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Autophagy Paradox and Ceramide

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Abstract

Sphingolipid molecules act as bioactive lipid messengers and exert their actions on the regulation of various cellular signaling pathways. Sphingolipids play essential roles in numerous cellular functions, including controlling cell inflammation, proliferation, death, migration, senescence, tumor metastasis and/or autophagy. Dysregulated sphingolipid metabolism has been also implicated in many human cancers. Macroatuophagy (referred to here as autophagy) “self-eating”, is characterized by nonselective sequestering of cytosolic materials by an isolation membrane, which can be either protective or lethal for cells. Ceramide (Cer), a central molecule of sphingolipid metabolism, has been extensively implicated in the control of autophagy. The increasing evidence suggests Cer is highly involved in mediating two opposing autophagic pathways, which regulate either cell survival or death, autophagy paradox. However, the underlying mechanism that regulates the autophagy paradox remains unclear. Therefore, this review focuses on recent studies with regard to the regulation of autophagy by Cer and elucidate the roles and mechanisms of action of Cer in controlling autophagy paradox.

Keywords

Sphingolipids; ceramide; autophagy; mitophagy; cell death

1. Autophagy

Autophagy (“self-eating” in Greek) is a self-digestive process that targets internal or damaged organelles and misfolded proteins to lysosomal degradation[1]. To date, three types of autophagy have been described, including macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), which distinguish from each other as to the functions and mechanisms[2–4]. Autophagy consists of several distinct processes[5]. It initiates with the formation of an isolation membrane, namely a phagophore, which elongates to engulf cytoplasmic components. With LC3B-II protein (phosphatidyl ethanolamine, PE-conjugated microtubule-associated protein 1 light chain 3b) localized to the isolation membrane, it encloses to form an autophagosome. Fusing of autophagosome with lysosome forms autophagolysosome, in which contents sequestered in autophagosome vesicles are degraded by lysosomal hydrolases (Fig. 1) [6–9]. Autophagy not only results in

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the removal of damaged proteins and organelles, but also precisely regulates the normal turnover of the intracellular components to ensure the cellular quality control[10]. It primarily acts as a survival mechanism under stress conditions, such as nutrition starvation, via self-cannibalism to provide cellular energy and produce metabolic precursors[8,11,12]. However, under various different stress conditions, dysregulated and/or persistent autophagy may also lead to cell death through different molecular mechanisms[13–15]. Autophagic defects have been implicated in various human diseases, including neurodegenerative diseases and aging, infection, pulmonary and cardiovascular diseases, metabolic stress and cancer[12,16–22].

2. Autophagy in cell survival and cell death

2.1 Autophagy acts as a pro-survival and cytoprotective mechanism

Basal autophagy primarily regulates cellular homeostasis[8,11]. It usually occurs at a low rate and serves its housekeeping function to remove aggregated proteins and damaged organelles as they are toxic to the cell[23]. In response to most forms of cellular stress, such as metabolic stress or nutrition deprivation, autophagy supplies cells with energy and essential metabolic materials to maintain normal cellular functions[1,10,24]. Mutant mice, in which ATG (Autophagy-related protein)5 or ATG7 was depleted, developed neurodegenerative disease with the accumulation of polyubiquitylated protein aggregates[25,26]. ATG5 deficient mice failed to survive the neonatal period, which showed severe metabolic crisis with low amino acid and ATP levels[27]. Moreover, mitophagy, a form of autophagy that selectively targets damaged mitochondria, is particularly important for the regulation of cell death, as defective mitophagy causes mitochondrial dysfunction, leading to mitochondrial damage and toxic ROS (reactive oxygen species) production, which eventually may result in cell death[28,29]. Moreover, autophagy is also induced under the context of metabolic stress or survival nutrient withdrawal, which stabilized cyclin-dependent kinase inhibitor 27, leading to cell death inhibition[30].

2.2 Autophagy acts as a pro-death mechanism

Autophagic cell death is also known as Type II programmed cell death, in which autophagy per se serves as a cell death mechanism[31]. It is defined as cell death by autophagy, not cell death with autophagy[32]. Autophagic cell death should meet the following standards: (1) the cell death occurs independent of apoptosis; (2) cell death should be rescued by suppression of autophagy via both pharmacological inhibitors (e.g.,3-methyladenine (3-MA)) and genetic approaches, such as gene knockdown/mutation or gene silencing of autophagic regulators; (3) not only the autophagy markers but also autophagy flux is present in the dying cells[33]. On one hand, sustained upregulation of autophagic activity beyond a certain threshold could contribute to irreversible cellular atrophy and cause catastrophic cellular dysfunction due to the huge loss of cytosol and organelles[31]. For example, $Bax^{-/-}Bak^{-/-}$ cells activated autophagy in response to growth factor withdrawal, which caused progressive atrophy and eventually resulted in irreversible cell death[34]. Over-activated autophagy may compromise the recovery ability of a cell if autophagic clearance leads to a complete elimination of an essential organelle[28]. One the other hand, autophagy may also mediate its cell killing effect through the selective degradation of essential proteins in the cell[31]. It was shown that autophagy selectively degraded catalase, which plays an essential role in cellular antioxidant defense. Moreover, catalase deficiency contributed to ROS accumulation in cells, which succumbed to autophagic mediated cell death[35]. Moreover, autophagy may also mediate its cell killing effect through the trigger of apoptosis and/or necrosis[28,31,36]. One prominent example is ATG5, which, under certain conditions, was proteolytically activated to mediate apoptosis via its translocation to mitochondria, triggering mitochondrial outer membrane permeabilization (MOMP)[37]. As

previously mentioned, Lenardo and coworkers showed that catalase degradation by autophagy promoted necrotic cell death, which could be prevented by autophagy suppression[35]. Autophagic cell death was also shown to be required in salivary glands during Drosophila development, in which overexpression of ATG1 induced caspase-independent degradation of salivary glands[38]. In addition, Beclin1 mutant, which didn't bind anti-apoptotic protein Bcl-2, resulted in uncontrolled upregulation of autophagy, and accelerated cell death[13].

3 Autophagy and cancer

3.1 Autophagy is a tumor suppressor mechanism

In early stages of cancer development, quality control of autophagy suppresses tumor growth and exerts its anti-carcinogenic function by preventing metabolic or oxidative stress, maintaining normal mitochondrial function and safeguarding against DNA damage and genetic instability[39]. Beclin 1, one essential protein of autophagy, is monoallelically deleted in 40 to 70% of human prostate, ovarian and breast cancers[40–42]. Also, monoallelic disruption of beclin 1 in mice promoted spontaneous tumorigenesis, indicating that Beclin 1 primarily functions as a tumor suppressor[43,44]. Other studies also identified additional autophagy-related proteins (e.g., UVRAG, Bif1 and ATG5) as tumor suppressor proteins[11,12,33]. Moreover, autophagy regulation is closely associated with some oncogenic signaling modulation[33]. For example, PI3K-mTOR-Akt signaling axis is constitutively activated in many human cancers and promotes tumor growth and proliferation, which, by contrast, suppress autophagy[45,46]. Some common oncogenic proteins (e.g., Bcl-2, PI3K and PKB) inhibit autophagy induction; however, several well-known tumor suppressors (e.g., p53, PTEN and TSC1/2) invariably activate autophagy[11].

Autophagy may also prevent tumorigenesis as the guardian of the genome[22]. Metabolic stress may lead to ROS accumulation, inducing mutations and DNA strand breaks, which causes tumor suppressor inhibition and oncogene activation[22]. Autophagy acts as a ROS scavenger, maintaining genomic integrity, and prevents tumorigenesis[47,48]. Thus, defective autophagy is highly likely to increase gene mutations and amplification, survive metabolic stress, which susbsequently promotes tumorigenesis[49]. For example, the mitochondrial DNA mutations in autophagy-deficient yeast suggested that autophagy was required to maintain the regular turnover of mitochondria and keep cells from DNA damage and genotoxic stress[8,50]. Failure of p62 clearance or p62 accumulation accounts for another important mechanism of autophagy-mediated tumor suppression[49,51]. Defective autophagy contributed to p62 accumulation, which induced nuclear factor erythroid 2-related factor 2 (NRF2) activation[52,53]. Activated NRF-2 translocated to the nucleus, where it stimulated anti-oxidant defense system and promoted cell survival[51,54]. In addition, p62 is also known as an activator of NF-kB signaling pathway to accelerate tumorigenesis[49,55,56]. In another study, autophagy was significantly elevated in Ras oncogene-induced senescence (OIS) and restricted cell proliferation[57]. Inhibition of autophagy delayed the senescence phenotype[49].

3.2. Autophagy induces tumor promotion/proliferation

In the late stage of oncogenesis or established tumors, autophagy confers tumor cells survival ability by meeting increased metabolic and energetic demands, and executing cellular quality control to eliminate toxic intracellular damages in the aggressive tumor microenvironment[12,33,39]. Pancreatic cancer primary tumors and cell lines showed elevated autophagy under basal conditions[58]. Autophagy inhibition by genetic and pharmacological means (chloroquine treatment) led to elevated DNA damage and impaired mitochondria oxidative phosphorylation, which suppressed pancreatic tumor growth[49].

Autophagy was also shown to be upregulated and promoted the tumorigenicity of the cells expressing activated Ras oncogene[59]. Also, Ras-expressing ATG5$^{-/-}$ and ATG7$^{-/-}$ cells showed reduced tumor growth in mice while Ras-expressing p62$^{-/-}$ cells displayed decreased viability under stress, as well as carcinogenesis inhibition[59]. Moreover, numerous studies showed that autophagy was utilized by cancer cells as an adaptive mechanism to induce chemotherapy resistance and promoted their survival[60]. Thus, inhibition of autophagy by pharmacological inhibitors and genetic approaches may greatly enhance anti-cancer drug cytotoxicity and sensitize cancer cells to various cancer therapies[12,33,49,51]. For example, in a recent study, combination of vinblastine and nanoliposomal C_6-ceramide synergistically inhibited cancer growth/progression via attenuation of autophagy maturation, leading to apoptosis and tumor suppression[61]. Autophagy has also been implicated to facilitate the use of cellular glucose toward glycolysis, which is essential for cancer cell transformation and proliferation[62–64].

4. Regulation of autophagy by Cer

4.1 Metabolism of Cer

Sphingolipids are a family of membrane lipids that have structural roles in the regulation of the fluidity and the subdomain structure of the lipid bilayers[65,66]. Cer, a central molecule of sphingolipid metabolism, is composed of a sphingosine base and amide-linked acyl chains varying in length from C_{14} to C_{26}[67]. Cer then serves as the metabolic and structural precursor for complex sphingolipids, which are composed of hydrophilic head groups, such as sphingomyelin (SM), Cer-1-phosphate and glucosylceramide (GlcCer), which is the precursor for glycolipids and gangliosides[65]. Endogenous Cer can be generated via a *de novo* pathway, which begins with condensation of serine and palmitoyl CoA by serine-palmitoyl CoA transferase (SPT)[68,69]. Cer can be also generated by the metabolism of other complex sphingolipids, which is tightly regulated by many specialized enzymes[65,66,70]. For example, Cer can be produced by either sphingomyelinases (SMases) mediated sphingomyelin (SM) hydrolysis[71] or cerebrosidase mediated GlcCer and galactosylceramide (GalCer) breakdown[72]. Cer can also be hydrolyzed by ceramidases (CDases) to yield sphingosine[73,74], which can be phosphorylated by sphingosine kinase (SK)1 or SK2, producing sphingosine-1-phosphate (S1P)[75]. Cer is also utilized as a precursor by ceramide kinase (CK), or SM synthase to generate Cer-1-phosphate (C1P) and SM, respectively (Fig. 2) [67,76,77]. Cer is converted into GlcCer by glucosylceramide synthase (GCS), which is Cer transporter (CERT) independent[78]; by contrast, Cer mediated SM synthesis is dependent on CERT, which is responsible for Cer transport from the endoplasmic reticulum (ER) to the Golgi via a non-vesicular mechanism[79–82]. Importantly, non-vesicular transport of GlcCer from its site of synthesis (early Golgi) to distal Golgi compartments is carried out by FAPP2, four-phosphate adaptor protein, controlling the synthesis of glycosphingolipids, which might play crucial roles in determining the lipid composition of the plasma membrane[83].

4.2 Ceramide synthases(CerS) and *de novo* generation of Cer

CerS and SPT primarily function to generate Cer de novo[69,84]. CerS was originally identified as the yeast longevity assurance gene 1 (LAG1), known to regulate life-span/longevity in *Saccharomyces cerevisiae*, and its deletion prolonged the replicative life-span of yeast[85,86]. Moreover, LAC1, a LAG1 homologue, was known to be crucial for yeast Cer synthesis and postulated to perform the same function of CerS[87,88]. The discovery of a mouse homologue of LAG1, also known as LASS1 or the upstream of growth and differentiation factor 1 (UOG1)[87,88], demonstrated that it specifically regulated the synthesis of C_{18}-Cer with a high degree of fatty-acid chain length specificity[86,89]. There exist six LASS proteins (LASS1-6) that were renamed CerS1-6[86]. They all possess a

domain of five predicted transmembrane helices termed the TLC (TRAM, LAG1 and CLN8 homology) domain, which contribute to the CerS activity and Cer generation[90]. They also differ in their specificity, and thus produce dihydroceramides(DHCs) with differing chain lengths, which are then desaturated by desaturase(DES) to generate Cer with different fatty acid chain lengths[92,93]. Importantly, CerS1/4 mainly produce C_{18}-Cer, to a lesser extent C_{20}-cermides, whereas CerS5/6 selectively mediates C_{16}-cermide, to a lesser extent C_{12}- and C_{14}-cermides[89]. Moreover, CerS2 generates very long-chain Cers, predominantly C_{24}-cermides and the ultra-long-chain Cers are generated by CerS3[93,94]. There is evidence that Cer with different fatty acid chain lengths produced by CerS1-6 might have distinct functions, and/or mechanism of action in the regulation of cell death and/or autophagy. These recent studies suggest that not all Cers are created equal, and distinct fatty acid chain lengths, subcellular localization/transport, and/or down-stream protein/lipid targets, help define their specific roles in various different stress conditions and/or cell/tissue types. Recently, it has also been postulated that changes in the long chain sphingoid bases of ceramides with 18 versus 16 carbons also play distinct roles in inducing autophagy versus cell death in cardiomyocytes[95].

4.3 Cer and autophagy induction

Cer has been implicated in the autophagy induction[96]. Class I PI3K and Akt are well-established autophagy suppressors, and Cer functions to activate PP2A, which blocks Akt activation[97–99]. Various treatments, leading to long-chain Cer accumulation, such as long-chain endogenous Cer generation by exogenous short chain Cer recycling[100]. PDMP (GlcCer synthase inhibitor) or tamoxifen, invariably suppressed Akt activity to stimulate autophagy in human colon (HT-29) and breast cancer (MCF-7) cell lines[98]. Moreover, amino acid deprivation resulted in an increase in Cer levels, which suppressed mTOR activity and induced autophagy in a PP1/PP2A dependent manner. Cer-mediated autophagy induction is also closely linked with its influence on nutrient transporters[101– 103]. Edinger *et al*. demonstrated that Cer caused a rapid and substantial downregulation of amino acid transporter proteins in a number of cell types[103]. Similarly, Cer suppressed nutrient transporter protein expression, which induced starvation, leading to the AMPK-dependent autophagy induction[104]. Beclin1, the mammalian orthologue of yeast ATG6, plays a central role in autophagy, which is upregulated under stress, and it is mutated or monoallelically deleted in many human cancers[105,106]. Scarlatti *et al*. showed that exogenous C_2-Cer treatment enhances Beclin1 expression and autophagy induction, which was blocked by Cer synthase inhibitor myriocin, indicating Cer regulates Beclin1 expression at the transcriptional or post-transcriptional level[98]. C_2-Cer also activated JNK in human cancer cell lines CNE2 and Hep3B, and mediated activation and phosphorylation of c-Jun, a transcription factor, which induced Beclin1 expression at the transcription level[107].

Also, increased levels of Beclin1 can originate from the dissociation of the Beclin1-Bcl2 complex[108]. On the one hand, Cer stimulated stress-activated protein kinase JNK1, leading to Bcl2 phosphorylation, which liberated Beclin1 from its association with Bcl2[109]. On the other hand, Cer mediated activation of Forkhead box protein O3 (FOXO3), which is negatively regulated by Akt, significantly upregulated BH3-only protein BNIP3 expression[96,110]. Elevated BNIP3 expression dissociated Beclin1 from binding to Bcl2 through its competitive binding of Bcl2 and Bcl-xL[111]. Cer-mediated ER stress also contributes to autophagy induction[96]. Disregulated Cer homeostasis in the ER by CerS2 downregulation interfered with intracellular Cer trafficking and distribution[112]. CerS2 downregulation lead to accumulation of long-chain Cers (C_{14}-and C_{16}-Cers) and induces ER-stress related cytoprotective autophagy[112]. Russo *et al*. demonstrated that dietary myristate oversupply mainly depended on CerS5 to generate Cer, which consequently induced autophagy, resulting in the cardiomyocytes hypertrophy phenotype[113]. Recently,

we showed that C_{18}-pyridinium-Cer treatment or endogenous C_{18}-Cer generation by CerS1 expression mediated lethal autophagy induction, independent of apoptosis in human head and neck cancer cells[114]. C_{18}-Cer–induced lethal autophagy was regulated via LC3B-II, and selective targeting of mitochondria by LC3B-II– containing autophagolysosomes (mitophagy) through direct interaction between Cer and LC3B-II upon Drp1-dependent mitochondrial fission[114].

4.4 DHC and autophagy regulation

DHCs are the immediate precursors for *de novo* synthesis of Cer[65]. Also, DHC is always deemed as biologically inactive molecules[108]. Although its precise physiological role is still controversial and remains to be determined, DHC is shown to be involved in autophagy induction in a number of studies[115– 118]. First, in DU145 prostate cancer cells, autophagy was induced by addition of exogenous C_2-DHC while 4-HPR (N-(4-hydroxyphenylretinamide), which elevated DHC level, also induced autophagy, suggesting that a large increase of endogenous DHCs might be responsible for autophagy induction[119]. Second, Signorellli *et al*. demonstrated that resveratrol-induced autophagy occurred with elevated intracellular DHC levels due to inhibition of DHC desaturase(DEGS) activity and that DHC accumulation led to autophagy induction in human gastric cancer cells HGC-127[120]. Third, under a hypoxia condition, DHC accumulation led to protective autophagy induction, which enhances cell survival and suppress cell proliferation[117]. It was proposed that DHC might function to regulate cell fate by switching from protective autophagy to Cer-mediated apoptosis under stress[96]. Moreover, celecoxib (a selective COX-2 inhibitor) has been recently shown to induce autophagy[121], which predominantly produces C16:0, C24:0 and C24:1 DHCs in human cancer cell lines by inhibiting DEGSs activity[122]. These data suggest that DHCs are involved in autophagy induction, and at least endogenously generated DHCs are not simply inactive sphingolipid molecules[123]. Further studies should dissect and elucidate the signaling pathways that are regulated by DHCs to exert pro-autophagic effects.

5. Cer is involved in the regulation of paradoxical roles of autophagy

5.1 Cer induces cytoprotective autophagy

Cer, especially long chain Cer, is known to be involved in cell death signaling regulation[124]. However, under certain conditions, Cer may induce protective autophagy and utilize autophagic machinery to prevent cells from programmed cell death. Guenther *et al*. demonstrated that C_2-Cer or endogenous Cer starved cells to death by limiting intracellular nutrients subsequent to nutrient transporter downregulation[104]. However, ceramide also triggered homeostatic protective autophagy to prevent cell death as blocking autophagy with chloroquine (CQ) sensitized cells to ceramide exposure[104]. Spassieva *et al*. demonstrated that down-regulation of CerS2 resulted in no significant decrease of very long chain Cer(VLC) (e.g. C_{24} or $C_{24:1}$ Cer), but led to 3-fold increase in long chain Cer (LC) (e.g. C_{14} or C_{16} Cer)[112]. SMS-KCNR neuroblastoma cells didn't undergo apoptosis with LC accumulation, which, by contrast, induced autophagy evidenced by LC3B-II lipidation and autophagy-related structures in electron microscope[112]. Mechanistically, CerS2 downregulation stimulated autophagy and UPR (unfolded protein response), which activated the pro-survival IRE1(inositol-requiring element 1), preventing induction of cell death[112]. Also, Park *et al*. showed that anti-cancer drugs vorinostat and sorafenib activated CD95 via the activation of acid sphingomyelinase(ASMase) and generation of Cer[125]. Cer-CD95 induced autophagy in a PERK dependent manner, which showed cytoprotective effects since suppression of ATG5 expression enhanced sorafenib and vorinostat lethality[125]. Similarly, cyclin-dependent kinase (CDK) inhibitor via Cer-CD95 signaling induced both pro-death/apoptotic signal and pro-survival/autophagic signal in

primary hepatocytes[126]. Blockade of protective autophagy induced by Cer-CD95, using 3-MA or siRNA against Atg5, further enhanced cell killing[126]. Tamoxifen, which is known to inhibit GlcCer synthase, elevated endogenous Cer levels and induced autophagic cell death in MCF-7 cells[127], but also triggered protective autophagy to delay cell death in other cancer cells (Fig. 3) [128,129].

Although the precise role of DHC is not fully elucidated, a number of studies showed that it is able to induce protective autophagy[115–117,121]. DEGS inhibitor XM462 delayed cell cycle G1/S transition via activation of ER stress and induction of autophagy in gastric carcinoma HCG27 cells[115]. Inhibition of XM462-induced autophagy resulted in significant reduction of metabolic activity measured by MTT assay, indicating DHC induced autophagy promotes survival[115]. Devlin *et al*. also demonstrated that direct treatment with DHC or indirect augmentation of DHC by siRNA blockade of DEGS1 or 2 decreased cell proliferation[117]. Celecoxib, primarily producing C16:0-, C24:0- and C24:1-DHCs, induced autophagy in human colorectal cancer cells. Inhibition of autophagy using 3-MA and wortmannin or siRNA against ATG8 significantly enhanced Vps34-mediated apoptotic cell death[121].

5.2 Cer induces lethal autophagy

Cer is associated with cell growth arrest and cell death induction[130]. Cer plays a well-established role in triggering programmed cell death in response to growth factor withdrawal, death receptor ligation, hypoxia, and chemotherapeutic drugs[104]. Although many studies confirmed the pivotal role of Cer in mediating lethal autophagy, the precise underlying mechanism is still not completely understood. An initial report from Dbaibo *et al*. showed that arsenic trioxide (As_2O_3) induced accumulation of cytotoxic levels of Cer in human leukemia cells through *de novo* Cer synthesis and inhibition of GluCer synthase activity[131]. Qian *et al*. demonstrated that As_2O_3 induced not only apoptosis but also autophagic cell death in leukemia cell lines. The lethal autophagy was attributed to upregulation of Beclin-1 protein and completely prevented by autophagy inhibitor 3-MA[132]. A research study in malignant glioma cells showed that Cer induced autophagic cell death, which was evidenced by occurrence of autophagic vacuoles, acidic vesicular organelles and LC3B-II lipidation[109]. The mechanism depicted was that Cer decreased mitochondrial membrane potential and activated death-inducing mitochondrial protein BNIP3[109]. In human cancer cell lines CNE2(nasopharyngeal carcinoma) and Hep3B(hepatocellular carcinoma), the lethal autophagy was mediated by Cer-induced c-Jun activation through JNK signaling, which transcriptionally upregulated Beclin-1 expression[107]. JNK activity inhibitor SP600125, as well as Beclin-1 siRNA rescued cancer cells from Cer-induced autophagic cell death[107]. Melanoma differentiation associated gene-7(mda-7)/interleukin-24(IL-24) was shown to induce ER stress, which triggered Cer and ROS generation in a PERK-dependent manner[133]. Autophagy-mediated cell death by mda-7/IL-24 depended on Cer and ROS generation, which could be blocked by PERK inhibitor treatment[133]. Patschan *et al*. exposed human umbilical vein endothelial cells (HUVECs) to glycated collagen I (GC), which led to autophagy induction and ASMase activation, leading to accumulation of ceramide[134], and premature cellular senescence[134]. In human leukemia cells (HL-60) and Chinese hamster ovary cells (CHO), Cer-CAPPs(Cer activated protein phosphatases) have inhibitory effects on Akt-mTOR pathway, which activated autophagy and induced autophagy-mediated cell death, whereas S1P-S1P3 signaling activated Akt-mTOR pathway, which offset autophagy and suppressed lethal autophagy[135]. Hou *et al*. found that C_6-pyridinium Cer preferentially promoted autophagy induction and induced mitochondrial permeabilization, which retarded MCF-7 cells growth and activated apoptosis[136]. Salazar *et al*. demonstrated that tetrahydrocannabinol (THC), which was known to induce Cer accumulation and eukaryotic

translation initiation factor 2α (eIF2) phosphorylation, activated ER stress, leading to autophagy induction via tribbles homolog 3 (TRB3) mediated mTOR inhibition. Cannabinoid-mediated lethal autophagy led to human glioma cell death and contributed to its anti-tumor action *in vivo*[137]. Our recent data suggested that $C1_8$-pyridinium Cer treatment or endogenous $C1_8$-Cer generation by CerS1 expression induced lethal autophagy, independent of apoptosis in human head and neck cancer cells[114]. Moreover, knockdown of CerS1 abrogated sodium selenite–induced mitophagy, and stable LC3B-II knockdown protected against CerS1- and C18-Cer–dependent mitophagy and blocked tumor suppression in vivo[114]. Mechanistically, our data suggested that ceramide-LC3-II interaction plays a key role to target autophagolysosomes to damaged mitochondria, which required Drp-1 mediated mitochondrial fission. This, then, results in reduced oxygen consumption rate, decreased ATP production, and subsequent cell death in response to ceramide stress in head and neck cancer cells (Fig. 4) [112].

DHC may play a similar role like Cer to induce autophagic cell killing. Gamma-tocotrienol (γTE) treatment led to marked increase of intracellular DHC and dihydrosphingosine and autophagy induction in human prostate PC-3 and LNCaP cancer cells, which caused fatality and decrease cell viability in vitro and inhibited xenograft growth *in vivo*[118]. This phenomenon was prevented by myriosin, a specific inhibitor of *de novo* sphingolipid synthesis, supporting the notion that DHC and dihydrosphingosine accumulation could be utilized as a novel anti-cancer mechanism of γTE[118].

6. Conclusion & future perspectives

Autophagy primarily regulates cellular homeostasis[11]. It functions not only to remove aggregated proteins and damaged organelles, but also supplies cells with energy and metabolites to maintain normal cellular functions under stressful conditions[24,39]. However, autophagy has paradoxical functions to regulate the cell fate. It may also induce cell death in two dependent mechanisms: one is caspase-independent (autophagic) cell death, the other is caspase-dependent (iDISC-mediated) cell death[96].

Autophagy is a tumor suppressor mechanism, supported by its role in preventing oxidative stress, maintaining normal mitochondrial function and safeguarding against DNA damage and genetic instability[39]. Autophagy is also a tumor promoting mechanism, which, established cancer cells exploit to meet increased metabolic demands. Also, autophagy is upregulated by cancer cells to resist cancer treatment, suppression of which enhances cytotoxicity of cancer therapy[22,33,49]

Sphingolipids are crucial regulators of autophagy. Various sphingolipids metabolites execute different functions in autophagy regulation. Based on the current literature, S1P-depedent autophagy has been mostly associated with cell survival[135,138–140], which is also comprehensively reviewed by many investigators[67,75,96,108,141,142]. Although Cer-mediated autophagy has also been found to be cytoprotective[104,112], it mainly functions to promote cell death[98,109,114]. Cer utilizes various mechanisms to induce autophagy. It is known to induce autophagy via the blockade of Akt and mTOR signaling[97–99]. It also leads to starvation-induced autophagy by downregulating nutrient transporters[104]. Moreover, it activates JNK/c-Jun pathway to upregulate Beclin1 or dissociates Beclin1 from its binding with Bcl2[98,107,109]. Cer-mediated ER stress is also involved in autophagy induction[96,112]. Our group demonstrated that mitochondrial ceramide directly binds and recruits LC3B-II labeled autophagosomes to damaged mitochondria forlysosomal degradation, also termed lethal mitophagy, [114]. In fact, generation of C_{18}-ceramide by CerS1 and ceramide localization to mitochondria were required for stress-induced lethal autophagy/mitophagy, which was Drp1-mitochondrial

fission dependent [114], placing mitochondrial ceramide in the center for selectively inducing lethal mitophagy (Fig. 4). Sphingolipid metabolism and related metabolites are closely linked to mitochondria function[96]. Further studies are needed to dissect the role of Cer in the regulation of lethal mitophagy induction, such as its involvement in manipulating the Parkin/Pink1 pathway. Interestingly, DHC is also involved in autophagy induction[115–117,121]. It may cooperate with Cer to regulate autophagy and determine cell fate. However, the biological activity of DHC is still controversial and needs to be warranted. Finally, the role and mechanisms of action of different Cer species or chain-length specific Cers in autophagy regulation remain unclear, and need to be elucidated[119,123].

Dysregulated Cer metabolism and/or trafficking has been observed in many cancer studies and is closely related to cancer progression and metastasis[143– 146]. Cer has been shown to act as a novel biomarker of cancer therapy response[147], as well as a potential target for the generation of chemotherapeutics[148,149]. In this review, we mainly described the role of Cer in lethal autophagy regulation, which might be an essential mechanism Cer utilize to induce cell killing. In fact, several autophagy-inducing, as well as Cer producing drugs, such as As_2O_3, tamoxifen and resveratrol, execute their anticancer function via autophagy modulation[108]. Therefore, those observations support the notion that Cer-mediated autophagic cell death may be a promising strategy to inhibit tumor growth. An enormous growth of Cer and DHC research will be anticipated to produce numerous advances in dissecting the roles and mechanisms of Cer and DHC in the regulation of autophagy and cancer pathogenesis. Cer-mediated autophagic cell death pathways may help develop novel therapeutic and/or prevention strategies against various human cancers.

In summary, although there are solid studies to establish a role for Cer in inducing autophagy, more mechanistic studies are needed to clarify how Cer regulates autophagy-mediated cell death versus protection (autophagy paradox) in response to various stress conditions in different cancer types. This regulation seems to be context dependent with regard to subcellular localization of ceramides (mitochondria versus ER), fatty acid chain length composition of ceramides (C_{18}-ceramide versus C_{16}-ceramide), and/or presence/absence of downstream targets of ceramides (Drp1 and LC3B-II) for induction of lethal versus survival autophagy. To this end, it is important to develop new techniques for purification of autophagolysosomes versus lysosomes or mitochondria, and measure their sphingolipid composition using mass spectrometry-based lipidomics, which will help define the roles of specific sphingolipids (and their subcellular localization) in the regulation of autophagy paradox. In addition, defining the molecular details of sphingolipid-protein binding, such as ceramide-LC3B-II complex [114], will be important to understand the mechanisms by which ceramide regulates lethal versus survival autophagy.

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Highlights

- Sphingolipids, including ceramide and sphigosine 1-phosphate are key bioactive molecules that regulate autophagy.
- This review article focuses on the roles of ceramide on the regulation of autophagy.
- Ceramide and dihydro-ceramide induce macroautophagy in various cell types under different stress conditions.
- Ceramide-mediated autophagy can be protective or lethal in cancer cells, which is context dependent.
- Ceramide-induced autophagy includes lethal mitophagy that results in tumor suppression.

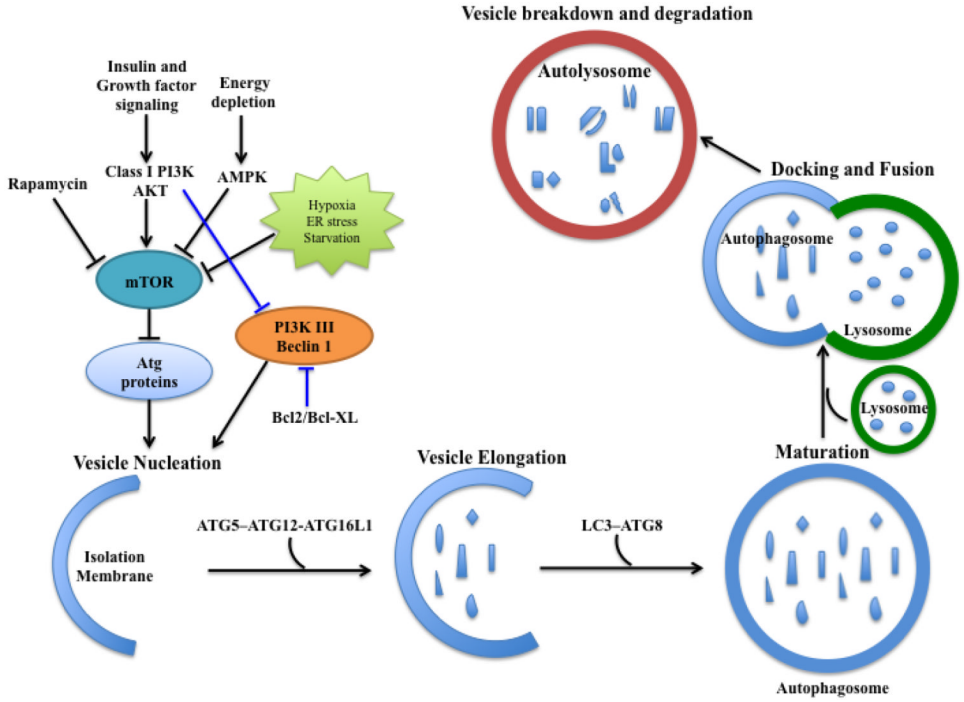

Fig 1. Schematic view of the autophagic pathway

Autophagic cellular events have distinct stages: vesicle nucleation (formation of the isolation membrane/phagophore), vesicle elongation and maturation, autophagosome-lysosome fusion, and vesicle breakdown and degradation. Autophagy occurs at a basal level and responds to various environmental signals and stress. The well-characterized regulatory modulators include PI3K-AKT-mTOR and Beclin1-Class III PI3K complexes. Insulin and other growth factors activate PI3K-AKT-mTOR, which negatively regulates autophagy. Environmental stress and energy depletion inhibit mTOR, relieving its inhibitory effects on autophagy induction. Both PI3K-AKT and Bcl2/Bcl-XL suppress Beclin1-Class III PI3K complex to inhibit autophagy. Autophagosomal elongation requires ubiquitin-like conjugation system, ATG5-ATG12-ATG16L1 complex. However, the conversion of LC3-I to LC3-II (PE-conjugated form) is a key regulatory step in autophagosome maturation.

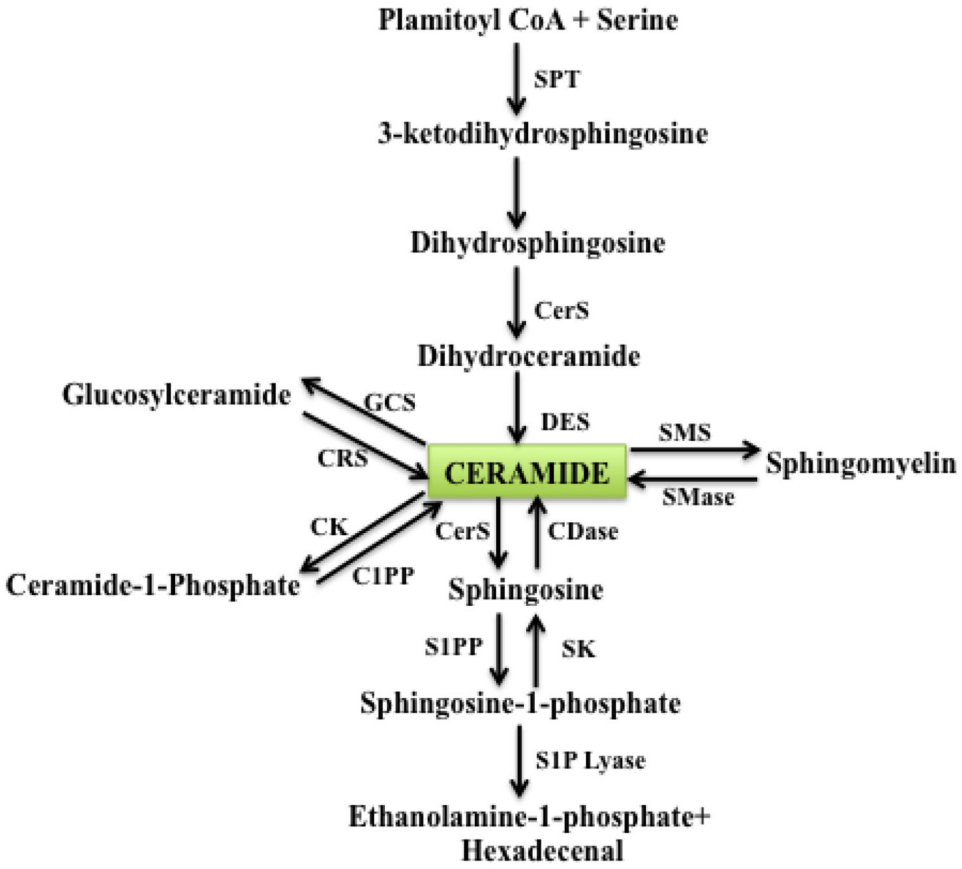

Fig 2. Pathways of sphingolipid metabolism

Ceramide can be formed *de novo* or from hydrolysis of sphingomyelin or cerebrosides. Conversely, ceramide can be phosphorylated by ceramide kinase to yield ceramide-1-phosphate, or can serve as a substrate for the synthesis of sphingomyelin or glycolipids. Ceramide can be metabolized by ceramidases (CDases) to yield sphingosine, which in turn is phosphorylated by sphingosine kinases (SKs) to generate sphingosine-1-phosphate (S1P). S1P can be cleared by the action of specific phosphatases that regenerate sphingosine or by the action of a lyase that cleaves S1P into ethanolamine-1-phosphate and a C_{16}-fatty-aldehyde. C1PP, ceramide-1-phosphate phosphatase; CRS, cerebrosidase; CK, ceramide kinase; CerS, ceramide synthase; DES, dihydroceramide desaturase; GCS, glucosylceramide synthase; S1PP, S1P phosphatase; SMS, sphingomyelin synthase; SMase, spingomyelinase; SPT, serine palmitoyl transferase.

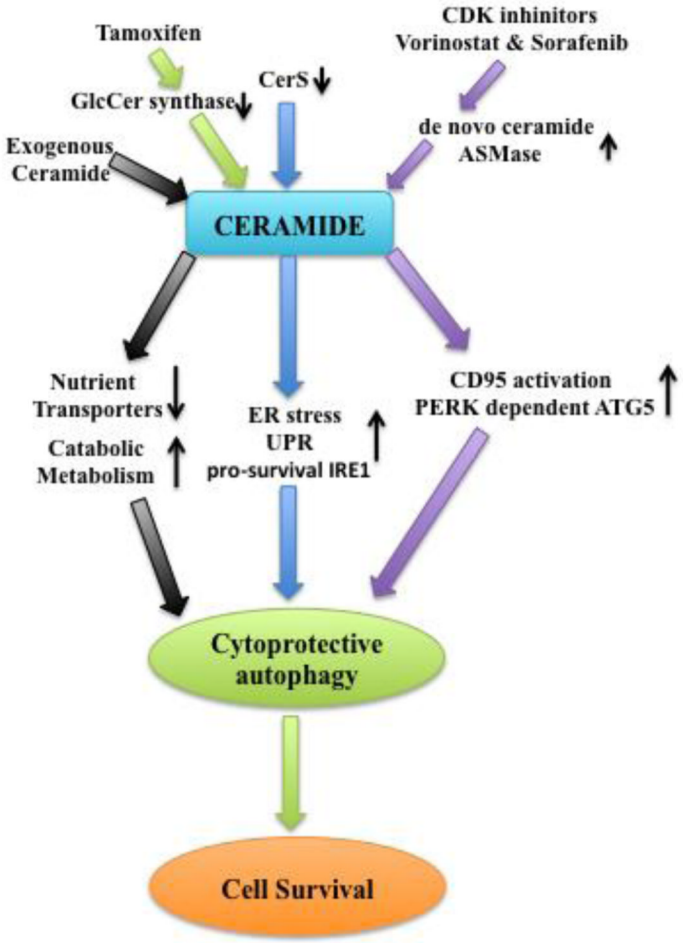

Fig 3. Signaling pathways of protective autophagy that are regulated by ceramide

Exogenous ceramide induces mild nutrient transporter downregulation, which triggers protective autophagy and enhances catabolic metabolism to maintain cell viability. Ceramide synthase downregulation, resulting in long chain ceramide accumulation, stimulates ER stress and activates pro-survival IRE1 (inositol-requiring element1), which leads to protective autophagy induction and cell death inhibition. Anti-cancer drugs vorinostat and sorafenib, as well as cyclin-dependent kinase (CDK) inhibitor, contribute to upregulation of de novo ceramide generation and acid sphingomyelinase (ASMase) activation, which leads to ceramide accumulation. Ceramide accumulation results in CD95 and PERK-dependent ATG5 activation, leading to protective autophagy induction and inhibition of cell death. Tamoxifen, which is known to inhibit GlcCer synthase, not only elevated endogenous Cer levels, but also triggered protective autophagy to delay cell death.

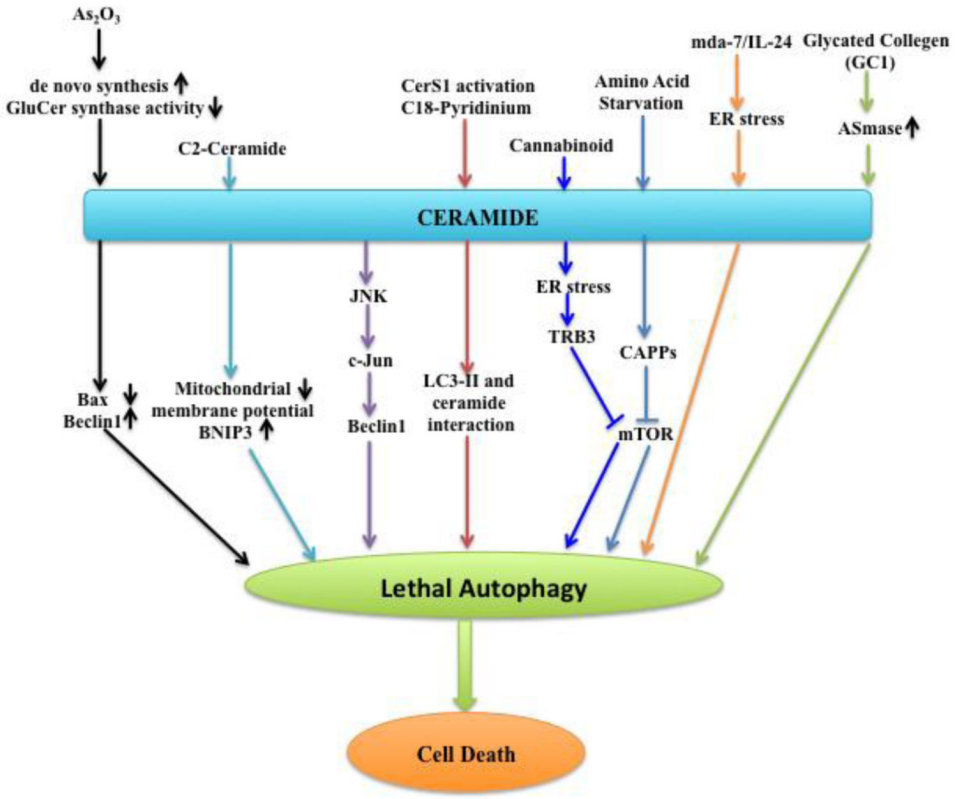

Fig 4. Signaling pathways of lethal autophagy that are regulated by ceramide

Under most conditions, ceramide leads to lethal autophagy induction and cell death. Arsenic trioxide (As_2O_3) induces cytotoxic ceramide accumulation via de novo synthesis upregulation and inhibition of GluCer synthase activity, which stimulates Beclin1 expression, leading to lethal autophagy. Ceramide mediated autophagic cell death may be attributed to mitochondrial membrane potential reduction and death-inducing mitochondrial protein BNIP3 activation. Ceramide also induced c-Jun activation through JNK signaling, which transcriptionally upregulated Beclin-1 expression, leading to autophagic cell death. C_{18}-pyridinium ceramide treatment or CerS1 activation induces lethal autophagy, independent of apoptosis, in which ceramide/LC3II interaction plays a key role to target autophagolysosomes to damaged mitochondria. Cannabinoid, which was known to induce ceramide accumulation, activated ER stress, leading to lethal autophagy induction via tribbles homolog 3 (TRB3) mediated mTOR inhibition. Amino acid deprivation also induces CAPPs (ceramide activated protein phosphatases), which have inhibitory effects on Akt/mTOR pathway, resulting in autophagy-mediated cell death. Melanoma differentiation associated gene-7(mda-7)/interleukin-24(IL-24) was shown to induce ER stress, which triggered ceramide-mediated lethal autophagy. Glycated collagen1 (GC1) exposure, which leads to ceramide accumulation and acid sphingomyelinase (ASMase) activation, induces autophagy mediated cellular senescence.