Sphingoid bases from sea cucumber induce apoptosis in human hepatoma HepG2 cells through p-AKT and DR5

ZAKIR HOSSAIN[1,2], TATSUYA SUGAWARA[1] and TAKASHI HIRATA[1]

[1]Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan;
[2]Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

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Abstract. Biofunctional marine compounds have recently received substantial attention for their nutraceutical characteristics. In this study, we investigated the apoptosis-inducing effects of sphingoid bases prepared from sea cucumber using human hepatoma HepG2 cells. Apoptotic effects were determined by cell viability assay, DNA fragmentation assay, caspase-3 and caspase-8 activities. The expression levels of apoptosis-inducing death receptor-5 (DR5) and p-AKT were assayed by western blot analysis, and mRNA expression of Bax, GADD45 and PPARγ was assayed by quantitative RT-PCR analysis. Sphingoid bases from sea cucumber markedly reduced the cell viability of HepG2 cells. DNA fragmentation indicative of apoptosis was observed in a dose-dependent manner. The expression levels of the apoptosis inducer protein Bax were increased by the sphingoid bases from sea cucumber. GADD45, which plays an important role in apoptosis-inducing pathways, was markedly upregulated by sphingoid bases from sea cucumber. Upregulation of PPARγ mRNA was also observed during apoptosis induced by the sphingoid bases. The expression levels of DR5 and p-AKT proteins were increased and decreased, respectively, as a result of the effects of sphingoid bases from sea cucumber. The results indicate that sphingoid bases from sea cucumber induce apoptosis in HepG2 cells through upregulation of DR5, Bax, GADD45 and PPARγ and downregulation of p-AKT. Our results show for the first time the functional properties of marine sphingoid bases as inducers of apoptosis in HepG2 cells.

Correspondence to: Dr Zakir Hossain, Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada
E-mail: hossain5@cc.umanitoba.ca

Abbreviations: DR, death receptor; GADD, growth arrest DNA damage; PPAR, peroxisome proliferator-activated receptor; GlcCer, glycosylceramides

Key words: sea cucumber, sphingoid bases, apoptosis, human hepatoma cells

Introduction

The highest liver cancer rates are found in East and South East Asia and in Central and Western Africa. Developing countries contribute more than 80% of the cases. Chronic hepatitis B virus infection accounts for approximately 60% of the total liver cancer cases in developing countries and for approximately 23% of the cancer cases in developed countries, while hepatitis C virus infection accounts for approximately 33% of the total liver cancer cases in developing countries and for approximately 20% in developed countries (1,2). Diet is involved in the risk of hepatocellular carcinoma (HCC). It has been shown that red meat and saturated fat are associated with increased chronic liver disease and HCC. A diet that is rich in polyunsaturated fatty acids and, possibly, β-carotene can reduce the risk of HCC (3). Therefore, an appropriate diet might be a potential tool to prevent HCC.

Sphingolipids and their metabolites, including ceramides, sphingosine, and sphingosine-1-phosphate, play important roles in cellular signaling, such as in proliferation, apoptosis, cellular senescence, growth arrest and differentiation in a variety of cells (4,5). Ceramide is generated from sphingomyelin upon diverse apoptotic stimuli and transfers the death signal, which is the so-called second messenger. Ceramide induces apoptosis by inhibiting a variety of pro-growth signal kinases, including AKT and PKC, with direct activation of protein phosphatases (6). Sphingolipids have a dual role within the cell, acting as key structural components of membranes as well as serving as bioactive second messengers. Central to the chemical backbone of all sphingolipids, sphingosine represents an intermediate linking the metabolism of ceramide to sphingosine-1-phosphate. Cellular levels of sphingosine are controlled by the action of ceramide synthase or sphingosine kinase, which are responsible for the formation of ceramide and sphingosine-1-phosphate, respectively. Conversely, sphingosine may be formed by the action of ceramidase from ceramide or sphingosine-1-phosphatase from sphingosine-1-phosphate (7,8).

Physiologically active substances including glycosylceramide (GlcCer) and related compounds have been extracted from a variety of sea cucumber species (9,10). Dry sea cucumber contains ~200 mg glycosylceramides per 100 g (11). GlcCer used as a food ingredient has been isolated from various plant sources, but their content is very low (1-40 mg/100 g

dry weight) (12). Thus, sea cucumber may be suitable as a dietary source of sphingolipids. However, the sphingoid base structures in sea cucumber are more complicated than those in mammals and there is little information regarding the nutritional function of these sphingoid bases that are not found in mammals (13,14). Sphingoid bases prepared from wheat flour cerebroside were found to suppress the growth of human colorectal cancer DLD-1 cells through apoptosis (15). Apoptosis was also induced in human colon cancer cells by plant and fungus sphingoid bases (16). In addition, sphingosine induced apoptosis in hippocampal neurons and astrocytes by activating caspase-3/-9 (17).

Peroxisome proliferator-activated receptor γ (PPARγ) has been identified and extensively studied. Several recent studies have shown that most human tumors express PPARγ, and there is evidence that PPARγ ligands have antitumor activity (18-25). PPARγ has been the focus of research as a target molecule to prevent cancer (26-28). The upregulation of PPARγ by bitter gourd seed oil containing 9-cis, 11-trans, 13-trans-conjugated linolenic acid was observed (29). In addition, the Bax and the growth arrest and DNA damage inducible gene 45 (GADD45) are known to have important roles in the pathways of growth inhibition and apoptosis induction in many types of cancer cells (30,31). Increased expression of GADD family genes is frequently observed in response to agents that trigger apoptosis (32,33). In this study, the possible pathways of apoptosis induced by sphingoid bases from sea cucumber in human hepatoma HepG2 cells were examined.

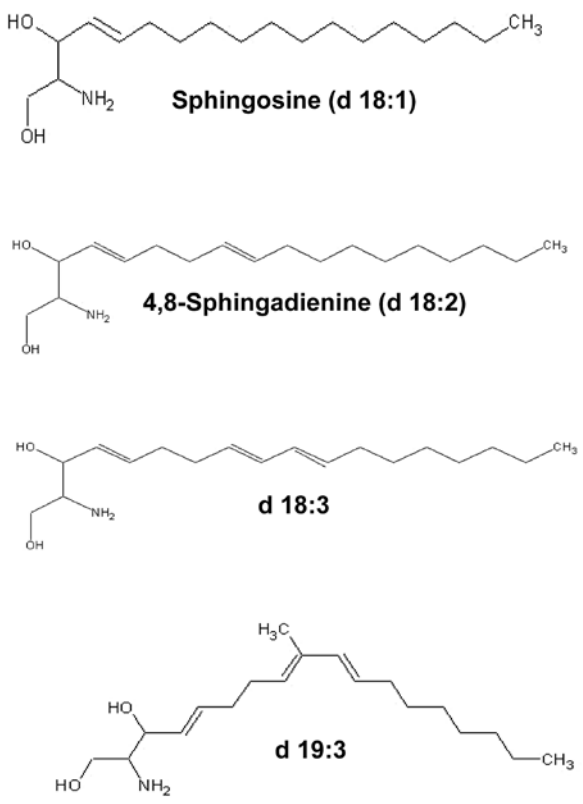

Figure 1. Chemical structure of sphingoid bases extracted from the total lipid of sea cucumber.

Materials and methods

Preparation of sphingoid bases. Sphingoid bases were prepared from sea cucumber and maize by a silica gel column after lipid extraction and saponification as previously described (11,34). 4,8-Sphingadienine (d18:2) from maize sphingoid bases and 4,8,10-sphingatrienine (d18:3) and 9-methyl-4,8,10-sphingatrienine (d19:3) from sea cucumber sphingoid bases (Fig. 1) were purified by HPLC (11,34).

Cell culture. HepG2 cells were obtained from the Human Science Research Resources Bank, Osaka, Japan. HepG2 cells were grown in 100-mm dishes containing Dulbecco's modified Eagle's medium with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-incubated fetal bovine serum in a 95% air-5% CO_2 atmosphere at 37˚C. Subculturing was carried out every 3 days at a concentration of $5x10^4$ cells/ml.

Cell viability assay. Aliquots of $1x10^5$ HepG2 cells were seeded into 96-well microplates containing 100 μl of growth medium/well. The plates were preincubated without any treatment for 24 h at 37˚C. The sphingoid bases were dissolved in ethanol and diluted into the culture medium (the final concentration was 0.1%). Control was maintained with 0.1% ethanol as vehicle. The plates were incubated for another 24 h at 37˚C with the sphingoid bases. The WST-1 reagent (3.3 mg/ml phosphate-buffered saline including 7% 1-methoxy PMS) was added at 10 μl in each well containing 100 μl of medium with the cells. The plates were incubated an additional 2 h at 37˚C. Absorbance was measured at 650 nm on a microplate reader (Bio-Rad, Sunnyvale, CA, USA).

Enzyme-linked immunosorbent assay (ELISA) for apoptosis. DNA fragmentation was measured by a biotin-labeled anti-histone-antibody and a peroxidase-conjugated anti-DNA antibody cell death detection ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany). In brief, HepG2 cells ($2x10^3$ cells/well) were plated in 96-well plates with 100 μl of medium and incubated for 24 h. After a 24-h incubation, cells were treated with the sphingoid bases for 24 h. The level of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in HepG2 cells was expressed as an enrichment factor, calculated using the following formula: Enrichment factor = Absorbance of sample (treated)/Absorbance of corresponding control.

Caspase-3 and -8 activities. Caspase-3 and -8 activities were measured using a caspase-3 and -8 colorimetric assay kit (BioVision Research Products, Mountain View, CA, USA). HepG2 cells were treated with the sphingoid bases for 24 h, then harvested and lysed with chilled cell lysis buffer and incubated on ice for 10 min. Cell lysates were clarified by centrifugation at 10,000 x g for 1 min at 4˚C. The protein concentration in the supernatant was quantified following the method of Lowry *et al* (35), and 100 μg of protein was added to 50 μl of lysis buffer for each assay. Fifty microliters of 2X reaction buffer (containing 10 mM dithiothreitol, DTT) was added to each sample. For caspase-3 and -8 assays, samples were incubated with Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) substrate for 2 h at 37˚C. Optical density reading was taken at a wavelength of 405 nm on a microplate reader.

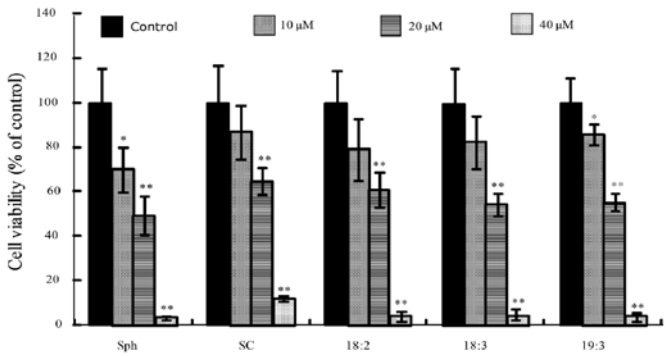

Figure 2. Cell viability of HepG2 cells treated for 24 h with different concentrations of sphingosine (Sph), sphingoid bases extracted from sea cucumber (SC), 4,8-sphingadienine (18:2), d18:3 and d19:3. Values are means ± SD. Statistical significance; *P<0.05 and **P<0.01 as compared to the non-treated control.

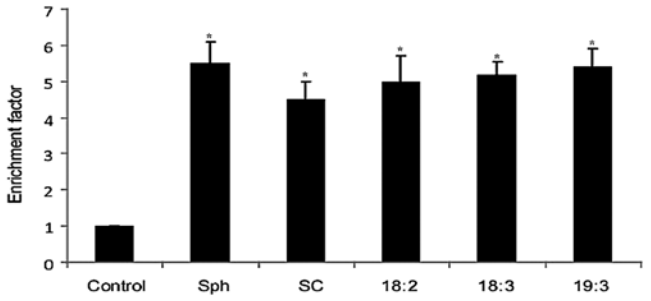

Figure 3. Enrichment factor increment in HepG2 cells treated with sphingosine (Sph), sphingoid bases extracted from sea cucumber (SC), 4,8-sphingadienine (18:2), d18:3 and d19:3. Cells were preincubated without any treatment for 24 h followed by treatment with Sph, SC, 4,8-sphingadienine (18:2), d18:3 and d19:3 for 48 h. Apoptosis was quantified using an ELISA assay. Results are represented as the relative fold increase in apoptosis as compared with the control. The bars represent ± SD (n=4). *P<0.01 vs. control.

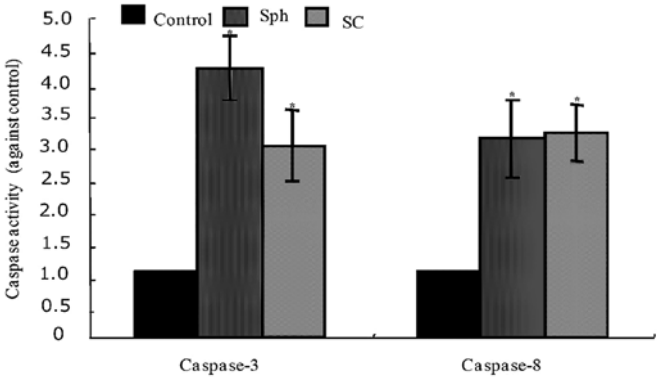

Figure 4. Activity of caspase-3 and -8 in HepG2 cells treated for 24 h with sphingosine (Sph), and sphingoid bases extracted from sea cucumber (SC). Values are means ± SD. Statistical significance; *P<0.01 as compared to the non-treated control.

Quantitative real-time RT-PCR for mRNA analysis. Quantitative real-time PCR was performed to measure the mRNA levels of the studied genes using a Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR-Green PCR reagents. Total RNA was extracted from the HepG2 cells treated with sphingoid bases using Sepasol-RNA I Super (Nacalai Tesque Inc., Kyoto, Japan). The following primers were used: PPAR-γ, 5'-AAGGCCATTTTC TCAAACGA-3' (sense), 5'-TGCAACCACTGGATCTGT TC-3' (antisense); GADD45, 5'-CAGAAGACCGAAAGG ATGGA-3' (sense), 5'-CAGAAGACCGAAAGGATGGA-3' (antisense); Bax, 5'-TCTGACGGCAACTTCAACTG-3' (sense), 5'-CCTGTAATCCCAGGTCCTTG-3' (antisense). The primer pair of GAPDH was 5'-TGGGATCGAGTGAAG GACCT-3' (sense) and 5'-CTCCTCCTGCCACTTCTTC TG-3' (antisense). The reaction solution (20 μl final volume) contained 6 μl of sample, 10 μl SYBR-Green dye and 2 μl of each primer. The thermal cycling conditions were as follows: 48˚C for 30 min to prevent carrying over of DNA, an initial denaturation of 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 1 sec and an annealing temperature of 55˚C for 1 min.

Western blot analysis. Cells were grown in 100-mm culture dishes, starved of serum for 48 h and treated with sphingoid bases. They were then lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Roche, Mannheim, Germany)], 1 mM Na_3VO_4, 50 mM NaF and 10 mM EDTA. Ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes, which were then saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween-20. The blots were incubated with the anti-AKT and anti-phospho-AKT antibodies at a dilution of 1:1,000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. The blots were also incubated with the anti-DR5 and anti-actin antibodies at a dilution of 1:1,000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an Enhanced Chemiluminescence Plus kit (Amersham International, Little Chalfont, UK). The results were analyzed using a Fujifilm visualizer (LAS-3000, Fujifilm Corp., Tokyo, Japan).

Results

Inhibition of HepG2 cell proliferation by sphingoid bases. The effect of sphingoid bases from sea cucumber on the inhibition of HepG2 cell proliferation at various concentrations was tested at 24 h. Sphingoid bases inhibited cell proliferation in a dose-dependent manner. There was significant growth inhibition with 20 or 40 μM of the sphingoid bases when compared with the control (Fig. 2). The growth inhibition with sphingosine and sphingoid bases from sea cucumber was comparable (Fig. 2).

Apoptosis induction in HepG2 cells treated with sphingoid bases. To characterize the apoptosis induced by sphingoid bases, an experiment was conducted using a quantitative cell death ELISA. The enrichment factors in cells treated with 20 μM sphingoid bases (18:3 or 19:3) were increased by 5.0-fold in comparison to the control (Fig. 3). Sphingosine and sphingoid bases from sea cucumber significantly increased caspase-3 and -8 activities compared to the control (Fig. 4).

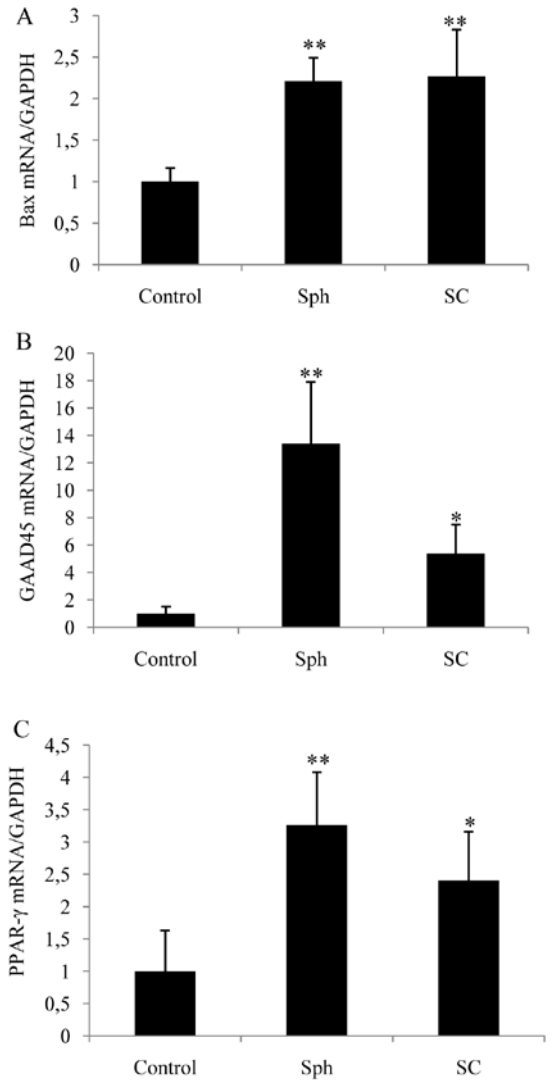

Figure 5. Effect of treatment with sphingosine (Sph) and sphingoid bases extracted from sea cucumber (SC) on the expression level of Bax (A), GADD45 (B) and PPARγ (C) mRNA in HepG2 cells. HepG2 cells were incubated in cultured medium with Sph and SC for 24 h. Expression of Bax, GADD45 and PPARγ mRNA was determined by real-time quantitative RT-PCR analysis. Data from 3 independent experiments were normalized to GAPDH mRNA levels and are shown as the means ± SD. *P<0.05 vs. control, **P<0.01 vs. control.

Bax, GADD45 and PPARγ expression in HepG2 cells treated with sphingoid bases. The expression level of Bax, GADD45 and PPARγ mRNA in HepG2 cells was increased following a 24-h incubation with 20 μM sphingoid bases. Treatment with the sphingoid bases of sea cucumber increased the expression of Bax, GADD45 and PPARγ by only 3.0-, 2.0 and 2.5-fold in the HepG2 cells whereas sphingosine increased their expression levels by 6.0-, 2.0- and 3.0-fold respectively, compared with the control (Fig. 5).

Expression of p-AKT decreased and expression of DR5 increased due to the effects of treatment with the sphingoid bases. To clarify whether the expression levels of p-AKT are associated with the effects of sphingoid bases, we measured expression of AKT and p-AKT by immunoblot analysis. AKT, which is the downstream effector of PI3-kinase was found to be phosphorylated. We found that sphingoid bases induced p-AKT inhibition and triggered apoptosis (Fig. 6A). To clarify whether the expression levels of DR5 are associated with the effects of sphingoid bases, we measured the expression of DR5 by immunoblot analysis. We found that treatment with the sphingoid bases increased expression of DR5 in the HepG2 cells (Fig. 6B).

Discussion

Dietary sphingolipids have gained attention for their potential to protect against the development of colon cancer. Sphingolipids are a family of compounds that have a long-chain (sphingoid) base backbone and include free sphingoid bases (sphingosine and sphinganine), ceramides, sphingomyelins, cerebrosides, sulfatides and gangliosides (36). Several studies have been conducted with milk sphingomyelin, synthetic sphingomyelin, synthetic dihydrosphingomyelin, as well as milk glycosphingolipids to determine 1,2-dimethylhydrazine (DMH)-induced colon cancer protection in CF1 mice. Sphingomyelin at 0.1% of the diet caused a higher percentage of adenomas and a lower percentage of more advanced adenocarcinomas. Notably, synthetic dihydrosphingomyelin (N-palmitoyl dihydrosphingomyelin) more potently reduced the number of aberrant colonic foci than synthetic sphingomyelin (N-palmitoylsphingomyelin) and milk sphingomyelin, suggesting that the 4,5-trans double bond, which is absent in dihydrosphingomyelin, is not required for the suppression of colon carcinogenesis (37). In the current study, we found that the growth suppression effects of sphingosine and sphingoid bases from sea cucumber (mixture of bases, 18:3 and 19:3), and a maize sphingoid base (18:2) in HepG2 cells were comparable. The long chain structure and 3 double bonds present in the bases of sea cucumber may be indispensable for the apoptotic effect of sphingoid bases on cancer cells.

Activation of the caspase cascade, a family of cysteine proteases, is required for apoptotic signaling. In addition to their crucial role as the energy-producing center of the cell, the mitochondria are central to the convergence of the intrinsic or extrinsic cellular signal transduction pathways that result in apoptosis. Essential to this process is the release of cytochrome *c* and other mitochondrial-related proteins that are normally confined to the mitochondrial intermembrane space by antiapoptotic Bcl-2 family members (38,39). Recent data suggest that oligomeric Bax is a component of the mitochondrial apoptosis-induced channel, and following their interaction, a selective cytochrome *c* release pore is formed (40). However, the consensus of these theories includes a requirement for Bax/Bak activation to promote pore opening and cytochrome *c* release. Our data showed an increased mRNA expression of Bax. The release of cytochrome *c* and other apoptogenic factors from injured mitochondria have been shown to activate caspases (41), and the mitochondrial integrity appears to be regulated, in part, by members of the Bcl-2 family. In response to apoptotic signals, Bax, a proapoptotic member of this family, is redistributed from the cytosol to the mitochondria, where it decreases membrane potential leading to cytochrome *c* release and caspase activation (42).

Little is known concerning the direct action of sphingolipids on the expression of genes in hepatocytes. In the

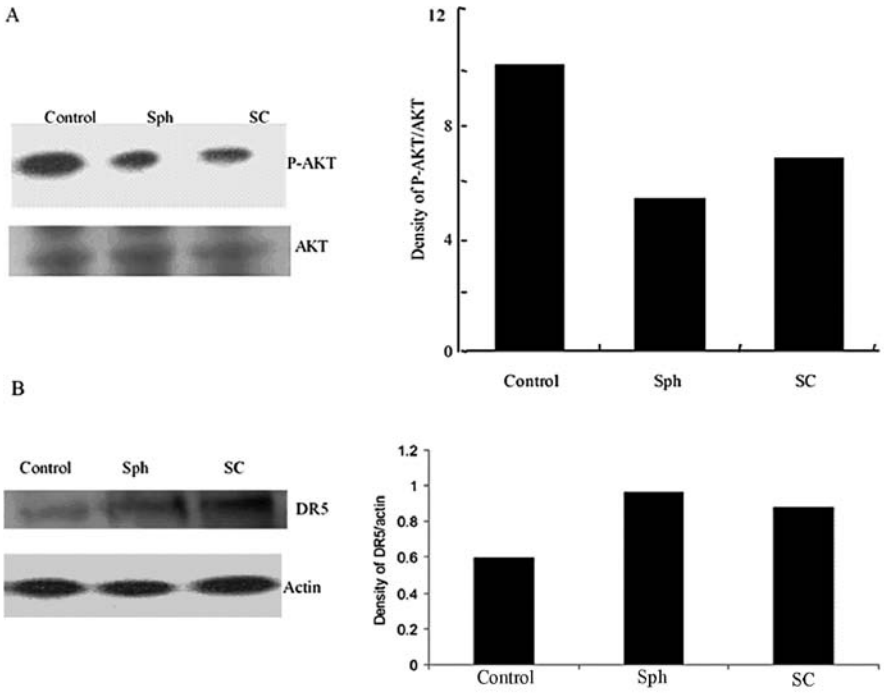

Figure 6. Effect of treatment with sphingosine (Sph) and sphingoid bases extracted from sea cucumber (SC) on the expression of p-AKT and DR5 in HepG2 cells. (A) HepG2 cells were incubated in cultured medium with Sph and SC for 24 h. Expression of p-AKT was determined by western blot analysis. Data from the experiment was normalized to AKT as a control. (B) HepG2 cells were incubated in cultured medium with Sph and SC for 24 h. Expression of DR5 was determined by western blot analysis. Data from the experiment was normalized to actin as a control.

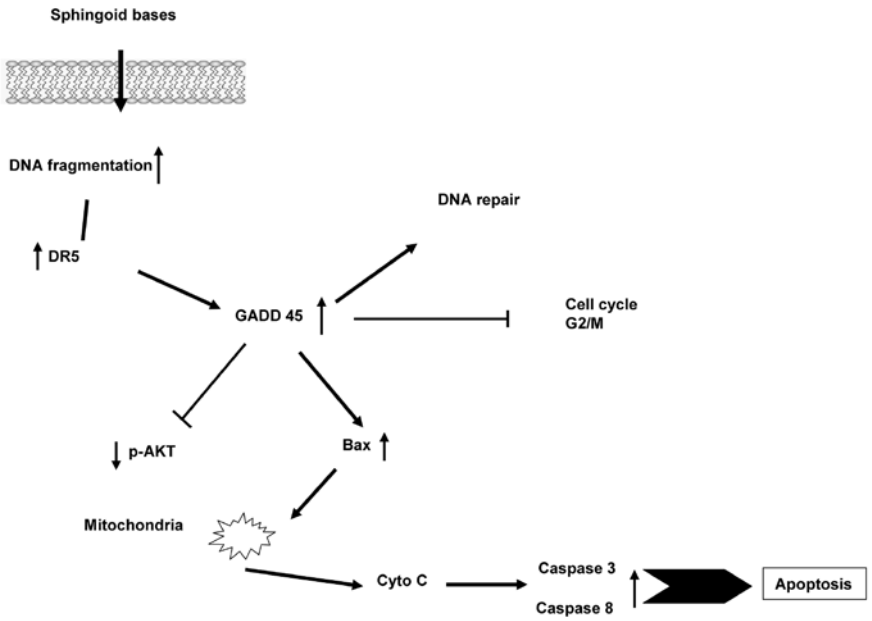

Figure 7. Illustration of the pathway of apoptosis induced by sphingoid bases extracted from sea cucumber (SC). SC upregulates DR5, GADD45, Bax, caspase-3 and -8 and downregulates p-AKT.

present study, we also found the increased mRNA expression of PPARγ in HepG2 cells. PPARγ displayed proapoptotic activity, as has been demonstrated in the HL-60 cell line (43) and in the lymphoblastic leukemia cell line (44). PPARγ liagands 15d-PGJ2 and troglitazone suppress DNA synthesis and induce apoptosis in HT-29 colon cancer cells (45). Troglitazone inhibited the growth of liver cancer cells PLC/PRF/5, HepG2 and HuH-7, by inducing apoptosis through caspase-3 activation (46). GADD45α overexpression in normal human fibroblasts and human cancer cells was found to cause G2-M arrest (47-49). GADD45α-dependent induction of apoptosis has been observed frequently in cancer cell lines (50,51), but its role in apoptosis induction in normal cells has been controversial. GADD45α has been

shown to induce cell cycle arrest in normal fibroblasts but failed to induce apoptosis (48). Others, however, have shown Gadd45α-dependent induction of apoptosis in normal epithelial cells (52). In the present study, we showed that GADD45 expression correlated with a significant increase in apoptosis in HepG2 cells. In this study, we characterized the functional role of AKT in the apoptotic pathway related to the sphingoid bases and we found that inhibition of the activation of AKT is one of the mechanisms by which sphingoid bases induced apoptosis. Our findings concurred with the results of Chang *et al* (5) who found that sphingosine induced apoptosis by inhibiting AKT expression. AKT is known to inhibit apoptosis by inactivating proapoptotic proteins such as Bad, procaspase-9, and forkhead and by activating antiapoptotic proteins such as NF-κB and cyclic adenosine monophosphate (cAMP)-response element binding protein (53,54). A previous study showed that dephosphorylation of AKT activates the proapoptotic function of Bax (55). Based on these observations, inhibition of AKT phosphorylation by sphingoid bases may lead to inhibition of Bax phosphorylation that promotes Bax translocation into the mitochondrial membrane.

Based on our results, we propose an outline of the sphingoid base-induced apoptotic signaling pathway (Fig. 7). Sphingoid bases induce DNA fragmentation resulting in the upregulation of GADD45, which induces cell cycle arrest in the G2/M phase giving cells the chance to repair DNA damage. In contrast, when DNA damage is not repaired, cells execute the apoptotic pathway. GADD45, DR5 and Bax are upregulated and p-AKT is downregulated, leading to the disruption of the mitochondrial membrane, which in turn causes cytochrome *c* release from the intramitochondria into the cytosol, thus activating caspase-3 and -8, which then cleaves the death substrates, leading to apoptosis. Sea cucumber is consumed as a food in different regions of the world. Prevention of cancer by supplementing the diet with naturally occurring compounds is considered as a potential approach to reduce cancer.

Acknowledgements

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