

Calcium and mitochondrial metabolism in ceramide-induced cardiomyocyte death



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ABSTRACT

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. Ca²⁺ 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 Ca²⁺ levels, mitochondrial function and cardiomyocyte death. Our data show that C₂-ceramide induces apoptosis and necrosis in cultured cardiomyocytes by a mechanism involving increased Ca²⁺ influx, mitochondrial network fragmentation and loss of the mitochondrial Ca²⁺ buffer capacity. These biochemical events increase cytosolic Ca²⁺ levels and trigger cardiomyocyte death via the activation of calpains.

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1. Introduction

Thudichum (1884) referred to sphingolipids (from the Greek Σφιγγίς; 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].

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; C₂-ceramide, D-erythro-sphingosine-N-acetate; CsA, cyclosporin A; cyt-c, cytochrome-c; DH-C₂, dihydro-D-erythro-sphingosine-N-acetate; ER, endoplasmic reticulum; LDH, lactic dehydrogenase; MP, methyl pyruvate; TMRM, tetramethylrhodamine methyl ester; PI, propidium iodide; ψ_{mt} , mitochondrial membrane potential; TNF- α , tumor necrosis factor alpha

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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, C₂-ceramide stimulates apoptosis in cardiomyocytes by reducing mitochondrial membrane potential (ψ_{mt}) 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]. C₂-ceramide alters ψ_{mt} and promotes cytochrome-c (cyt-c) release and channel formation in the mitochondrial outer membrane [12,13]. In a previous study, we showed that C₂-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, C₂-ceramide also stimulates mitochondrial network fragmentation in HeLa cells, which was linked to ER Ca²⁺ release and cell death [15].

The contribution of Ca²⁺ to cell death processes has been extensively described [16,17]. Ca²⁺ overload is a common feature of all types of cell

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 lactate 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 $1-8 \times 10^3/\text{mm}^2$ 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 (ψ_{mt}) 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 ($\lambda_{\text{excitation}} = 543 \text{ nm}$; $\lambda_{\text{emission}} = 560 \text{ nm}$) 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 Luminescent 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 electrotransferred 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

in NADH (10 mg/mL) spectrophotometrically at 340 nm for 2 min, in the presence of lactate (2.5 mg/mL). The fraction of LDH released was determined by comparing LDH activity in the culture medium relative to total LDH activity.

2.11. Cardiomyocyte transfection

Small interfering RNAs (siRNA) for calpains I and II and negative control (Mission, Sigma-Aldrich Corp., St Louis, MO) were used following the manufacturer's instructions. Cells were grown and after 48 h, transfected with siRNAs at a concentration of 25 nM with Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA). After 6 h incubation in Opti-mem®, the medium was removed and replaced with 10% FBS in DMEM/M199. Calpain I/II down-regulation was assessed 48 h post-transfection. The siRNAs used for knockdown experiments were as follows:

- Negative control, catalog number SIC001;
- Calpain Ia, sense (5'-GUUGUGACCUUUGAUCUAU-3'), antisense (5'-AUAGAUCAAAGGUCACAAC-3')
- Calpain Ib, sense (5'-CUGACUAUGUUUGCAUGAA-3'), antisense (5'-UUCAUGCAAACAUAGUCAG-3')
- Calpain IIa, sense (5'-GAACGUACGGGAACAUUUA-3'), antisense (5'-UAAAUGUCCCGUACGUUC-3')
- Calpain IIb, sense (5'-GUUUCAAGCUGCCUGUCA-3'), antisense (5'-UGACAGGGCAGCUUGAAAC-3')

Different combinations of two siRNAs against each calpain were tested, obtaining the best results with the siRNA mixture containing the specific siRNAs Ia and IIa, which was used for all the subsequent experiments (Supplementary Fig. 1A).

2.12. RNA purification and real-time reverse transcription-PCR (RT-PCR)

Total RNA was isolated using TRIzol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Random hexamers were used for reverse-transcription reactions using superscript II (Invitrogen, Carlsbad, CA). RT-PCR was performed using SYBR green (Invitrogen, Carlsbad, CA). Data for each transcript were normalized to both GAPDH and 18S rRNA as internal controls using the 2- $\Delta\Delta C_t$ method. The primers used are listed below:

Calpain 1 Rat Forward 5'-TGGAGATCAGTGTCAAGGAGTTAC-3'
 Calpain 1 Rat Reverse 5'-GCACTCATGCTGCCAGACTGTCCAGGTCA-3'
 Calpain 2 Rat Forward 5'-GGGCAGACCAACATCCACCTCAGCAAAAAC-3'
 Calpain 2 Rat Reverse 5'-ATCTCCGCATCTCTCCAGCCAGC-3'

2.13. Expression of results and statistical analysis

Data are presented either as means \pm SD of a number (n) of independent experiments or as examples of representative experiments performed on at least three separate occasions. Data were analyzed by analysis of variance and comparisons between groups were performed using a protected Tukey *t*-test. A value of $p < 0.05$ was chosen as the limit of statistical significance.

3. Results

3.1. C₂-ceramide induces apoptosis and necrosis in cardiomyocytes

Currently, there are conflicting views concerning the role of ceramides as second messengers in several cardiac pathologies [28]. One possibility to explain the death-promoting functions of ceramides invokes damage to mitochondria and alterations in calcium homeostasis. Whether such mechanisms may be relevant in cardiomyocytes remained to be determined. Thus, we investigated the types of cell death induced by C₂-ceramide in cultured rat cardiomyocytes and the role of Ca²⁺

and mitochondria in these events. For these experiments, we used a serum-free culture model to enhance sensitivity and detect more readily the cell death induced by C₂-ceramide than previously described [14]. As shown in Fig. 1A the treatment of cardiomyocytes with C₂-ceramide 20 μ M during a 6 h period induces a significant increase in the sub G1 population (from 8.0% \pm 1.6 to 24.2% \pm 2.0), indicative of apoptosis, which however does not account for the total cell death observed under these conditions, as evaluated by PI incorporation (from 7.8% \pm 0.9 to 41.4% \pm 5.1). Importantly, this effect was not observed when the cells were treated with the inactive analog, dihydro-C₂-ceramide, discarding a detergent effect of these short-chain lipids. The same treatment with C₂-ceramide also stimulated LDH release, as determined by measuring the activity of this enzyme in the culture medium (from 10.0% \pm 0.5 to 33.3% \pm 6.8), as well as a decrease in the ψ_{mt} and the intracellular levels of ATP ($-26.8\% \pm 3.4$ and $-30.3\% \pm 2.3$, using CCCP 50 μ M or oligomycin 2 μ M as negative and positive controls, respectively) (Fig. 1B–D). Moreover, the treatment of cardiomyocytes with C₂-ceramide also induced substantial release of cyt-c after 1–3 h of treatment. This effect decreased upon exposure to C₂-ceramide for longer periods of time (Fig. 1E). Collectively, these results suggest that the treatment of cardiomyocytes with C₂-ceramide induces cell death by a mechanism that involved time-dependent early apoptosis followed by a delayed necrosis.

3.2. Ca²⁺ influx triggered by C₂-ceramide stimulates cardiomyocyte death

Ceramide-induced cell death has often been associated with increments in the levels of two intracellular second messengers, Ca²⁺ and ROS [29–31]. To study the role of both messengers, two different approaches were used. ROS were measured by electron spin resonance (ESR) using the spin trap DMPO, whereas Ca²⁺ influx was assayed by confocal microscopy using the fluorescent probe Fluo 3-AM. C₂-ceramide (20 μ M) did not change the intracellular levels of ROS in cultured cardiomyocytes (Fig. 2A). Moreover the presence of antioxidants, including N-acetyl cysteine (NAC), Trolox (a water-soluble derivative of vitamin E) and Tiron did not reduce the C₂-ceramide-induced cell death (Fig. 2B). On the other hand, ceramide increased cytoplasmic Ca²⁺ levels in a manner that was dependent on the presence of external Ca²⁺ (Fig. 3A). To investigate whether this Ca²⁺ signal contributes to cell death triggered by ceramide, cardiomyocytes were pretreated either with the Ca²⁺ chelating agent EGTA (2 mM) or with GdCl₃ (10 μ M), a potent but nonselective antagonist of nonselective cation currents [32]. Both agents decreased PI incorporation into the cells after treatment with C₂-ceramide and also prevented decreases in ψ_{mt} and intracellular ATP levels (Fig. 3B–D), whereby Gd³⁺ was much more effective than EGTA. Additionally, both EGTA and Gd³⁺ delayed cyt-c redistribution, which under these conditions was no more longer significant at 3 h of treatment (Fig. 3E).

3.3. Calpains but not caspases are involved in cardiomyocyte death induced by C₂-ceramide

To shed light on mechanisms underlying cell death induced by C₂-ceramide, activation of two important downstream effectors and key cell death mediators was evaluated in cardiomyocytes, namely the cysteine protease caspases and Ca²⁺-dependent calpains [33]. The activation of these proteases was evaluated by determining the cleavage of pro-caspase 3 to active caspase-3 and of α -spectrin, an important target of activated calpains [34]. As shown in the Supplementary Fig. 1B–C, under our experimental conditions only α -spectrin cleavage was detected, suggesting that calpains are activated in our experimental model. Moreover, the inhibitors of caspases Z-VDAD-fmk (50 μ M) or Z-VAD-fmk (50 μ M) did not prevent cell death induced by C₂-ceramide, whereas the calpain inhibitors leupeptin (100 μ M) or E64D (10 μ M) did (Fig. 4A–B). Interestingly, leupeptin also attenuated

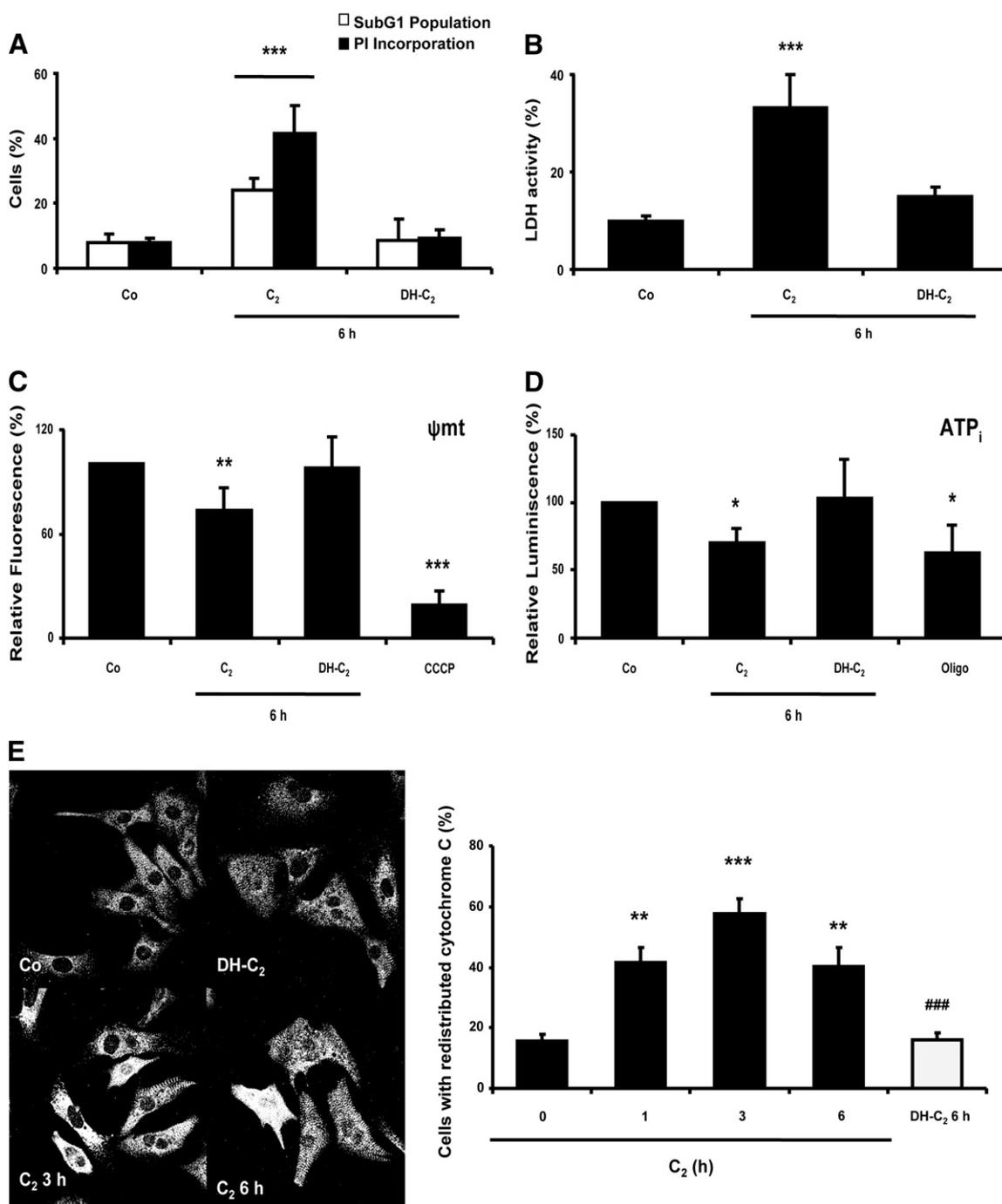


Fig. 1. C_2 -ceramide induces apoptosis and necrosis in cultured cardiomyocytes. (A) Cells were treated with C_2 -ceramide (C_2 , 20 μ M) or the inactive analog dihydroceramide (DH- C_2) at the same concentration. After 6 h, PI incorporation and the subG1 population were quantified by FACS analysis. Values are shown as the mean \pm SD of 3 independent experiments, *** P < 0.001. (B) Cells were incubated with C_2 -ceramide or DH- C_2 (20 μ M for 6 h) and necrosis was determined by the extent of LDH release to the culture medium. Data represent means \pm SD (n = 3), **** P < 0.001 vs. Control (Co). (C–D) Quantification of the mitochondrial membrane potential (ψ_{mt}) and the intracellular levels of ATP in cells treated with C_2 -ceramide or DH- C_2 (20 μ M, 6 h). Values are means \pm SD of n = 5 and 6 experiments, respectively, * P < 0.05, * P < 0.005 and **** P < 0.001 vs. Co. (E) Cells were incubated with C_2 -ceramide (C_2 , 20 μ M) or DH- C_2 for the indicated period of time and cytochrome-c was detected by indirect immunofluorescence analysis. Cytochrome-c redistribution was quantified by counting the cells with a diffuse pattern of fluorescence. (n = 7), ** P < 0.01, *** P < 0.001 vs. control and ## P < 0.01 vs. C_2 -ceramide (C_2) 3 h.

the decrease in the intracellular levels of ATP induced by C_2 -ceramide. However, neither E64D nor leupeptin prevented the decrease in ψ_{mt} in cells exposed to C_2 -ceramide (Supplementary Fig. 2).

Given that some calpain inhibitors are rather non-specific [35] and that calpains I and II (also known as μ -calpain and m-calpain, respectively) are both expressed in the heart requiring high Ca^{+2} concentration to be activated [36], both calpain isoforms were knocked

down using siRNA technology. Fig. 4C shows that siRNAs against calpains I and II prevented the cell death induced by C_2 -ceramide. These results were similar to those obtained by the use of calpain inhibitors. Also, this strategy did not revert the decrease in the ψ_{mt} and the intracellular levels of ATP (Fig. 4D–E). Altogether, these results suggest that mitochondrial dysfunction occurs prior to the onset of the calpain-dependent cell death induced by ceramides.

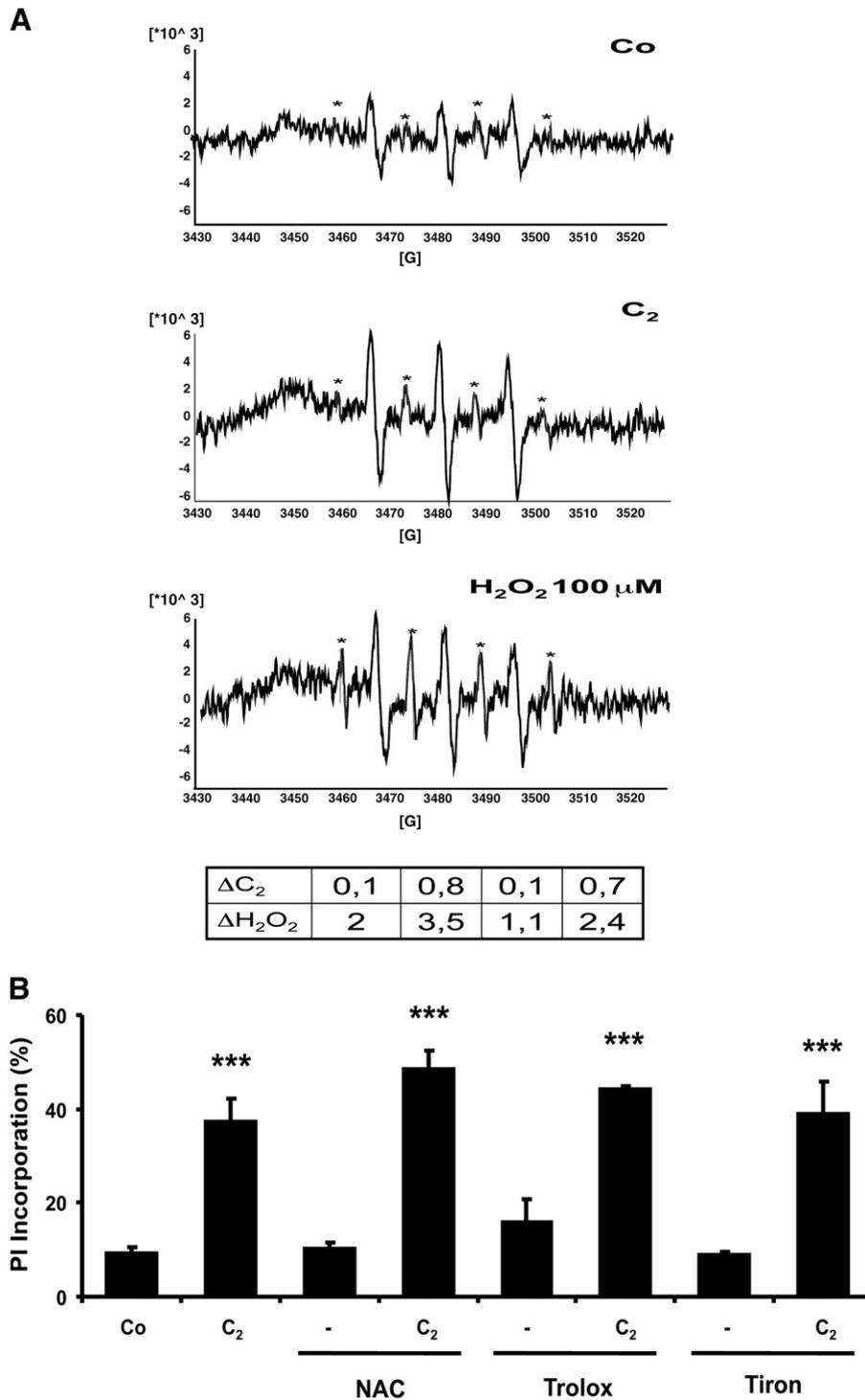


Fig. 2. Ceramide does not stimulate ROS generation in cardiomyocytes. (A) Cells were incubated with C_2 -ceramide (C_2 , 20 μ M) for a 6 h period in the presence of DMPO (200 mM) for 30 min. Cells were lysed and extracts were analyzed by electron spin resonance (ESR). Spectrometer conditions: microwave frequency 9.83 GHz, microwave power 20 mW, modulation amplitude 0.2 G, scan rate 1.25 G/s, time constant 0.5 s, and number of scans: 20. Plots are representative of 3 independent experiments ($n = 3$). (B) Effect of the antioxidant agents N-acetylcysteine (NAC, 5 mM), Trolox (5 mM) and Tiron (5 mM) on PI incorporation in cardiomyocytes treated with C_2 ceramide (C_2 , 20 μ M) for 6 h. ($n = 3$), *** $P < 0.001$ vs. control (Co).

3.4. C_2 -ceramide induces cardiomyocyte death by modulating mitochondrial function

We have previously shown that C_2 -ceramide induced fragmentation of the mitochondrial network in cultured rat cardiomyocytes. For this reason, we evaluated here whether mitochondrial fission was triggered under our experimental conditions and whether this process was also regulated by the Ca^{+2} influx. Following the treatment for 6 h with

C_2 -ceramide (20 μ M) in serum-free medium, substantial mitochondrial network fragmentation was detected (Fig. 5A–B), as evidenced by an increase in the number of mitochondria per cell and a decrease in mean mitochondrial volume, both considered as criteria to document mitochondrial fission [14,37,38]. Moreover, this mitochondrial fragmentation process was prevented by the pre-incubation of cardiomyocytes with EGTA and Gd^{+3} (Fig. 5A–B), thereby connecting our previous results (Fig. 3) with the onset of mitochondrial dysfunction.

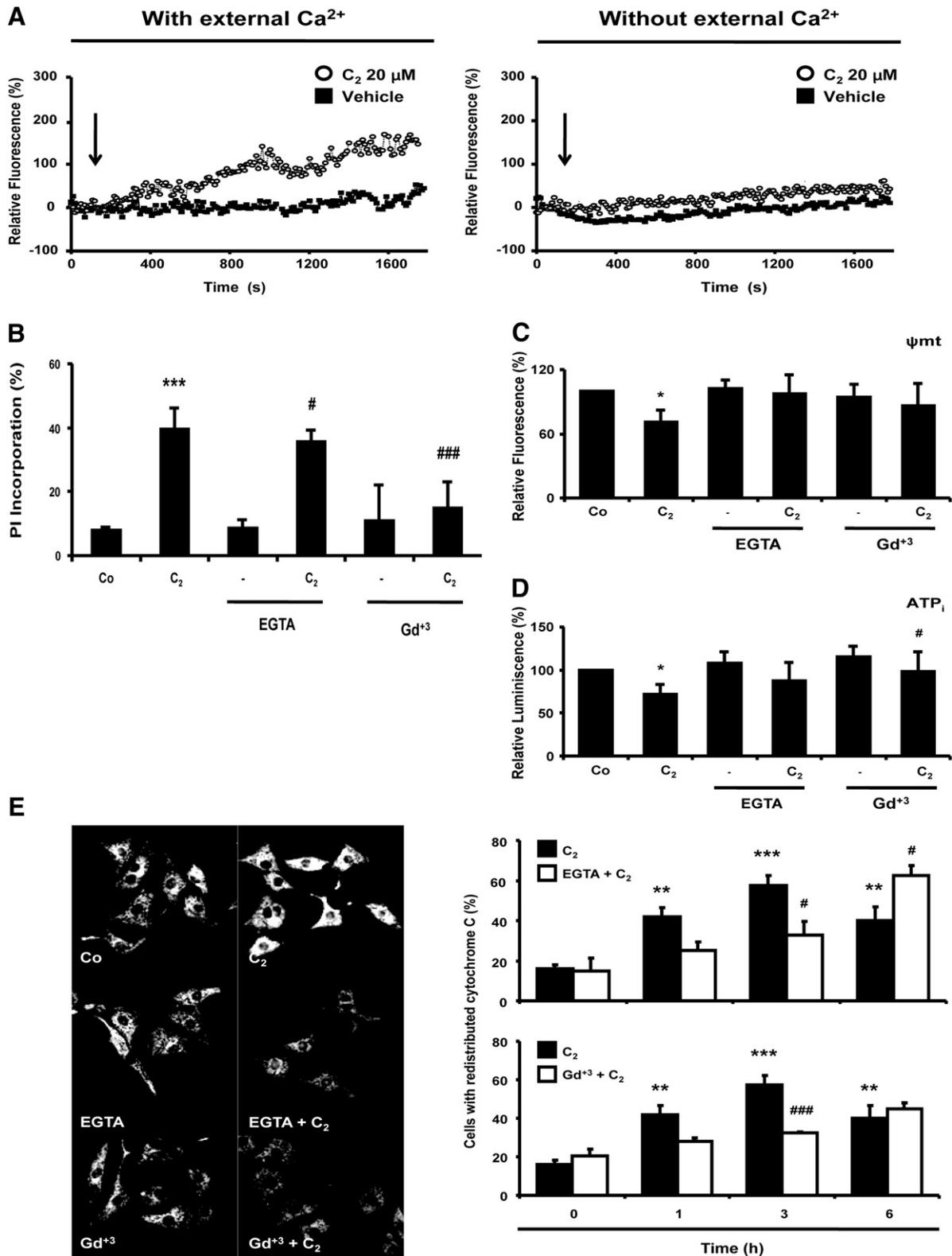


Fig. 3. Ca^{2+} influx induced by C_2 -ceramide modulates cardiomyocyte death. (A) Changes in internal cytosolic Ca^{2+} were investigated in individual, fluo3-AM-loaded cells maintained in medium with or without Ca^{2+} . Cells were stimulated with C_2 -ceramide (C_2 , 20 μM) or the control (DMSO vehicle) for the time indicated by the arrow. Results are expressed as relative total fluorescence [ratio of fluorescence difference ($F - F_0$), to basal value (F_0)] $\times 100$ as a function of time and are representative of 5 independent experiments in which at least 20 cells were analyzed in each case. The effect of the chelating agent EGTA (1 mM) and Gd^{+3} (10 μM) were studied in cells treated with C_2 -ceramide (C_2 , 20 μM) for 6 h on (B) PI incorporation, $n = 3$; (C) mitochondrial potential, $n = 5$; and (D) intracellular levels of ATP, $n = 7$, * $P < 0.05$ vs. Control (Co) and # $P < 0.05$ vs. C_2 6 h. (E) Effect of EGTA (1 mM) and Gd^{+3} (10 μM) pre-incubation on the redistribution of cytochrome-c in cells treated with C_2 -ceramide (C_2 , 20 μM), $n = 7$, ** $P < 0.01$, *** $P < 0.001$ vs. Control (Co), # $P < 0.05$ and ### $P < 0.001$ vs. C_2 3 h.

Although the relationship between mitochondrial fission and apoptosis is still controversial [39,40], our previous data [14] established a model whereby loss of ψ_{mt} precedes mitochondrial fission, which is

then followed by the release of cyt-c. These results suggested that ceramide-induced mitochondrial fission is required to trigger apoptosis. Moreover, the pre-incubation of cardiomyocytes with cyclosporine A

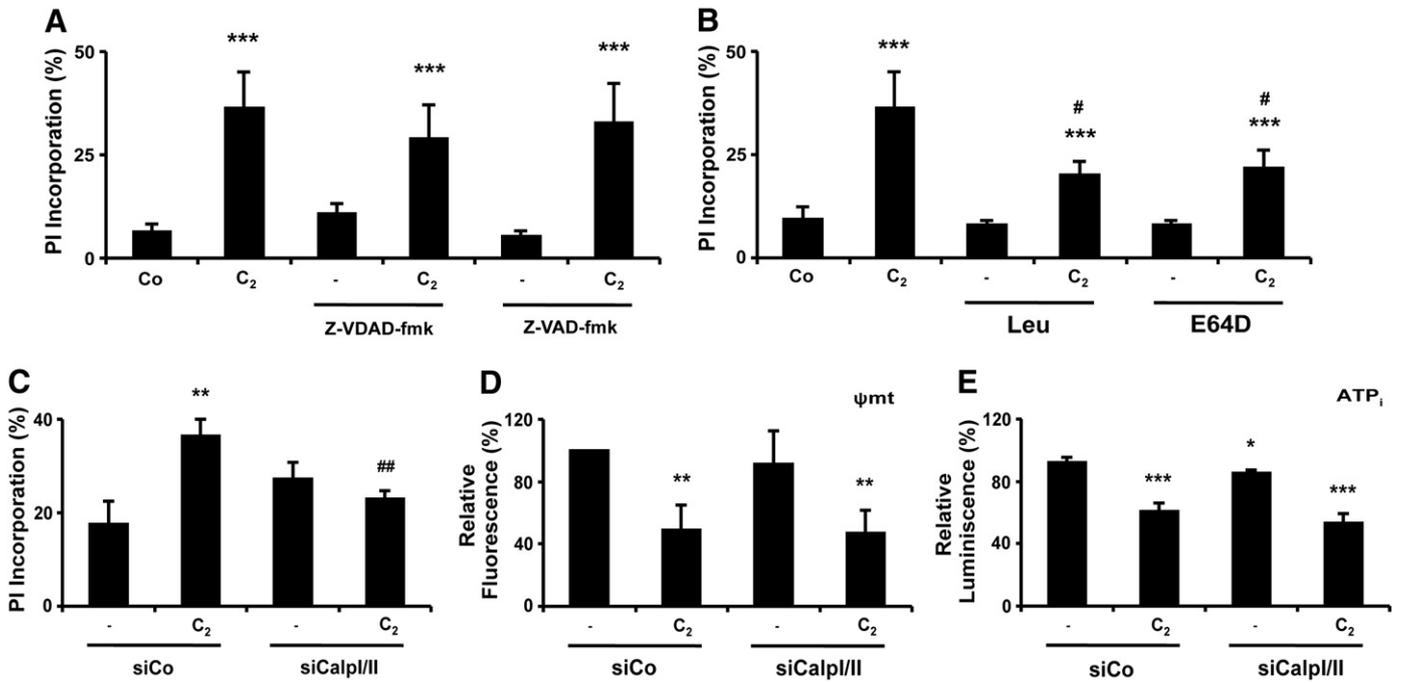


Fig. 4. Calpains but not caspases are involved in cardiomyocyte death induced by C₂-ceramide. PI incorporation analysis in cells treated with C₂-ceramide (C₂, 20 μM) for 6 h and pre-incubated (30 min) with (A) the caspase 2 (Z-VDAD-fmk) and pan-caspase (Z-VAD-fmk) inhibitors (50 μM), n = 3 or (B) the calpain inhibitors leupeptin (Leu, 100 μM) and E64D (10 μM), n = 5, ***P < 0.001 vs. Control (Co) and #P < 0.05 vs. C₂ 6 h. Effect of the calpain I and II knockdown (siCalp/II) in cells treated with C₂-ceramide (C₂, 20 μM 6 h) on the (C) PI incorporation, n = 3; (D) mitochondrial potential, n = 3; and (E) intracellular levels of ATP, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001 vs. a negative siRNA Control (siCo) and ##P < 0.01 vs. siCo C₂ 6 h.

(250 nM), an inhibitor of the mitochondrial transition pore, a key step in the development of mitochondrial dependent cell death, almost completely suppressed C₂-ceramide-enhanced PI incorporation, as well as decreased ψ_{mt} and intracellular ATP levels (Fig. 5C–E).

Subsequently, we sought to establish a direct relationship between the increase in cytosolic Ca²⁺ influx observed in cardiomyocytes treated with C₂-ceramide and decreased mitochondrial Ca²⁺ buffer capacity, which was previously described for fragmented mitochondria [41,42]. To that end, we loaded the cells with the mitochondrial Ca²⁺ dye Rhod-FF and then added histamine, a classical agent that evokes a Ins(3,4,5)P₃-dependent Ca²⁺ release from the ER [43]. As shown in Fig. 5F, histamine-stimulated mitochondrial Ca²⁺ uptake was significantly lower in ceramide-pretreated cardiomyocytes than controls. These results suggest that fragmented mitochondria in C₂-ceramide-treated cells display decreased ER-mitochondria coupling. Moreover, these observations are indicative of reduced Ca²⁺ buffering capacity that may explain the increase in the cytosolic Ca²⁺ and subsequent activation of calpains as effectors of cell death.

Finally, it is well known that mitochondrial Ca²⁺ uptake stimulates mitochondrial metabolism, in terms of mitochondrial respiration and ATP production because the activities of dehydrogenases in the Krebs cycle are stimulated by Ca²⁺ either directly (isocitrate and α-ketoglutarate dehydrogenases) or indirectly (pyruvate dehydrogenase) [44]. If C₂-ceramide causes mitochondrial fragmentation and as a consequence affects mitochondrial Ca²⁺ uptake and metabolism, we hypothesized that stimulating mitochondrial metabolism should decrease ceramide-induced cell death, by precluding decreased ψ_{mt} and ATP levels. To test this idea, we sought to bypass the requirement for mitochondrial Ca²⁺ by enhancing the availability of TCA cycle intermediates and increasing the production of reducing equivalents. To this end, cells were pre-incubated with membrane-permeable methyl-pyruvate (MP) that is oxidized to provide reducing equivalents (NADH), which then drive oxidative phosphorylation and ATP production [45]. Fig. 6A–C shows that MP partially prevented ceramide-

induced cardiomyocyte death, as assessed by PI incorporation, the decrease in the ψ_{mt} as well as the decrease in the intracellular levels of ATP.

4. Discussion

Our data summarized in the graphical abstract strongly suggest that C₂-ceramide induces cardiomyocyte death through a molecular mechanism involving enhanced Ca²⁺ influx, increased mitochondrial fragmentation and a reduction in the mitochondrial Ca²⁺ buffer capacity. These changes lead to an increase in cytosolic Ca²⁺ levels, which is depicted here as responsible for the activation of calpains. Interestingly, some of these effects were partially overcome by medium supplementation with membrane-permeable methyl-pyruvate. Our data also show that C₂-ceramide induces both early apoptosis and delayed necrosis. The former is dependent on Ca²⁺ influx (Fig. 3E) and thus can be blocked by preventing either the rise in cytoplasmic Ca²⁺ or loss of mitochondrial function and both approaches protect cardiomyocytes against ceramide-induced cell death.

4.1. Ceramide-induced cardiomyocyte death

A considerable body of evidence implicates ceramides in apoptotic cardiomyocyte death [28]. However, several recent reports in other cell types suggest that cell death with necrotic features may occur due to increased endogenous ceramide levels, as well as in response to treatment with cell-permeable ceramides [46–50]. In this study, we sought to define the molecular mechanisms by which cell-permeable ceramides promote cardiomyocyte death. Our current study relies heavily on the use of a flow cytometry assay that identifies different cell populations on the basis of fluorescence intensity due to DNA staining by PI that enters the cell as a consequence of augmented membrane permeability [3,46]. In order to evaluate whether cardiomyocyte membrane permeability to PI triggered by exposure to cell-permeable C₂-ceramide correlated with release of

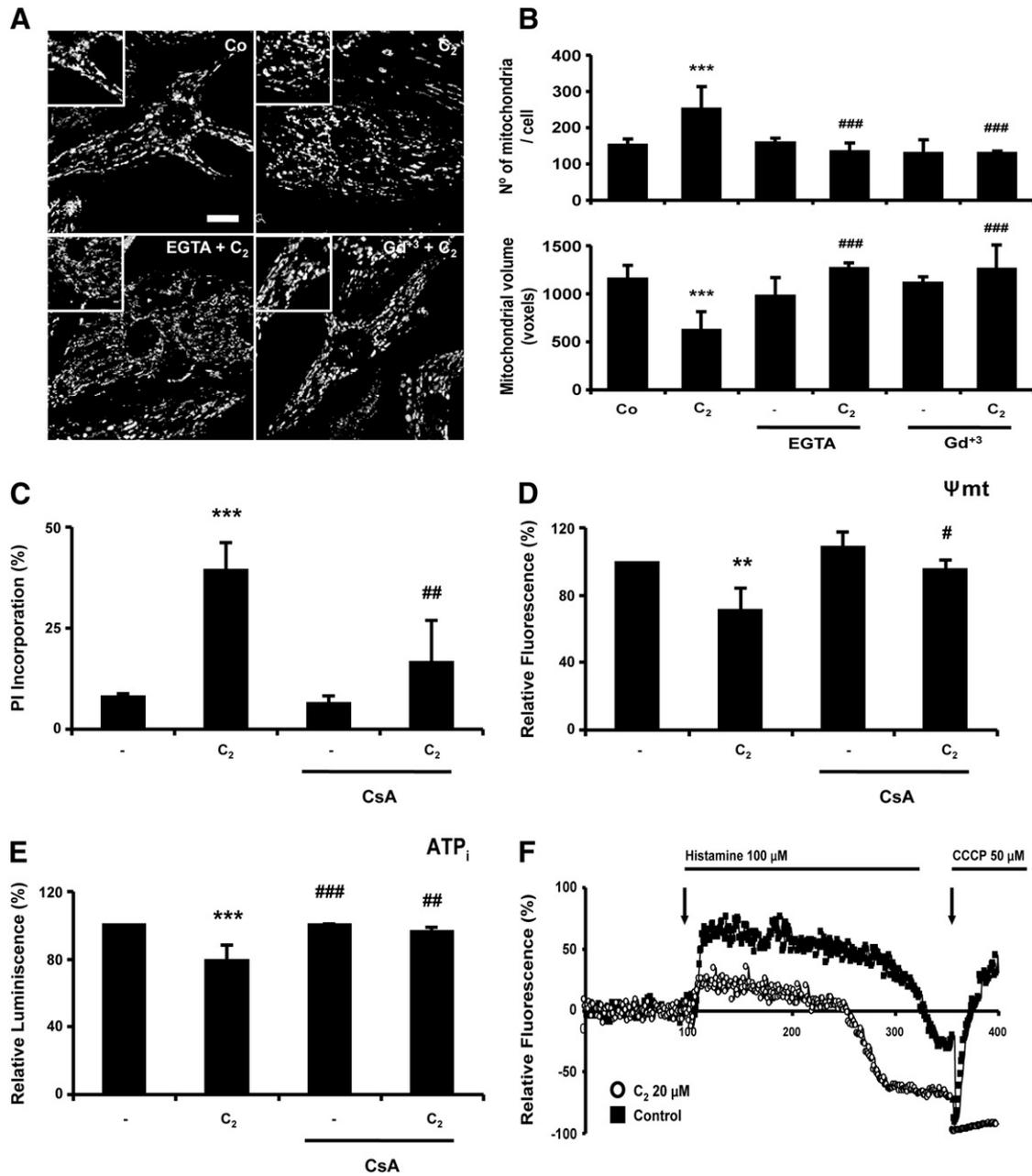


Fig. 5. C₂-ceramide induces cardiomyocyte death by modulating mitochondrial function. (A) Mitochondrial fragmentation induced by C₂-ceramide (C₂, 20 μM) was studied in cardiomyocytes pre-incubated (30 min) with or without EGTA (1 mM) and Gd³⁺ (10 μM). Cells were incubated with C₂-ceramide for 6 h and then loaded with mitotracker green. Multi-slice imaging reconstitution was obtained by confocal microscopy to show mitochondrial morphology. The scale bar is 20 μm. (B) The mean mitochondrial volume and the number of mitochondria per cell were determined. Values shown are mean ± SD, n = 4, ***P < 0.001 vs. Control (Co) and ###P < 0.001 vs. C₂ 6 h. The effect of the mitochondrial transition pore inhibitor, cyclosporine (CsA, 250 nM) was studied in cells treated with C₂-ceramide (C₂, 20 μM) for 6 h on (C) PI incorporation, n = 3; (D) mitochondrial potential, n = 4 and; (E) intracellular levels of ATP, n = 5. **P < 0.01, ***P < 0.001 vs. Control (Co), #P < 0.05, ##P < 0.01 and ###P < 0.001 vs. C₂ 6 h. (F) Representative traces of mitochondrial calcium ([Ca²⁺]_{mt}) obtained from cardiomyocytes loaded with the Rhod-FF calcium probe, either untreated (Control) or treated with C₂-ceramide (C₂, 20 μM) for 6 h prior to histamine addition, which is indicated by an arrow. The mitochondrial uncoupler CCCP (50 μM) was used as a control. Results are expressed as relative total fluorescence [ratio of fluorescence difference (F – F₀), to basal value (F₀)] × 100 over time and are representative of 3 independent experiments in which at least 10 cells were analyzed in each case.

cytoplasmic content from cells, LDH release, a criterion employed to identify necrotic cell death, was used in parallel. Unexpectedly as shown in Fig. 1, 6 h of treatment with C₂-ceramide induced both early apoptosis and delayed necrosis. Whereas necrosis was only detected by LDH release after 6 h treatment, early apoptotic markers, such as cyt-c re-distribution were detected earlier (3 h). Ceramide-induced cell death was apparently Ca²⁺-dependent (Fig. 2E) and cyt-c redistribution was delayed upon addition of either EGTA or Gd³⁺. Consequently, we only observed decreases in ATP after 6 h of ceramide treatment, correlating with the increased LDH release, which is in agreement with the general perception that apoptosis requires ATP while necrosis occurs

in its absence. Moreover, in contrast to those models where primary necrosis occurs, secondary necrosis usually follows apoptosis if the removal of apoptotic bodies is delayed or absent, as is the case of cell culture models [51]. In these cases, necrotic events, such as LDH release, occur coincident with or after cyt-c release [52], observations that strongly correlate with the results described in the present study.

4.2. Ceramide, second messengers and executors of cell death

It has been shown that ceramides increase cytoplasmic Ca²⁺ concentrations in different types of cells. Indeed, cytoplasmic Ca²⁺ levels

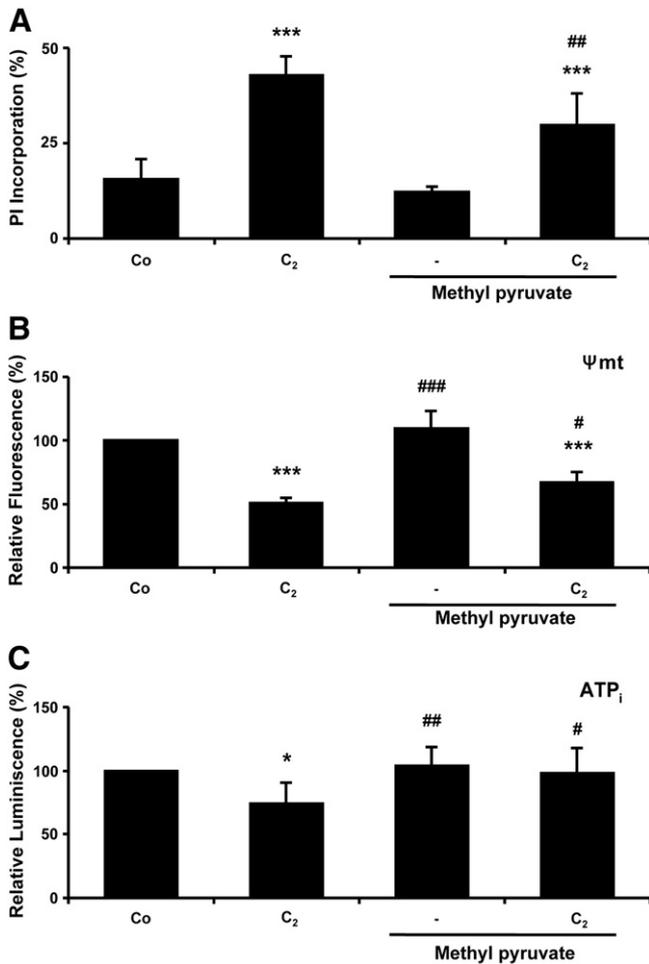


Fig. 6. Improved mitochondrial function prevents cardiomyocyte death induced by C₂-ceramide. Effects of methyl-pyruvate (5 mM) in cells treated with C₂-ceramide (C₂, 20 μ M) 6 h on (A) PI Incorporation, n = 5; (B) mitochondrial potential, n = 5 and; (C) intracellular levels of ATP, n = 7. *P < 0.05, ***P < 0.001 vs. Control (Co) and #P < 0.05, ##P < 0.01 and ###P < 0.001 vs. C₂ 6 h.

are increased in cardiomyocytes exposed to C₂-ceramide (Fig. 3A). In addition to ATP-dependent Ca²⁺ influx, ceramide increases Ca²⁺ from a thapsigargin-sensitive Ca²⁺ pool and triggers a subsequent capacitative Ca²⁺ entry in Jurkat T cells [53]. In the aforementioned studies, increased cytoplasmic Ca²⁺ concentrations were linked to the liberation of Ca²⁺ from intracellular stores and the activation of a store-operated Ca²⁺ channel [53]. Similarly, in our study ceramide increased cytoplasmic Ca²⁺ was necessary for the subsequent cardiomyocyte death because both, EGTA and Gd³⁺ decreased PI incorporation and also prevented the deleterious effects of ceramide on ψ_{mt} and intracellular ATP levels (Fig. 3C–D). Activation of calpains due to increased intracellular Ca²⁺ concentrations has been linked to cardiomyocyte death [54]. Our data agree with these observations because two different calpain inhibitors (leupeptin and E64D) and siRNAs for these proteases prevented the ceramide-induced cardiomyocyte death. However, they did not exert the same inhibitory effect on ψ_{mt} and the intracellular levels of ATP (Fig. 4). These results suggest that calpain activation represents an event that occurs downstream of mitochondrial dysfunction during cardiomyocyte death. Interestingly, caspases seem not to be involved in cardiomyocyte death induced by C₂-ceramide (Fig. 4A). These results are in agreement with studies in neuroblastoma cells where synthetic ceramides induced a caspase-independent, necrosis-like morphology [55]. Moreover, ceramides kill normal lymphocytes and lymphoid cell lines in the absence of caspase activation, triggering a nonapoptotic

morphology in dying cells [47]. Thus, ceramide-induced cell death is apparently not regulated by caspase activation, although the type of death triggered by these lipids is strongly dependent on the cell type and context.

4.3. Ceramide, mitochondrial morphology and function

Different studies have shown that ceramides alter the homeostasis of several organelles, including particularly mitochondria [56]. According to this evidence, C₂- and C₁₆-ceramides form channels in planar lipid bilayers allowing the release of cyt-c [56]. On the other hand, C₂-ceramide also decreases ψ_{mt} in isolated heart mitochondria [57]. Our previous study established that C₂-ceramide regulates mitochondrial dynamics through the stimulation of mitochondrial fission, involved among other events in the activation of cardiomyocyte apoptosis [14]. This fission process was accompanied by early translocation of the fission protein Drp-1 to the mitochondria [14]. However, the mechanisms that trigger this process remained unknown.

In the present study, we show that C₂-ceramide not only induces mitochondrial fission, but also decreases ψ_{mt} and the intracellular levels of ATP (Fig. 5A–E). Also, histamine-induced mitochondrial Ca²⁺ uptake in cardiomyocytes is reduced (Fig. 5F), suggesting that the fragmented mitochondria observed in ceramide-treated cells display a lower Ca²⁺ buffering capacity. Moreover, this may explain the increase in cytosolic Ca²⁺ observed following C₂-ceramide treatment and the subsequent activation of calpains as effectors of cell death. These data agree with previous reports establishing that a fragmented mitochondrial network affects mitochondrial Ca²⁺ buffering capacity and the spatial and temporal development of cytoplasmic Ca²⁺ signals [41]. Increases in cytosolic Ca²⁺ trigger the translocation of the protein Drp1 to the mitochondria, thereby unleashing mitochondrial fragmentation [58]. In our graphical abstract we propose a model where C₂-ceramide induces an increase in cytosolic Ca²⁺, which triggers mitochondrial network fragmentation and a decrease in the function of this organelle. In this scenario, mitochondrial fragmentation reduces the mitochondrial Ca²⁺ buffering capacity, increases cytosolic Ca²⁺ levels and activates calpains.

Finally, one of the most interesting findings of this study was the ability of MP to partially prevent the decrease in mitochondrial metabolism and preclude cardiomyocyte death (Fig. 6). Constitutive InsP₃R-dependent Ca²⁺ release to mitochondria is an essential event required for efficient mitochondrial respiration and the maintenance of normal cell bioenergetics [45,59]. This process seems to be altered in cardiomyocytes treated with exogenous ceramides, given that histamine stimulation results in reduced mitochondrial Ca²⁺ uptake following ceramide treatment. Future work should clarify whether metabolic rescue by MP prevents the reduction in mitochondrial Ca²⁺ buffering capacity and also whether this may represent a beneficial approach to treat other pathological states where ceramides are implicated.

4.4. Concluding remarks

Our data provide novel evidence that cardiomyocyte mitochondria are primary targets of cell-permeable ceramides. These sphingolipids promote cytosolic Ca²⁺ influx, mitochondrial fragmentation and dysfunction, as well as cell death through apoptosis and necrosis. Our results also show that mitochondrial dysfunction following C₂-ceramide-induced mitochondrial ψ_{mt} and ATP decrease is a major driving force for cardiomyocyte apoptosis. Thus, metabolic rescue observed upon addition of MP may represent an appropriate strategy to avoid cardiomyocyte death in a broad range of cardiac pathologies where ceramides and calpain activation may play a major role, such as in ischemia-reperfusion [36,60] and doxorubicin treatment [61,62].

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbadis.2013.04.009>.

Disclosure statement

No potential conflicts of interest are reported for any of the authors.

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