



Functional and structural alterations in the endoplasmic reticulum and mitochondria during apoptosis triggered by C₂-ceramide and CD95/APO-1/FAS receptor stimulation

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ABSTRACT

C₂-ceramide (C₂-cer) and binding of the CD95/APO-1/FAS (aCD95) receptor are acknowledged inducers of apoptosis. In spite of that, their effects on the endoplasmic reticulum (ER) and mitochondria during early phases of apoptotic onset are poorly characterized. Here, by employing various approaches, we followed structural and functional modifications of these organelles at the beginning of cellular demise.

In detail, we observed that C₂-cer, but not CD95 activation, markedly modifies the morphology of the ER and promotes Ca²⁺ release. Accordingly, mitochondria of C₂-cer-treated, but not of CD95-stimulated, cells are fragmented, show reduced Ca²⁺ uptake, and collapsed membrane potential ($\Delta\Psi_m$). Most notably, C₂-cer-mediated morphological aberrations of the ER are prevented neither by the pan-caspase inhibitor Z-VADfmk nor by the cell cytoskeleton dissembler cytochalasin-D, while on the contrary they are reduced by incubation in the presence of the intracellular Ca²⁺ chelator 1,2-bis(O-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). We conclude that initiation of apoptosis via the intrinsic (i.e. C₂-cer mediated) pathway causes an early structural and functional alteration of both ER and mitochondria, thus underlying a final “non return” point in the apoptotic pathway.

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Introduction

Apoptosis is a form of cell death that can be pre-programmed, such as during development [1], or stimulated by a variety of stress or signals [2]. It is characterized by morphologic and biochemical changes orchestrated by a family of cysteine proteases known as caspases [3]. Caspase activation executes the apoptosis process by cleaving various intracellular substrates, eventually leading to plasma membrane blebbing, genomic DNA fragmentation, and exposure on the cell surface of phagocytosis signalling molecules [4]. Apoptosis can be triggered by the engagement of plasma membrane receptors belonging to a subgroup of the TNF-receptor superfamily known as death receptors [5] among which CD95/APO-1/Fas is probably the best known [6]. Interaction of CD95 with its agonistic antibody (aCD95) induces receptor oligomerisation,

followed by the recruitment, through homophilic interactions, of adaptor proteins to the death domain and activation in a transcription-independent manner of the death-inducing caspase cascade, which causes cytochrome *c* release, and further activation of caspases and DNases. The CD95 pathway plays an important role in regulating the immune system [7], and has been implicated in cancer, human immunodeficiency, virus infection, stress, ageing and even drug abuse [8].

Ceramide is a lipid second messenger generated by *de novo* synthesis or by the hydrolysis of sphingomyelin [9] that plays a role in several biological phenomena such as differentiation, growth suppression, senescence [10], cell stress [11] and apoptosis [12].

Changes in cytosolic Ca²⁺ concentration ([Ca²⁺]_c) trigger key cellular functions, ranging from muscle contraction to secretion of neurotransmitters and modulation of metabolism [13]. Ca²⁺ also plays a major role in the regulation of mitochondrial physiology and cell death [14,15]. Notably, although the central role of mitochondria in programmed cell death is widely recognised [16], a major role of the ER in apoptosis emerged later and particularly in decoding apoptotic signals involving alterations in cellular

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Ca²⁺ homeostasis [17]. The Ca²⁺ storage capacity of the ER is therefore the target of pharmacological agents that facilitate apoptotic cell death [18].

A major involvement of the ER has been demonstrated in NO-induced apoptosis [19] and ER stress has been shown to induce procaspase and calpain activation with the subsequent degradation of different substrates both in the ER and in the cytosol [20]. Disturbances in cellular redox regulation, glucose deprivation and viral infections are all cellular conditions leading to ER stress. This may be associated with an accumulation of unfolded proteins to trigger the so called unfolded protein response (UPR) [21]: an evolutionarily conserved physiological response that may lead to cell demise via apoptosis or autophagy. In this process, Ca²⁺ deregulation within the ER may be key [22].

Aim of this work was to investigate by a combination of morphological and functional approaches the involvement of the ER and mitochondria in apoptosis triggered by C₂-cer and aCD95, two prototypic examples of initiation of apoptosis via the intrinsic and extrinsic pathway, respectively. Our data suggest that these two classical apoptotic stimuli have dramatically different impact on the morphology and physiology of the ER and mitochondria.

Materials and methods

Cells, solutions, and reagents. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Euroclone, I), supplemented with 10% FCS (Life Technologies, I), in 75 cm² flasks (Falcon, Franklin Lakes, USA). Krebs–Ringer modified Buffer (KRB) contained: 125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM Na₂HPO₄, 5.5 mM glucose, 1 mM CaCl₂, 20 mM NaHCO₃, 2 mM L-glutamine and 20 mM Hepes (pH 7.4). For permeabilized cells, an intracellular buffer (IB) was employed: 140 mM KCl, 10 mM NaCl, 1 mM K₃PO₄, 5.5 mM glucose, 2 mM MgSO₄, 1 mM ATP, 2 mM sodium succinate, 20 mM Hepes (pH 7.05 at 37 °C).

Ionomycin, histamine, digitonin, N-acetyl-D-sphingosine (C₂-ceramide) and D-erythro-N-acetylsphinganine (C₂-dihydroceramide), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA), were purchased from Sigma–Aldrich (I), coelenterazine and Fura-2/AM from Molecular Probes (NL).

Transfection. Transfection was done with the Ca²⁺-phosphate technique [23]. For the aequorin measurements, cells seeded onto 13 mm coverslips (BDH, I) were transfected with 4 µg mtAEQ; for GFP experiments, cells were seeded onto 24 mm coverslips and transfected with 8 µg mtGFP or erGFP.

Aequorin measurements. Thirty-six hours after transfection the cells were incubated with 5 µM coelenterazine for 1–2 h in DMEM supplemented with 1% FCS and then transferred to the perfusion chamber. All aequorin measurements were carried out in KRB, supplemented with 1 mM Ca²⁺. Agonists and other drugs were added to the same medium. Experiments were terminated by lysing cells with 100 µM digitonin in a hypotonic Ca²⁺-rich solution (10 mM CaCl₂ in H₂O). The light signal was collected and calibrated into [Ca²⁺]_i values [23].

Microscopic analyses. The coverslips with the cells were placed in a thermostatted Leyden chamber, (model TC-202A, Medical Systems Corp., NY, USA) on the stage of an inverted Nikon Eclipse TE300 microscope (Nikon, Tokyo, JP) equipped with epifluorescence and a piezoelectric motorization of the objective (Physik Instrumente, GmbH & Co., DE). The images were captured by a back-illuminated CCD camera (Pricenton Instruments, AZ, USA) using the Metamorph software (Universal Imaging Corporation, PA, USA). In the computationally deblurred images of Fig. 3 a stack of images through the z plane was acquired and processed using the EPR software developed by the Biomedical Imaging group (Worcester, MA, USA).

[Ca²⁺]_c measurements. Changes in the [Ca²⁺]_c were measured with the fluorescent indicator Fura-2/AM using an LS50 Perkin Elmer fluorometer (Perkin Elmer Ltd., Beaconsfield, UK). The cells (1 × 10⁷/ml) were suspended in 1 mM Ca²⁺/KRB containing 2 µM Fura-2/AM and 250 µM sulfinpyrazone (Sigma) at 37 °C for 15 min. After washing changes in [Ca²⁺]_c were determined in a thermostated, magnetically stirred cuvette, with the 340/380 excitation ratio at an emission wavelength of 505 nm.

Imaging mitochondrial membrane potential (ΔΨ_m). Tetramethyl rhodamine methyl ester (TMRM) (Invitrogen) was used in “redistribution mode” and was present continuously at concentration of 50 nM distributing between cellular compartments in response to different potentials. TMRM fluorescence was quantified by removing all background signals by “thresholding” and measuring the mean fluorescence intensity in the pixels containing mitochondria.

Results

CD95 activation and C₂-ceramide differently affect ER morphology and intracellular Ca²⁺ homeostasis

HeLa cells treated with C₂-cer or aCD95 undergo apoptotic cell death with similar efficiency and a comparable time course [24]. We confirmed this by counting the percentage of apoptotic cells after 6 h incubation with either 1 µg/ml aCD95 or 20 µM C₂-cer. Specifically, cells were seeded onto coverslips and grown in DMEM. When 70% confluence was reached, the incubation medium was switched to KRB modified buffer supplemented with either aCD95 or C₂-cer. With both stimulants, 6 h after the challenge approximately 90% of the cells underwent apoptosis (data not shown) together with typical shrinkage and plasma membrane blebbing (Fig. 1A, B). In parallel, we dynamically followed changes in ER morphology. For this purpose, HeLa cells were transiently transfected with an ER-targeted GFP construct (erGFP) and positive cells were analysed at different time points with a high-resolution digital imaging system [25]. The experimental protocol itself did not affect ER morphology, as in the absence of the stimulants no changes in morphology were detected during a 6 h incubation in the microscopy chamber under repeated (12) short (1 s) light pulses (Supplementary Fig. 1A). Notably, aCD95 treated cells did not show appreciable modifications of the overall ER structure even after a 5 h treatment (length of time coinciding with caspase activation), nor the challenge caused any detectable increase in [Ca²⁺]_i (Fig. 1C, Ci).

In striking contrast, C₂-cer dramatically affected both ER morphology and [Ca²⁺]_i homeostasis. As shown in Fig. 1D C₂-cer caused detectable modifications of ER morphology as early as 10–20 min after its addition, while after 3 h the fine ER network was almost completely replaced by large round aggregates (Fig. 1D, see also Supplementary Fig. 1B). At variance with aCD95, C₂-cer addition promoted a clear [Ca²⁺]_i rise consistent with a release of Ca²⁺ from the ER [26] (Fig. 1Di). In agreement with this interpretation, the subsequent addition of ionomycin (iono) did not cause a further [Ca²⁺]_i increase.

[Ca²⁺]_i buffering attenuates ER changes

We then explored whether the morphological modifications of the ER were a direct consequence of C₂-cer or a downstream effect due to caspase activation, and whether they were specific for the pro-apoptotic moiety of the lipid. For this purpose, HeLa cells expressing erGFP were pre-treated with the broad-spectrum caspase inhibitor Z-VADfmk (100 µM) before incubation with C₂-cer (Fig. 2C) and compared to cells treated with either C₂-cer alone (Fig. 2A), or its inactive form, di-C₂-cer (20 µM) (Fig. 2B). As shown in Fig. 2A, Z-VADfmk (100 µM) does not inhibit C₂-cer-induced

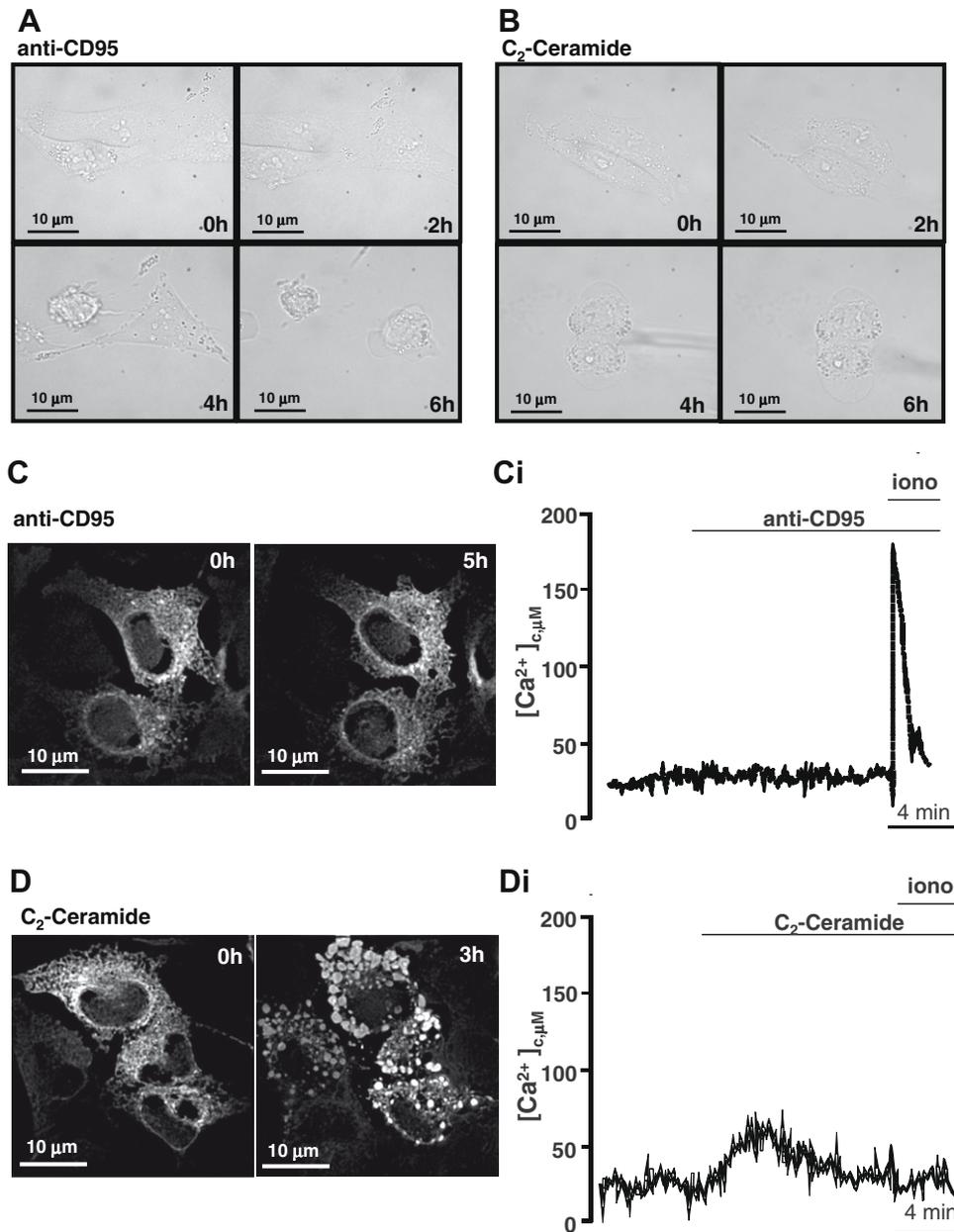


Fig. 1. C_2 -ceramide alters endoplasmic reticulum morphology and promotes Ca^{2+} release. Time laps of HeLa cells treated with $1 \mu\text{g/ml}$ aCD95 (A) or $20 \mu\text{M}$ C_2 -cer (B). (C) Representative images of eRFP expressing HeLa cells taken at time 0 and 5 h after treatment with aCD95 ($n = 30$, 2 days of experiments). (Ci) HeLa cells, loaded with $2 \mu\text{M}$ Fura-2/AM and $250 \mu\text{M}$ sulfinpyrazone (37% for 15 min), are challenged with $1 \mu\text{g/ml}$ aCD95 and iono (100 nM). (D) Images of ER morphology at 3 h after C_2 -cer treatment are presented ($n = 20$, 3 days of experiments) and in Di the evoked changes in $[Ca^{2+}]_c$ reported.

modifications and di- C_2 -cer has no effect on ER morphology. This suggested that ER disassembly is not a downstream effect in the apoptotic process but rather an early, specific event occurring upstream of caspase activation.

We then searched for potential effectors mechanisms. To test whether the cytoskeleton was targeted by C_2 -cer, eRFP-expressing HeLa cells were pre-treated with $20 \mu\text{M}$ cytochalasin-D, an inhibitor of actin assembly. Fig. 2D shows that cytochalasin does not prevent the ER structural aberrations caused by C_2 -cer. We then investigated whether Ca^{2+} itself is responsible for ER changes. Firstly, we discharged ER Ca^{2+} by incubating HeLa cells in KRB medium supplemented with 100 nM iono and 0.5 mM EGTA. Notably, depletion of ER Ca^{2+} stores by this procedure reduced C_2 -cer-triggered ER alterations, albeit not completely (Fig. 2E). While this experiment calls for a primary role of ER Ca^{2+} , we further verified if

also Ca^{2+} release into the cytosol may play a facilitator role for the morphological changes. We previously showed that the intracellular Ca^{2+} chelator BAPTA reduces the apoptotic efficacy of C_2 -cer, very likely by preserving mitochondrial integrity [16]. Here, we recorded that BAPTA loading [27] markedly reduced the ER modifications (Fig. 2F) thus suggesting a $[Ca^{2+}]_i$ dependent step in the mechanism of action of C_2 -cer.

Mitochondrial Ca^{2+} and $\Delta\Psi_m$ are altered in C_2 -ceramide treated cells

After assessing the modifications occurring in the ER, we tested the effects of the C_2 -cer and aCD95 on mitochondrial morphology and physiology. HeLa cells were transfected with a mitochondrially targeted GFP (mtGFP) and the organelle structure evaluated [25]. In control cells, mitochondria appeared tubular and interconnected in

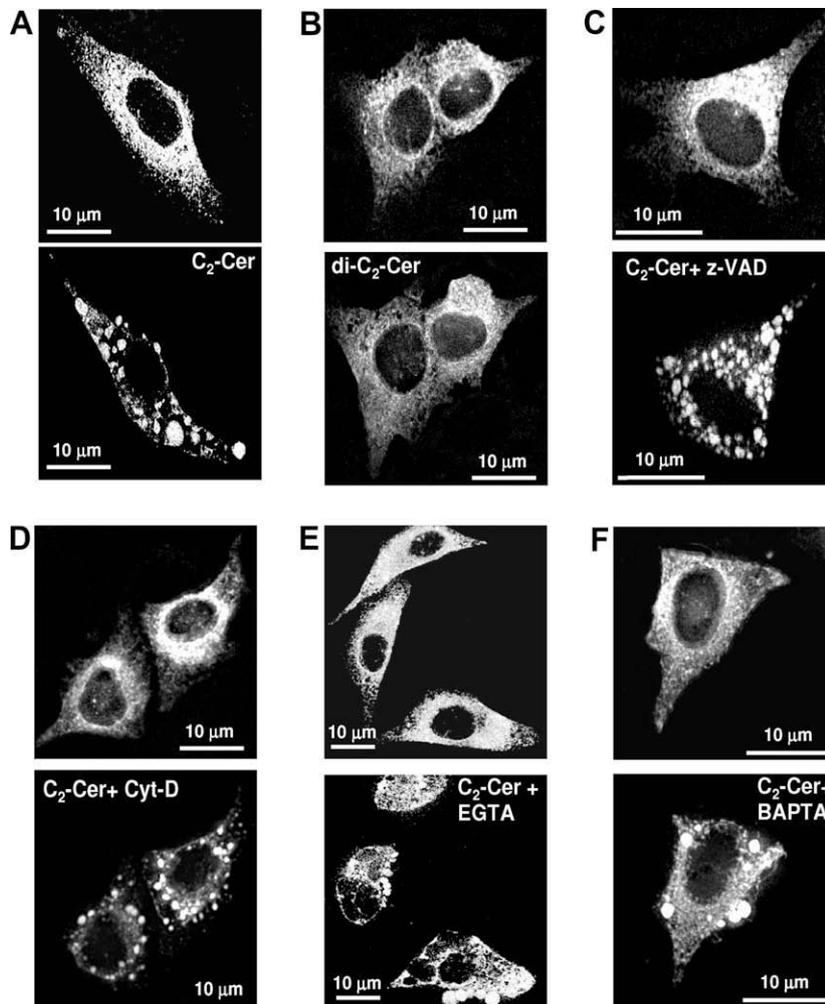


Fig. 2. C₂-ceramide elicited structural aberrations are avoided by chelating cytosolic Ca²⁺. Representative images of eGFP expressing HeLa cells taken at time 0 and 2 h after treatment with 20 μM C₂-cer (A, *n* = 10), 20 μM di-C₂-cer (B, *n* = 8) or pre-incubated with 100 μM Z-VADfmk (C, *n* = 3), 20 μM cytochalasin-D (D, *n* = 3), 20 μM C₂-cer in 0.5 mM EGTA Ca²⁺-free medium (E, *n* = 15) or 5 μM BAPTA for 30 min at 37 °C (F, *n* = 4).

a complex network that undergoes only marginal and localised morphological modifications even after 1 h incubation in the microscopy chamber (Fig. 3A). No changes were appreciable in cells stimulated with aCD95 antibodies (Fig. 3Ai), whilst mitochondrial morphology changed dramatically in C₂-cer stimulated cells [27]. As soon as 30 min after C₂-cer addition, the mitochondrial network began to break-down, and after 1 h most of the mitochondria were rounded, shrunken and fragmented (Fig. 3Aii). We then investigated whether challenge with either C₂-cer or aCD95 affects mitochondrial Ca²⁺ homeostasis. To this aim, by using a chimeric aequorin targeted to the mitochondrial matrix (mtAEQ), we monitored changes in mitochondrial [Ca²⁺]_m ([Ca²⁺]_m) elicited by an IP₃-producing stimulus: histamine. Fig. 3B shows that in control cells histamine rapidly and transiently increases [Ca²⁺]_m reaching maximum values (80 ± 19.4 μM Ca²⁺) similar to those detected in cells stimulated with aCD95 antibodies (87.4 ± 29 μM Ca²⁺) (Fig. 3Bi). Thus, CD95 stimulation did not induce any change in the mitochondrial Ca²⁺ uptake capacity. Although the kinetics of the Ca²⁺ transients in C₂-cer-stimulated cells were similar to those of control and aCD95-treated cells, Ca²⁺ increases were strongly reduced (49.7 ± 4.3 μM Ca²⁺). This result was not surprising, considering the substantial Ca²⁺ depletion of the ER and the major morphological alterations of the mitochondria caused by C₂-cer. We investigated however whether the intrinsic mitochondrial Ca²⁺ uptake capacity were reduced, e.g. due to a reduction of the electrochemical proton gradient. HeLa cells

expressing mtAEQmut were perfused with a saline solution mimicking the ionic composition of the intracellular milieu (IB). The plasma membrane was then permeabilized by perfusing the cells for 1 min with IB/EGTA supplemented with 100 μM digitonin. After a 2 min wash with IB/EGTA, Ca²⁺ accumulation into mitochondria was initiated by switching the perfusion medium to IB solution containing a free [Ca²⁺] of 1 μM. The rate and amplitude of mitochondrial Ca²⁺ accumulation was almost identical in control and aCD95 treated cells (12.2 ± 5.2 μM Ca²⁺/s vs 11.7 ± 3.5 μM Ca²⁺/s, respectively) while C₂-cer-treated cells Ca²⁺ accumulation was significantly slower (6.6 ± 3.5 μM Ca²⁺/s, **p* < 0.05) (Fig. 3C). In addition, we verified whether this was consistent with modifications in the mitochondrial membrane potential (ΔΨ_m) which provides the driving force for organelle Ca²⁺ uptake. HeLa cells were incubated with TMRM [28], at 50 nM for 30 min and placed on the stage of a confocal microscope and challenged with the lipid solvent DMSO for controls, or with the 1 μg/ml aCD95 or 20 μM C₂-cer. Images were acquired every 10 min. Fluorescence emission recorded in each experimental condition 1 h after the addition of the agonist, normalized to control emission at time 0, is shown in Fig. 3D (Time 0, Control:1; aCD95:1.029 ± 0.037; C₂-Cer:1.0056 ± 0.03; Time 1 h, Control:0.98 ± 0.072 *n* = 84; aCD95:1.091 ± 0.03, *n* = 114; C₂-Cer:0.638 ± 0.039 *n* = 120, **p* < 0.05). Overall, the data show a decrease in ΔΨ_m that well matches the reduction in mitochondrial Ca²⁺ uptake observed both in intact and permeabilized cells [16].

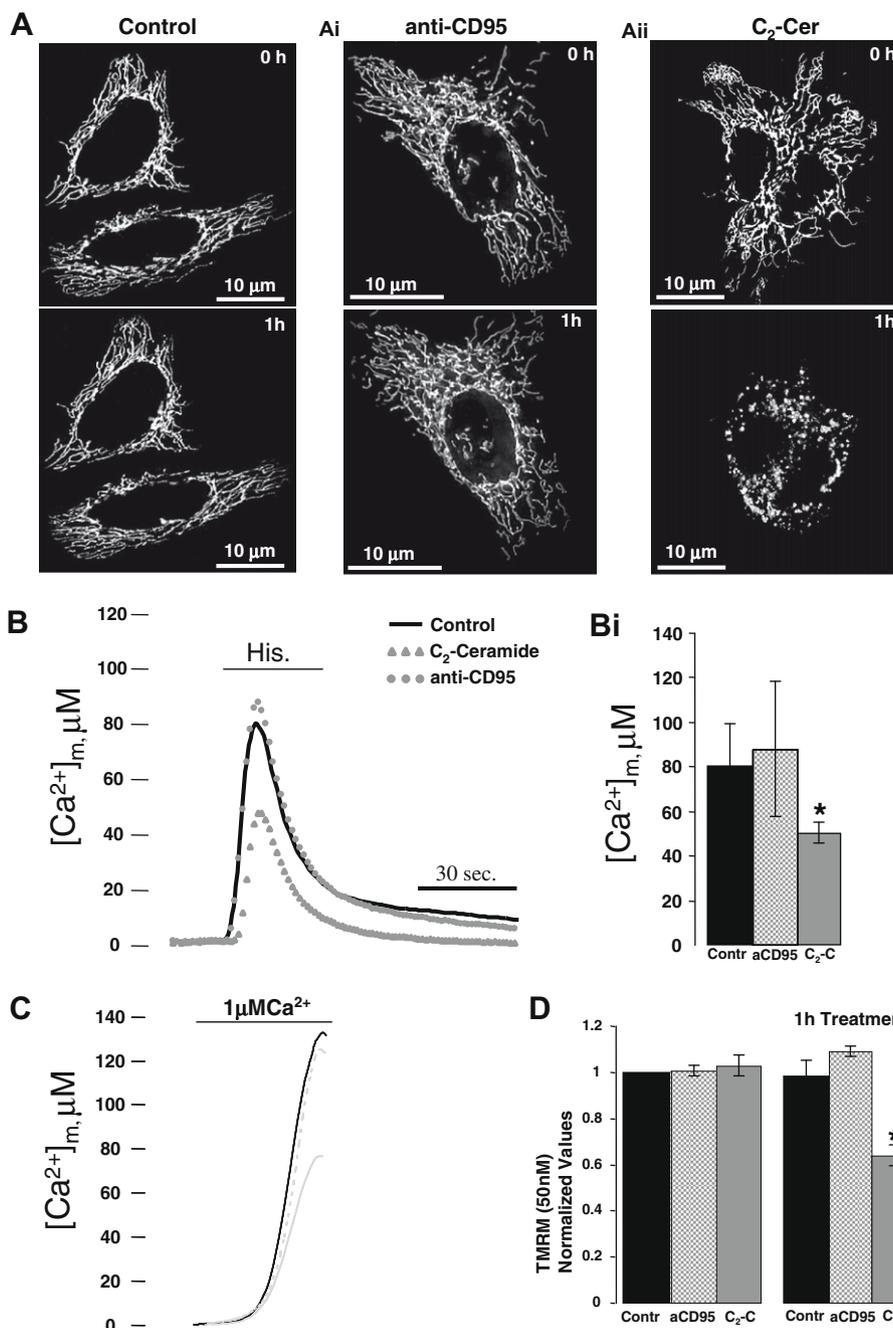


Fig. 3. C₂-ceramide induces dramatic changes in mitochondrial morphology, Ca²⁺ uptake and $\Delta\Psi_m$. mtGFP expressing HeLa cells at time 0 and 1 h after the challenge with solvent (A), 20 μ M C₂-cer (Ai), or 1 μ g/ml aCD95 (Aii). (B) Representative records of mitochondrial Ca²⁺ uptake in HeLa cells treated with solvent (black trace), 1 μ g/ml aCD95 (grey dots), or with 10 μ M C₂-cer (grey triangular dots) for 1 h. After the treatment, $[Ca^{2+}]_m$ was measured with mtAEQmut in response 100 μ M histamine and relative values are summarized in Bi. The mitochondrial driving force for Ca²⁺ was also assessed. mtAEQmut expressing cells treated with solvent (black trace) or with 1 μ g/ml aCD95 (black dots), or with 20 μ M C₂-cer (black triangular dots) were permeabilized with 100 μ M digitonin and perfused with IB containing 1 μ M $[Ca^{2+}]_i$ (C). Panel D shows values normalized to control of the $\Delta\Psi_m$ measured with TMRM at time 0 and 1 h after treatment with the apoptotic stimuli.

Discussion

Apoptosis is a fundamental process involved in the regulation of organism development, tissue homeostasis, and defence against pathogens [29]. Deregulation of this process concurs to the onset of acute or chronic pathological states the most representative of which is probably neoplasia [30,31]. Apoptotic cell death is induced by a variety of stimuli that ultimately lead to the activation of a stereotyped series of intracellular events causing cell death and disassembling of the cell structures [32]. Nowadays, there is increasing interest in dissecting the intracellular pathways leading

to cell death, trying to uncover metabolic differences in the apoptotic mechanism activated by diverse although equally effective stimuli. Interestingly, attention has been put on organelles such the endoplasmic reticulum, the Golgi apparatus and the mitochondria, as putative damage sensors and points of generation of local pro-apoptotic intracellular mediators [33]. Previously, it has been shown that short chain ceramide (e.g. C₂-cer) induces Ca²⁺ release from the ER thus increasing $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, leading to cell death with a mechanism involving mitochondrial structural and functional alterations [27]. Noteworthy, the reduction of $[Ca^{2+}]_er$ as well as the buffering of cytoplasm Ca²⁺ changes [16], efficiently

prevented mitochondrial damages and protected the cells from apoptosis [34]. Here, we show that C₂-cer causes dramatic changes also on the ER morphology in parallel to a concomitant release of Ca²⁺ (Fig. 1B, D) that concurs to the degree of the damage.

Although we employed alternative strategies to counteract C₂-cer induced modifications of the ER structure, none of them proved to be efficient except for the buffering of cytosolic Ca²⁺ level. Thus, both the inhibition of caspase activity by Z-VAD and the disassembly of the actin cytoskeleton by cytochalasin D did not impair the apoptotic effect of C₂-cer. This indicated that modifications in the ER morphology were not depending on events downstream of apoptosis initiation but is likely linked to a triggering step almost concomitant with the lipid mediated toxicity.

Ceramide is well known for its ability to alter the permeability state of the mitochondrial outer membrane by promoting the formation of channels that render the organelles permeable to the pro-apoptotic factors such as cytochrome c [35]. Although the level of permeability of the mitochondrial outer membrane correlates with the amount of ceramide present in the membrane [36,37], Ca²⁺ is known to be a critical sensitizing factor in the opening of the permeability transition pore (PTP), the molecular mechanism underlying the mitochondrial apoptotic transition [38]. Therefore, reduction or sequestering of free Ca²⁺ levels ameliorates C₂-cer effect on the membranes. Our data suggest that morphological alterations occur also in the ER, and also this process is causally linked to alteration in cellular Ca²⁺ homeostasis, as the C₂-cer mediated membrane damage is mitigated by chelating cytosolic Ca²⁺ with BAPTA (Fig. 2F). It can be envisioned that the gradual release of Ca²⁺ into the cytosol, and the activation of cytosolic effectors, impinge directly on the ER membrane already compromised by C₂-cer thus provoking further release of Ca²⁺, establishing a vicious cycle that erodes the integrity of the whole structure.

Mitochondria, central players in the apoptotic process by integrating death signals through mitochondrial proteins belonging to the Bcl-2/Bax family [2,30,39], are generally thought to remain intact and functional during the early events of apoptosis. The data presented here indicate that the signalling route operated by the lipid mediator C₂-cer greatly modifies structure and function of mitochondria. Initiation of cells death by C₂-cer induced a fast fragmentation of the mitochondrial network accompanied by dramatic changes of the organelle morphology and decreased capacity to accumulate Ca²⁺ released by the ER (Fig. 3B). This can be explained by two concomitant events. (1) In healthy cells the mitochondrial and ER networks have many sites of close contact, thus facilitating the transfer of the Ca²⁺ ions between the two compartments. (2) Alteration of the ER morphology and fragmentation of mitochondria could separate mitochondria from the ER network thus reducing the possibility of mitochondria to sequester ER-released Ca²⁺. Another not mutually exclusive hypothesis to explain the reduced Ca²⁺ uptake of mitochondria is linked to a direct damage of the organelle by C₂-cer itself. This event is confirmed by experiments performed in permeabilized cells whereas [Ca²⁺]_{er} of the extracellular/intracellular buffer is superimposed (Fig. 3C). In these experimental conditions mitochondria sense a Ca²⁺ concentration that is independent from the [Ca²⁺]_{er}, giving the possibility to measure the real ion uptake capacity of the mitochondria. Yet, changes in mitochondrial membrane potential ($\Delta\Psi_m$) (Fig. 3D) suggest an intrinsic functional damage of the organelles independent of the ER. Interestingly, mitochondria of aCD95 stimulated cells show Ca²⁺ uptake capacity and $\Delta\Psi_m$ (Fig. 3C, D) that resemble that of control cells, indicating that CD95 pathway causes cell death without interfering with mitochondrial physiology.

Altogether, these data demonstrate that organelles involved in intracellular Ca²⁺ homeostasis are differently affected in the cell death pathways controlled by C₂-cer and aCD95 during apoptotic induction. Interestingly, mitochondria are completely unaffected,

both morphologically and functionally, throughout the apoptotic route triggered by the engagement of the death receptor, whereas a major effect on Ca²⁺-mediated signalling, and activity of both organelles, is detected in the pathway operated by the lipid mediator, well before any biochemical or morphological sign of apoptosis. This notion implies radically different control mechanisms that can be explored, also pharmacologically, in further studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.101.

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