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Sphingosine-1-phosphate Mobilizes Intracellular Calcium and Activates Transcription Factor NF-κB in U937 Cells

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Sphingosine-1-phosphate (SPP), a metabolite of sphingolipids, has been implicated as a second messenger in cell growth regulation and signal transduction via calcium mobilization from internal stores. This study shows that SPP mobilizes intracellular calcium in U937 cells and demonstrates for the first time the ability of SPP to activate the transcription factor NF-κB in these cells. Furthermore, calcium release from the internal stores by thapsigargin (TG), an inhibitor of the endoplasmic reticulum Ca^{2+} pump, was associated with activation of NF-κB. Moreover, we have shown that while an intracellular calcium chelator BAPTA/AM was able to inhibit both SPP- and TG-induced NF-κB activation, it had no effect on TNF-induced NF-κB activation. In addition, SPP-induced NF-κB activation was blocked both by cyclosporin A, known to inhibit calcineurin phosphatase activity, and by the antioxidant butylated hydroxyanisole. These observations suggest that intracellular calcium mobilization is required for SPP-induced NF-κB activation, which may involve calcineurin- and redox-dependent mechanisms.

Sphingosine-1-phosphate (SPP), the initial product of sphingosine degradation by sphingosine kinase, is known to play an important role in cell growth regulation and signal transduction and is served as an intracellular second messenger (1,2,3,4). Ghosh et al. (5,6) demonstrated that sphingosine and SPP induced a rapid release of Ca^{2+} from internal stores in smooth muscle cells. They have shown that endoplasmic reticulum membrane itself contains the sphingosine kinase activity required to convert inactive sphingosine to SPP as well as the site at which Ca^{2+} release was activated. Studies by Spiegel and co-workers revealed that SPP acts as a potent mitogen on Swiss 3T3 fibroblasts by a pathway that is independent of protein kinase C and can cause mobilization of Ca^{2+} from internal calcium stores through a mechanism independent of inositol lipid hydrolysis and arachidonic acid release (1,7,8). However, little is known about the effects of SPP on transcription factors. Recent studies have shown that SPP markedly increased specific DNA binding activity of AP-1 in Swiss 3T3 fibroblasts (9).

The inducible transcription factor NF-κB resides in the cytoplasm as an inactive form complexed to the inhibitory subunit IκB. Upon stimulation of cells with a large variety of agents including cytokines, mitogens, UV irradiation, LPS and viruses, NF-κB is rapidly activated by its release from IκB, which allows translocation of the p50/p65 complex into the nucleus where it activates transcription of target genes such as interferons, cytokines, cell adhesion molecules and hematopoetic growth factors (10). Here we demonstrate that SPP mobilizes intracellular calcium and induces activation of the transcription factor NF-κB in U937 cells. Our observations suggest that intracellular calcium release, calcineurin- and redox-dependent mechanisms may play a role in SPP-induced NF-κB activation.

MATERIALS AND METHODS

Materials. SPP was purchased from BIOMOL (Plymouth, USA). Thapsigargin (TG), BAPTA/AM, and Indo-1 were obtained from CALBOICHEM (La Jolla, USA). Recombinant human TNF-α was provided by BASF (Ludwigshafen, Germany). All other reagents were purchased from SIGMA (Munich, Germany).

Cell culture. U937 monocytic cells were cultured in RPMI 1640 (GIBCO, Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum (FCS), HEPES (0,02 M) and 50 μg/ml gentamycin.

Ca^{2+} fluorescence measurements. The assay of cytosolic Ca^{2+} was performed using Indo-1. Cells ($1 \cdot 10^6$/ml) were loaded with 5 μM Indo-1 at 37°C for 30 min and resuspended in calcium free medium containing 2 mM EGTA. After a base-line calcium level was measured, SPP, TG or TNF were added, and changes in free calcium levels were measured by flow cytometry at 37°C, as previously described (11).

Electrophoretic mobility shift assay (EMSA). Preparation of nuclear and cytosolic extracts and conditions for electrophoresis mobility shift (gel shift) assay have been previously described in detail (12,13). Equal amounts of extracts were incubated with an NF-κB-

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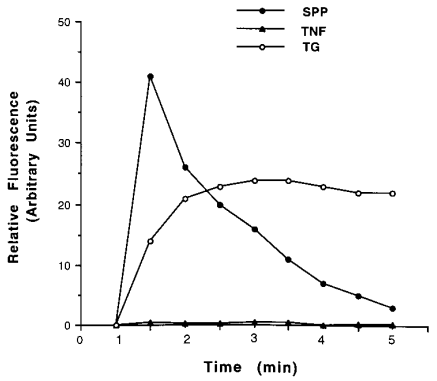

FIG. 1. Effects of sphingosine-1-phosphate, thapsigargin and TNF on intracellular calcium mobilization in U937 cells. Cells were loaded with Indo-1 as described in Materials and Methods. Changes in cytoplasmic Ca^{2+} were monitored by measuring the changes in ratio (FL400/FL486) over time. SPP (5 μM), TG (100 nM) and TNF (10 ng/ml) were added at the point 1 min in the calcium free medium (2 mM EGTA) after establishing a baseline level of cell calcium.

specific ^{32}P-labeled oligonucleotide. The sequence of the double-stranded 30-bp probe comprising the NF-κB site and the flanking sequences from the mouse kappa light chain enhancer was shown by Zabel et al. (14). For control of specificity, competition experiments were performed with a 100-fold molar excess of either unlabeled wild-type oligonucleotide or oligonucleotide mutated in the NF-κB-binding site by two point mutations. In some experiments NF-κB activation was determined by an 2 $\times$ NF-κB - LUC reporter assay. Briefly, cells were transfected with 2 $\times$ NF-κB - LUC reporter. After 12 hr, some of the cultures were incubated for an additional 12 hr with SPP, TG or TNF, and luciferase activities were determined as described previously (15,16).

RESULTS AND DISCUSSION

Using ratio cytofluorimetry and the internalized Ca^{2+} chelating dye, Indo-1, intracellular calcium mobilization was measured in U937 cells incubated in Ca^{2+}-free medium (2mM EGTA). As shown in Fig. 1, the addition of 5 μM SPP induced a rapid Ca^{2+} release from the internal stores, whereas TG, an irreversible inhibitor of the endoplasmic reticulum Ca^{2+} ATPase, caused a slow increase in calcium. This observation is consistent with previous reports (2,6,7,8). In contrast, TNF was unable to mobilize calcium from the internal stores in U937 cells (Fig. 1). Recently, Pahl and Baeuerle (17) have shown that several endoplasmic reticulum stress-inducing agents, such as TG, triggered NF-κB activation in HeLa cells. However, the precise mechanism by which such stimuli (TG) induce NF-κB activation remains to be elucidated (18).

To investigate the effect of SPP on NF-κB activity, electrophoretic mobility shift assays were performed. U937 cells were treated for 2 hr with SPP, as well as with TG and TNF, both of which have been reported to induce NF-κB activation (17,19). Subsequently, cell extracts were prepared and analysed for DNA-binding activity to a NF-κB-specific oligonucleotide. Compared with the medium control, SPP, as well as TG and TNF, taken as positive controls, led to strong induction of NF-κB binding activity in U937 cells (Fig.2A,B). The specificity of NF-κB DNA binding, induced by SPP, was confirmed in competition analysis. As shown in Fig.2A, incubation with an excess of mutated oligonucleotide

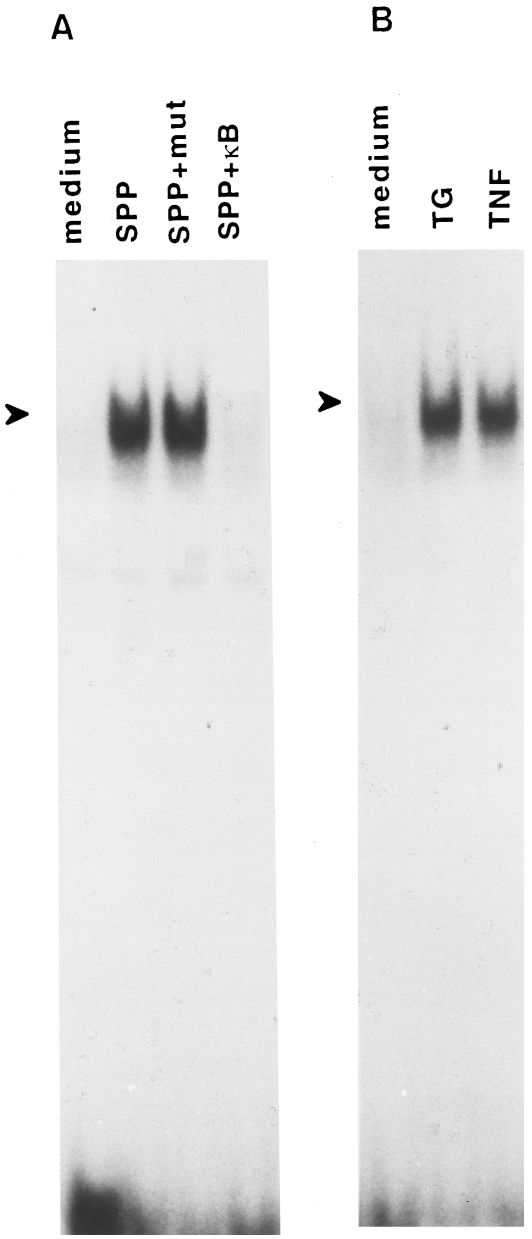

FIG. 2. Effects of sphingosine-1-phosphate, thapsigargin and TNF on NF-κB activation. U937 cells were treated for 2 hr with medium alone, 5 μM SPP (A), 100 nM TG (B) and 10 ng/ml TNF (B). Cell extracts (10 μg of protein) were analysed subsequently for NF-κB activity by EMSA. In competition analysis (A), a 100-fold excess of unlabeled mutated (mut) or wild-type oligonucleotide (κB) was added to the extracts and mixed with ^{32}P labeled probes specific for the κB enhancer. The position of the NF-κB DNA complex is marked by an arrowhead.

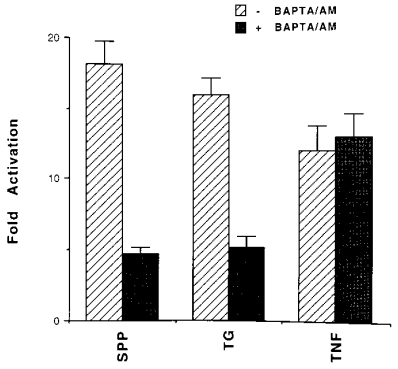

FIG. 3. Effects of intracellular Ca^{2+} chelator BAPTA/AM on sphingosine-1-phosphate-, thapsigargin- and TNF-induced NF-κB activation. Cells were transfected with 2 × NF-κB LUC reporter (15,16); 12 hr after transfection, U937 cells were treated for additional 12 hr with 5 μM SPP, 100 nM TG and 10 ng/ml TNF in the absence or presence of 25 μM BAPTA/AM, as indicated. Luciferase activities were determined as described previously (15,16). The results shown are means of three independent experiments.

did not antagonize NF-κB binding, whereas competition with a 100-fold molar excess of unlabeled oligonucleotide led to inhibition of binding activity. In order to investigate whether intracellular calcium is required for SPP-induced NF-κB activation we used BAPTA/AM, an effective intracellular calcium chelator (20). Pretreatment of cells with 25 μM BAPTA/AM for 1 hr markedly suppressed SPP- or TG-mediated increase in intracellular calcium level (data not shown). U937 cells were treated with SPP, TG or TNF in the absence or presence of BAPTA/AM, and NF-κB activation was determined by the luciferase reporter gene assay (15,16). As seen in Fig.3, treatment of cells with intracellular calcium chelator BAPTA/AM resulted in inhibition of SPP- and TG-induced NF-κB activation but had no effect on TNF-induced NF-κB activation. BAPTA/AM by itself had no effect on NF-κB activity (data not shown). These observations suggest that release of intracellular Ca^{2+} from internal stores is required for NF-κB activation induced by SPP and TG, and confirms previous reports that calcium does not play a role in TNF-induced NF-κB activation in U937 cells. Our findings also suggest that Ca^{2+} may act as a second messenger in the signal transduction pathways for SPP-induced NF-κB activation. A variety of events in mammalian cells are dependent on an increase in the concentration of intracellular calcium (21,22,23). One potential mediator of such calcium-dependent events is calcineurin, a Ca^{2+}- and calmodulin-dependent protein phosphatase (24). NFAT, an activated T cell-specific factor, requires calcium signal pathway, and its activity is sensitive to cyclosporin A (CsA), which blocks the calcium-dependent pathway by inhibiting the protein phosphatase calcineurin (22,25). Rincon and Flavell (26) have shown that while protein kinase C activation was sufficient to induce AP-1 DNA-binding activity in primary T lymphocytes, an additional intracellular calcium increase was required to induce transcriptional activity of AP-1 and this transcriptional activity was cyclosporin sensitive. Paya and co-workers (27) have found that activation of calcineurin could stimulate NF-κB DNA binding activity in monocytic U937 cells. Therefore we studied next the role of calcineurin in SPP-induced NF-κB activation in U937 cells. As shown in Fig. 4, NF-κB activation induced by SPP was markedly inhibited by CsA, an immunosuppressive drug which blocks calcium dependent signaling by inhibiting the Ca^{2+}/calmodulin-dependent phosphatase. This observation suggests the involvement of the phosphatase calcineurin in the SPP-induced NF-κB activation. In contrast, CsA was unable to inhibit TNF-induced NF-κB activation (Fig. 4), which is known to involve Ca^{2+}-independent pathways (28,29).

Recently, it has been proposed that activation of NF-κB is regulated by the formation of reactive oxygen intermediates that may be utilized as second messenger molecules integrating the diverse variety of NF-κB inducing agents in the final pathway. It has been demonstrated that NF-κB was activated after exposure to hydrogen peroxide, and that activation in response to diverse stimuli such as cytokines, LPS and phorbol esters was commonly inhibited by antioxidants (10,30). Consistent with these findings, Pahl and Baeuerle (17) have shown that TG-induced NF-κB activation was inhibited by the antioxidant PDTS. Antioxidants such as hydroxyanisole (BHA) have been described by us and others as potent inhibitor of NF-κB activation (12,28). We examined whether BHA, which acts as a radical scavenger, is capable of blocking SPP-induced NF-κB activation. U937 cells were preincubated with 100 μM of the antioxidant BHA for 1 hr prior to stimulation

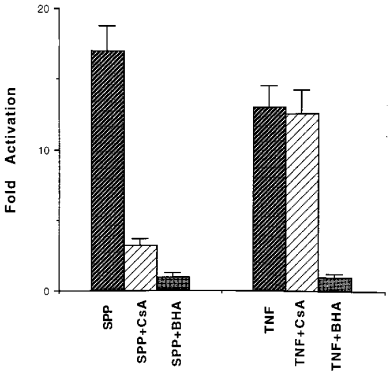

FIG. 4. Effects of calcineurin phosphatase inhibitor (cyclosporin A) and radical scavenger (BHA) on NF-κB activation induced by sphingosine-1-phosphate and TNF. U937 cells were transfected with 2 × NF-κB LUC reporter, and after 12 hr cells were incubated with 5 μM SPP and 10 ng/ml TNF in the absence or presence of inhibitors: 1 μM CsA or 100 μM BHA, as indicated. Luciferase activities were determined as described previously (15,16). The results shown are averages of three separate experiments.

with SPP or TNF. As shown in Fig. 4, BHA led to a marked inhibition of SPP- and TNF-induced NF-κB activation in U937 cells. BHA alone did not play any significant effect on NF-κB activation (data not shown). Our observation that SPP-induced activation of NF-κB was inhibited by the antioxidant BHA further indicates the involvement of redox-dependent processes.

Similarly, TG-induced NF-κB activation was strongly inhibited by CsA and BHA (data not shown).

Several reports have indicated that NF-κB may play an essential role in preventing TNF-induced cell death (31,32,33). Recent paper of Spiegel and co-workers (34) demonstrated that SPP was able to suppress ceramide- and TNF-induced apoptosis in U937 cells. SPP has been shown to counteract the ceramide- and TNF-induced activation of stress-activated protein kinase (SAPK/JNK), which is known to play a crucial role in ceramide-mediated apoptosis. Based on these findings and on data presented here it is tempting to speculate that SPP-induced NF-κB activation may contribute to the suppression of TNF- and ceramide-mediated apoptosis by SPP.

In conclusion, our results suggest that the release of calcium from the internal stores in response to SPP is required for SPP-induced NF-κB activation which most likely involve colcineurin- and redox-dependent mechanisms.

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