Sphingosine 1-Phosphate Inhibits Activation of Caspases that Cleave Poly(ADP-ribose) Polymerase and Lamins during Fas- and Ceramide-mediated Apoptosis in Jurkat T Lymphocytes*

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Physiologic cell death occurs through an evolutionary conserved suicide process, termed apoptosis, which plays a considerable role in early development and homeostasis of adult tissues (1). Ceramide has recently emerged as a critical component of apoptosis (2, 3). A variety of stress stimuli, such as tumor necrosis factor α (TNFα),1 Fas ligand, growth factor withdrawal, anticancer drugs, oxidative stress, heat shock, ionizing radiation, and ultraviolet light increase cellular ceramide which, in turn, is capable of inducing apoptosis (3, 4). This apoptotic effect is blocked by addition of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) or diacylglycerol, both activators of protein kinase C (PKC) (2, 5), suggesting that PKC activation counteracts ceramide-mediated apoptosis. Activation of PKC in diverse cell types stimulates sphingosine kinase activity resulting in intracellular accumulation of sphingosine 1-phosphate (SPP) (6, 7). Recently, we showed that SPP prevented the hallmarks of apoptosis resulting from elevated levels of ceramide induced by TNFα, anti-Fas antibody, sphingomyelinase, or cell-permeable ceramide (7). Furthermore, inhibition of PKC, as well as inhibition of sphingosine kinase, induces apoptosis, which can be overcome by the addition of SPP (7). These results indicate that PKC may inhibit ceramide-induced apoptosis by activating sphingosine kinase.

Recently, attention has been focused on the role of a novel family of aspartate-specific cysteine proteases, called caspases, which are intimately associated with apoptosis (reviewed in Ref. 8). Genetic studies of the nematode Caenorhabditis elegans have led to the identification of two genes, ced-3 and ced-4, that are required for apoptotic cell death (9). The ced-3 gene encodes a caspase similar to the prototype mammalian interleukin 1β-converting enzyme (ICE) (9). Ten other homologs of ICE/CED-3 have been identified and phylogenetically classified into three subfamilies: (i) the CED-3 subfamily, consisting of caspase-3 (CPP32/Yama/apopain), caspase-6 (Mch2), caspase-7 (Mch3/ICE-LAP6/Mch6), caspase-8 (FLICE/MACH/Mch5), caspase-9 (ICE-LAP6/Mch6), and caspase-10 (Mch4); (ii) the ICE subfamily, which includes caspase-1 (ICE); caspase-4 (TX/ICH2/ICE rel-I), caspase-5 (TY/ICE rel-III), and caspase-11 (ICh3); and (iii) the Nedd-2 subfamily, which is represented by caspase-2 (IChl/murine Nedd-2).

Previous studies have identified a number of substrates for the caspases, particularly the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (10) and the nuclear lamins (11). Proteolysis of these substrates may account for many of the biochemical and morphological nuclear changes associated with apoptosis. The early cleavage of PARP can be catalyzed by several caspases belonging to the CED-3 subfamily (10, 12–14). Of these, caspase-3/CPP32 is thought to be the most efficient PARP protease (10, 12), followed by caspase-7/Mch3 (13). The degradation of nuclear lamins is required for packaging of the condensed chromatin into apoptotic bodies, a typical morphology observed during the late stages of apoptosis (11). Caspase-6/Mch2 is the only known caspase capable of cleaving lamins (14, 15).

Although the relative intracellular levels of ceramide and SPP have been proposed to be a critical gauge of cell fate (7), the molecular mechanism of actions of these sphingolipid me-
tabolites are not well understood. Recently, ceramide has been implicated in PARP cleavage (16, 17) and caspase-3/CPP32 activation (18). In addition, it appears that ceramide can be generated via a CrmA-inhibitable caspase, most likely caspase-8/FLICE, since overexpression of CrmA blocks both cell death and ceramide accumulation induced by TNFa, while exogenous ceramide can bypass this sensitive step and induce apoptosis (16).

In this study, the relationship between sphingolipid metabolites and the caspasases was examined. We demonstrated that both SPP and TPA decrease PARP cleavage by inhibiting activation of caspase-3/CPP32 and caspase-7/Mch3 induced by Fas ligation or cell-permeable ceramide. Furthermore, we also report that SPP and TPA inhibit activation of caspase-6/Mch2 and subsequent lamin B cleavage. Finally, ceramide, in contrast to Fas ligation, did not induce activation of caspase-8/FLICE and neither SPP nor TPA were able to prevent this activation.

EXPERIMENTAL PROCEDURES

Materials—Anti-Fas monoclonal antibody (clone CH-11) was from Upstate Biotechnology (Lake Placid, NY). C6-ceramide and TPA were from Biomol (Plymouth Meeting, PA). Ac-DEVD-AMC was from Bachem (King of Prussia, PA). [methyl-3H]Thymidine (70–90 Ci/mmol) was from NEN Life Science Products. Peroxidase-conjugated anti-goat IgG (Mch2) was from Boehringer Mannheim. Anti-mouse (lamin B1) and anti-rabbit (CPP32, Mch3 and FLICE) IgG conjugated to peroxidase was from Bio-Rad.

Cell Culture—Jurkat T cells (clone E6–1, ATCC, Rockville, MD) were grown in RPMI 1640 supplemented with 10% fetal bovine serum. On the day of the experiment, the cells were washed twice in RPMI 1640 containing 5 μg/ml transferrin and 5 μg/ml insulin in place of serum and resuspended in this serum-free medium (0.75–1 × 10^6 cells/ml).

DNA Fragmentation Assay and Staining of Apoptotic Nuclei—DNA fragmentation was measured using a full-length cDNA clone for PARP (gift of Dr. Donald Nicholson) that was used to drive the synthesis of PARP labeled with [35S]methionine (NEN Life Science Products) by coupled T7 transcription/translation in a reticulocyte lysate system (Promega, Madison, WI). [35S]PARP was purified by gel filtration chromatography on a Superdex-75 FPLC column (Pharmacia Biotech Inc.) using 10 mM HEPES-KOH (pH 7.4) buffer containing 10 mM EDTA, 0.2% (v/v) Triton X-100 and incubated for 15 min on ice. Fragmented DNA and intact chromatin were separated by centrifugation at 12,000 × g for 10 min. Pellets were resuspended in TTE, and trichloroacetic acid was added to a final concentration of 12.5% (w/v). DNA fragmentation was calculated as follows: percent DNA fragmentation = (fragmented/fragmented + intact chromatin) × 100. All results were determined in triplicate and expressed as means ± standard deviations.

Apoptosis was also assessed by staining cells with bisbenzimide trihydrochloride (8 μg/ml in 30% glycerol/PBS; Hoechst 33258, Calbiochem, La Jolla, CA) for 10 min on ice. Cells were then examined with a Zeiss Photoscope II fluorescent microscope (Petersburg, VA).

Cleavage of in Vitro Translated Poly(ADP)-ribose Polymerase—A full-length cDNA clone for PARP (gift of Dr. Donald Nicholson) was used to drive the synthesis of the PARP labeled with [35S]methionine by coupled T7 transcription/translation in a reticulocyte lysate system (Promega, Madison, WI). [35S]PARP was purified by gel filtration chromatography on a Superdex-75 FPLC column (Pharmacia Biotech Inc.) using 10 mM HEPES-KOH (pH 7.4) buffer containing 10 mM EDTA, 0.1% (w/v) CHAPS, and 5 mM dithiothreitol. DNA fragmentation was measured by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. DNA bands were quantitated by densitometry.

RESULTS

Sphingosine 1-Phosphate and TPA Inhibit Fas- and C2-Ceramide-Mediated Apoptosis—As described previously (7, 18, 20), Jurkat cells treated with Fas antibody underwent extensive cell death within 3 h as measured by the quantitative DNA fragmentation assay (Fig. 1A). Blebbing of cell membrane was observed and cells were fragmented into characteristic condensed nuclei and apoptotic bodies (Fig. 1C). In agreement with our previous study (7), when cells were treated simultaneously with TPA, DNA fragmentation was completely inhibited, while co-treatment with SPP reversed apoptosis by ~50% (Fig. 1, A, D, and E). Ceramide has been proposed to play a role in the Fas signaling pathway (20, 21). Treatment of Jurkat T cells with the cell-permeable C2-ceramide (10 μM) resulted in cell death that was overcome either by co-treatment with TPA or to a lesser extent by SPP (Fig. 1F). Stimulation of sphingosine kinase leading to intracellular accumulation of SPP has been shown to be one of the effects triggered by activation of PKC (6, 7). Moreover, activation of PKC by phorbol esters limits ceramide production (7, 22). In agreement, the PKC inhibitors, staurosporine, chelerythrine chloride, and calphostin C, induce sphingomyelin hydrolysis to generate ceramide (23, 24). These studies suggest that PKC activation may oppose the Fas apoptotic pathway, both by activation of sphingosine kinase and by reducing the levels of ceramide.

Sphingosine 1-Phosphate and TPA Inhibit Activation of PARP Cleavage Induced by Fas Ligation or Ceramide—Because Fas ligation leads to proteolytic cleavage of PARP (12, 25–27), it was of interest to determine whether SPP and TPA could inhibit this cleavage. In agreement with previous results (25–27), extracts prepared from cells exposed to anti-Fas for 3 h were able to cleave an in vitro translated [35S]PARP substrate into the characteristic 89-kDa fragment (Fig. 2A). Densitometric analysis revealed that almost 70% of the 116-kDa full-length PARP was converted to the 89-kDa fragment, while only 38% was cleaved by extracts from control cells. SPP treatment reduced cleavage of PARP induced by Fas ligation by over 50% and as expected, treatment with TPA completely prevented Fas-induced PARP proteolysis. Ceramide generation has recently been shown to precede PARP cleavage in TNFa-mediated apoptosis (16). In addition, co-treatment of Molt-4 or MCF-7 cells with exogenous C2-ceramide results in cleavage of PARP (16, 17), suggesting that ceramide activates a caspase responsible for PARP cleavage. In accordance with these studies (16, 17),
we found that C2-ceramide also induced PARP cleavage (Fig. 2B) and co-treatment with TPA fully prevented this cleavage (Fig. 2B). These results clearly indicate that SPP can inhibit PARP cleavage induced either by Fas ligation or exogenous ceramide.

Sphingosine 1-Phosphate and TPA Prevent Activation of Caspase-3-like Proteases

Caspase-3 is known as the most efficient PARP-cleaving caspase, with a $K_m$ of $-10 \mu M$ (10, 12). Caspase-3 is synthesized as a 32-kDa precursor (p32) that is cleaved to generate the mature form composed of 17-kDa (p17) subunit, through an intermediary 20-kDa (p20) form, and 12-kDa (p12) subunit (10, 12, 28). In agreement with previous studies (25, 27), following Fas activation, the p32 precursor was cleaved into active caspase-3 (Fig. 3B). Caspase-3 maturation was completely blocked by TPA, as active subunit was not detected in cells treated with both anti-Fas and TPA. SPP also prevented Fas-mediated caspase-3/CPP32 processing by over 60% as measured by densitometry (Fig. 3B).

A similar approach was used to assess the protective effects of SPP and TPA on exogenous C2-ceramide-mediated caspase-3/CPP32 activation. In accordance with a previous study (18), extracts from cells treated with C2-ceramide displayed an increase in caspase-3-like activity (Fig. 4A), corresponding with the appearance of the active p20 and p17 subunits of caspase-3 (Fig. 4B). TPA effectively blocked both the caspase-3-like activity increase (Fig. 4A) and the processing of caspase-3/CPP32 into p20 and p17, triggered by ceramide (Fig. 4B). Cytosolic extracts from cells co-treated with SPP displayed significantly decreased CPP32-like activity (Fig. 4A). Western blotting also demonstrated the same strong inhibition (60–90%) of p20 and p17 expression (Fig. 4B).

Caspase-7/Mch3, which is also capable of cleaving PARP (13), is expressed as a 35-kDa precursor, and upon activation is processed into 20-kDa (p20) and 12-kDa (p12) subunits (25, 29). Fas ligation leads to the generation of the p20 and p12 subunits (25, 29). Thus, it was of interest to examine whether ceramide was also capable of inducing activation of Mch3 and if SPP could affect this processing. As expected, Fas induced the ap-
pearance of the activated p20 form, which was completely abolished by pretreatment with TPA and to a lesser extent with SPP (Fig. 5A). Similarly, processing of Mch3 to the p20 form induced by ceramide was markedly decreased by co-treatment with TPA, and SPP diminished C2-ceramide-induced Mch3 processing by over 50%. Thus, SPP is able to counteract proteolytic cleavage of caspase-3 and caspase-7, and subsequent cleavage of PARP triggered by Fas or exogenous ceramide.

**SPP and TPA inhibit FAS- and Ceramide-mediated Caspase-6/Mch2 Activation and Subsequent Lamin B Degradation**—In addition to the breakdown of the nuclear enzyme PARP, another event that is common to apoptosis is the cleavage of lamins, which play major roles in nuclear envelope integrity (11). To date, caspase-6/Mch2 is the only known laminase (14, 15). Engagement of Fas in Jurkat T cells has been shown to activate caspase-6 (14) and consequently trigger the cleavage of lamin B (26). Thus, we determined whether ceramide treatment could also induce caspase-6 activation and lamin B cleavage. Lamin B is cleaved into a characteristic 28-kDa fragment after treatment with Fas or exogenous C2-ceramide (Fig. 6, A and B). Western blotting of extracts from cells co-treated with TPA and Fas antibody (Fig. 6A) or C2-ceramide (Fig. 6B) displayed only intact lamin B, indicative of inhibition of cleavage. When cells were treated with SPP during Fas- (Fig. 6A) or C2-ceramide-mediated apoptosis (Fig. 6B), lamin B proteolysis was markedly reduced. We next determined if caspase-6 was protected by SPP and TPA from activation caused by Fas- and C2-ceramide treatment. In accordance with previous studies (14), Fas antibody was able to trigger caspase-6 cleavage, generating 21-, 18-, and 14-kDa fragments (Fig. 7A). Treatment with C2-ceramide similarly led to processing of caspase-6 (Fig. 7B). When cells were co-treated with TPA or SPP, expression of active forms of caspase-6, especially p18 and p14, was strongly reduced (Fig. 7, A and B). Therefore, SPP, like TPA, can attenuate proteolytic cleavage of caspase-6 and its target, nuclear lamins, during Fas- or C2-ceramide-induced apoptosis.

**Fas-induced Caspase-8/FLICE Activation Is Not Inhibited by SPP or TPA**—Apoptosis and ceramide accumulation induced by TNF-α or Fas are completely inhibited by CrmA, a product of the cowpox virus, while exogenous ceramide is able to bypass this block and induce apoptosis by activating downstream caspases (16). CrmA is a potent inhibitor of ICE (30). However, the importance of caspase-1/ICE itself in Fas-mediated apoptosis is controversial (Refs. 31 and 32 versus Refs. 27 and 33). Instead, caspase-8/FLICE, the most upstream caspase implicated in Fas- and TNFα-mediated apoptosis, appears to be the target of CrmA in vivo (34). Thus, it was of interest to determine the involvement of sphingolipid metabolites in activation of caspase-8. Fas-ligation induced the appearance of a p20 band indicative of cleavage and corresponding to the mature form of FLICE (Fig. 8). In contrast, C2-ceramide treatment did not induce cleavage of caspase-8 (Fig. 8). This is in agreement with
the concept that ceramide acts downstream of a CrmA-inhibitable caspase. Finally, neither SPP nor TPA have an inhibitory effect on Fas-triggered caspase-8/FLICE cleavage (Fig. 8).

**FIG. 5.** Fas antibody- and C$_2$-ceramide-induced proteolytic cleavage of pro-caspase-7/Mch3 is inhibited by SPP and TPA. Extracts prepared from Jurkat cells treated for 3 h in serum-free conditions with 50 ng/ml Fas antibody (A) or 10 µM C$_2$-ceramide (B), in the absence or the presence of 50 nM TPA or the indicated concentrations of SPP, were resolved by SDS-PAGE and probed with anti-caspase-7/Mch3 p20 antibody. The arrow indicates the mobility of mature p20 form of caspase-7/Mch3.

**FIG. 6.** Lamin B cleavage induced by Fas antibody- or C$_2$-ceramide is inhibited by SPP and TPA. Jurkat cells were treated for 3 h in serum-free conditions with 50 ng/ml Fas antibody (A) or 10 µM C$_2$-ceramide (B), in the absence or presence of 50 nM TPA or the indicated concentrations of SPP. Cytosolic extracts were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-lamin B1 antibody. Mobilities of intact and cleaved forms are indicated by arrows.

**FIG. 7.** Cleavage of Mch2/caspase-6 proenzyme induced by Fas antibody or C$_2$-ceramide is inhibited by SPP and TPA. Cell extracts from Jurkat cells treated for 3 h in serum-free conditions with 50 ng/ml Fas antibody (A) or 10 µM C$_2$-ceramide (B) in the absence or presence of 50 nM TPA or the indicated concentrations of SPP, were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with goat antiserum specific for the p21 subunit of caspase-6/Mch3. The arrows indicate the mobilities of the processed mature forms (p21, p18, and p14).

**FIG. 8.** Fas-induced proteolytic cleavage of pro-caspase-8/FLICE is not induced by ceramide nor prevented by SPP or TPA. Extracts from Jurkat cells treated for 3 h in serum-free conditions with 50 ng/ml Fas, in the absence or presence of 50 nM TPA or the indicated concentrations of SPP, or 10 µM C$_2$-ceramide, were analyzed by SDS-PAGE with anti-caspase-8/FLICE p20 antibody. The arrow represents the mobility of the p20 fragmented mature form. Similar results were observed in three independent experiments.

**DISCUSSION**

Within the last few years, new studies have been reported, which have enhanced our understanding of how cell surface events are communicated to the cell suicide machinery (21, 35, 36). Protein-protein interactions link death domains of cell surface receptors for TNF-α (TNF receptor-1) or Fas ligand (CD95/Fas/APO-1) to a cascade of ICE/CED-3-homologous pro-
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Fig. 9. Scheme illustrating the relationship between the proteolytic cascade and SPP in Fas-mediated apoptosis. Solid lines indicate established pathways, and dotted lines indicate incomplete or still not well defined pathways. See “Discussion” for more details.

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