Sphingosine-induced c-jun expression: differences between sphingosine- and C2-ceramide-mediated signaling pathways

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Abstract Sphingolipids such as ceramide and sphingosine are putative intracellular signal mediators in cell differentiation, growth inhibition and apoptosis. Previously, we reported that C2-ceramide induced c-jun expression in apoptosis of human leukemia HL-60 cells. Here we report that sphingosine also induced c-jun expression in apoptosis of HL-60 cells. Sphingosine-induced c-jun expression was stimulated by H-89, a protein kinase A inhibitor, whereas C2-ceramide-induced c-jun expression was inhibited by protein kinase C inhibitors. Furthermore, H-89 potentiated sphingosine-induced but not C2-ceramide-induced growth inhibition. These results suggest that sphingosine and C2-ceramide might induce c-jun expression and apoptosis in distinct signaling pathways.

Key words: Sphingosine; Ceramide; c-jun; Apoptosis; Protein kinase A; Protein kinase C

1. Introduction

Sphingolipids such as ceramide and sphingosine have emerged as novel intracellular signal mediators [1]. Various studies have shown that ceramide is involved in cell death (apoptosis) and growth inhibition (for reviews see [2]). Increase of ceramide and/or activation of (acidic and neutral) sphingomyelinases have been detected in apoptosis induced by many stimuli, including tumor necrosis factor-α, anti-Fas antibody, anti-cancer drugs, and irradiation [3–6]. We previously showed that C2-ceramide induced c-jun expression at the transcriptional level and increased DNA binding activity of AP-1 [7]. We also reported that antisense oligonucleotides of c-jun suppressed C2-ceramide-induced apoptosis [7]. Another group showed that overexpression of a dominant negative form of c-Jun inhibited ceramide-induced apoptosis [8]. These results suggested that activation of AP-1 might be required for ceramide-mediated apoptotic signaling pathway.

Sphingosine, an *N*-deacylated form of ceramide, is a potent inhibitor of protein kinase C (PKC) [9]. Sphingosine also induces apoptosis and growth inhibition in various cell types [10,11]. However, the mechanisms by which sphingosine induces apoptosis remain unclear.

Here we show that sphingosine induces c-jun expression in HL-60 cells. Furthermore, we show that H-89, a protein kinase A (PKA) inhibitor, potentiates sphingosine-induced c-jun expression and growth inhibition, whereas staurosporine, a PKC inhibitor, suppresses C2-ceramide-induced c-jun expression and apoptosis. These results suggest the requirement of c-jun expression for sphingosine-induced apoptosis and growth inhibition, and also suggest different mechanisms between sphingosine- and C2-ceramide-induced c-jun expression.

2. Materials and methods

2.1. Materials

Sphingosine was purchased from Sigma. D-Erythro-C2-ceramide and C18-ceramide were purchased from Matreya (USA). H-89 and H-7 were purchased from Seikagaku (Japan). Diacylglycerol kinase was a gift from Dr. Yusuf A. Hannun (Medical University of South Carolina, USA). Other chemicals were purchased from Sigma (USA). Sphingosine and C2-ceramide was dissolved in ethanol. C18-ceramide was dissolved in ethanol/dodecane (98:2).

2.2. Cell culture

Human myelogenous leukemia HL-60 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO_2 incubator. HL-60 cells in exponentially growing phase were washed once with RPMI 1640 medium containing 5 μg/ml transferrin and 5 μg/ml insulin instead of serum, resuspended in the serum-free medium overnight at a concentration of 5×10^5 cells/ml, and then used for experiments.

2.3. Cell counting

Viable cell number was determined under microscope by trypan blue dye exclusion test.

2.4. Apoptosis

Apoptotic cells were determined under microscope by typical morphological changes such as nuclear fragmentation and formation of apoptotic body. At least 200 cells were examined in each experiment.

2.5. Northern blot analysis

Total RNA was extracted by using ISOGEN (Nippon Gene, Japan) according to the manufacturer's protocol. Northern blotting was performed as described previously [7]. Briefly, human c-jun oligonucleotide probe (Oncogene Science, USA) was 5′-end labeled by using a Kination kit (Toyobo, Japan), and purified through Sephadex G-25 spun column twice. Hybridizations were performed at 42°C for 24 h, and then the membranes were washed in 2×saline sodium citrate (0.15 M NaCl, 15 mM sodium citrate)/0.1% sodium dodecyl sulfate at room temperature for 30 min and at 50°C for 20 min. The membranes were exposed to X-ray films with intensifying screens at −80°C for 2 days. Equal loading of RNA on each lane was confirmed by methylene blue staining of the membrane.

2.6. Ceramide measurement

The levels of ceramide were determined by using diacylglycerol kinase as described [12].

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Abbreviations: PKC, protein kinase C; PKA, protein kinase A

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3. Results

3.1. Induction of c-jun mRNA expression by sphingosine

We reported that C2-ceramide induced c-jun expression and apoptosis in HL-60 cells [7]. As previously reported [10], sphingosine (5 μM) induced apoptosis in HL-60 cells (data not shown). Therefore, we examined whether the expression of c-jun mRNA was induced by sphingosine. As shown in Fig. 1A, Northern blot analysis demonstrated that the levels of c-jun mRNA (2.7 and 3.2 kb) were increased by treatment with sphingosine. The expression of c-jun mRNA increased at 30 min, peaked at 1 h, and then gradually decreased. The time course of c-jun induction by sphingosine was similar to that by C2-ceramide. Fig. 1B showed that c-jun mRNA was induced by sphingosine in a dose-dependent manner up to 10 μM. It seemed that C2-ceramide induced c-jun expression more strongly (approximately three- to five-fold) than sphingosine at the same concentration (5 μM).

Recently, it was reported that natural long-chain ceramide could induce apoptosis by usage of dodecane [13]. Therefore, we examined the effect of natural ceramide with dodecane on c-jun induction. Increase of c-jun expression was observed 4 h after treatment with natural ceramide (Fig. 1C). The level of c-jun mRNA was further increased at 8 h. Because the time-course of c-jun induction by natural ceramide was much slower than that by sphingosine or C2-ceramide, natural ceramide was not used in the experiments described below.

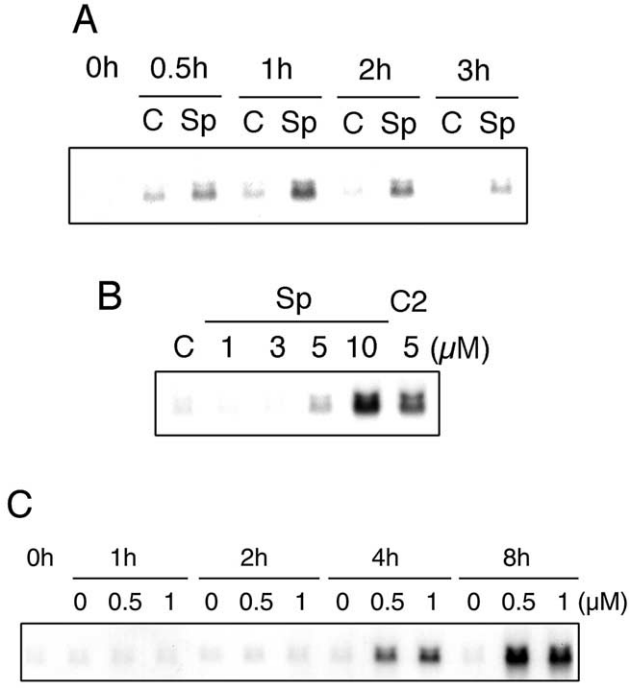

Fig. 1. Sphingosine-induced c-jun expression. A: Time-course of sphingosine-induced c-jun expression. HL-60 cells were treated with either 0.1% ethanol vehicle (C) or 10 μM sphingosine (Sp) for the indicated times. B: Dose dependence of sphingosine-induced c-jun expression. HL-60 cells were treated with the indicated concentrations of sphingosine for 1 h. C2, treated with 5 μM C2-ceramide for 1 h. C: Natural ceramide-induced c-jun expression. HL-60 cells were treated with either 0.1% ethanol/dodecane (98:2) vehicle or 0.5 or 1 μM C18-ceramide dissolved in ethanol/dodecane (98:2) for the indicated times. Northern blot experiments were performed as described in Section 2. The results are representative of three different experiments. Equal loading of RNA on each lane was confirmed by methylene blue staining of the membrane.

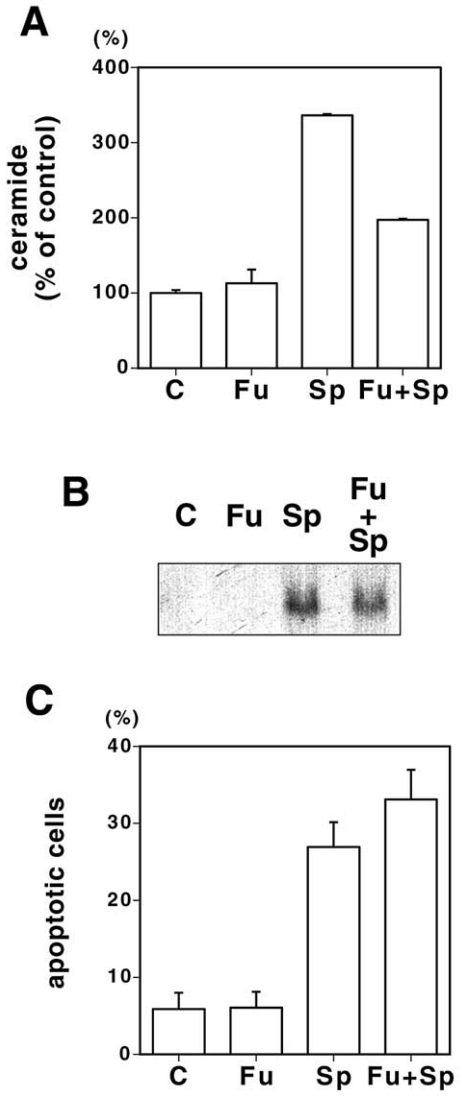

Fig. 2. No effect of fumonisin B_1 on sphingosine-induced c-jun expression and apoptosis. A: HL-60 cells were pretreated with or without 100 μM fumonisin B_1 (Fu) for 30 min and then treated with 0.1% ethanol vehicle or 10 μM sphingosine (Sp) for 1 h. The levels of ceramide were determined as described in Section 2. The results are representative of two different experiments. The data show means plus S.D. of duplicate counts. B: The levels of c-jun mRNA were determined as described in Section 2. The results are representative of two different experiments. C: HL-60 cells were pretreated with or without 100 μM fumonisin B_1 (Fu) for 30 min and then treated with 0.1% ethanol vehicle or 10 μM sphingosine (Sp) for 4 h. Apoptosis was assessed under microscope by typical morphological changes such as nuclear fragmentation and formation of apoptotic bodies. At least 200 cells were examined in each experiment. The data show means plus S.D. of three different experiments.

3.2. No effect of fumonisin B_1 on sphingosine-induced c-jun expression and apoptosis

Because sphingosine could be converted to ceramide by *N*-acylation and ceramide could induce c-jun expression, we used fumonisin B_1, an inhibitor of ceramide synthase, to investigate whether sphingosine itself induced c-jun expression. As shown

in Fig. 2A, treatment with sphingosine greatly increased the level of ceramide, presumably by *N*-acylation of sphingosine in cells. When cells were treated with both sphingosine and fumonisin B_1, generation of ceramide from sphingosine was inhibited by more than 50%. However, fumonisin B_1 did not significantly inhibit sphingosine-induced c-jun expression (Fig. 2B). Furthermore, sphingosine-induced apoptosis was not suppressed by co-treatment with fumonisin B_1 (Fig. 2C). These results suggest that sphingosine itself could induce c-jun expression and apoptosis without conversion to ceramide.

3.3. Effects of kinase inhibitors on c-jun induction by sphingosine and C2-ceramide

Because it has been reported that activation of PKA and PKC induced the expression of c-jun mRNA, we examined whether PKA and PKC were involved in c-jun induction by sphingosine. As shown in Fig. 3, sphingosine-induced c-jun expression was stimulated by H-89 (a PKA inhibitor). The level of c-jun mRNA treated with sphingosine and H-89 was approximately three-fold compared to that with sphingosine alone. H-7 or staurosporine (PKC inhibitors) did not significantly affect sphingosine-induced c-jun expression. These results suggest that inhibition of PKA might be involved in c-jun induction by sphingosine.

To investigate whether C2-ceramide induced c-jun expression by a similar mechanism with sphingosine, we examined the effects of PKA and PKC inhibitors on c-jun induction by C2-ceramide. As shown in Fig. 3, H-89 did not affect C2-ceramide-induced c-jun expression, whereas H-7 and staurosporine strongly inhibit c-jun induction by C2-ceramide. These results suggest that activation of PKC might be involved in c-jun induction by C2-ceramide, demonstrating the difference between the mechanisms by which sphingosine and C2-ceramide induced c-jun expression.

3.4. Potentiation of sphingosine-induced growth inhibition by H-89

We previously reported that C2-ceramide induced apoptosis and inhibited cell growth through induction of c-jun and activation of AP-1. Because H-89 stimulated sphingosine-induced c-jun expression, we examined whether H-89 potentiated sphingosine-induced growth inhibition. As shown in Fig. 4, treatment with sphingosine and H-89 significantly inhibited cell growth compared to treatment with sphingosine alone. Furthermore, H-89 did not affect C2-ceramide-induced growth inhibition. As we previously reported [14], staurosporine inhibited C2-ceramide-induced apoptosis and growth inhibition, whereas sphingosine-induced growth inhibition was not affected by staurosporine (data not shown). These results further suggest that c-jun induction might be required for C2-ceramide- and sphingosine-induced apoptosis and growth inhibition.

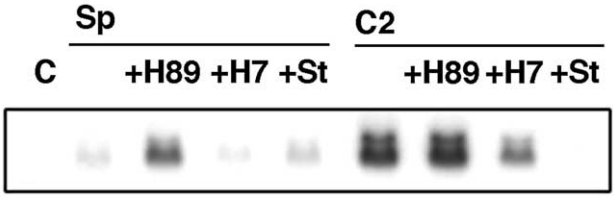

Fig. 3. Effects of PKA or PKC inhibitors on sphingosine- or C2-ceramide-induced c-jun expression. HL-60 cells were treated with either 10 μM sphingosine (Sp) or C2-ceramide (C2) alone or plus 1 μM H-89 (H89), 10 μM H-7 (H7), or 10 μM staurosporine (St) for 1 h. C: treated with 0.1% ethanol vehicle. Northern blot experiments were performed as described in Section 2. The results are representative of three different experiments. Equal loading of RNA on each lane was confirmed by methylene blue staining of the membrane.

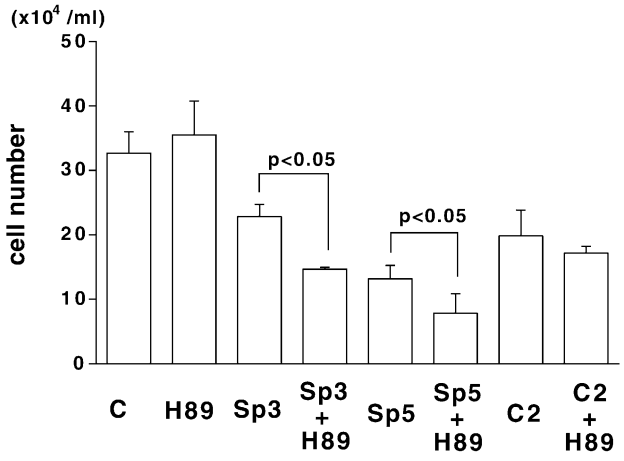

Fig. 4. Effect of H-89 on sphingosine- or C2-ceramide-induced growth inhibition. HL-60 cells at a concentration of 2×10^5 cells/ml were treated with either 3 or 5 μM sphingosine or 2 μM C2-ceramide alone or plus 0.5 μM H-89 for 24 h. Viable cell numbers were counted under microscope by trypan blue dye exclusion test. C: treated with 0.1% ethanol vehicle. The results are representative of three different experiments. The data show means plus S.D. of triplicate counts.

4. Discussion

It has been proposed that ceramide may function as a biostat regulating cell growth and apoptosis. Sphingosine, an *N*-deacylated form of ceramide, is also a potent inducer of apoptosis and growth inhibition. However, the mechanisms by which ceramide and sphingosine exert these effects remain to be elucidated. Here we demonstrated that (i) sphingosine (as well as ceramide) induced c-jun expression, (ii) sphingosine-induced c-jun expression was stimulated by H-89, a PKA inhibitor, whereas C2-ceramide-induced c-jun expression was not affected by H-89, (iii) sphingosine-induced c-jun expression was only slightly affected by PKC inhibitors, whereas C2-ceramide-induced c-jun expression was strongly inhibited by PKC inhibitors. These results suggested that inhibition of PKA might be involved in c-jun induction by sphingosine, whereas activation of PKC might be involved in c-jun induction by C2-ceramide. It seemed quite surprising that sphingosine and C2-ceramide induced c-jun expression by distinct mechanisms, considering the similarities in structure between sphingosine and C2-ceramide.

Furthermore we showed that H-89, which stimulated sphingosine-induced c-jun expression, potentiated sphingosine-induced growth inhibition. Previously, we demonstrated the requirement of c-jun/AP-1 activation in C2-ceramide-induced apoptosis and growth inhibition. Consistent with this, staurosporine, which inhibited C2-ceramide-induced c-jun expression, suppressed C2-ceramide-induced apoptosis. These results suggested that c-jun induction might be required in sphingosine-induced apoptosis and growth inhibition.

Further studies will be needed to elucidate the signaling

pathways in c-jun expression induced by sphingosine and ceramide.

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