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Ceramide Induces Release of Pro-Apoptotic Proteins From Mitochondria by Either a Ca^{2+}-Dependent or a Ca^{2+}-Independent Mechanism

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Several observations have been reported in the last years indicating that ceramide may activate the mitochondrial route of apoptosis. We show here that on addition of either C_2- or C_{16}-ceramide to mitochondria isolated from rat heart and suspended in a saline medium, release of cytochrome *c* and apoptosis-inducing factor (AIF) from the intermembrane space takes place. The release process is Ca^{2+}-independent and is not inhibited by Cyclosporin A (CsA). For the protein release process to occur, the presence of an oxidizable substrate is required. When mitochondria are suspended in sucrose instead of potassium medium, only short chain C_2-ceramide causes cytochrome *c* release through a Ca^{2+}-dependent and CsA sensitive mitochondrial permeability transition (MPT) mechanism. The latter effect appears to be related to the membrane potential dissipating ability exhibited by short chain C_2-ceramide.

KEY WORDS: Ceramide; heart mitochondria; apoptosis; cytochrome *c* release; AIF release.

INTRODUCTION

Mitochondria play a central role in cell death through several mechanisms including i) energy failure owing to inhibition of oxidative phosphorylation; ii) alteration of the inner membrane permeability with consequent drop of membrane potential; iii) generation of reactive oxygen species (ROS), following the interaction with the electron transfer chain of various poisons; iv) release of a number of proteins, usually confined in the intermembrane space, into the cytosol, where they initiate the apoptotic cascade. Among the proteins released are the so-called apoptosis-inducing factor (AIF), smac-DIABLO, adenylate kinase-2 (AK-2), and cytochrome *c* (Loeffler and Kroemer, 2000; Kohler *et al.*, 1999; Du *et al.*, 2000; Verhagen *et al.*, 2000). Once in the cytosol cytochrome *c* interacts with apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9. The activation of the latter causes in turn activation of caspase-3 and -7, which drive the biochemical execution of apoptosis (Cai *et al.*, 1998; Zou *et al.*, 1997).

As far as the mechanism of cytochrome *c* release is concerned, two general possibilities have been proposed: i) matrix swelling, with consequent outer membrane rupture, caused by either mitochondrial permeability transition (MPT) or increased potassium uptake into mitochondria (Vander Heiden *et al.*, 1997; Marzo *et al.*, 1998); ii) formation of specific conducting channels provided by proteins, such as Bax, Bad, and tBid, which make the outer membrane permeable to cytochrome *c* (Desagher and Martinou, 2000). MPT is a Ca^{2+}-dependent mechanism initiated by the opening of the permeability transition pore (PTP) (reviewed in Gunter and Pfeiffer, 1990; Zoratti and Szabo, 1995; Bernardi, 1999). The process, which can be followed by monitoring the absorbance decrease of the mitochondrial suspension, is inhibited by a set of inhibitors

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including cyclosporin A (CsA), bongkrekic acid, Mg^{2+} ions, *N*-ethylmaleimide (NEM) as well as adenine nucleotides (Zoratti and Szabo, 1995; Bernardi, 1999).

Ceramide is generally recognized to promote apoptosis. It is a sphingolipid molecule which mediates the action of inflammatory cytokines or growth factors such as tumor necrosis factor-α (TNF-α), interleukin 1-β, γ-interferon, and nerve growth factor (reviewed in Hannun, 1996; Kolesnick and Golde, 1994). Ceramide can be generated within the cell by de novo synthesis or sphingomyelin hydrolysis. In apoptosis the deregulation of ceramide generating and utilizing processes causes a net increase of cellular ceramide level (Hannun, 1996; Kolesnick and Kronke, 1998).

Several observations indicate that ceramide may activate the mitochondrial route of apoptosis: i) mitochondria isolated from TNF-α-treated hepatocytes exhibited a two- to threefold increased content of ceramide (Garcia-Ruiz *et al.*, 1997); ii) a ceramide synthase activity associated to mitochondria has been identified and characterized (El Bawab *et al.* 2001); iii) mitochondrial ceramide levels are elevated during apoptosis caused by radiation as well as chemotherapeutic drugs (Bose *et al.* 1995). These agents promote reactive oxygen species (ROS) generation which, in turn, are likely to stimulate ceramide synthesis (Radin, 2001; Pautz *et al.*, 2002). Since ceramide itself causes an enhancement of ROS generation in mitochondria, this provides a feedback amplification loop leading to apoptosis.

Addition to isolated mitochondria of cell permeable ceramide analogue *N*-acetyl-D-erytrosphingosine (C_2-ceramide) was reported to cause inhibition of the electron transfer activity with consequent increase of ROS generation (Gudz *et al.*, 1997; Garcia-Ruiz *et al.*, 1997; Di Paola *et al.*, 2000b). C_2-ceramide did also cause cytochrome *c* release from mitochondria (Di Paola *et al.*, 2000b; Ghafourifar *et al.*, 1999). Since C_2-ceramide is an inner membrane permeabilizing agent, able to dissipate the mitochondrial membrane potential ($\Delta\Psi$), thus the question arose whether cytochrome *c* release could be a consequence of C_2-ceramide-dependent collapse of $\Delta\Psi$. However, our group has shown that long-chain naturally occurring *N*-palmitoyl-D-erytrosphingosine (C_{16}-ceramide) caused cytochrome *c* release from heart mitochondria, without affecting the membrane potential (Di Paola *et al.*, 2000b).

Different hypotheses have been put forward for the mechanism of ceramide-dependent cytochrome *c* release from mitochondria. According to Siskind *et al.* (2002), oligomeric aggregates of ceramide molecules may form channels in the outer membrane, through which the permeation of a number of intermembrane proteins, including cytochrome *c*, is allowed. On the contrary, data have been reported recently suggesting that ceramide does not permeabilize the mitochondrial outer membrane, rather it would just promote dissociation of cytochrome *c* from its interaction sites with respiratory complexes III and IV and with anionic phospholipids (Yuan *et al.*, 2003).

In this paper, we report that both C_2- and C_{16}-ceramide cause the release of cytochrome *c*, AIF, and AK-2 from mitochondria isolated from rat heart and suspended in a saline medium. The release process was Ca^{2+} independent and was not inhibited by either CsA or other known PTP inhibitors. For the protein release process to occurr, the presence of an oxidizable substrate was required. Under different experimental conditions, that is in a sucrose, instead of potassium based medium, only C_2-ceramide caused cytochrome *c* release, through a Ca^{2+}-dependent and CsA-sensitive MPT mechanism. The latter effect appears to be related to the membrane potential dissipating capacity exhibited by short chain C_2-ceramide.

MATERIALS AND METHODS

Preparation of Mitochondria

Heart mitochondria from adult male Wistar rats were isolated by differential centrifugation, as described elsewere (Di Paola *et al.*, 2000b). The final pellet was resuspended in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 0.25 mM phenylmethylsulphonyl fluoride (PMSF), 10 μM EGTA, at a protein concentration of 50–60 mg/mL, as determined by the Biuret method. The isolated mitochondria exhibited respiratory control ratios routinely higher than seven (with NADH-linked substrate). The integrity of outer membrane was assessed by the lack of uncoupled respiration increase upon addition of exogenous cytochrome *c*. Determination of mitochondrial cytochrome *c* content was carried out as reported in Williams (1964). Typically, a content of around 0.6 nmol mg protein^{-1} of cytochrome *c* was found in our preparations.

Mitochondrial Swelling

Changes in absorbance of rat heart mitochondria were monitored at 540 nm in a Beckmann DU 7400 spectrophotometer, equipped with a thermostatted and magnetically stirred automatic sampling unit. Mitochondria were suspended at 0.1 mg/mL either in a potassium-based medium (75 mM sucrose, 50 mM KCl, 30 mM Tris-Cl, pH 7.4, 5 mM KH_2PO_4, 10 μM EGTA, 10 mM succinate, 1 μg/mL rotenone), or in a sucrose medium (250 mM

sucrose, 10 mM Tris-Cl, pH 7.4, 5 mM KH_2PO_4, 10 μM EGTA, 10 mM succinate, 1μg/mL rotenone), at 25°C. Swelling was triggered after 1 min of Ca^{2+} loading by the addition of ceramides or other effectors.

Detection of Mitochondrial Protein Release

Mitochondria were suspended under the same conditions described for swelling experiments and, after 10 min incubation at 25°C, spun down at 14000*g* for 10 min at 4°C. The pellets were solubilized at 1 mg/mL in 5% SDS, 15% glycerol, 50 mM Tris-Cl, pH 6.8, 2% mercaptoethanol and subjected to SDS-PAGE according to Schagger *et al.* (1986), while the resulting supernatants were centrifuged at 100,000*g* for 15 min, at 4°C. The supernatants of the second centrifugation were concentrated 200 times using Millipore ultrafree-4 centrifugal filter and subjected to electrophoresis. After electrophoresis, cytochrome *c*, AIF, and AK-2 detection was performed by immunoblotting with the corresponding antibodies according to Andreyev *et al.* (1998). Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-antibodies using enhanced chemiluminescence Western Blotting reagents (NEN). Relative optical densities and areas of bands were quantified using a Camag TLC scanner II densitometer equipped with a D-2000 Cromatointegrator (Merck-Hitachi). Quantitative analysis of cytochrome *c* release was performed using linear concentrations of cytochrome *c* as standard. Matrix glutamate dehydrogenase activity in the supernatants was measured as described in Morii *et al.* (1991).

Chemicals

N-acetylsphingosine (C_2-ceramide), *N*-acetylsphinganine (C_2-dihydroceramide), *N*-palmitoylsphingosine (C_{16}-ceramide), and cyclosporin A were purchased from Calbiochem (La Jolla, CA). Arachidonic acid, rotenone, PMSF, horse-heart cytochrome *c* (type VI), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma (St. Louis, MO). Monoclonal anti-cytochrome *c* antibody (7H8-2C12) was purchased from Pharmingen (San Diego, CA). Polyclonal anti-AIF and anti-AK-2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were of the highest purity grade commercially available.

RESULTS

In Fig. 1 the results of an experiment are reported in which the effect of ceramides on mitochondrial matrix swelling and intermembrane protein release is analyzed. On addition of C_2-ceramide to heart mitochondria supplemented with Ca^{2+}, only a small decrease of mitochondrial suspension turbidity was observed (trace a). The C_2-ceramide effect, which was insensitive to CsA (trace b), was not exhibited by either C_{16}-ceramide (trace c) or

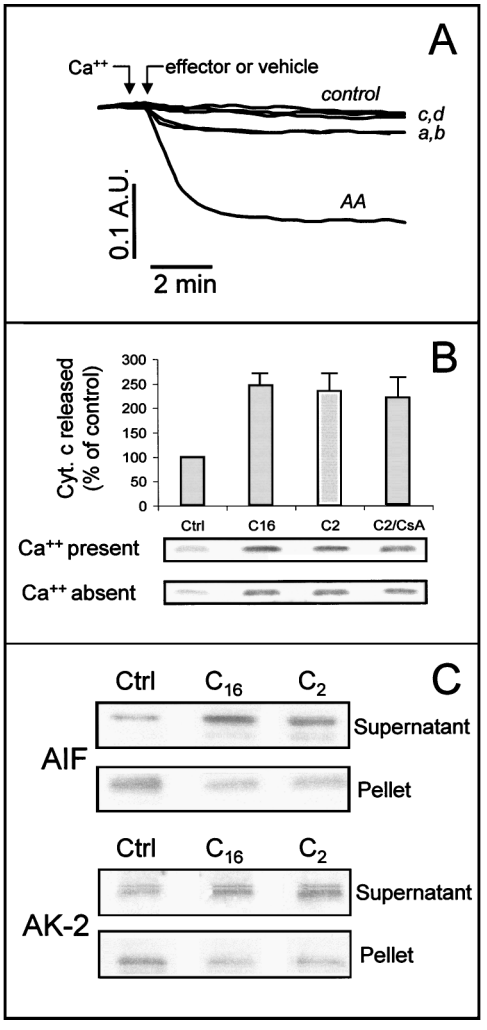

Fig. 1. Mitochondrial swelling and intermembrane protein release: effect of ceramide. Freshly isolated heart mitochondria were incubated in the potassium medium described under Materials and Methods section. (A) Swelling measurements of the mitochondrial suspension: where indicated, 30 μM Ca^{2+} and 20 μM effector (vehicle in the control) were added. (a) C_2-ceramide; (b) C_2-ceramide in the presence of 2 μM CsA; (c) C_{16}-ceramide; (d) DHC; AA: arachidonic acid. (B) Western blot analysis of cytochrome *c* released into the supernatant. Mitochondria were incubated as described in 1(A), with or without Ca^{2+}, for 10 min. Values (mean ± SD from five different experiments) represent percent variations of cytochrome *c* content measured by scanning densitometry of the blots from supernatant fractions. (C) AIF and AK-2 release from mitochondria caused by 20 μM ceramides. The experimental conditions are those described for the measurement of cytochrome *c* release.

C_2-dihydroceramide (DHC) (trace d), a closely related analogue of C_2-ceramide, lacking the 4,5-trans double bond in the sphingoid backbone. Saturating concentrations of the uncoupler CCCP did not cause any effect as well (not shown). The observation that C_2-ceramide effect was not inhibited by CsA, as well as by other typical MPT inhibitors such as *N*-ethyl-maleimide, Mg^{2+} ions, and adenine nucleotides, together with the finding that the same absorption change was observed even in the absence of Ca^{2+} (not shown), indicates that under these conditions C_2-ceramide does not induce MPT. The effect of arachidonic acid (AA), which has been shown to induce MPT (Di Paola *et al.*, 2000a; Scorrano *et al.*, 2001) was used as positive control.

In the experiment shown in Fig. 1(B), we measured directly the effect of ceramide on cytochrome *c* release from mitochondria. Both short- and long-chain ceramides caused a two- to threefold increase of cytochrome *c* release. Interestingly, the release process was CsA insensitive and Ca^{2+} independent. Scanning densitometry determinations of the blots from supernatants of ceramide-treated heart mitochondria revealed that cytochrome *c* released under these conditions amounted to 0.33 ± 0.04 nmol mg protein^{-1}, that is around 55% of its total mitochondrial content. The protein release process was not specific for cytochrome *c*, since the release of other proteins residing in the intermembrane space was also promoted by ceramides. Figure 1(C) shows, in fact, that C_{16}- and C_2-ceramide induced also the release of AIF and AK-2 from mitochondria. Their concentration increment in the supernatant was more than twofold and represented around 40% of the amounts released after treatment of mitochondria with alamethicin, a channel forming antibiotic which causes maximal release of intermembrane proteins (Andreyev and Fiskum, 1999). Consistently with the release pattern, a definite decrease of their content in the pellet was clearly observed. Again, the release of both AIF and AK-2 was Ca^{2+} independent and CsA insensitive (not shown). The activity of the matrix enzyme glutamate dehydrogenase measured in the supernatants of the same samples was undetectable.

The effect of ceramide on the release of intermembrane proteins was dependent on the energized state of mitochondria. As shown in Fig. 2(A), in the absence of the oxidizable substrate succinate, no appreciable release of cytochrome *c*, AIF, and AK-2 in the supernatant of ceramide-treated mitochondria was detected. Importantly, the effect of ceramide on protein release from energized mitochondria was sensitive to the uncoupler CCCP (Fig. 2(B)).

Under different experimental conditions, that is with mitochondria suspended in sucrose instead of potassium medium, addition of C_2-ceramide caused a definite absorption decrease of Ca^{2+}-loaded mitochondrial suspension (Fig. 3(A)). This effect, which was strictly Ca^{2+} dependent and CsA sensitive, was not exhibited by C_{16}-ceramide. However, saturating concentrations of the uncoupler CCCP did cause a similar CsA sensitive effect. The above findings are suggestive of C_2-ceramide-dependent induction of permeability transition with outer membrane rupture and depletion of cytochrome *c*. The results presented in Fig. 3(B) show, in fact, that under these conditions C_2-ceramide caused a CsA sensitive release of cytochrome *c* from mitochondria. Remarkably, C_{16}-ceramide as well as the uncoupler CCCP did not promote significant cytochrome *c* release. Again, no evidence for the presence of glutamate dehydrogenase activity in the supernatants was obtained.

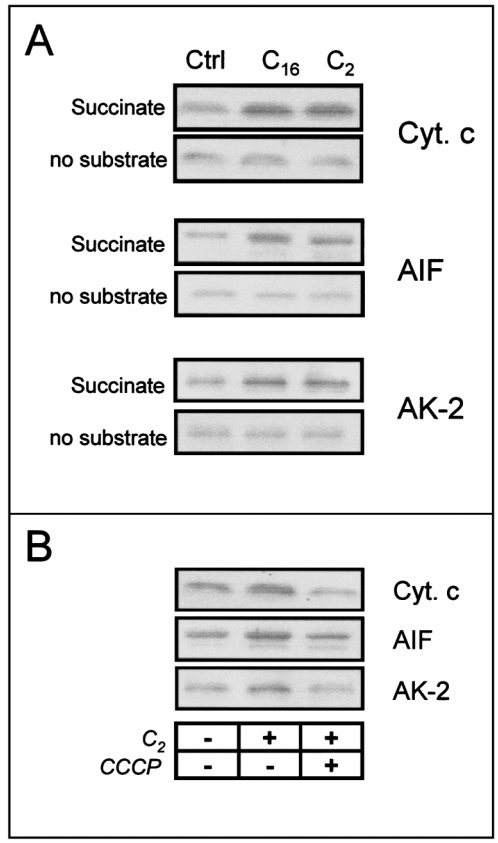

Fig. 2. Dependence of ceramide effect on mitochondrial energized state. Mitochondria were incubated as described in the legend to Fig. 1. Succinate was used as substrate in the experiments reported in Fig. 2(B) and, where indicated, in Fig. 2(A). Ceramides were added to a final concentration of 20 μM and reacted for 10 min at 25°C. CCCP was present at 0.25 μM. Immunoblot analysis of cytochrome *c*, AIF, and AK-2 release was carried out as reported under Materials and Methods section.

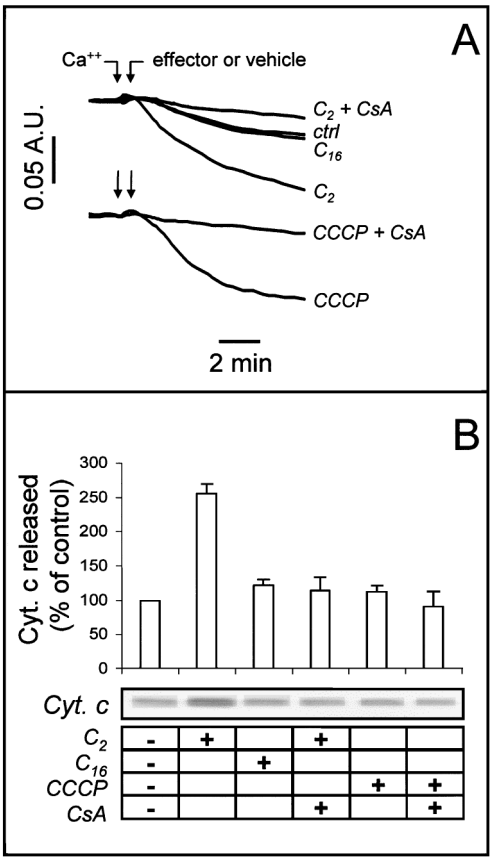

Fig. 3. Influence of medium composition on mitochondrial swelling and cytochrome *c* release caused by ceramide. Heart mitochondria were suspended in the sucrose medium described under Materials and Methods section. (A) Swelling measurements of the mitochondrial suspension: where indicated, Ca^{2+}, ceramides, and CCCP were added to a final concentration of 30, 20, and 0.25 μM, respectively. CsA was present at a final concentration of 2 μM. (B) Immunoblot analysis of cytochrome *c* released: values, as percent of control, were obtained by scanning densitometry of the bands and are mean ± SD from three different experiments.

DISCUSSION

Short-chain permeable ceramides, such as C_2-ceramide, have been extensively used in experiments with cells and isolated mitochondria. However, their use has been questioned (Hofmann and Dixit, 1998) since they cause perturbation of membrane structure, owing to their disordering effect towards the membrane lipid components as compared to naturally occurring long-chain ceramide (see van Blitterswijk *et al.*, 2003 for review). Our group has indeed shown that, in contrast with C_{16}-ceramide, C_2-ceramide was able to dissipate the mitochondrial inner membrane potential (Di Paola *et al.*, 2000b). Thus, the results reported in Fig. 3 can be interpreted as follows: i) under the reported experimental conditions, C_2-ceramide causes initiation of MPT by decreasing $\Delta\Psi$ below the gating potential; ii) CCCP dissipates $\Delta\Psi$ as well; iii) for cytochrome *c* to be released, in a medium at low ionic strength, the protein needs to be dissociated from the membrane components facing the intermembrane space (Ott *et al.*, 2002); iv) C_2-ceramide, but not CCCP, appears able, as suggested by Yuan *et al.* (2003), to promote dissociation of cytochrome *c* from the membrane.

In the intermembrane space the ionic strength is quite high. Thus, the C_2-ceramide MPT triggering effect, we showed here to occur at low ionic strength, does not appear of physiological relevance. However, such observations may help to understand the mechanism and the circumstances under which the VDAC-ANT-Cyp-D complex may deform into MPT pore, which can be now susceptible to be opened by a given effector.

As discussed by various authors (Crompton, 2000; Doran and Halestrap, 2000), MPT pore opening is difficult to be considered as a precise mechanism for outer membrane permeabilization, since it causes $\Delta\Psi$ collapse and ATP dissipation, that are conditions leading to necrosis. From this point of view naturally occurring long-chain ceramide appears as an orthodox proapoptotic effector, since it causes permeabilization of outer membrane to intermembrane proteins, triggering a mechanism MPT independent and without affecting the membrane potential.

We report here, for the first time, that AIF, in addition to cytochrome *c*, is released upon interaction of both C_2- and C_{16}-ceramide with intact heart mitochondria in a saline medium (Fig. 1). No MPT inducing effect was exerted by either ceramides or by CCCP under these conditions. The small absorbance decrease observed upon interaction of C_2-ceramide with mitochondria (Fig. 1(A)) may represent the consequence of the fluidizing effect exerted by the short chain analogue, in contrast with natural long-chain ceramide which exhibits an ordering/packing effect on membrane phospholipids (see van Blitterswijk *et al.*, 2003 for review).

As shown in Figs. 1 and 2, a number of proteins are released in the supernatant of ceramide-treated mitochondria suspended in a saline medium. Thus, a particular release mechanism does not appear to be involved in outer membrane permeabilization. The proposed capability of ceramides to form conducting channels for small proteins in the outer membrane (Siskind *et al.*, 2002) may then explain the present results. In vivo, cytochrome *c* release depends on the involvement of proteins, such as Bax, Bad, and tBid that localize at mitochondrial contact sites (Lutter *et al.*, 2001; Marzo *et al.*, 1998), where ANT and VDAC assemble to form junctional complexes and where they can recruit other proteins depending on the function to be executed. Bax, Bad, and tBid are believed to catalyze

the restructuring of lipids to induce conducting channel formation (Basanez *et al.*, 1999). It is conceivable that ceramides, whose membrane lipid restructuring ability is considered of physiological relevance (van Blitterswijk *et al.*, 2003), may give rise to channel formation at contact sites. This ability is apparently limited under MPT conditions. In fact, under these conditions, C_{16}-ceramide did not cause cytochrome *c* release whereas, as discussed above, short-chain C_2-ceramide may promote a Ca^{2+}-dependent and CsA-sensitive cytochrome *c* release only because of its uncoupling properties (Fig. 3).

It is known that changes in the interactions between the mitochondrial membranes correlate with the functional state of the inner membrane (Knoll and Brdiczka, 1983). Furthermore, the actual number of contact sites, which increases in apoptosis (He *et al.*, 2003), is in correlation with the cell energy requirement (Ziegelhoffer-Mihalovicova *et al.*, 2002). On this ground and from the results of the experiments reported in Fig. 2, we would conclude that the membrane lipid restructuring effect of ceramide and the consequent channel formation, as far as the intermembrane proteins leakage in the cytosol is concerned, requires mitochondrial energized conditions. Support to this view results from the work by Doran and Halestrap (2000), who provided evidence that in energized mitochondria a pathway for cytochrome *c* release can be activated by protein–protein interaction related destructuration of contact sites.

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