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Sphingosine mediates FTY720-induced apoptosis in LLC-PK$_1$ cells

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Abbreviations: DHS, *dl-threo*-dihydrosphingosine; DMS, *N,N*-dimethylsphingosine; FBS, fetal bovine serum; FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3propanediol; OPA, o-phthalaldehyde; PBS, phosphate buffered saline; SK, sphingosine kinase; S1P, sphingosine 1-phosphate; S1PRs, S1P receptors

Abstract

FTY720, a synthetic sphingoid base analog, was examined as a new sphingosine kinase inhibitor, which converts endogenous sphingosine into its phosphate form. With 20 μM of FTY720, sphingosine accumulated in the LLC-PK$_1$ cells in a time- and dose-dependent manner. The FTY720 treated cells showed a high concentration of fragmented DNA, a high caspase-3 like activity and TUNEL staining cells. It was also found that the sphingosine and sphinganine level increased in a time- and dose-dependent manner within 12 h after the FTY720 treatment. The sphingosine kinase activity was reduced by FTY720 as much as other sphingosine kinase inhibitors, N, N-dimethylsphingosine (DMS), *dl-threo*-dihydrosphingosine (DHS). The fragmented DNA content as a result of the 20 μM of FTY720 treatment and by 5 μM of the exogenously added BSA-sphingosine complex indicated typical apoptosis. Under similar conditions, the accumulated sphingosine concentration in all the cells was almost identical even though the sphingosine distribution inside the cells was somewhat different. These results indicate that the FTY720 induced apoptosis is associated with the inhibition of the sphingosine kinase activity and is strongly associated with the successive accumulation of sphingosine.

Keywords: apoptosis; FTY720; LLC-PK$_1$ cells; sphingosine; sphingosine kinase

Introduction

Sphingosine kinase (SK) is activated by various growth factors to produce sphingosine-1-phosphate (S1P), which regulates a wide spectrum of biological processes including calcium mobilization, cell growth, survival, differentiation, motility and cytoskeletal organization (Pyne and Pyne, 2000; Spiegel and Milstien, 2002).

Recent studies have clarified the SK activation mechanism where the SK family was characterized in humans and other species including macaques, mice, rats, *Drosophila*, and *C. elegans*, has a conserved phosphorylation site in the amino acid sequence surrounding Ser225 (SKTPAS^{225}PVVVQ) by ERK1/2 for SK activation (Pitson *et al.,* 2003).

Sphingosine, which is a natural SK substrate, acts as a negative regulator of cell proliferation and promotes apoptosis (Cuvillier, 2002). Because SK is highly conserved in Swiss 3T3 fibroblasts and human platelets, exogenously added sphingosine rapidly changes into S1P and induces cell proliferation in Swiss 3T3 fibroblasts and platelet aggregation. However, at high concentrations ($>$10 μM), sphingosine becomes cytotoxic and induces apoptosis in most cultured cells. Its enigmatic properties are strongly related to the difference in the sphingosine metabolic fate depending on the cell types.

A newly developed immunosuppressant, FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol, is a modified synthetic analog of ISP-1/myriocin, which is a fungal metabolite in *Isaria Scinclariii* (Miyake *et al.,* 1995). The apoptosis induced by FTY720 decreases the bcl-2 to Bax ratio in human lymphocytes (Suzuki

et al., 1996; Li *et al.,* 1997) and increases the intracellular concentration of calcium ions in HL-60 cells (Shinomiya *et al.,* 1997). FTY720-induced apoptosis has been suggested to be mediated *via* the activation of phospholipase C (Shinomiya *et al.,* 1997), Fas-independent, and bcl-associated signaling (Azuma *et al.,* 2003). In addition, phosphorylated FTY720 has been reported to be a potent agonist on the S1P receptors (S1PRs), suggesting that FTY720 is phosphorylated by SK1 and/or SK2 (Paugh *et al.,* 2003).

Previously, it was reported that endogenous sphingosine accumulation by FTY720 in LLC-PK_1 cells and the increasing population of floating cells indicated typical apoptosis as a result of an altered sphingolipids metabolic pathway (Lee and Lee, 1999).

This study proposes that FTY720 inhibits SK. Therefore, it is the accumulated sphingosine that induces apoptosis in LLC-PK1 cells, where its cell lysates have a high SK activity.

Materials and Methods

Cell culture

The LLC-PK1 porcine kidney endothelial cells were obtained from ATCC (Manassas, VA) and maintained in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine albumin (FBS), 100 μg/ml streptomycin and 100 IU/ml penicillin in a humidified atmosphere of 5% CO_2 at 37°C. The number of cells was counted using a hemocytometer. The cell viability was assessed using trypan blue exclusion analysis.

FTY720 supplementation and induction of apoptosis

FTY720 was chemically synthesized and its designed structure was confirmed by ^{1}H-and ^{13}C-NMR, mass spectrometry and FT-IR, respectively. The cells were incubated at a density of 1.0-2.0×10^8 cells/ml in complete medium supplement for 24 h. After incubation, the cells were washed, suspended at a density of 2×10^8 cells/ml in serum-free medium containing transferrin (5 μg/ml) and insulin (5 μg/ml), and were allowed to re-equilibrate for 1 h. The cells were then treated with 20 μM of FTY720 for 4-24 h, as described in the Figure legends and text. A standard stock solution of FTY720 was made by dissolving it in ethanol, with the final concentration of ethanol in the test samples being $< 0.1\%$.

DNA electrophoresis

The FTY720-treated or untreated LLC-PK_1 cells were treated with 0.5% trypsin, collected by centrifugation and washed with PBS. The washed cells were suspended in a lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS] containing 0.1% RNase A and incubated for 60 min at 50°C. The lysate was incubated in the presence of 1 mg/ml proteinase K for an additional 60 min at 50°C. The resulting DNA preparations were analyzed by DNA gel electrophoresis on 1.5% agarose gel in a Tris-acetate buffer (40 mM Tris-acetate, 1 mM EDTA). After electrophoresis for 60 min at 50 V, the DNA was visualized by ethidium bromide staining.

Flow cytometry

Flow cytometry was carried out using a FACS Caliber (Becton Dickinson, San Jose, CA). The cells were grown at 1×10^8 cells/ml density and incubated with FTY720 or the indicated agents, and then were harvested with trypsin and fixed with 70% ice-cold ethanol. Prior to flow cytometry, the cells were washed with 40 μl PBS at room temperature, centrifuged, and stained with 1 ml of a propidium iodide solution (0.05 mg/ml), 0.05 mg RNase A/ml and 0.1 mM EDTA in PBS (pH 7.4). After gentle re-suspension in this solution, the cells were left at 4°C for 30 min at least, in the absence of light, prior to analysis. The area below the G_0/G_1 diploid peak represents the apoptotic cells. The percentage of cells in each stage of the cell cycle was estimated using ModFit LT software (Verity Software House, Inc., Topsham, Maine).

Microscope observation with TUNEL staining

The apoptotic cells were stained by DNA nick end labeling (TUNEL), pre-fixed in 4% neutral buffered formalin, treated with proteinase K (20 mg/ml) for 15 min at 25°C, and stained with propidium iodide and successively with an *In Situ* Cell Death Detection Kit, Fluorescence (Roche Molecular Biochemicals, Mannheim, Germany). An image of stained cells suspension was taken using fluorescence microscopy (Olympus AX-70) equipped with Universal Photo System.

Measurement of caspase-3 like activity

A cell pellet was re-suspended in an extraction buffer (50 mM PIPES-NaOH pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 20 μM cytochalasin B, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 50 μg/ml antipain, 10 μg/ml chymopapain). The cells were disrupted by five freeze-thaw cycles in liquid nitrogen. For the assay of the Ac-DEVD-AMC cleaving activity, the cell lysate was mixed with an ICE buffer (100 mM HEPES-KOH pH 7.5, 10% sucrose, 10 mM DTT and 0.1 mg/ml ovalbumin) containing Ac-DEVD-AMC, caspase substrate. The

enzyme reactions were performed in 96-well plates at 30°C for 1 h. The free AMC released was then measured at a wavelength of 320 nm (excitation) and 444 nm (emission) using a Molecular Devices fluorescence reader (Sunnyvale, CA). The enzyme activity was calculated from the AMC standard curve.

BCA protein assay

The total cellular protein concentration was determined in order to both normalize the results and estimate the level of cell growth. A cell lysate was mixed with a BCA reagent and incubated for 30 min. The protein content was determined using Molecular Devices ELISA reader (Sunnyvale, CA) at 562 nm based on a standard curve.

Sphingoid bases analysis

The procedure for extracting the sphingolipids from the cells involved o-phthalaldehyde (OPA) derivatization of the sphingoid bases, and HPLC determination of the OPA derivatives from the sphingoid bases in each of the treated dishes, as described by Merrill *et al.,* with a slight modifications. Briefly, the lipids were extracted with chloroform/methanol (1:2, v/v), and the phases were separated. Aliquots of the organic layer were added to 20 pmol of C_{20}-sphinganine as an internal standard, and saponified for 60 min at 37°C by adding 1 ml of 0.1 M KOH in chloroform/methanol (1:2, v/v). The hydrolyzed lipids were re-extracted by phase separation between chloroform and ammonium based alkaline water. The organic layer was dried in vacuo and then processed by OPA derivatization. The fluorescent derivatives of the sphingoid bases were eluted on an ODS column within 20 min using 90% acetonitrile as the eluent.

Measurement of sphingosine kinase activity

The cells were plated overnight in a 60 mm Petri dish (0.5×10^6 cells/ml / 5 ml). The next morning, the cells were washed twice with 3 ml of a siraganian buffer, pre-incubated in 2.5 ml of the same buffer for 10 min and stimulated with 20 μM of FTY720, 20 μM of DMS and 15 μM of DHS for 60 min at 37°C, respectively. After incubation, 80 μl of 0.1 M phosphate buffer (pH 6.8) containing 10 mM $MgCl_2$, 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM Na_3VO_4, 15 mM NaF, 10 μg/ml leupeptin and aprotinin, 1 mM PMSF and 0.5 mM 4-deoxypyridoxine, was added, and cells were scraped. The cells were disrupted by freezing and thawing three times, and the cytosolic fractions were prepared by ultracentrifugation at 105,000 *g* for 90 min. Reaction mixtures containing 42.5 μl of the supernatant, 2.5 μl of a 100 μM stock solution of sphingosine-BSA complex; final 5 μM) and 5 μl of ^{32}P-ATP (final 1 mM, 0.2 mCi/ml) was incubated for 20 min at 30°C. After incubation, the lower phase was collected and 10 μl of each sample was loaded onto the activated TLC plate. The ^{32}P-labeled lipids were separated using 1-butanol: methanol: acetic acid: water (80:20:10:20, v/v) and visualized by autoradiography.

Preparation of sphingosine stock solution

For the stock solution of fatty acid free BSA, 100 mM sphingosine in ethanol was diluted into a cell buffer solution containing 2.5 mM BSA to a final concentration of 2 mM. The solution was then incubated in a shaking water bath at 37°C for 2 h, prior to use. The solution was frozen and was re-dispersed by an additional 2 h of incubation using the above methods prior to use.

Statistical analyses

Unless otherwise noted, the analyses were carried out in triplicate, and the statistical significance of the differences between the groups was evaluated using a Student's *t* test.

Results

Effect of FTY720 on apoptosis

LLC-PK1 cells have been used to study the sphingolipid metabolism because of the rapid sphingolipid biosynthesis and high growth rate. The typical morphology of this cell shows the appearance of a flat and boundless shape reflecting strong attachment to

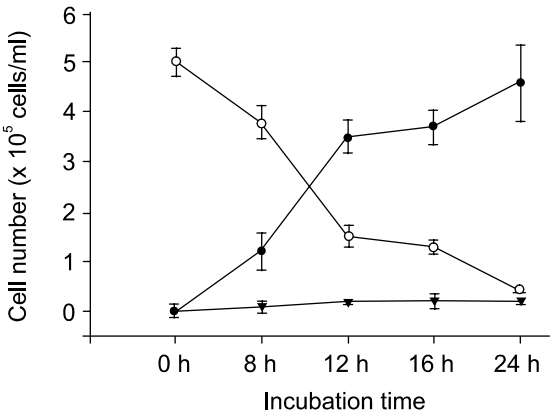

Figure 1. The increase in the number of floating cells with 20 μM of FTY720. The cells were counted using the exclusion of 4% trypan blue staining after the trypsin treatment. The symbols represent ● the floating cells, ○ the adherent cells, ▼ the floating cells without FTY720 treatment. Each result was obtained from 3 independent experiments and is expressed as the mean ± SD.

the coated plastic wall. Treatment of the cells with a 20 μM of FTY720 induced alterations in cells where it began to detach after 8 h of incubation in a time dependent manner (Figure 1). However, the floating cells responded to trypan blue exclusion during the analysis. The detached cells did not re-attach to the wall but can be re-grown by removing FTY720 from the cultured media (data not shown). This result and the observation of DNA ladder formation on gel electrophoresis within 12 h indicate that the activity of FTY720 is closely associated with apoptotic state of the cells (Figure 2A).

In order to understand the induction of apoptosis by FTY720, this study further investigated the frag-

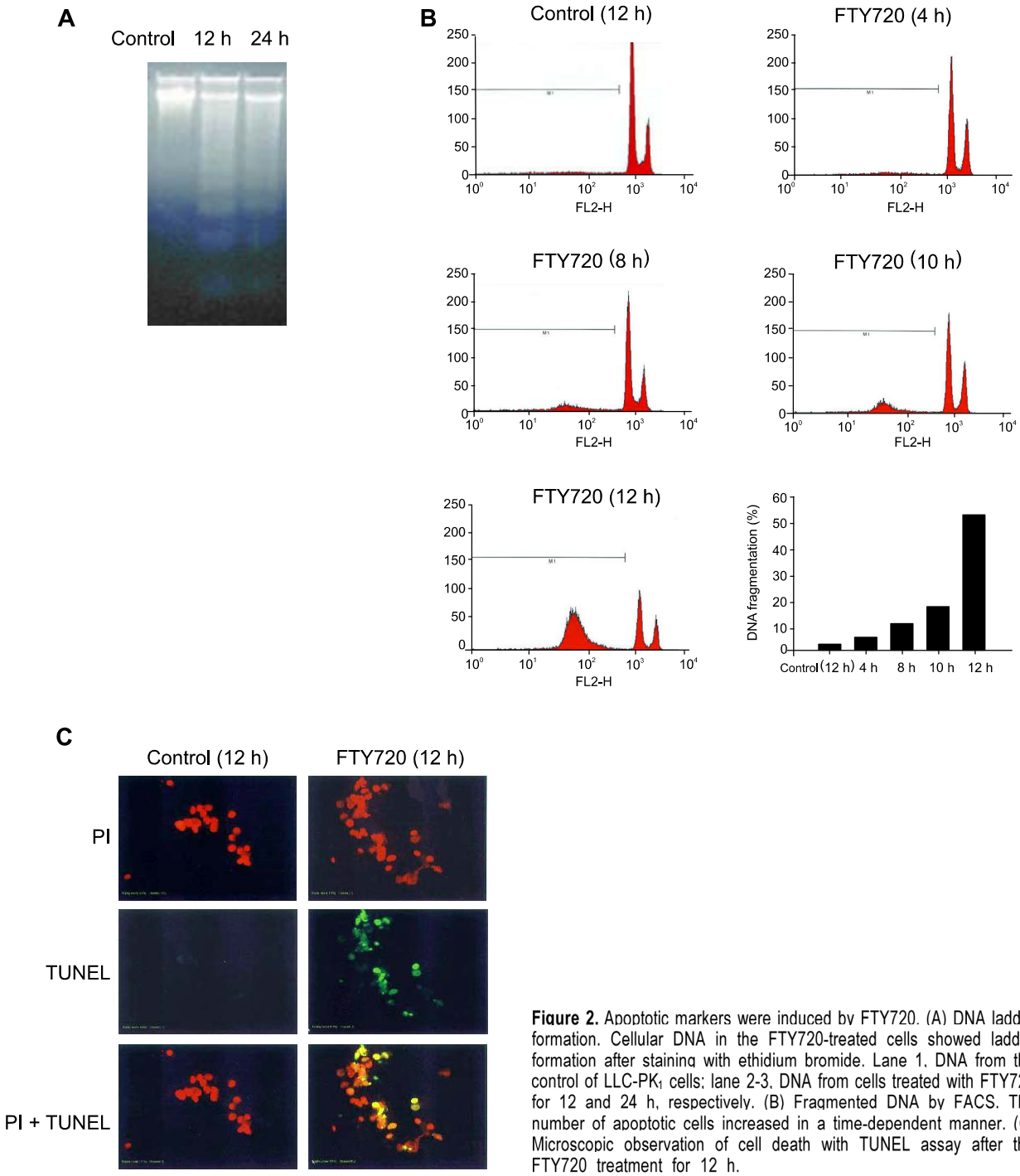

Figure 2. Apoptotic markers were induced by FTY720. (A) DNA ladder formation. Cellular DNA in the FTY720-treated cells showed ladder formation after staining with ethidium bromide. Lane 1, DNA from the control of LLC-PK_1 cells; lane 2-3, DNA from cells treated with FTY720 for 12 and 24 h, respectively. (B) Fragmented DNA by FACS. The number of apoptotic cells increased in a time-dependent manner. (C) Microscopic observation of cell death with TUNEL assay after the FTY720 treatment for 12 h.

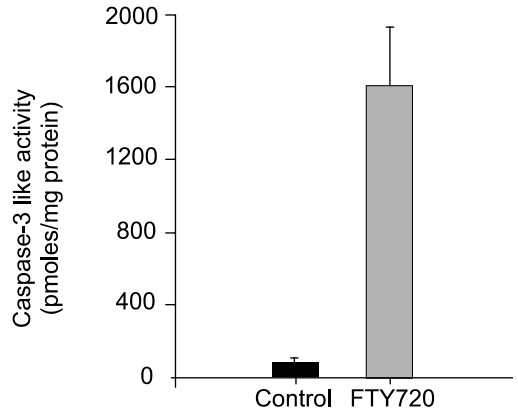

Figure 3. FTY720 activates caspase-3 like activity after 12 h. The LLC-PK_1 cells treated with 20 μM of FTY720 were harvested and the activity was to that of compared the control (without FTY720).

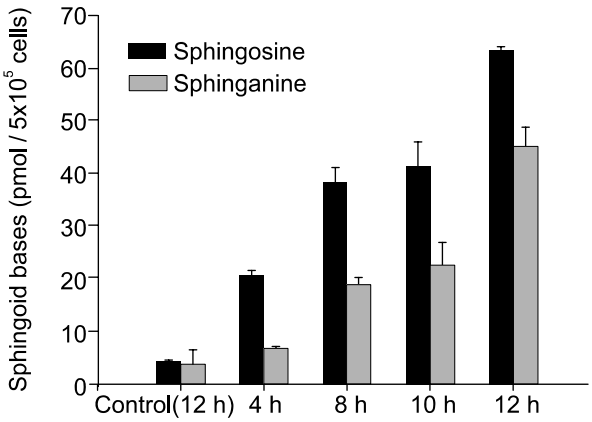

Figure 4. FTY720 increases the level of sphingoid bases 10 times higher than the control after 12 h.

mented DNA content using FACS analysis, DNA nick end labeling (TUNEL) and caspase-3-like activity. The hypodiploid region of the DNA gradually increased as a result of the FTY720 treatment (Figure 2B). The time course of the fragmented DNA content showed a good correlation with the detached-cell population shown in Figure 1. However, the relative cell population in the G_0/G_1, S and G_2/M phase was relatively unchanged. The apoptotic cells by DNA nick end labeling with FITC and the additional staining with propidium iodide (PI) were clearly observed as a yellow-green spot in Figure 2C. The caspase-3 like activity was also 20 times higher in 12 h after the 20 μM of FTY720 treatment (Figure 3).

These results indicate that FTY720 caused the typical apoptosis process on the LLC-PK1 cells, and is effective in triggering the apoptosis signaling that is located at a site up-stream of caspase-3. In view of the fact that the structure of FTY720 is similar to those of the sphingoid bases, particularly sphingosine and sphinganine, FTY720 modulating the sphingolipid metabolism is a definitive possibility.

Effects of FTY720 on levels of sphingoid bases

FTY720 was synthesized as a new immunosuppressant based on the chemical structure of ISP-1, which is a natural product of the sphingoid base structure extracted from Isaria sinensis, and is immunosuppressive towards T cells (Miyake *et al.,* 1995). Based on its structural similarity to sphingoid bases, it is suggested that FTY720 might be involved in the initiation of apoptosis signal triggered from the metabolites of the sphingolipid.

The changes in the concentration of sphingoid bases in the cells suggest a possible mechanism for the induction of apoptosis by FTY720. The measurement of the sphingoid bases reported by Merrill *et al.* was partially modified during the process of direct lipid extraction with the organic KOH solution in $CHCl_3$-methanol (2:1, v/v) and was applied to this study. Generally, 5-10 pmol of sphingosine in 1×10^6 cells was observed in the intact LLC-PK1 cells. FTY720 treatment induced a time-dependent increment of the endogenous sphingosine concentration over 60 pmol in 5×10^5 cells after 12 h, which is a 15-fold increase compared with the control (Figure 4). FACS analysis showed that this increase in sphingosine coincided with the increase in fragmented DNA (Figure 2B) relating the integral cellular processes leading to cell apoptosis.

Effects of FTY720 on sphingosine kinase activity

In a study evaluating FTY720 as an inhibitor of sphingosine kinase which results in increase of the sphingosine levels, FTY720 was compared with the other sphingosine kinase inhibitors, *dl-threo*-dihydrosphingosine (DHS) and *N*, *N*-dimethylsphingosine (DMS). As shown in Figure 5A, FTY720 and the two sphingosine kinase inhibitors elevated the endogenous sphingosine and sphinganine. The strong increase in the sphingosine level was observed in the order of DMS, FTY720 and DHS coinciding with the result that DMS is the strongest inducer of apoptosis. The increased sphinganine level after the DHS treatment produced a strong background due to the condition of HPLC system where DHS itself, a stereoisomer of sphinganine is not separated from the cellular sphinganine. DMS, a strong sphingosine kinase inhibitor also increases the sphinganine level. Although sphingosine accumulation by FTY720 is lower than that level induced by DMS, FTY720 has a higher specificity to accumulate sphingosine rather than sphin-

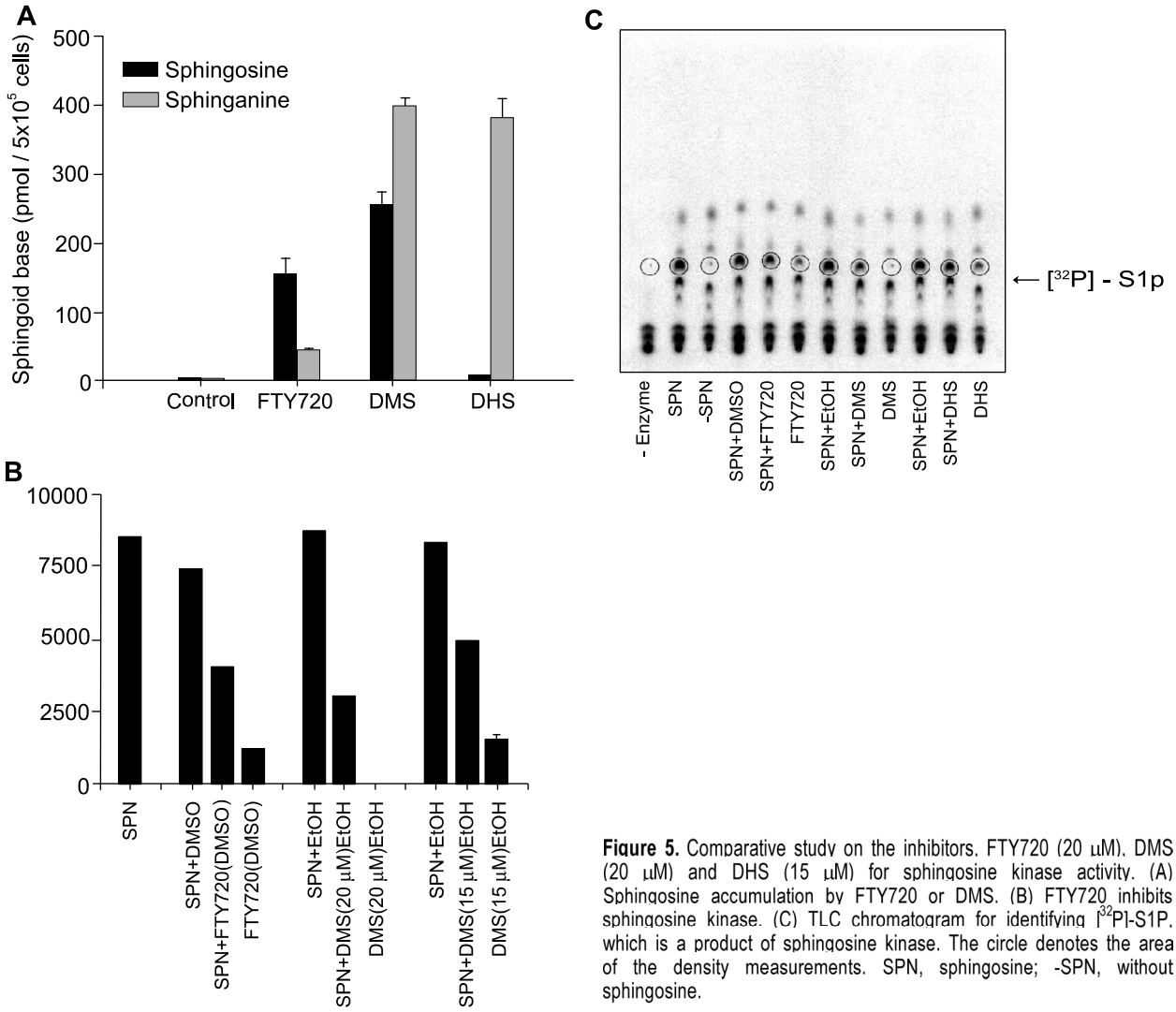

Figure 5. Comparative study on the inhibitors, FTY720 (20 μM), DMS (20 μM) and DHS (15 μM) for sphingosine kinase activity. (A) Sphingosine accumulation by FTY720 or DMS. (B) FTY720 inhibits sphingosine kinase. (C) TLC chromatogram for identifying [^{32}P]-S1P, which is a product of sphingosine kinase. The circle denotes the area of the density measurements. SPN, sphingosine; -SPN, without sphingosine.

ganine.

From the result showing that FTY720 causes the accumulation of the cellular sphingoid bases, FTY720 was speculated to inhibit sphingosine phosphorylation. FTY720 also has phosphorylation sites in its structure and is likely a competitive inhibitor of the sphingosine phosphorylation in the sphingosine kinase catalyzed reaction (Figure 5B). With the exception of DMS, phosphorylated spots of FTY720 and DHS by sphingosine kinase were observed on the TLC plate (Figure 5C).

Exogenous sphingosine increases DNA fragmentation

The sphingosine-BSA complex was introduced to the cell to examine its role in inducing apoptosis. During 12 h incubation with FTY720, 5 μM and 10 μM of the sphingosine-BSA complex, the level of fragmented DNA as an apoptotic marker increased by 43, 55 and 58%, respectively. (Figure 6A) The fragmented DNA content reached 55% when the cells were incubated with 5 μM of the sphingosine-BSA complex, which suggests that the incorporation of exogenous sphingosine is effective in triggering apoptosis.

The levels of sphingosine accumulated when the cells were treated with the various concentrations of sphingosine-BSA complexes was dose-dependent and comparable to that of the FTY720 treatment (Figure 6B). These results suggest that the sphingosine accumulation effected by inhibiting sphingosine kinase might be partially involved in the transduction of the apoptosis signal of FTY720.

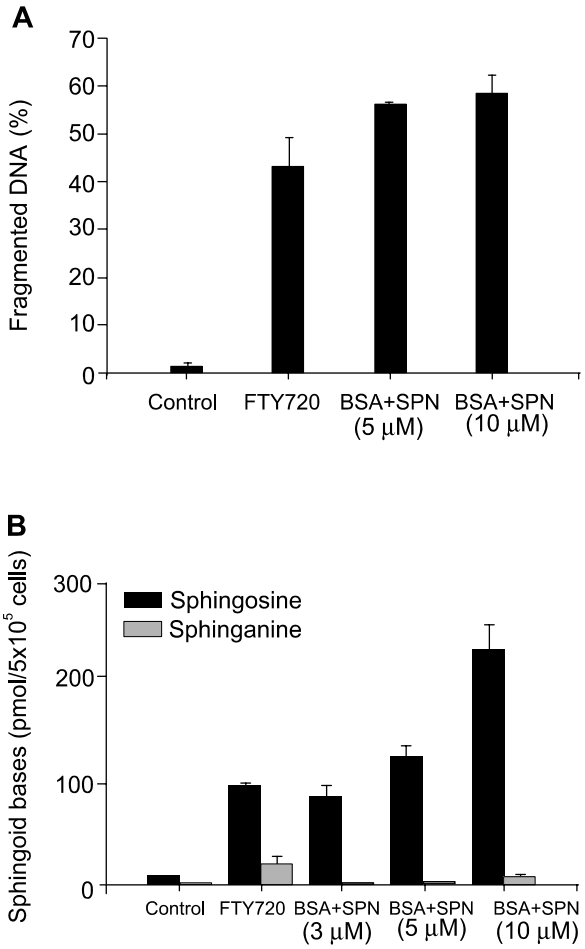

Figure 6. Correlation between apoptosis and increases of sphingosine. (A) Exogenous sphingosine induces DNA fragmentation. (B) Exogenous sphingosine increases endogenous level of the sphingosine concentration. BSA + SPN means the bovine serum albumin complex with sphingosine for exogenous application to the cells.

Discussion

This study showed that FTY720 inhibits sphingosine kinase in the LLC-PK_1 cells resulting in the elevation of cellular sphingosine, which induces apoptosis. The FTY720-induced apoptosis strongly correlated with the inhibition of sphingosine kinase in both a time- and dose-dependent manner. In a recent study, the ceramide / S1P ratio in the cells may be used as an apoptosis marker and as an indicator of sphingosine kinase inhibition (Edsall *et al.,* 1998). Intracellular sphingosine might induce apoptosis either by itself or by the re-acylation to ceramide. This study demonstrated that the accumulation of intracellular sphingosine might be a valuable marker of apoptosis, even though the concentrations of ceramide or S1P in the cells were not measured. In comparing the elevated sphingoid bases among the sphingosine kinase inhibitors, DMS increased the sphingosine and sphinganine levels, and revealed to be a strong inducer of apoptosis while DHS and FTY720 inhibited the sphingosine kinase activity to a lesser degree. The BSA-sphingosine complex enhanced its introduction into cells, and showed strong endogenous accumulation resulting cell apoptosis. These results suggest that an increase in the endogenous sphingoid bases is a major factor for inducing apoptosis. This hypothesis may be controversial in that the induction of apoptosis was attenuated by a pre-treatment with fumonisin B_1, which interrupts the de novo sphingolipid synthesis including ceramides (Bose *et al.,* 1995). Fumonisin B_1 selectively inhibits sphinganine N-acyltransferase and rapidly accumulates sphinganine quite early. Among sphingosine kinase inhibitors tested, FTY720 preferentially increased the sphingosine level rather than sphinganine. Unlike the other inhibitors, the specific increase in sphingosine by FTY720 demonstrates its different regulation of the sphingolipid metabolism. Although FTY720 was revealed to be an effective sphingosine kinase inhibitor, its immunosuppressive activity will require a further study. The sphingolipid metabolism might also be involved in the immunosuppressive activity.

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