Ceramide Induces Caspase-Dependent and -Independent Apoptosis in A-431 Cells

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We investigated the ceramide-induced apoptosis and potential mechanism in A-431 cells. Ceramide treatment causes the round up and the death of A-431 cells that is associated with p38 activation and can be observed in 10 h. Short-time ceramide treatment-induced cell death is not associated with the typical apoptotic phenotypes, such as the translocation of phosphatidylserine (PS) from inner layer to outer layer of the plasma membrane, loss of mitochondrial membrane potential, DNA fragmentation, caspase activation, and PARP or PKC-δ degradation. SB202190, a specific inhibitor of p38 mitogen-activated protein (MAP) kinase, but not caspase inhibitor, blocks the cell death induced by short-time ceramide treatment (within 12 h). Whereas neither inhibition of p38 MAP kinase nor inhibition of caspases blocks cell death induced by prolonged ceramide treatment. Moreover, incubation of cells with ceramide for a long time (over 12 h) results in the reduction of proportion of S phase accompanied with typical apoptotic cell death phenotypes that are different from the cell death induced by short-time ceramide treatment. Our data demonstrated that ceramide-induced apoptotic cell death involves both caspase-dependent and caspase-independent signaling pathways. The caspase-independent cell death that occurred in relatively early stage of ceramide treatment is mediated via p38 MAP kinase, which can progress into a stage that is associated with changes of cell cycle events and involves both caspase-dependent and -independent mechanisms. J. Cell. Physiol. 199: 47–56, 2004.

Apoptosis is a form of cell death with unique cellular phenotypes including appearance of phosphatidylserine (PS) on the external cell membrane, loss of mitochondrial transmembrane potential, the activation of the caspase, cleavage of the poly (ADP) ribose polymerase (PARP), and protein kinase C-δ (PKC-δ), nuclear chromatin condensation, and DNA fragmentation (Blatt and Glick, 2001). Apoptosis induced by ceramide through either caspase-dependent or -independent mechanisms has been reported in recent years. However, the mechanism of caspase-independent apoptosis was still poorly understood. In lymphoblast cells, it was shown that ceramide and radiation exposure can induce distinct types of apoptosis via different mechanisms which can be determined from their phenotypes (Shi et al., 2001). In human glioma cells, Akt protein kinase, a well-known factor for cell survival, was found to be involved in the negative regulation of ceramide-induced caspase-independent apoptosis (Mochizuki et al., 2002). Ceramide-induced cell death of T lymphocyte and U937 was also shown to be a caspase-independent apoptosis (Belaud-Rotureau et al., 1999). In human cervix carcinoma cells (Lopez-Marure et al., 2002) and prostate cancer cell line LNCaP (Engedal and Saatcioglu, 2001), ceramide induces necrosis-like cell death without the biochemical and morphological markers characteristic of apoptosis. Nevertheless, in contrast to caspase-dependent apoptosis where the central mechanism and molecules involved in the transduction of death signals have been extensively studied and largely delineated, information regarding the players participating in the regulation of caspase-independent apoptosis is very limited.

Sphingolipids are not only structural components of cell membrane but also a ubiquitous and evolutionarily

Abbreviations: ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; PARP, poly (ADP) ribose polymerase; PKC-δ, protein kinase C-δ.

Sheng Zhao and Ya-Nan Yang contributed equally to this work.

Contract grant sponsor: The Chinese Academy of Sciences; Contract grant number: KSCX2-SW-203; Contract grant sponsor: The Virtual Research Institute of Aging of Nippon Boehringer Ingelheim; Contract grant sponsor: The “973” Project of China; Contract grant number: 2002CB513000.

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Received 6 May 2003; Accepted 3 September 2003
DOI: 10.1002/jcp.10453

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conserved signaling system in which ceramide and other sphingolipid derivatives can be formed. Ceramide can be generated through the hydrolysis of sphingomyelin or de novo synthesis. Inhibition of the degradation of ceramide by ceramidase also has an impact on the intracellular level of ceramide (Testi, 1996; Hannun and Luberto, 2000; Ohanian and Ohanian, 2001; Gomez et al., 2002). In addition, the cellular compartmentalization restricts the site of ceramide production and subsequent interaction with target proteins (Kolesnick and Goni, 2000; Ohanian and Ohanian, 2001; Vielhaber et al., 2001). Increasing evidence suggests that branching pathways of sphingolipid metabolism mediate either cell death or mitogenic responses depending on cell type and the nature of the stimulus. Although the main biological function of ceramide appears to be linked to its potency to induce cell death, its actual relevance as a regulator of cell death has been the subject of controversial discussions. Previous reports have shown that the mitogen-activated protein (MAP) kinase cascade, a key signal transduction pathway, contributes to ceramide-induced physiologic effects and usually determines the fate of the cells. The extracellular signal-regulated kinase (ERK) pathway, activated by growth factors and hormones, is involved in mediating cellular proliferation, transformation, and differentiation (Grewal et al., 1999). In contrast, the c-Jun N-terminal kinase (JNK) and the p38 cascades are implicated in cell death triggered by cytokines, growth factor withdrawal, and environmental stresses (Davis, 2000; Martin-Blanco, 2000). Ceramide was shown to inhibit ERK activity but activate JNK and p38 MAPK in some types of cell lines (Westwick et al., 1995; Kitatani et al., 2001; Willaime et al., 2001; Won et al., 2001). While in other cases, it was reported that ERK can be activated by ceramide (Raines et al., 1993; Reunanen et al., 1998; Subbaramaiah et al., 1998; Chen et al., 2001). The implications of JNK and p38 in ceramide-induced cell death in various cell types have been shown through some pharmacological and molecular assays (Brenner et al., 1997; Hida et al., 1999; Caricchio et al., 2002). Nevertheless it has also been demonstrated that p38 may be not essential for ceramide-induced cell death in U937 and MC/9 cells (Jarvis et al., 1997; Scheid et al., 1997; Hida et al., 1999; Caricchio et al., 2002). In addition, the cellular compartmentalization restricts the site of ceramide production and subsequent interaction with target proteins (Kolesnick and Goni, 2000; Ohanian and Ohanian, 2001; Vielhaber et al., 2001).

### Materials and Methods

#### Materials

A-431 human epidermoid carcinoma cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell culture reagents and fetal bovine serum (FCS) were purchased from Life Technologies (Grand Island, NY). [3H-methyl]-thyridine (83.60 Ci/mmol) was from NEN Life Science Products (Boston, MA). PD98059, SB202190, C8-ceramide, caspase inhibitor I (Z-VAD-fmk, a specific caspase inhibitor), caspase inhibitor III (BD-fmk, a broad caspase inhibitor), caspase substrate set I (Colorimetric), and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-goat secondary antibodies were purchased from Calbiochem (La Jolla, CA). Nitrocellulose membrane was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Mouse monoclonal antibody against phosphorylated ERK (p-ERK) and rabbit polyclonal antibodies against ERK, p38, PARP, PFK-5, and goat polyclonal antibody against actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody against phosphorylated p38 (p-p38) was obtained from New England Biolabs, Inc. (Beverly, MA). Super signal reagents were purchased from Pierce (Rockford, IL). Annexin V-FITC apoptosis detection kit I was obtained from Pharmingen (San Diego, CA). 3,3'-Dihexyloxacarbocyanine iodide (DiOC6(3)), propidium iodide (PI), hydroxyurea, nocodazole, and daunorubicin were from Sigma (St. Louis, MO).

#### Cell culture

A-431 cells cultured in 100-mm master plates in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO2 at 37°C. Medium were renewed every 2–3 days until confluence was reached. For experiment, cells were seeded into 60 or 35-mm plates and cultured under the same condition. The photographs of cell morphology were taken directly under the inverted phase-contrast microscope after indicated treatments.

#### Detection of the loss of plasma membrane asymmetry

In the early stage of apoptosis, the membrane PS is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35–36 kDa Ca2+-dependent phospholipid-binding protein that has a high affinity for PS, which can bind to cells with exposed PS (Raynal and Pollard, 1994). Annexin V-FITC staining precedes the loss of membrane integrity that accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes, which can be determined via PI staining. Thus, we used the annexin V-FITC in conjunction with PI staining to identify the.
early apoptotic cells (Annexin V-FITC positive and PI negative) as per Vendor’s instruction of the kit.

Detection of the loss of mitochondrial membrane potential

The loss of mitochondrial membrane potential is another early event of apoptosis. This phenomena can be examined by several potent sensitive dyes such as DiOC₆(3) to which normal cells will be stained (positive). It can also be used in conjunction with PI staining to determine the early apoptotic cells (DiOC₆(3) negative and PI negative) (Joza et al., 2001). After indicated treatment, cells were trypsinized and stained by 40 nM DiOC₆(3) and 500 ng/ml PI in phosphate-buffered saline (PBS) at 37°C for 30 min and then analyzed by FACS (Becton Dickinson FACScan, Franklin Lakes, NJ).

Cell cycle analysis

The cell cycle was quantitatively determined by flow cytometry analysis (Blatt and Glick, 2001; Shi et al., 2001). The percentage of cells with sub-G₁ DNA content was taken as a measure of the apoptotic rate of cell population. After indicated treatment, cells were trypsinized and fixed with 70% ethanol for over 1 h. Cells were then pelleted and washed with PBS plus 20 mM EDTA. RNA was removed by adding RNase (1 mg/ml) at 37°C for at least 2 h. Cells were finally stained with PI (final concentration: 30 μg/ml), and the DNA contents of cells were then analyzed by FACS. To synchronize the cells to the entry of S phase, we first incubated the cells in serum-free medium for 24 h, followed by incubating the cells in 10% FCS containing medium plus 1 mM hydroxyurea for another 24 h. When the medium was replaced again with the normal culture medium, the cells were released from the entry of S phase. Six hours later, cells that were going to enter the G₂ phase were treated with nocodazole (50 μg/ml) for 4 h. The cells were then synchronized in the entry of M phase.

Preparation of cell lysates and immunoblotting

A-431 cells cultured in 60-mm plates were treated with C₈-ceramide as indicated, trypsinized, and washed once with PBS. Cells were re-suspended in ice-cold lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA) for 5 min and centrifuged at 10,000 g for 10 min. The supernatants (cytosolic extract) were collected as the sample for determining the caspase activity. Each assay contains 10 μl samples with 10–30 μg proteins, 10 μl substrate (2 mM), and 80 μl assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, and 10% glycerol). The reactions were proceeded at 37°C for 120 min, followed by measuring the optical density (OD) at 405 nm.

Statistical analysis

Results are presented as means ± standard deviations (SD) for the number of experiments indicated. For statistical analysis, Student’s t test was performed. Differences were considered significant at a level of P < 0.05.

RESULTS

C₈-ceramide induces the apoptosis in A-431 cells

Ceramide was shown to induce the time-dependent apoptosis of A-431 cells as detected by several different methods. By morphological examination, we observed that, in response to C₈-ceramide treatment, cells became firstly rounded up and looked much brighter than the untreated cells. Further incubation of cells with ceramide (10 h and longer) resulted in the death of cells (Fig. 1A). By using Annexin V or DiOC₆(3) staining, two classical early events of apoptotic cell death, the translocation of PS from inner side of the cytoplasm membrane to the outer side and the loss of mitochondrial membrane potential were detected. In Figure 1B, the annexin V positive but PI negative cells (the right lower square) were detected 14 h after treatment, and then moved upward into the annexin V and PI positive square (end stage apoptosis and death) when C₈-ceramide treatment was prolonged. In Figure 1C, the DiOC₆(3) and PI negative cells (the left lower square) were also observed 14 h after the treatment, which then moved upward into the DiOC₆(3) negative but PI positive square (end stage apoptosis and death) when ceramide treatment was continued. In addition, there is an increase in the sub G₁ DNA content and decrease in

DNA fragmentation assay

DNA fragmentation of apoptotic cells was detected as previously described (Chen et al., 2000) with minor modifications. The cells were rinsed with PBS twice and lysed on ice for 30 min in 10 mM Tris-Cl (pH 8.0), 25 mM EDTA, and 0.25% Triton X-100. After centrifugation at 13,800g for 15 min, the supernatant was incubated with RNase at 37°C for 60 min and then with proteinase K at 56°C overnight. The contents were extracted sequentially with phenol, phenol:chloroform (1:1), and chloroform. The DNA in aqueous phase was precipitated and analyzed by 1.5% agarose gel electrophoresis. Gel was visualized and photographed under transmitted UV light.

Caspase activity assay

A-431 cells cultured in 60-mm plates with 10% FCS were treated with C₈-ceramide as indicated, trypsinized, and washed once with PBS. Cells were re-suspended in ice-cold lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na₃VO₄, 10 mM Na₄P₂O₇·10H₂O, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) followed by shearing using 2 ml needle for three times. The lysates were centrifuged at 14,000g for 10 min. The supernatants were analyzed by Western blotting after SDS–PAGE. The proteins were transferred onto nitrocellulose membrane followed by blocking with 5% BSA in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h at room temperature and subsequently incubated with the primary antibody (1:2,000 dilution) overnight at 4°C. After being washed for another 1 h at room temperature, the membrane was further incubated with a horseradish peroxidase conjugated secondary antibody for 2 h and washed for 1 h. The immunoreactive bands were visualized by super signal reagents (Pierce) and the protein level was detected under the same condition after stripping the signal reagents (Pierce) and the protein level was detected under the same condition after stripping
the proportion in cells of S phase (Fig. 1D). The increase in the sub G1 DNA content became highly pronounced 20 h after the C8-ceramide treatment.

Inhibition of p38 MAP kinase blocks the apoptosis induced by ceramide in the early stage of the treatment

To investigate the potential mechanism of ceramide-induced apoptosis, we first examined the effect of ceramide on MAPK activation. As shown in Figure 2A, ceramide stimulates a rapid increase in the level of phosphorylated p38 which can be observed from 30 min to 8 h after the treatment. Incubation of cells with C8-ceramide also resulted in an activation of ERK as shown by increased forms of phosphorylated ERK1/ERK2 (Fig. 2B). We further investigated the role of these MAPK in ceramide-induced cell death. As shown in Figure 2C, inhibition of p38 by specific inhibitor SB202190 suppressed cell apoptosis induced by ceramide in the early period of treatment (within 12 h), which can be easily observed through cell morphological examination. Nevertheless, SB202190 is unable to inhibit the cell death induced by long-term ceramide treatment (24 h). In contrast, inhibition of ERK1/ERK2 by the MEK1/MEK2 inhibitor PD 98059 has no effect on ceramide-induced apoptosis. We also observed that

![Image](https://via.placeholder.com/150)

**Fig. 1.** Ceramide induced cell death. A-431 cells cultured in medium containing 10% FCS were treated with C8-ceramide (25 μM) for indicated times. **A:** The morphological changes of cells were examined by microscopy (400× magnifications). **B:** After treatment with ceramide, cells were trypsinized and collected, then stained with annexin V/PI. The deaths of cells were determined by FACS. **C:** Cells were treated as in B and stained with DiOC6(3)/PI, which exhibited the early apoptotic events induced by ceramide after 14 h. **D:** After the ceramide treatment, cells were stained with PI and fixed with ethanol. The sub G1 and S phase DNA contents of cells were determined by FACS. The data represent one of three independent experiments with same results.
treatment of cells with Z-VAD-fmk does not inhibit ceramide-induced cell death. The same results were obtained with BD-fmk (data not shown). In addition, inhibition of p38 does not prevent the PS translocation, and the loss of mitochondrial potential (Fig. 3A,B), and DNA fragmentation induced by long-term ceramide treatment (Fig. 3C), implying that p38 activation may not be involved in the classic apoptotic cell death which occurred only in long-term ceramide treatment, but is implicated in atypical apoptotic cell death which played a role in the early period of ceramide treatment.

**Prolonged treatment with ceramide induces the classic caspase-dependent cell apoptosis**

We examined the ceramide-induced cellular caspase activities with several different caspase substrates for a better understanding of the mechanism of ceramide-induced apoptosis. Ceramide-induced caspase activities were also determined by measuring the degradation of PARP and PKC-δ. As shown in Figure 4A, C₈-ceramide was shown to induce various types of caspase activities, which can be inhibited efficiently by Z-VAD-fmk and BD-fmk. Treatment of cells with C₈-ceramide also induces a time-dependent PARP (Fig. 4B) and PKC-δ (Fig. 4C) degradation, which became evident after 16 h, further indicating the ceramide-induced activation of caspases. Daunorubicin, which was previously shown to induce the caspase-dependent apoptosis and the degradation of PARP and PKC-δ as shown previously (Chen et al., 2000) was used here as a positive control.

To investigate the involvement of caspases in long-term ceramide treatment-induced apoptosis, cells were treated in the presence or absence of BD-fmk, and then treated with C₈-ceramide for 24 h. As shown in Figure 5,
BD-fmk, but not the p38 MAP kinase inhibitor SB202190, is able to block the DNA fragmentation (Fig. 5A) and PKC-δ or PARP degradation (Fig. 5B) of cells induced by prolonged ceramide treatment. In the case of PARP or PKC-δ, degradation was only partially inhibited by BD-fmk, which is consistent with our recent observation that PARP can also be hydrolyzed by caspase-independent pathway (Yang et al., 2003). Unexpectedly, BD-fmk either used alone or together with SB202190, failed to block the cell death induced by prolonged ceramide treatment (Fig. 5C), suggesting that caspase- and p38-independent mechanisms were also implicated in ceramide-induced apoptosis which occurred in the later period of the treatment.

C8-ceramide-induced caspase-dependent programmed cell death is associated with the decrease in the S phase proportion

By synchronization of cell cycle, we were able to further observe the effect of ceramide in a condition in which all the cells are in the same phase of cell cycle. As shown in Figure 6, the prolonged treatment of cells with C8-ceramide (24 h) resulted in an obvious decline of the S phase proportion, but an increase of the sub G1 DNA content, which is consistent with Figure 1D. Moreover, it seems that ceramide does not cause the cell cycle arrest but just delays the progress of the cell cycle, suggesting that the ceramide-induced programmed cell death are related with the alterations of signaling events controlling the cell cycle.

**DISCUSSION**

Apoptosis is mediated by various cellular events including protein synthesis and degradation, the alteration in protein phosphorylation states, the activation of lipid second messenger systems, and disruption of normal mitochondrial function. It has been shown that ceramide is able to activate a genetically active and regulatable suicide program (Belaud-Rotureau et al., 1999; Engedal and Saatcioglu, 2001; Shi et al., 2001; Lopez-Marure et al., 2002; Mochizuki et al., 2002), which is distinct from the classic caspase-dependent apoptotic cell death. In this study, we demonstrated that caspase-independent apoptosis was induced by short-time ceramide treatment, which was followed by both caspase-dependent and -independent apoptosis. The caspase-independent apoptosis that occurred in the early stage of the ceramide-treatment cannot be determined by classic phenotypes of apoptosis. It thus appears to be an atypical apoptotic cell death. The caspase-dependent apoptosis was determined by unique cellular phenotypes including the translocation of PS from inner membrane to the outer membrane, loss of mitochondrial transmembrane potential, activation of caspase, and DNA fragmentation. Since ceramide-induced cell death varies with cell types and the cellular environment, it is likely that the cellular context and the environment may contribute to the types of the cell death induced by ceramide.

It has been reported by several groups that p38 MAPK was involved in the mediation of ceramide-induced cell death (Brenner et al., 1997; Hida et al., 1999; Shimizu et al., 1999; Willaime et al., 2001). In ultraviolet B irradiation-induced apoptosis of human keratinocyte HaCaT cells (Shimizu et al., 1999), p38 MAP kinase was considered as a signaling component upstream of the caspase. In contrast, reported evidence that does not support the role of p38 MAPK in the mediation of ceramide-induced apoptosis has also emerged (Jarvis et al., 1997; Scheid et al., 1999). Although ERK MAP kinase can be down-regulated by ceramide in some cell lines, our data demonstrated that C8-ceramide can induce the activation of both ERK and p38 MAP kinases in A-431 cells, which is consistent with the reports that sphingomyelinase and ceramide activate ERK and p38 in HL-60 cells (Baines et al., 1993), dermal fibroblasts (Reunanen et al., 1998), 184B5/HER cells (Subbaramaiah et al., 1998), and NCI-H292 cells (Chen et al., 2001). Since the inhibition of ERK activation by MEK inhibitor PD98059 has no effect on ceramide-induced cell death, whereas inhibition of p38 MAP kinase by specific inhibitor SB202190 suppressed the ceramide-induced death in A431 cells, it thus appears that the activation of p38 but not ERK pathway
plays an important role in cell apoptosis induced by short-term ceramide treatment. Short-time ceramide treatment induces the cell death without the appearance of the typical apoptotic phenotypes including the DNA fragmentation, caspase activation, PKC-δ and PARP degradation, and cannot be inhibited by caspase inhibitor, suggesting that this type of cell death is a caspase-independent atypical apoptosis. Since the inhibition of p38 MAP kinase strongly suppresses the short-time ceramide treatment-induced cell death, the result indicates that p38 MAP kinase plays an important role in the caspase-independent cell death induced by short-time ceramide treatment. Longer time treatment of cells with ceramide (over 12 h) will induce the classic apoptotic phenotypes, such as PS translocation from inner side to the outer side of the plasma membrane, loss of mitochondrial potential, caspase activation, and DNA fragmentation. Because caspase inhibitor effectively inhibits the long-term ceramide treatment-induced DNA fragmentation and partially inhibits PKC-δ and PARP degradation, but has no effect on the ceramide-induced cell death, it suggests that both caspase-dependent and -independent mechanisms were involved in the apoptosis induced by long-term ceramide treatment. The fact that ceramide-induced caspase-independent cell death can be blocked by inhibiting p38 MAP kinase also implies that such cell death is not a passive process (necrosis) but an active and regulated apoptotic cell death.

It has been reported that ceramide acted as a cell cycle suppressor which can lead to G_1/G_0 arrest (Dbaibo et al., 1995; Hannun, 1996; Lee et al., 1998). In this study, we observed a reduction in S phase proportion of cells when ceramide treatment was conducted after cell cycle synchronization. Moreover, cells that decreased in S phase proportion in response to ceramide treatment was associated with an increase in sub-G_1 DNA contents, indicating that DNA cleavage and therefore the caspase-dependent apoptosis was induced. The dying of cells in response to ceramide may contribute to the drop of S phase proportion because the cell cycle was just delayed but not arrested. The results imply that the G_1 cells cannot pass through the G_1/S check point normally, which may render the cells more sensitive to ceramide stimuli and the subsequent cell apoptosis. The data presented by two groups on the doxorubicin-induced apoptosis in SH-SY5Y cells (Di Bartolomeo et al., 2000) and the C_2-ceramide-induced apoptosis in HL-60 cells (Bartova et al., 1997) also suggested these possibilities. It has been reported recently that p38 and ERK MAP kinases are activated in ceramide-, taxol-, etoposide-, and

![Fig. 3. Effect of MAP kinase inhibitors on ceramide-induced cell apoptosis. A and B: The effect of SB202190 on the C_8-ceramide-induced cell apoptosis as determined by FACS assays. Cells were treated with C_8-ceramide (25 μM) for indicated time in the presence or absence of SB202190, PD98059. Cell apoptosis were determined by annexin V/PI (A) or DoC6(3)/PI (B) staining assay. C: The effect of p38 inhibitor on ceramide-induced apoptosis as measured by DNA fragmentation. Cells were treated with C_8-ceramide (25 μM) in the presence or absence of selective p38 MAP kinase inhibitor SB202190 (μM) for indicated time. DNA was extracted and DNA fragmentation was examined. The data are the represented results from two or three independent experiments.](image-url)
Fig. 4. Ceramide induced caspase activation and PARP and PKC-δ cleavage. A-431 cells cultured in DMEM containing 10% FCS were treated with C_{16}-ceramide (25 μM) for 24 h in the presence or absence of caspase inhibitors pre-added 10 min before the treatment. A: The effects of different caspase inhibitors on ceramide-induced caspase activity. Data are the means ± SD of three independent experiments. Statistic analysis was performed by comparing the last two bars with the second bar for each groups. *, **, significant at P < 0.05 and 0.01 respectively. B: Time-dependent PARP degradation induced by C_{16}-ceramide. C: Time-dependent PKC-δ degradation induced by C_{16}-ceramide. The data represents one of two or three independent experiments with the same results. Daunorubicin (DNR, 2.5 μM for 6 h) was used as a positive control.
whereas short-term ceramide treatment-induced caspase-independent apoptosis was mediated via mechanism in which p38 MAPK plays an important role.

ACKNOWLEDGMENTS

We thank Dr. Hehua Chen and Yiran Zhou for their helpful discussions.

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