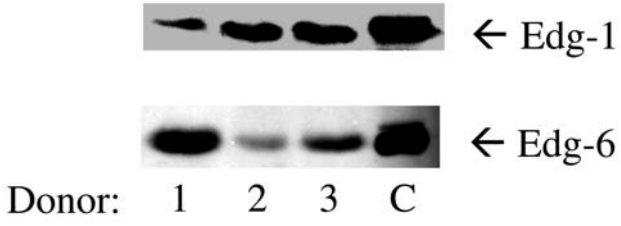

Figure 6. Protein expression of Edg-1 and Edg-6 receptors in human neutrophils. Neutrophils were isolated from three different volunteers and 4×10^6 cells from each of these isolates were lysed and loaded in each lane. The different samples were subjected to immunoblot analysis with a specific Ab against either the Edg-1 receptor (44 kDa) or the Edg-6 receptor (45 kDa). The lane marked C was loaded with a sample containing a positive control for the respective Edg protein.

in caspase-3 activity (138.6 ± 17.1% of the control). This effect of SB203580 agrees with previously published results [16], and further supports the idea that p38 MAPK acts as a survival factor in neutrophils. Our results suggest that SPP inhibits neutrophil apoptosis by a mechanism that results in increased p38 MAPK activity, and the present demonstration that Edg receptors are expressed also at the protein level in human neutrophils (fig. 6), opens up the possibility that SPP initiates its effect on neutrophil apoptosis via such cell surface receptors.

Discussion

Neutrophils have a high sphingosine kinase activity and a significant percentage of the SPP that is formed inside the cell is released [31]. This could then cause a continuous autocrine or paracrine stimulation, in particular in situations of high neutrophil densities such as at inflammatory foci. Nevertheless, only a few studies concerning the effects of SPP on neutrophils have been published, and they have shown that this sphingosine derivative acts as a regulator of neutrophil motility [45], and induces intracellular calcium mobilization in differentiated neutrophil-like HL-60 cells [46]. In both these studies, optimal effects were obtained when micromolar concentrations of SPP were added to the isolated neutrophils. The need for such concentrations in modulating neutrophil responses are different from the nanomolar concentrations normally used to activate Edg receptors on endothelial cells. A possible explanation for this discrepancy is the fact that neutrophils exhibit an endogenous production of SPP [31] that might cause a desensitization of neutrophil responsiveness, similar to that caused by chemotactic factors [47], therefore generating a need for micromolar concentrations of ex-

ogenous SPP to trigger a cellular response. Complementary to the cited findings in neutrophils, our results demonstrate that SPP inhibits spontaneous, sphingosine- and anti-Fas-effected apoptosis in human neutrophils. Furthermore, we observed that the anti-apoptotic effect of SPP is mediated by a PTX-sensitive G protein, suggesting the involvement of a G protein-coupled receptor. Certain members of the Edg family of G protein-coupled receptors (which includes Edg-1, -3 -5, -6 and -8) have been shown to specifically bind SPP [32, 34, 35], and mRNA for Edg-1 to -6 has been detected in human neutrophils [33]. Here we complement the latter findings by demonstrating that Edg receptors are also expressed at the protein level in human neutrophils. In addition, the receptors for Edg-1 and -3 have been reported to interact with the PTX-sensitive G_i/G_o proteins in endothelial and smooth muscle cells [34, 35]. These findings could imply that all ligands such as fMLP, which bind to a receptor coupled to a PTX-sensitive G protein, should be able to protect neutrophils from apoptosis. However, fMLP does not affect apoptosis in human neutrophils [3]. The fMLP receptor is rapidly turned off by lateral segregation and therefore sends signals for only a short time [48, 49]. Compared to the fMLP-induced calcium signal, we found that the SPP-induced PTX-sensitive calcium signal is greatly prolonged, suggesting that a more sustained G protein signal is triggered by SPP and that this might be an essential feature for SPP-mediated survival in neutrophils.

SPP *per se* may function as an intracellular second messenger, as suggested by investigators who noted that overexpression of sphingosine kinase inhibited apoptosis in fibroblasts and Jurkat T cells [30]. However, the finding that a significant part of endogenous SPP is released from neutrophils [31], and our observation that the effect of SPP is sensitive to PTX, implies that the anti-apoptotic action of SPP in human neutrophils is triggered at the cell surface.

In previous studies performed by us [16], and by other investigators [14], freshly isolated human neutrophils were found to spontaneously express the active phosphorylated form of p38 MAPK. Moreover, in our earlier investigation [16], we noticed that a transient inhibition of p38 MAPK occurs during both spontaneous and anti-Fas-induced apoptosis, and that the activity of p38 MAPK constitutes a survival signal in human neutrophils. The latter finding agrees well with the observation that pharmacological inhibition of p38 MAPK potentiates the apoptotic response in eosinophils [50], and with the recent discovery that p38 MAPK signalling contributes to the survival of mice granulocytes [15]. This concept is further supported by the present observations that SPP reduced the inactive phase of p38 MAPK and concomitantly protected neutrophils from apoptosis. We also noted that the p38 MAPK inhibitor SB203580 counteracted the SPP-induced reductions of caspase-3 activity and nuclear condensation. These results suggest that the survival mechanism induced by SPP in neutrophils is, at least in part, mediated by a p38 MAPK signalling pathway that is different from the mechanisms proposed for a variety of other cell systems, which are believed to involve ERK [27, 34]. The demonstration that SPP can increase the activity of myosin light-chain phosphatase [51], and the observation that activation of the SH2 domain-containing tyrosine phosphatase-1 promotes cell death in myeloid cells [52] suggest that the presently observed SPP-induced increase in p38 MAPK activity could be mediated via inhibition of a phosphatase rather than promoting a kinase-induced phosphorylation of p38 MAPK. Our conclusion that p38 MAPK plays an essential role in the action of SPP is supported by studies showing that GM-CSF, a well-known survival factor for neutrophils, triggers activation of p38 MAPK [53], and the more recent finding that SPP activates NF-κB [54], a potential mediator of the effect of p38 MAPK on neutrophil survival [16]. In addition, we found that SPP increased the amount of phosphorylated active ERK, but pharmacological inhibition of this kinase did not significantly affect neutrophil apoptosis, which shows that, in contrast to p38 MAPK, ERK is not involved in mediating the effect of SPP on spontaneous or anti-Fas-induced apoptosis. This also indicates that the signalling pathway utilized by SPP differs from those of LPS and calcium pyrophosphate dihydrate, both of which have been reported to modulate neutrophil apoptosis through activation of ERK [55, 56].

In further support of our conclusion regarding the role of p38 MAPK, we found that other signals that could potentially mediate the effect of SPP on neutrophil apoptosis were not activated. More specifically, neither in the absence nor the presence of SPP did we detect the phosphorylated active form of Akt during neutrophil apoptosis. These results uphold the above-stated conclusion that SPP attenuates neutrophil apoptosis by a mechanism distinct from the Akt signalling pathway. The finding that SPP does not cause an activation of Akt is most readily explained by the demonstration that p38 MAPK-dependent activation of MAPKAPK2 functions as PDK2, an enzyme necessary for PI 3-kinase-induced activation of Akt, in human neutrophils [57]. This can explain why we did not observe activation of Akt in the present study despite the fact that Fas triggers an activation of PI 3-kinase that peaks after 1 h [16], since even in the presence of SPP, the activity of p38 MAPK is very low after 1 h. In addition, we performed experiments to ascertain whether the JNK kinase participates in the SPP-induced modulation of spontaneous and anti-Fas-mediated apoptosis. Despite results from other cell types [11, 27], we found no indication of the involvement of JNK, since the basal activity of this kinase was not affected by SPP, which again emphasizes the role of p38 MAPK in SPP-induced attenuation of neutrophil apoptosis.

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