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Ceramide induced mitophagy and tumor suppression

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Abstract

Sphingolipids are bioactive lipid effectors, which are involved in the regulation of various cellular signaling pathways. Sphingolipids play essential roles in controlling cell inflammation, proliferation, death, migration, senescence, metastasis and autophagy. Alterations in sphingolipid metabolism has been also implicated in many human cancers. Macroautophagy (referred to here as autophagy) is a form of nonselective sequestering of cytosolic materials by double membrane structures, autophagosomes, which can be either protective or lethal for cells. Ceramide, a central molecule of sphingolipid metabolism is involved in the regulation of autophagy at various levels, including the induction of lethal mitophagy, a selective autophagy process to target and eliminate damaged mitochondria. In this review, we focused on recent studies with regard to the regulation of autophagy, in particular lethal mitophagy, by ceramide, and aimed at providing discussion points for various context-dependent roles and mechanisms of action of ceramide in controlling mitophagy.

Keywords

Ceramide; sphingolipids; mitophagy; tumor suppression

1. Introduction

Sphingolipids are membrane lipids with important functions in regulating membrane fluidity and subdomain structures. Advances in sphingolipid research suggest that sphingolipids are highly bioactive molecules and have a great impact on cellular signaling and disease pathogenesis [1]. Ceramide is one of the central molecules of sphingolipid metabolism and plays a key role in the regulation of various cellular functions, including cell proliferation, death, migration, and senescence [2]. Ceramide is intimately involved in cancer pathogenesis; alterations in its metabolism are involved in controlling cancer initiation,

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progression, and/or response of cancer cells to chemotherapeutic agents and radiation. Endogenous levels of ceramide, especially C₁₈-ceramide, are suppressed in many types of tumor tissues compared to non-cancerous counterparts. Additionally, ceramide levels increase upon exposure of cancer cells to stress-causing agents, such as cytokines, anticancer drugs, and radiation, leading to cancer cell death and tumor suppression [2, 3]. Ceramide-mediated tumor suppression is regulated by various mechanisms such as apoptosis, necroptosis, lethal autophagy, and mitophagy [4-8].

Mitophagy is a form of autophagy that results in selective degradation of mitochondria through the autophagic machinery [9]. Ceramide plays a key role in the regulation of general autophagy at many levels from initiation to formation of autophagosomes [10]. Recently, we have begun to better understand ceramide's role in inducing selective lethal mitophagy. Several recent studies showed the ability of endogenous or exogenous ceramides to cause cell death after accumulating in the mitochondria [11-13]. Our recent study showed that mitochondrial ceramide acts as a receptor for LC3-II [14]. Ceramide binding directly to LC3-II protein leads to the recruitment of autophagosomes to damaged mitochondria, resulting in lethal mitophagy [14]. This review will focus on the roles and mechanism of action of ceramide in the regulation of mitophagy and tumor suppression.

2. Metabolism and biological roles of ceramide

a. Structure and metabolism of ceramide

Ceramide is a bioactive sphingolipid with a peculiar structure. It is composed of a sphingosine backbone that is esterified to a fatty acyl chain via an amide linkage at carbon 3 [1, 2]. The variety in the length of the fatty acyl chain generates many different ceramides, such as C₁₄ to C₂₆-ceramides. A trans-double bond between carbons 4 and 5 in the sphingosine backbone is required for its biological activity, as the loss of the double bond generates dihydro-ceramide [1].

Ceramide lies at the center of sphingolipid metabolism: acting as a substrate for the generation of more complex sphingolipids, or as a product of the breakdown of complex sphingolipid molecules (Figure 1). As a substrate, ceramide is converted to sphingomyelin, ceramide-1-phosphate, hexosylceramides, and other complex glycosphingolipids and gangliosides [1]. As a product, ceramide can be generated by the breakdown of these more complex sphingolipids by specialized enzymes such as sphingomyelinases (SMAse), which hydrolyze sphingomyelin (SM), and cerebrosidases which hydrolyze hexosylceramides [15].

De novo generation of ceramide begins with the condensation of serine and palmitoylCoA to form 3-ketosphinganine, and then dihydrosphingosine (sphinganine) [16], involving multiple metabolic enzymes. This is followed by the action of ceramide synthases 1-6 (CerS1-6) (also known as dihydro-ceramide synthases) that esterify the dihydrosphingosine to generate dihydroceramide [17]. Ceramide is then generated by the action of (dihydroceramide)-desaturase that irreversibly inserts a double bond between carbons 4 and 5 [18].

Ceramide catabolism is regulated mainly by ceramidases, which cleave ceramide to generate sphingosine. Sphingosine then gets phosphorylated by sphingosine kinases 1 or 2 (SphK1 or SphK2) to produce sphingosine 1-phosphate (S1P). S1P can be lysed to hexadecanal and ethanolaminephosphate by S1P lyase or dephosphorylated by S1P phosphatases [19]. Hence, endogenous levels of ceramide increase through *de novo* synthesis, activation of sphingomyelinases, or decreased clearance through the inhibition of glucosylceramide synthase (GCS), sphingomyelin synthase (SMS), or ceramidase (CDase) [20].

Sphingolipid metabolism is highly compartmentalized in cells, as the subcellular localization of different sphingolipid molecules and the metabolic enzymes play key roles in this process [1]. For example, CerS1-6 enzymes are localized in the endoplasmic reticulum (ER) where *de novo* synthesis of ceramide occurs. For the synthesis of SM, ceramide is transported from the ER to the Golgi apparatus by ceramide transporter protein (CERT) in a non-vesicular fashion [21, 22]. Similarly, for the synthesis of glucosylceramide (GlcCer), ceramide is transported to the Golgi by Fabb2 transporter [22]. Recently, a role for a lipid transporter protein GLTPD1 for ceramide 1-phosphate, referred to as CPTP, has been discovered, which plays a critical role in the regulation of inflammation [23]. Ceramide is also found in the mitochondria where it can be generated by neutral sphingomyelinase (N-SMase) in response to increased reactive oxygen species (ROS) production [1]. Ceramide accumulation in the mitochondria can lead to ceramide stress-induced mitochondrial fragmentation, and decrease in ATP production [14]. In lysosomes, ceramide is mainly produced by acid sphingomyelinase (A-SMase), and in the plasma membrane, ceramide is localized within lipid rafts, which are specialized membrane microdomains that regulate various signaling pathways [24].

b. Biological functions of Ceramide Synthases 1-6

Ceramide Synthases 1-6 (CerS1-6) were first identified in yeast as longevity assurance gene 1 (LAG1). LAG1 regulates longevity in yeast in a way that its deletion prolongs the replicative lifespan [17]. The mouse homologue of LAG1 is LASS1, also known as the upstream of growth and differentiation factor 1 (UOG1), which was discovered to specifically regulate the synthesis of C₁₈-ceramide [25, 26]. There are at least six mammalian LASS proteins that are currently known as CerS1-6 [17]. All of the CerS enzymes share a domain required for their enzymatic activity to generate ceramide called TLC domain (TRAM, LAG1, and CLN8 homology), which is made up of five predicted transmembrane helices [27, 28]. CerS2-6 also contain a HOX domain which CerS1 lacks [29, 30].

CerS1-6 exhibit some specificity for the generation of endogenous ceramides with different fatty acid chain lengths [31, 32]. For example, CerS1 mainly generates ceramide with an 18-carbon containing fatty acid chain (C₁₈-ceramide), CerS4 generates both C₁₈-ceramide and C₂₀-ceramide, and CerS5 and CerS6 generate mainly C₁₆-ceramide, and to a lesser extent, C₁₂- and C₁₄-ceramides. CerS2 and CerS3 are known to generate very long chain C₂₂₋₂₄- and C₂₆-ceramides, respectively [1-3, 7, 15, 33].

Different species of ceramide with distinct fatty acyl chain lengths have diverse biological functions (Table 1). For example, CerS1 and CerS6, generating C₁₈-ceramide and C₁₆-

ceramide respectively, have opposing roles in cell death and proliferation in head and neck cancer cells and tumors. CerS1/C₁₈-ceramide axis leads to cancer cell death and decreases head and neck tumor growth [34-36]. Increased levels of serum C₁₈-ceramide act as a potential biomarker to monitor patients' response to chemotherapy [37]. On the other hand, C₁₆-ceramide promotes head and neck cancer cell proliferation and its increased serum levels associate with a positive lymph node status in breast cancer patients [38, 39]. On the other hand, there are studies showing that C₁₆-ceramide is pro-apoptotic, whereas C₂₄-ceramide protects from cell death [40, 41]. Thus, overall, distinct roles of ceramides appear to be context dependent, especially that knockout mice for multiple CerS enzymes exhibit different phenotypes: mice that express a mutant CerS1 (toppler mice) exhibit neurological disorders associated mainly with alterations in Purkinjee cells; mice with genetic loss of CerS2 results in liver damage; mice with CerS6 knockout exhibit neurological/behavioral alterations; and CerS4 knockout mice exhibit severe alopecia with alterations in sebaceous glands and sebum contents [42-43,204]. The demonstration of the distinct roles of CerS-generated ceramides in the regulation of cell death is also consistent with studies performed in *Drosophila* and *C. elegans* that showed the distinct roles of CerS-generated ceramides in these organisms [45-47]. Moreover, recent evidence suggests that changes in the carbon length of the sphingoid base of ceramides with 18 versus 16 carbons play roles in inducing survival autophagy versus cell death in cardiomyocytes [48].

c. Cancer suppressing role of ceramide

Ceramide induces a variety of anti-proliferative responses such as programmed cell death, cell cycle arrest, senescence, and differentiation. Indeed, studies show that ceramide is involved in apoptosis, necroptosis, lethal autophagy, and mitophagy, all of which decrease cancer cell viability [14, 49-51]. Exogenously supplied ceramides (C₂-C₁₈-ceramides), also have anti-proliferative activities when added to cells in culture [11, 12, 52]. Thus, ceramide metabolism has a significant role in suppressing cancer progression, and ceramide is emerging as a tumor suppressor lipid.

Ceramide is generated during stress conditions such as hypoxia, growth factor withdrawal, hyperthermia, and DNA damage which then mediates cell death [53]. In addition, there is an increase in ceramide levels during the activation of extrinsic apoptosis via FAS/FAS ligand pathway or tumor necrosis factor (TNF)-alpha pathway [54]. Ionizing radiation causes ceramide formation by activating A-SMase while androgen ablation in prostate cancer cells induces *de novo* generation of ceramide, leading to cell death [55-57].

One mechanism by which ceramide leads to cell death is by activating protein phosphatases PP1 and PP2A [58]. PP2A is a tumor suppressor protein that acts as a phosphatase to regulate signaling of many targets including Akt, c-Myc, and Bcr-Abl [59, 60]. Ceramide binds to the biological inhibitor of PP2A, I2PP2A or SET oncoprotein, leading to PP2A reactivation. PP2A can then dephosphorylate and inactivate several anti-apoptotic proteins such as Bcl-2 and AKT, or c-Myc. [50, 61]. Interestingly, SET/I2PP2A oncoprotein preferentially associates with endogenous C₁₈-ceramide compared with other ceramide species [62]. This specificity for binding to a specific species of ceramide is also evident in CERT binding preferentially to C₁₆- and C₁₈-ceramides but not very long chain ceramides

[63]. Ceramide interacts with another phosphatase, PP1, which inactivates the pro-apoptotic protein Bid [54]. Moreover, ceramide-PP1 is involved in the regulation of retinoblastoma protein (RB), a tumor suppressor protein that plays an important role in cell cycle regulation. Ceramide treatment can dephosphorylate and activate RB leading to cell cycle arrest in cancer cells [64]. In addition, ceramide generated in lysosomes by A-SMase activates cathepsin D by inducing autocatalytic proteolysis, resulting in Bid cleavage and caspase activation [65, 66].

Another mechanism that associates with the tumor suppressor roles of ceramide is its regulation of telomere length by acting as an upstream regulator of telomerase. Studies showed that ceramide accumulation in lung cancer cells inhibits telomerase expression by inactivating c-Myc transcription factor, which is an activator of the human telomerase reverse transcriptase (hTERT) promoter, via increased ubiquitination and proteasome mediated degradation. The inactivation of telomerase prevents the cancer cell from elongating the telomeric ends of the chromosomes after each replication cycle, leading eventually to cell death [67, 68].

Ceramide is also involved in pathways that lead to quiescence and senescence in cancer cells. For example, ceramide inactivates cyclin dependent kinase 2 (CDK2), and upregulates the expression of CDK inhibitors p21 and p27 in Wi-38 fibroblasts and nasopharyngeal carcinoma cells, respectively [69-71]. In addition, exogenous supply of ceramide to fibroblasts cultured at low passage induces the biochemical and morphological features of senescence [1, 72]. By inducing senescence, ceramide helps in the suppression of key mitogenic pathways, leading to tumor suppression [73].

Because of these pro-death characteristics, ceramide analogues or mimetics have the potential to act as anti-cancer agents. Endogenous accumulation of ceramide might also be beneficial to suppress tumor growth. One example is the group of ceramide analogues, Ceramidoids or pyridinium ceramides, which preferentially accumulate in the mitochondria/nuclei, and suppress the tumor growth of lung, breast, and head and neck squamous cell carcinomas [52, 74]. The mitochondrial accumulation of pyridinium ceramides results in mitochondrial permeability transition and either caspase-dependent apoptosis or mitophagy [14, 74]. Another example is the group of glucosylceramide synthase inhibitors (PPMP and PPPP) that lead to the accumulation of ceramide, which decrease glucosylceramide generation, and suppress solid tumor growth [75, 76]. Ceramide can also be delivered exogenously in PEGylated nanoliposomes. Studies using these liposomes show that this delivery method of ceramide decreases phosphorylation of AKT, stimulates the activity of caspase 3/7, and prevents the growth of breast cancer cells *in vitro* and *in vivo* [77, 78].

3. General autophagy and its regulation by ceramide

a. Progression of autophagy

Autophagy, or self-eating in Greek, describes the mechanism utilized by the cell to self-digest internal organelles and misfolded proteins using lysosomal hydrolytic enzymes [79]. Autophagy starts with the formation of cup shaped structures called phagophores, also known as isolation membranes, which will elongate to engulf organelles and other

cytoplasmic components. The maturation of the phagophores leads to the formation of an autophagosome that fuses with lysosomes for the formation of autophagolysosomes [80]. It is at this stage that lysosomal enzymes start the digestion process and the rate of breakdown of the cellular components is referred to as lysosomal flux. Autophagy plays a critical role in physiology and cellular homeostasis, allowing the cells to recycle nutrients from digested organelles at times of starvation and remove aberrantly folded proteins [81-84].

Dysregulation in autophagy has been implicated in various human diseases such as neurodegenerative disorders, cardiovascular diseases, and cancer [84-89].

The discovery of autophagy genes (Atg) in yeast had a great impact on our understanding of the mechanisms of autophagy process. Most of the Atg genes are conserved in humans and play various roles including autophagy initiation, autophagosome formation and maturation [90-92]. During initiation, Atg1 forms a complex with ULK (Unc-51 like kinase) that integrates inputs from mTOR signaling [87, 93-95]. During autophagosome formation, Atg9 allows for membrane addition and retrieval to and from sites of autophagosome biogenesis [83, 96], while Atg6 (or Beclin 1 in mammals) forms a multimeric complex with Atg14, Vps34/PI3kinase, and Vps15 [94, 96]. In addition, during autophagosome formation, Atg12 is activated by the enzyme Atg7 (E1-like ubiquitin ligase) and then transferred to Atg10 (E2-like ubiquitin ligase) to be conjugated to Atg5 (E3-like ubiquitin ligase), forming an autophagosomal precursor. Finally, Atg8 is required during the maturation phase [93, 95, 97, 100, 101].

LC3, mammalian homologue of Atg8, which plays important roles for the maturation of autophagosomes, has three isoforms: LC3A, LC3B and LC3C. GABARAP and GATE16 are also mammalian homologues of Atg8 [102]. LC3 is synthesized in the cell in its cytosolic form, LC3-I, with a carboxy terminal glycine (Gly120) [102]. During autophagy, LC3 is cleaved by Atg4 protease and then activated by Atg7 to be transferred to Atg3 (E2-like ubiquitin ligase) [80, 103]. This allows the Gly120 residue on the carboxyl terminal to be conjugated to phosphatidylethanolamine (PE) to form LC3-II. This allows its docking to the membranes, leading to membrane closure and formation of a mature autophagosome [104]. This sets LC3-II as a well-established marker of autophagosomes.

b. Autophagy paradox: cell survival or death regulation

Autophagy was initially discovered as a mechanism occurring at a low rate to remove protein aggregates and damaged organelles that are otherwise toxic for the cell [81]. In addition, autophagy is considered as a vital process during metabolic stress or nutrient deprivation, in which the degradation of organelles provides macromolecules and nutrients that maintain energy production and the basic cellular functions [79, 82, 99].

Mechanistically, several reports show that upon cell starvation, LC3-I is modified to LC3-II to promote autophagy [80]. *In vivo*, GFP-LC3 localizes in punctate structures in heart and skeletal muscle tissues in transgenic mice when the animals were under fasting conditions [102, 105]. These functions of autophagy provide pro-survival and cyto-protective mechanisms.

Recently, autophagy was found to be a pro-death mechanism especially if it occurs for a sustained period of time with a high intensity. Cells with sustained upregulation of

autophagic activity became atrophic with loss of vital organelles and cellular functions [106]. This suggests that over-activated autophagy can lead to cell death when associated with major elimination of essential organelles. Moreover, studies indicate that autophagy can lead to cell death via its ability to degrade cyto-protective proteins such as catalase, an anti-oxidant enzyme [107]. Autophagy can also result in cell death by upregulating apoptosis or necroptosis [108-110]. One particular example is the case of Atg5, which during autophagy can translocate to the mitochondria to induce mitochondrial membrane depolarization and caspase dependent cell death [111]. However, autophagic cell death, also known as lethal autophagy, or autosis, can be achieved independent of apoptosis or necroptosis. This type of cell death is rescued by suppression of autophagy by pharmacological or genetic approaches, and involves the action of autophagy genes and lysosomal flux during the death process [106, 108, 112-114].

c. Autophagy in cancer

There are several lines of evidence supporting that autophagy is a tumor-suppressor mechanism: Cancer cells have increased oxidative and metabolic stress that cause chromosomal abnormalities, DNA strand breaks, and gene mutations. Autophagy helps in scavenging reactive oxygen species by removing the damaged organelles, thus preventing the genetic abnormalities that might otherwise lead to oncogene activation or tumor suppressor gene inactivation [115-118]. Some cancer cells suppress autophagy as a mechanism to avoid the quality control during oxidative stress, DNA damage, and genetic instability [119]. One example to illustrate this is the role of autophagy in the turnover of p62 protein. When autophagy is suppressed, p62 protein clearance is prevented, leading to its accumulation, which in turn activates NRF2 (nuclear factor erythroid 2 related factor 2). NRF2 can then translocate to the nucleus, where it activates an anti-oxidant and pro-survival response [117, 120-122].

Some of the Atg proteins act as tumor suppressor genes. Beclin 1 (Atg6) expression is suppressed in malignant breast epithelial cells, and it is monoallelically deleted in 40-70% of prostate, ovarian, and breast cancers. Overexpression of Beclin 1 in breast cancer cells promoted autophagy and inhibited the malignant phenotype [123-125]. *In vivo*, targeted deletion of Beclin 1 in mice led to early embryonic death. Heterozygous disruption of Beclin 1 resulted in an increased risk of spontaneous tumor development, even though the other allele is intact. This suggests that the pro-autophagic Beclin 1 is a haplo-insufficient tumor suppressor protein [126]. Further studies then highlighted that other pro-autophagic proteins also act as tumor suppressors, such as Atg5 and Bif1 [83, 84, 112]. Autophagy can also be suppressed due to its regulation by signaling pathways that are up- or down-regulated in the cancer cells [112]. For instance, cancer cells with upregulation of PI3K-AKT-mTOR signaling cascade, or downregulation of PTEN activity, will have suppressed autophagy, promoting tumor growth/proliferation [83, 127, 128].

As cancer progresses to late stages, autophagy can act as a mechanism to help the cancer cells meet metabolic demands and repair intracellular damages inflicted by the aggressive tumor environment [84, 112, 119]. Pancreatic cancer cell lines demonstrate a high basal rate of autophagy, and upon pharmacological inhibition of autophagy by chloroquine, pancreatic

cancer cell growth was diminished mainly due to increased DNA damage and oxidative stress [117, 129]. Additionally, cancer cells expressing the Ras oncogene have a higher basal rate of autophagy, such that Ras expressing Atg5^{-/-} and Atg7^{-/-} cells, have suppressed autophagy levels and showed reduced tumor growth *in vivo* [130].

The implication of autophagy in cancer pathogenesis gives insight into new pharmacological therapies for cancer. If autophagy is required for the survival of cancer cells in the late stages, then pharmacological inhibition of autophagy can enhance the anti-cancerous effect of chemotherapeutic drugs [84, 112, 112, 117, 120]. For instance, combining vinblastine with C₆-ceramide attenuated autophagy and inhibited cancer cell growth in a synergistic fashion [131]. In contrast, if autophagy induction leads to cancer cell death via lethal autophagy, then drugs that induce autophagy will lead to tumor suppression. For example, pyridinium ceramide treatment leads to cancer cell death via in part activating autophagy [11].

d. Role of ceramide in mediating general autophagy

Ceramide is known to induce both survival and lethal autophagy via several mechanisms that are outlined in Figure 2 [4, 132, 133]. One mechanism by which ceramide can induce survival autophagy is by regulating cellular nutrient transporters [134-136]. Transporter proteins are required by cells to move nutrients across the plasma membrane. Since these transporters control the cellular fuel supply, alterations of the expression of the nutrient transporters is one way to affect survival and cell growth. Ceramide was shown to down-regulate the expression of amino acid and nutrient transporters leading to starvation, a state that induces survival autophagy by reducing mTOR signaling or activating AMPK [135, 137]. Survival autophagy was also induced in the context of CerS2 downregulation that dysregulated the normal trafficking of ceramide in the ER, leading to long chain ceramide accumulation, and activation of pro-survival IRE-1 (inositol requiring element 1) to prevent induction of cell death [133].

Moreover, ceramide is shown to induce lethal autophagy by affecting the expression of pro-autophagic protein Beclin 1 whereby exogenous treatment of cells with C₂-ceramide increased Beclin 1 expression and induced lethal autophagy [138]. This is due to the conversion of C₂-ceramide to long chain ceramide as the effect was inhibited when *de novo* ceramide synthesis was blocked using myriocin, the pharmacological inhibitor of SPT [138]. Further evidence indicated that ceramide increases Beclin 1 expression by activating JNK kinase, which in turn activates c-Jun, a known transcription factor for Beclin 1 expression [139]. In addition, JNK activation by ceramide leads to Bcl2 phosphorylation allowing it to dissociate from Beclin 1 [140]. In addition, chemotherapeutic drugs and arsenic trioxide lead to ceramide production that increase Beclin1 expression and promote lethal autophagy [141]. Other drugs such as cannabinoids also lead to ceramide accumulation to induce ER stress, mTOR inhibition via TRB3 (tribbles homolog 3), and lethal autophagy [142].

Ceramide can be hydrolyzed for the generation of S1P, which plays important roles in the regulation of autophagy [19, 143]. When cells were subjected to starvation, the activity of sphingosine kinase 1 (SphK1) increased, leading to increased accumulation of S1P [144]. SphK1 overexpression was able to induce autophagy by inhibiting the mTOR pathway, but

unlike the case of ceramide, this mechanism was independent of AKT dephosphorylation [143-145]. In addition, SphK1 downregulation enhanced ER stress and induced autophagy in an mTOR independent fashion [146].

4. Ceramide-mediated mitophagy

a. Mitophagy: selective autophagy of the mitochondria

Autophagy was considered to be a general process whereby the autophagosomes engulf many cytoplasmic elements, including mitochondria, endoplasmic reticulum, and peroxisomes [147]. However, recent findings suggest that autophagy can be selective to a specific organelle. The findings are based on the discovery of specific proteins of the organelles that are required to instigate the autophagy process. These include pexin 14 of peroxisomes in yeast for pexophagy, the autophagic degradation of peroxisomes; and Uth1p, an outer mitochondrial membrane protein required for selective mitochondrial autophagy, also known as mitophagy [148-150].

Aged and dysfunctional mitochondria are removed from cells to prevent the harm of unhealthy mitochondria, which generate reactive oxygen species, and release pro-apoptotic proteins [151]. This turnover process involves the action of autophagosomes and lysosomal hydrolytic enzymes. The term mitophagy has been suggested to refer to such process that selectively removes the mitochondria by autophagy [151-153].

It has been shown that mitochondria in hepatocytes that had undergone a mitochondrial permeability transition or a depolarization of the mitochondrial membrane potential are selectively removed by autophagosomes [153]. These studies showed that upon loss of mitochondrial membrane potential or during mitochondrial permeability transition, mitochondria are engulfed by GFP-LC3 positive autophagosomes [9, 154]. Photo-damaged mitochondria also recruited GFP-LC3 positive structures to the damaged areas [155]. It is suggested that reactive oxygen species (ROS) act as a signal in damaged mitochondria to recruit the LC3 positive autophagosomes. ROS can activate Atg4B, the protease that is required for LC3-I to be converted to LC3-II [156, 157].

b. Types of mitophagy

It is proposed that there are at least three types of mitophagy depending on the cellular mechanism of sequestration of mitochondria into autophagosomes [152].

Type 1 mitophagy refers to the mitophagy process that occurs during nutrient deprivation. The process occurs in coordination with mitochondrial fission, which starts with the formation of phagophores that enlarge to surround mitochondria to form structures called mitophagosomes. Mitophagosomes are then acidified to activate the hydrolytic enzymes of lysosomes [152, 155, 158].

Type 2 mitophagy refers to the degradation of mitochondria during photo damage. In this case, depolarized mitochondria recruit LC3 positive structures to aggregate onto their surface. These structures then fuse together to sequester the mitochondria into a mitophagosome [159-161]. This type of mitophagy is not coordinated with mitochondrial

fission, unlike Type 1. Another difference is that Beclin-1 protein is required for Type 1 mitophagy but not for Type 2. This came from studies showing that Type 1 mitophagy can be prevented by pharmacological inhibition of PI3K using 3-methyladenine or wortmannin. On the other hand, Type 2 mitophagy was shown to be independent of Beclin-1 and PI3K [152].

Type 3 mitophagy, also known as micromitophagy, involves the formation of mitochondria-derived vesicles, which translocate to the lysosomes [152, 162, 163]. The release of mitochondrial derived vesicles depends on oxidative stress in the mitochondria and involves pink 1 and parkin proteins. Micromitophagy does not involve LC3 or Atg5, and it is independent of mitochondrial depolarization or mitochondrial fission [164]. This process allows the cell to selectively remove damaged or oxidized components of the mitochondria without total degradation.

c. Progression of mitophagy and the involvement of mitochondrial fission/fusion

The signaling pathways involved in the progression of mitophagy share many similarities with general autophagy. At baseline, LC3 is dispersed throughout the cytosol, and some LC3 is found in pre-autophagic structures close to the mitochondrial membrane [80]. During mitophagy, LC3 is conjugated to phosphatidylethanolamine (PE), forming LC3-II. During type 1 mitophagy, already existing preautophagic structures enlarge in size to envelope and sequester the mitochondria. This event forms the mitophagosome, which then fuses with a lysosome or a late endosome to form a mitophagolysosome that digests the mitochondrial content [152, 155].

The molecular events contributing to mitophagy initiation were first identified in yeast. Studies showed that there are three yeast proteins participating in mitophagy initiation: outer mitochondrial protein Uth1, intermembrane space protein phosphatase Aup1, and inner membrane protein required for K^+/H^+ exchange Mdm38p [9]. Atg32 was identified as the main signal to direct autophagosomes to mitochondria after interacting with Atg8 and Atg11 [165, 166, 202]. There are no mammalian homologues for Atg32; however, studies showed that there are some receptors on the mitochondrial outer membrane that signal for the mitophagy process. For instance, optineurin acts as an autophagy receptor in parkin-mediated mitophagy, and FUNDC1 mediates hypoxia induced mitophagy [196,197]. Autophagy receptors can also be lipids in the mitochondrial membrane such as cardiolipin and ceramide [14,198,199].

Moreover, proteins involved in mitochondria fission/fusion are key regulators for the selective elimination of mitochondria in mammalian cells [167]. Mitochondria are dynamic mobile organelles continuously dividing or fusing [147]. The processes of fusion and fission are intrinsic for mitochondrial viability, and they are important in the regulation of calcium homeostasis and the generation of ATP and ROS [168, 169]. In addition, fission and fusion of the mitochondria are important during mitophagy [9]. Fission, or mitochondrial division, involves the translocation of DRP-1 (dynamin related protein 1) to the mitochondria, where it oligomerizes to bind to Fission 1 (Fis1) in the outer mitochondrial membrane [170]. Fusion, a process that fuses two mitochondria together, involves mitofusin 1 and mitofusin 2, located in the outer mitochondrial membrane, and OPA1 (optic atrophy protein 1) located

in the inner mitochondrial membrane [171]. OPA1 is processed by mitochondrial peptidase OMA1 and i-AAA protease YME1L and is regulated by mitofusin 1 during inner mitochondrial membrane fusion [200,201].

The role of fission and fusion during the mitophagy process is illustrated in several studies. Cells with a knockdown of DRP-1 had suppressed rates of mitophagy, whereas cells with overexpression of DRP-1 had excessive mitochondrial disappearance [172-174]. In addition, mitochondria going through a round of fusion followed by fission generate two populations of mitochondria: those that re-fuse and are healthy, and those that never re-fuse, have a depolarized membrane potential, and get degraded by mitophagy [173, 175]. The loss of the pro-fusion OPA-1 is a key process for mitophagy, such that OPA-1 overexpression was able to decrease mitophagy [173, 175].

The pro-fission function of DRP-1 makes it an important player during mitophagy. DRP-1 is a cytosolic GTPase with three domains: GTP binding domain, bundle signaling element (BSE), and a stalk that allows for stable dimerization and oligomerization [176]. Upon its activation, DRP-1 translocates to the mitochondria to form dimers and oligomers that are necessary for fission. It is believed that DRP-1 translocation requires adapter proteins, such as mitochondrial fission factor (MFF), mitochondrial elongation factor 1/mitochondrial dynamics proteins of 49 and 51 kDa (MIEF1/MiD49/MiD51), and mitochondrial fission protein Fis1 [177-181]. DRP-1 is regulated by several post-transcriptional modifications such as phosphorylation. There are two sites of phosphorylation for DRP-1: Ser637 and Ser616. DRP-1 is activated when it is phosphorylated by cyclin B1-CDK1 at Ser616 and dephosphorylated by calcineurin at Ser637 [170, 182]. DRP-1 is inactivated when it is phosphorylated by protein kinase A at Ser637 [170, 182]. Another form of regulation of DRP-1 is nitrosylation by nitric oxide in the mitochondria, and deSUMOylation by SENP5 protease, both of which promote DRP-1 activation and dimer formation [182].

Two other proteins, which are associated with Parkinson's disease, are involved in DRP-1 mediated mitophagy: pink1 and parkin. Pink1 is a serine/threonine protein kinase located inside the mitochondria, while parkin is an E3 ubiquitin protein ligase located mainly in the cytosol [183-186]. It is believed that Parkin and Pink1 promote mitochondrial fission such that silencing their expression leads to mitochondrial defects due to lack of fission [184, 187]. Pink 1 and Parkin also regulate other mitochondrial functions, including mitochondrial biogenesis, mitochondrial transport, and calcium homeostasis [188-192]. In healthy mitochondria, Pink1 is cleaved and exported to the cytosol where it is rapidly degraded by proteasomes [200]. Upon uncoupling or depolarization of the mitochondria, Pink1 is stabilized, and Parkin is translocated from the cytosol to the mitochondria in a Pink 1-dependent fashion. This leads to ubiquitin mediated proteasomal degradation of outer mitochondrial proteins such as mitofusin 1 and mitofusin 2, leading to mitochondrial fragmentation and initiation of mitophagy [177, 184, 193]. DRP-1 is recruited to mitochondrial sites in close proximity of Pink1 and Parkin highlighting their importance in DRP-1-dependent mitophagy. In addition, Parkin can induce mitochondrial fission independent of Pink1 by affecting DRP-1 phosphorylation [184].

Moreover, ROS serve as candidates to initiate mitophagy. Cells supplied exogenously with hydrogen peroxide or superoxide showed evidence of autophagosomal structure formation [166]. It has been shown that upon ROS generation in the mitochondria, the mitochondrial membrane was depolarized, leading to Parkin translocation and initiation of mitophagy [195]. Interestingly, overexpression of the anti-oxidant enzyme superoxide dismutase 2 or pre-treatment with antioxidants prevented ROS-induced mitophagy [194, 195]. This suggests that oxidative stress may be an important signal to initiate mitophagy.

d. Ceramide mediated mitophagy as a tumor suppressor mechanism

There is evidence to support a role for mitophagy in cell survival or death, which appears to be context dependent. Mitophagy promotes cell survival under circumstances where it degrades the mitochondria that are about to activate caspase dependent apoptosis. In this case, disrupting the autophagic and lysosomal processes will prevent survival and lead to apoptosis [152, 153]. On the other hand, when mitophagy occurs excessively or for a sustained period of time, enzymes from the lysosomal flux such as cathepsins can leak to the cytosol where they initiate caspase dependent cell death [79, 83, 152]. Therefore, the functional outcome of mitophagy inducing cell death or survival depends on the intensity and duration of the stress as well as cellular contact [152].

Moreover, mitophagy can serve as a programmed cell death mechanism independent of apoptosis. This mechanism of cell death depends on ceramide synthase 1 (CerS1) and its metabolic product C₁₈-ceramide. Sentelle et al. showed that CerS1 and C₁₈-ceramide selectively induce non-apoptotic lethal mitophagy independent of Bax, Bak, or caspase activity, in head and neck squamous cell carcinoma cells and tumors. Ectopic expression of CerS1 or treatment with C₁₈-pyridinium-ceramide resulted in LC3-II formation, and promoted its direct binding to ceramide on the mitochondrial membranes. This lipid-protein binding then allowed the mitochondria to be targeted by the LC3-II containing autophagosomes. This report was the first to describe the role of ceramide signaling in mediating lethal mitophagy through ceramide-LC3-II binding (Figure 3). Interestingly, endogenous C₁₆-ceramide generated by CerS6 did not show any mitophagy promoting function in these cells. However, treatment with C₁₆-pyridinium ceramide, which accumulates in the mitochondria, induced mitophagy. This suggested that the subcellular localization of endogenous ceramides, and not their fatty acid chain length *per se*, is of great importance to determine their distinct biological actions during mitophagy [14].

The binding of ceramide to LC3-II indicates that ceramide acts as a tumor suppressor lipid that can directly bind proteins. Ceramide is shown to have a higher affinity to the PE-conjugated LC3-II than LC3-I. This interaction was proposed to involve the central hydrophobic domain of LC3 that has structural similarities to the domain of CERT (ceramide transporter protein) that binds C₁₆- and C₁₈-ceramides. Within this hydrophobic domain, the Ile35 and Phe52 residues of LC3-II were required for ceramide binding. Computational docking simulations and molecular modeling suggested that conjugation of LC3-I to phosphatidylethanolamine hides a low-affinity ceramide-binding sites allowing ceramide to bind selectively to the opposite end of the protein [14, 49]. More importantly, although point mutations at the Ile35 and/or Phe52 for conversion to Ala did not prevent

ceramide-mediated LC3 lipidation, and inhibition of ceramide binding modulated mitophagy and resulted in resistance to ceramide-mediated tumor suppression. Thus, these data support that ceramide plays a novel receptor role at the mitochondrial membranes to recruit LC3-II-containing autophagosomes to the mitochondria, which have been subjected to DRP-1-mediated fission. These data also suggest that targeting LC3 containing autophagosomes to mitochondria by ceramide at the mitochondrial membranes results in cancer cell death and tumor suppression, which seems to be regulated downstream of DRP1-mediated mitochondrial fission. Interestingly, in the absence of CerS1 overexpression, tumor cells with knockdown of LC3 had a reduced growth *in vivo* suggesting that at baseline, LC3 mediated autophagy is required for tumor growth in head and neck squamous cell carcinoma tumors [14, 49].

5. Conclusions and future perspectives

Ceramide, as a bioactive sphingolipid, plays key roles in the regulation of general and selective autophagy and/or mitophagy. This role of ceramide is of great importance in tumor biology as in most cases ceramide mediated autophagy leads to cell death and is thus called lethal autophagy or autosis [108, 114]. One example by which ceramide regulates lethal autophagic signaling pathways is its activation of c-Jun through JNK signaling, causing upregulated Beclin 1 expression and autophagic cell death [138]. Exogenous supply of C₁₈-pyridinium ceramide or overexpression of CerS1 resulted in caspase independent mitophagy where ceramide acts as a mitochondrial receptor for LC3-II-containing autophagosomes by interacting directly with LC3-II, recruiting autophagolysosomes to damaged mitochondria [14]. Exogenous ceramide mediated autophagic cell death is believed to involve BNIP3 activation after a reduction in mitochondrial membrane potential [140]. Chemotherapeutic drugs and arsenic trioxide lead to ceramide production that increase Beclin1 expression and promote lethal autophagy [141]. Other drugs such as cannabinoids also lead to ceramide accumulation to induce ER stress, mTOR inhibition via TRB3 (tribbles homolog 3), and lethal autophagy [142]. Amino acid deprivation is known to induce lethal autophagy in a ceramide dependent manner by activating CAPPs (ceramide activated protein phosphatases), which inhibits Akt/mTOR pathway [8]. The knowledge of the role of ceramide in autophagy/mitophagy sets an example to the importance of sphingolipid metabolism and signaling in these cellular mechanisms. Thus, more studies should be invested in this area of research to define the roles and mechanisms of how ceramide and/or other bioactive sphingolipid molecules mediate mitophagy and their relation to mitochondrial dynamics. Ceramide's role in recruiting autophagosomes specifically to mitochondria gave support to the findings that removal of mitochondria by autophagy can be selective rather than inadvertent. Ceramide acts as a receptor in the mitochondria binding the LC3-II in the autophagosomes to direct them specifically to mitochondria. However, the relationship between ceramide and the fission/fusion machinery is still not clear. DRP-1 is required for ceramide-mediated mitophagy, however the mechanism underlying DRP-1 activation remains unknown. Other studies looking at the interplay of ceramide and Pink1 or Parkin are also important for the field. Importantly, studies should consider the compartmentalized roles of ceramides with different fatty acyl chain lengths, involved in the regulation of the mitophagy process. We expect that there will be key discoveries to dissect the mechanisms

of how ceramide regulates lethal mitophagy and tumor suppression during the next few years, as analytical, molecular, pharmacologic and/or genetic tools are now available to define these roles of ceramides/sphingolipids in various disease models.

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6. References

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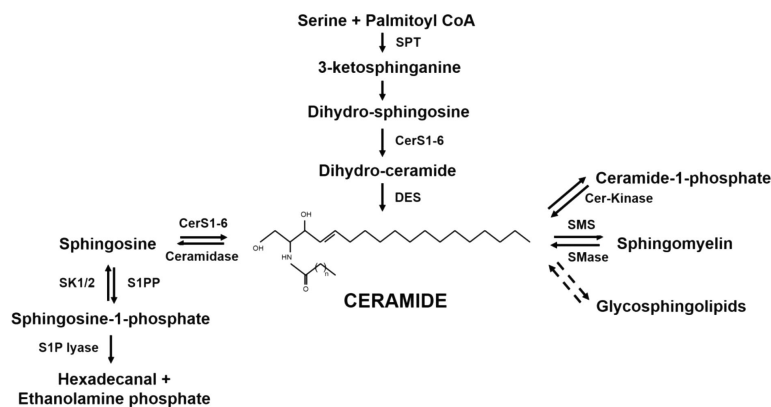
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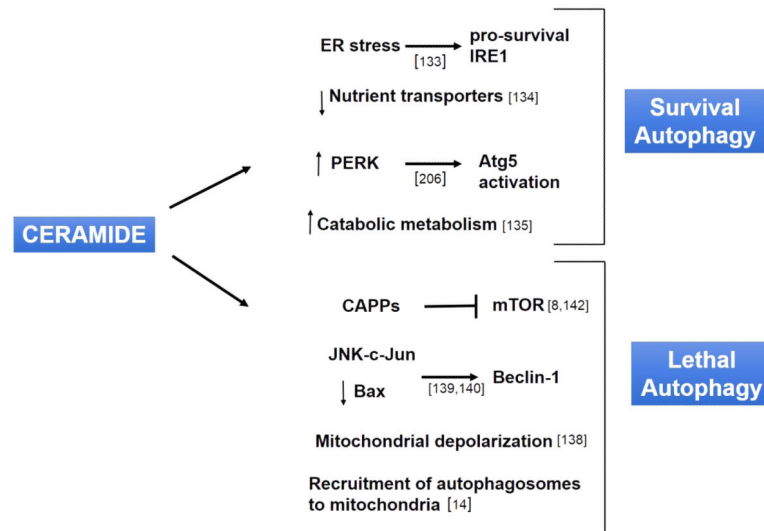
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Highlights

- This review focuses on the roles and mechanisms of ceramide-induced mitophagy.
- Ceramide is a key bioactive sphingolipid molecule, which is involved in the regulation of mitophagy.
- Ceramide-mediated mitophagy involves ceramide-LC3B-II binding and Drp1-mediated mitochondrial fission.
- Ceramide-induced mitophagy results in cell death and tumor suppression.

**Fig. 1.**

Ceramide metabolic pathways. Ceramide lies at the center of sphingolipid metabolism. Ceramide de novo generation starts with a condensation reaction involving Serine and Palmitoyl CoA by the enzyme Serine Palmitoyl CoA Transferase (SPT), to generate 3-ketosphinganine, which is then converted to sphinganine or dihydrosphingosine. Then, Ceramide Synthases (CerS1-6) transfer a fatty acyl CoA to the amino group yielding dihydroceramide which gets desaturated to ceramide by Dihydro-ceramide Desaturase enzyme (DES). Ceramide can be generated back from Sphingosine-1-phosphate with the help of S1P phosphatase (S1PP) and ceramide synthase (salvage pathway) or from Sphingomyelin by Sphingomyelinase (SMase), from Glycosphingolipids, or from Ceramide-1-phosphate by Ceramide kinase (Cer-Kinase). As a metabolic outlet for ceramide, it is converted to sphingosine by the action of ceramidase. Sphingosine kinases 1 and 2 (SK1/2) phosphorylate sphingosine to sphingosine-1-phosphate that can be further degraded by S1P lyase to hexadecanal and ethanolamine phosphate.

**Fig. 2.**

Ceramide's role in survival and lethal autophagy. Ceramide regulates several signaling pathways in autophagy, some of which lead to cytoprotective autophagy and survival while some lead to cell death through lethal autophagy. In the context of survival autophagy, ceramide can induce ER stress that activates the pro-survival IRE1 (inositol-requiring element 1), downregulate nutrient transporters and improves catabolic metabolism, and can result in activation of Atg5 via CD95 and PERK. In the context of lethal autophagy, ceramide can induce ER stress that inhibits mTOR through TRB3, activate Ceramide associated phosphatases (CAPPs) that also inhibit mTOR by inactivating Akt, increase the expression of Beclin-1 by activating JNK-c-Jun axis or by inactivating Bax, leading to loss of mitochondrial membrane potential and activation of BNIP3, and bind to LC3-II to recruit autophagosomes to engulf mitochondria.

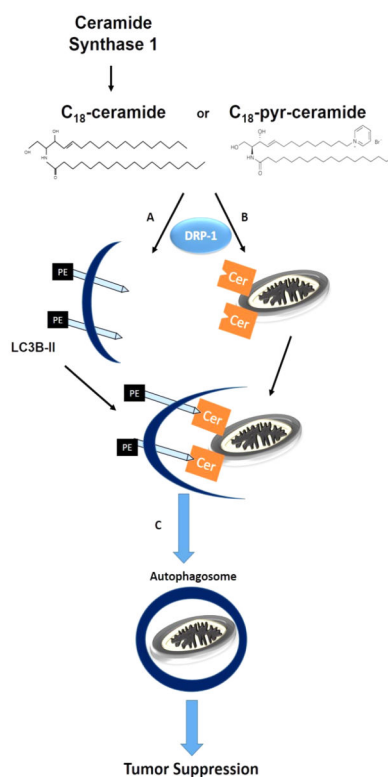


Fig. 3.

Regulation of mitophagy by ceramide. Endogenous generation of C₁₈-ceramide via CerS1 or exogenous treatment by C₁₈-pyridinium-ceramide is followed by two processes: A. conjugation of LC3-I to phosphatidylethanolamine on the carboxy terminal to form LC3-II and B. accumulation of ceramide in the mitochondrial outer membrane. Ceramide in the mitochondrial membrane acts as a receptor to LC3-II by binding to its amino terminal, opposite to where PE is conjugated. This results in C. recruiting the autophagosome to engulf the mitochondria. Lysosomes then fuse with the autophagosomes (D) for hydrolytic degradation of the contents.

Table 1

Diverse biological roles of Ceramide Synthases 1-6.

Ceramide Synthase isoform	Ceramide product	Biological Role
Ceramide Synthase 1	C ₁₈ -ceramide	- Induces cancer cell death [34] - Decreases tumor growth [35] - CerS1 mutant mice have neurological disorders [42]
Ceramide Synthase 2	C ₂₂₋₂₄ -ceramide	- C24-ceramide can protect from cell death [40,41] - CerS2 knockout mice exhibit liver damage [44]
Ceramide Synthase 3	Ultra long chain ceramides >C ₂₆	CerS3 knockout leads to trans-epidermal water loss leading to death after birth [203]
Ceramide Synthase 4	C ₁₈ - and C ₂₀ -ceramide	CerS4 knockout mice exhibit severe alopecia with alterations in sebaceous glands and sebum contents [43]
Ceramide Synthase 5	C ₁₂ -, C ₁₄ -, and C ₁₆ -ceramide	Implicated in induction of autophagy mediated hypertrophy of cardiac myocytes [204]
Ceramide Synthase 6	C ₁₂ -, C ₁₄ -, and C ₁₆ -ceramide	- Increases cancer cell proliferation [38] - Increased expression associates with positive lymph node status [39] - Induces chemotherapy mediated apoptosis [41] - CerS6 knockout mice exhibit neurological/behavioral alterations [205]