Ceramide-induced Translocation of Protein Kinase C-δ and -ε to the Cytosol

IMPLICATIONS IN APOPTOSIS*

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Ceramide is now recognized as an intracellular lipid signal mediator, which induces various kinds of cell functions including apoptosis. Ceramide-induced apoptosis was reported to be blocked by 12-O-tetradecanoylphosphol 13-acetate, a protein kinase C (PKC) activator, but its mechanism remains unclear. Therefore, we investigated whether ceramide has any effect on PKC in the induction of apoptosis. We here report that N-acetyl-sphingosine (synthetic membrane-permeable ceramide) induced translocation of PKC-δ and -ε isozymes from the membrane to the cytosol within 5 min in human leukemia cell lines. Treatment with sphingomyelinase, tumor necrosis factor-α, or anti-Fas antibody, all of which can induce apoptosis by generating natural ceramide, similarly induced cytosolic translocation of PKC-δ and -ε. In Fas-resistant cells anti-Fas antibody did not induce cytosolic translocation of PKC-δ and -ε because of no generation of ceramide, whereas N-acetyl-sphingosine induced apoptosis with cytosolic translocation of PKC-δ and -ε. Furthermore, both 12-O-tetradecanoylphosphol 13-acetate and a nonspecific kinase inhibitor, staurosporine, prevented ceramide-induced apoptosis by inhibiting cytosolic translocation of PKC-δ and -ε. These data suggest that cytosolic translocation of PKC-δ and -ε plays an important role in ceramide-mediated apoptosis.

Sphingolipids have recently emerged as intracellular signal mediators in a variety of cell functions (1). Hannon et al. (2, 3) reported that sphingosine, the backbone of sphingolipids, and lysosphingolipids inhibit PKC in vitro and in vivo. Thereafter, the “sphingomyelin cycle,” transient hydrolysis of sphingomyelin and concomitant generation of ceramide, was discovered in the early phase of monocytic differentiation of human leukemia HL-60 cells induced by 1α,25-dihydroxy-vitamin D₃ (4, 5). Many reports have supported the idea that ceramide is an intracellular signal mediator transducing the effects of various extracellular stimulants including tumor necrosis factor-α (TNF-α) (6, 7), γ-interferon (6), interleukin-1 (8), and nerve growth factor (9). Recent studies have shown that ceramide plays an important role in apoptosis (10). Besides TNF-α, apoptosis-inducing stimuli such as cross-linking of Fas (11, 12), ionizing radiation (13), glucocorticoid (14), anti-immunoglobulin antibody (15), anti-cancer drugs (16, 17), and serum deprivation (18) have been reported to induce the sphingomyelin hydrolysis and/or generation of ceramide.

PKC is a family of serine/threonine kinases that takes part in various cellular responses (19). Molecular cloning and biochemical studies have revealed the presence of at least 10 PKC isozymes that can be classified into three subgroups. The classical PKC members (α, β, βII, and γ) are activated by Ca²⁺, phophatidylserine, and diacylglycerol (DAG) or phorbol esters. The novel PKC members (δ, ε, γL, and θ), which lack the C₂ region, are activated by phosphatidylserine and DAG or phorbol esters without Ca²⁺. The atypical PKC members (ζ and λ), which have only one cysteine-rich region, are dependent on phosphatidylserine but are not affected by DAG, phorbol esters, or Ca²⁺. The role of different PKC isozymes in cellular functions remains unclear.

It has been reported that ceramide has no effect on PKC activity in vitro (2), but it remains unclear whether ceramide has any effect on PKC in vivo. It is known that activation of PKC by DAG or phorbol esters induces the translocation of PKC from the cytosol to the membrane fraction (20) and inhibits ceramide-induced apoptosis (10, 18, 21). Although it was recently reported that ceramide inhibited the membranous translocation of PKC induced by PKC-activating stimuli (22, 23) and inactivated PKC-α (24), the mechanisms by which PKC activators inhibit ceramide-induced apoptosis are still not known. We therefore investigated the change of the subcellular distribution of each PKC isozyme in the apoptosis-inducing process by ceramide and ceramide-generating signals. We here show that N-acetylsphingosine (C₂-ceramide), membrane-permeable synthetic ceramide, induced the translocation of PKC-δ and -ε from the membrane to the cytosol fraction in three human leukemia cells (HL-60, U-937, and HPB-ALL cells). Induction of apoptosis by neutral bacterial sphingomyelinase, TNF-α, or anti-Fas antibody, all of which generate natural ceramide, induced cytosolic translocation of PKC-δ and -ε as well. Furthermore, we show the following lines of evidence...
Ceramide-induced Cytosolic Translocation of PKC-δ and -ε

Demonstrating how ceramide-induced cytosolic translocation of PKC-δ and -ε is indispensable in leukemic cell apoptosis: 1) HPB-αFR cells are resistant to anti-Fas antibody inducing apoptosis because of no generation of ceramide by anti-Fas antibody and the consequent failure of cytosolic translocation of PKC-δ and -ε; 2) ceramide can induce both apoptosis and cytosolic translocation of PKC-δ and -ε in HPB-αFR cells; 3) translocation of PKC-δ and -ε to the membrane by 12-O-tetradecanoylphorbol 13-acetate (TPA) or staurosporine inhibits ceramide-induced apoptosis as a consequence of prevention of cytosolic translocation of PKC-δ and -ε. These data suggest that ceramide-induced cytosolic translocation of PKC-δ and -ε plays an important role in the signaling pathway leading to apoptosis. We also discuss the topological meanings of PKC translocation in apoptotic signals.

EXPERIMENTAL PROCEDURES

Materials—α-erythro-C2-ceramide was purchased from Matreya, Inc. α-erythro-N-Acetylhydrophospho sine (α-erythro-C2-dihydroceramide) was kindly provided by Dr. Y. A. Hannan (Duke University). p-Amidophenyl methanesulfonyl fluoride hydrochloride was purchased from Wako (Osaka, Japan). Recombinant neutral bacterial sphingomyelinasewas purchased from Higeta-Shoyu (Choshi, Japan). Other chemicals were obtained from Sigma.

Cell Culture—Human myelogenous leukemia HL-60 cells, human monoblastic leukemia U-937 cells, and human T-lymphoblastic leukemia HL-60 cell growth (5). Because ceramide induced apoptosis in monoblastic leukemia U-937 cells and T-lymphoblastic leukemia HL-60 cell growth (5), we examined the effect of 10 μM C2-ceramide on the cellular distribution of PKC isozymes within 1 h before the cells showed apoptotic characteristics. As shown in Fig. 1, A and B, PKC-δ and -ε, which initially existed more abundantly in the membrane than in the cytosol fraction, showed translocation from the membrane to the cytosol fraction, reaching a maximum within 2–5 min by treatment with C2-ceramide. The increased levels of PKC-δ and -ε in the cytosol fraction subsequently decreased near the control levels after 30 min, whereas the levels of PKC-δ and -ε in the membrane fraction once recovered after 15 min but decreased after 30 min (Fig. 1B). The reason for the transient nature of cytosolic translocation of PKC-δ and -ε may not be due to the metabolism of ceramide to other inactive compounds because the metabolism of C2-ceramide is not so rapid in HL-60 cells as we reported before (5), whereas another report showed the extensive metabolism of C2-ceramide in Chinese hamster ovary cells (28). Although it remains to be elucidated why PKC-δ and -ε disappeared from the cytosol, one possibility is that PKC-δ and -ε may be degraded after translocation to the cytosol by some proteases as mentioned in a recent report (29). In terms of the brief reappearance of PKC-δ and -ε in the membrane, the increase of DAG by treatment with ceramide might be the cause, but it is at present unclear. The classical PKC (α, βII, and γ) and the atypical PKC-ζ did not show significant changes in the subcellular distribution by treatment with C2-ceramide (Fig. 1A). Further experiments were performed to determine whether ceramide translocated PKC-δ and -ε to the nucleus, but no significant changes of PKC-δ and -ε were detected in the nuclear fraction (data not shown).

C2-ceramide induced the cytosolic translocation of PKC-δ and -ε in a dose-dependent manner (Fig. 1C). The increase of PKC-δ and -ε in the cytosol fraction was detected at the concentration as low as 1 μM C2-ceramide, which began to inhibit HL-60 cell growth (5). Because ceramide induced apoptosis in monoblastic leukemia U-937 cells and T-lymphoblastic leukemia HL-60 cells as well (data not shown), we investigated whether ceramide could induce translocation of PKC-δ and -ε to the cytosol fraction in these cell lines. Translocation of PKC-δ and -ε from the membrane to the cytosol was also detected in these cell lines (Fig. 1, D and E), suggesting that this translocation of PKC-δ and -ε might be a general phenomenon in ceramide-induced leukemic cell apoptosis.

Specificity of Cytosolic Translocation of PKC-δ and -ε Induced by Ceramide—C2-dihydroceramide, which has almost the same chemical structure as C2-ceramide except for lacking the C4–C5 double bond of sphingoid backbone, did not induce apoptosis in HL-60 cells in contrast to C2-ceramide (30). C2-
dihydroceramide did not induce significant translocation of PKC-δ and -ε (Fig. 2A), demonstrating the biological specificity of ceramide effects on the cytosolic translocation of PKC-δ and -ε. The effects of natural ceramide on translocation of PKC-δ and -ε were investigated by treating HL-60 cells with exogenous neutral bacterial sphingomyelinase (SMase). Because SMase purified from bacteria may contain some other phospholipases, we used recombinant neutral SMase (Higeta-Shoyu, Japan) (31, 32). The cytosolic translocation of PKC-δ and -ε was detected 60 min after treatment with 100 milliunits/ml SMase (Fig. 2B), which induced DNA fragmentation within 3 h in HL-60 cells (data not shown). SMase-induced translocation occurred later and more weakly than C2-ceramide-induced translocation, presumably due to insufficient action of ceramide generated in the outer membrane by exogenous SMase. In order to examine the specificity of SMase action, we checked the effects of other phospholipases on the subcellular localization of PKC-δ and -ε (Fig. 2B). Treatment with the phospholipases other than SMase did not induce apoptosis within at least 6 h in HL-60 cells, whereas 30–50% of the cells showed apoptotic changes by treatment with SMase in 6 h. PKC-δ in the cytosol fraction was decreased within 5 min of treatment with phosphatidylinositol-specific phospholipase C, which generates DAG and inositol trisphosphate from phosphatidylinositol, and remained under the control until 60 min after treatment. It was likely that translocation of PKC from the cytosol to the membrane was induced by phosphatidylinositol-specific phospholipase C-generated DAG. Neither phospholipase D, which generates phosphatidic acid mainly from phosphatidylcholine, nor phospholipase A2, which generates arachidonic acid and lysophosphatidylcholine, induced significant changes in the subcellular localization of PKC-δ. No significant changes of translocation of PKC-ε were detected by phosphatidylinositol-specific phospholipase C, phospholipase D, or phospholipase A2. These results suggest that natural ceramide generated by SMase was specifically involved in translocation of PKC-δ and -ε to the cytosol in contrast to other lipid second messengers including DAG, phosphoinositide, phosphatidic acid, and arachidonic acid.

**TNF-α and Anti-Fas Antibody Induced Cytosolic Translocation**

![Fig. 1. Cytosolic translocation of PKC-δ and -ε induced by ceramide.](image-url)

The results are representative of at least three different experiments. A, effects of C2-ceramide on the subcellular localization of PKC-α, -βII, -γ, -δ, -ε, and -ζ isozymes in HL-60 cells. The cells were treated with 10 μM C2-ceramide for 0, 5, 15, and 30 min. A single band was detected at the position corresponding to each recombinant PKC isozyme except PKC-γ, detected as double bands. The position of PKC-ζ is indicated by the arrow. B, time course of the ceramide-induced cytosolic translocation of PKC-δ and -ε. HL-60 cells were treated with 10 μM C2-ceramide for the indicated times. The densities were assessed using NIH Image version 1.47, and fold induction was calculated by comparing each density with that of the control in the cytosol (left) or in the membrane (right) fraction. Squares, PKC-δ; circles, PKC-ε. C, dose dependence of the ceramide-induced cytosolic translocation of PKC-δ and -ε. HL-60 cells were treated with the indicated concentrations of C2-ceramide for 5 min. D and E, effects of C2-ceramide on the subcellular localization of PKC-δ and -ε in U-937 cells (D) or in HPB-ALL or HPB-αFR cells (E). The cells were treated with the indicated concentrations of C2-ceramide for 5 min.
Generation of ceramide has been reported to be induced by various stimuli including TNF-α (6, 7) and anti-Fas antibody (11, 12). We investigated whether these biological stimuli translocated PKC-δ and -ε to the cytosol in the process of apoptosis. As shown in Fig. 3, TNF-α in U-937 cells induced cytosolic translocation of PKC-δ and -ε more evidently than in HL-60 cells, probably because U-937 cells were more susceptible to the apoptosis-inducing effect of TNF-α than HL-60 cells (data not shown). Cytosolic translocation of PKC-δ and -ε was observed within 5–15 min after treatment with TNF-α in U-937 cells. Because HL-60 and U-937 cells were not very sensitive to anti-Fas antibody (data not shown), we used HPB-ALL cells, highly sensitive to anti-Fas antibody, to examine its effect on PKC translocation. Treatment with anti-Fas antibody induced cytosolic translocation of PKC-δ and -ε within 15–60 min (Fig. 4A). In order to confirm the role of translocation to the cytosol in induction of apoptosis, we used a Fas-resistant HPB-ALL subline (HPB-αFR) in which anti-Fas antibody hardly induced apoptosis (Fig. 4B) and did not generate ceramide at least within 3 h, whereas in HPB-ALL cells anti-Fas antibody induced ceramide generation (Fig. 4C). In HPB-αFR cells, treatment with anti-Fas antibody did not induce cytosolic translocation of PKC-δ and -ε (Fig. 4A), whereas treatment with C2-ceramide translocated PKC-δ and -ε from the membrane to the cytosol and subsequently induced apoptosis (Figs. 1E and 4B). These results suggest that physiological apoptotic stimuli including TNF-α and anti-Fas antibody may require ceramide-mediated cytosolic translocation of PKC-δ and -ε.
Inhibition of Both Ceramide-induced DNA Fragmentation and Cytosolic Translocation of PKC-δ and -ε by Treatment with TPA and Staurosporine—Previous reports have shown that phorbol esters inhibited ceramide-induced apoptosis (10, 18). We confirmed that TPA inhibited DNA fragmentation induced by C2-ceramide in a dose-dependent manner in HL-60 cells (Fig. 5A). The cytosolic translocation of PKC induced by C2-ceramide was also inhibited by treatment with TPA at the concentrations that blocked ceramide-induced apoptosis (Fig. 5B). Because higher concentrations of TPA induced rapid down-regulation of PKC-δ, the amount of PKC-δ in the membrane fraction was decreased within 5 min by treatment with C2-ceramide and 10 nM TPA and remained down-regulated at least after 30 min. PKC-ε was also down-regulated within 15 min of treatment with TPA (data not shown). Conversely TPA-induced translocation of PKC-δ and -ε from the cytosol to the membrane was competed by C2-ceramide dose-dependently (Fig. 5C), and higher concentrations of C2-ceramide induced apoptosis against treatment with TPA (Fig. 5D). Furthermore, we investigated the effects of a synthetic DAG analogue, 1,2-dioctanoyl-sn-glycerol (diC8) on the ceramide-induced translocation of PKC-δ and -ε to the cytosol, because DAG is a critical physiological activator of PKC. We found that diC8 inhibited the translocation of PKC-δ and -ε to the cytosol by C2-ceramide as well as TPA did (Fig. 5E). Although it was reported that diC8 inhibited ceramide-induced apoptosis in HL-60 cells (18, 21), in our hands it did not inhibit ceramide-induced DNA fragmentation, presumably because of the short duration of action due to its rapid metabolism compared with TPA and because a higher concentration (20 μM) of diC8 induced DNA fragmentation itself (data not shown).

Because staurosporine, a nonspecific kinase inhibitor, was reported to induce the translocation of PKC-ε from the cytosol to the membrane in SH-SYSY human neuroblastoma cells (33), we examined its effect on the ceramide-induced cytosolic translocation of PKC-δ and -ε. We found that staurosporine translocated PKC-δ as well as PKC-ε in HL-60 cells and inhibited the cytosolic translocation of PKC-δ and -ε induced by C2-ceramide (Fig. 6A). Furthermore, we investigated the effect of staurosporine on ceramide-induced apoptosis and found that ceramide-induced DNA fragmentation was suppressed by staurosporine (Fig. 6B). These results demonstrated that ceramide-induced apoptosis and cytosolic translocation of PKC-δ and -ε was inhibited by both PKC activator and non-PKC activator, which translocated PKC-δ and -ε from the cytosol to the membrane, and therefore membrane translocation (rather than activation) of PKC-δ and -ε might be necessary to inhibit ceramide-induced apoptosis. In other words, it is suggested that cytosolic translocation of PKC-δ and -ε plays an important role in ceramide-mediated apoptosis.

DISCUSSION
Although it is known that sphingosine or lysosphingolipids inhibit PKC activity (2, 3), ceramide has been considered to have no direct effect on PKC. Recent studies have shown that treatment with C2-ceramide (or sphingomyelinase) inhibits membrane translocation of PKC by PKC-activating stimuli (22, 23) and inactivates PKC-α (24), but there have been no reports that demonstrate the biological effect of ceramide on PKC in induction of apoptosis. We showed the treatment with C2-ceramide translocated PKC-δ and -ε from the membrane to the cytosol fraction in three different human leukemia cells (promyelocytic HL-60, monoblastic U-937, and T cell HPB-ALL cells). Neither treatment with dihydroceramide lacking the C4–C5 double bond of ceramide structure nor exogenous phospholipases (PLC, phospholipase D, and phospholipase A2, except SMase) induced cytosolic translocation of PKC-δ and -ε, demonstrating the specificity of ceramide effect on the cytosolic
translocation of PKC-δ and -ε. Furthermore, treatment with TNF-α or anti-Fas antibody, which induced apoptosis in the consequence of generating ceramide by hydrolysis of sphingomyelin (6, 7, 11, 12), translocated PKC-δ and -ε to the cytosol as well as ceramide. These data suggest that cytosolic translocation of PKC-δ and -ε may be closely related to the induction of apoptosis by ceramide and ceramide-generating stimuli including TNF-α and anti-Fas antibody.

In order to confirm that ceramide and ceramide-generating stimuli required the translocation of PKC-δ and -ε to the cytosol for completing apoptotic signals, we investigated 1) whether cytosolic translocation of PKC-δ and -ε was blocked by the failure of ceramide generation and 2) whether apoptosis was affected by the inhibition of ceramide-induced cytosolic translocation of PKC-δ and -ε. The results showed that in HPB-aFR cells neither cytosolic translocation of PKC-δ and -ε nor apoptosis was induced because of no generation of ceramide by the anti-Fas antibody and that the resistance to apoptosis in HPB-aFR cells was overcome by ceramide treatment accompanied by cytosolic translocation of PKC-δ and -ε. We also showed that ceramide-induced apoptosis was suppressed by competitive inhibition of cytosolic translocation of PKC-δ and -ε with TPA or a nonspecific kinase inhibitor staurosporine, both of which induced translocation to the membrane of PKC-δ and -ε. Taken together, cytosolic translocation of PKC-δ and -ε seemed to be indispensable to ceramide-mediated apoptosis in leukemia cells.

It has been reported that phorbol esters and DAG analogues, both activators of PKC, inhibited apoptosis induced by ceramide or ceramide-generating stimuli (10, 18, 21). As shown in Fig. 5, ceramide-induced apoptosis and cytosolic translocation of PKC-δ and -ε were overcome by TPA, and increasing doses of ceramide induced apoptosis and cytosolic translocation of PKC-δ and -ε against TPA. These data suggested that TPA and ceramide performed competitively in terms of the translocation of PKC-δ and -ε between the cytosol and the membrane. Although diC₈ inhibited ceramide-induced cytosolic translocation of PKC-δ and -ε, apoptosis was not suppressed by diC₈ possibly due to its rapid metabolism and because higher concentrations of diC₈ induced apoptosis with DNA fragmentation. On the other hand, we here investigated the effects of staurosporine on the ceramide-induced cytosolic translocation of PKC-δ and -ε because Jalava et al. (33) showed translocation to the membrane of PKC-ε by staurosporine. Surprisingly, staurosporine suppressed both ceramide-induced cytosolic translocation of PKC-δ and -ε and DNA fragmentation. Whereas higher concentrations of staurosporine (100 nM or more) were reported to induce apoptosis, conceivably due to nonspecific inhibition of various kinases (34, 35), at the concentration (3 nM) used in our experiments staurosporine might inhibit PKC more effectively than cyclic AMP-dependent kinase or tyrosine kinase, judging from IC₅₀ values (2.7, 8.2, and 6.4 nM, respectively). These results suggest that translocation of PKC-δ and -ε to the membrane may be more strongly related to the inhibition of ceramide-induced apoptosis than to the activation of PKC. More complex modulation of PKC activity related to its topological changes may be critical to dissect the signal transduction between apoptosis and proliferation. In other words, the increase (possibly activation) of cytosolic PKC-δ and -ε may be the decisive signal to ceramide-mediated apoptosis as discussed below.

It therefore remains to be elucidated how PKC-δ and -ε translocated to the cytosol are involved in the signal transduction pathway leading to apoptosis. It was reported that overexpression of PKC-δ induced morphological change and growth inhibition by treatment with TPA in Chinese hamster ovary cells and NIH 3T3 cells, whereas overexpression of PKC-ε increased the growth rate in NIH 3T3 cells and induced malignant transformation in Rat 6 fibroblasts (36–38). Although these data suggest that activation of PKC-δ may inhibit cell growth or cell cycle progression, whereas that of PKC-ε may have the opposite effects, neither the mechanism of topological changes of PKC between the cytosol and the membrane nor the relation between the activity of each PKC isozyme and the induction of apoptosis is known. Recently, proteolytic activation of PKC-δ to a cytosolic 40-kDa fragment in apoptotic cells treated with radiation, TNF-α, or anti-Fas antibody was reported (29). These findings may be a clue to clarifying the biological meanings of ceramide-induced cytosolic translocation of PKC-δ and -ε in induction of apoptosis, because we found that treatment with TNF-α or anti-Fas antibody degraded not only PKC-δ but also PKC-ε in the cytosol fraction. These suggest that ceramide-induced cytosolic translocation of PKC-δ and -ε may be a prerequisite for their proteolytic activation in TNF-α or anti-Fas antibody-induced apoptosis. The more precise implications of ceramide-induced cytosolic translocation of PKC-δ and -ε in apoptosis will be defined by the further biochemical and biological investigations on the relation between cytosolic translocation and the activation of PKC-δ and -ε consequent to their degradation in the cytosol.  

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