ARTICLE

Protein Phosphatase 1-Dependent Dephosphorylation of Akt Is the Prime Signaling Event in Sphingosine-Induced Apoptosis in Jurkat Cells

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ABSTRACT

Sphingosine (SPH) is an important bioactive lipid involved in mediating a variety of cell functions including apoptosis. However, the signaling mechanism of SPH-induced apoptosis remains unclear. We have investigated whether SPH inhibits survival signaling in cells by inhibiting Akt kinase activity. This study demonstrates that treatment of Jurkat cells with SPH leads to Akt dephosphorylation as early as 15 min, and the cells undergo apoptosis after 6 h. This Akt dephosphorylation is not mediated through deactivation of upstream kinases, since SPH does not inhibit the upstream phosphoinositide-dependent kinase 1 (PDK1) phosphorylation. Rather, sensitivity to the Ser/Thr protein phosphatase inhibitors (calyculin A, phosphatidic acid, tautomycin, and okadaic acid) indicates an important role for protein phosphatase 1 (PP1) in this process. In vitro phosphatase assay, using Akt immunoprecipitate following treatment with SPH, reveals an increase in Akt-PP1 association as determined by immunoprecipitation analysis. Moreover, SPH-induced dephosphorylation of Akt at Ser^{473} subsequently leads to the activation of GSK-3β, caspase 3, PARP cleavage, and ultimately apoptosis. Pre-treatment with caspase 3 inhibitor z-VAD-*fmk* and Ser/Thr phosphatase inhibitor abrogates the effect of SPH on facilitating apoptosis. Altogether, these results demonstrate that PP1-mediated inhibition of the key anti-apoptotic protein, Akt, plays an important role in SPH-mediated apoptosis in Jurkat cells. J. Cell. Biochem. 112: 1138–1153, 2011.   © 2011 Wiley-Liss, Inc.

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Programmed cell death or apoptosis plays an important role in pathophysiological conditions such as: differentiation, control of cell number and removal of damaged cells, autoimmune and neurodegenerative diseases, and cancer [Ellis et al., 1991; Bauer et al., 1995]. Apoptosis is characterized at a cellular level by cell shrinkage, cytoplasmic/nuclear and chromatin condensation, membrane blebbing, protein fragmentation, DNA degradation, finally leading to the breakdown of the cell into smaller units or apoptotic bodies [Thompson, 1995]. Fundamental to these cellular events are caspase activity. Caspases, a family of cysteine-dependent aspartate-directed proteases, play a critical role in the initiation and execution of apoptosis. Among this family of caspases, caspase 3, in particular, is believed to be the ultimate caspase in the execution of apoptosis in various cell types [Izban et al., 1999].

A number of previous studies have suggested that sphingolipids, such as ceramide (Cer) and sphingosine (SPH), are key regulators of apoptosis, cell-proliferation, and differentiation [Jarvis et al.,

1996; Phillips et al., 2007; Yoon et al., 2009]. Indeed, it has been previously reported that SPH is rapidly generated during apoptosis mediated by TNF-α, FAS (CD95) receptor ligation, irradiation, or doxorubicin treatment [Ohta et al., 1994; Cuvillier et al., 2000, 2001; Nava et al., 2000]. However, the mechanism of action of SPH is still not clear. At the biochemical level, SPH has been shown to be a potent inhibitor of protein kinase C (PKC) [Hannun et al., 1986], calmodulin-dependent kinase [Jefferson and Schulman, 1988], and insulin receptor tyrosine kinase [Arnold and Newton, 1991]. Furthermore, SPH has been shown to stimulate other protein kinases such as JNK and p38 [Cuvillier, 2002]. Recently, it has been reported that p38 mitogen-activated protein kinase activation is critical for SPH-induced cell death [Yoon et al., 2009]. It has been reported that SPH down-regulates the expression of the anti-apoptotic protein Bcl-2 [Cuvillier, 2002]. Importantly, SPH activates various caspases and stimulates poly (ADP-ribose) polymerase (PARP) cleavage, a well-known target for caspases [Hung et al., 1999]. Moreover, caspase inhibitors have been shown to block SPH-induced apoptosis in HL-60 cells [Sweeney et al., 1998], Jurkat cells [Cuvillier et al., 2000], rhabidomyosarcoma cells [Phillips et al., 2007], and Hep3B hepatoma cells [Hung et al., 1999]. In contrast to the proposed apoptotic role of SPH, in some studies, SPH has been reported to actually induce cell proliferation, an effect that may be due to its rapid conversion to sphingosine-1-phosphate by the action of sphingosine kinase [Olivera and Spiegel, 1993].

Protein kinase B (PKB), commonly referred to as Akt, is a member of the family of phosphatidylinositol 3-OH-kinase regulated Ser/Thr kinases (Akt 1, 2, 3) [Franke et al., 1995]. Full activation of Akt requires phosphorylation at Ser[473] and Thr[308] by phosphoinositide-dependent kinase 1 (PDK1). Once activated, Akt regulates a wide range of target proteins such as Bad [del Peso et al., 1997], caspase-9 [Cardone et al., 1998], glycogen synthase kinase (GSK)-3β [Cross et al., 1995], and XIAP [Dan et al., 2004], which regulate cell survival, proliferation, and growth. In contrast to the activation of Akt associated with its phosphorylation by PDK1, inactivation of Akt is still not fully understood. Recently, Akt has been shown to be dephosphorylated at both Ser[473] and Thr[308] and inactivated by Ser/Thr phosphatases [Xu et al., 2003]. The Ser/Thr phosphatases are usually classified as type 1 (PP1) or type 2 (PP2A), depending on their substrate specificity and sensitivity to inhibitors.

Protein phosphatase 1 (PP1) is a major eukaryotic Ser/Thr phosphatase that regulates diverse cellular processes such as cell cycle progression, proliferation, protein synthesis, muscle contraction, carbohydrate metabolism, transcription, and neuronal signaling [Shenolikar, 1994]. Recently, PP1 has been shown to be involved in the regulation of apoptosis by affecting the phosphorylation of Bad protein [Ayllon et al., 2000]. Moreover, PP1 plays a key role in the mitotic transition by dephosphorylating proteins that are essential in these cellular functions [Axton et al., 1990]. During the cell cycle PP1 activity is regulated by phosphorylation [Liu et al., 1999]. It has been shown that phosphorylation of PP1-α at Thr[320] by cyclin-dependent kinase blocked the enzymatic activity of PP1-α [Dohadwala et al., 1994]. PP1 has also been reported as a target for natural ceramides in vitro [Chalfant et al., 1999].

In this study, we demonstrate for the first time that SPH induces PP1-dependent rapid Akt dephosphorylation at Ser[473]. This Akt dephosphorylation leads to the activation of caspase 3 and cleavage of PARP, finally leading to apoptosis. Additionally, inhibition of PP1 activity with calyculin A and phosphatidic acid (PA) protected PP1-dependent dephosphorylation of Akt and apoptotic signaling pathways in Jurkat cells.

MATERIALS AND METHODS

MATERIALS

D-erythro sphingosine, L-erythro-sphingosine, L-threo-sphingosine, and D-erythro-C_6-Cer were from Avanti Polar Lipid. RPMI 1640+ GlutaMAX medium and fetal bovine serum (FBS) were obtained from GIBCO BRL (Grand Island, NY). Akt kinase assay kit was purchased from Cell Signaling Technologies (Beverly, MA). CaspASE Assay System Colorimetric and ProFluor Ser/Thr fluorometric PPase Assay system were purchased from Promega (Madison). WST-8 cell counting kit, fumonisin B1 and z-VAD-fmk were from Alexis. Annexin V-FITC apoptosis detection kit was from BD Pharmingen (San Diego, CA). Calyculin A and okadaic acid (OA) were from Calbiochem (La Jolla, CA). Low-melting agarose were from BRL, Life Technology (MD). Egg yolk PA and other fine chemicals were purchased from Sigma Chemicals Co (St. Louis, MO).

ANTIBODIES

Anti-actin, anti-PP1, anti-GSK3$\alpha\beta$, and donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PARP, anti-Akt, anti-phospho Akt (Ser[473]), anti-phospho Akt (Thr[308]), anti-phospho GSK3β (Ser[9]), anti-phospho GSK3β (Ser[9/21]), anti-PDK1, and anti-phospho PDK1 (Ser[241]) were from Cell Signaling Technology. Anti-rabbit IgG and anti-mouse IgG were purchased from Sigma Chemicals Co.

CELL CULTURE AND SPHINGOSINE TREATMENT

Jurkat (Acute lymphocytic T-cells leukemia) (ATCC) were grown in RPMI 1640 containing GlutaMAX medium supplemented with 10% (v/v) heat-inactivated FBS without antibiotics in the humidified atmosphere of 95% air and 5% CO_2 at 37°C. Sphingosine was dissolved in ethanol at a concentration of 10 mM and was stored in a dark colored bottle at -20°C. The stock was diluted to the required concentration with ethanol when needed. Prior to SPH treatment, cells were grown about 60–70% confluence, and then exposed to SPH at different concentrations (0–10 μM) and for a different period of time (0–6 h). Cells grown in a medium containing an equivalent amount of ethanol without SPH served as control.

Calyculin A and OA were dissolved in DMSO made up as concentrated stocks of 10 and 20 μM, respectively. PA was prepared and delivered to the cells as previously reported [Kishikawa et al., 1999].

CYTOTOXICITY ASSAYS

Jurkat cells were incubated with SPH at concentrations ranging from 0 to 8 μM for 6 h, and the number of viable cells was evaluated with WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay. WST-8 is converted to reduced yellow formazan by dehydrogenase activities in viable cells. According to manufacturer's instruction,

WST-8 solution was added at 1:100 in culture medium. After 2 h incubation, the light absorbance (test wavelength OD 450 nm, reference wavelength 655 nm) was measured using Anthos Labtec HT2 96-well plate reader. The cytotoxicity was expressed as percentage over control.

COMET ASSAY

Jurkat cells were seeded in 60 mM dishes. After 24 h, cells were treated with different concentrations of SPH for 6 h. Cells were washed with PBS and pelleted at $500g$ for 2 min at 4°C. Then the cells were resuspended in 1% low-melting agarose in PBS pre-warmed to 37°C. An aliquot of 50 µl of cells suspension was laid on the agarose pre-coated slide. Quickly, the drop was covered with a coverslip and left at 4°C for 10 min. Then the coverslip was removed, and the cells were lysed by immersion of the slide in pre-cooled, freshly prepared lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris–HCl, pH 7.5, 1% Triton X-100) for 1 h. After lysis, nuclei were submitted to alkaline pre-treatment in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA pre-cooled at 4°C) for 40 min and electrophoresed for 15 min at 25 V. The samples were neutralized by three washes of 5 min with 0.4 M Tris, pH 7.5. The nuclei were stained by immersion in a solution containing 50 µg/ml ethidium bromide in PBS for 5 min and gently washed three times with PBS, and the slides were dried. The analysis was performed on an Olympus DP71 fluorescent microscope using standard UV filter.

APOPTOSIS ASSAY

For detection of apoptosis, after SPH treatment, cells were washed twice with ice cold PBS and stained simultaneously with FITC-conjugated Annexin V and propidium iodide (PI) according to the manufacturer's instructions (BD PharMingen) for 20 min at room temperature in the dark with a binding buffer containing 10 mM HEPES-NaOH, 140 mM NaCl, and 2.5 mM $CaCl_2$. Within the next hour, cells were analyzed for apoptosis. Total numbers of apoptotic cells were determined by calculation of Annexin V^+ and PI^- cells (reflecting early apoptosis) together with Annexin V^+ and PI^+ cells (reflecting late apoptosis/secondary necrosis). Analysis of data was performed using the CellQuest (BD Biosciences) acquisition software.

IN VITRO AKT KINASE ASSAY

The kinase activity of immunoprecipitated Akt protein from SPH-treated or -untreated Jurkat cells was determined using the Akt kinase assay kit. Briefly, Jurkat cells in a 10-cm plate, treated with 8 µM SPH or the same volume of vehicle (ethanol) for 0–6 h, were washed with cold PBS and lysed with 300 µl of lysis buffer. Akt protein was immunoprecipitated, and its kinase activity was assayed using GSK-3 as the substrate, according to the manufacturer's protocol, with the exception that the reaction tubes contained either 8 µM SPH or the same volume of ethanol and incubated at 30°C for 1 h. The reaction was stopped by adding 6× SDS-sample buffer and heating at 100°C for 5 min, and the samples were then run on a SDS–PAGE and transferred onto nitrocellulose membrane and then probed with pGSK3αβSer[9/21].

ENZYMATIC CASPASE 3 ASSAY

The enzymatic assay of caspase induced by SPH was measured by using the manufacture's protocol (Promega). Briefly, cells were lysed in a lysis buffer by freeze and thawing. The lysed cells were centrifuged at 14,000 rpm for 15 min. Fifty micrograms of protein was incubated with 30 µl of caspase assay buffer and 2 µl of caspase 3 (DEVD-pNA) colorimetric substrate at 37°C for 4 h. The optical density of the reaction mixture was quantitated by spectrophotometrically at a wavelength of 405 nm by using Athos Labtec HT2 96-well plate reader.

MEASUREMENT OF PARP CLEAVAGE

To determine the activation of caspase 3, we examined the cleavage of PARP as previously reported [Thayyullathil et al., 2008]. Thirty micrograms of whole cell extract were resolved on 10% polyacrylamide gel, transferred to nitrocellulose membrane, blocked with 5% non-fat milk protein probed with PARP antibodies (1:2,000), followed by secondary antibody and protein were detected by using enhanced chemiluminescence reagent (Pierce Biotech).

IMMUNOPRECIPITATION OF AKT AND PP1

After the SPH treatment, Jurkat cells were lysed in immunoprecipitation buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin. Jurkat cell extract was incubated overnight at 4°C with Akt rabbit polyclonal antibody or mouse monoclonal PP1 antibody. Protein A was added, and the incubation was continued for further 3 h at 4°C. Samples were washed four times with immunoprecipitation buffer, separated by SDS–PAGE, and Western blot analysis was performed.

WESTERN BLOTTING

Cells were washed twice with phosphate-buffered saline (PBS) and lysed in a RIPA lysis buffer [50 mM Tris–HCl (pH 7.4), 1% NP-40, 40 mM NaF, 10 mM NaCl, 10 mM Na_3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM dithiothreitol (DTT) and EDTA-free protease inhibitor tablets per 20 ml buffer]. The cell lysates were centrifuged at 14,000 rpm for 15 min. Total protein, determined by Bio-Rad protein assay, were mixed with 6× loading buffer and boiled at 100°C for 3 min samples at 40 µg/lane were resolved by SDS–PAGE and the separated proteins were transferred on to nitrocellulose membrane by wet transfer method using Bio-Rad electro transfer apparatus. Following transfer, the blot were blocked with 5% non-fat milk in tris-buffer saline containing 0.1% Tween-20 were then incubated with primary antibodies followed by secondary antibody. Proteins were visualized using enhanced chemiluminescence system.

IN VITRO AKT DEPHOSPHORYLATION ASSAY

Jurkat cells were lysed in immunoprecipitation buffer. Cell extract was incubated overnight at 4°C with mouse monoclonal PP1 antibody. Protein A was added, and the incubation was continued for further 3 h at 4°C. The pelleted beads were washed three times with immunoprecipitation buffer and twice with PP1 assay buffer

(50 mM Tris (pH 7.5), 0.1 mM EDTA, and 1 mM $MnCl_2$). Then the pellet was resuspended in 50 μl of PP1 assay buffer containing 5 mM DTT, 25 ng of phosphorylated Akt protein (Upstate Biotechnology) with/or without 8 μM SPH or calyculin A. Tubes were incubated 30°C for 1 h with occasional vortexing. The reaction was stopped by the addition of 6× sample buffer and heating 100°C for 3 min. The reaction products were separated by SDS–PAGE, and Western blot analysis was performed.

PROTEIN PHOSPHATASE-1 ASSAY

A fluorometric assay was used to measure PP1 activity. The ProFluor Ser/Thr Phosphatase assay kit was used to measure the activity of PP1. After the SPH treatment, Jurkat cells were lysed in immunoprecipitation lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin. Jurkat cell extract was incubated for overnight at 4°C with Akt rabbit polyclonal antibody. Protein A beads were added and the incubation was continued for further 3 h at 4°C. The immune complexes were washed four times with immunoprecipitation buffer and re-suspended in PP1 assay buffer and performed the assay according to the manufacturer's instruction. Akt immunoprecipitates (25 μl) were mixed with 25 μl of 10 μM phosphorylated bisamide rhodamine-110 peptide substrate, 10 μM AMC substrate, and incubated in a 96-well black plate for 15 min at room temperature. Subsequently, a protease reagent (1 mU/μl of a protease reagent provided with the kit) was mixed with the cell lysates and incubated for 90 min at room temperature. After terminating the reaction, PP1 activity was determined using a fluorescent plate reader (Perkin Elmer spectrofluorometer, Victor 3×) set at wavelengths 485/535 nm to measure the fluorescence of the rhodamine-110 peptide substrate. Thus, the increase in fluorescence intensity is correlated with increased PP1 activity. Protein phosphatase activity was expressed as percentage over control.

INTRACELLULAR CER MEASUREMENT

After SPH treatment, cells were washed in PBS and lysed in 50 mM Tris (pH 7.4) containing 0.4% IGEPAL CA 630 by the freeze and thaw method. The final concentration of IGEPAL CA 630 in the assay was
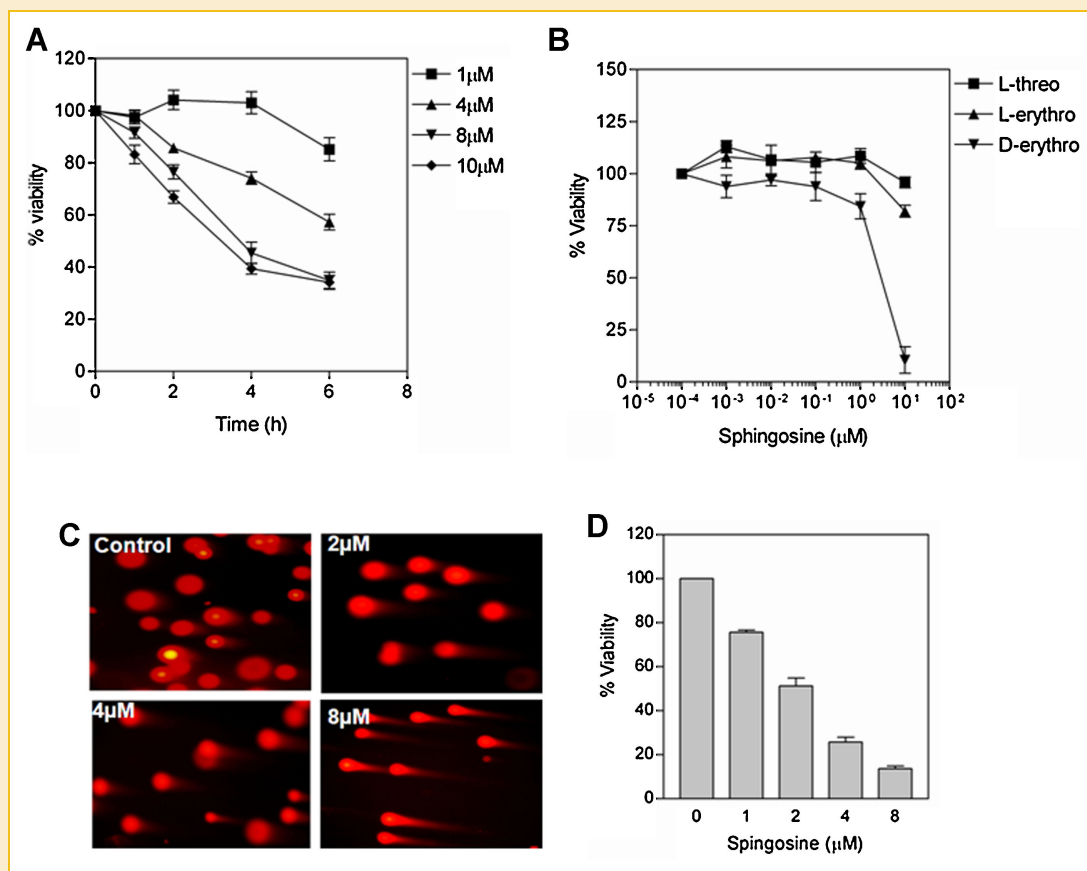

Fig. 1. Effects of SPH on viability and DNA damage of Jurkat cells. A: WST cell viability assay for Jurkat cells following treatment with different concentrations of SPH (0–10 μM) for 0–6 h. B: WST cell viability assay for Jurkat cells following exposed to D-erythro-sphingosine (SPH), L-erythro-sphingosine, or L-threo-sphingosine for 0–6 h. Data shown are means ± SD (n = 3). C: DNA damage induced by SPH. Jurkat cells were treated with the indicated concentrations of SPH for 6 h, embedded in agarose, and electrophoresed according to the comet assay. The length of the comet tail reflects the extent of DNA damage. Plates shown here are representatives of three independent experiments. D: WST cell viability assay for molt-4 cells following treatment with different concentrations of SPH (0–8 μM) for 0–6 h. Data shown are means ± SD (n = 3). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

0.2%. The lysate were then heat at 70°C for 5 min in a water bath, and centrifuged at 12,000 rpm for 10 min at 4°C. The reaction was started by adding 10 μl of supernatant to the tube containing 20 ng recombinant human neutral ceramidase enzyme (10 μl), and incubated for 1 h at 37°C. The reaction was stopped by adding 55 μl of stopping buffer (1:9, 0.07 M potassium hydrogen phosphate buffer/methanol). The released SPH was derivatized with o-phthaladehyde (OPA) reagent. This was achieved by adding 25 μl of freshly prepared OPA reagent (12.5 mg OPA dissolved in 250 μl ethanol and 12.5 μl mercaptoethanol and made up to 12.5 ml with 3% (w/v) boric acid). The mixture was allowed to stand for 30 min. An aliquot of 25 μl was used for the Cer analysis using Waters 1525 binary pump HPLC system. Waters XTerra C18 column (5 μm, 3.9 mm × 150 mm) column was equilibrated with the mobile phase (20% methanol, 80% 1:9, 0.07 M potassium hydrogen phosphate buffer/methanol) at a flow rate of 0.5 ml/min. The fluorescence detector (Waters 2475) was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

RESULTS

SPHINGOSINE TREATMENT SIGNIFICANTLY DECREASES JURKAT CELL VIABILITY

Initially, to investigate the effect of SPH on the viability of Jurkat cells, we treated Jurkat cells with different doses of SPH for various times and analyzed the viability using WST assay (Fig. 1A). Sphingosine caused reduction of Jurkat cell number stereospecifically. Only the naturally occurring stereoisomer, D-erythro-sphingosine (SPH), was biologically active, while exposure of the cells to the other stereoisomers of SPH, L-erythro-sphingosine and L-threo-sphingosine, was not significant (Fig. 1B). Next, the effect of SPH on DNA damage was checked by using the comet assay, a sensitive method for measuring the extent of DNA strand breaks in individual cells. As shown in Figure 1C, SPH treatment induced comet formation dose dependently. These results provide direct molecular evidence for SPH-induced DNA strand breaks in Jurkat cells. In order to establish whether Jurkat cell response to SPH is cell
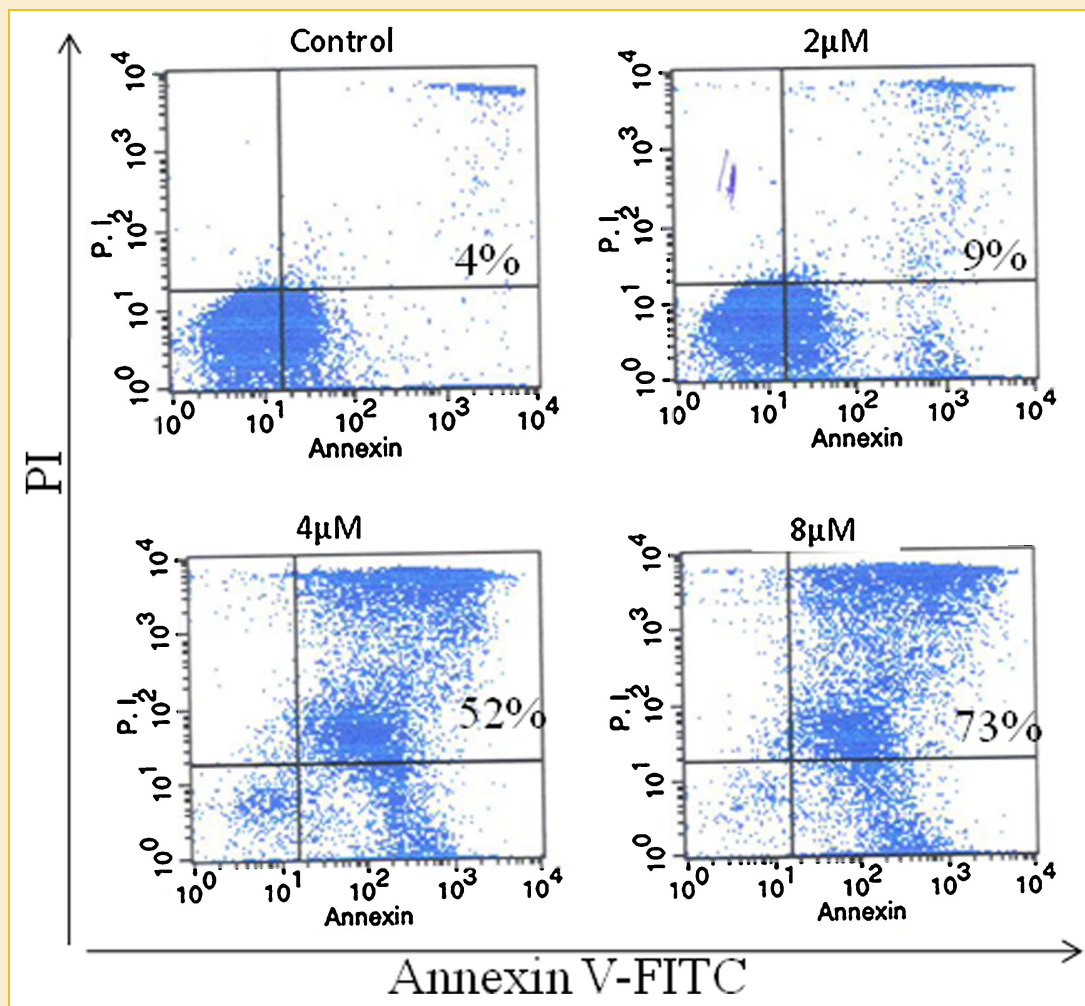

Fig. 2. Treatment of Jurkat cells with SPH leads to increased Annexin V and PI staining in a dose–dependent fashion. Jurkat cells were treated with the indicated concentrations of SPH for 6 h and subjected to apoptosis assay as described in the Materials and Methods Section. Data shown are representative of two independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

specific, the effect of SPH on Molt-4 cells, another human leukemic cells, were examined. Molt-4 cells were exposed to various concentrations of SPH for 6 h and cell viability was checked by WST assay. As with Jurkat cells, exposure of Molt-4 cells to SPH led to a significant decrease in the cell viability (Fig. 1D).

MODE OF SPH–INDUCED CELL DEATH IN JURKAT CELLS

In order to identify the mode of Jurkat cell death (apoptosis or necrosis) following SPH treatment, a combination of PI and Annexin V staining and FACS analysis was used. This procedure distinguishes between cells that are dying from apoptosis, which stain positively for Annexin V (used to detect early apoptotic-associated translocation of phosphatidylserine from the inner to the outer leaflet of cell membrane), and negatively for PI (used to assess plasma membrane integrity); cells already dead from apoptosis, which stain positively for both markers; and cells that are dying by necrosis, which stain positively only for PI. Jurkat cells were treated with different concentrations of SPH for 6 h. The cells were then stained with Annexin V-FITC and PI, and finally analyzed by flow cytometry. As shown in Figure 2, flow cytometric analysis demonstrated that treatment of Jurkat cells with SPH decreased the number of normal cells (Annexin V-FITC$^-$/PI$^-$) dose dependently. The early apoptotic cells (Annexin V-FITC$^+$/PI$^-$) and late apoptotic cells (Annexin V-FITC$^+$/PI$^+$), were increased in a dose-dependent manner confirming the mode of cell death to be apoptotic.
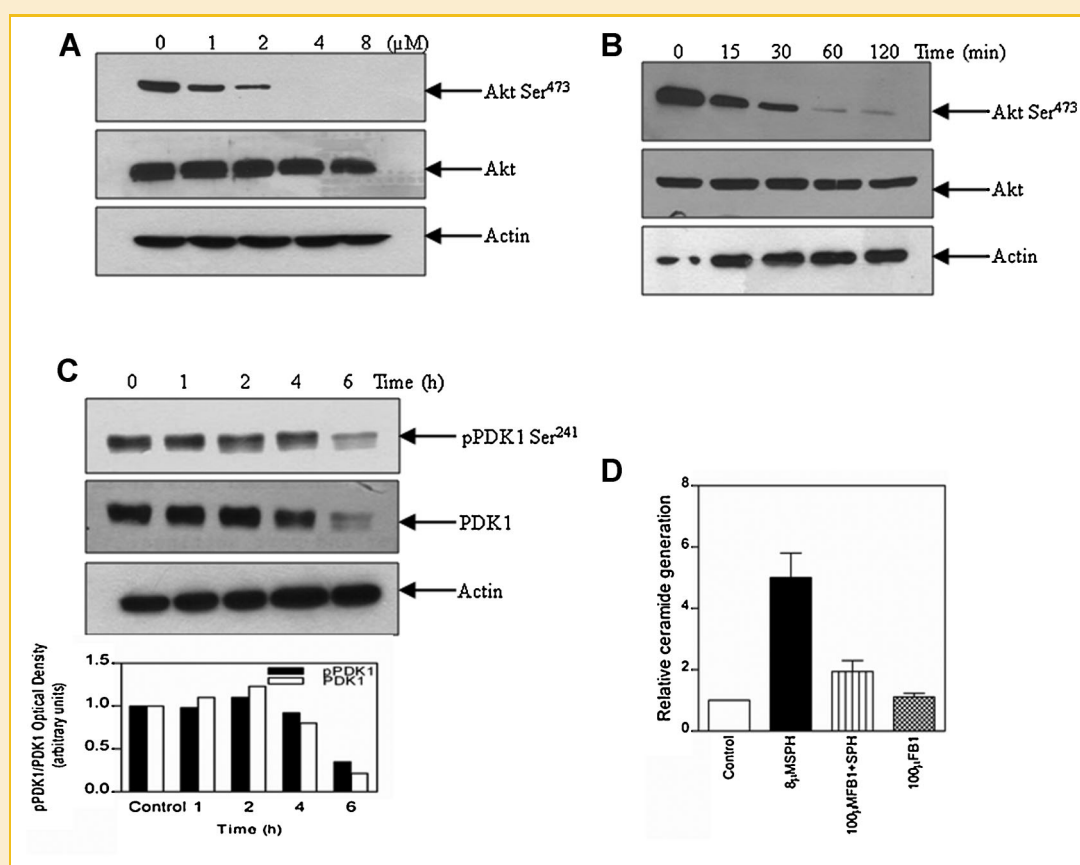

Fig. 3. Sphingosine induces the dephosphorylation of Akt at Ser^{473}. A: Jurkat cells were treated with the indicated concentrations of SPH for 6 h and (B) treatment of Jurkat cells with 8 μM SPH for the indicated time. Cells were lysed as described under the Materials and Methods Section, and were fractionated by SDS–PAGE. Western blot were probed with antibodies specific for phospho-Akt (Ser^{473}) and Akt. Actin was used as a loading control. C: Jurkat cells incubated with 8 μM concentration of SPH for indicated time. Lysed cells were subjected to Western blot analysis by using the antibodies specific for phospho-PDK1 (Ser^{241}) and PDK1. Actin was used as a loading control. Bar graph represents densitometric analysis of both phospho-PDK1 (Ser^{241}) and PDK1 (D) Jurkat cells were pre-treated with FB1 (100 μM) 1 h before SPH treatment for further 3 h and cells were lysed and subjected to ceramide analysis as described in the Materials and Methods Section. E: Jurkat cells were pre-treated with FB1 (100 μM) 1 h before SPH treatment for further 6 h and cells were lysed and subjected Western blot analysis. Actin was used as a loading control. F: Jurkat cells were pre-treated with FB1 (100 μM) 1 h before SPH (8 μM) treatment for further 6 h and cell viability assay were performed by using WST cell viability assay kit. Data shown are means ± SD (n = 3). SPH inhibits Akt kinase activity. G: Jurkat cells were treated with 8 μM SPH for indicated time period. Cells were lysed with lysis buffer. Akt proteins were immunoprecipitated by using bead immobilized with mouse anti-Akt monoclonal antibody. Akt kinase activity was determined by detecting the ability to phosphorylate the substrate protein GSK-3. The phosphorylation of GSK-3 protein was detected by Western blotting with anti-phospho-GSK-3Ser$^{9/21}$ antibody. The density of each band was quantified using Bio-Doc-Analyze and plotted with Prism Graph Pad-3 software. H: Jurkat cells were treated with the indicated concentrations of SPH for 6 h and (I) treatment of Jurkat cells with 8 μM SPH for indicated time, cell lysate were subjected to Western blot and probed with antibodies specific for phospho-GSK-3β (Ser^9) and GSK-3αβ. Actin was used as a loading control. Blots shown here are representative of two independent experiments.
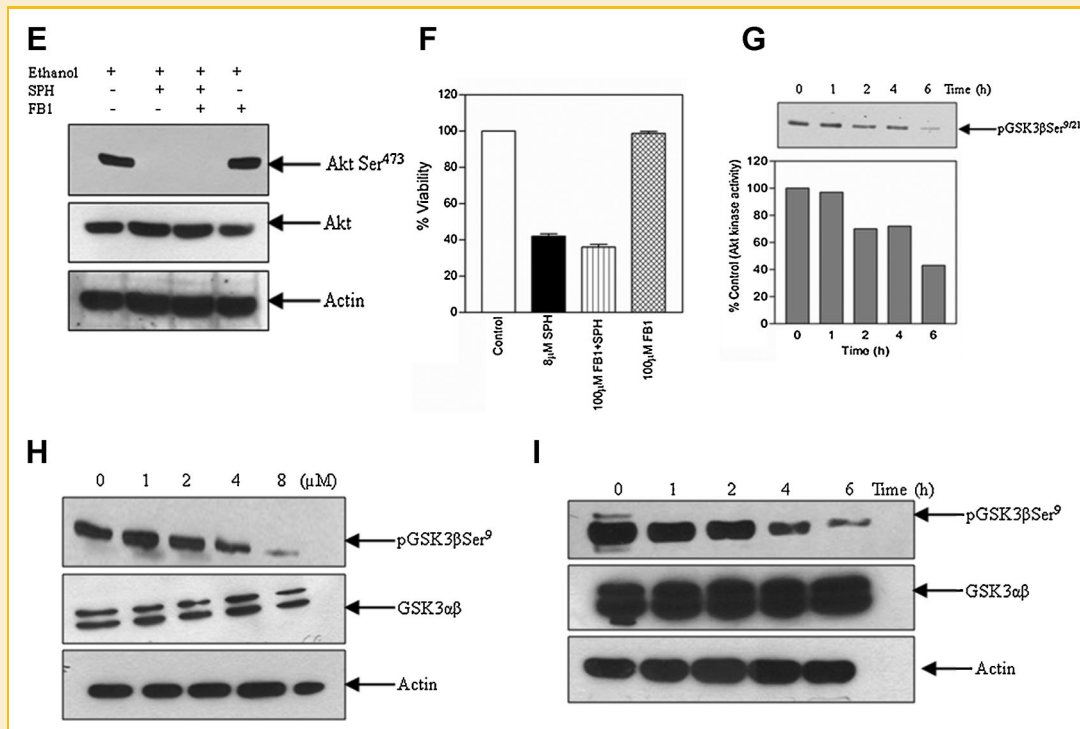
Fig. 3. (*Continued*)

SPHINGOSINE–INDUCED DEPHOSPHORYLATION OF AKT AT SER^{473}

It is now well established that activation/inactivation of Akt depends on the phosphorylation state of the two major regulatory phosphorylation sites Ser^{473} and Thr^{308} [Alessi et al., 1996; Meier et al., 1998]. Previous studies have demonstrated that constitutively activated Akt significantly protected Hep3 B and HMNI cells from both SPH and C_2-Cer-induced apoptosis [Chang et al., 2001; Matsuoka et al., 2003]. Therefore, the degree of Akt phosphorylation was determined by using an antibody specific for phospho-Ser^{473}. In untreated control cells, Akt was found to be constitutively phosphorylated at Ser^{473} (Fig. 3A). However, when cells were treated with different concentrations (0–8 μM) of SPH for 6 h, a dramatic dephosphorylation of Akt at Ser^{473} was observed (Fig. 3A). A time course study with 8 μM of SPH showed that dephosphorylation at Ser^{473} started as early as 15 min, which intensified with time (Fig. 3B). This result demonstrated that SPH-induced dephosphorylation of Akt at Ser^{473} is dose and time dependent. Intriguingly, no detectable phosphorylation at Thr^{308} of Akt were observed neither in SPH-treated nor -untreated cells (data not shown).

PDK1 is an upstream kinase, which activates Akt by phosphorylation. The activity of PDK1 was examined by measuring the phosphorylation status of this protein. As shown in the (Fig. 3C), SPH did not have any effect on the expression level of phosphorylated PDK1 and total PDK1. These results indicate that reduction in the phosphorylation status of Akt at Ser^{473} is not associated with PDK1 activation, rather, it may be associated with increased protein phosphatase activity towards phosphorylated Akt.

The effect of SPH on Molt-4 cells were tested and a dose-dependant dephosphorylation of Akt at Ser^{473} were observed (data not shown).

Previously, rapid generation of Cer following SPH treatment of Jurkat cells have been reported [Jarvis et al., 1996; Cuvillier et al., 2000]. Indeed, in this study FB1 (100 μM), a known inhibitor of ceramide synthase, inhibited SPH-induced Cer generation in Jurkat cells (Fig. 3D). However, FB1 did not inhibit SPH-induced dephosphorylation of Akt (Fig. 3E). Furthermore, similar to previous reports in Jurkat cells [Cuvillier et al., 2000], HL-60 cells [Jarvis et al., 1996], and Hep3B cells [Hung et al., 1999], FB1 by itself did not induce apoptosis, nor did it affect the extent of SPH-induced apoptosis (Fig. 3F). All together, these results suggest that SPH itself is likely to be inducing dephosphorylation of Akt and apoptosis, rather than being converted first to Cer.

In order to examine whether the inhibitory effect of SPH on Akt phosphorylation is associated with Akt kinase activity, we performed in vitro Akt kinase assay. We treated Jurkat cells with SPH or vehicle, following cellular lysis, Akt protein was immunoprecipitated from an equivalent amount of cell lysate, the Akt substrate GSK-3β was added, and the mixture was incubated at 30°C for 1 h. The reaction was stopped by the addition of SDS-sample buffer, and phosphorylation of GSK-3β was detected with anti-phospho-GSK-3β antibody. The data indicate that exposure of Jurkat cells to SPH caused a dramatic decrease in Akt kinase activity (Fig. 3G). However, SPH (10 μM) and C_6-Cer (20 μM) had no effect on Akt kinase when added to the in vitro assay (data not shown).

Next we examined the effect of SPH on the expression and phosphorylation level of GSK-3β in Jurkat cells following treatment

with SPH. As shown in the Figure 3H,I, SPH-induced GSK-3β dephosphorylation at Ser[9] in a dose- and time-dependent manner. However, SPH had no effect on the total level of GSK-3αβ expression. Thus, reduction in Akt kinase activity is correlating with those of phosphorylated (at ser[9]) GSK-3β level.

SPHINGOSINE-INDUCED AKT DEPHOSPHORYLATION IS PREVENTED BY INHIBITION OF PROTEIN PHOSPHATASE 1

Previously, it has been reported that molecules such as C_2-Cer, 4-hydroxynonenal, N-ethylmaleimide and the SPH analogue FTY720, facilitate Akt dephosphorylation by activating PP2A or PP2A-like phosphatase [Salinas et al., 2000; Schubert et al., 2000; Matsuoka et al., 2003]. Consequently, we investigated a potential link between SPH-mediated Akt dephosphorylation and protein phosphatase activation by pre-treating cells with phosphatase inhibitor prior to SPH addition. Pre-treatment of Jurkat cells with Ser/Thr protein phosphatase inhibitor calyculin A at 10 nM inhibited SPH-induced Akt dephosphorylation, whereas, treatment with the inhibitor alone increased the phosphorylation level of Akt protein (Fig. 4A).

PA, a preferential and potent inhibitor of PP1, has been used to inhibit ceramide and docosahexanoic acid-induced cytotoxicity in Molt-4 and Jurkat cells, respectively [Kishikawa et al., 1999; Siddiqui et al., 2001]. Pre-treatment of Jurkat cells with PA, completely abolished SPH-induced Akt dephosphorylation (Fig. 4B).
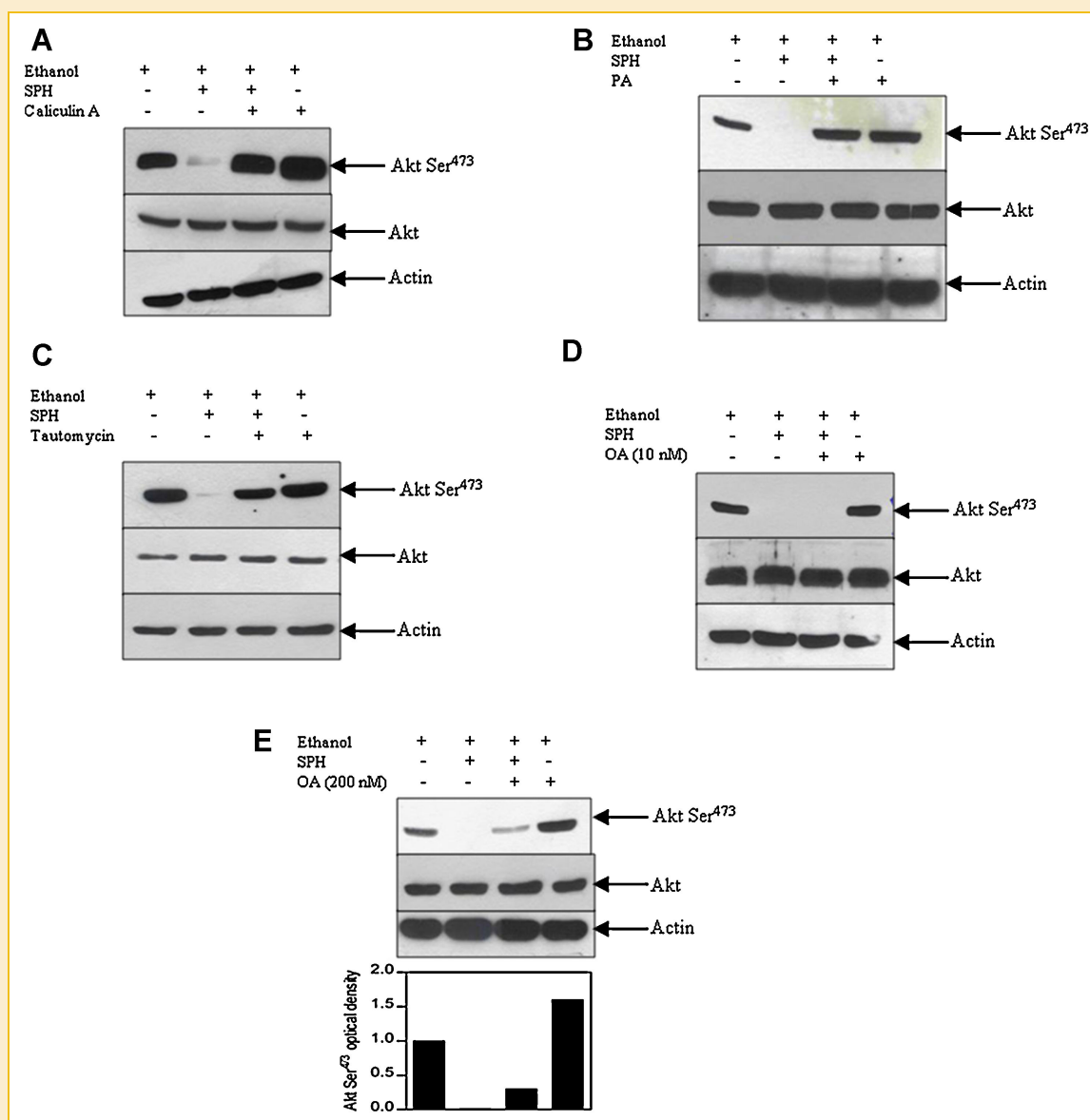

Fig. 4. Effects of protein phosphatase inhibitor on SPH-induced dephosphorylation of Akt at Ser[473]. A: Jurkat cells were pre-treated 1 h with 10 nM calyculin A, (B) 30 μM PA, (C) 100 nM tautomycin, (D) 10 nM OA, and (E) 200 nM OA followed by 6 h treatment with SPH. Cells were lysed as described under the Materials and Methods Section, and were fractionated by SDS–PAGE. Western blot were probed with antibodies specific for phospho–Akt (Ser[473]) and Akt. Bar graph represents densitometric analysis of both phospho Akt (Ser[473]). Actin was used as a loading control.

Next we used tautomycin, a highly specific inhibitor of PP1 [Chen et al., 2005]. Pre-treatment of tautomycin (100 nM) could completely abrogate the effect of SPH on phospho-Akt, further supporting the involvement of PP1 in SPH-induced dephosphorylation of Akt (Fig. 4C). This was additionally verified by using the unique activity profile of OA. OA inhibits PP2A at low concentrations ($IC_{50} < 0.1$ nM), but inhibits both PP2A and PP1 at higher concentrations ($IC_{50} = 150$ nM). Jurkat cells were pretreated with low concentration of OA (10 nM) for 1 h, followed by treatment with SPH for 6 h. As shown in Figure 4D, OA did not affect Akt dephosphorylation at 10 nM concentration. However, when high concentrations of OA (200 nM) were used, there was approximately 30% protection from SPH-induced Akt dephosphorylation (Fig. 4E). These data support a role for PP1, but not PP2A, in mediating SPH-induced Akt dephosphorylation in Jurkat cells.

SPHINGOSINE INDUCES AKT DEPHOSPHORYLATION THROUGH SER/THR PROTEIN PHOSPHATASE 1

The inhibitor experiments above suggested that PP1 or PP1-like phosphatase might dephosphorylate Akt. This suggestion would be strongly supported by demonstration of a direct interaction between Akt and PP1. To address whether PP1 is associated with Akt, and whether PP1 directly dephosphorylates Akt, we investigated the interaction of PP1 and Akt in intact cells through co-immunoprecipitation studies. Reciprocal co-immunoprecipitation of cytoplasmic proteins was performed under SPH-treated and -untreated conditions (Fig. 5A,B). High levels of Akt protein were observed in anti-PP1 immunoprecipitates of SPH-treated Jurkat cells. Reprobing the membrane with an anti-PP1 antibody showed that there were similar levels of PP1 in both untreated and treated immunoprecipitates (Fig. 5A). In reciprocal experiments, high levels of PP1 were detected in anti-Akt immunoprecipitates from SPH-
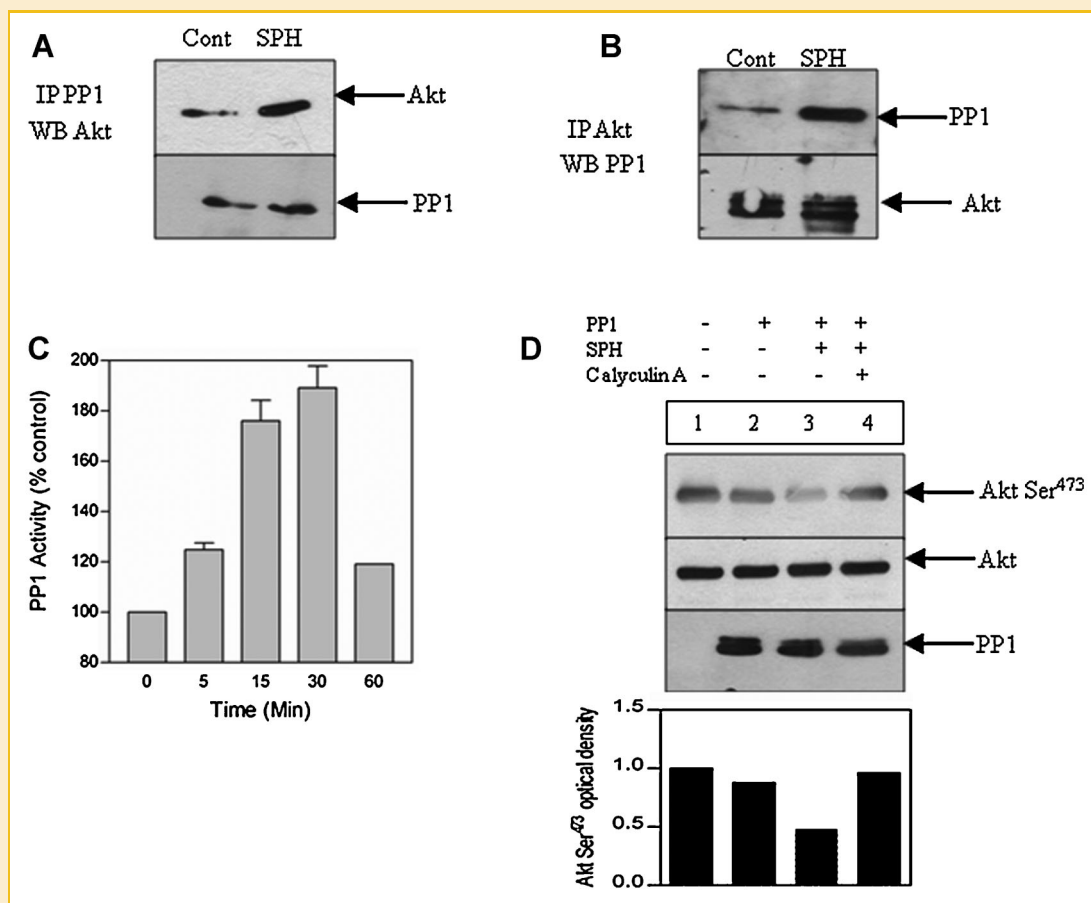

Fig. 5. Interactions of Akt and PP1. A: Jurkat cells were treated with 8 μM SPH for 30 min, cells were lysed, and then immunoprecipitated with anti-PP1 antibody, transferred to nitrocellulose membrane, and immunoblotted with anti-Akt and anti-PP1. B: Similarly, Akt was immunoprecipitated from Jurkat cell lysate after treatment with 8 μM SPH for 30 min and immunoblotted with anti-PP1 and anti-Akt. Similar results were obtained in two independent experiments. C: Estimation of Ser/Thr phosphatase 1 activity. After treating Jurkat cells with 8 μM SPH for the indicated time, Akt was immunoprecipitated. The PP1 activity was estimated by using phosphorylated bisamide rhodamine-110 peptide fluorescent substrate according to manufacturer's instructions. Data shown are means ± SD (n = 3). D: SPH treatment of Jurkat cells increases the activity of PP1 toward AKT. Jurkat cells were treated or untreated with 8 μM SPH for 1 h. Immunoprecipitated PP1 from SPH-treated Jurkat cells was mixed with phosphorylated AKT protein with (lane 4) or without (lane 3) 5 nM calyculin A. Lane 2 represent immunoprecipitated PP1 from untreated Jurkat cells mixed with phosphorylated Akt. Lane 1 represent phosphorylated Akt mixed with protein A-agarose beads without PP1. After incubation, AKT phosphorylation and protein level were determined by Western blotting with specific antibodies. Bar graph represents densitometric analysis of both phospho Akt (Ser[473]).

treated cells when compared to control (Fig. 5B). Similarly, reprobing the membrane with anti-Akt antibody showed that there were equal levels of Akt in both SPH-treated and -untreated cells (Fig. 5B). These data imply that PP1 directly interacts with Akt, and hence, likely regulates its dephosphorylation.

In order to confirm that PP1 is the active phosphatase associated with Akt, we immunoprecipitated Akt from SPH-treated and -untreated cells, following which phosphatase activity was measured by using a fluorescent substrate (phosphorylated bisamide rhodamine-110 peptide). As shown in Figure 5C, a rapid increase in the

phosphatase activity reached a peak after 15–30 min, then declined to the control level after 1 h of treatment with SPH. Indeed, this was further confirmed by an in vitro Akt dephosphorylation assay in which we used the endogenous PP1 immunoprecipitated from SPH-treated/untreated Jurkat cells with phospho-Akt as a substrate. We found immunoprecipitated PP1 (SPH-treated Jurkat cells) can dephosphorylate Akt significantly when compared to immunoprecipitated PP1 from untreated Jurkat cells (control), see Figure 5D. Furthermore, the PP1-dependant nature of phosphatase activity in the immunoprecipitate was confirmed by the addition of
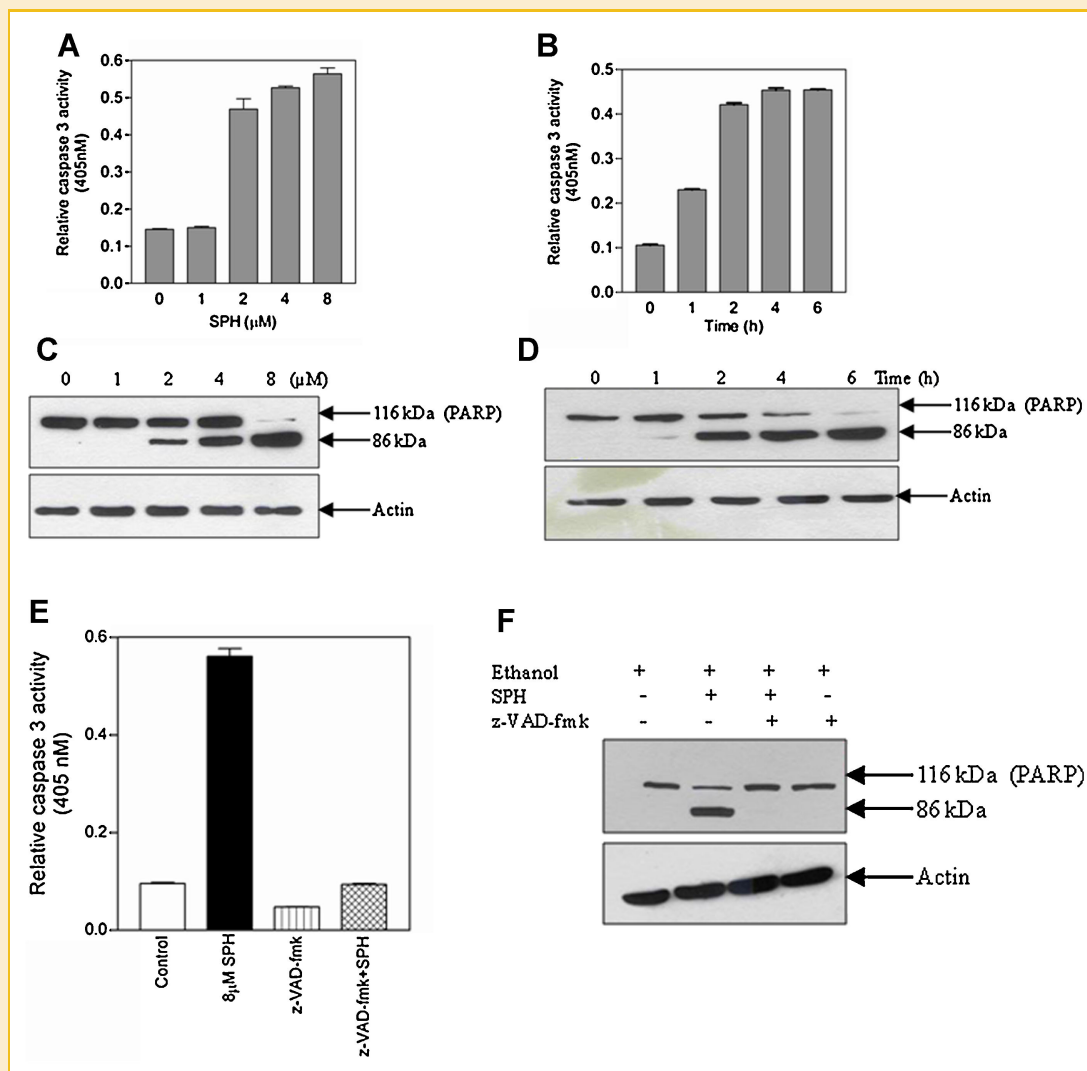

Fig. 6. Effects of SPH on caspase activity and caspase-3-specific PARP cleavage. A: Jurkat cells were treated with the indicated concentrations of SPH for 6 h and (B) treatment of Jurkat cells with 8 μM SPH for the indicated time and harvested in lysis buffer. Enzymatic activity of caspase-3 was determined by incubation of 50 μg of total protein with 200 μM chromogenic substrate (DEVD-pN) in a 100 μl assay buffer for 3 h at 37°C. The release of chromophore pNA was monitored spectrophotometrically (405 nm). Data shown are means ± SD (n = 3). C: Jurkat cells were treated with the indicated concentrations of SPH for 6 h and (D) treatment of Jurkat cells with 8 μM SPH for indicated time. Equal amounts of lysates (30 μg) were subjected to electrophoresis and analyzed by Western blot for PARP. The 85 kDa proteolytic cleavage of PARP is indicated by the arrow. E: Jurkat cells were pre-treated with z-VAD-*fmk* (50 μM) for 1 h before SPH (8 μM) treatment for further 6 h, and caspase-3 assay was performed as described in the Material and Methods Section. F: Jurkat cells were pre-treated with z-VAD-*fmk* (50 μM) for 1 h before SPH (8 μM) treatment for further 6 h and cell lysate were subjected Western blot analysis and probed with antibodies specific for PARP antibody. Actin was used as a loading control. Blots shown here are representative of three independent experiments. G: Jurkat cells were pre-treated with z-VAD-*fmk* (50 μM) 1 h before SPH (8 μM) treatment for further 6 h, and apoptosis was determined by apoptosis assay as described in the Materials and Methods Section. Data shown are means ± SD (n = 3). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
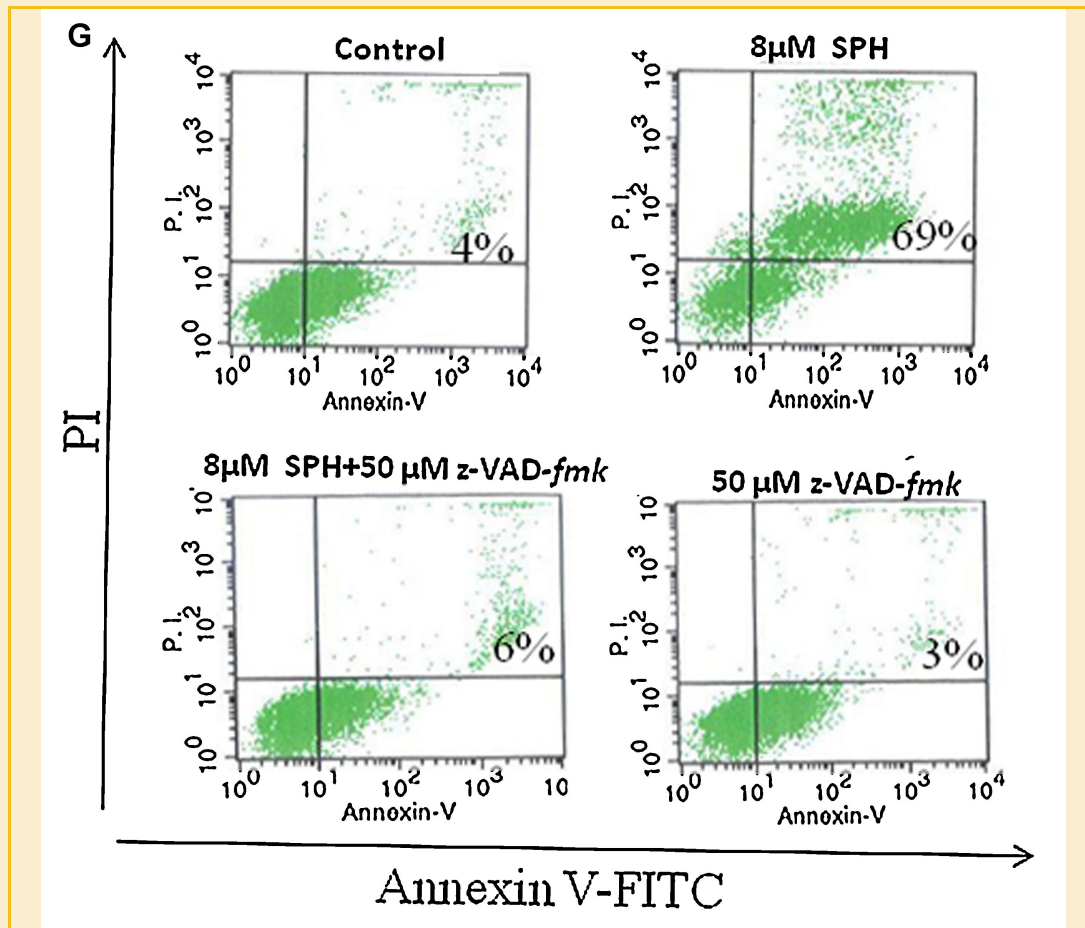
Fig. 6. (*Continued*).

5 nM calyculin A (Fig. 5D). However, SPH did not affect PP1 activity directly since addition of SPH in the phosphatase assay did not affect Akt dephosphorylation (data not shown). These data demonstrated conclusively that the PP1, and not other phosphatases, were involved in the Akt dephosphorylation.

SPHINGOSINE–INDUCED DEPHOSPHORYLATION OF AKT LEADS TO CASPASE–3 ACTIVATION AND PARP CLEAVAGE

Caspases are important mediators of apoptosis induced by various apoptotic stimuli [Izban et al., 1999]. Therefore, the effects of SPH on caspase activation were examined. As can be seen in Figure 6A,B, SPH-activated caspase-3 in a dose- and time-dependent manner. Indeed, SPH can activate caspase-3 within 2 h of SPH treatment. In order to confirm caspase-3 activation, the caspase-3 substrate PARP and its cleavage product were examined by Western blot analysis. As can be seen in Figure 6C,D, a dose- and time-dependent clear accumulation of the 86 kDa PARP cleavage fragment is present. Hence, confirming the activation of caspase-3, the main protease responsible for PARP cleavage. Furthermore, pre-treatment of cells with 50 μM z-VAD-*fmk*, a universal inhibitor of caspases abrogated caspase-3 activation, PARP cleavage, and also prevented apoptosis induced by SPH (Fig. 6E–G). These data clearly indicate that SPH-

induced Akt dephosphorylation leads to the activation of caspase-3 and PARP cleavage.

INHIBITION OF PROTEIN PHOSPHATASE 1 PROTECTS AGAINST SPH–INDUCED APOPTOSIS IN JURKAT CELLS

In order to determine whether Akt dephosphorylation, and the resultant caspase 3 activation, had any relevance to SPH-induced cell death, the effect of protein phosphatase inhibitors on SPH-induced cell viability were examined using the WST assay. Earlier, it was observed that both calyculin A and PA prevented SPH-induced Akt dephosphorylation (Fig. 4A,B), whilst OA at low concentration did not prevent Akt dephosphorylation (Fig. 4D). Jurkat cells were treated with 10 nM calyculin A, or 30 μM PA, prior to treatment with 8 μM SPH. Both Calyculin A and PA pre-treatment significantly prevented the cytotoxic effect of SPH (Fig. 7A,B). However, pre-treatment of Jurkat cells with 10 nM OA did not protect against SPH-mediated cytotoxicity (Fig. 7C). Next, we examined the effect of pre-treatment with calyculin A, PA, and OA on apoptosis using FACS analysis (calyculin A-treated cells were clumped, so we were unable to do the FACS analysis of that sample). Figure 7D shows that pre-treatment of Jurkat cells with PA totally protected against SPH-
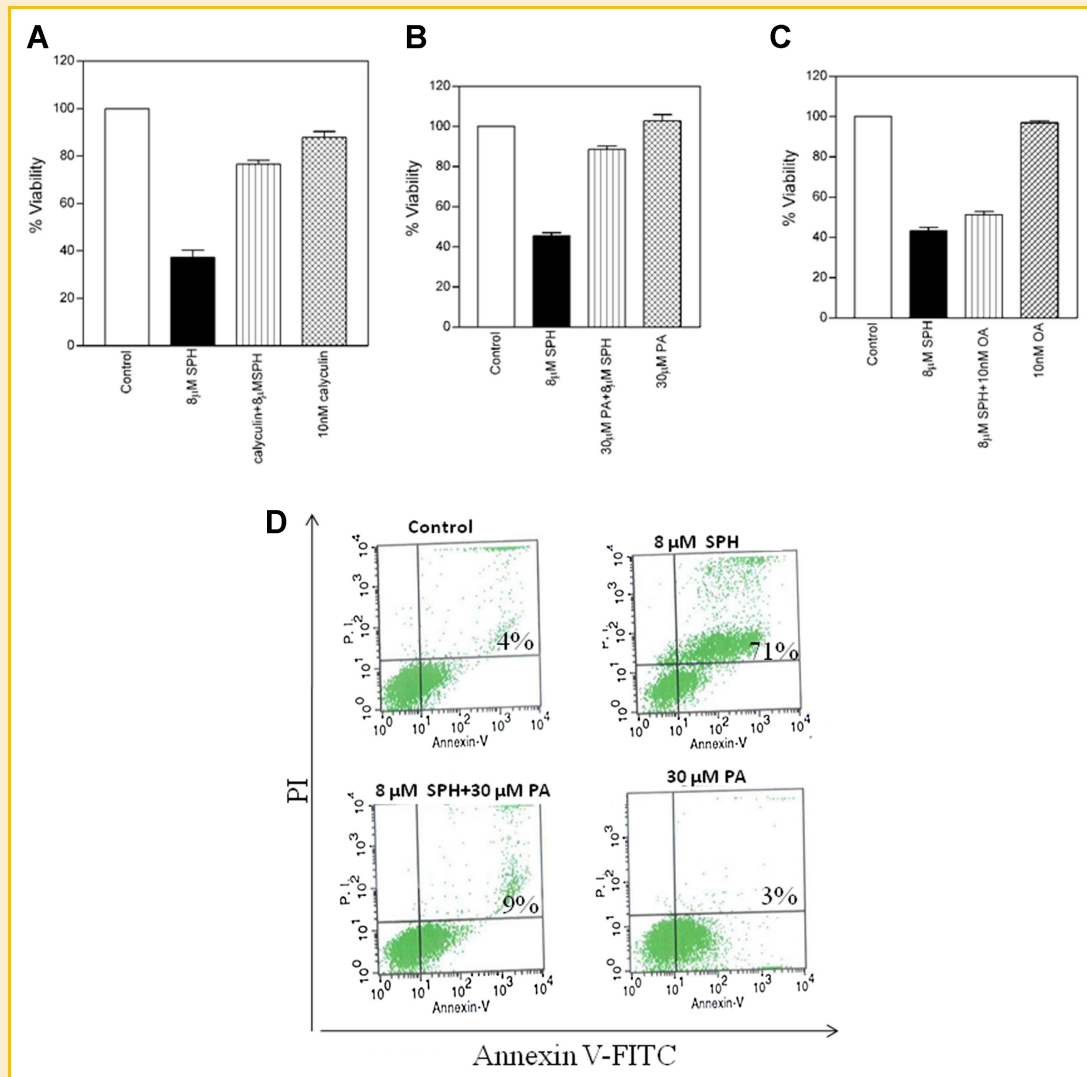
Fig. 7.   Effect of protein phosphatase inhibitors on SPH-induced apoptosis and PARP cleavage. A: Jurkat cells were pre-treated 1 h with 10 nM calyculin A, (B) 30 μM PA, (C) 10 nM OA followed by 6 h treatment with SPH. Cell viability assay was performed by using WST cell viability assay kit. Data shown are means ± SD (n = 3). Jurkat cells were pre-treated 1 h with (D) 30 μM PA, (E) 10 nM OA followed by 6 h treatment with SPH, apoptosis was determined by apoptosis assay as described in the Materials and Methods Section. Data shown here are representative of three independent experiments. F: Jurkat cells were pre-treated 1 h with 10 nM calyculin A, (G) 30 μM PA, (H) 10 nM OA followed by 6 h treatment with SPH then cell lysates were subjected Western blot analysis and probed with antibodies specific for PARP antibody. Actin was used as a loading control. Blots shown here are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

induced apoptosis, whereas, OA pre-treatment was unable to protect Jurkat cells from SPH-induced apoptosis (Fig. 7E).

Additionally, the effect of pre-treatment with calyculin A, PA, and OA on PARP cleavage were examined. As can be seen in Figure 7F,G, pre-treatment of Jurkat cells with calyculin A and PA strongly inhibited SPH-induced and caspases-mediated PARP cleavage. However, OA did not protect against SPH-induced caspase activation and PARP cleavage (Fig. 7H). These findings clearly indicate that PP1 activation leads to the caspase-dependent apoptosis in Jurkat cells.

Based on our finding in this study, we propose a relationship between PP1-dependent Akt dephosphorylation and caspase activation, which ultimately leads to DNA damage and Jurkat cell apoptosis following SPH treatment.

DISCUSSION

This study for the first time demonstrates that SPH induces PP1-dependent Akt dephosphorylation which leads to capsase-3 activation, PARP cleavage, and ultimately apoptosis. Moreover, inhibition of PP1 activity using calyculin A and PA protects against PP1-dependent Akt dephosphorylation and induction of apoptotic signaling pathway in Jurkat cells.

Sphingolipids have come to prominence as bioactive lipids. It is now well established that Cer may function as an important signaling molecule in the regulation of cell growth and apoptosis. Indeed, Cer generation has been implicated in Fas- and TNF-α-induced apoptosis. Recently, SPH, a Cer metabolite, has been shown to be a critical mediator in TNF-α-, Fas-, phorobol ester-, and
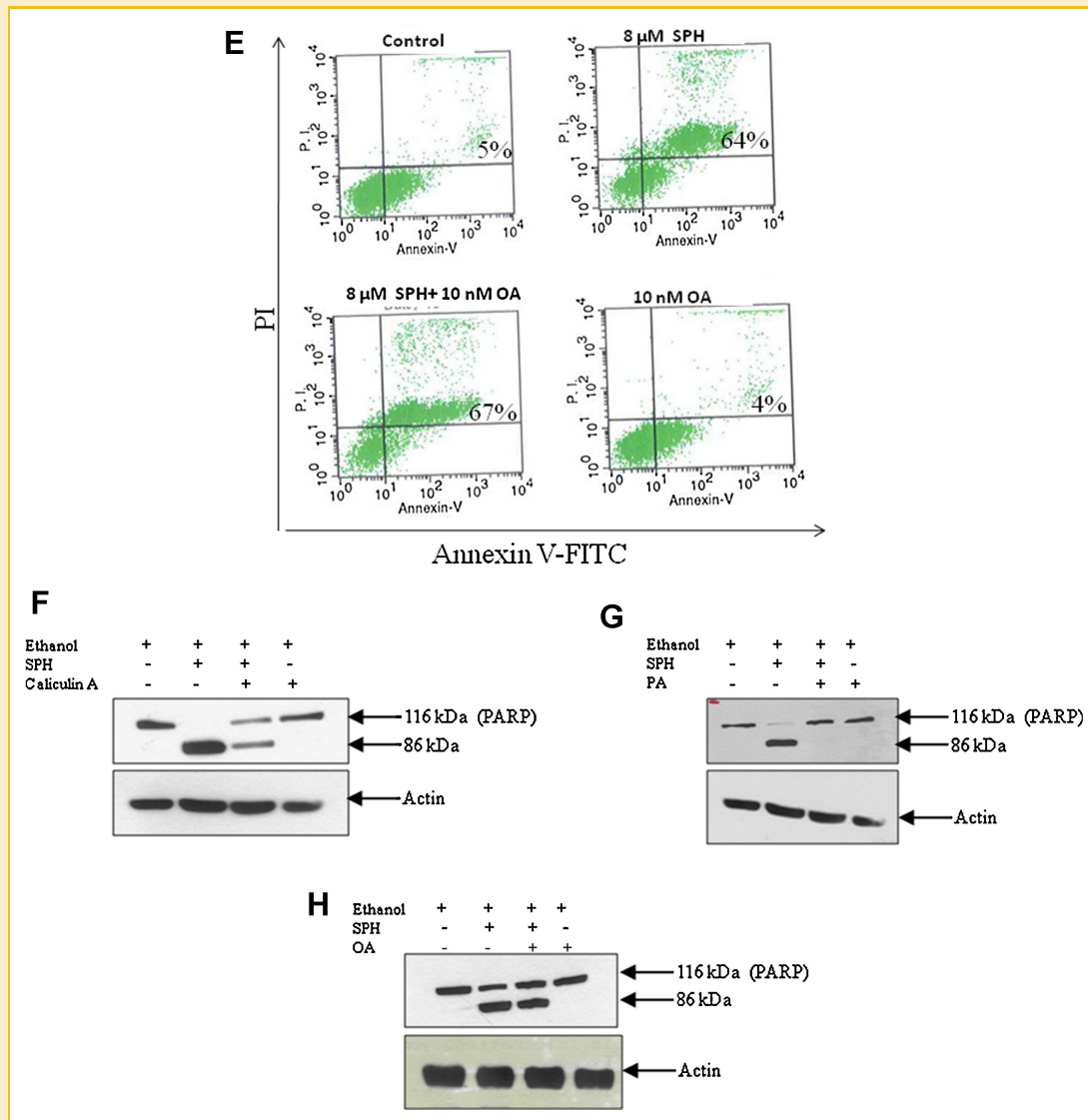
Fig. 7. (Continued).

doxorubicin-induced apoptosis in a variety of cell types [Ohta et al., 1995; Jarvis et al., 1996; Cuvillier et al., 2000]. Additionally, exogenous SPH also induces apoptosis in different cancer cells [Jarvis et al., 1996; Phillips et al., 2007].

In this study Jurkat cells are used as a cell model and as can be seen in Figure 1, SPH has a dose, time, and stereospecific effect on cell viability. Moreover, this effect of SPH on cell viability is further confirmed by FACS analysis (Fig. 2). SPH may induce apoptosis without being converted into Cer, inasmuch as the ceramide synthase inhibitor FB1 does not affect SPH-induced apoptosis (Fig. 3F). Thus, there may be a SPH-activated apoptotic signaling pathway, and this study attempts to address that possibility. Exposure of Jurkat cells to SPH induces a rapid Akt dephosphorylation that is completed within 1 h (Fig. 3B). This exposure leads to caspase-3 activation, DNA damage, ultimately leading to apoptosis. Akt dephosphorylation and caspase-dependent apoptosis

by SPH were blocked by pre-treatment of Jurkat cells with PP1 inhibitors such as calyculin A and PA. This finding suggests that phospho-Akt becomes a PP1 rather than a PP2A substrate following SPH treatment of Jurkat cells.

Akt has been identified as a key downstream effector of PI 3-kinase that blocks apoptosis in a variety of cell types [Dudek et al., 1997]. Akt is activated when cells are exposed to growth factors, and its activation occurs via a pathway that includes PI3-kinase activation [Dudek et al., 1997]. Subsequently, Akt is phosphorylated at Thr^{308} and Ser^{473} by 3-phosphoinositide-dependent protein kinase 1 (PDK1), and 3-phosphoinositide-dependent protein kinase 2 (PDK2) [Downward, 1998]. Akt, coupled with its phosphorylation at Thr^{308} and Ser^{473}, is known to prevent apoptosis by catalyzing the phosphorylation of a number of downstream targets, including GSK-3β [Cross et al., 1995], BAD [del Peso et al., 1997], caspase-9 [Cardone et al., 1998], XIAP [Dan et al., 2004], ASK-1 [Kim et al.,

2001], and Mdm2 [Zhou et al., 2001]. Akt also prevents the release of cytochrome c from mitochondria. Accumulating evidence indicates that Akt plays a critical role in tumorigenesis and tumor progression. In fact, increased Akt phosphorylation has been reported in various cancers [Wang et al., 2000; Yamamoto et al., 2004]. Following its activation, Akt is inactivated by dephosphorylation, which is mediated by PP1 or PP2A like phosphatases [Chen et al., 2005]. This study demonstrates that SPH dephosphorylates Akt at Ser^{473} in a dose- and time-dependent manner (Fig. 3A,B). Additionally, these studies demonstrate that SPH does not inhibit PDK1 phosphorylation; it is likely that SPH does not inhibit Akt activation through modulating PDK1 kinase activity. These results indicate that reduction in the phosphorylation state of Akt at Ser^{473} is not associated with the inhibition of PDK1-induced phosphorylation. Rather, it is due to enhanced protein phosphatase activity. Since, GSK-3β is a downstream target for activated Akt [Cross et al., 1995], we examined the phosphorylation levels of GSK-3β in SPH-treated and -untreated Jurkat cells. We find that SPH induces a time- and dose-dependant dephosphorylation of GSK-3β in Jurkat cells (Fig. 3H,I).

It has been reported that the histone deacetylase (HDAC) inhibitor trichstatin A increases PP1 association with Akt and thereby causes Akt dephosphorylation [Chen et al., 2005]. The present study demonstrates a mechanistic link between PP1- and SPH-mediated Akt dephosphorylation. Indeed, the immunoprecipitation assay illustrates that SPH treatment leads to an enhanced association of PP1 with Akt and vice versa, resulting in Akt dephosphorylation (Fig. 5A,B). Since this study indicates that PP1 activity in Akt immunoprecipitates is increased (peaking at 30 min) following SPH treatment, which corresponds to the increase in Akt dephosphorylation, this suggests a correlation between PP1 phosphatase activity and Akt dephosphorylation. These results illustrate that PP1 associates with and directly dephosphorylates Akt, and the PP1-Akt interaction provides a mechanism for controlling Akt phosphorylation, and as a consequence, its dephosphorylation then may mediate SPH-induced apoptosis. Furthermore, calyculin A and PA, two well established inhibitors of PP1, completely blocked SPH-induced Akt dephosphorylation, while 10 nM OA, the concentration at which OA specifically inhibits PP2A activity in cells, had no effect. Therefore, the data reveal that SPH treatment causes an increased association between PP1 and Akt, which leads to dephosphorylation of Akt. It has been shown that activation of PP1 plays an important role in Fas-induced apoptosis by stimulating mitochondrial release of cytochrome c and activation of caspase cascade in HL-60 and Jurkat cells [Wolf and Eastman, 1999]. Several pieces of evidence involve PP1 in the induction of apoptosis: by dephosphorylation of the proapoptotic Bcl-2 family member Bad [del Peso et al., 1997], retinoblastoma protein [Wang et al., 2001], and caspase 9 [Dessauge et al., 2006]. The dephosphorylation of these proteins by PP1 may be critical for the initiation of apoptosis.

Caspase 3, a member of the cysteine protease family, plays a central role in regulating and executing mammalian cell apoptosis, as the activation of this protease is shown to be an early and essential step in multiple apoptotic signaling pathways [Tewari et al., 1995]. Recently, from this laboratory we have reported the existence of caspase-independent mechanism of apoptosis in

mouse fibroblast cells [Thayyullathil et al., 2008]. In the present study, we examined the activation of caspase-3 following Akt dephosphorylation. In agreement with previous report, SPH is found to activate caspase-3, as well as causing PARP cleavage (Fig. 6A–D). Indeed, the pan caspase inhibitor z-VAD-fmk prevented both caspase-3 activation and Jurkat cell apoptosis (Fig. 6E–G). These results suggest that SPH induces caspase-dependent apoptosis in Jurkat cells. In this study the role of PP1 in SPH-induced apoptosis was analyzed by investigating the mechanism by which calyculin A and PA inhibit SPH-induced activation of caspase-3 and PARP cleavage. Since caspase 3 activation occurs more than 1 h after the Akt dephosphorylation, it is conceivable that PP1-dependent dephosphorylation of Akt is required for the activation of caspase-mediated apoptosis in Jurkat cells.

In summary, we have demonstrated that SPH induces PP1-dependent Akt dephosphorylation as well as caspase-dependent apoptosis in Jurkat cells. Furthermore, both caspase inhibitor (z-vad-fmk) and PP1 inhibitors (calyculin A, PA, and tautomycin), completely reverse SPH-induced apoptosis in Jurkat cells. These data suggest that SPH-induced apoptosis occurs via PP1-dependent dephosphorylation of Akt at Ser^{473} and subsequent activation caspase-3 and PARP cleavage.

CONCLUSION

In conclusion, we have demonstrated that SPH exposure elicits a rapid increase in PP1-dependent Akt dephosphorylation at Ser^{473}, and a subsequent activation of GSK-3β, caspase 3, and PARP cleavage. Moreover, calyculin A and PA inhibition of SPH-induced Akt dephosphorylation at Ser^{473}, and thereby, prevention of caspase 3 activation and PARP cleavage, and ultimately apoptosis, underscore the role of PP1 as the prime signaling event in the SPH-induced apoptosis in Jurkat cells. The possible effect on other proteins that may regulate the modulation of apoptosis will have to be investigated in the future.

ACKNOWLEDGMENTS

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