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J Bioenerg Biomembr. Author manuscript; available in PMC 2017 May 17.

Published in final edited form as:

J Bioenerg Biomembr. 2016 April ; 48(2): 153–168. doi:10.1007/s10863-015-9602-3.

Sphingolipids and mitochondrial apoptosis

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Abstract

The sphingolipid family of lipids modulate several cellular processes, including proliferation, cell cycle regulation, inflammatory signaling pathways, and cell death. Several members of the sphingolipid pathway have opposing functions and thus imbalances in sphingolipid metabolism result in deregulated cellular processes, which cause or contribute to diseases and disorders in humans. A key cellular process regulated by sphingolipids is apoptosis, or programmed cell death. Sphingolipids play an important role in both extrinsic and intrinsic apoptotic pathways depending on the stimuli, cell type and cellular response to the stress. During mitochondrial-mediated apoptosis, multiple pathways converge on mitochondria and induce mitochondrial outer membrane permeabilization (MOMP). MOMP results in the release of intermembrane space proteins such as cytochrome c and Apaf1 into the cytosol where they activate the caspases and DNases that execute cell death. The precise molecular components of the pore(s) responsible for MOMP are unknown, but sphingolipids are thought to play a role. Here, we review evidence for a role of sphingolipids in the induction of mitochondrial-mediated apoptosis with a focus on potential underlying molecular mechanisms by which altered sphingolipid metabolism indirectly or directly induce MOMP. Data available on these mechanisms is reviewed, and the focus and limitations of previous and current studies are discussed to present important unanswered questions and potential future directions.

Keywords

Sphingolipid; mitochondria; apoptosis; ceramide; cancer; Bcl-2 proteins

Sphingolipids, sterols and glycerolipids are three classes of lipids that constitute the majority of eukaryotic cell membranes. Sphingolipids were initially discovered in brain extract in 1870 and their nomenclature was established by J.L.W. Thudichum in 1884 (Blaschko 1975; Hawthorne 1975; McIlwain 1975). Their structure, metabolism and cellular functions have been extensively investigated and reported. Sphingolipids provide structural integrity to cell membranes, and modulate numerous important cellular processes including apoptosis, proliferation, endocytosis, transport, migration, senescence, and inflammation through signal transduction and gene regulation (Patwardhan and Liu 2011). The function of sphingolipids

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depends largely on their level, the cell type, and the subcellular location in which they are generated. Imbalances in sphingolipid levels result in aberrant cellular processes, which contribute to the onset and progression of various diseases such as COPD, Gaucher disease, Alzheimer's disease, inflammatory bowel disease, autoimmune diseases, chronic heart failure, asthma, diabetes, sepsis, and cancer (Baranowski and Gorski 2011; Kolter 2011; Russo et al. 2013; van Echten-Deckert and Walter 2012; Yang and Uhlig 2011). Such disease states may be triggered by deviant intracellular apoptotic signaling regulated by alterations in the levels of individual sphingolipids.

Sphingolipid family members generally contain a common sphingoid base backbone. Structural and functional diversity of sphingolipids is introduced through alterations of the backbone, including the introduction of a double bond(s), differing length (sphingoid base backbones of 16 and 18 carbons are possible), addition of a N-linked fatty acyl chain, and additions to the terminal hydroxyl group. The length of the N-linked fatty acyl chain varies as well as its degree of saturation and hydroxylation. Groups that can be added to the C1-hydroxyl group include a phosphate, carbohydrates, and phosphorylcholine moieties, giving rise to thousands of different sphingolipid species ((Hannun and Obeid 2008); Fig. 1).

Sphingolipid Metabolism

Sphingolipid metabolism (Fig. 2) centers on ceramide, which can be generated in cells by sphingomyelin (SM) hydrolysis, *de novo* synthesis, the salvage pathway, or from breakdown of more complex sphingolipids (Fig 2). Sphingomyelinases (SMases) catalyze the hydrolysis of SM to form ceramide and phosphocholine and are classified by their pH optima. The acid and Mg^{+2}-dependent neutral sphingomyelinases have been implicated in stress-induced ceramide generation. There is also a secretory form of acid sphingomyelinase that has been implicated in stress responses (Jenkins et al. 2009). *De novo* ceramide synthesis occurs in the ER. The first step in *de novo* synthesis is catalyzed by serine palmitoyl transferase (SPT) and involves the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine, which is then reduced to form sphinganine. Ceramide synthases catalyze the N-acylation of sphinganine to form dihydroceramide (DH). A dihydroceramide desaturase catalyzes the conversion of dihydroceramide to ceramide through the insertion of a *trans* double bond at the 4-5 position of the sphingoid base backbone.

Ceramide synthases catalyze the formation of ceramide in two distinct pathways: *de novo* synthesis and the recycling/ salvage pathway. In mammals, there are 6 ceramide synthases (CerS1-6) that have preferential activity for adding fatty acyl CoAs of particular chain lengths. The different CerS are expressed in a cell-type specific manner and their expression changes during development (Mullen et al. 2012). CerS activity is regulated at least in part by homo- and heterodimerization between the different CerS family members (Laviad et al. 2012). For instance, catalytic activity of CerS2 depends on dimer formation between CerS2 and CerS5 (Laviad et al. 2012). *In vitro* data shows that CerS2 activity is lower by itself compared to other CerS (15 μM V/S 12 μM Km for sphinganine), but increases significantly when co-expressed with other CerS or expressed as a constitutive dimer (Laviad et al. 2012). Data indicates that in addition to homo- and heterodimerization, the specific CerS are regulated at the post-translational level by other mechanisms. For example, a variety of

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cellular stresses induce proteosomal processing of CerS1 thereby reducing its activity and inducing its translocation from the ER to the Golgi (Sridevi et al. 2010; Sridevi et al. 2009). This may be one mechanism by which cells have evolved to survive particular cellular stresses. Recent data implicates the involvement of proteins in the Bcl-2 family in regulating the activity of specific CerS at the post-translational level during cell death (Beverly et al. 2013; Jensen et al. 2014; Siskind et al. 2010). Thus, CerS activity in cells is determined by many factors such as availability of other CerS for dimerization, allosteric binding of substrate to monomer or dimer, differential regulation by the different transcript variants, proteolytic processing, translocation, and post-translational regulation by proteins in the Bcl-2 family. Ascribing distinct cellular functions to the individual CerS is at present not well established (Park et al. 2014) at least in part because of a lack of specific inhibitors against each of the individual CerS, but data suggest that individual CerS have distinct roles in regulating cellular processes. Studies utilizing CerS knockout mice (single and double knockout mice) are beginning to become available and are uncovering specific pathologies associated with the deletion of specific CerS (Ebel et al. 2014; Ebel et al. 2013; Ginkel et al. 2012; Imgrund et al. 2009; Jennemann et al. 2012; Park et al. 2013b; Petrache et al. 2013; Pewzner-Jung et al. 2010a; Pewzner-Jung et al. 2010b; Zigdon et al. 2013). The interpretation of data from knockout mice requires one to keep in mind the potential compensatory metabolic pathways that may result from perturbing one arm of sphingolipid metabolism (Park et al. 2013a; Park et al. 2013b; Petrache et al. 2013; Pewzner-Jung et al. 2010a; Pewzner-Jung et al. 2010b; Silva et al. 2012). For example, data in cell culture studies indicate that siRNA mediated knockdown of a single CerS, induces a compensatory increase in the expression of other CerS-family members (Mullen et al. 2011). In addition, some CerS may be coupled to specific downstream metabolic arms of ceramide metabolism whereas other CerS may not be coupled to these same processes.

Glycosphingolipids are a very heterogeneous class of lipids, varying in both the hydrophilic and hydrophobic region of the molecule. The hydrophilic portion of glycosphingolipids consists of a sugar headgroup and can contain neutral or charged groups. The hydrophobic portion can vary in the length of the *N*-linked fatty acyl chain and sphingoid base as well as the degree of saturation, hydroxylation, and branching. In general, glycosphingolipids are divided into two main classes based on the first sugar moiety linked to the ceramide backbone, namely galactose or glucose to form either galactosylceramide (GalCer) or glucosylceramide (GluCer), respectively (collectively known as hexosylceramides). GalCer is formed in the ER via the enzyme UDP-Gal:ceramide galactosyltransferase or GalCer synthase (CGalT) via the transfer of galactose from UDP-galactose to ceramide. GalCer is then transported to the Golgi where sulfatide or galactose groups can be added.

Glucosylceramides are generated from ceramide synthesized in the ER that are transported to the Golgi where UDP-Glc:ceramide glucosyltransferase (glucosylceramide synthase) catalyzes the addition of glucose onto ceramide from an activated nucleotide precursor (UDP-glucose). In mammals, a galactose group is then added from UDP-galactose to generate lactosylceramide, a reaction catalyzed by lactosylceramide synthases. Several sequential modifications to lactosylceramides can then occur to give rise to the more complex gangliosides, lactosylsulfatides, globotriosylceramides, and fucosylactosylceramides. Thus, LacCer is essential to the formation of complex

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glycosphingolipids. Gangliosides are a class of complex glycosphingolipids that arise from the addition of sialic acid residues to lactosylceramide and are highly abundant in the kidney. Degradation of complex glycosphingolipids leads to the accumulation of “simple” glycosphingolipids such as lactosylceramide and glucosylceramide. This degradation occurs mainly in the lysosome, but is known to occur at the plasma membrane and mitochondrion as well.

Ceramide synthesized in the ER can be transported to the Golgi for synthesis of sphingomyelin (SM). The ceramide transport protein CERT is located in the cytosol and interacts with both the ER and Golgi to extract ceramide from the ER and deliver it to the Golgi for synthesis of SM (Hanada 2006; Hanada et al. 2007; Kawano et al. 2006). CERT function is essential for embryogenesis, specifically for proper heart development (Wang et al. 2009). Interestingly, deletion of CERT in primary mouse embryonic fibroblasts does not results in the accumulation of ceramide, but rather hexosylceramides in the ER and mitochondria, resulting in decreased retrograde transport of plasma membrane proteins to the Golgi, increased levels of ER stress, mitochondrial dysfunction, increased levels of mitophagy, and eventual cell senescence (Rao et al. 2014). These data indicate the key role that CERT plays in maintaining balanced sphingolipid metabolism as well as the toxic impact when it becomes unbalanced.

Ceramidases catalyze the breakdown of ceramide via the removal of the *N*-linked fatty acyl chain to form sphingosine. Three different types of mammalian ceramidases have been identified. Acid ceramidases are lysosomal (Ferlinz et al. 2001; Park and Schuchman 2006), neutral ceramidases (Hwang et al. 2005; Ito et al. 2014; Tani et al. 2005) are localized to the plasma membrane, mitochondria, and nuclear membranes, and alkaline ceramidases reside in the Golgi and ER (Mao et al. 2001; Mao et al. 2003; Sun et al. 2010). Neutral/alkaline ceramidase has also been shown to catalyze the reverse reaction to generate ceramide from sphingosine and fatty acids (El Bawab et al. 2001; Novgorodov et al. 2011; Tani et al. 2000). Mitochondria have been shown to be capable of generating ceramide via the action of reverse ceramidase activity (Bionda et al. 2004; El Bawab et al. 2000; Novgorodov et al. 2011) and mitochondria isolated from the livers of neutral ceramidase deficient mice have significantly decreased generation of ceramide from sphingosine and palmitoyl-CoA or palmitate; a mitochondrial thioesterase has been shown to hydrolyze palmitoyl-CoA to palmitate for reverse synthesis of ceramide within the mitochondria by a neutral ceramidase (Novgorodov et al. 2011).

Sphingosine generation, *via* ceramidase catalyzed hydrolysis of ceramide, can also be used to generate sphingosine-1-phosphase (S1P). Phosphorylation of sphingosine is catalyzed by sphingosine kinase (SK) (Heffernan-Stroud and Obeid 2013; Lima and Spiegel 2013; Pitson 2011; Pyne et al. 2012; Siow et al. 2011; Snider 2013; Wattenberg 2010). There are two sphingosine kinases, namely SK1 and SK2. SK1 is thought to be cytosolic and can translocate to the plasma membrane, where it generates S1P that is secreted outside the cell where it can act as a ligand for certain G-coupled protein S1P receptors (Pitson 2011; Pitson et al. 2005; Wattenberg 2010). The subcellular location of SK2 is highly debated, but data suggests that it could be localized to the ER, mitochondria, and nuclei (Antoon et al. 2011; Don and Rosen 2009; Hait et al. 2007; Igarashi et al. 2003; Liu et al. 2000; Liu et al. 2003;

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Weigert et al. 2010). S1P has been shown to be protective/ anti-apoptotic, although data suggests that S1P may also exert a pro-apoptotic role under certain circumstances (Gandy and Obeid 2013; Heffernan-Stroud and Obeid 2013; Pitson 2011; Zhang et al. 2014). The subcellular localization in which S1P is generated and the ratio of S1P to ceramide/ sphingosine most likely dictate whether S1P exerts a pro- or anti-apoptotic function. S1P is further irreversibly metabolized to hexadecenal and phosphoethanolamine by S1P lyase (S1PL). S1PL is localized in ER and controls S1P signaling (Ikeda et al. 2004). S1P can also be dephosphorylated to regenerate sphingosine by lipid phosphate phosphatases or S1P phosphatase (SPP). SPPs are lipid phosphohydrolases that are predominantly localized in the ER; two SPPs, SPP1 and SPP2 have been identified that demonstrated high sphingolipid species specificity. Both S1PL and SPPs are implicated in cancer underscoring their role in cell death.

Mitochondrial Apoptosis

There are two main pathways for apoptosis, namely the extrinsic receptor-mediated pathway and an intrinsic mitochondrial pathway. The extrinsic pathway of apoptosis involves the binding of a death ligand (for example tumor necrosis factor-α, TNFα) to its receptor (for example, TNF receptor-1) on the external surface of the plasma membrane, which leads to the formation of a death-inducing signaling complex (DISC). The DISC recruits multiple effectors and these are responsible for the execution of the cell. Sphingolipids have been implicated as being key regulators in the extrinsic pathway of apoptosis (Bollinger et al. 2005; Grassme et al. 2003; Grassme et al. 2001a; Grassme et al. 2001b; Gulbins and Kolesnick 2003; Heinrich et al. 2000; Schneider-Brachert et al. 2004). This data will not be discussed here as the current review focuses on the role of sphingolipids specifically in the intrinsic pathway of apoptosis that is mediated by mitochondria.

Mitochondria play a major role in the intrinsic apoptotic pathway whereby stress signals converge on mitochondria to induce mitochondrial outer membrane permeabilization (MOMP). MOMP allows the release of proteins from the intermembrane space of mitochondria to the cytosol where they activate the caspase cascade. Upon activation of caspases, cells display several features of apoptosis, including cleavage of PARP, externalization of plasma membrane phosphatidylserine, chromatin condensation and rapid cell death within minutes. Thus, MOMP is thought to be an irreversible event that is tightly regulated (Galluzzi et al. 2012). MOMP is controlled largely by pro and anti-apoptotic members of Bcl-2 family (Chipuk et al. 2006; Chipuk and Green 2008; Chipuk et al. 2010). Both pro- and anti-apoptotic members in the Bcl-2 family share homology at one or more specific domains referred to as Bcl-2 homology domains (BH domains)(Chipuk et al. 2010). The anti-apoptotic proteins include Bcl-2, Bcl-xL, Mcl-1, Bfl1, Bcl-b and Bcl-w and their over-expression often rescues cells from cell death induced by several stimuli (Chipuk et al. 2010). The pro-apoptotic proteins are subdivided into two classes: the multi-domain proteins such as Bax and Bak, and BH3-only proteins like Bid, Bim, Bad, Puma, Noxa, and Bik (Chipuk et al. 2010). The Bcl-2 family can be regulated by p53, which transcriptionally activates proapoptotic proteins (Bax, PUMA) or directly binds and inhibits activity of anti-apoptotic proteins (Bcl-2) (Chipuk and Green 2004; Chipuk and Green 2006; Chipuk et al. 2004; Chipuk et al. 2003; Follis et al. 2013; Perfettini et al. 2004). The proteins in the Bcl-2

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family are present in various cellular compartments, including mitochondria, ER, cytosol, and nuclei (Annis et al. 2001; Malina and Hess 2004; Scorrano et al. 2003; Zong et al. 2003). Some Bcl-2 family members change their intracellular localization in response to apoptotic stimuli. For example, Bax is present as a soluble cytosolic protein and following an apoptotic signal it changes conformation, translocates to mitochondria, and forms a proteolipidic channel in the MOM to induce MOMP (Nomura et al. 1999; Wolter et al. 1997). Bak on the other hand is an integral membrane protein localized to the ER and the MOM. Bax and Bak form homo and hetero-oligomers with other Bcl-2 proteins to regulate MOMP. The interaction of Bax and/ or Bak and with anti-apoptotic Bcl-2 proteins inhibits their ability to induce MOMP, while interactions with particular pro-apoptotic BH3-only members promotes MOMP (Chipuk and Green 2008; Chipuk et al. 2010). Cells deficient in both Bax and Bak are extremely resistant to mitochondrial-mediated apoptosis and thus one of these proteins is thought to be necessary for MOMP to be induced (Cartron et al. 2003; Lindsten et al. 2000; Rathmell et al. 2002; Wei et al. 2001). The exact nature of the interactions between the various members of the Bcl-2 family and the manner in which these interactions regulate MOMP are still under intense investigation. It is clear, however, that MOMP is regulated by more than just Bcl-2 proteins. Data indicate that lipids, including sphingolipids, derived from ER and mitochondrial-associated membranes are also required for Bax and Bak activation and channel formation in the MOM (discussed below). Sphingolipids have numerous other effects on ER and mitochondria that may lead to MOMP induction. These potential mechanisms by which sphingolipids alter mitochondrial function and induce MOMP are discussed below.

Sphingolipids and mitochondrial apoptosis

Obeid *et al.* in their pioneering 1993 paper in *Science* illustrated for the first time that ceramide could induce apoptosis when present endogenously in tissues or when exogenously added to cells (Obeid et al. 1993). Countless studies have since validated this initial discovery and indicate that cellular ceramide levels increase early in apoptosis in a variety of cell types and in response to a multitude of apoptotic stimuli including TNFα (Garcia-Ruiz et al. 1997; Geilen et al. 1997), UV-irradiation (Charruyer et al. 2007; Chatterjee and Wu 2001; Deng et al. 2002; Siskind et al. 2010; Uchida et al. 2010; Yao et al. 2013), IL3 withdrawal (Siskind et al. 2010), ionizing radiation (Alphonse et al. 2013; Chmura et al. 1997; Deng et al. 2008; Haimovitz-Friedman et al. 1994; Lu and Wong 2004; Mesicek et al. 2010; Moeller et al. 2009; Vit and Rosselli 2003), serum withdrawal (Caricchio et al. 2002; Fernandez-Ayala et al. 2000), etoposide (Chen et al. 2006; Lin et al. 2005; Perry et al. 2000; Sawada et al. 2000), staurosporine (Wiesner and Dawson 1996), daunorubicin (Bettaieb et al. 1999; Bose et al. 1995; Chen et al. 2000; Come et al. 1999; Jaffrezou et al. 1996; Mansat et al. 1997), taxol (Charles et al. 2001), and cisplatin (Min et al. 2007; Noda et al. 2001; Siskind et al. 2010). Thus, ceramide generation appears to be a common cellular response to apoptosis-inducing agents. Increases in cellular ceramide levels have been shown to occur prior to the mitochondrial phase of apoptosis (Kroesen et al. 2001; Rodriguez-Lafrasse et al. 2001; Thomas et al. 1999) and to be accompanied by the release of proteins from the mitochondrial intermembrane space, increased mitochondrial reactive oxygen species (ROS) generation, and collapse in the mitochondrial inner membrane potential (Fig. 3) (Andrieu-Abadie et al. 2001; Gentil et al. 2003; Hearps et al. 2002; Lin et al. 2004). Interventions that

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suppress mitochondrial dysfunction also suppress ceramide-induced apoptosis, including bongkrekic acid (Lin et al. 2004; Stoica et al. 2003) cyclosporine A (Pacher and Hajnoczky 2001), Bcl-2 (Karasavvas et al. 1996; Zhang et al. 1996), and Bcl-xL (Wiesner et al. 1997). Thus, ceramide has been established as a *bona fide* pro-death molecule.

Metabolism of ceramide is one mechanism by which cells evade the apoptotic process. The enzyme glucosylceramide synthase (GCS) converts ceramide to glucosylceramide, which is utilized to generate downstream complex glycosphingolipids, including gangliosides. GCS not only enables cells to escape ceramide-induced apoptosis but also renders cells resistant to many of the aforementioned apoptotic stimuli. For example, Liu *et al.* showed that MCF-7 breast cancer cells with upregulated GCS are resistant to ceramide induced apoptosis when exposed to TNF-α and doxorubicin (Liu et al. 2008). Complex glycosphingolipids derived from ceramides (for example gangliosides) have been reported to increase cell growth. For example, GD1a increases EGFR dimerization and phosphorylation and increases activation of downstream EGFR signaling pathways, which results in cell proliferation (Li et al. 2001; Liu et al. 2004). Metabolites of ceramide such as S1P are thought to help cells evade ceramide-induced apoptosis through activation of ERK and other anti-apoptotic signaling pathways (Shida et al. 2008; Stevenson et al. 2011; Takabe and Spiegel 2014). However, S1P and its breakdown product hexadecanal have also been proposed to exert a pro-apoptotic role when generated in mitochondrial-associated membranes (MAMs) (Chipuk et al. 2012). S1P and hexadecanal were shown *in vitro* to activate the pro-apoptotic Bcl-2 proteins Bak and Bax, respectively (Chipuk et al. 2012). Thus, levels of a particular sphingolipid, changes in enzymes involved in sphingolipid metabolism, and behavior of these sphingolipids according to site of generation/metabolism (e.g. lipid rafts) determines whether cells will undergo apoptosis or proliferation.

There are several reports implicating ceramide in mitochondrial-induced apoptosis. Ceramide either directly participates in apoptosis by having direct effects on mitochondria to induce MOMP or activates proteins that regulate MOMP. In cells, elevated ceramide levels have been shown to modulate the function of a number of proteins involved in the apoptotic process, including ceramide-activated protein kinase (CAPK), ceramide-activated protein phosphatases PP1 and PP2A, protein kinase C-ζ (PKCζ), and the lysosomal protease cathepsin D (Hannun and Obeid 2008). Besides these indirect mechanisms by which sphingolipds could contribute to mitochondrial apoptosis, a plethora of data suggests that sphingolipids such as ceramide may directly alter mitochondrial function to induce apoptosis. Generation of ceramide within mitochondria via mitochondrial targeting of bacterial sphingomyelinase results in MOMP and apoptosis (Birbes et al. 2001; Birbes et al. 2002; Birbes et al. 2005). There are pools of ER and mitochondrial sphingolipids such as ceramide, sphingomyelin, and glycosphingolipids (see below) and data suggests and that when the levels of these sphingolipids are altered, mitochondrial function declines and mitochondrial-mediated apoptosis is induced. There are several ways by which sphingolipids contribute to apoptosis. Here, we will focus only on their impact on mitochondrial-mediated events.

Sphingolipid metabolism, mitochondria, ER, and Mitochondrial-associated Membranes—Mitochondria are highly dynamic and organized organelles that are closely

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associated with the ER through mitochondrial-associated membranes (MAMs) that tether the ER and mitochondria. It is estimated that 5-20% of the mitochondrial surface is in close contact with ER networks (Rizzuto et al. 1998) and the distance between ER and mitochondria at the MAM is estimated to be only 10-25 nm (Csordas et al. 2006). MAMs contain enzymes for lipid synthesis and proteins for lipid transfer (Csordas et al. 2006) and ER-mitochondrial cross talk is crucial for the regulation of apoptosis (Fig. 3) (Iwasawa et al. 2011; Pinton et al. 2011). The ER is a major site for the synthesis of sphingolipids (see above) and data indicates that sphingolipids generated in the ER can transfer to mitochondria (Stiban et al. 2008), possibly by way of vesicles or MAMs. In addition, sphingolipids are present in both MAMs and mitochondria (Bionda et al. 2004). Several enzymes in the sphingolipid metabolic pathway have been localized to mitochondria, including ceramide synthase, ceramidase, sphingosine kinases, and neuraminidase 4 (Bigi et al. 2010; Bionda et al. 2004; Novgorodov and Gudz 2009; Novgorodov et al. 2014; Novgorodov et al. 2011; Strub et al. 2011; Yamaguchi et al. 2005). Bionda *et al.*, confirmed the presence of ceramide synthase in ultrapure MAM-free mitochondria and showed that both mitochondrial outer and inner membranes are capable of ceramide synthesis (Bionda et al. 2004). Data indicate that the levels of MAM and possibly mitochondrial outer or inner membrane sphingolipids are altered during apoptosis (Babiychuk et al. 2011; Kujjo et al. 2013; Lee et al. 2011; Novgorodov and Gudz 2011; Novgorodov et al. 2011; Rego et al. 2012; Siskind 2005). Thus, sphingolipid metabolism can occur in and is altered during apoptosis in ER, MAMs, and/ or mitochondria during apoptosis. Functionally, sphingolipids have been documented to have numerous effects on mitochondrial function, permeabilization of the outer membrane and release of intermembrane space proteins. Further, sphingolipids can alter mitochondrial morphology, mitophagy, and mitochondrial respiration via impacting individual components of the mitochondrial electron transport chain. These data are discussed below (Fig. 3).

Alterations in ER function by sphingolipids that could play a role in mitochondrial-mediated apoptosis: *De novo* ceramide synthesis occurs in the ER (see above). Sphingolipids are also known to play a key role in ER Ca^{+2} homeostasis and ER stress responses, which also can play a role in mitochondrial-mediated apoptosis. For example, inhibition of neutral sphingomyelinase protected retinal epithelial cells from ER stress-induced apoptosis (Kucuksayan et al. 2014). In addition, knockdown of CERT in mouse embryonic fibroblasts induced ER stress that was linked to an accumulation of hexosylceramide, rather than ceramide (Rao et al. 2014). Interestingly, decreased CerS6 expression actually induced ER stress via induction of calcium release from the ER and activation of transcription factor 6 (ATF6) (Senkal et al. 2010; Senkal et al. 2011). Dihydroceramides have also been implicated in ER stress; treatment of cells with a dihydroceramide desaturase inhibitor induced ER stress as determined by induction of eIFalpha and Xbp1 (Gagliostro et al. 2012). These data suggest that dysregulated sphingolipid metabolism plays a role in ER stress responses. Since it is known that ER stress can induce MOMP and apoptosis via altering ER Ca^{+2} homeostasis, sphingolipids could indirectly induce MOMP via their effects on the ER (Fig. 3). Alternatively, sphingolipids generated in the ER could directly regulate mitochondrial events via transfer to mitochondrial membranes via MAMs or an unidentified transfer protein. Stiban *et al.* showed that ceramide generated in the ER and MAMs are rapidly

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transferred to the mitochondrial outer membrane in sufficient amounts to allow for the induction of MOMP (Stiban et al. 2008). Mesicek *et al.* showed that ionizing radiation of HeLa cells induces CerS activity in MAM, elevating ceramide levels eventually in MOM and inducing MOMP; these effects were inhibited by ceramide synthase inhibitor fumonisin B1 (FB_1)(Mesicek et al. 2010). Further, data indicate that CerS5 and CerS6 are indispensable for IR-induced CerS activity and apoptosis. Interestingly, Mesicek *et al.*, proposed that IR-induced CerS5 mediated C_{16}-ceramide is pro-apoptotic whereas CerS2 mediated $C_{24:1}$- and $C_{24:0}$-ceramides may be pro-survival (Mesicek et al. 2010). The ER-resident sphingomyelin synthase-related protein (SMSr, also known as sterile a-motif domain-containing protein; SAMD8) synthesizes the sphingomyelin analogue ceramide phosphoethanolamine (CPE); SMSr appears to act as a suppressor of ceramide-mediated cell death as its downregulation increases ceramides in the ER and in turn in mitochondria, triggering MOMP (Tafesse et al. 2014). Thus, dysregulated sphingolipid metabolism may contribute to MOMP and apoptosis indirectly via effects on the ER or directly via transfer to mitochondria or generation directly within mitochondria.

Impacts of sphingolipids on the mitochondrial outer membrane (MOM) that to could contribute to apoptosis: As discussed above mitochondrial outer membrane permeabilization (MOMP) plays a pivotal role in the induction of mitochondrial-mediated apoptosis. Sphingolipids have been proposed to play a key role in the regulation and/ or induction of MOMP. Data suggests that if sufficient quantities of ceramides accumulate within the MOM, then MOMP and apoptosis would result. There are two main mechanisms by which sphingolipids have been implicated to alter the MOM to induce MOMP, namely ceramide channel formation and the interaction with Bcl-2 proteins.

Ceramide channels: In 2000, Siskind and Colombini first reported that ceramides form channels in membranes as determined by their ability to induce vertical increments in current under voltage clamp conditions (Siskind and Colombini 2000). Since their initial discovery, ceramide channels have been extensively characterized (see reviews (Colombini 2010; Colombini 2013) for further information). The size of the ceramide channels was estimated to be large enough to allow the passage of proteins (Samanta et al. 2011; Siskind et al. 2003; Siskind et al. 2002). A typical ceramide channel is reported to have pore diameter approximately of 10 nm based on the molecular weight of proteins released from mitochondria, conductance of single channels and visualization of pores by electron microscopy (Samanta et al. 2011; Siskind et al. 2003; Siskind et al. 2002; Siskind et al. 2006). Ceramide channels are directly inhibited by anti-apoptotic Bcl-2 proteins (Siskind et al. 2008) and ceramides can form hybrid channels with the pro-apoptotic Bcl-2 protein Bax (Ganesan and Colombini 2010). While ceramides are capable of forming a channel that could allow for MOMP to occur, the evidence for their formation *in vivo* is still lacking. In an attempt to determine whether ceramides could induce MOMP *in vivo*, we utilized cells deficient in Bax and Bak as one of these proteins is required for the induction of MOMP *in vivo*. Our data indicated a much more complicated situation in that cells deficient in Bax and Bak were incapable of generating ceramides in response to apoptotic stimuli (Siskind et al. 2010). Data indicate that the protein Bak regulates ceramide synthases and thus is required for ceramide generation in mitochondrial-mediated apoptosis (Siskind et al. 2010). Activated Bak is more efficient at activating CerS (Beverly et al. 2013). In fact, ABT263, which

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directly activates Bax and/ or Bak by facilitating their release from anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-xL, induces CerS activation, ceramide generation, and apoptosis of cells, which is enhanced if the downstream metabolism of ceramide to glucosylceramide or S1P is inhibited (Beverly et al. 2013; Casson et al. 2013). Thus, data indicate a complicated system of cross-talk between sphingolipids and Bcl-2 proteins which is further discussed below.

Interaction with Bcl-2 proteins: Interaction of Bcl-2 proteins with sphingolipids is intriguing, given the redundancy and involvement of Bcl-2 family members in various stages and pathways of apoptosis. In general, *in vitro* data from studies utilizing isolated mitochondria or planar phospholipid membranes indicate that anti-apoptotic Bcl-2 family proteins disassemble ceramide channels whereas pro-apoptotic Bcl-2 family members directly enhance them (Ganesan and Colombini 2010; Ganesan et al. 2010; Perera et al. 2012). Ganeshan *et al.* demonstrated that activated Bax and ceramide act synergistically to permeabilize MOM and the amount of Bax required for MOMP was inversely proportional to amount of ceramide available (Ganesan et al. 2010). In fact, very small amounts of Bax facilitate the growth of ceramide channels, but once the optimal channel size is achieved, additional Bax ceases to affect the channel (Ganesan et al. 2010). Synergistic channel formation by ceramides and Bax is very exciting given that in cells, ceramide generation has been shown to be required for Bax activation in response to particular apoptotic stimuli (Belaud-Rotureau et al. 2000; Jin et al. 2008; Kim et al. 2001; Kim et al. 2008; Lee et al. 2011; Martinez-Abundis et al. 2009; von Haefen et al. 2002). Anti-apoptotic proteins in the Bcl-2 family inhibit both Bax and ceramide induced apoptosis. It is possible that anti-apoptotic Bcl-2 proteins bind to ceramide and Bax/ Bak to prevent their interaction and synergistic channel formation in the MOM (Fig. 3).

Interestingly, other sphingolipids can activate Bcl-2 family mediated MOMP, possibly independent of ceramide. Phillips *et al.* reported that sphingosine activates Bax causing its translocation to MOM promoting release of mitochondrial cytochrome c and Smac/Diablo, but not other mitochondrial proteins (Phillips et al. 2007). Whilst cytochrome c and Smac/ Diablo were released by sphingosine and Bax, there was no decrease in mitochondrial inner membrane potential or enhanced production of reactive oxygen species (Phillips et al. 2007). *Chipuk et al.,* presented a possibility that S1P and its metabolite hexadecenal activate Bak and Bax, respectively, depending on the cell death stimulus; data suggest that these sphingolipid products specifically cooperate with pro-apoptotic BH3-only proteins to coordinate MOMP and cytochrome c release (Chipuk et al. 2012). The levels of total cellular levels of S1P and hexadecenal generally exceedingly low, but local concentrations within or near mitochondria may be higher. Thus, future studies will need to determine if sufficient levels of mitochondrial S1P and hexadecanal are achieved in mitochondria during the early stages of apoptosis to induce Bak and Bax activation.

Impacts of sphingolipids on the mitochondrial inner membrane (MIM) that could contribute to apoptosis: The mitochondrial inner membrane contains two small electron carriers (cytochrome c and ubiquinol) and five multimeric protein complexes (complexes I-V) that produce an electrochemical proton gradient ($\Delta\Psi_m$) across the MIM necessary for complex V (F1F0-ATP synthase) to generate ATP in a process commonly referred to as oxidative

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phosphorylation. ATP is required for the activation of caspases and the execution phase of apoptosis. Thus, it is still highly debated whether the collapse in $\Delta\Psi_m$ is a cause or a consequence of MOMP and apoptosis. Disruption in the functioning of the complexes of the MIM electron transport chain can lead to the generation of free radicals and reactive oxygen species (ROS) as well as changes in $\Delta\Psi_m$. Generation of ROS is an important factor in mitochondrial-mediated apoptosis. Numerous data implicate sphingolipids as playing a role in changes in $\Delta\Psi_m$ or mitochondrial ROS generation. Early *in vitro* data utilizing isolated mitochondria or whole cells treated with sphingolipids shed light on potential *in vivo* effects of sphingolipids. Gudz *et al.* showed that ceramide addition to isolated mitochondria inhibits complex III (Gudz et al. 1997). Garcia-Ruiz *et al.* illustrated that TNF treatment of cells increased mitochondrial ceramide levels, which was upstream of induced mitochondrial ROS production (Garcia-Ruiz et al. 1997). The involvement of ceramide in mitochondrial apoptosis through ROS is bolstered by studies by Quillet *et al.* that demonstrated that in human myeloid leukemia U937 cells, addition of the short chain ceramide analogue C_6–ceramide (but not dihydroceramide) rapidly induced H_2O_2 at the ubiquinone step of the mitochondrial respiratory chain, resulting in apoptosis (Quillet-Mary et al. 1997). Park *et al.* showed that in mesenchymal stem cells derived from human adipose tissue (hASCs), ceramide generates ROS and disrupts mitochondrial potential ($\Delta\Psi_m$), and the effect was inhibited by addition of antioxidant NAC (Park et al. 2011). This is an important finding as mesenchymal stem cells (MSCs) are being examined as a therapy for regenerative medicines and tissue engineering. Although MSCs have shown some promise in pre-clinical and early clinical trials, apoptosis of MSCs may hamper the repair of the tissue. Other sphingolipids have been implicated in ROS production. For example, sphingosine induced a similar effect as ceramide, although much higher concentrations were required. Further, there are reports that sphingosine induces mitochondrial dysfunction via inhibition of complex IV (cytochrome c oxidase) of the electron transport chain (Abrahan et al. 2010; Novgorodov et al. 2014). Gorria *et al.* demonstrated that exogenously added monosialoganglioside GM1 protects rat hepatic F258 epithelial cells from benzo[a]pyrene (B[a]P) induced apoptosis by preventing the collapse in $\Delta\Psi_m$ and mitochondrial ROS production . Interestingly, GM1 does not affect activation of the p53 pathway by B[a]P, indicating that it prevents apoptosis at the mitochondrial level (Gorria et al. 2006). *In vitro* effects of sphingolipids on the function of the MIM are bolstered by several *in vivo* studies. First, Fabry disease, which is characterized by altered function of components of the electron transport chain, is cause by a defect in α-galactosidase leading to an accumulation of specific species of glycosphingolipids (Lucke et al. 2004). Second, mice deficient in ceramide synthase 2 have an altered sphingolipid profile in liver mitochondria, concomitant with reduced complex IV activity and progressive hepatopathy (Pewzner-Jung et al. ; Pewzner-Jung et al. ; Zigdon et al. 2013). A third example comes from mice deficient for the ceramide-ER transport protein (CERT) where hexosylceramide accumulates in mitochondria that have defects in oxygen consumption, resulting in degeneration of mitochondria, and embryonic lethality (Kujjo et al. 2013). *In vitro* data from studies utilizing isolated mitochondria and whole cells grown in culture when combined with *in vivo* studies in humans and mice provide powerful evidence for a role of sphingolipids in altered functions of the MIM. These changes in the structure and function of the MIM may play a pivotal role in the ability of sphingolipids to induce apoptosis. For additional details of the effects of sphingolipids on the mitochondrial electron

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transport chain and ROS generation see an extensive review written by Kogot-Levin and Saada (Kogot-Levin and Saada 2014).

Mitochondrial lipid rafts and mitochondrial morphology: Sphingolipids are thought to be a major component of lipid rafts. In plasma membranes, lipid rafts play a role in receptor clustering to facilitate signaling cascades. Lipids rafts containing glycosphingolipid enriched microdomains (GEMs), have been found in the mitochondrial outer membrane. In normal rat hepatocytes, GD3 is primarily present in plasma membrane. In response to TNF-α exposure, GD3 undergoes rapid redistribution depleting its levels in the plasma membrane and instead accumulating in mitochondrial domains through actin dependent vesicle trafficking (Garcia-Ruiz et al. 2002). Similar results were seen in human colon carcinoma cells HT-29 (Garcia-Ruiz et al. 2002). GD3 accumulation selectively in mitochondrial domains is derived by decrease in Glutathione (GSH) levels in mitochondria; low levels of GSH themselves does not lead to decrease in mitochondrial apoptosis, but attracts GD3 to mitochondria making it a two way process (Garcia-Ruiz et al. 2002). These domains are also home to mitochondrial fission proteins (hFis1, DLP1) and pro-apoptotic Bcl-2 family proteins (Bid, Bax). Assembly of these proteins together in lipid microdomains may promote mitochondrial fission and apoptosis. Ciarlo *et al.* observed that upon anti CD95/Fas stimulation of HeLa cells there was significant co-localization of fission protein DLP1 in GD3 enriched microdomains in mitochondria undergoing fragmentation, as compared to control cells (Ciarlo et al. 2010). Similar results were seen for hFis1 and GD3 in MOM, emphasizing the fact that fission proteins may selectively be recruited to GD3 enriched domains (Ciarlo et al. 2010). Disruption of these rafts by fumonisin-B1 and PDMP led to reduced recruitment of fission proteins to microdomains, decreased mitochondrial fission, and inhibition of apoptosis (Ciarlo et al. 2010; Garofalo et al. 2007; Martinez-Abundis et al. 2009). Gangliosides such as GD3 are broken down by the action of sialidases (neuraminidases, Neu) that catalyze the removal of the sialic acid residues. Interestingly, neuraminidase 4 is localized to mitochondria (Bigi et al. 2010; Yamaguchi et al. 2005). Thus, a system exists to decrease ganglioside levels directly in mitochondria and potentially regulate mitochondrial morphology and apoptosis.

In addition to gangliosides, ceramides have been reported to alter mitochondrial morphology, which may play a role in their ability to induce MOMP. Ceramides may either directly or indirectly manipulate mitochondrial morphology by altering expression of mitochondrial fusion and fission proteins. C_2-ceramide (a short-chain cell permeable ceramide analogue) treatment in neonatal rat cardiomyocytes led to increased mitochondrial fission proteins Drp-1 and Fis-1 in mitochondrial fractions, mitochondrial fragmentation and apoptosis (Parra et al. 2008). Recently, Smith *et al.* demonstrated that C_2-ceramide increased expression of Drp-1 and induced ROS generation in C2C12 cells (Smith et al. 2013). Galectin-1 (Gal-1) induces fission via increased expression of Drp-1 and Fis-1; data indicate that Gal-1 regulates induction of mitochondrial fission requires generation of ceramide (Matarrese et al. 2005). Alternatively, data indicate that ceramide may also regulate mitochondrial fusion through influencing expression of mitochondrial fusion protein Mfn2 (Rojas-Charry et al. 2014). Lastly, sphingolipids such as ceramides are known to alter membrane curvature, which plays a role in fission and fusion events (Holopainen et al. 2000; Rogasevskaia and Coorssen 2006; Silva et al. 2012; Sonnino et al. 2007; Sot et al. 2005).

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Thus, sphingolipids alter mitochondrial morphology via direct biophysical effects on mitochondrial outer and inner membranes, in addition to direct regulation of known mitochondrial fission and fusion proteins.

Future directions/challenges—Apoptosis is a vital process in normal development and homeostasis. Manipulating or targeting apoptotic pathways is a cornerstone for prospective therapy of many diseases. Sphingolipids form an essential and integral part of cell system influencing apoptosis, potentially through induction of MOMP. There are still several unanswered questions regarding mechanisms of sphingolipids mediated mitochondrial apoptosis. The first and foremost question pertains to the formation of ceramide channels *in vivo*. While it is clear that ceramides do indeed form channels (Colombini 2010), there is lack of solid evidence to show ceramide channel formation *in vivo*, due to a lack of adequate analytical methods. *In vitro* work with isolated mitochondria and electrophysiological studies of ceramide channels in planar phospholipid membranes indicate that these channels have the required characteristics to facilitate MOMP (reviewed (Colombini 2010)). However, the immediate ceramide metabolites dihydroceramide and sphingosine as well as anti-apoptotic Bcl-2 proteins inhibit ceramide channels (Elrick et al. 2006; Ganesan and Colombini 2010). Thus, cells appear to have evolved a variety of mechanisms to ensure that channel formation by ceramides is prevented.

The mechanism of transport between the ER and mitochondria is unknown. Ceramide is transported from the ER to the Golgi and plasma membrane by vesicular transport and via transport proteins. It is not known if such a system exists for transport to mitochondria. In the context of mitochondrial ceramide levels, the location (ER or mitochondria) in which the specific ceramides are generated may not be as important as we think if a mechanism for rapid transport of ceramide between ER and mitochondria exists. Recently, a mitochondrial associated neutral sphingomyelinase (manSMase) was identified (Wu et al. 2010). In some cell types, this enzyme showed localization at the periphery of mitochondria (consistent with MAMs), whereas in other cell types it co-localized with mitochondria (Wu et al. 2010). A role for this enzyme in apoptosis is unknown and requires further study.

The presence of sphingolipid metabolism enzymes in the vicinity of mitochondria certainly hints at *in situ* generation of ceramide within mitochondria, and raises the question of regulation of these enzymes. It would be interesting to determine if enzymes like manSMase and nCDase are localized to specific mitochondrial subcompartments that may define their mechanism of apoptosis. This would be very interesting as elevated ceramide levels specifically in the MOM could lead to formation of pathways for release of intermembrane space proteins, whereas increases in ceramide in the MIM would result in reduction of mitochondrial inner membrane potential and generation of ROS. Data indicate that ceramides do not form channels in the MIM, nor do they from channels in the plasma membrane (Siskind et al. 2002). Determining the factors that influence the propensity for ceramide to form channels within specific membrane environments will be important for understanding the physiological significance of ceramide channel formation with regards to apoptosis. Thus, the submitochondrial location of ceramide generation/accumulation is an important determinant of the impact of ceramides on mitochondrial function.

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Of particular importance for future studies is elucidation of the complex system of cross talk between sphingolipids and proteins within the Bcl-2 family. Bcl-2 and Bcl-xL involvement in ceramide-induced MOMP has been well deduced by several studies (Geley et al. 1997; Wiesner et al. 1997; Zhang et al. 1996). For instance, Chen *et al.* in 1995, showed that IR exposure in HL-60 and U-937 human leukemia cells suppresses Bcl-2 levels and induces ceramide mediated apoptosis (Chen et al. 1995). Siskind *et al.* demonstrated that Bcl-2 actually dissembles ceramide channels in isolated rat mitochondria and mitochondria isolated from yeast lacking Bcl-2 proteins (Siskind et al. 2008). Ganeshan *et al.* showed that ceramide and Bax synergistically lead to MOMP (Ganesan and Colombini 2010; Ganesan et al. 2010). It is possible that Bax-ceramide hybrid channels are capable of inducing MOMP at levels far below that required for either factor alone. Data suggests that the individual Bcl-2 family members may have distinct mechanisms in regulating ceramide-induced apoptosis. Recently, data suggests that members of the Bcl-2 family also regulate enzymes involved in ceramide generation during apoptosis. The Bcl-2 family protein Bcl-2L13 inhibits CerS activity and prevents ceramide-induced apoptosis (Jensen et al. 2014). In glioblastoma cells and other solid tumors, Bcl-2L13 is over-expressed and contributes to drug resistance. Suppressing Bcl-2L13 expression or developing drugs to interfere with its inhibition of CerS2 and CerS6 could help sensitize glioblastomas to conventional therapies. The pro-apoptotic Bcl-2 family member Bak activates CerS during apoptosis (Beverly et al. 2013; Siskind et al. 2010). Again, understanding the specific mechanism for this activation will facilitate the development of drugs that can interfere with or mimic the activation of CerS by Bak for regulation of apoptosis. Once elevated, ceramide may itself form channels in the MOM or interact with Bax to form synergistic channels. Data also indicate that downstream ceramide metabolites such as S1P and hexadecanal may be important for activation of Bak and Bax and induction of MOMP (Chipuk et al. 2012). These precise sphingolipid/ Bcl-2 protein interactions need further elucidation as they represent novel drug targets.

For cancer treatment, sphingolipid mediated mitochondrial apoptosis is a highly exploited area in *in vitro* and *in vivo* studies. Many chemotherapeutic agents have been shown to induce apoptosis through sphingolipids and in the case of drug resistance, altering levels of sphingolipids has resulted in encouraging outcomes. Several studies indicate that prevention of ceramide metabolism to either glucosylceramides or S1P via inhibition of glucosylceramide synthase or sphingosine kinase sensitizes cancer cells to standard of care therapeutics (for example (Casson et al. 2013)). Ceramide analogues are also being exploited for potential use in combinatorial therapies. For example, a cationic analogue of ceramide LCL124 that specifically accumulates within mitochondria induces apoptosis in gemcitabine and 5-FU resistant pancreatic cancer cells, whilst sparing normal cells (Beckham et al. 2013). Thus LCL124 has promise for treatment of rare and difficult cancers like pancreatic cancer with potentially minimal side effects (Beckham et al. 2013). Liu *et al.* developed C_6-ceramide nanoliposomal formulations for specific delivery to NK-LGL leukemia cells resulting in the induction of disease remission in animal models (Liu et al. 2010). Thus, novel methods for delivery of ceramides or drugs that regulate ceramide metabolism specifically to tumor cells could prove to highly fruitful for the development of targeted therapies utilized alone or in combination with standard of care therapeutics.

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Conclusion

Sphingolipids are metabolized in the vicinity of mitochondria and regulating mitochondrial structure and function. It is clear from the many studies cited within this review (and those that the authors inadvertently failed to cite) that deregulated sphingolipid metabolism contributes to mitochondrial dysfunction and disease. However, the exact molecular mechanisms by which specific sphingolipids alter mitochondrial biology are not fully understood. Determining the specific protein-lipid interactions that occur *in vivo* is challenging but is required for more adequately targeting this pathway for the development of novel therapeutics for treatment of a variety of diseases.

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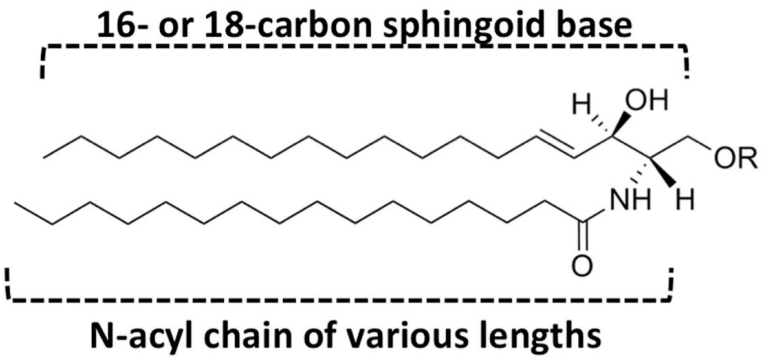

Figure 1.

Basic structure of a sphingolipid. Sphingolipid family members generally contain a common sphingoid base backbone, which can be altered in a number of ways including the introduction of a double bond(s), differing length (sphingoid base backbones of 16 and 18 carbons are possible), addition of a N-linked fatty acyl chain, and additions to the terminal hydroxyl group. The length of the N-linked fatty acyl chain varies as well as its degree of saturation and hydroxylation. Groups that can be added to the C1- hydroxyl group of the sphingoid base backbone include a phosphate, carbohydrates, and phosphorylcholine moieties, giving rise to thousands of different sphingolipid species.

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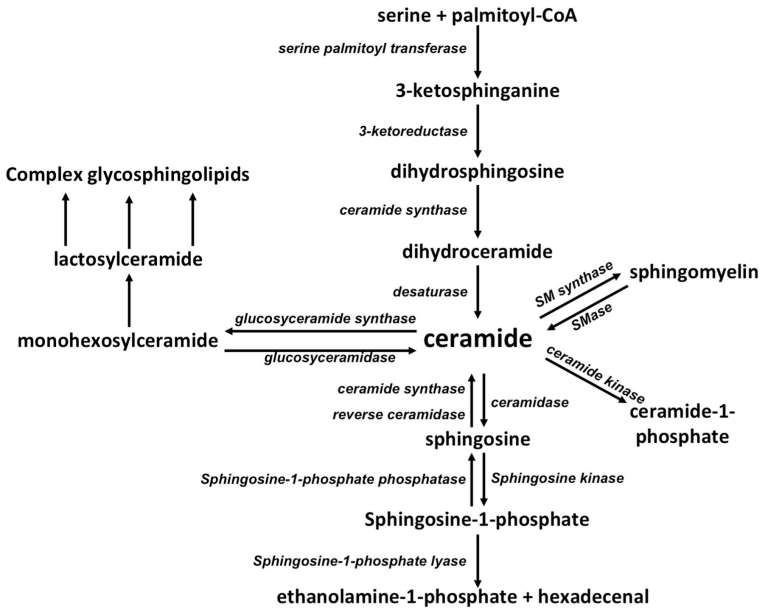

Figure 2.

Schematic of sphingolipid metabolism. Ceramide is the center of sphingolipid metabolism and can be synthesized de novo from serine and palmitoyl CoA, the hydrolysis of sphingomyelin, breakdown of glycosphingolipids, or via salvaging free sphingosine. Once synthesized, ceramides can be utilized to form glycosphingolipids, ceramide-1-phosphate, or sphingomyelin. Alternatively, the N-linked fatty acyl chain can be cleaved off of ceramide to generate sphingosine that can be utilized to form sphingosine-1-phosphate. Sphingosine-1-phosphate can be turned back into sphingosine or can broken down to form hexadecenal and phosphoethanolamine (ethanolamine-1-phosphate).

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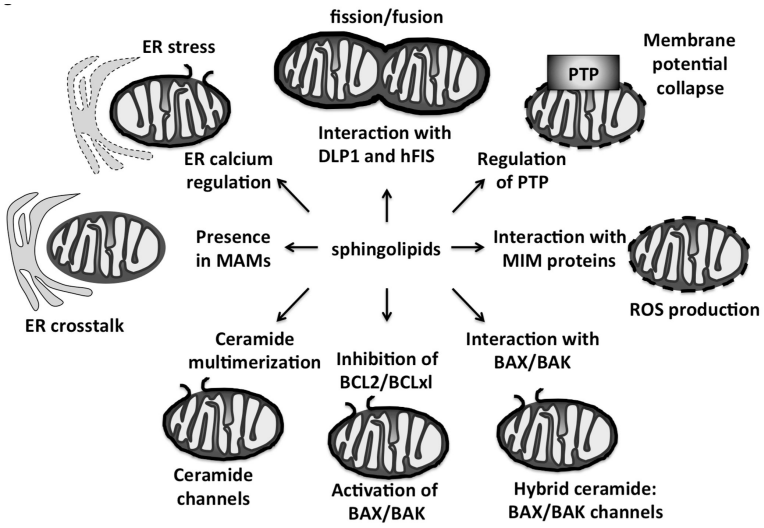

Figure 3.

Diagram illustrating the proposed mechanisms by which sphingolipids alter mitochondrial structure and function to induce mitochondrial outer membrane permeabilization (MOMP) and apoptosis.