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Calcium and mitochondrial metabolism in ceramide-induced cardiomyocyte death

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Ceramides are important intermediates in the biosynthesis and degradation of sphingolipids that regulate numerous cellular processes, including cell cycle progression, cell growth, differentiation and death. In cardiomyocytes, ceramides induce apoptosis by decreasing mitochondrial membrane potential and promoting cytochrome-c release. Ca^{2+} overload is a common feature of all types of cell death. The aim of this study was to determine the effect of ceramides on cytoplasmic Ca^{2+} levels, mitochondrial function and cardiomyocyte death. Our data show that C_2-ceramide induces apoptosis and necrosis in cultured cardiomyocytes by a mechanism involving increased Ca^{2+} influx, mitochondrial network fragmentation and loss of the mitochondrial Ca^{2+} buffer capacity. These biochemical events increase cytosolic Ca^{2+} levels and trigger cardiomyocyte death via the activation of calpains.

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1. Introduction

Thudichum (1884) referred to sphingolipids (from the Greek Σφιγξ: sphinx) as a peculiar group of lipids because of their enigmatic nature [1]. Ceramides represent the structural backbone common to all sphingolipids [2]. Moreover, they regulate several key cellular processes, including cell cycle, cell growth and differentiation, as well as cell death. Intracellular de novo synthesis of ceramides occurs in response to cytokines, environmental stress and chemotherapeutic agents [2]. Ceramides have also been shown to trigger different forms of cell death, depending on cell context. In this respect, it should be noted that they stimulate necrosis in lymphoid cells by increasing ROS formation and promoting ATP depletion [3].

Sphingolipid alterations occur in obesity and metabolic diseases where biological processes such as insulin sensitivity, lipid metabolism, inflammation, and immune responses are deregulated [4]. Particularly, ceramide accumulation has been implicated in the pathogenesis of multiple diseases including diabetes, cardiomyopathies and atherosclerosis [5]. More specifically, in the cardiovascular system, ceramides regulate cardiac contractility, vasomotor responses and endothelial function [6] and they also mediate cardiomyocyte apoptosis triggered by ischemia/reperfusion [7] or by TNF-α [8]. Moreover, C_2-ceramide stimulates apoptosis in cardiomyocytes by reducing mitochondrial membrane potential (ψmt) and by activation of caspases 8 and 3 [9]. In addition, the anti-apoptotic effect of cardiac preconditioning may be due to reduced ceramide production during sustained ischemia in the rabbit heart [10].

Mitochondria play an important role in apoptotic cell death produced by multiple conditions [11]. C_2-ceramide alters ψmt and promotes cytochrome-c (cyt-c) release and channel formation in the mitochondrial outer membrane [12,13]. In a previous study, we showed that C_2-ceramide promotes a rapid fragmentation of the mitochondrial network in a concentration- and time-dependent manner and, as a consequence, the early activation of apoptosis [14]. Accordingly, C_2-ceramide also stimulates mitochondrial network fragmentation in HeLa cells, which was linked to ER Ca^{2+} release and cell death [15].

The contribution of Ca^{2+} to cell death processes has been extensively described [16,17]. Ca^{2+} overload is a common feature of all types of cell

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; C_2-ceramide, D-erythro-sphingosine-N-acetate; CsA, cyclosporin A; cyt-c, cytochrome-c; DH-C_2, dyhidro-D-erythro-sphingosine-N-acetate; ER, endoplasmic reticulum; LDH, lactic dehydrogenase; MP, methyl pyruvate; TMRM, tetramethylrhodamine methyl ester; PI, propidium iodide; ψmt, mitochondrial membrane potential; TNF-α, tumor necrosis factor alpha

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death [17]. Several reports have shown a rise in cytoplasmic Ca^{2+} at both early and late stages of the apoptotic process [18]. Ca^{2+} release from ER and capacitative Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels have also been implicated in apoptosis [15,19,20]. In addition, ceramides cause the release of Ca^{2+} from the ER, increasing Ca^{2+} in both the cytosol, and the mitochondrial matrix. Accordingly, buffering cytoplasmic Ca^{2+} prevents mitochondrial damage and protects HeLa cells from apoptosis [15].

The aim of the current study was to investigate the molecular mechanisms involved in ceramide-dependent cardiomyocyte death and more specifically the role of cytoplasmic Ca^{2+} and mitochondria in this process. Our data show that C_2-ceramide induces apoptosis and necrosis in cultured cardiomyocytes by increasing cytoplasmic Ca^{2+} and promoting loss of mitochondrial function.

2. Materials and methods

2.1. Reagents

Cell culture reagents, Hanks medium, Dulbecco's modified Eagle's medium (DMEM), M199 medium, pancreatin, gelatin, Triton X-100, 5-bromo-2′-deoxyuridine, gadolinium chloride, propidium iodide (PI), methyl pyruvate (MP), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), oligomycin, FITC-conjugated anti-IgG mouse antibody and the chemical inhibitors E64D, leupeptin, Z-VAD-fmk, Z-VDAD-fmk and the lactic dehydrogenase (LDH) kit were purchased from Sigma-Aldrich Co (St. Louis, MO). The sphingolipids D-erythro-sphingosine-N-acetate (C_2) and dihydro-D-erythro-sphingosine-N-acetate (DH-C_2), cyclosporin A (CsA) and HRP-conjugated secondary antibodies anti-IgG rabbit and mouse were from Calbiochem (La Jolla, CA). Antibodies against cytochrome-c (cyt-c) and anti-α-spectrin were from BD Pharmingen (San Diego, CA) and EMD Millipore (Billerica, MA), respectively. Anti-caspase 3 antibody was purchased in Cell Signaling (Danvers, MA). Fluo 3-AM and tetramethylrhodamine methyl ester (TMRM) were from Invitrogen (Carlsbad, CA). Organic and inorganic compounds, salts, acids and solvents were purchased from Merck (Darmstadt, Germany). CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega (Madison WI).

2.2. Animals

Rats were bred in the Animal Breeding Facility, Faculty of Chemical and Pharmaceutical Sciences, University of Chile (Santiago, Chile). Studies were approved by the Institutional Bioethical Committee, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* published by the US (NIH Publication No. 85-23, revised 1996).

2.3. Culture of cardiomyocytes

Cardiomyocytes were prepared from hearts of 1- to 3-d-old Sprague–Dawley rats as described previously [21]. Cells were maintained in DMEM/M199 (4:1) medium containing 10% FBS and serum-starved for 24 h prior stimulation. Cells were plated at a density of $1–8 \times 10^3/mm^2$ on gelatin-coated 6-, 12-, 24- and 96-wells or 35-mm Petri dishes. For Ca^{2+} and immunofluorescence studies, cells were plated on gelatin-coated 25- and 18-mm glass coverslips in 35 mm or 12-well Petri dishes, respectively.

2.4. Cell viability and apoptosis assays

The integrity of the plasma membrane was assessed by determining the ability of cells to exclude propidium iodide (PI). The level of PI incorporation was quantified in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Cell size was evaluated by forward-angle light scattering (FSC). PI-negative cells of normal size were considered viable [22,23]. Sub-G1 population was determined in cardiomyocytes permeabilized with methanol and labeled with propidium iodide (PI). Then the cells were analyzed using flow cytometry [22,24].

2.5. Mitochondrial membrane potential assay

Mitochondrial membrane potential (ψmt) was measured as described previously [24]. Briefly, cells were loaded with 200 nM TMRM (used in nonquenching mode) for 30 min at 37 °C. Afterwards, cells were trypsinized and fluorescence was assessed by flow cytometry ($\lambda_{excitation} = 543$ nm; $\lambda_{emission} = 560$ nm) using a FACScan system (Becton-Dickinson, San Jose, CA,). CCCP 50 μM for 30 min was used as positive control of mitochondrial depolarization.

2.6. Intracellular and mitochondrial Ca^{2+} measurements

Intracellular and mitochondrial Ca^{2+} signals were determined as described previously [25–27]. Images were obtained from cultured cardiomyocytes preloaded with Fluo3-AM or RhodFF using an inverted confocal microscope (Carl Zeiss LSM-5, Pascal 5 Axiovert 200 microscope). Coverslips were mounted in a 1-mL capacity chamber and placed in the microscope for fluorescence measurements after excitation with a laser line of 488-nm for Fluo3-AM or 543-nm for RhodFF. The fluorescent images were collected every 0.4–2.0 s for fast signals and analyzed frame by frame with Image J software (NIH, Bethesda, MD).

2.7. Immunofluorescence studies for cytochrome-c release

Cyt-c release was evaluated by immunofluorescence as described previously [14]. Cardiomyocytes grown on coverslips were fixed with PBS containing 4% paraformaldehyde for 10 min, permeabilized with 0.3% Triton X-100 for 10 min, and blocked for 1 h with 5% BSA in PBS. Cells were then incubated with anti-cyt-c antibody at 1:400 and revealed with anti-mouse IgG-Alexa 488. Coverslips were mounted in DakoCytomation fluorescence mounting medium (DakoCytomation, Carpinteria, CA) and visualized by confocal microscope (Carl Zeiss Axiovert 135, LSM Microsystems).

2.8. Intracellular ATP levels

Cellular ATP content was measured using the luciferin/luciferase based ATP detection kit CellTiter-Glo Luminiscent Cell Viability Assay (Promega) following the manufacturer's instructions. Briefly, cardiomyocytes were cultured in 96-well Petri dishes and washed 3 times with PBS before incubation with the reagent. Sample luminescence was quantified in a TopCount NXT microplate luminescence counter (Perkin-Elmer, Waltham, MA). Data was normalized as fold over control.

2.9. Western blot analysis

Equal amounts of protein from cells were separated by SDS-PAGE (10% polyacrylamide gels) and electrotransfered to nitrocellulose. Membranes were blocked with 5% milk in Tris-buffered saline, pH 7.6, containing 0.1% (v/v) Tween 20 (TBST). Membranes were incubated with primary antibodies at 4 °C and re-blotted with horseradish peroxidase-linked secondary antibody [1:5000 in 1%(w/v) milk in TBST]. The bands were detected using ECL reagent followed by exposure to Kodak film and then quantified by scanning densitometry. Proteins were normalized to β-tubulin levels.

2.10. LDH release assay

Necrosis was measured by quantifying LDH activity in culture medium. LDH activity was determined by measuring the decrease

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