Involvement of Caspase 3- and 8-Like Proteases in Ceramide-Induced Apoptosis of Cardiomyocytes

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ABSTRACT

Ceramides are the metabolic products of sphingolipids of the eukaryotic cell membranes and are believed to function as signaling molecules in a variety of biological processes. Ceramide induces apoptosis in cultured cardiomyocytes. However, the molecular pathway underlying ceramide-induced apoptosis is not clear. In this study, we investigated the role of the cysteinyl aspartate–specific proteases (caspases) in cardiomyocyte apoptosis induced by ceramide. Treatment of in vitro cultured rat neonatal cardiomyocytes with ceramide results in robust cell death, of which the majority is apoptotic, as shown by positive staining for terminal deoxyribonuclease transferase–mediated deoxyuridine triphosphate nick end-labeling and the appearance of pyknotic nuclei with Hoechst staining. Caspase 3- and 8-like protease activities are induced in cardiomyocytes by ceramide treatment. Addition of the tetrapeptide inhibitors for caspases attenuated ceramide-induced apoptosis. The nonselective caspase inhibitor (B-D-FMK) and the caspase 3 (Z-DEVD-FMK) and caspase 8 (Z-IETD-FMK) inhibitors reduced ceramide-induced cardiomyocyte death and significantly inhibited the activation of caspase 3. However, the inhibitors specific for caspases 1, 2, 4, 6, and 9 have no significant effects on cardiomyocyte survival under the same conditions. These data suggest that caspases 3- and 8-related proteases are involved in ceramide-induced cardiomyocyte apoptosis.

Key words: cardiomyocytes, apoptosis, Sphinogosine, caspase.

Programmed cell death of the myocardium contributes significantly to cardiac cell loss during myocardial infarction and the progression of congestive heart failure (1,2). However, the molecular mechanisms that regulate cardiomyocyte apoptosis remain largely unknown. Ceramide is a sphingosine metabolite that is believed to function as a secondary messenger for many cytokines. The cell-permeable ceramide analogues inhibit cell proliferation and induce cell differentiation in certain cell types (3,4). They have also been shown to induce programmed cell death in many cell types, including primary cardiomyocytes (5). It has been reported that ceramide may mediate angiotensin II and tumor necrosis factor-α (TNF-α)–induced cardiomyocyte apoptosis (5). Additionally, ceramide level is elevated in both cardiomyocyte cultures exposed to hypoxia and animal models of myocardial infarction (6).

In this study, we investigated the role of the cysteinyl aspartate–specific protease (caspase) family of proteases in cardiomyocyte apoptosis induced by ceramide. We found that caspase 3- and 8-like protease activity is induced in cardiomyocytes treated with ceramide. In addition, caspase inhibitors specific for caspase 3 (Z-DEVD-FMK) and caspase 8 (Z-IETD-FMK) significantly reduced ceramide-induced cardiomyocyte apoptosis. Our data suggest that caspase 3- and 8–related proteases are involved in ceramide-induced cardiomyocyte apoptosis.
Methods

Cell Culture

Isolation of rat neonatal cardiomyocytes was performed according to published protocol (7), with minor modifications. Briefly, hearts from 1- to 3-day-old neonatal Harlan-Sprague rats were minced and dissociated with 0.1% trypsin (Life Technologies, Gaithersburg, MD). After preplating in regular Petri dishes to reduce the number of nonmyocytes, remaining cell mixtures were plated on specially coated culture dishes (PRIMARIA, Life Technologies, Gaithersburg, MD) at a density of 5 × 10^3/cm^2. The cells were incubated in a mixture (1:1) of Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12, 2 mmol/L of L-glutamine, 50 μg/mL of gentamycin, 5% horse serum, and 0.1 mmol/L of bromodeoxyuridine (BrdU) for 24 hours. They were then changed to medium without serum and BrdU. Typical preparation results in cultures in which approximately 90% of the cells are myocytes (data not shown).

Cell Treatment and Cell Survival Curve

To induce cell death, cardiomyocyte cultures were incubated with C2-ceramide or C2-dihydroceramide (Calbiochem-Behring Corp, American Hoechst Corp, San Diego, CA) for various times. After treatment with these reagents, cardiomyocyte cultures in 6-well plates were washed twice with phosphate-buffered saline, incubated with 0.5 ml of 0.25% trypsin for 5 minutes at 37°C, and neutralized with 0.5 mL of fetal bovine serum. The cells were collected into an eppendorf tube and stained with 0.4% trypan blue. Surviving cells, assessed by trypan blue exclusion, were counted with a hemocytometer under light microscopy. The assay was performed in triplicate wells for each condition, and the experiments were performed at least twice.

To evaluate the role of caspases in cardiomyocyte apoptosis, substrate-based caspase inhibitors (Enzyme System Inc, Richmond, CA) were added to the cell culture medium simultaneously with ceramide, and surviving cells were counted 24 hours later.

Assessment of Cardiomyocyte Apoptosis Assessment of apoptotic myocytes was determined using a combination of the in situ terminal deoxyribonucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) and Hoechst staining. Myocytes grown on 24-well plates were fixed with 4% paraformaldehyde and permeated with ice-cold methanol. TUNEL assay was performed with the in situ cell death detection kit (Boehringer Mannheim, Indianapolis, IN) following the manufacturer’s recommendations. After TUNEL staining, cells were counterstained with 0.5 μg/mL of Hoechst 33285 and monitored with fluorescence microscopy. Cells positive for TUNEL and showing the morphological features of apoptosis (cell shrinkage, chromatin condensation, and fragmentation) were counted as apoptotic.

Measurement of Caspase Activity

Measurement of caspase activity was performed with the caspase-3 assay kit using fluorogenic substrates (Biosource International, Camarillo, CA). After treatment, cardiomyocytes were collected by scraping with a plastic cell lifter (Life Technologies) and centrifuged. Cell pellet was resuspended in ice-cold lysis buffer (50 μL for each 1 million cells) and incubated on ice for 10 minutes. The cell lysate was centrifuged at 12,000 rpm for 3 minutes at 4°C, and the supernatant was transferred to a new eppendorf tube and stored at −80°C. To measure the protease activity, 50 μL of cell lysate was mixed with 50 μL of 2× reaction buffer and 5 μL of 1 mol/L of fluorogenic substrate (DEVD-AFC, 7-amino-4-trifluoromethyl coumarin) in a 96-well plate and incubated at 37°C for 1 hour. The release of AFC from substrate was read in a fluorometer (Molecular Devices Corp, Sunnyvale, CA) with a 400-nm excitation filter and 505-nm emission filter (for AMC substrate) or 360-nm excitation filter and 490-nm emission filter (for AMC substrate). Protease activity (unites) was calculated according the formula:

\[ \text{Unites} = (\Delta \text{FU/h}) \times (\text{cal. curve slope})^{-1} \times (\text{n mole AFC/FU}) \]

where ΔFU is the difference of FU between T0 and T1, and curve slope is calculated from the standard curve of AFC or AMC.

Results

C2-Ceramide Induces Apoptosis in Rat Neonatal Cardiomyocytes

To investigate the effects of ceramide on cultured rat neonatal cardiomyocytes, cultures were incubated with different concentration of C2-ceramide for 24 hours, and surviving cells remaining on the culture surface were collected with trypsin and counted (Fig. 1A). C2-ceramide reduced cardiomyocyte survival in a dose-dependent manner. Only approximately 50% of the cardiomyocytes survived after 24 hours of treatment with 50 μmol/L of C2-ceramide, whereas less than 25% of the myocytes survived with 100 μmol/L of C2-ceramide. A biologically inactive form of ceramide, dihydroceramide, was used as a negative control. As shown in Fig. 1A, C2-dihydroceramide did not significantly affect cardiomyocyte survival at concentrations similar to that of ceramide.
Observation by phase-contrast microscopy showed that ceramide-treated cardiomyocytes began to round up, shrink, and detach from the culture dish at 4 to 6 hours after treatment with C₂-ceramide, with maximum cell detachment reached at approximately 12 to 16 hours after C₂-ceramide treatment (Figs. 1B and 2A and B).

To determine whether ceramide induced programmed cell death in cardiomyocytes, cardiomyocyte cultures treated with either vehicle or C₂-ceramide for 16 hours were subjected to the in situ TUNEL (Fig. 2E and F) and counterstained with Hoechst 33258 (Fig. 2C and D). Nuclei of the dying cells in the C₂-ceramide–treated cultures stained positive for TUNEL (Fig. 2F) and showed chromatin condensation and fragmentation (Fig. 2D), which are characteristic of apoptosis. These data are consistent with a previous report and indicate that C₂-ceramide induces apoptosis in cultured rat neonatal cardiomyocytes.

The percentage of TUNEL-positive nuclei in cardiomyocyte cultures treated with different concentrations of ceramide was also measured. As shown in Fig. 2G, treatment with 20 μmol/L, 50 μmol/L, and 100 μmol/L of ceramide resulted in approximately 15%, 30%, and 75% TUNEL-positive nuclei, respectively. Thus, ceramide induces cardiomyocyte apoptosis in a dose-dependent manner.

**Selective Caspase Inhibitors Prevent Ceramide-Induced Cardiomyocyte Apoptosis**

To investigate the role of different caspases in ceramide-induced cardiomyocyte apoptosis, we tested whether substrate-based peptide caspase inhibitors can prevent apoptosis in cultured rat neonatal cardiomyocytes treated with ceramide. Cardiomyocyte cultures were incubated with different substrate-based peptide caspase inhibitors (50 μmol/L) in addition to ceramide (75 μmol/L) for 24 hours. Surviving cells from each of the treated cardiomyocyte cultures were counted. As shown in Fig. 3, ceramide (75 μmol/L) treatment of cardiomyocytes for 24 hours resulted in approximately 50% cell survival compared with untreated cell cultures. Coincubation of ceramide with pan-caspase inhibitors, Z-VAD-fmk and B-D-fmk, increased cell survival rates to 80% and 90%, respectively. Inhibitors for caspase 3-like proteases (Z-DEVD-fmk) and caspase 8-like proteases (Z-IETD-fmk) also increased cell survival rates to 81% and 89%, respectively. Conversely, peptide inhibitors specific for caspase 1 and 4 (Z-YVAD-fmk), caspase 2-like proteases (Z-VDVAD-fmk), caspase 6 (Z-VEID-fmk), or caspase 9 (Z-LEHF-fmk) did not significantly affect cardiomyocyte survival after ceramide treatment. At greater concentrations (100 μmol/L), caspases 1, 2, 4, 6, and 9 also failed to block ceramide-induced cardiomyocyte apoptosis (data not shown). A negative control for these inhibitors, Z-PA-fmk, also had no effect on ceramide-induced cell death in cardiomyocytes.
Fig. 2.
Activation of Caspase 3- and 8-Like Protease in Ceramide-Treated Cardiomyocytes

We further measured the relative protease activity toward different caspase substrates in cardiomyocytes treated with ceramide. Cardiomyocytes were treated with C2-ceramide or vehicle for various times before cell lysates were collected. Protease activities toward different fluorogenic caspase substrates in the cell lysates were measured. As shown in Fig. 4A, there is a significant elevation of caspase 3-like protease activity (toward DEVD-afc) and caspase 8-like protease activity (IETD-afc), with maximum activity reached at 14 hours after ceramide treatment. Conversely, the levels of caspase 6-like protease (VEID-amc) and caspase 1- and 4-like protease (YDVD-afc) activity are very low and did not change significantly after ceramide treatment. There is no detectable level of protease activity toward YDVAD-afc (caspase 2), WEHD-afc (caspase 5), and LEHD-afc (caspase 9) before and after ceramide treatment.

To investigate whether the caspase inhibitors rescued ceramide-induced cardiomyocyte apoptosis through the inhibition of caspase activation, we measured caspase activity in cardiomyocytes treated with ceramide plus different caspase inhibitors. As shown in Fig. 4B, caspase 3-specific inhibitor, DEVD-fmk, and pan-caspase inhibitor, B-D-fmk (at 50 μmol/L), completely inhibited ceramide-induced caspase 3 activity. At 50 μmol/L, caspase 8 inhibitor, IETD-fmk, and nonselective caspase inhibitor, VAD-fmk, partially blocked ceramide-induced caspase 3 activation. At greater concentrations, VAD-fmk and IETD-fmk showed greater inhibition (data not shown). YVAD-fmk, VEID-fmk, VDVAD-fmk, LEHD-fmk, and FA-fmk did not inhibit caspase 3 activity in ceramide-treated cardiomyocytes.

**Discussion**

We report that ceramide induces a dose-dependent apoptosis in cultured rat neonatal cardiomyocytes. We also show that caspase 3- and 8-like protease activity is induced in ceramide-treated cardiomyocytes. In addition, peptide inhibitors specific for caspase 3- and 8-like proteases, as well as pan-caspase inhibitors, can protect cardiomyocytes from ceramide-induced apoptosis. However, inhibitors specific for caspases 1, 2, 4, 6, and 9 have no significant effect on ceramide-induced apoptosis in cardiomyocytes. These data indicate that caspase 3- and 8-like proteases are involved in ceramide-induced apoptosis in cardiomyocytes.

The caspase family of proteases is involved in the signaling or execution of apoptosis. These proteases all contain an active-site pentapeptide of QACXG and cleave their target after specific aspartic acid residues. To date, 13 members of the caspase family proteins have been identified in mammals, and they have distinct roles in apoptosis and inflammation. Caspases 2, 3, 6, 7, 8, 9, and 10 are believed to be involved in apoptosis and can be further divided into 2 classes: those directly responsible for proteolytic cleavages that lead to cell...
disassembly (effector) and those involved in the upstream regulatory events (initiator). The effectors include caspases 2, 3, 6, and 7, and the initiators are caspases 8, 9, and 10 (8).

Caspase 3 has been shown to be required in apoptosis of many cell types and is considered a key executioner of apoptosis (9,10). Yue et al (11) have shown that caspase 3 activation has a role in staurosporine-induced apoptosis in rat neonatal cardiomyocytes. We show here that caspase 3–like protease activity is induced in cardiomyocytes treated with ceramide, and that caspase 3 inhibitor can prevent ceramide-induced cardiomyocyte apoptosis. Therefore, our data are consistent with the central role for caspase 3 in apoptosis and further suggest that caspase 3 is also required for ceramide-induced apoptosis in cardiomyocytes. Caspase 7 recognizes the same tetrapeptide sequence (DEVD) and shares similar substrate spectrum (12). The caspase 3 inhibitor, DEVD-fmk, can also inhibit the protease activity of recombinant caspase 7 toward DEVD-afc in an in vitro assay (data not shown). Therefore, our current data do not exclude a role of caspase 7 in ceramide-induced cardiomyocyte apoptosis. Like caspase 3, caspase 7 is also highly expressed in the heart (13). Thus, it is likely that caspase 7 is also involved in cardiomyocyte apoptosis.

Caspase 2 and 6 also function as effector caspases. However, we were unable to detect protease activity toward caspase 2–specific peptide (VDVAD-afc) and caspase 6–specific peptide (VEID-amc) in ceramide-treated cardiomyocytes. Moreover, caspase 2– and 6–specific inhibitors are not able to inhibit ceramide-induced cardiomyocyte apoptosis at concentrations effective for caspase 3 inhibitor (50 μmol/L) or greater concentrations (100 μmol/L). Collectively, these data suggest that caspases 2 and 6 are not involved in cardiomyocyte apoptosis induced by ceramide.

Caspase 8 is involved in the signaling of TNF-α receptor (TNFR)– and CD95-mediated apoptosis. These cell-surface cytokine receptors contain a homologous cytoplasmic domain known as death domain (DD). Activation of these receptors results in the recruitment of the adapter proteins, TNFR-1–associated DD protein (TRADD) or Fas-associated protein with death domain (FADD), to the DDs. Aggregated FADD or TRADD can bind pro-caspase 8 through the N-terminal death effector domain and activate caspase 8. Activated caspase 8 will initiate apoptosis, either directly or through activation of downstream caspases (9,14). A previous report has also shown that the sphingolipid cascade is involved in TNF-α–induced cardiomyocyte apoptosis (5). Here, we showed that caspase 8 is activated by ceramide in cardiomyocytes. In addition, substrate-based caspase 8 inhibitor can inhibit the activation of caspase 3 protease and protect cardiomyocytes from ceramide-induced apoptosis. However, we were unable to detect caspase 9–like

Fig. 4. (A) Protease activity toward different caspase substrates in ceramide-treated cardiomyocytes. Cultured cardiomyocytes were treated with ceramide, and cell lysates were collected at times indicated. Cell lysates were incubated with different fluorogenic caspase substrates at 37°C for 1 hour, and the release of fluorogenic reagent was recorded with a fluorometer (molecular devise). The cell lysates were also incubated with caspase substrates, YDVAD-afc, WEHD-afc, and LEHD-afc, with no detectable enzyme activity recorded. Cell lysates were collected from these cells, and caspase activities toward DEVD-amc were measured. Caspase activity in untreated cell lysate (lane 1) was set as 100%. Mean values and SDs are from 3 independent experiments. TUNEL, terminal deoxyribonuclease transferase–mediated deoxyuridine triphosphate nick end-labeling.
protease activity in ceramide-treated cardiomyocytes, and caspase 9–specific inhibitor was not effective in preventing ceramide-induced cardiomyocyte apoptosis. These data are inconsistent with the notion that caspase 8 is upstream of caspase 3 in the cascade of caspase activation (8) and suggest that caspase 8 functions as the initiator caspase in ceramide-induced cardiomyocyte apoptosis. Conversely, the caspase 9/ADAM1 pathway may not be involved in ceramide-induced apoptosis. However, the signaling pathway by which ceramide activates caspase 8 remains to be elucidated.

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References
