

# Changes in mitochondrial dynamics during ceramide-induced cardiomyocyte early apoptosis

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Cell death;  
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Cardiomyocytes

**Aims** In cells, mitochondria are organized as a network of interconnected organelles that fluctuate between fission and fusion events (mitochondrial dynamics). This process is associated with cell death. We investigated whether activation of apoptosis with ceramides affects mitochondrial dynamics and promotes mitochondrial fission in cardiomyocytes.

**Methods and results** Neonatal rat cardiomyocytes were incubated with C<sub>2</sub>-ceramide or the inactive analog dihydro-C<sub>2</sub>-ceramide for up to 6 h. Three-dimensional images of cells loaded with mitotracker green were obtained by confocal microscopy. Dynamin-related protein-1 (Drp-1) and mitochondrial fission protein 1 (Fis1) distribution and levels were studied by immunofluorescence and western blot. Mitochondrial membrane potential ( $\Delta\Psi_m$ ) and cytochrome c (cyt c) distribution were used as indexes of early activation of apoptosis. Cell viability and DNA fragmentation were determined by propidium iodide staining/flow cytometry, whereas cytotoxicity was evaluated by lactic dehydrogenase activity. To decrease the levels of the mitochondrial fusion protein mitofusin 2, we used an antisense adenovirus (AsMfn2). C<sub>2</sub>-ceramide, but not dihydro-C<sub>2</sub>-ceramide, promoted rapid fragmentation of the mitochondrial network in a concentration- and time-dependent manner. C<sub>2</sub>-ceramide also increased mitochondrial Drp-1 and Fis1 content, Drp-1 colocalization with Fis1, and caused early activation of apoptosis. AsMfn2 accentuated the decrease in  $\Delta\Psi_m$  and cyt c redistribution induced by C<sub>2</sub>-ceramide. Doxorubicin, which induces cardiomyopathy and apoptosis through ceramide generation, also stimulated mitochondrial fragmentation.

**Conclusion** Ceramides stimulate mitochondrial fission and this event is associated with early activation of cardiomyocyte apoptosis.

## 1. Introduction

Mitochondria are complex interconnected organelles that display particular morphology and distribution in different cell types.<sup>1,2</sup> In cardiomyocytes, mitochondrial activity has a key role in energy generation through oxidative phosphorylation and in the regulation of calcium homeostasis, contraction, production of reactive oxygen species, and cell death.<sup>3,4</sup>

The mitochondrial network exists in a continuous balance between local fission and fusion events.<sup>5,6</sup> Mitochondrial fission entails fragmentation of tubular interconnected mitochondria into several smaller individual organelles. The outer

mitochondrial membrane protein fission 1 (Fis1) and the GTPase dynamin-related protein-1 (Drp-1) are the main elements of the mitochondrial fission machinery.<sup>7,8</sup> When the fission process occurs, cytosolic Drp-1 is recruited into the mitochondrial fission foci where it interacts with Fis1.<sup>9</sup> In contrast, mitochondrial fusion promotes the assembly of individual mitochondria that combine their membranes. This process is also controlled by GTPases, including the mitofusins (Mfns) 1 and 2 and dynamin-related protein OPA1.<sup>2,10</sup> Although Drp-1, Fis1, and Mfn2 are all highly expressed in whole cardiac tissue,<sup>11–13</sup> how and when mitochondrial fission events take place in cardiomyocytes remain unknown. Likewise, there are no reports concerning the mechanism of mitochondrial fission in cardiomyocytes undergoing loss of mitochondrial network integrity. Conversely, interconnected mitochondria facilitate energy delivery from the cell periphery

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to the cell core and organize protonic potential to form ATP.<sup>14</sup> Moreover, Benard *et al.*<sup>15</sup> have recently proposed that there is a bidirectional relationship between mitochondrial network organization and bioenergetics. In skeletal muscle cells, Mfn2 repression causes fragmentation of the mitochondrial network, reduces mitochondrial metabolic response, and inhibits expression of oxidative phosphorylation enzymes.<sup>13,16</sup>

Mitochondria have a central role in apoptotic cell death produced by multiple conditions.<sup>17</sup> Activation of the mitochondrial fission machinery and further mitochondrial fragmentation have been associated with apoptosis, albeit this point remains contentious.<sup>6,18,19</sup> Under some specific conditions, Drp-1 interacts with Bax in mitochondrial scission loci, and mitochondrial fragmentation is a prerequisite for mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* (cyt *c*) release.<sup>19</sup> In contrast, mitochondrial fragmentation in other models is independent of the occurrence of apoptosis.<sup>18,20</sup>

In HeLa cells, ceramide induces mitochondrial network fragmentation and calcium release from the endoplasmic reticulum.<sup>21</sup> Moreover, through Drp-1 over expression Szabadkai *et al.*<sup>22</sup> demonstrated that mitochondrial network integrity is necessary for C<sub>2</sub>-ceramide-induced death. Ceramides, produced either by *de novo* synthesis or by the action of acid or neutral sphingomyelinases, interact with different organelles, especially with mitochondria, and both exogenous and endogenous ceramides can induce cell arrest and death.<sup>23</sup> C<sub>2</sub>-ceramide alters mitochondrial membrane potential ( $\Delta\Psi_m$ ) and promotes cyt *c* release and channel formation in the mitochondrial outer membrane whereas dihydro-C<sub>2</sub>-ceramide (DH-C<sub>2</sub>-ceramide) is inactive.<sup>23–25</sup> In cardiomyocytes, ceramides mediate both ischaemia-reperfusion and TNF $\alpha$ -induced cell death.<sup>26,27</sup> C<sub>2</sub>-ceramide triggers apoptosis in cultured cardiomyocytes, stimulating  $\Delta\Psi_m$  decrease through p38-MAPK and the activation of caspases 8 and 3.<sup>28,29</sup> In addition, ischaemic preconditioning prevents ceramide accumulation.<sup>30,31</sup> Yet, C<sub>2</sub>-ceramide also has positive inotropic effects and enhances contraction in adult cardiomyocytes.<sup>32</sup>

Given that (a) mitochondrial dynamics are associated with metabolism and cell death and (b) ceramides change mitochondrial homeostasis and trigger apoptosis, and considering that mitochondrial dynamics in cardiomyocytes has received little attention until now, we investigated whether ceramides alter mitochondrial dynamics and through these effects promote cell death in cultured rat neonatal cardiomyocytes. To this aim, we analysed the effects of ceramides on mitochondrial network,  $\Delta\Psi_m$ , the expression of the mitochondrial fission proteins Fis1 and Drp-1, and cell death. In addition, we studied whether decreasing mitochondrial connectivity (by a decrease of Mfn2 expression) enhanced the effects of C<sub>2</sub>-ceramide.

We show here for the first time that C<sub>2</sub>-ceramide promotes fragmentation of the mitochondrial network in cultured neonatal cardiomyocytes. This fragmentation correlated with mitochondrial fission. C<sub>2</sub>-ceramide also promoted rapid  $\Delta\Psi_m$  decrease and cyt *c* release. Decreasing Mfn2 expression accentuates the effects of C<sub>2</sub>-ceramide on mitochondrial fragmentation and in the  $\Delta\Psi_m$  decrease. Collectively, our data show that C<sub>2</sub>-ceramide stimulates mitochondrial fission events linked to an early activation of apoptosis.

## 2. Methods

### 2.1 Materials

Antibodies against Drp-1 and cyt *c* were purchased from BD Biosciences. Fis1 antibody was from Alexis Biochemicals and mt-Hsp70 antibody was from Affinity BioReagents. Anti- $\beta$ -myosin heavy chain antibody was from Novocastra Labs. Tetramethylrhodamine methyl ester (TMRM) and mitochondrial tracker green were from Molecular Probes. FBS was from Invitrogen. TRITC conjugated anti-IgG mouse polyclonal antibody, FITC conjugated anti-IgG rabbit, C<sub>2</sub>-ceramide, DH-C<sub>2</sub>-ceramide, anti- $\beta$ -actin antibody, propidium iodide (PI), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Dulbecco's modified Eagle's medium (DMEM), M199 medium, doxorubicin, and other reagents were purchased from Sigma-Aldrich Corp. Protein assay reagents were from Bio-Rad. The generation and use of AsMfn2, an adenovirus expressing Mfn2 antisense mRNA, was previously described.<sup>13</sup> Cardiomyocytes were transduced with adenoviral vectors at a multiplicity of infection (MOI) of 1000, 48 h before ceramide treatment. An empty adenovirus was used as control (mock).

### 2.2 Culture of cardiomyocytes

Cardiomyocytes were isolated from hearts of neonatal Sprague-Dawley rats as described previously.<sup>33</sup> Rats were bred in the Animal Breeding Facility of the University of Chile. All studies conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and it was approved by our Institutional Ethics Review Committee. Cardiomyocytes were plated at a final density of  $1-8 \times 10^3/\text{mm}^2$  on gelatin-coated 35-, 60- or 100 mm Petri dishes. For fluorescence measurements, cells were plated on gelatin-precoated 25 mm glass coverslips in 35 mm Petri dishes. Our initial studies showed that serum deprivation stimulated fragmentation of the mitochondrial network (data not shown). Accordingly, primary cell cultures were incubated with or without C<sub>2</sub>-ceramide or DH-C<sub>2</sub>-ceramide (0–40  $\mu\text{M}$ ) for 0–6 h or Doxo (1  $\mu\text{M}$  for 24 h) in DMEM/M199 (4:1) medium containing 10% FBS. Cultured cardiomyocytes were identified using an anti- $\beta$ -myosin heavy chain antibody as previously described<sup>23</sup> and cell cultures were at least 95% pure.

### 2.3 Subcellular fractionation

Mitochondrial and cytosolic fractions were obtained by differential centrifugation of cardiomyocyte homogenates. Cells were scraped, pelleted, and re-suspended in ice-cold buffer containing 250 mM sucrose, 1 mM EGTA, and 10 mM Hepes, pH 7.4; and the protease inhibitors PMSF, leupeptin, pepstatin A, and aprotinin. Cells were homogenized using a homogenizer with a tight fitting Teflon pestle. The homogenates were centrifuged (750 *g*, 10 min) to remove nuclei and unbroken cells, and supernatants were centrifuged (10 000 *g*, 25 min) to obtain a pellet highly enriched in mitochondria. The protein content was determined by Bradford's method.<sup>34</sup> The purity of mitochondrial fraction, assessed by mt-Hsp70 levels,<sup>35</sup> was 85%.

## 2.4 Western blot analysis

Equal amounts of protein were separated by SDS-PAGE (12% 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 with exposure to Kodak film and quantified by scanning densitometry. Protein contents were normalized by  $\beta$ -actin and mt-Hsp70 levels.

## 2.5 Mitochondrial dynamics analysis

Cells were preincubated with mitotracker green FM (400 nM) and maintained in Krebs solution. Confocal image stacks were captured with a Zeiss LSM-5, Pascal 5 Axiovert 200 microscope, using LSM 5 3.2 image capture and analysis software and a Plan-Apochromat 63x/1.4 Oil DIC objective. Images were deconvolved with Image J (NIH), Z-stacks analysis of the thresholded images were volume-reconstituted using the VolumeJ plug-in, and changes in number (objects) and volume of individual mitochondria were quantified using the ImageJ-3D Object counter plug-in. Each experiment was done at least four times and 16–25 cells per condition were quantified. Within cells, two to three regions of interest (ROI) of equal area were defined, and mitochondria counts and volume were measured for each ROI. The ROI sampling criteria considered only cytoplasmic mitochondria, excluding condensed perinuclear mitochondria (Supplementary material online, *Figure A*). Fragmentation criteria were: mitochondria individual volume decrease and increase in number of mitochondria.<sup>22,36</sup> Percentage of cells with a fragmented pattern was also determined.<sup>36</sup>

## 2.6 Immunofluorescence studies for Drp-1 and Fis1 and colocalization analysis

Cells grown on coverslips were fixed with PBS containing 4% paraformaldehyde and incubated in ice-cold 0.3% Triton X-100 for permeabilization. Nonspecific sites were blocked with 5% BSA in PBS for 1 h. Cells were then incubated with Drp-1, Fis1 or mt-Hsp70 antibodies (1:1000). Nuclei were stained with 5  $\mu$ g/mL Hoechst 33342 (Invitrogen). Secondary antibodies were anti-mouse IgG-TRITC and anti-rabbit IgG-FITC (1:1000). For details in image acquisition and colocalization analysis, Supplementary material online, *Materials and methods*.

## 2.7 Cell death assays

Cells were incubated with either C<sub>2</sub>-ceramide or DH-C<sub>2</sub>-ceramide at the concentrations indicated for up to 6 h at 37°C. DNA fragmentation was used as a marker of apoptosis. Cells were collected and permeabilized with methanol for 24 h. Later permeabilized cells were treated with RNAase for 2 h and 2  $\mu$ L PI (25  $\mu$ g/mL) was added prior to flow cytometry analysis.<sup>37</sup> For determination of cell viability, cells were harvested and stained with PI (10  $\mu$ g/mL).<sup>38</sup> Samples were analysed by a FACS Scan and data were evaluated using Cell Quest software (Becton Dickinson). Cytotoxicity was also quantified by lactate dehydrogenase (LDH) activity in cell supernatant using the LDH kit from Sigma following the manufacturer's instructions. To determine  $\Delta\Psi_m$  dissipation, cells

were loaded with 100 nM TMRM for 20 min at 37°C.<sup>39</sup> Fluorescence imaging of cells was conducted in a confocal microscope (excitation 543 nm, emission 560 nm). Cyt c release in cardiomyocytes undergoing ceramide-induced apoptosis was determined as previously described.<sup>38</sup>

## 2.8 Statistical analysis

Data shown are given as mean  $\pm$  SEM of the number of independent experiments indicated (*n*) and represent experiments performed on at least three separate occasions with similar outcomes. Data were analysed by ANOVA and comparisons between groups were performed using a protected Tukey's test. Statistical significance was defined as  $P < 0.05$ .

## 3. Results

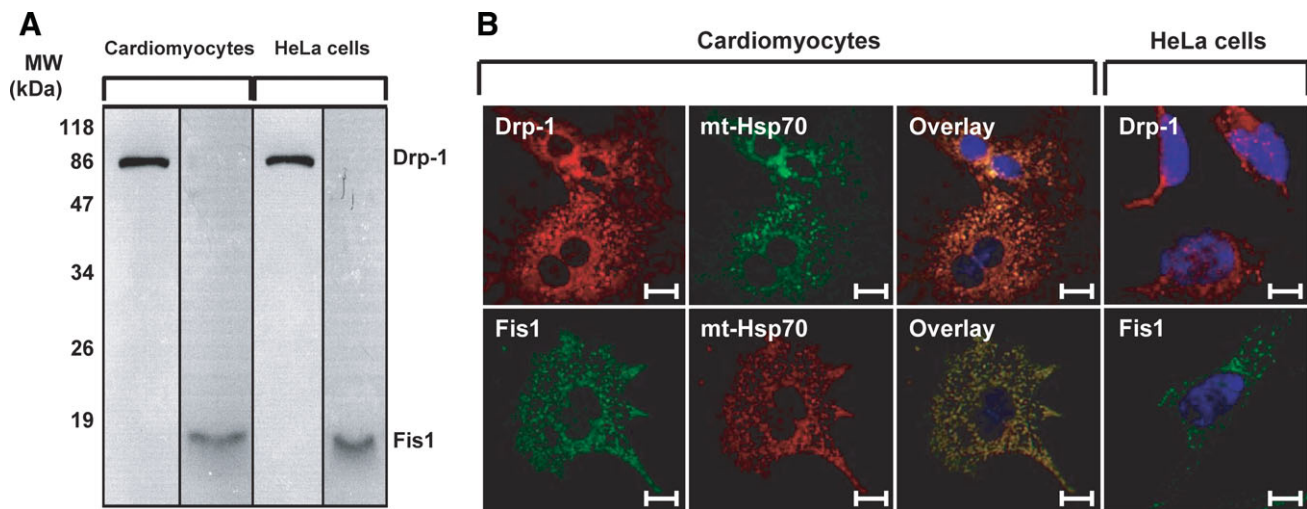
### 3.1 Ceramides stimulate mitochondrial fragmentation in cultured cardiomyocytes

As shown in *Figure 1A*, cardiomyocytes contain two proteins (80 and 17 kDa immunoreactive bands) corresponding to Drp-1 and Fis1, which are fully coincident with the protein bands found in HeLa total cell extracts.<sup>8,22</sup> Images of the subcellular distributions of Drp-1 and Fis1 in cardiomyocytes, illustrated in *Figure 1B*, show that Drp-1 was present throughout the cytoplasm but presented particular punctuate accumulations that partially overlapped with mt-Hsp70, used as a mitochondrial marker.<sup>35</sup> In contrast, Fis1 was mostly distributed in the mitochondrial network and presented a similar 'spaghetti-like' distribution as that described in HeLa cells.<sup>40</sup> Neither Drp-1 nor Fis1 were detected in the nucleus.

*Figure 2A* shows that C<sub>2</sub>-ceramide, but not the inactive analogue DH-C<sub>2</sub>-ceramide, gradually converted the mitochondrial tubular shape to a spherical conformation in a time-dependent manner. Exposure of cultured cardiomyocytes to 20, 30 and 40  $\mu$ M C<sub>2</sub>-ceramide for 6 h resulted in significant increases in the percentage of cells that displayed fragmented mitochondria, from control values (in %) of  $11 \pm 7$  to  $46 \pm 4$ ,  $65 \pm 2$ , and  $80 \pm 2$ , respectively (*Figure 2B*). To assess mitochondrial network integrity, we determined after 3D reconstitution both the average individual volume of each object (mitochondria) and the number of mitochondria per cell.<sup>22,36</sup> *Figure 2C* indicates that C<sub>2</sub>-ceramide gradually decreased mitochondrial volume in a time- and concentration-dependent manner. Individual mitochondrial volume decreased 20, 40 and 60% after 2, 4 and 6 h, respectively, of incubation with C<sub>2</sub>-ceramide. *Figure 2D* shows that the number of mitochondria per cell increased significantly from  $150 \pm 20$  (control) to  $280 \pm 20$  in cells incubated with 40  $\mu$ M C<sub>2</sub>-ceramide for 6 h. Incubation with 40  $\mu$ M DH-C<sub>2</sub>-ceramide for 6 h, however, did not modify any of the three parameters described above (*Figure 2B–D*). On the basis of these results, we used 40  $\mu$ M C<sub>2</sub>-ceramide in all subsequent experiments.

### 3.2 Ceramides increase the mitochondrial contents of Drp-1 and Fis1

According to some authors,<sup>9</sup> the initial step in mitochondrial fission is migration of Drp-1 to Fis1-containing fission



**Figure 1** Detection of Drp-1 and Fis1 in cultured cardiomyocytes and HeLa cells. (A) Protein levels were determined by western blot ( $n = 4$ ). (B) The subcellular distributions of Drp-1, Fis1, and mt-Hsp70 were studied by confocal microscopy. Nuclei were stained with Hoechst (blue). Images are representative of three independent determinations. The scale bar is 10  $\mu\text{m}$ .

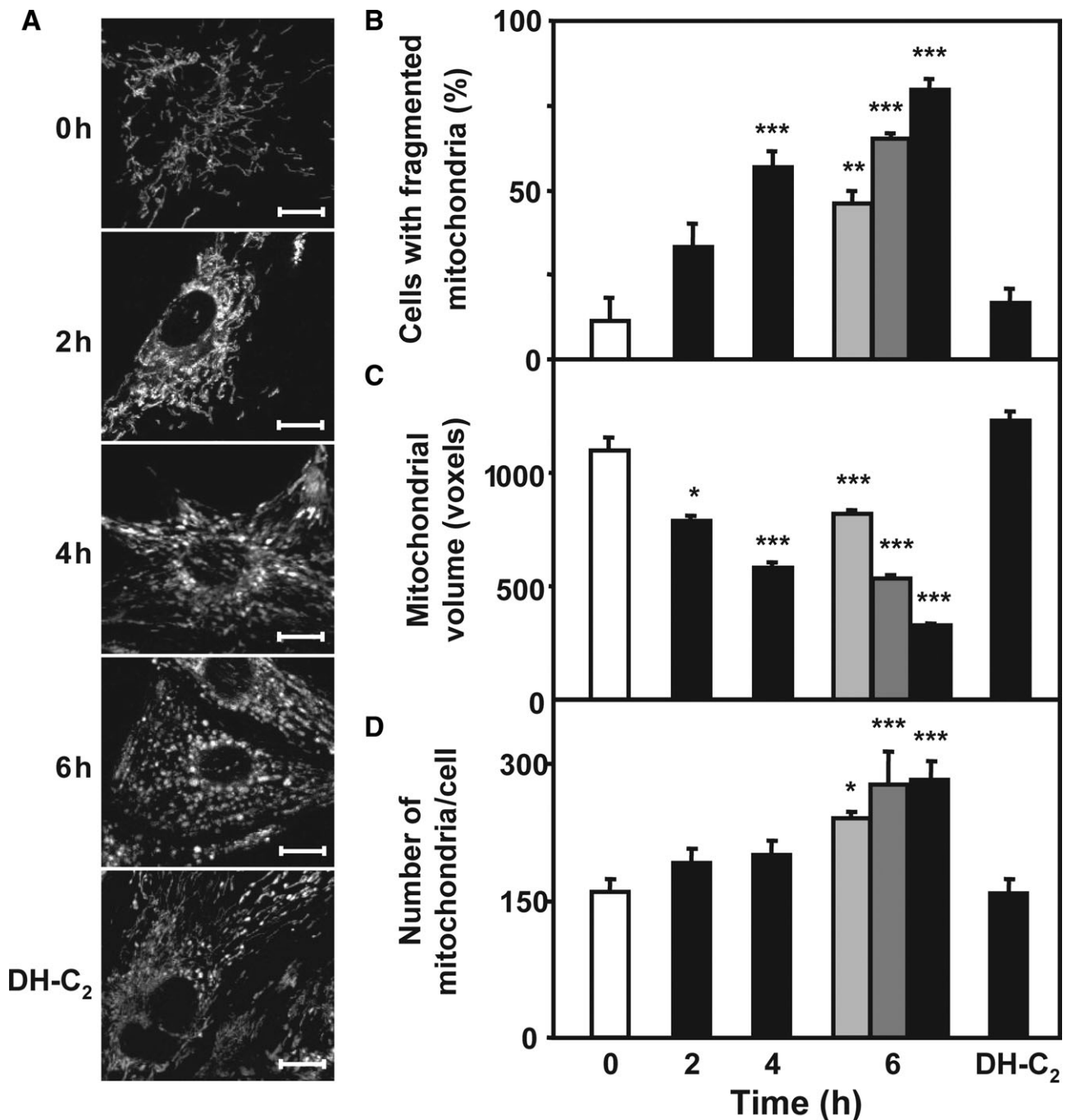
points. Thus, we evaluated next whether mitochondrial fragmentation triggered by  $\text{C}_2$ -ceramide was associated with changes in the distribution of Drp-1 and Fis1. Western blot analysis revealed that during the time period studied, Drp-1 levels did not change in total cell extracts from cardiomyocytes incubated with  $\text{C}_2$ -ceramide, whereas Fis1 content increased after 6 h of incubation with 40  $\mu\text{M}$   $\text{C}_2$ -ceramide (Figure 3A). Subsequent subcellular fractionation of  $\text{C}_2$ -ceramide-incubated cardiomyocytes into mitochondria revealed that Drp-1 and Fis1 levels increased transiently in the mitochondrial fraction after 2 h of incubation with  $\text{C}_2$ -ceramide and decreased at longer times to control levels (Figure 3B). These effects were specific to  $\text{C}_2$ -ceramide because DH- $\text{C}_2$ -ceramide did not modify Drp-1 and Fis1 total protein levels (Figure 3A) or their levels in the mitochondrial fraction (Figure 3B). Using immunofluorescence and confocal microscopy as a complementary approach revealed that Drp-1 displayed a punctuated distribution pattern, which was accentuated after 2 h of incubation with  $\text{C}_2$ -ceramide (Figure 3C). At the same time, an increase in Fis1 content was observed mainly in mitochondria. The extent of colocalization of both proteins increased two-fold in response to  $\text{C}_2$ -ceramide ( $P < 0.05$ ) (Figure 3C). We next quantified Fis1 fluorescence levels and changes in Drp-1 distribution. After stimulation with  $\text{C}_2$ -ceramide, the Fis1 coupled intensities increased  $\sim 1.75$ -fold relative to the Drp-1 coupled fluorescence ( $P < 0.001$ ) (Figure 3D).  $\text{C}_2$ -ceramide also increased the effective colocalization of Drp-1 with Fis1 ( $P < 0.001$ ) but not the effective colocalization of Fis1 with Drp-1 (Figure 3E). The increased size of the Fis1 related ROIs (green) 2 h after  $\text{C}_2$ -ceramide addition reflects the increased fluorescence intensities of the green channel. Only a very small fraction ( $\sim 5\%$ ) of the Fis1-labelled structures colocalized with Drp-1-labelled structures, independent on the stimuli or size of the segmented area. In contrast, Drp-1-labelled structures (red ROIs) increased significantly their effective colocalization

with Fis1-labelled structures and independent of the increasing size of the Fis1 related ROIs.

### 3.3 Down-regulation of Mfn2 levels alters the mitochondrial network but does not change the effects of ceramide

Mfns are key players in the mitochondrial fusion process. Mfn2 is particularly expressed in the heart.<sup>13</sup> As previously shown, down-regulation of Mfn2 causes a reduction in mitochondrial fusion; as a result, net mitochondrial fragmentation is detected.<sup>10</sup> In order to determine whether alterations in mitochondrial fusion modify the effects of ceramides on mitochondrial fragmentation, we studied whether a decrease in Mfn2 levels altered the cellular response to ceramides.<sup>13</sup> Overexpression of AsMfn2 produced a 75% decrease in Mfn2 protein levels in cultured cardiomyocytes (Figure 4A). This reduction in Mfn2 levels prompted mitochondrial fragmentation in cardiomyocytes and decreased the mitochondrial average volume by 25% ( $P < 0.05$ ). The cardiomyocytes transduced with AsMfn2 displayed a further decrease in mitochondrial volume and an increase in the number of cells with fragmented mitochondria following the incubation with  $\text{C}_2$ -ceramide (Figure 4B). The changes in mitochondrial volume and in the number of cells with fragmented mitochondria induced by AsMfn2 and  $\text{C}_2$ -ceramide were not synergistic but additive (Figure 4B). At every time studied,  $\text{C}_2$ -ceramide triggered equivalent mitochondrial volume reductions in mock and AsMfn2-transduced cardiomyocytes (Figure 4B). Yet, Drp-1 and Fis1 showed higher colocalization in AsMfn2-containing cardiomyocytes after 2 h of incubation with  $\text{C}_2$ -ceramide (Figure 4C).

Taken together these results suggest that addition of  $\text{C}_2$ -ceramide to cardiomyocytes undergoing Mfn2 down-regulation induced similar mitochondrial fragmentation than in controls, which was associated with an increase of mitochondrial Drp-1 and Fis1. Both  $\text{C}_2$ -ceramide and Mfn2 down-regulation showed an additive rather than a synergic



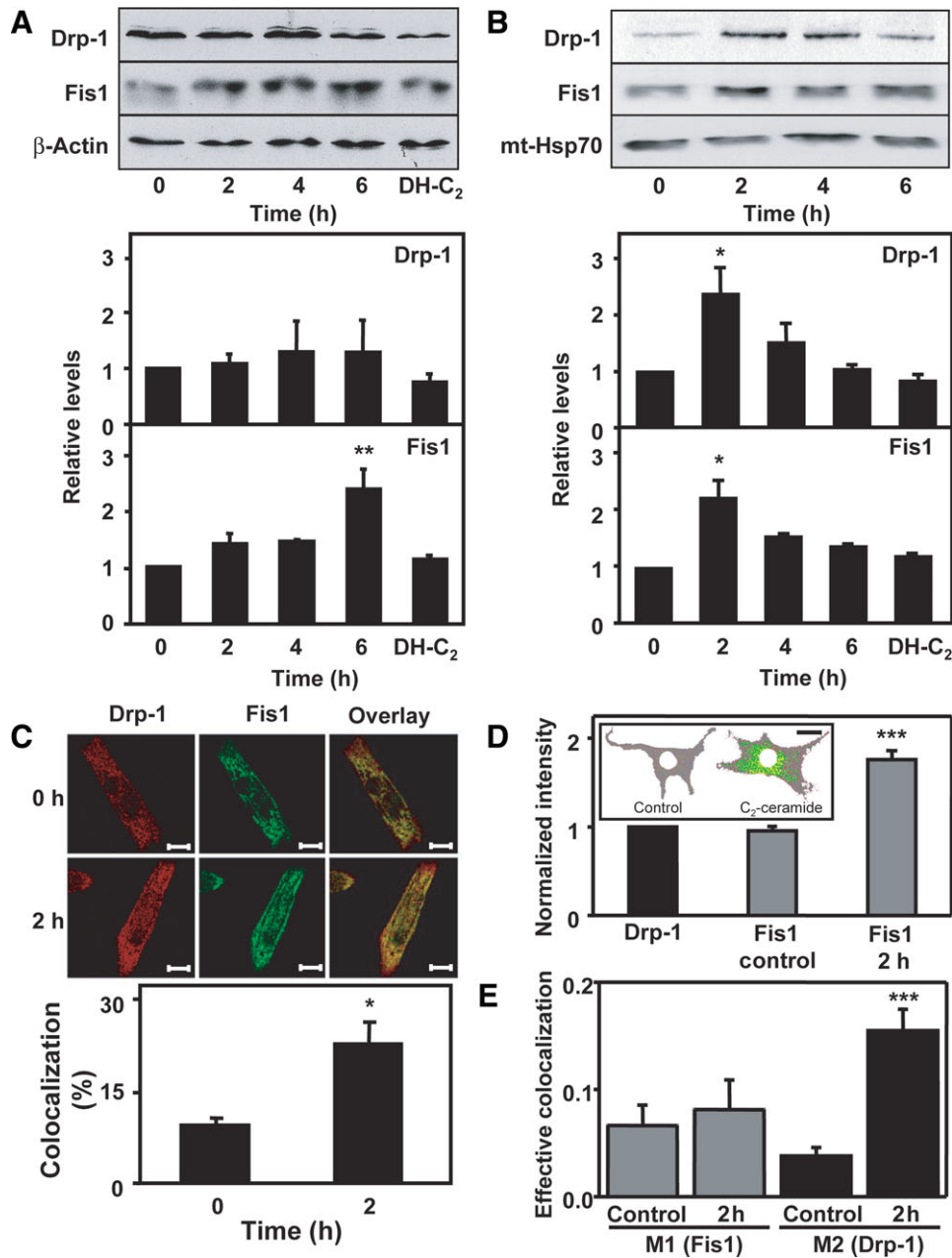
**Figure 2** Time-course effect of C<sub>2</sub>-ceramide on mitochondrial fragmentation in cultured cardiomyocytes. (A) Cells were incubated with C<sub>2</sub>-ceramide (40 μM) at indicated times or with dihydro-C<sub>2</sub>-ceramide (DH-C<sub>2</sub>, 40 μM) for 6 h and then loaded with mitotracker green. Multi-slice imaging reconstitutions were obtained by confocal microscopy to show mitochondrial morphology. The scale bar is 10 μm. (B) The percentage of cells with fragmented mitochondria, (C) the individual mitochondrial volume, and (D) the number of mitochondria per cell were determined in cells incubated without (white bars) or with C<sub>2</sub>-ceramide (grey bar: 20 μM for 6 h; dark grey bar: 30 μM for 6 h; black bars: 40 μM for 2–6 h) or DH-C<sub>2</sub> (black bars: 40 μM for 6 h). Values are given as mean ± SEM (n = 4), \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 vs. time 0 h.

effect on mitochondrial fission. These results further strengthen the view that ceramides largely affect mitochondrial fission, not fusion.

### 3.4 Mitochondrial fusion regulates mitochondrial outer membrane permeabilization and apoptosis triggered by ceramide

Figure 5A shows that transduction of cardiomyocytes with AsMfn2 but not with Mock increased the rate of  $\Delta\psi_m$

decrease induced by C<sub>2</sub>-ceramide (Figure 5A). A similar result in cyt c release from mitochondria was observed after C<sub>2</sub>-ceramide incubation (Figure 5B). AsMfn2 itself triggered cyt c redistribution (Figure 5B). C<sub>2</sub>-ceramide (40 μM for 6 h) or DH-C<sub>2</sub>-ceramide did not change cell viability in AsMfn2 or mock transduced cardiomyocytes (Figure 5C). As illustrated in Figure 5D–E, DNA fragmentation and LDH activity levels did not change with C<sub>2</sub>-ceramide. These data suggest that ceramides did not stimulate cell death by necrosis or the final execution of apoptosis until up 6 h.



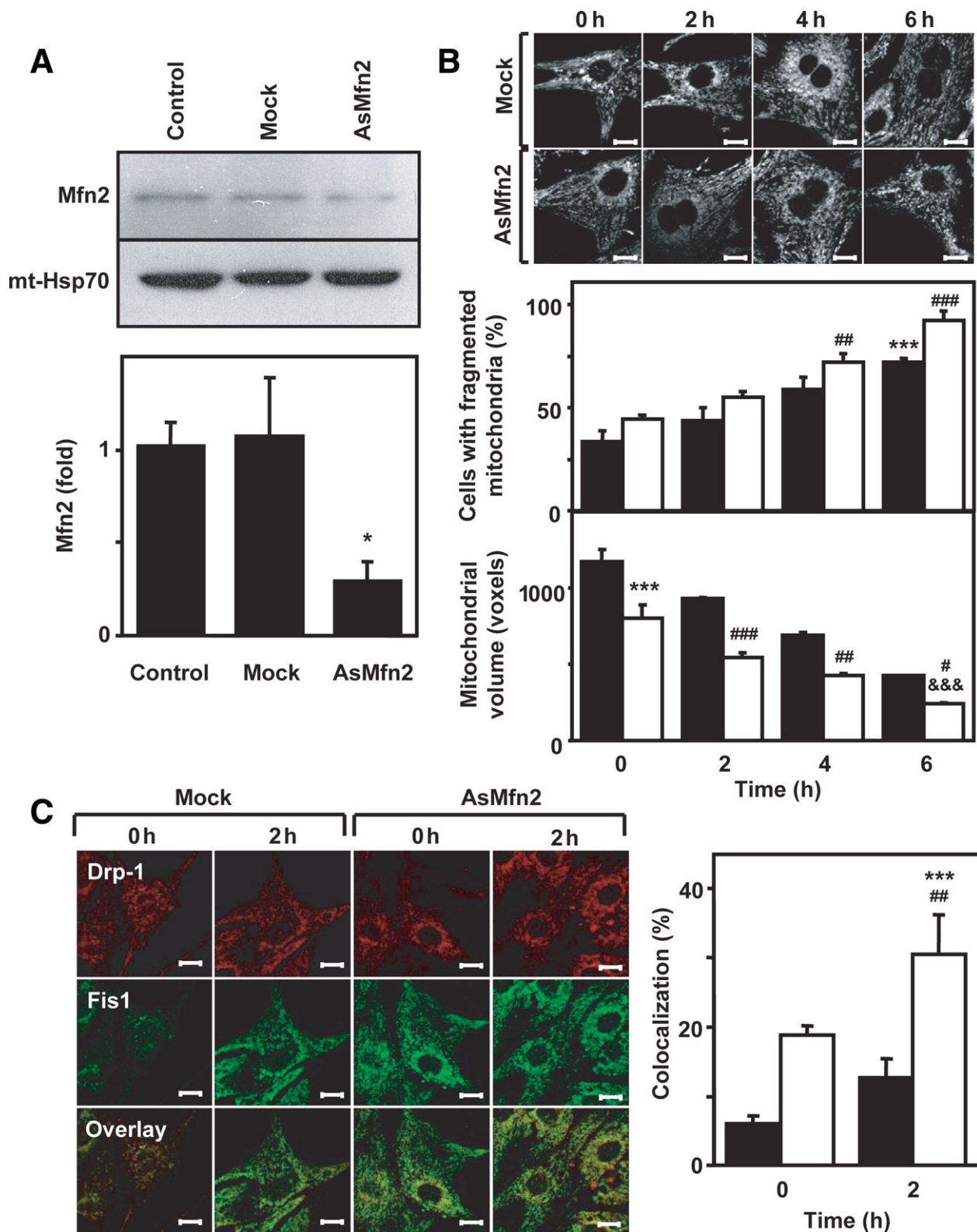
**Figure 3** C<sub>2</sub>-ceramide changes the subcellular distribution of Drp-1 and Fis1 in cultured cardiomyocytes. (A) Total extracts and (B) mitochondrial fraction were prepared from cells incubated with C<sub>2</sub>-ceramide (40 μM) for the indicated times. Drp-1 and Fis1 levels were determined by western blot. Representative western blots are shown. Protein contents were normalized using anti-β-actin and anti-mt-Hsp70 antibodies for total and mitochondrial fractions, respectively. Densitometric analysis for Drp-1 and Fis1 normalized levels are shown in left and right panels for total and mitochondrial fractions, respectively. Data correspond to mean ± SEM, *n* = 5, \**P* < 0.05 and \*\**P* < 0.01 vs. 0 h. (C) Control cells or cells incubated with C<sub>2</sub>-ceramide (40 μM) for 2 h were stained for Drp-1 (red) or Fis1 (green) to determine colocalization. The mean level of Drp-1 and Fis1 colocalization was determined in 20 cells (*n* = 3). The scale bar is 10 μm, \**P* < 0.05 vs. 0 h. (D) Fis1 coupled fluorescence intensity evaluation. Fis1 and Drp-1 coupled fluorescence intensities were determined inside the segmented contours of cultured cardiomyocytes (*n* = 12 for control and *n* = 7 for C<sub>2</sub>-ceramide-incubated cells; see representative segmentations on grey contours in the insert). Data represent the mean values ± SEM of the Drp-1 normalized Fis1 coupled intensities (grey bars) (\*\*\**P* < 0.001). The scale bar is 15 μm. (E) Effective colocalization of Drp-1 with Fis1. C<sub>2</sub>-ceramide also increased the effective colocalization of Drp-1 with Fis1 (black bars, \*\*\**P* < 0.001) but not the effective colocalization of Fis1 with Drp-1 (grey bars). M1 and M2: Manders colocalization coefficients for Fis1 and Drp-1, respectively. Mean values ± SEM are plotted for *n* = 12 (control) and *n* = 7 (C<sub>2</sub>-ceramide). For effective colocalization definition and quantification details, Supplementary material online.

Conversely, these results collectively indicate that mitochondrial fission modulates MOMP and early apoptosis triggered by C<sub>2</sub>-ceramide in cardiomyocytes.

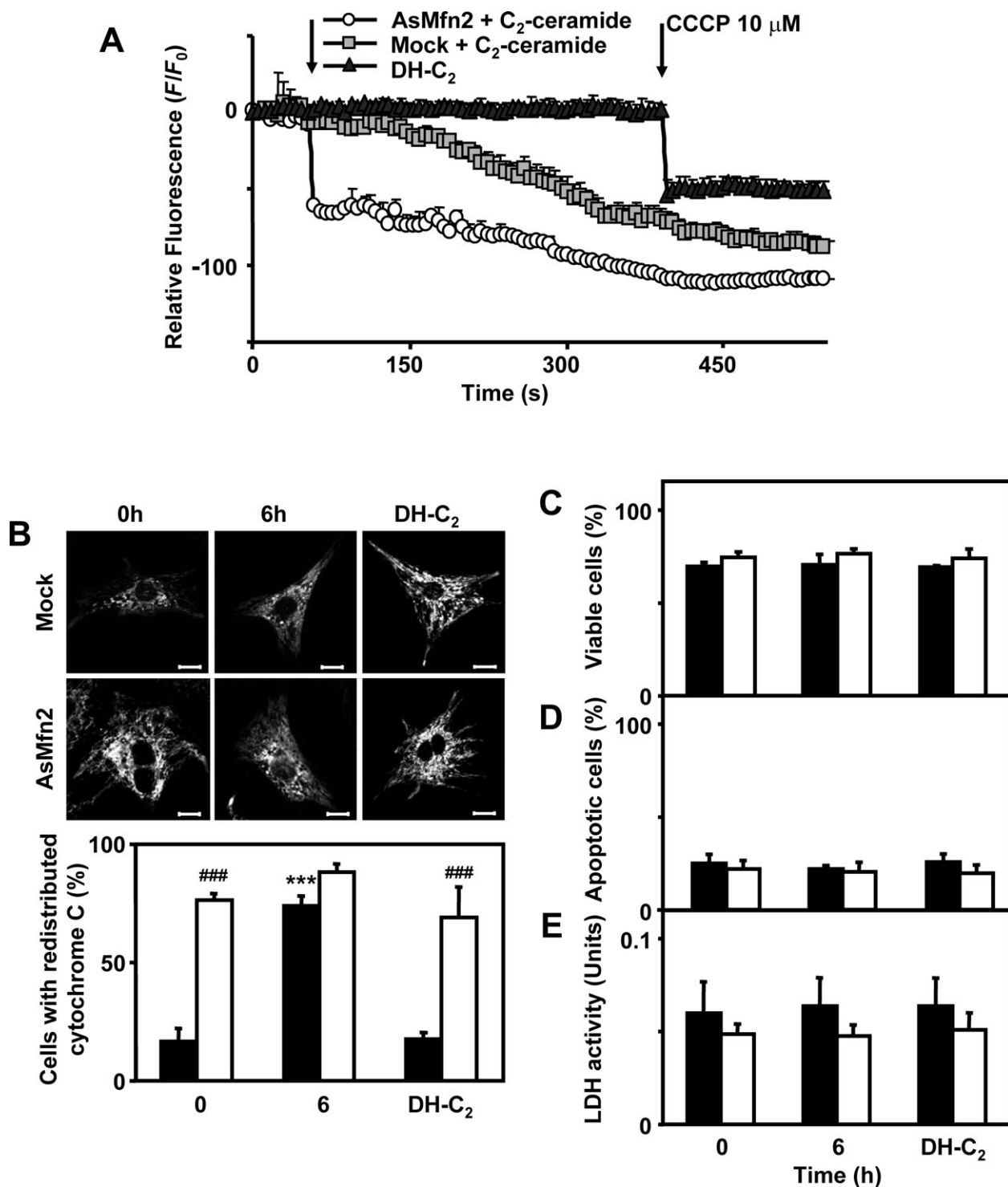
### 3.5 Doxorubicin also stimulates mitochondrial fragmentation in cardiomyocytes

Doxo is an antineoplastic agent widely used in cancer chemotherapy in spite of the fact that it causes cardiomyopathy.

Doxo induces cell death through activation of the intrinsic apoptotic pathway.<sup>41–44</sup> The work of Delpy *et al.*<sup>45</sup> demonstrated that Doxo induces cardiomyocyte apoptosis through ceramide generation. Accordingly, given our present results we investigated whether Doxo stimulates mitochondrial fragmentation in cardiomyocytes. *Figure 6A* shows that Doxo changed mitochondrial morphology from a tubular shape to a spherical conformation, resulting in significant increase in



**Figure 4** Down-regulation of Mfn2 levels does not increase mitochondrial fragmentation triggered by  $C_2$ -ceramide. Cells were transduced for 48 h with AsMfn2 to reduce Mfn2 protein levels or an adenovirus empty vector (mock). (A) The mitochondrial fraction was obtained and Mfn2 (122 kDa) and mt-Hsp70 (75 kDa) protein levels were determined by western blot (upper panel). The lower panel shows the quantitation by densitometric analysis. Values are mean  $\pm$  SEM ( $n = 4$ ), \* $P < 0.05$  vs. mock. (B) Analysis of mitochondrial dynamics was performed as described in Figure 2. Cells were transduced with AsMfn2 (MOI = 1000, white bars) or an adenovirus empty vector (mock, black bars) for 48 h before exposure to  $C_2$ -ceramide ( $40 \mu M$ ) for the time indicated. Images were used to evaluate percentage of cells with fragmented mitochondria (middle panel) or individual mitochondrial volume (lower panel). The scale bar is  $10 \mu m$ . Values are mean  $\pm$  SEM ( $n = 3$ ), \*\*\* $P < 0.05$  vs. mock (0 h), # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. mock in each time, and ### $P < 0.001$  vs. AsMfn2 at 0 h. (C) Cells were incubated with  $C_2$ -ceramide and stained for Drp-1 (red) or Fis1 (green) to determine colocalization (left panel). Colocalization (right panel) was determined for 20 cells (mean  $\pm$  SEM,  $n = 3$ ), \*\*\* $P < 0.001$  vs. mock (0 h) and ## $P < 0.01$  vs. mock (2 h).



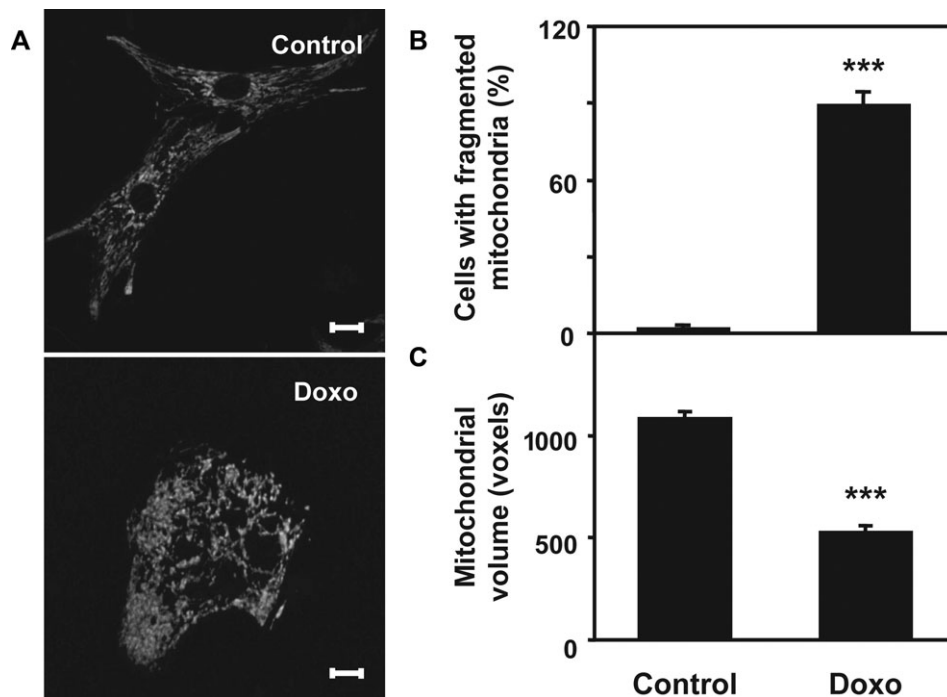
**Figure 5**  $C_2$ -ceramide stimulates early activation of apoptosis in cultured cardiomyocytes, which is accentuated by down-regulation of Mfn2 levels. Cells were transduced for 48 h with an adenovirus empty vector (mock, black bars) or with an adenovirus antisense against mitofusin-2 (AsMfn2, MOI = 1000, white bars) and incubated with  $C_2$ -ceramide ( $40 \mu$ M) at indicated times, or DH- $C_2$  ( $40 \mu$ M) for 6 h. (A) The mitochondrial membrane potential ( $\Delta\psi_m$ ) was determined in cells pre-loaded with TMRM and incubated for the indicated times with  $C_2$ -ceramide ( $40 \mu$ M) or DH- $C_2$ -ceramide ( $40 \mu$ M), added at the first arrow. The maximum  $\Delta\psi_m$  decrease was obtained following the addition of CCCP ( $10 \mu$ M). (B) Cyt c redistribution was evaluated by immunofluorescence. Images obtained by confocal microscopy are shown in upper panel. The percentage of cells with redistributed cyt c are shown in bottom panel. (C) Cell cytotoxicity was quantified using flow cytometry by incorporation of propidium iodide and (D) DNA fragmentation (in methanol-permeabilized cells) and (E) LDH activity in cell supernatants were determined as described in Section 2. Values are mean  $\pm$  SEM ( $n = 3$ ), \*\*\* $P < 0.001$  vs. mock 0 h and ### $P < 0.001$  vs. respective mock.

the percentage of cells with fragmented mitochondria respect to control (Figure 6B) and decreased individual mitochondrial volume (Figure 6C). Both Doxo and Mfn2 down-regulation also showed an additive effect on mitochondrial fission (Supplementary material online, Figure B).

#### 4. Discussion

In cardiomyocytes, mitochondrial dynamics is emerging as a fundamental cell biological process, important not only for control of the shape but also the function of mitochondria,





**Figure 6** Doxorubicin stimulates mitochondrial fragmentation in cultured cardiomyocytes. Cells were preloaded with mitotracker green and incubated with Doxo (1  $\mu$ M) for 24 h. Multi-slice imaging reconstitutions were obtained as described before and representative images are shown in (A). The percentage of cells with fragmented mitochondria (B) and mitochondrial volume (C) were determined as described in Section 2. The scale bar is 10  $\mu$ m, \*\*\* $P$  < 0.001 vs. control.

which have a central role in determining the life or death of cells. Our results strongly suggest that  $C_2$ -ceramide regulates mitochondrial dynamics through the stimulation of mitochondrial fission, among other events involved in the early activation of cardiomyocyte apoptosis. This proposal is based on the following evidences: (a)  $C_2$ -ceramide, but not the inactive analog DH- $C_2$ -ceramide, gradually modified mitochondrial tubular shape to a spherical conformation in a time- and concentration-dependent manner, and decreased individual mitochondrial volume while increasing the number of mitochondria per cell. (b) The mitochondrial fission proteins Drp-1 and Fis1 were detected in cardiomyocytes. Total Drp-1 levels did not change following incubation with  $C_2$ -ceramide whereas total Fis1 levels only increased after 6 h; after 2 h, however,  $C_2$ -ceramide induced Drp-1 migration to mitochondria where it colocalized with Fis1. (c)  $C_2$ -ceramide triggered rapid activation of apoptosis, evidenced by  $\Delta\Psi_m$  dissipation and redistribution of cyt *c*, although without DNA fragmentation (the final step of apoptosis), necrosis (evaluated by LDH release) and cell viability. (d) Mfn2 down-regulation caused  $\Delta\Psi_m$  decay and cyt *c* redistribution, but did not increase  $C_2$ -ceramide-dependent mitochondrial fragmentation. (e) Doxo, an anticancer drug with cardiotoxic properties linked to ceramide generation and activation of the apoptotic intrinsic pathway, also stimulated mitochondrial fragmentation in cultured cardiomyocytes.

#### 4.1 Ceramide-induced mitochondrial fission

Most of the current knowledge concerning mitochondrial fission comes from tumour cell lines and little information exists in normal cells.<sup>46</sup> In fact, there are few studies on mitochondrial fission/fusion in cardiac cells. One exception

is the work of Terman *et al.*<sup>47</sup> who investigated this process in senescent cardiomyocytes, reporting an accumulation of big and defective mitochondria with age. In the present work, we evaluated the induction of mitochondrial fission in cultured cardiomyocytes incubated with ceramide, a condition that stimulates cell death.<sup>26–29</sup> Previous studies showed that Drp-1 and Fis1 are expressed in the whole heart,<sup>11,12</sup> yet their expression in specific cardiac cell types remained unknown. We show here that both proteins are present in cardiomyocytes with a subcellular localization coincident with other reports.<sup>7,8</sup> Our data also revealed an increase in Drp-1 and Fis1 levels in mitochondrial fractions obtained from  $C_2$ -ceramide-treated cells, which occurred prior to mitochondrial fragmentation.<sup>9</sup> These findings agree with a previous study reporting that  $C_2$ -ceramide also stimulated mitochondrial network fragmentation, linked to endoplasmic reticulum calcium release and HeLa cell death.<sup>21</sup> Likewise, Brady *et al.*<sup>48</sup> showed that mitochondria undergo extensive fragmentation during simulated ischaemia in the HL-1 cardiac cell line, albeit the nature of the proteins involved in mitochondrial fragmentation was not reported. Interestingly, our results indicate that the decrease in Mfn2 levels induced by AsMfn2 or incubation with Doxo also triggered mitochondrial fission in cultured cardiomyocytes. Collectively, these results indicate that cardiomyocyte mitochondrial dynamics can be regulated with different stimuli, including ceramide, Doxo and by the manipulation of the balance between fission/fusion proteins.

Various studies have shown that ceramides alter the homeostasis of distinct organelles, particularly mitochondria.<sup>23</sup> In this regard,  $C_2$ - and  $C_{16}$ -ceramides form channels in planar lipid bilayers that are large enough to allow release of cyt *c*,<sup>23</sup> whereas  $C_2$ -ceramide (but not

C<sub>16</sub>-ceramide) decreases  $\Delta\Psi_m$  in isolated heart mitochondria.<sup>49</sup> Ceramides have been linked with cardiomyocyte apoptosis.<sup>26–29</sup> Our results agree with these reports, since we found that C<sub>2</sub>-ceramide decreased  $\Delta\Psi_m$  and stimulated cyt c release from the mitochondria to the cytoplasm before the final execution of programmed cardiomyocyte death. The relationship between mitochondrial fission and apoptosis has been controversial.<sup>6,18,19</sup> Our results show that loss of  $\Delta\Psi_m$  precedes mitochondrial fission, which is in turn followed by cyt c release, suggesting that ceramide-induced mitochondrial fission is a prerequisite to the ensuing apoptosis. Further studies should clarify whether the loss of mitochondrial connectivity induced by C<sub>2</sub>-ceramide as a cause or a consequence of the cell death process.

#### 4.2 Mitochondrial fission and apoptosis

Cells deficient in Mfn1 or Mfn2 have aberrant mitochondrial morphology, decreased mitochondrial fusion and altered dynamics.<sup>10</sup> Cellular repression of Mfn2 in primary skeletal myotubes decreases  $\Delta\Psi_m$  and metabolism, indicating that Mfn2 plays a key role in mitochondrial homeostasis.<sup>13</sup> Our results show that the 75% decrease in Mfn2 protein levels observed in ASMfn2-cardiomyocytes was accompanied by altered mitochondrial dynamics, with increased fission. In these cells, an imbalance between fusion and fission events may have promoted mitochondrial fragmentation. Attenuation of Mfn2 levels also increased the rate of  $\Delta\Psi_m$  loss in cardiomyocytes and promoted cyt c release from mitochondria, but did not prompt to the apoptosis execution during the first 6 h. These results imply that loss of mitochondrial connectivity predisposes cardiomyocytes to early phase of the apoptotic programme. These results differ from the work of Shen *et al.*<sup>50</sup> who reported that Mfn2 is a major determinant of oxidative stress-mediated cardiomyocyte apoptosis. Yet, attenuation of Mfn2 levels did not modify significantly the mitochondrial fragmentation triggered by C<sub>2</sub>-ceramide, suggesting that ceramide promotes apoptosis by affecting fission.

#### 4.3 Concluding remarks

The use of cultured neonatal rat cardiomyocytes is one of the main limitations of the present study. Although cultured neonatal rat cardiomyocytes have represented a very useful model for the understanding of the cellular aspects of the electrophysiological, contractile, morphological, metabolic, and molecular properties of the myocardium,<sup>51,52</sup> they exhibit important differences respect to adult cardiomyocytes, specially in metabolism and mitochondrial architecture.<sup>53</sup> Hence, further work in cultured adult cardiomyocytes will be required to investigate the role of ceramides on mitochondrial dynamics and apoptosis in fully differentiated cardiac cells. Additionally, we analysed only Drp-1 and Fis1 response after C<sub>2</sub>-ceramide stimulation; yet, other known fusion proteins, such as Opa-1 and Mfn1, can also regulate mitochondrial dynamics in cardiomyocytes.

Our present findings raise the possibility that, in addition to ceramides, other cell death-inducing stimuli may also act by altering mitochondrial dynamics. Given that perturbations in mitochondrial fusion/fission could result in significant loss in cardiac function, *in vivo* studies are necessary to determine the role of mitochondrial network dynamics in the apoptotic cell death observed in some cardiac diseases.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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