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ORIGINAL ARTICLE

Regulation of ceramide channel formation and disassembly: Insights on the initiation of apoptosis

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Abstract Sphingolipid research has surged in the past two decades and has produced a wide variety of evidence supporting the role of this class of molecules in mediating cellular growth, differentiation, senescence, and apoptosis. Ceramides are a subgroup of sphingolipids (SLs) that are directly involved in the process of initiation of apoptosis. We, and others, have recently shown that ceramides are capable of the formation of protein-permeable channels in mitochondrial outer membranes under physiological conditions. These pores are indeed good candidates for the pathway of release of pro-apoptotic proteins from the mitochondrial intermembrane space (IMS) into the cytosol to initiate intrinsic apoptosis. Here, we review recent findings on the regulation of ceramide channel formation and disassembly, highlighting possible implications on the initiation of the intrinsic apoptotic pathway.

1. Introduction

Whether a cell proliferates or perishes requires a complex set of cellular decisions that depend on the environment and its physical and nutritional states. Signaling in cells is regulated optimally through a wide array of macromolecules and messengers that control the fate of the cell creating many intertwined networks of regulators and effectors. Until recently, SLs were merely considered structural components of cellular membranes. We now know of a plethora of functions these molecules perform in cell signaling, stress and death (Hannun, 1996; Saba et al., 1996; Chalfant et al., 2001; Jenkins and Hannun, 2001). Multiple novel agents that modulate SL metabolism have been studied and at least in one instance applied therapeutically for cancer treatment (Adan-Gokbulut et al., 2013). Ceramide (Cer), is responsible for several intracellular signals and is considered the parent SL

Abbreviations: Bcl-2, B cell CLL/lymphoma-2; Cer, ceramide; CerS, ceramide synthase; DES, dihydroceramide desaturase; DHCer, dihydroceramide; ER, endoplasmic reticulum; IMS, intermembrane space; KSR, 3-ketosphinganine reductase; MOMP, mitochondrial outer membrane permeability; SLs, sphingolipids; So, sphingosine; SM, sphingomyelin; SPT, serine palmitoyl transferase

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molecule (Hannun, 1996; Jayadev and Hannun, 1996; Perry et al., 1996; Lee et al., 1996).

Ceramides are a family of lipids with a sphingosine (So) backbone *N*-acylated with a variety of fatty acids, creating a diverse group of molecules (Fig. 1). They are involved in cell signaling in various contexts (Hannun, 1996; Saba et al., 1996; Jayadev and Hannun, 1996; Lee et al., 1996; Hayakawa et al., 1996; Yoon et al., 2002; Shin et al., 2002; Obeid et al., 1993). In this review we focus on one aspect of Cer cell signaling: the regulation of cell death by Cer channel formation in the outer membranes of mitochondria (Siskind et al., 2002). In addition to this pathway of protein release, there are many proposed pathways to explain how mitochondrial IMS proteins are discharged from mitochondria: the opening of the permeability transition pore (Crompton, 1999; Crompton et al., 1999); the oligomerization of Bax monomers to achieve channel activity (Antonsson, 2001; Antonsson et al., 2000, 2001; Saito et al., 2000); the opening of the mitochondrial apoptosis-induced channels (Pavlov et al., 2001); the interactions of BH3/Bax/cardiolipin (Kuwana et al., 2002), and the interactions between Bax and Cer (Belaud-Rotureau et al., 2000). Regardless of the pathway, it is clear that Bcl-2 (B cell CLL/lymphoma) family proteins play a central role in the regulation of mitochondrial permeability. Here, we review the effects of these proteins, and other metabolites, on Cer channel formation and disassembly highlighting possible implications on the onset of intrinsic apoptosis.

2. Ceramide

Cer is a condensation product of the amino alcohol So and a fatty acid in an acylation reaction. The range of acylation is wide, creating ceramides that contain fatty acids varying from 6 to 34 or even more carbons. D-erythro-N-palmitoyl-sphingosine (C_{16}-Cer) is an example of one of the naturally occurring forms of Cer (Fig. 1). Another key aspect of the Cer molecule is the presence of a 4,5-*trans* double bond that clearly has a profound impact on the biophysical characteristics of Cer and on cell survival pathways, as will be presented later.

Of the various roles of Cer inside cells, the ability to induce apoptosis is the clearest. Ceramides have been shown to induce apoptosis directly and indirectly (Saba et al., 1996; Obeid et al., 1993; Linardic et al., 1996; Danial and Korsmeyer, 2004; Wiesner and Dawson, 1996a,b). MCF7 breast cancer cells experienced mitochondrial outer membrane permeabilization and apoptosis when bacterial sphingomyelinase (a Cer-generating enzyme) was targeted to mitochondria and Cer was generated specifically in mitochondria (Birbes et al., 2001). When bacterial sphingomyelinase was targeted to other organelles, apoptosis and mitochondrial permeabilization did not occur (Hannun et al., 2001). In leukemia cells, Cer levels were increased significantly upon the addition of the chemotherapeutic agent vincristine resulting in growth suppression and marked apoptosis (Zhang et al., 1996).

The mechanisms by which Cer causes mitochondrial outer membrane permeabilization which results in apoptosis are diverse (reviewed in Siskind (2005)). Remarkably, Cer is able to permeabilize mitochondrial outer membranes through the formation of channels that are large enough to allow the egress

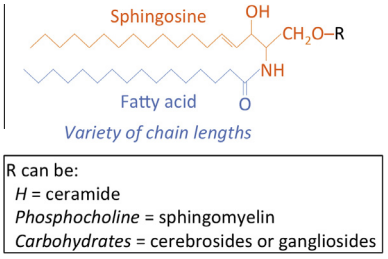

Figure 1 The basic structure of sphingolipids. SLs have a So backbone (orange) that is *N*-acylated with fatty acids of a variety of chain lengths (blue). The C-1 hydroxyl can be as simple as a hydrogen atom (in ceramides) or as complex as multiple carbohydrate subunits in cerebrosides and gangliosides. The *trans* double bond at C-4 is characteristic of Cer and when it is saturated the molecule is DHCer.

of IMS proteins into the cytosol (Siskind et al., 2002, 2006; Stiban et al., 2008). Thus, channel formation by Cer is an upstream event to the induction of apoptosis (reviewed in Colombini (2010)). The permeability of the mitochondrial outer membrane to proteins including cytochrome *c* can be increased by the incubation of the isolated mitochondria with ceramide in a time- and dose-dependent manner (Siskind et al., 2002). This was the first indication that a lipid can form pores in a biological membrane. Different groups observed similar effects of ceramide channel formation in protein-free systems (Siskind and Colombini, 2000; Montes et al., 2002; Pajewski et al., 2005; Stiban et al., 2006). Increasing evidence (topped by the visualization of the channels by transmission electron microscopy (Samanta et al., 2011) demonstrated that a channel formed by a lipid is possible and valid (Stiban et al., 2008; Siskind et al., 2003, 2005; Ganesan et al., 2010; Siskind et al., 2008). Since it is inherently different than a protein channel, Cer channel formation depends on the steady state level of Cer in the membrane. Thus, the formation of Cer channels is controlled mainly by the metabolism of Cer in the membrane.

3. Ceramide biosynthesis

Ceramides are central molecules in sphingolipid synthesis. *In vivo*, there are three pathways that lead to the generation of Cer (Fig. 2). The *de novo* Cer synthesis pathway starts in the endoplasmic reticulum (ER) with the condensation of palmitoyl-CoA with serine to form 3-ketosphinganine catalyzed by serine palmitoyl transferase (SPT). The ensuing product is then reduced by 3-ketosphinganine reductase (KSR) to sphinganine which is acylated by a family of Cer synthases (CerS) generating dihydroceramides (DHCer) with varying fatty acyl chain lengths. In the final step of this pathway DHCer desaturase (DES) facilitates the formation of ceramide inserting a double bond between C4 and C5 of the sphingoid base. A variety of evidence suggests that this pathway occurs in the endoplasmic reticulum (Hirschberg et al., 1993) however, some enzymes were localized in mitochondria of some cell types (Yu et al., 2007; Novgorodov et al., 2011) but not others (Stiban et al., 2008; Mesicek et al., 2010), indicating that this localization might be cell-type specific. Even though under normal conditions liver mitochondria were shown to be devoid of DES activity (Stiban et al., 2008), *N*-myristoylation targeted the