Sphinganine Causes Early Activation of JNK and p38 MAPK and Inhibition of AKT Activation in HT-29 Human Colon Cancer Cells

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Abstract. *The sphingoid base sphinganine induces apoptosis in HT-29 human colon cancer cells more potently than other bioactive sphingolipid metabolites sphingosine and C_2-ceramide tested in our previous study. The objective of this study was to investigate the effect of sphinganine, at a concentration that induces apoptosis, on the mitogen activated protein kinases (MAPKs) including ERK1/ERK2, JNK2/JNK1, and p38 MAPK and AKT (protein kinase B), which regulate cell proliferation and apoptosis. HT-29 cells were cultured with sphinganine at 35 μM and the protein expression and phosphorylation status of ERK1/ERK2 (p44/p42), JNK2/JNK1 (p54/p46), p38 MAPK, and AKT were determined using Western blot analysis. Sphinganine clearly increased the active phosphorylated forms of JNK2/JNK1 and p38 MAPK after 15, 30, and 60 min treatment, with minimal effects on activation of ERK1/ERK2. Sphinganine weakly inhibited the phosphorylation of AKT at ser473 after 30 and 60 min. Sphinganine had little or no effect on the protein expression level of any of the kinases. The findings are consistent with a mechanism by which sphinganine induces apoptosis in HT-29 cells via early and strong activation of JNK and p38 MAPK and weak inhibition of AKT activation.*

Sphingolipids are found in all eukaryotic cell membranes, some prokaryotes, and also in a variety of foods including dairy and soy products (1, for review: 2). Sphingolipids have gained much attention for their potential to protect against the development of colon cancer (for review: 2). Complex dietary sphingolipids including sphingomyelin, dihydrosphingomyelin, glucosylceramide, lactosylceramide, and ganglioside GD_3 reduced aberrant colonic foci in CF1 mice treated with DMH (3-8) and the number of tumors in all regions of the intestine in Multiple Intestinal Neoplasia (Min) mice with a truncated Adenomatous Polyposis Coli (APC) gene product (8, 9).

This protective role of sphingolipids against colon carcinogenesis may be the result of conversion of complex sphingolipids to bioactive metabolites including sphingoid bases (sphingosine and sphinganine) and ceramide, which inhibit proliferation and induce apoptosis (programmed cell death) in various cancer cells (10, 11). For example, in HT-29 and HCT-116 human colon cancer cells, ceramide and sphingoid bases induce apoptosis with sphinganine being the most potent of the metabolites studied (11).

A possible mechanism by which sphingoid bases and ceramide could inhibit proliferation and induce apoptosis is by altering the balance of anti-apoptotic and pro-apoptotic signaling through mitogen activated protein kinases (MAPKs) and AKT. MAPKs are a family of serine/threonine kinases with major isoforms including extracellular signal-regulated kinase (ERK1/ERK2, p44/p42), c-Jun NH_2-terminal kinase (JNK2/JNK1, p54/p46, also called stress-activated protein kinase (SAPK)), and p38 MAPK (12-16). JNK and p38 MAPK appear to have pro-apoptotic roles. Though ERK activation has most often thought of as mitogenic and anti-apoptotic, recent evidences suggest that ERK activation is not always required for mitogenic (17) and anti-apoptotic (18) effects. Jarvis *et al.* (19) reported that sphingosine-mediated lethality is largely associated with weak stimulation of JNK2/JNK1, strong inhibition of ERK1/ERK2, and enhanced p38 MAPK activity in U937 human monoblastic leukemia cells.

Alternatively, sphingosine could induce apoptosis by inhibiting AKT (protein kinase B). AKT is a cytoplasmic

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serine/threonine kinase that promotes cell survival and inhibits apoptosis (for review: 20, 21). Enhanced AKT activity is associated with cancer progression in various tissues (22-25). Chang *et al.* (18) demonstrated that sphingosine induced apoptosis by suppressing phosphorylated/active forms of AKT in human hepatoma cells.

The hypothesis to be tested by this study is that sphinganine may inhibit proliferation and induce apoptosis by activation of MAPKs and suppression of AKT activation in HT-29 human colon cancer cells. The effect of sphinganine on protein expression and phosphorylation of MAPKs (ERK1/ERK2, JNK2/JNK1, p38) and AKT was thus examined at a concentration that induces apoptosis in HT-29 cells.

Materials and Methods

Sphinganine preparation. Sphinganine (D-*erythro*-dihydrosphingosine) was obtained from Matreya Co. (cat. no. 1831, State College, PA). Stock solutions of sphinganine were prepared by dissolving sphinganine in ethanol at 50 mM and stored at 4˚C. A working solution of sphinganine was prepared as a 1:1 complex with 1 mM bovine serum albumin (BSA) dissolved in phosphate buffered saline (PBS) and stored at 4˚C up to one month. Prior to use of sphinganine for experiments, sphinganine was sonicated for 1 h at room temperature to ensure sphinganine-BSA complex formation.

Cell culture. HT-29 human colon cancer cells were purchased from American Type Culture Collection (Rockville, MD). HT-29 cells were cultured in 100 mm dishes (Corning, Cambridge, MA) containing Dulbeco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA), 3.5 g/L glucose and 2.5 mL/L penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA) at 37˚C supplied with 5% CO_2 and humidified air. All experiments were performed with cell passage ≤20.

Isolation and quantitation of cellular protein. Confluent HT-29 cells were trypsinized and seeded at 2 x 10^5 cells/mL (*i.e.* 1.8 x 10^4/cm^2 growth area) in 100 mm dish and cultured in 5 mL of DMEM supplemented with 10% FBS, 3.5 g/L glucose and 2.5 mL/L penicillin-streptomycin for 24 h before treatment with sphinganine. Then the medium was replaced with 5 mL of DMEM supplemented with 1% FBS, 3.5 g/L glucose, and 2.5mL/L penicillin-streptomycin and sphinganine was added directly to each dish for various culture periods. Cells on ice were extracted with lysis buffer (1.5 M Tris-Cl, pH 8.8, 100mM sodium vanadate, 20% SDS) and cell lysates were sonicated for 5-10 sec with a probe sonicator and stored at -80˚C until use. The cellular protein was quantitated *via* the Bradford assay (Bio-Rad Laborotories, Hercules, CA) (26).

Western blot analysis for protein expression and phosphorylated forms of ERK1/ERK2, JNK2/JNK1, p38 MAPK, and AKT. Protein (20 μg for both phospho-ERK, JNK, p38 and ERK, JNK, p38; 60 μg for phospho-AKT-ser473 and AKT) was applied and separated on 10% SDS-PAGE gel under reducing conditions at 80 V for 20 min and 120 V for 1.5 h and transferred to PVDF membranes at 30 V overnight. The blots were washed three times and blocked with TBST (100 mM Tris-Cl pH 7.5, 0.9% (=150 mM) NaCl, 0.1% Tween-20) containing 5% BSA for 8 h at 4˚C and washed twice with TBST. The blots were incubated with primary antibodies (Cell signaling/New England Biolabs, Beverly, MA), (1:10,000 for anti-rabbit IgG polyclonal antibodies against phospho-ERK1/ERK2, phospho-JNK2/JNK1, phospho-p38, ERK1/ERK2, JNK2/JNK1, and p38; 1:1,000 for phospho-AKT-ser473 and AKT) in blocking buffer (5% BSA in TBST) with gentle shaking overnight (~18 h) at 4˚C. After removal of the primary antibodies and washing four times with TBST, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Cell signaling, Beverly, MA), (1:20,000 for phospho-ERK1/ERK2, phospho-JNK2/JNK1, phospho-p38 and ERK1/ERK2, JNK2/JNK1, and p38; 1:2,000 for phospho-AKT-ser473 and AKT) for 1 h at room temperature. The blots were detected with western blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or Supersignal West Dura Extended Duration Chemiluminescent Substrate (Pierce Biotechnology Inc., Rockford, IL). The blots were exposed to X-ray film for 1-10 min.

Densitometry. The density of bands was estimated using the GelExpert (San Mateo, CA) or Kodak ID 3.6 computer software program (Kodak Scientific Imaging System, New Haven, CT) and was expressed in % of time zero. Then each value for phosphorylated forms of ERK1, ERK2, JNK2, JNK1, p38, and AKT-ser473 was divided by the corresponding control for total ERK1, ERK2, JNK2, JNK1, p38, and AKT, respectively.

Results

To determine the effects of sphinganine on the status of protein expression and phosphorylation/activation of ERK1/ERK2, JNK2/JNK1, p38 MAPK, and AKT, sub-confluent HT-29 cells were cultured with sphinganine at 35 μM, which induced apoptosis in our previous study (11). Cellular protein was isolated and the kinases were evaluated using Western blot analysis.

Sphinganine has small effects on early activation of ERK1 and ERK2. Sphinganine at 35 μM had small effects on the increase of active phosphorylated forms of ERK1/ERK2 (p44/p42) at 15, 30 and 60 min compared to the corresponding control, while having little or no effect on protein expression levels of ERK1/ERK2 (Figure 1).

Sphinganine causes early activation of JNK2 and JNK1. Phosphorylated forms of JNK2/JNK1 were virtually undetectable in controls (Figure 2). Sphinganine at 35 μM clearly increased the active phosphorylated forms of JNK2/JNK1 (p54/p46) in time-dependent manner compared to the corresponding control, while having little or no effect on protein expression levels of JNK2/JNK1. The strongest activation/phosphorylation of JNK2/JNK1 was observed at 60 min (Figure 2).

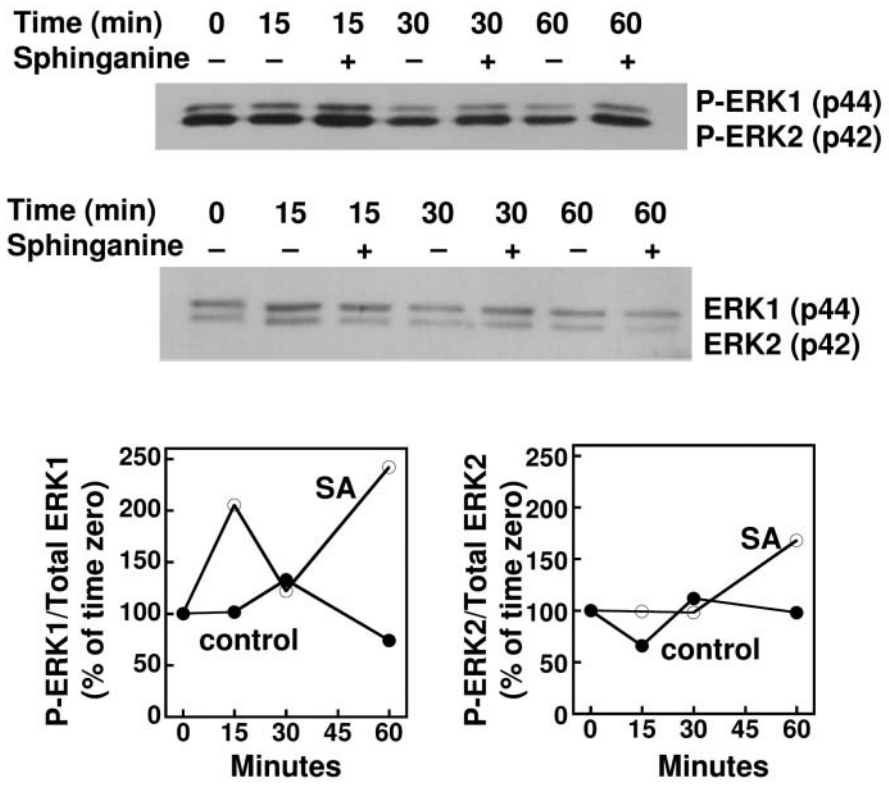

Figure 1. *Effects of sphinganine on activation of ERK1/ERK2 in HT-29 cells. Sub-confluent cells were treated without (●) or with sphinganine (○, SA) at 35 μM for 0, 15, 30, and 60 min. Cellular proteins were isolated, quantitated via Bradford assay and analyzed via Western blotting. The density of bands was calculated using Kodak ID 3.6 computer software program. Each value for phosphorylated forms of ERK1 and ERK2 (P-ERK1 and P-ERK2) was divided by the corresponding control for total ERK1 and ERK2 and then expressed in % of time zero. The two independent experiments were performed and consistent results were observed and representative data are shown.*

Sphinganine causes early activation of p38 MAPK. Phosphorylated forms of p38 MAPK were almost undetectable in controls (Figure 3). Sphinganine at 35 μM clearly increased the active phosphorylated forms of p38 MAPK at 15, 30 and 60 min compared to the corresponding control, while having little or no effect on protein expression levels of p38 MAPK. The strongest activation/phosphorylation of p38 MAPK was observed at 60 min (Figure 3).

Sphinganine causes weak inhibition of phosphorylation of AKT-ser473. Sphinganine at 35 μM weakly inhibited the active phosphorylated forms of AKT-ser473 at 30 and 60 min, while had little or no effect on protein expression level of AKT (Figure 4).

Discussion

Most studies examining sphingolipids have focused on the pro-apoptotic actions of ceramide (acylated form of sphingoid bases) and sphinganine. Furthermore, it is uncertain whether the sphingosine or sphinganine backbones of ceramides mediate anti-proliferative and pro-apoptotic activities of ceramide on cancer cells (27, 28; for review: 29, 30).

In the current study, we focused on effects of sphinganine since we previously found that sphinganine most potently induced apoptosis within 24 h among the metabolites sphingosine, sphinganine, and ceramide tested in HT-29 human colon cancer cells (11). HT-29 cell line is a useful model to study cancer chemotherapy because this cell line possesses adherent growth property and epithelial morphology and is tumorigenic in nude mice (31) and carries mutations in genes related to carcinogenesis and apoptosis such as APC (32) and p53 (33, 34) tumor suppressor genes. HT-29 cells express *c-myc, Myb, sis*, and *fos* oncogenes (35) and catalytically inactive cyclooxygenase 2 (36, 37).

We found that sphinganine at an apoptosis-inducing concentration causes strong activation of JNK and p38 MAPK after 15, 30 and 60 min with maximum activation at 60 min in HT-29 cells. The relatively greater activation of p38 MAPK at 15 min than at 30 min might be associated with the "burst" of sphinganine and sphingosine that occurs when cells in culture are changed to fresh medium (38). In

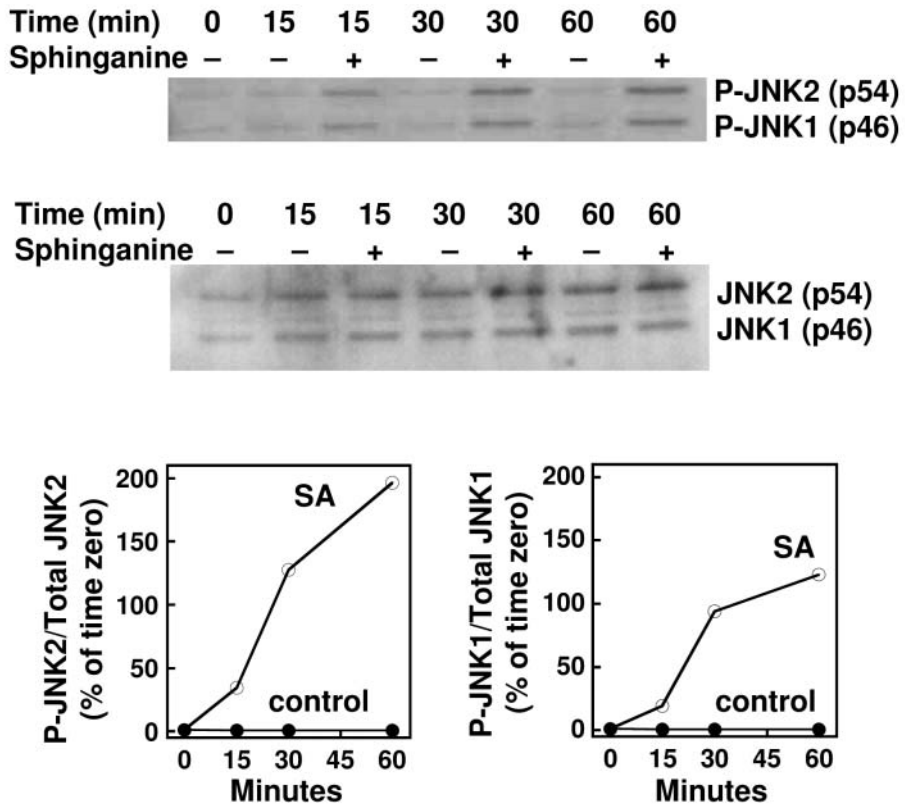

Figure 2. *Sphinganine causes early activation of JNK2/JNK1 in HT-29 cells. Sub-confluent cells were treated without (●) or with sphinganine (○, SA) at 35 μM for 0, 15, 30, and 60 min. Cellular proteins were isolated, quantitated via Bradford assay and analyzed via Western blotting. The density of bands was calculated using Gel Expert and Kodak ID 3.6 computer software program. Each value for phosphorylated forms of JNK2 and JNK1 (P-JNK2 and P-JNK1) was divided by the corresponding control for total JNK2 and JNK1 and expressed in % of time zero. The two independent experiments were performed and consistent results were observed and representative data are shown.*

order to establish the causative relationship of JNK and p38 activations by sphinganine with induction of apoptosis, future study can co-treat the HT-29 cells with sphinganine and the inhibitors of JNK or p38 to determine if the treatment of the inhibitors can abolish the induction of apoptosis by sphinganine. In previous studies by others (19, 39), C_2-ceramide increased JNK activity with maximum activity at 15 min in HT-29 cells (39). Sphingosine-mediated lethality of U937 human monoblastic leukemia cells was largely associated with weak stimulation of JNK2/JNK1 and enhanced p38 MAPK activity (19).

It is noteworthy that several chemotherapeutic drugs including indomethacin (a nonsteroidal anti-inflammatory drug) (39) and tamoxifen (40) cause turnover of complex sphingolipids to bioactive sphingolipid metabolites, such as ceramide and/or sphingoid bases, and induce apoptosis by stimulating JNK activity. Our findings might suggest that the sphingoid base sphinganine might mediate the apoptotic effect of certain chemotherapeutic drugs in cancer cells, in part, by activating JNK.

In the present study, sphinganine had a small effect on ERK activation. The relatively greater activation of ERK1/ERK2 at 15 min for both control and sphinganine-treated cultures compared to other groups at 0, 30 and 60 min might be associated with the "burst" of sphinganine and sphingosine that occurs when cells in culture are changed to fresh medium (38). Our results are in contrast to a previous study by Kim *et al.* (39) who found that 50 μM C_2-ceramide inhibited ERK activity in HT-29 cells as early as 15 min and inhibition was sustained for up to 3 h. In a recent study (41), phenethylisothiocyanate sphinganine (a sphinganine derivative) was found to inhibit ERK1/ERK2 activity after 15 through 90 min treatment in HL-60 human promyelocytic leukemia cells. Differing culture conditions during the treatment (*e.g.* 1% FBS in the present study *versus* 10% FBS in other studies by Kim *et al.* (39) and Johnson *et al.* (41)) may have contributed to the disparity in results between the studies.

Alternatively, sphingosine could induce apoptosis by inhibiting AKT (protein kinase B). AKT activation is

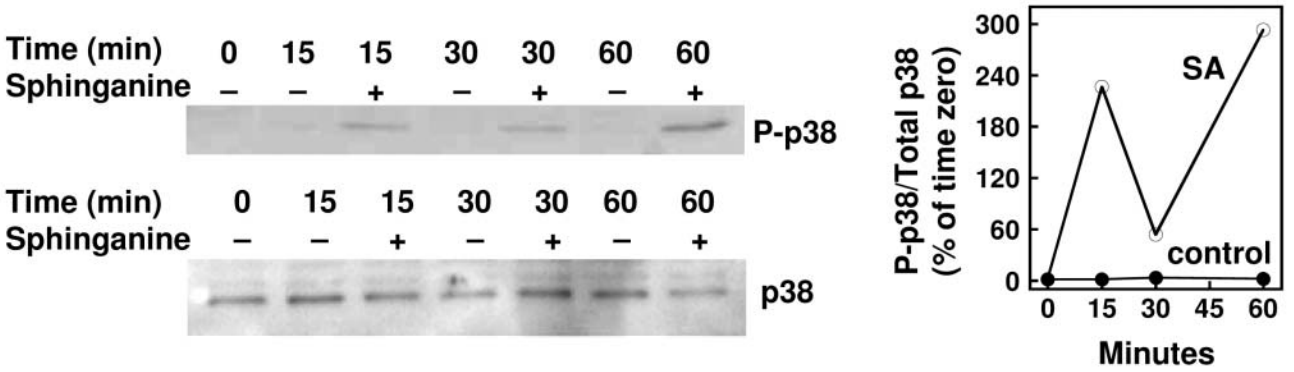

Figure 3. *Sphinganine causes early activation of p38 MAPK in HT-29 cells. Sub-confluent cells were treated without (●) or with sphinganine (○, SA) at 35 μM for 0, 15, 30, and 60 min. Cellular proteins were isolated, quantitated via Bradford assay and analyzed via using Western blotting. The density of bands was calculated using Gel Expert and Kodak ID 3.6 computer software program. Each value for phosphorylated forms of p38 (P-p38) was divided by the corresponding control for total p38 and then expressed in % of time zero. The two independent experiments were performed and consistent results were observed and representative data are shown.*

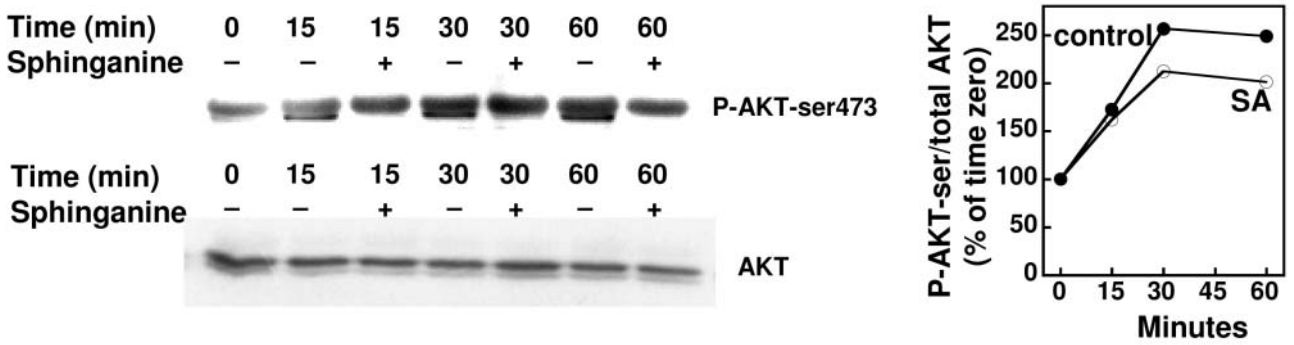

Figure 4. *Sphinganine causes early and weak inhibition of phosphorylation of AKT-ser473 in HT-29 cells. Sub-confluent cells were treated without (●) or with sphinganine (○, SA) at 35 μM for 0, 15, 30 and 60 min. Cellular proteins were isolated, quantitated via Bradford assay and analyzed using Western blotting. The density of bands was calculated using Kodak ID 3.6 computer software program. Each value for phosphorylated forms of AKT-ser473 (P-AKT-ser473) was divided by the corresponding control for total AKT and then expressed in % of time zero. The two independent experiments were performed and consistent results were observed and representative data are shown.*

achieved by phosphorylations at ser473 and thr308 residues (20). The current study showed that sphinganine weakly inhibited the phosphorylated/active forms of AKT-ser473 in HT-29 cells. Our findings are consistent with those of Chang *et al.* (18) who showed that sphingosine induced apoptosis and suppressed activation of AKT in human hepatoma cells. Moreover, apoptosis induced by sphingosine was attenuated in cells transfected with constitutively active AKT (18). Similarly, a future study can transfect HT-29 cells with constitutively active AKT to determine if the induction of apoptosis by sphinganine can be reduced. This will answer if the inhibition of AKT activation by sphinganine is directly responsible for inducing apoptosis.

Future studies should determine the cellular concentration of sphingoid bases and ceramide after addition of exogenous sphinganine over the similar time periods to the times tested for ERK, JNK, p38, and AKT in the current study. This will answer how much sphinganine added to cell culture are actually bound to cellular membrane and taken up by cells. In addition, it will be interesting to compare the effect of fumonisin B_1 (a mycotoxin that inhibits ceramide synthase in the *de novo* biosynthetic pathway of sphingolipids causing depletion of complex sphingolipids and accumulation of sphinganine) (42) on apoptosis and these kinases in order to know whether fumonisin B_1 mimics the effect of sphinganine or not. It is suggested to co-treat HT-29

cells with fumonisin B_1 and sphinganine to determine whether fumonisin B_1 can be synergetic with sphinganine or interfere the action of sphinganine by blocking its incorporation into ceramide. This approach will clarify if sphinganine is indeed the major player inducing apoptosis by regulating JNK, p38, and AKT and it is not through the conversion of sphinganine into ceramide.

Based on studies of uptake and partitioning of short-chain ceramides, Hannun and Luberto (43) suggested that short-chain ceramides are active at physiologically relevant concentrations (43). Specifically, when short-chain ceramides at 1-20 μM are added to $2x10^6$ - $10x10^6$ cells/mL, they reach cellular concentrations that are within an order of magnitude of the endogenous levels of ceramide (43). Abnet *et al.* (44) reported that median serum concentrations for the study population (265 residents of Linxian, People's Republic of China) were 52 nmol/L for sphinganine and 64 nmol/L for sphingosine. Further investigations are needed to know if the physiologically relevant and apoptosis-inducing concentration of sphingoid bases can be achieved by consumption of dietary sphingolipids for colon cancer patients.

In conclusion, the current study demonstrates that sphinganine at a concentration-inducing apoptosis causes early (15 through 60 min) and strong activation of JNK and p38, with small effect on ERK activation, and weak inhibition of AKT activation in HT-29 cells. The findings suggest that JNK, p38 MAPK, and AKT might mediate the action of sphinganine to induce apoptosis of human colon cancer cells.

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