Inter-organellar communication with mitochondria regulates both the intrinsic and extrinsic pathways of apoptosis

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One of our major research interests is to define BCL-2 family function in the cellular decision to induce mitochondrial outer membrane permeabilization and apoptosis. Curiosity in BCL-2 family - mitochondrial interactions led to the identification that the sphingolipid pathway plays a crucial role in BCL-2 family function. For approximately 20 years, sphingolipid biology and apoptotic mechanisms have criss-crossed, but remained distinct, as neither literature could explain the observable interplay between these pathways. We recently identified that two products within the sphingolipid pathway, sphingosine-1-PO_4 and hexadecenal, directly regulate BAK and BAX activation, respectively. Furthermore, our work suggests that mitochondrial communication with heterotypic membrane and/or organelles may be an important regulatory mechanism for apoptotic sensitivity.

Throughout the decades of BCL-2 (B-cell lymphoma 2) family research, the majority of effort focused on defining the individual BCL-2 family members.[1] As such, the BCL-2 family has been parsed into anti-apoptotic (e.g., BCL-2) and pro-apoptotic proteins (e.g., BAX, BCL-2 associated protein X). Furthermore, the functional cooperation between pro-apoptotic members has also been defined via elegant biochemical and cellular studies that revealed the mechanisms leading to apoptosis.[2-8]

Pro-apoptotic BCL-2 family members are divided into two subclasses: the "effector" proteins (e.g., BAX and BAK, BCL-2 antagonist killer) and the "BH3-only"

proteins (e.g., BID, BH3 interacting-domain death agonist).[1] The effector proteins are responsible for targeting the outer mitochondrial membrane (OMM), leading to its permeabilization and the release of pro-apoptotic factors in a process referred to as mitochondrial outer membrane permeabilization (MOMP).[2] Once MOMP happens, the apoptotic proteases are activated and cell death rapidly ensues.[9]

As suggested above, the pro-apoptotic BCL-2 family members activate at the OMM leading to its disruption, but the mechanisms that allow for mitochondrial targeting are highly debated. There are suggestions that OMM proteins allow for specificity, along with evidence that mitochondrial lipids contribute to BAK/BAX activation and MOMP.[10-12] Using an unbiased approach, we biochemically isolated and defined an activity that supported the activation of pro-apoptotic BCL-2 proteins at the OMM.[13] Our experiments identified that a neutral sphingomyelinase activity was responsible to maintain mitochondrial responses to BID-induced BAK/BAX activation and apoptosis.[13] The identified pathway was no stranger to the apoptosis field as an extensive literature supports a role for sphingolipids in the cell death pathways, but our work provided a novel interpretation of how the BCL-2 family and sphingolipid pathways mechanistically intersect to engage MOMP and apoptosis.[14-16]

Our recent studies highlighted a role for two products within a sphingomyelin metabolic pathway as essential regulators of BAK/BAX-mediated MOMP:
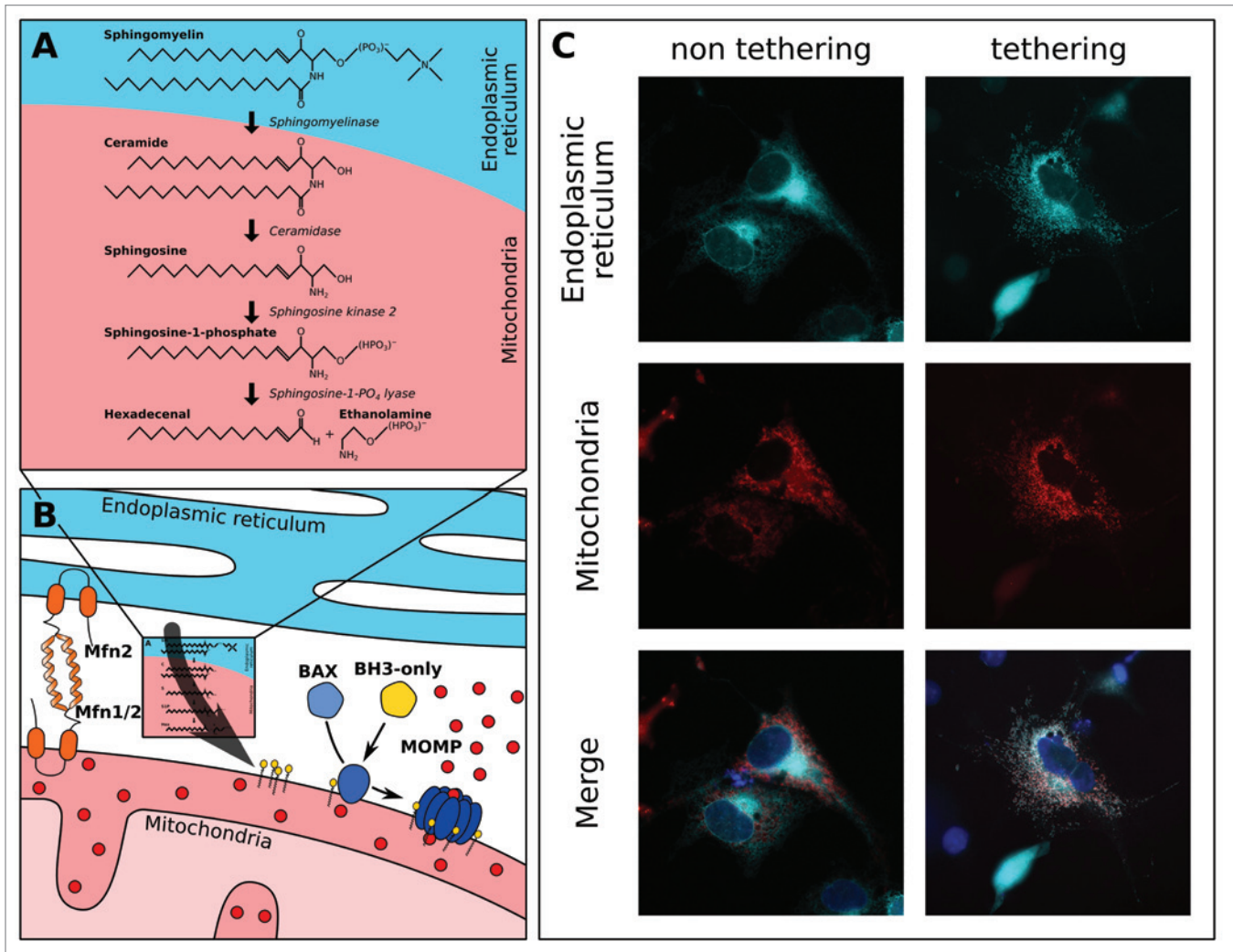
Figure 1. Inter-organellar sphingolipid metabolism with mitochondria regulates both the intrinsic and extrinsic pathways of apoptosis. (**A**) The relevant sphingolipid substrates, products and enzymes (italics), are shown. Our work suggests that sphingomyelin originates from a heterotypic membrane (e.g., the endoplasmic reticulum), and its hydrolysis product, ceramide, is transferred to mitochondria.[13] (**B**) Communication between the ER and mitochondria is suggested to be regulated by proteins that tether the two organelles, such as mitofusins 1 and 2 (Mfn1/2).[28] Biochemical data show that mitochondria contain numerous sphingolipid metabolism enzymes allowing for the generation of sphingosine-1-PO$_4$ and hexadecenal to support BH3-only protein induced BAK and BAX activation, respectively, and MOMP.[13] (**C**) Recombinant tethers have been generated that lead to marked increases in ER - mitochondrial communication.[30] For example, here we show that recombinant tethers expressed in mouse embryonic fibroblasts cause reorganization of the ER (cyan, top) and mitochondrial (red, middle) networks and induce marked co-localization (merge, bottom). As control, non-tethering controls maintain distinct ER and mitochondrial networks.

sphingosine-1-PO$_4$ and its degradation product, hexadecenal (**Fig. 1A**). Through a series of biochemical approaches, we determined that S1P and hexadecenal promoted the activation of BAK and BAX, respectively, and that these sphingolipid products could specifically cooperate with pro-apoptotic BH3-only proteins to coordinate MOMP and cytochrome c release. As purified full length, functional BAX can be examined in vitro, we demonstrated that high micromolar concentrations of hexadecenal directly activated BAX leading to conformational changes within BAX monomers that supported oligomerization and the permeabilization of liver mitochondria and defined liposomes.[17] We speculate that cellular signaling events that lead to increased hexadecenal production (or decreased hexadecenal degradation/metabolism) could promote BAX activation, MOMP and apoptosis.

Importantly, we were able to demonstrate that pharmacological inhibitors to sphingolipid metabolism that prevent sphingosine-1-PO$_4$ and hexadecenal production block the mitochondrial pathway of apoptosis, along with death receptor mediated apoptosis that depends on mitochondrial contributions.[13] Moreover, the exogenous expression of catalytically active neutral sphingomyelinases in cells could sensitize to both the mitochondrial pathway of apoptosis (e.g., induced by BCL-2 interacting mediator of cell of death, short isoform; BIM-S) and death receptor-induced apoptosis (e.g., tumor necrosis factor α, TNFα).[13] These cellular

data could be recapitulated biochemically by the addition of bacterial neutral sphingomyelinase or recombinant human neutral sphingomyelinase to isolated mitochondria, which also caused marked sensitization to pro-apoptotic stimuli.[13]

The novelty in our work is focused on the notion that sphingolipid metabolism directly regulates individual pro-apoptotic BCL-2 members to allow for MOMP. The majority of literature suggests that pro-apoptotic sphingolipids, such as ceramide, are necessary and sufficient to engage cell death; and a more recent literature provides evidence that sphingolipids allow for pro-apoptotic BCL-2 function by creating membrane environments that also support and synergize with BAX activation.[16,18-21] Our studies provide evidence that sphingolipid metabolic products directly engage pro-apoptotic proteins like BAK and BAX to coordinate appropriate sensitivity to BH3-only proteins.[13]

Directly upstream of sphingosine-1-PO$_4$ and hexadecenal production is two enzymes that coordinate metabolism: the sphingosine kinases (SPHK1 and SPHK2) and sphingosine-1-PO$_4$ lyase (**Fig. 1A and B**). We investigated the localization of these enzymes and provided evidence that SPHK2 and lyase activities were biochemically detected in mitochondrial fractions.[13] We find these results of importance as it suggests that mitochondria regulate the production of sphingosine-1-PO$_4$ and hexadecenal, which directly impacts on sensitivity to pro-apoptotic BCL-2 family function and subsequent apoptosis (**Fig. 1B**). There is also intriguing evidence that both of these enzymes impact on cellular sensitivity to apoptosis via changes in the expression of various BCL-2 family, and vice versa;[22-25] and interesting data suggesting that mitochondrially localized pro-apoptotic BAK is required for stress-induced production of long chain ceramide through ceramide synthase.[26] Moreover, the addition of hexadecenal has recently been implicated within the mitochondrial pathway of apoptosis via BIM- and BAX-dependent apoptosis.[27]

Our observations suggested that mitochondria contain many sphingolipid-metabolizing enzymes, yet they appear to lack significant neutral sphingomyelinase

activity compared with other cellular compartments (**Fig. 1A**). This focused our efforts to investigate how the mitochondrial sphingolipid environment is actively maintained, which lead to identify that a protease-sensitive mitochondrial-associated organelle or compartment likely contributes to the sphingolipid environment of the OMM (**Fig. 1B and C**).[13] Indeed, the majority of mitochondria are actively tethered to the endoplasmic reticulum, and this may be responsible for sphingolipid metabolism and/or transfer to the OMM.[28-30] At present, we are investigating the influence of mitochondrial tethering to the endoplasmic reticulum on sphingolipid metabolism, mitochondrial composition and cellular sensitivity to pro-apoptotic stimulation (**Fig. 1C**); all of which will surely provide compelling data to highlight the marked influence of inter-organellar communication on cell fate decisions. These results may reveal unique pharmacological opportunities to enhance or inhibit cell death in various human pathologies.

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