Research Communication

Functional Role of Caspases in Sphingosine-Induced Apoptosis in Human Hepatoma Cells

Hui-Chiu Chang, Chin Hsu, Hseng-Kuang Hsu and Rei-Cheng Yang

Department of Physiology, Kaohsiung Medical University, Kaohsiung 807, Taiwan, Republic of China

Summary

We have previously shown that sphingosine increased caspase-3 activity and induced apoptosis in human hepatoma cells. Our data also suggest that other caspases may be involved in sphingosine-triggered apoptosis. In order to clarify this issue, we used different approaches to study the functional role of several initiator or executioner caspases in apoptosis induced by sphingosine. Activation of procaspases-2, -7, and -8, was clearly demonstrated during sphingosine-triggered apoptosis. Pretreatment with chemical inhibitors for caspase-7 and -8, attenuated apoptotic cell death induced by sphingosine. Conversely, pretreatment with specific caspase-2 inhibitor Z-VDVAD-FMK did not show any protective effect. In addition, enforced expression of constitutively activated AKT kinase which is known to inhibit apoptosis induced by sphingosine, potently suppressed activation of procaspases-7 and -8. In summary, these data suggest that in addition to caspases-3, caspase-7 and -8 are involved in the apoptosis induced by sphingosine.

IUBMB *Life*, 55: 403–407, 2003

Keywords Sphingosine; caspase; apoptosis; AKT kinase; hepatoma.

INTRODUCTION

Recent studies have indicated that sphingolipids, including ceramide, sphingosine, sphingosine1-phosphate etc. are highly bioactive lipids. These molecules play important roles in cell-cell interaction, apoptosis, proliferation and differentiation (1, 2). Our previous results indicated that sphingosine and a number of long-chain bases potently induced apoptosis in human hepatoma cells (3). Additionally, we found that caspase-3, an aspartate-directed cysteine protease, which was

shown to be involved in the apoptosis induced by different apoptotic signals, was activated in sphingosine-induced apoptotic cell death (3). Our recent results indicated that sphingosine-triggered apoptosis is associated with inhibition of AKT kinase and overexpression of constitutively activated AKT may suppress apoptosis induced by sphingosine (4).

To date, at least 10 caspases have been identified in mammalian cells. These cysteine proteases may be divided into 'initiator' caspases, with long prodomains (like caspases-8, -9, and -10) which may activate downstream caspases, and 'executioner' caspases, with short prodomains (such as caspases-3, -6, and -7) which may cleave intracellular substrates (5). Our previous results suggested that in addition to caspase-3, other caspases may also participate in sphingosine-induced apoptosis in human hepatoma cells. Interestingly, recent work demonstrated that exogenous sphingosine induced cytochrome *c* release from mitochondria and activated a number of caspases simultaneously in human T cells (6). However, whether all of the caspases play a role in the induction of apoptosis by sphingosine is not clear.

In the present work, we studied the functional role of caspases by different approaches and our results suggest that caspases-7 and -8, but not caspase-2, are involved in the induction of apoptosis by sphingosine.

MATERIALS AND METHODS

Reagents and Cell Culture

D-Erythro-sphingosine was from Calbiochem-Novabiochem International (San Diego, CA, USA) and was dissolved in DMSO at a stock concentration of 20 mM. Anti-procaspase- 2, -7 and -8 antibodies were obtained from BD Transduction Laboratories. Hep3B human hepatoma cells were cultured in DMEM/F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml streptomycin and 100 IU/ml penicillin in a 5% CO_2 incubator at 37°C. Hep3B cells transfected with constitutively activated (myristoylated) form of AKT were generated as described

previously *(7)* and provided by Dr. R.H. Chen. Caspase-8 inhibitor Ac-AAVALLPAVLLALLAPIETED-CHO and caspase-7 inhibitor Ac-DEVD-FMK were obtained from Calbiochem-Novabiochem International. Caspase-2 inhibitor Z-VDVAD-FMK was purchased from R&D systems (Minneapolis, USA).

Apoptosis Assays

Cells were seeded at a density of 2×10^5 cells/well in 6-well plates in 10% FCS medium. After 24 h, cells were washed with phosphate-buffered saline (PBS) and cultured in 0.5% FCS medium containing different caspase inhibitors for 2 h. Sphingosine (20 μM) was then added and incubated for another 24 h. Apoptotic cells were determined by fluorescent staining as described previously *(3)* and expressed as a percentage of the total cell number counted.

Western Blot Analysis

After different treatments, cells were harvested in an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin A) and protein concentrations were determined by a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Cell lysates (20 μg protein/sample) were subjected to SDS-polyacrylamide gel electrophoresis and western blot analysis was performed to analyze the expression and proteolysis of different procaspases as described previously *(4)*.

Assessment of AKT Activation

To investigate the AKT activity, Hep3B and Hep3B cells transfected with a constitutively activated (myristoylated) form of AKT (Hep3B-AKT) were harvested as described above. Cellular proteins were transferred to nitrocellulose blots and probed with an anti-phosphoAKT antibody which specifically recognized phosphorylated Ser-473 to reveal the presence of activated AKT. To confirm equivalent loading and to verify the identity of the phosphorylated AKT, blots were stripped and re-probed with an anti-AKT antibody.

RESULTS

Activation of Different Procaspases was Found During Sphingosine-induced Apoptosis in Hep3B Cells

Our previous data demonstrated that sphingosine at a concentration of 20 μM potently induced apoptosis in Hep3B cells *(3)*. Therefore, we routinely used this concentration of sphingosine to trigger cell death in this work. Cells were treated with sphingosine for different times and cellular proteins were harvested for western blot analysis. We studied different caspases. Caspase-8 is an initiator caspase which is shown to be activated early in cells receiving apoptotic stimuli and may induce processing of executioner caspases to trigger apoptosis.

This caspase also plays a pivotal role in death receptor-mediated apoptosis. Caspase-2 shares the peptide recognition motif with the executioner caspase-3 and is involved in the initiation or execution phase of apoptosis. We also examined the activation of a well-characterized executioner caspase, caspase-7. The antibodies used in this study can recognize the procaspase form of caspases-2 and -7, but not the active form of these two caspases. Therefore, reduction of procaspase level is considered to be an indication of caspase activation. The anti-caspase-8 antibody recognizes the procaspase-8 (56–58 kDa) and an intermediate cleavage product (41–43 kDa) in apoptotic Hep3B cells. As shown in Fig. 1, activation of different caspases was detected in sphingosine-treated Hep3B cells in a time-dependent manner. Reduction of procaspases-2 and -7 was demonstrated about 8 h after sphingosine addition. Our data also demonstrated that activation of caspase-8 was observed in a similar time course. Because these caspases were activated prior to induction of apoptosis, which could be clearly detected about 12 h after addition of sphingosine, it is suggested that these caspases may play a role in the induction of apoptosis by sphingosine.

Inhibitions of Caspases-7 and -8, but not Caspase-2, may Suppress Sphingosine-induced Apoptosis

In order to clarify whether all of these caspases are needed for induction of apoptosis by sphingosine, cells were preincubated with different caspase inhibitors for 2 h and then treated with sphingosine for another 24 h. Apoptotic cells were determined as described in the Materials and methods section and expressed as a percentage of the total cell number counted. Our results showed that a caspase-8 inhibitor, Ac-AAVALLPAVLLALLAPIETED-CHO, suppressed sphingosine-induced apoptosis in a dose-dependent manner (Fig. 2). Because caspase-7 shows strong substrate specificity with caspase-3, no specific inhibitors for caspase-7 are available at present. We used a general inhibitor Ac-DEVD-FMK, which blocks activities of caspases-3 and -7 simultaneously, to suppress the activation of caspases-7 in this work. We found that this inhibitor potently inhibited apoptosis triggered by sphingosine (Fig. 2). Conversely, a caspase-2 specific inhibitor Z-VDVAD-FMK could not prevent sphingosine-induced apoptosis. In summary, these data suggest that caspases-7 and -8, but not caspase-2, participate in the induction of apoptosis by sphingosine.

Overexpression of Constitutively Activated AKT Kinase Inhibited Activation of Caspase-7 and -8

Our previous data have shown that inhibition of the kinase activity of the anti-apoptotic AKT kinase and induction of cytochrome *c* release from mitochondria is one of the mechanisms by which sphingosine induced apoptosis in Hep3B hepatoma cells. We also demonstrated that overexpression of the constitutively activated AKT kinase could rescue cells from sphingosine-induced apoptosis by inhibiting
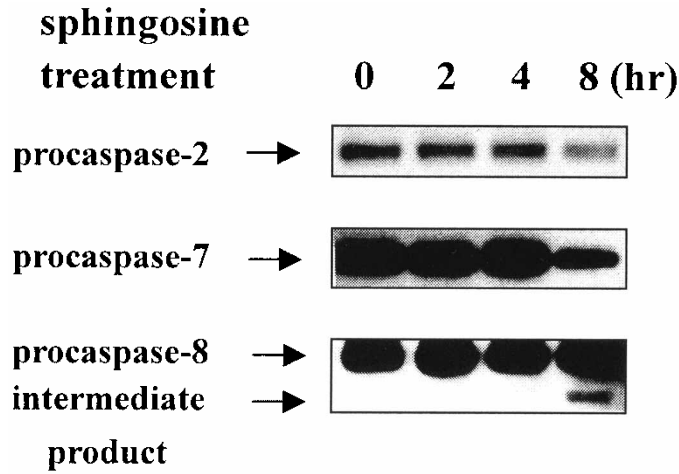
sphingosine
treatment 0 2 4 8 (hr)

procaspase-2 ➡

procaspase-7 ➡

procaspase-8 ➡
intermediate ➡
product

Figure 1. Sphingosine induces activation of different caspases in a time-dependent manner. Hep3B human hepatoma cells were treated with 20 μM sphingosine for different times and cellular proteins were harvested. Expression and processing of different procaspases was analyzed by immunoblotting as described in the Materials and methods section.
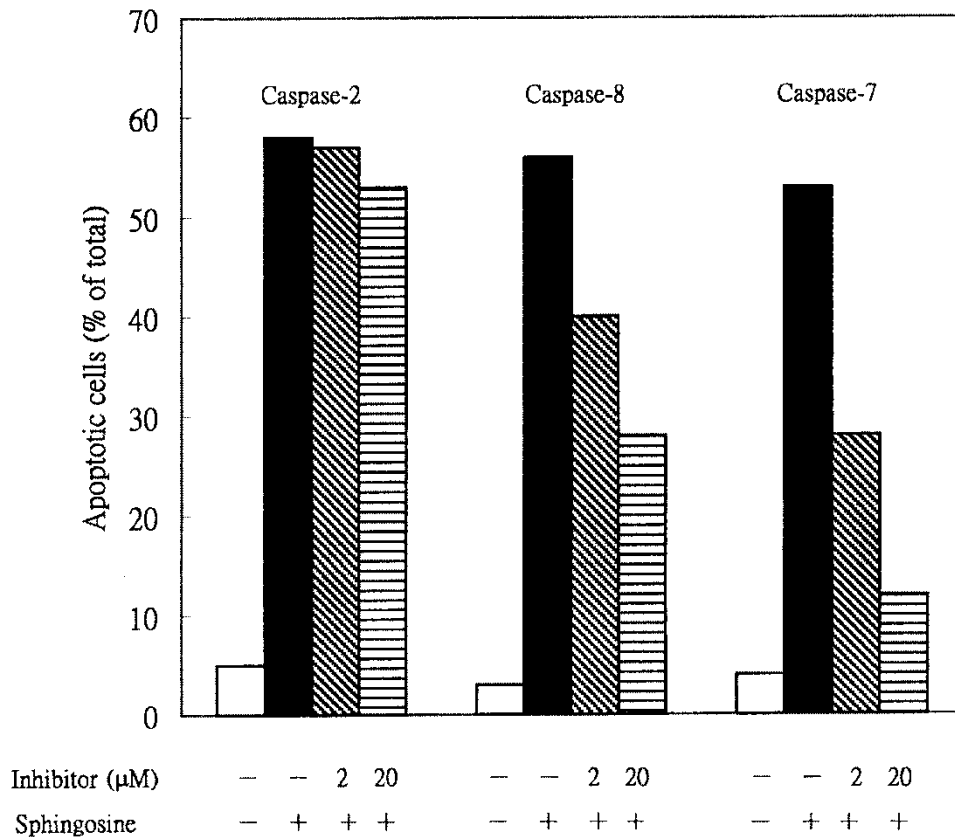
Figure 2. Suppression of sphingosine-induced apoptosis by inhibitors of caspases-7 and -8, but not by an inhibitor of caspase-2. Cells were pre-incubated with different concentrations of caspase inhibitors for 2 h and then treated without ($-$) or with ($+$) 20 μM sphingosine for another 24 h. Apoptotic cells were determined by fluorescent staining and expressed as a percentage of the total cell number counted.

cytochrome *c* release and caspases-3 activation. Therefore, we investigated whether overexpression of AKT could inhibit activation of caspase-7 and -8. We addressed this question by comparing the effect of sphingosine on parental Hep3B cells and Hep3B-AKT cells. Hep3B-AKT cells were generated by transfecting Hep3B cells with a constitutively activated AKT (myristoylated form of AKT). Figure 3A shows that Hep3B-AKT cells indeed exhibited a high level of activated AKT kinase. We found that reduction of procaspase-7 protein level and increase of the 41–43 kDa intermediate cleavage product of caspase-8 was significantly suppressed in Hep3B-AKT cells treated with sphingosine (Fig. 3B). These data suggest that inhibition of activation of caspases-7 and -8 may attenuate sphingosine-induced apoptosis.
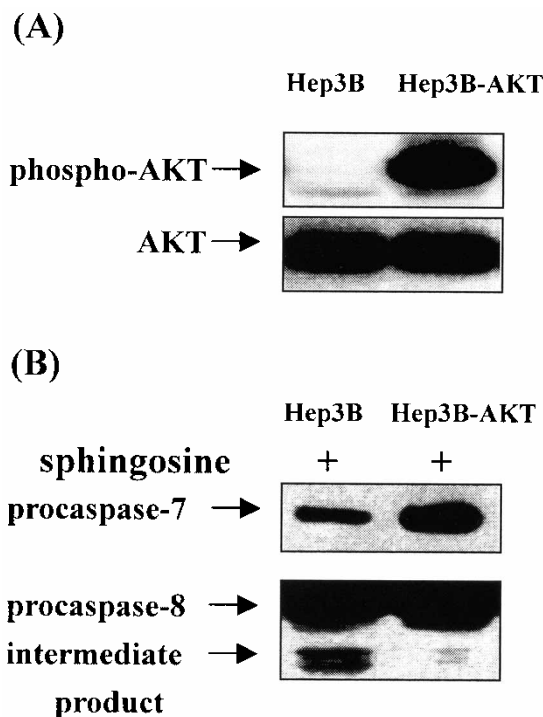
(A)

(B)

Figure 3. Overexpression of AKT attenuates activation of procaspases-7 and -8 in sphingosine-treated cells. (A) AKT activity was significantly elevated in Hep3B cells transfected with a constitutively activated form of AKT (Hep3B-AKT). Cellular proteins were extracted and immunoblotting was performed to detect the presence of activated AKT by probing nitrocellulose membranes with an anti-phosphoAKT antibody which specifically recognized the phosphorylated Ser-473 of AKT kinase. To confirm equivalent loading and to verify the identity of the phosphorylated AKT, blots were stripped and re-probed with an anti-AKT antibody. (B) Hep3B or Hep3B-AKT cells were treated with 20 μM sphingosine for 8 h and cellular proteins were harvested. Expression and processing of different procaspases was analyzed by immunoblotting as described in Fig. 1.

DISCUSSION

A number of sphingolipids were found to be important mediators in apoptosis triggered by different extracellular stimuli. Ceramide is a typical representative. Generation of this sphingolipid by activation of a sphingomyelinase has been implicated in Fas- and TNF-α-induced apoptosis *(8, 9)*. Recently, another sphingolipid metabolite, sphingosine, has been shown to be a critical mediator in TNF-α- and phorbol ester-induced apoptosis *(10, 11)*. In addition, exogenous sphingosine may induce apoptosis in many types of cancer cell *(12–14)*. Our studies and those of others have indicated that sphingosine induces apoptosis without being converted into ceramide, inasmuch as the ceramide synthase inhibitor fumonisin B1 did not affect sphingosine-triggered apoptosis *(4, 15)*. Thus, the apoptotic signaling pathway activated by sphingosine is unique and is not well-defined at present.

In this study, we tried to elucidate the functional role of different caspases in sphingosine-induced apoptosis. Our previous results have shown that a downstream caspase, caspase-3, participated in this process. We now report that two other caspases, caspases-7 and -8, also play a critical role in sphingosine-induced apoptosis. Several important features found in this study are the following. First, a number of reports indicate that activation of different caspases either simultaneously or sequentially can be detected in cells undergoing apoptosis. However, whether all of the caspases participate in the induction of apoptotic cell death is not known. Results from the present study support the notion that not all of the activated caspases play a role in the execution of cells death because we found that pretreatment of caspase-2 inhibitor could not prevent sphingosine-induced apoptosis in Hep3B hepatoma cells. Interestingly, a recent study also indicated that although activities of caspases-1, -3, -8 and -9 were significantly elevated in Fas-triggered apoptosis in rat submandibular acinar cells, only caspase-1 inhibitor could effectively rescue cells from apoptosis *(16)*. Secondly, our results demonstrate that sphingosine induced caspase-8 activation in Hep3B cells, pre-incubation with caspase-8 inhibitor suppressed sphingosine-induced apoptosis. It is of note that caspase-8 is an apical caspase and is activated quickly in cells treated with diverse apoptotic agents *(17, 18)*. However, activation of this caspase could only be detected at 8 h after addition of sphingosine. Our data suggest that this caspase may not act as an initiator caspase in sphingosine-induced apoptosis. Similar observations have also been reported in human type II Jurkat T cells after sphingosine treatment *(6)*. Further work will be needed to clarify the mechanism by which sphingosine induces caspase-8 activation. Thirdly, our results suggest that release of cytochrome *c* from mitochondria is a critical step in the induction of apoptosis by sphingosine. We have previously shown that sphingosine-induced apoptosis is linked with cytochrome *c* release and may lead to subsequent caspase activation *(4)*. In addition, we

found that activation of the anti-apoptotic AKT kinase could rescue sphingosine-induced apoptosis by inhibiting cytochrome *c* release. Our present results demonstrate that constitutively activated AKT potently suppresses activation of caspases-7 and -8 induced by sphingosine. These data strongly suggest that sphingosine-triggered apoptosis and caspase activation is cytochrome *c*-dependent. Taken together, the results from this study suggest that caspases-7 and -8, but not caspase-2, are involved in induction of apoptosis by sphingosine.

ACKNOWLEDGEMENTS

REFERENCES

1. Spiegel, S., and Merrill, A. H. Jr. (1996) Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10**, 1388–1397.

2. Hannun, Y. A., and Obeid, L. M. (1995) Ceramide: an intracellular signal for apoptosis. *Trends Biochem. Sci.* **20**, 73–77.

3. Hung, W. C., Chang, H. C., and Chuang, L. Y. (1999) Activation of caspase-3-like proteases in apoptosis induced by sphingosine and other long-chain bases in Hep3B hepatoma cells. *Biochem. J.* **338**, 161–166.

4. Chang, H. C., Tsai, L. H., Chuang, L. Y., and Hung, W. C. (2001) Role of AKT kinase in sphingosine-induced apoptosis of human hepatoma cells. *J. Cell. Physiol.* **188**, 188–193.

5. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) Human ICE/CED-3 protease nomenclature. *Cell* **87**, 171.

6. Cuvillier, O., Edsall, L., and Spiegel, S. (2000) Involvement of sphingosine in mitochondria-dependent Fas-induced apoptosis of type II Jurkat T cells. *J. Biol. Chem.* **275**, 15691–15700.

7. Chen, R. H., Su, Y. H., Chuang, R. L. C., and Chang, T. Y. (1998) Suppression of transforming growth factor-beta-induced apoptosis through a phosphatidylinositol 3-kinase/Akt-dependent pathway. *Oncogene* **17**, 1959–1968.

8. Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O'Rourke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J. Biol. Chem.* **271**, 4961–4965.

9. Redondo, C., Flores, I., Gonzalez, A., Nagata, S., Carrera, A. C., Merida, I., and Martinez-A, C. (1996) Linomide prevents the lethal effect of anti-Fas antibody and reduces Fas-mediated ceramide production in mouse hepatocytes. *J. Clin. Invest.* **98**, 1245–1252.

10. Ohta, H., Yatomi, Y., Sweeney, E. A., Hakomori, S., and Igarashi, Y. (1994) A possible role of sphingosine in induction of apoptosis by tumor necrosis factor-alpha in human neutrophils. *FEBS Lett.* **355**, 267–270.

11. Ohta, H., Sweeney, E. A., Masamune, A., Yatomi, Y., Hakomori, S., and Igarashi, Y. (1995) Induction of apoptosis by sphingosine in human leukemic HL-60 cells: a possible endogenous modulator of apoptotic DNA fragmentation occurring during phorbol ester-induced differentiation. *Cancer Res.* **55**, 691–697.

12. Ahn, E. H., and Schroeder, J. J. (2002) Sphingoid bases and ceramide induce apoptosis in HT-29 and HCT-116 human colon cancer cells. *Exp. Biol. Med.* **227**, 345–353.

13. Jarvis, W. D., Fornari, F. A., Traylor, R. S., Martin, H. A., Kramer, L. B., Erukulla, R. K., Bittman, R., and Grant, S. (1996) Induction of apoptosis and potentiation of ceramide-mediated cytotoxicity by sphingoid bases in human myeloid leukemia cells. *J. Biol. Chem.* **271**, 8275–8284.

14. Sweeney, E. A., Sakakura, C., Shirahama, T., Masamune, A., Ohta, H., Hakomori, S., and Igarashi, Y. (1996) Sphingosine and its methylated derivative N,N-dimethylsphingosine (DMS) induce apoptosis in a variety of human cancer cell lines. *Int. J. Cancer* **66**, 358–366.

15. Sweeney, E. A., Inokuchi, J., and Igarashi, Y. (1998) Inhibition of sphingolipid induced apoptosis by caspase inhibitors indicates that sphingosine acts in an earlier part of the apoptotic pathway than ceramide. *FEBS Lett.* **425**, 61–65.

16. Aiba-Masago, S., Masago, R., Vela-Roch, N., Talal, N., and Dang, H. (2001) Fas-mediated apoptosis in a rat acinar cell line is dependent on caspase-1 activity. *Cell. Signal.* **13**, 617–624.

17. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997) FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. *J. Biol. Chem.* **272**, 2952–2956.

18. Stennicke, H. R., Jurgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., *et al.* (1998) Pro-caspase-3 is a major physiologic target of caspase-8. *J. Biol. Chem.* **273**, 27084–27090.