EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 35, No. 3, 181-188, June 2003

Role of sphingomyelin-MAPKs pathway in heat-induced apoptosis

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Accepted 12 May 2003

Abbreviations: DAG, diacylglycerol; ERK, extracellular signal-related protein kinase; JNK, Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; PC, phosphatidylcholine; PKC, protein kinase C; PIP2, phosphatidyl inositol 4,5-bisphosphate; SMase, sphingomyelinase; TPA, 12-o-tetradecanoylphorbol-13-acetate

Abstract

The role of sphingomyelinase (SMase) activation and mitogen activated protein kinases (MAPKs) activation in cellular apoptosis was investigated during the hyperthermic treatment of HL-60 human leukemia cells. Treating the cells for 1 h at 43°C caused more than 50% of cellular apoptosis within several hours. The neutral-SMase activity in the cells treated for 1 h at 42°C was slightly increased but decreased in the cells treated at 43°C or 44°C for the same period whereas the acid SMase activity was slightly increased after heating the cells at 42°C and 43°C and markedly increased at 44°C for 1 h. Treatment of cells with inhibitors of SMase activation and ceramide formation significantly reduced the heat-induced apoptosis. Three major families of mitogen-activated protein kinases (MAPKs), *i.e.* ERK1/2, p38 and JNK, were activated by the hyperthermic treatment of cells. Inhibition of ERK1/2 with PD98059 exerted little effect on the heat-induced apoptosis and p38 inhibition with SB203580 slightly lessened apoptosis whereas, inhibition of JNK with SP600125 markedly suppressed the heat-induced apoptosis. These results indicate that heat-shock induced the activation of SMase, particularly acid-SMase, thereby causing apoptosis and that JNK played a pivotal role in heat-induced apoptosis in HL-60 leukemia cells.

Keywords: apoptosis; heat-shock; MAPKs; SMase

Introduction

Hyperthermia is becoming an established regimen in treating various human cancers (Overgaard *et al.*, 1995; Venon *et al.*, 1996; Sneed PK *et al.*, 1998; van der Zee *et al.*, 2000). It has been known that thermosensitivity of mammalian cells is greatly affected by environmental factors such as acidity (Hahn and Shin, 1979). We have observed that an acidic environment markedly increases the heat-induced apoptosis in a variety of cancer cells including HL-60 cells (Takasu *et al.*, 1998; Ohtsubo *et al.*, 2000; Ohtsubo *et al.*, 2001). However, the mechanisms underlying the heat-induced apoptosis and also the mechanisms for the increase in thermosensitivity in an acidic environment have not yet been understood.

When cells are stressed or stimulated, sphingomyelinase (SMase) is rapidly activated and the SMase accelerates cleavage of sphingomyelin to ceramide. Ceramide then acts as a secondary messenger stimulating ceramide activated protein kinase, ceramide-activated protein phosphatase and protein kinase C (Javid *et al.*, 1996; Liu *et al.*, 1997; Levade and Jaffrezou, 1999). The activation of these kinases and phosphatase leads to a variety of biological consequences such as growth arrest, proliferation, differentiation and inflammation (Basu and Kolesnick, 1998; Okazaki *et al.*, 1998; Huwiler *et al.*, 2000). It has become increasingly evident in recent years that sphingomyelin-ceramide signal transduction pathway also plays a pivotal role in induction of apoptosis caused by diverse exogenous stresses (Haimovitz-Friedman *et al.*, 1994; Verhij *et al.*, 1996; Mathias *et al.*, 1998; Perry and Hannun, 1998). Among several SMases that have been known, acid-SMase and neutral-SMase are

most abundant. The ceramide generated by SMase initiates the signaling pathway leading to activation of mitogen-activated protein kinases (MAPKs) (Haimovitz-Friedman *et al.*, 1994; 1996; Verhij *et al.*, 1996; Haimovitz-Friedman *et al.*, 1997; Pena *et al.*, 1997; Basu and Kolesnick, 1998; Mathias *et al.*, 1998; Perry and Hannun, 1998; Ichijo 1999). The activated MAPKs then activate caspases and other proteins that are directly involved in apoptosis (Haimovitz-Friedman *et al.*, 1994; Jarvis *et al.,* 1994; Verhij *et al.*, 1996; Zanke *et al*, 1996; Chmura *et al.*, 1997; Mansat *et al.*, 1997; Mosser *et al.*, 1997; Bettaieb *et al.*, 1999; Cifone *et al.*, 1999; Levade and Jaffrezou, 1999; Dai *et al.*, 2000). Like other stresses, heat stress has been demonstrated to trigger hydrolysis of sphingomyelin to ceramide (Verhij *et al.*, 1996; Punyiczki and Fesus, 1998; Kundo *et al.,* 2000). In other studies, heat shock has been demonstrated to activate MAPKs (Chu *et al.*, 1996; Zanke *et al*, 1996; Mosser *et al.*, 1997; Dai *et al.*, 2000). Taking these observations together, one may speculate that when cells are exposed to heat-shock, SMases are activated causing hydrolysis of sphingomyelin to ceramide, which then acts as a secondary messenger and activates MAPKs followed by apoptosis.

It has been reported that activation of acid-SMase requires diacylglycerol (DAG) (Haimovitz-Friedman *et al.*, 1994; Jarvis *et al.*, 1994; Javid *et al.,* 1996; Chmura *et al.*, 1997; Liu *et al.,* 1997; Bettaieb *et al.*, 1999; Cifone *et al.*, 1999; Levade and Jaffrezou, 1999). We therefore investigated whether inhibition of DAG formation during heating suppresses heat-induced apoptosis. It is known that hydrolysis of sphingomyelin to ceramide is inhibited by protein kinase C (PKC) and thus activation of PKC suppresses apoptosis caused by various cytotoxic agents (Haimovitz- Friedman *et al.*, 1994; Javid *et al.*, 1996; Verhij *et al.*, 1996; Liu *et al.*, 1997; Levade and Jaffrezou, 1999). Therefore, we have investigated whether heat-induced apoptosis can be suppressed by activation of PKC with TPA (12-o-tetradecanoylphorbol-13-acetate), a known PKC activator.

There have a number of reports on the role of SMase-ceramide-MAPKs signal cascade in induction of apoptosis following heat-shock. However, the past studies on the signal cascade responsible for the heat-induced apoptosis have been fragmental. For example, there have been few attempts to reveal the activation of SMase and the activation of all three family members of MAPKs, *i.e.* ERK1/2, JNK and p38, simultaneously in the same cells. In the present report, we have demonstrated circumstantial evidence that acid-SMase may be the major SMase responsible for the heat-induced apoptosis and that JNK is the major MAPKs that plays a pivotal role in heat-induced apoptosis.

Materials and Methods

Cell line and culture conditions

The human promyelocytic leukemia HL60 cell line was used. The cells were grown in RPMI supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were cultured in 25 cm^2 plastic tissue culture flasks at 37°C in a humidified 5% CO_2/ 95% air atmosphere.

Heat treatment of the cells

The flasks were closed, and the screw area was tightly sealed with wax paper. The flasks were then immersed for 1 h in a temperature regulated water bath preset at 42, 43 or 44°C. After heating, the screws were loosened, the flasks were returned to a 37°C incubator and the cells were cultured in 5% CO_2 atmosphere.

Effects of inhibition of DAG synthesis and activation of PKC on heat-induced apoptosis

The effect of inhibition of DAG formation on the heat-induced apoptosis was elucidated using PIP_2-phospholipase C inhibitor (U73122) and phosphatidyl choline (PC)-phospholipase C inhibitor (D609). Cells were incubated with 0.65 μM U73112 or 30 μg/ml D609 for 30 min and then heated. The role of PKC in sphingomyelinase-apoptosis pathway was investigated by exposing the cells to 100 nM TPA (a known PKC inhibitor; 12-o-tetradecanoylphorbol-13-acetate) from 30 min before heating and during heating.

Determination of sphingomyelinase activity

Cells were heated at 42, 43 or 44°C for 1 h as described above and the sphingomyelinase activity in the cells was determined using Amplex Red Sphingomyelinase assay kit (Molecular Probes Inc., Eugene, OP.#A-12220). In this enzyme-coupled assay, SMase activity was monitored indirectly using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2. The fluorescence intensity was read with a fluorescence microplate reader. The activity of neutral SMase and acid SMase could be separately determined by adjusting the pH of reaction solution to 5.5 and 7.5 for acidic SMase and neutral SMase, respectively.

Western blot analysis

Cell lysates were prepared with solubilizing buffer (pH 7.4, 1% Triton X-100, 1% deoxycholic acid sodium salt, 0.1% SDS, 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 2 mM PMSF, 2 μg/ml aprotinin and 2 μg/ ml leupeptin). The cell extracts were loaded on 10%

polyacrylamide gel and subjected to electrophoresis using SDS-polyacrylamide gel. After SDS-PAGE was performed, proteins were transferred to a PVDF membrane (Amersham, Piscataway, NJ). The membrane was blocked with Tris-buffered saline supplemented with 0.05% Tween-20 (TBST) including 3% milk at 4°C overnight. The membrane was then incubated with anti-p38 (1 μg/ml; Cell Signaling Technology), anti-ERK1/2 (1 μg/ml; Cell Signaling Technology), anti-Phospho-ERK1/2 (1 μg/ml; Cell Signaling Technology), anti-JNK (1 μg/ml; Pharmingen International), anti-α-Tubulin (1 μg/ml; Oncogene Science), and secondarily incubated with HRP-conjugated antibody (1:2,000; Amersham, Piscataway, NJ). Subsequently, the membrane was developed with ECL Western blotting detection reagents.

Immune complex kinase assay

The JNK or p38 was immunoprecipitated from 0.2% Tween 20 extracts prepared from HL60 cells. The kinase reaction mixtures (50 mM Tris (pH 7.4), 10 mM MgCl2, 1 mM DTT, 10 M [γ-^{32}P] ATP and 25 ng/l substrate) were added to immunoprecipitates. Substrates were GST-cJun and ATF for JNK kinase and p38 kinase, respectively. The kinase mixtures were incubated for 30 min at 30°C. The kinase activity was then terminated by the addition of an equal volume of 2$\times$SDS-sample buffer. The reaction mixtures were resolved on 10% SDS-polyacrylamide gels and the kinase activity was visualized by autoradiograph and quantitated with a scintillation β-counter.

Effects of MAPK inhibitors on heat-induced apoptosis

The role of three different families of MAPKs in heat-induced apoptosis was investigated with the use of MAPK inhibitors. Cells were incubated with 20 μM PD98059 (inhibitor of ERK1/2, 2'-Amino-3'-methoxyflavone), 10 μM SB203580 (inhibitor of p38, 4-[4-Fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl] 1H-imidazole) or 20 μM SP600125 (inhibitor of JNK, anthra [1.9-cd] pyrazol-6 [2H]-one; 1,9-pyrazoloanthrone) at 37°C for 30 min and heated at 43°C for 1 h. Cells were harvested at different time points after heating, and apoptosis was determined.

Determination of apoptosis

Flow cytometery

Cells were collected by centrifugation, resuspended in staining solution (0.1% Triton X-100, 0.1 mM EDTA, 50 μg/ml propidium iodide (PI; Sigma Chemical Co.) and 50 μmg/ml DNase-free RNase (Amersham Pharmacia Biotech.). The DNA content in the cells was determined using a Becton Dickinson FACS Calibur flow cytometer with a 488 nm, and the fraction of cells undergoing apoptosis was calculated from the DNA content.

DNA Gel Electrophoresis

After the various treatments, the cells in the medium were collected by centrifugation at 5,000 rpm for 5 min, washed twice with ice-cold PBS, resuspended in lysis buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.3 mg/ml proteinase K], and incubated at 48°C for overnight. After cold 5 M NaCl solution added to a final concentration of 1 M, the mixture was vigorously shaken with a vortex and centrifuged at 15,000 rpm for 5 min. After precipitation with isopropanol, the pellets were resuspended in TE buffer [10 mM Tris-HCl (pH 7.4), and 1 mM EDTA] and incubated with 0.1 mg/ml DNase-free RNase and incubated at 37°C for 2 h. The DNA, 20 μg from each sample, was subjected to electrophoresis on 1% agarose gel in TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) and the DNA was stained with 0.5 μg/ml ethidium bromide and visualized under UV light.

Results

Sphingomyelinase activity

The effect of heat treatment of HL-60 cells on the SMase activity is shown in Figure 1. The enzyme activity immediately after heating is expressed as an arbitrary unit, *i.e.* fluorescence/μg of protein, which was read with fluorescence microplate reader. At

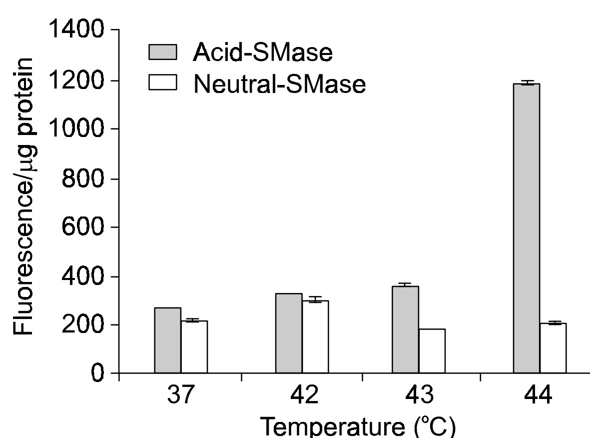

Figure 1. Changes in SMases activity in HL-60 cells following at 42-44°C for 60 min. SMases activities were determined with Amplex Red reagent-based assay containing 50 μM Amplex Red reagent, 1 U/ml HRP. Fluorescence was measured with a fluorescence microplate reader at 540 nm. Average of 3 independent experiments with $\pm$1 SE are shown.

37°C, acid-SMase activity was slightly greater than neutral-SMase activity. Heating the cells for 1 h at 42°C or 43°C slightly increased the acid SMase activity while heating at 44°C for 1 h markedly increased the acid SMase. The neutral SMase activity slightly increased upon heating the cells at 42°C for 1 h, but slightly decreased as the heating temperature was raised to 43°C or 44°C.

Heat-induced apoptosis

Figure 2A shows the histograms for cell populations with different DNA content, obtained with flow cytometry. Since DNA content in cells reflects the stage of the cells in cell cycle progression, the histogram shown in Figure 2A actually represents the distribution of cells in different cell cycle phases. The cells containing less DNA than G1 cells, *i.e.* sub-G1 cells, are those undergoing apoptosis. It can be seen that in the cells heat treated in regular medium, marked apoptosis occurred in 6 h after the cells heat treated for 1 h at 43°C. Increasing the temperature to 44°C further increased apoptosis. The result from the five separate experiments (Figure 2B) shows that close to 50% of cells underwent apoptosis in 6 h after heat treatment of the cells for 1 h at 43°C while less than 5% of control cells underwent apoptosis.

Effects of inhibition of DAG formation and activation of PKC on heat-induced apoptosis

DAG is known to require for the activation of acid-SMase (Haimovitz-Friedman *et al.*, 1994; Jarvis *et al.*, 1994; Javid *et al.,* 1996; Chmura *et al.*, 1997; Liu *et al.,* 1997; Bettaieb *et al.*, 1999; Cifone *et al.*, 1999; Levade and Jaffrezou, 1999). DAG is formed as a product of hydrolysis of phosphatidyl choline (PC) and phosphatidyl inositol 4,5-bisphosphate (PIP2) by PC-phospholipase C and PIP2-phospholipase C, respectively. A heat shock treatment of the cells is reported to stimulate phospholipase C, thereby enhancing the conversion of PIP2 to PIP3 and presumably DAG as well (Calderwood *et al.*, 1987; Calderwood and Stevenson, 1993). We investigated the effect of suppressing DAG formation using PC-phospholipase C inhibitor (D609), and PIP2 inhibitor (U73122) on the heat-induced apoptosis. When cells were pre-incubated with 30 μg/ml D609 or 0.65 μM U73122 for 30 min at 37°C and then heated for 1 h at 43°C, the heat-induced apoptosis was significantly reduced as shown in Figure 2 probably due to suppression of acid SMase activation. Activation of PKC has been known to reduce hydrolysis of sphingomyelin to ceramide and thereby reduced apoptosis (Haimovitz-Friedman *et al.*, 1994; Javid *et al.*, 1996; Verhij *et al.*, 1996; Liu *et al.*, 1997; Levade and Jaffrezou, 1999). Treating the cells with 100 nM of TPA, PKC activator,

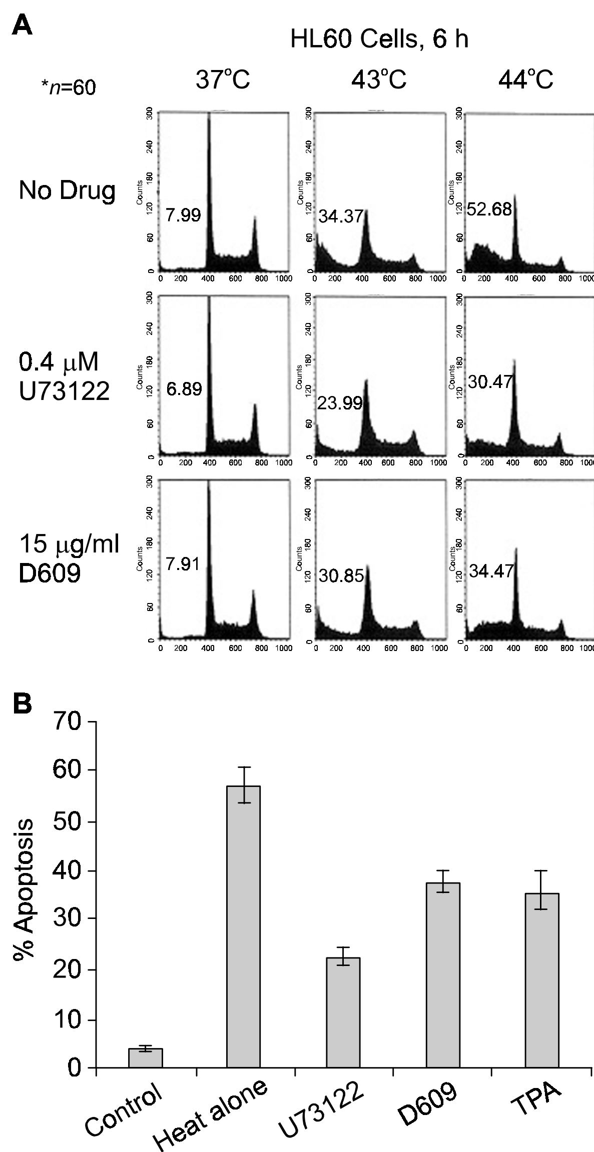

Figure 2. (A) Flow cytometric analysis of heat-induced apoptosis in HL-60 cells. The effects of U73122, an inhibitor of PIP2-phospholipase C, or D609, an inhibitor of PC-phospholipase C are shown. This is a representative of five independent experiments that produced similar results. (B) % apoptosis in HL-60 cells 6 h after heating at 43°C for 60 min with or without pre-incubation with U73122, D609 or TPA. The percentage of apoptotic cells was estimated from the area under the subdiploid (DNA $<$ 2N) peak in the DNA content histogram as shown in Figure 2A. Values shown are the means of five independent experiments $\pm$ 1 SE (10,000 cells were counted for each sample).

beginning 30 min prior to heating the cells for 1 h at 43°C significantly reduced the heat-induced apoptosis as shown in Figure 2B.

Activation of MAPKs

The effect of heat-shock treatment of HL-60 cells on

the expression of MAPKs proteins and their activities are shown in Figure 3. Heat treatment of the cells for 1 h at 43°C caused no significant change in the protein levels of MAPKs while it significantly increased the activity of the MAPKs. The kinetics and the magnitude of the changes in the activity of each MAPKs, *i.e.* JNK, p38 and ERK1/2 were considerably different. Specifically, the activity of JNK significantly increased immediately after heat treatment of the cells and it further increased reaching about 15-fold of control at 30 min post-heating. The activity of JNK remained elevated until 2 h after heat treatment of the cells and then recessed 4 h. The activity of p38 also increased immediately after heat-treatment and then it progressively declined thereafter. The activity of ERK1/2 was also markedly increased immediately after heat treatment of the cells, peaked at 1 h and then gradually declined to control level at 4 h.

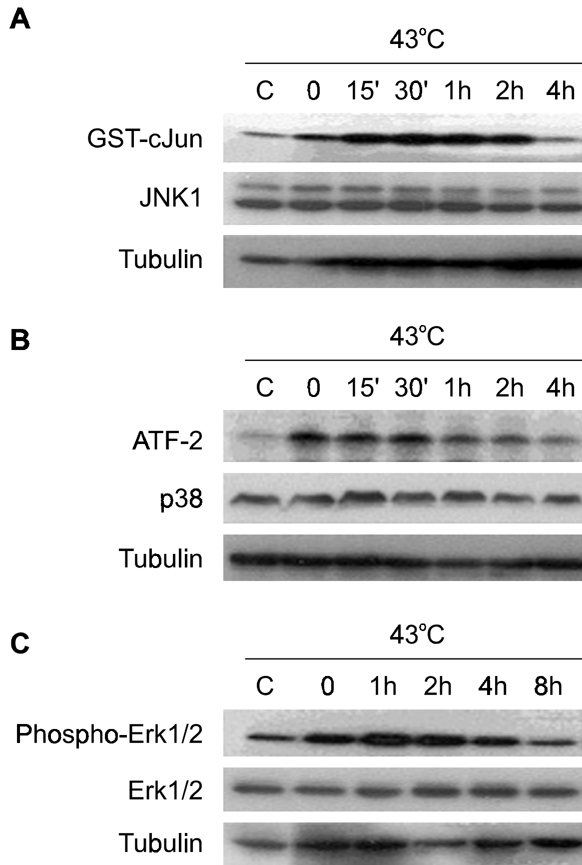

Figure 3. Heat-induced activation of MAPKs in HL-60 cells. After heating HL-60 cells at 43°C for 1 h, cells were harvested with lysis buffer and subjected to *in vitro* kinase assay for JNK kinase and p38 kinase assay. The expression of JNK, p38, phopho-ERK1/2, and ERK1/2 proteins were determined by Western blot analysis using specific antibodies.

Inhibition of MAPK activity on heated-induced apoptosis

The DNA fragmentation due to apoptosis in HL-60 cells caused by 1 h heat treatment at 43°C with or without MAPK inhibitors is shown in Figure 4. Apoptotic DNA fragmentation could be observed in 1 h after heat treatment of the cells in regular medium at 43°C for 1 h and it further progressed peaking at 2-4 h. The DNA fragmentation in cells heat treated in the presence of 20 μM PD98059, inhibitor of ERK1/2, was similar to the DNA fragmentation in the cells heat treated in the regular medium. On the other hand, the heat-induced DNA fragmentation was noticeably suppressed by 10 μM SB20358, inhibitor of p38. The treatment of cells with 20 μM SP600125, inhibitor of JNK, markedly suppressed the heat-induced DNA fragmentation. Figure 5 shows the effect of MAPK inhibitors on the heat-induced apoptosis, as determined from the DNA content using flow cytometry. The results shown in Figure 4 and 5 are essen-

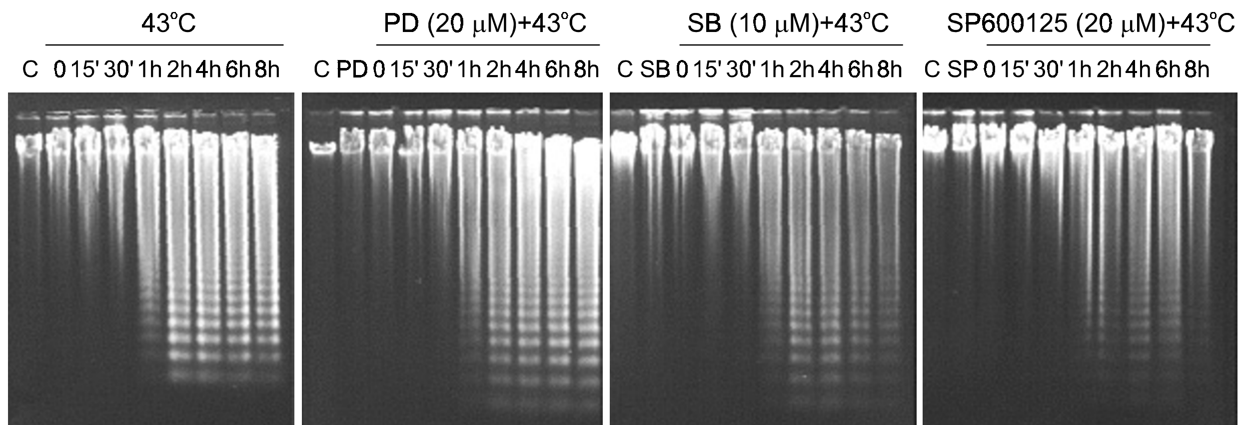

Figure 4. DNA fragmentation in HL-60cells heating HL-60 cells heated at 43°C for 1 h with or without MAPKs inhibitors. After heating the cells were incubated for the indicated time at 37°C, DNA was extracted and subjected to agarose gel electrophoresis.

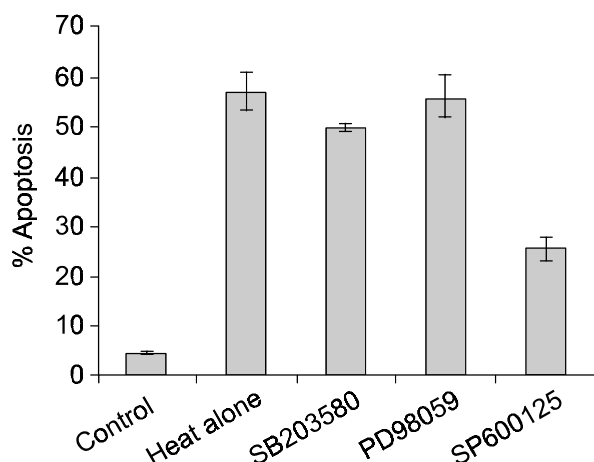

Figure 5. % apoptosis in HL-60 cells 6 h after heating at 43°C for 60 min with or without inhibitors of MAPKs. Values shown are the means of five independent experiments1 ± SE (10,000 cells were counted for each sample).

tially the same: whereas PD98059, inhibitor of ERK1/2, exerted no effect on heat-induced apoptosis, 20 μM SB203580, inhibitor of p38, slightly suppressed the heat-induced apoptosis and 20 μM SP600125, inhibitor of JNK, markedly suppressed the heat-induced apoptosis.

Discussion

In the present study, we have observed that heat-shock activates SMase, particularly acid-SMase and also demonstrated that although heat-shock activates all three major members of MAPKs, *i.e.* ERK1/2, JNK, and p38, JNK appears to play a major role in the heat-induced apoptosis.

The sphingomyelin-MAPK signal transduction pathway caused by diverse exogenous stresses has been well documented (Haimovitz-Friedman *et al.*, 1994; Verhij *et al.*, 1996; Perry and Hannun, 1998). However, relatively little is known about the heat-induced activation of sphingomyelin-MAPK pathway. It would be reasonable to speculate that, similar to the activation of MAPKs by other stimuli, the heat-induced activation of MAPKs initiated by the ceramide-induced activation of MAPK kinase kinase (MAPKKK), a Ser/Thr kinase, could cause phosphorylation and activation of MAPK kinases (MAPKKs). JNK, p38 and ERK1/2, can be activated by different MAPKKs although cross-talk among the different MAPKKs has been recognized (Basu and Kolesnick, 1998; Mathias *et al.*, 1998; Perry and Hannun, 1998; Ichijo 1999). It is generally agreed that JNK and p38 are involved mainly in stress response such as inflammation and apoptosis while ERK1/2 regulates differentiation, cell growth and cell survival. The marked increase in the activation of JNK relative to the activation of p38 (Figure 3) indicated that the apoptosis caused by heat-shock may be mediated mainly by the JNK pathway. This notion is supported by the results shown in Figs 4 and 5 that inhibition of JNK activity was far more effective than inhibition of p38 activity in suppressing heat-induced apoptosis. The IC_{50} for the inhibition of p38 kinase activity has been reported to be about 0.6 μM (Lee *et al.,* 1999; Wond *et al.,* 2000). We have observed in the present study that 20 μM SB203580 was no more effective than 10 μM SB203580 in inhibiting heat-induced apoptosis (data not shown). It is generally believed that ERKs are survival signals. Therefore, one may expect that an inhibition of ERK1/ 2 would result in an increase in apoptosis. However, inhibition of ERK1/2 activity with 25 μM PD98059 exerted no effect on heat-induced apoptosis (Figures 4, 5). It would be reasonable to conclude that the activation of ERK1/2 is not related to heat-induced apoptosis or its survival signal is overwhelmed by the JNK death signals. Taking these results together, it may be concluded that among MAPKs, JNK plays a pivotal role in heat-induced apoptosis.

The heat shock treatment of the cells is known to activate SMase, but the present results are the first one to demonstrate that heat shock preferentially activates acid SMase among many different SMases (Figure 1). It is probable that the well known phenomenon that an acidic environment sensitizes tumor cells to heat (Hahn and Shin, 1979; Takasu *et al.,* 1998; Ohtsubo *et al.,* 2000, 2001) may be due to the preferential activation of acid SMase. DAG has been reported to require for the activation of SMase (Haimovitz-Friedman *et al.*, 1994; Jarvis *et al.*, 1994; Javid *et al.,* 1996; Chmura *et al.*, 1997; Liu *et al.,* 1997; Bettaieb *et al.*, 1999; Cifone *et al.*, 1999; Levade and Jaffrezou, 1999). The results shown here suggest that heat-induced apoptosis could be significantly suppressed by the inhibitors of DAG formation *i.e.* U73122 or D608, (Figure 2) demonstrated that SMase activation is responsible, at least in part, for the heat-induced apoptosis in HL-60 cells. However, we could not exclude the possibility that U73122 or D608 suppressed the heat-induced apoptosis through mechanisms other than suppressing the SMase activation. Further investigation is in progress in our laboratory by determining the changes in SMase activation in the presence of the inhibitor of DAG formation. It has been well established that heat stress triggers hydrolysis of sphingomyelin to ceramide (Verhij *et al.*, 1996; Punyiczki and Fesus, 1998; Kundo *et al.,* 2000) and that activation of PKC suppresses the hydrolysis of sphingomyelin to ceramide in the cells exposed to various cytotoxic agents

(Haimovitz-Friedman *et al.*, 1994; Verhij *et al.*, 1996; Levade and Jaffrezou, 1999). It is thus highly likely that the suppression of heat-induced apoptosis by TPA, activator of PKC (Figure 2) was due to suppression of ceramide formation, thereby suppressing the ceramide-MAPKs-apoptosis pathway. Experiment is in progress to confirm this hypothesis by determining the effect of TPA on the ceramide formation in heated HL-60 cells.

In conclusion, we have demonstrated that heathock activates SMase, particularly acid SMase, thereby it activates MAPKs and induces apoptosis in HL-60 cells. We have also demonstrated first time that although both JNK and p38 are activated by heat-shock, JNK plays greater role than p38 in heat-induced apoptosis. The preferential activation of acid SMase relative to neutral-SMase may account for the well-known thermosensitization in an acidic environment.

Acknowledgement

We thank Mr. Brent Williams for technical assistance. This work was supported in part by grants NIH/NCI CA44114, KOSEF R01-1999-000-00107-0, and KISTEP Life Sci 2000.

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