Ceramide induces cytochrome c release from isolated mitochondria

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Abstract

This chapter addresses the role of mitochondria in apoptosis. Emphasis is put on the recently observed influence of ceramides on mitochondrial functions. We report here that N-acetylsphingosine (C\(_2\)-ceramide), N-hexanoylsphingosine (C\(_6\)-ceramide) and, to a much lesser extent, C\(_2\)-dihydroceramide, induce cytochrome c (cyt c) release from isolated rat liver mitochondria. Ceramide-induced cyt c release is prevented by a low concentration of Bcl-2. The release takes place when cyt c is oxidized, but not when it is reduced. Upon cyt c release mitochondrial oxygen consumption, mitochondrial transmembrane potential (\(\Delta \Psi_m\)) and Ca\(^{2+}\) retention are diminished. Bcl-2 prevents, and addition of cyt c reverses, the alteration of these mitochondrial functions. In ATP-energized mitochondria ceramides do not alter \(\Delta \Psi_m\), neither when cyt c is oxidized nor when it is reduced. This rules out a non-specific disturbance by ceramides of mitochondrial-membrane integrity. It is concluded that some of the apoptogenic properties of ceramides are mediated via their interaction with mitochondrial cyt c followed by its release.

Apoptosis

Apoptosis is an evolutionarily conserved form of physiological cell death important for tissue development and homoeostasis. Its hallmarks are distinct morphological alterations such as nuclear condensation, cell shrinkage and bleb formation, and the absence of inflammatory responses towards the affected cells. Deranged apoptosis is involved in diseases such as cancer, AIDS, autoimmune diseases and neurodegeneration (reviewed in [1]).

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The programme for apoptotic cell death appears to be present constitutively in virtually all eukaryotic cells and can be activated by a variety of extra- and intracellular signals. Although the various steps and biochemical mechanisms participating in apoptosis are not completely understood, it is clear from genetic studies in lower organisms and comparative investigations in mammalian systems that apoptosis generally comprises four distinct stages, namely the decision to die, the execution of death, the engulfment of dead cells or fragments thereof and their degradation (reviewed in [2]).

**Mediators of apoptosis**

Several conditions, molecules or organelles are considered participants of apoptosis, but at present it is not always clear whether they are required for, or are the consequence of, apoptosis. This is due to our incomplete knowledge of the cellular machinery and timing of the various stages of apoptosis. Also, studies with reconstituted systems have shown that characteristic apoptotic responses occur in isolated nuclei as well as in nucleus-free cell fractions. Thus it is conceivable that some of the conditions or molecules are required to direct the cell to undergo apoptosis, whereas others may be required for its execution. There may also exist cell-type-specific requirements.

**Oxidative stress**

There is ample evidence that apoptosis is accompanied by oxidative stress (reviewed in [3]). A valuable tool used to elucidate the importance of oxidative stress is the proto-oncogene Bcl-2 (see also below), which stimulates an anti-oxidative response in cells and prevents apoptosis [4,5]. The importance of oxidative stress as a cause for apoptosis was questioned because cells can undergo Bcl-2-inhibitable apoptosis also under very low oxygen tensions [6,7].

**Calcium**

The requirement of Ca\(^{2+}\) for apoptosis is controversial (see [8]). Early reports suggested that a rise of the intracellular Ca\(^{2+}\) leads to apoptosis via endonuclease activation, and more recent work indicated that apoptosis is accompanied by shifts of Ca\(^{2+}\) between various intracellular pools. It is worth noting that cellular Ca\(^{2+}\) handling and the production of reactive oxygen species (ROS) are related. Thus increased mitochondrial Ca\(^{2+}\) release, followed by re-uptake (Ca\(^{2+}\) cycling) driven by the mitochondrial transmembrane potential (ΔΨ\(_m\)), stimulates ROS production (see below).

**Proteases**

Presently, the role of proteases in apoptosis is receiving much attention (reviewed in [9]). Gene analysis in *Caenorhabditis elegans* identified a key pro-apoptotic gene, *Ced-3*, which encodes a putative cysteine protease, the nematode homologue of the mammalian protease interleukin-1β-converting enzyme. Related proteases cleave poly(ADP ribose) polymerase, which results in the activation of a Ca\(^{2+}/\text{Mg}^{2+}\)-dependent endonuclease implicated in internucleosomal DNA cleavage characteristic of apoptosis.
Ceramide

Recently, the importance of ceramides in cell regulation has become apparent. It is now evident that ceramides are involved as second messengers in what has become known as the sphingomyelin cycle (reviewed in [10]), apoptosis and differentiation in many cell types [11]. The mechanisms by which ceramide mediates apoptosis have not yet been fully addressed; however, it has been shown that ceramides have mitochondria as targets. Direct inhibition of mitochondrial respiratory chain complex III by ceramide [12], ceramide-induced generation of ROS in intact mitochondria [13] and in cell lines [14], and ceramide-induced cell death via disruption of mitochondrial functions [15] are lines of evidence for a strong influence of ceramide on mitochondria.

Cyt c

Cyt c plays a dual role in cell homoeostasis. It is needed, as part of the respiratory chain, for cell life and it is needed, as one of the triggers of apoptosis, for cell death. It is now well accepted that many apoptogenic factors run cells into apoptotic death via mitochondrial cyt c release [16]. The released cyt c switches on the death machinery in various ways, for example by activation of caspases [17]. The proto-oncogene Bcl-2 is known to interact with the apoptotic machinery at various levels, such as inhibition of cyt c release induced by a variety of stimuli [18]. It has also been shown that Bcl-2 acts downstream of cyt c release [19].

Tumour necrosis factor-α (TNFα)

A few years ago we reported [20,21] that the apoptogenic protein TNFα causes ROS formation in mitochondria, which itself induces Ca2+ cycling and as a consequence thereof apoptosis. When TNFα was added to isolated mitochondria no ROS formation was observed, suggesting the operation of a mediator transducing the message from the plasma-membrane-receptor-bound TNFα to mitochondria. In 1997 evidence was provided [13,14] that the mediator was ceramide: first, challenge of cells by TNFα caused an increase in the level of ceramide in mitochondria; and secondly, when ceramides were added to isolated mitochondria, an increase in ROS production was observed, and it was suggested that ceramides act at or near the antimycin a-binding site of the respiratory chain [17].

Bcl-2 links oxidative stress, Ca2+ and the ΔΨm to apoptosis

Given that Bcl-2 elicits an anti-oxidative response in cells, what are the biochemical mechanism(s) by which Bcl-2 prevents apoptosis? We showed [20,21] that one mechanism is the prevention of ROS-induced mitochondrial Ca2+ cycling, a process which results in a collapse of ΔΨm and in cellular ATP depletion. Thus Bcl-2 prevents disturbances of the cellular Ca2+ homoeostasis and ROS production at the mitochondrial level. Based on these and other findings we suggested [22] that a pro-oxidant-induced Ca2+ release from mitochondria, followed by Ca2+ cycling and ATP depletion, is a common cause of apoptosis. Accordingly, maintenance of ΔΨm stabilizes mitochondria
and thereby prevents apoptosis. Bcl-2 thus provides the link between the antioxidant defence system, $\text{Ca}^{2+}$ and $\Delta \Psi_m$ (reviewed in [23]).

**Ceramides induce cyt c release from mitochondria**

We have recently investigated in detail the action of ceramides on mitochondria, using ceramides of different apoptotic potencies and isolated rat liver organelles [24]. We found that $N$-acetylphosphatidylcholine (C$_{2}$-ceramide), $N$-hexanoylsphingosine (C$_{6}$-ceramide) and, to a much lesser extent, C$_{2}$-dihydroceramide induce cyt c release from isolated rat liver mitochondria. A low concentration of Bcl-2 (40 nM) prevents cyt c release. This release takes place when cyt c is oxidized and/or in the absence of $\Delta \Psi_m$, but not when cyt c is reduced or when $\Delta \Psi_m$ is present. Upon cyt c loss, mitochondrial functions are altered: mitochondrial oxygen consumption, $\Delta \Psi_m$ and $\text{Ca}^{2+}$ retention are diminished. Incubation with Bcl-2 prevents, and addition of cyt c reverses, these malfunctions. In ATP-energized mitochondria ceramide does not alter $\Delta \Psi_m$, neither when cyt c is oxidized, nor when it is reduced, ruling out any non-specific disturbance by ceramide of mitochondrial membrane integrity. Affinity chromatography with D- and L-ceramide columns (courtesy of Dr. J. Brunner, Eidgenössische Technische Hochschule, Zürich) shows that cyt c binds to ceramide in a stereoselective manner. Also, titration of horse heart cyt c with ascorbate reveals a diminished reducibility of the protein in the presence of ceramide.

**Conclusion**

We conclude that some of the apoptogenic properties of ceramides are mediated via their interaction with cyt c followed by release of the cytochrome from mitochondria. Since this interrupts electron flow towards cytochrome oxidase the respiratory chain members upstream of cyt c should be more reduced and therefore should more readily form superoxide radicals. Cyt c release weakens mitochondria, as shown by $\Delta \Psi_m$ and calcium-retention measurements. It is presently not clear whether other mitochondrial proteins such as proteases are released along with cyt c, or whether release of cyt c triggers the subsequent release of such proteins. Interestingly, ceramides are much more effective in cyt c release when the protein is oxidized. This finding may indicate that oxidative stress is indeed pro-apoptogenic and not simply an epiphenomenon during apoptosis.

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Mitochondria, ceramide and apoptosis

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