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Forum Review Article

Oxidative Stress and Autophagy in the Regulation of Lysosome-Dependent Neuron Death

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Abstract

Lysosomes critically regulate the pH-dependent catabolism of extracellular and intracellular macromolecules delivered from the endocytic/heterophagy and autophagy pathways, respectively. The importance of lysosomes to cell survival is underscored not only by their unique ability effectively to degrade metalloproteins and oxidatively damaged macromolecules, but also by the distinct potential for induction of both caspase-dependent and -independent cell death with a compromise in the integrity of lysosome function. Oxidative stress and free radical damage play a principal role in cell death induced by lysosome dysfunction and may be linked to several upstream and downstream stimuli, including alterations in the autophagy degradation pathway, inhibition of lysosome enzyme function, and lysosome membrane damage. Neurons are sensitive to lysosome dysfunction, and the contribution of oxidative stress and free radical damage to lysosome dysfunction may contribute to the etiology of neurodegenerative disease. This review provides a broad overview of lysosome function and explores the contribution of oxidative stress and autophagy to lysosome dysfunction–induced neuron death. Putative signaling pathways that either induce lysosome dysfunction or result from lysosome dysfunction or both, and the role of oxidative stress, free radical damage, and lysosome dysfunction in pediatric lysosomal storage disorders (neuronal ceroid lipofuscinoses or NCL/Batten disease) and in Alzheimer's disease are emphasized. *Antioxid. Redox Signal.* 11, 481–496.

Introduction

Lysosomes were discovered >50 years ago by Christian de Duve (41) in a series of serendipitous experiments aimed originally at characterizing liver glucose 6-phosphatase. De Duve discovered the association of glucose 6-phosphatase with a labile enzyme called acid phosphatase, which fractionated with populations of mitochondria and microsomes. On further optimization of their fractionation protocols, a "light mitochondrial" fraction was discovered that was intermediate in sedimentation to that of mitochondria and microsomes. Subsequent analysis of this purified fraction delineated several more enzymes, one of which was cathepsin D (CD), which had acid pH optima. Today the scientific community appreciates the lysosome as an organelle with the critical function of regulating the pH-dependent degradation of intracellular macromolecules. The ability of lysosomes to compartmentalize degradation within their lumen protects the rest of the cell from the transient induction of oxidative stress and cytoplasmic degradation. Under conditions of cell stress, however, lysosome function and integrity may become compromised and can trigger regulated cell death. Instrumental in this cell-death induction are alterations in the vesicular recycling pathway autophagy, which can induce lysosomal dysfunction or become compromised as a result of lysosomal dysfunction or both. In addition, oxidative stress may cause direct, intralysosomal damage or cause secondary lysosomal damage through the increased production of damaged macromolecules or organelles. This review provides an overview of lysosome function and the role that oxidative stress and autophagy play in lysosomal damage. Lysosomal death pathways are explored in great

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detail, with particular focus to their role in age-related neurodegenerative diseases including Alzheimer's disease and the pediatric neurodegenerative disease neuronal ceroid lipofuscinoses (NCL)/Batten disease.

Lysosome Structure, Function, and Assembly

Lysosomes serve an important intracellular role as the site for the terminal proteolytic degradation of damaged proteins and organelles, which is accomplished in the range of pH 4.5 to 5 *via* >50 lysosomal hydrolases with acidic pH optima (113). Morphologically, lysosomes are cytoplasmic dense bodies that are either spheroid, ovoid or occasionally tubular in appearance (113). Neuron lysosomes are typically <1 μm in size and are often situated in a perinuclear position (113). Lysosomal hydrolases are surrounded by a limiting membrane containing an abundance of glycosylated proteins (117). An intact lysosomal membrane provides the barrier necessary to maintain such a low pH compared with the neutral pH of the surrounding cytosol. Upward of two dozen cathepsins are known, with specificities for different peptide bonds, including the cysteine proteases cathepsins B (CB), H, and L or the aspartic acid protease CD. Lysosomal hydrolases catalyze the pH-dependent degradation of proteins into amino acid pools for intracellular recycling. As is discussed in subsequent sections, the increase in posttranslational oxidative modifications has been shown to decrease the effective degradation of proteins by lysosomal hydrolases and may lead to an increase in protein accumulation, which may contribute to the increase in autofluorescent lipopigment in postmitotic neurons (149).

Although lysosomal hydrolases reside at their terminal location in lysosomes, their synthesis and transport to lysosomes requires a complex series of events that carries them through many different organelles and vesicles (Fig. 1). Their localization to lysosomes must be confirmed either by colocalization with lysosomal membrane proteins such as LAMP-1 or LAMP-2 (49) or by subcellular fractionation. Lysosome synthesis begins initially in the endoplasmic reticulum (ER) (142), where newly synthesized hydrolases contain an N-terminal, 20- to 25-amino acid signal peptide, which allows their translocation into the ER lumen. On cleavage of the signal peptide, oligosaccharides are added onto the hydrolases, which allows the enzymes to be equipped with mannose-6-phosphate (M6P) recognition markers in the *trans* Golgi network (TGN). This M6P tag allows lysosomal hydrolases to recognize and bind to M6P receptors (M6PRs), and the receptor–ligand complex subsequently exits from the TGN in clathrin-coated vesicles as they deliver their contents directly to late endosomes or indirectly *via* delivery to early endosomes, which are thought to mature into late endosomes. Endosomes exhibit an acidic pH, as do lysosomes, but can be distinguished from lysosomes in that lysosomes are M6PR negative. The low pH of endosomes facilitates dissociation of lysosomal hydrolases from M6PRs, which allows the vesicle-mediated recycling of M6PRs back to the TGN. Concomitant with further maturation steps, including dephosphorylation, oligosaccharide trimming, and proteolytic activation, lysosomal hydrolases arrive at the lysosomes, events that are mediated most likely by a type of fusion event between the late endosome and lysosome (86).

Autophagy

Intracellular macromolecules and organelles are delivered to lysosomes for degradation and recycling by autophagy

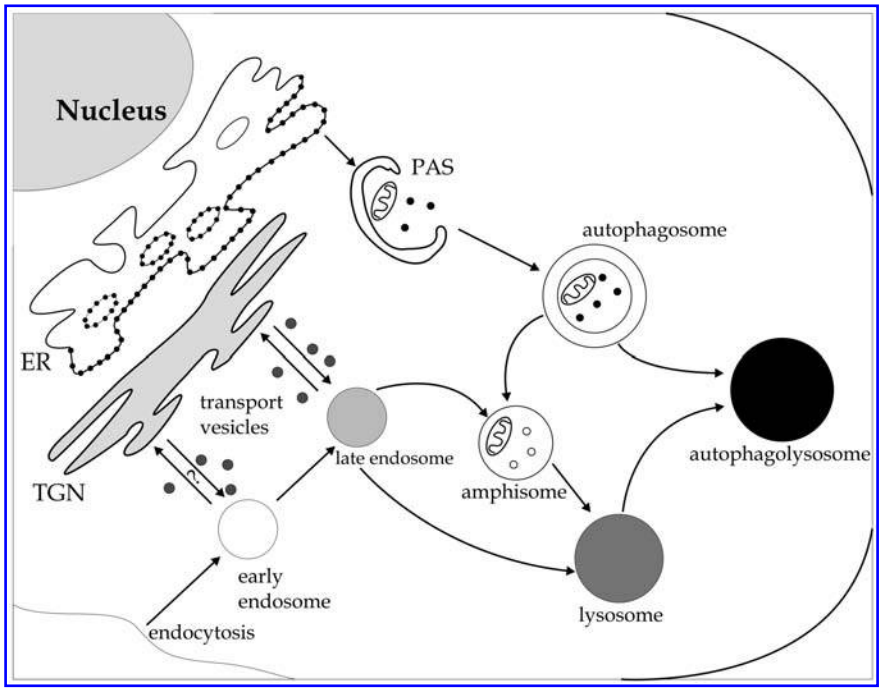

FIG. 1. Convergence of the endosomal–lysosomal and autophagy–lysosomal degradation pathways. Lysosomal hydrolases are produced in the endoplasmic reticulum (ER) and, on delivery to the trans-Golgi network (TGN), are transported in vesicles by recognition of mannose-6-phosphate receptors (M6PRs) to the late endosome (or to the early endosome, which then matures to form the late endosome). The late endosome is then thought to deliver lysosomal hydrolases *via* a type of fusion event to their terminal location, the lysosome, which is M6PR negative. Damaged organelles and macromolecules are surrounded by a limiting membrane from the ER to form a preautophagosomal structure (PAS), which matures to form the double membraned autophagosome. The pH of autophagosomes is not sufficient to degrade their intraluminal contents, and fusion with lysosomes (forming the autophagolysosome) or with endosomes (forming an amphisome), which both contain pH-dependent acid hydrolases, must take place for autophagosomal contents to be effectively degraded. Please refer to text for further details.

(Greek for "eat oneself"), and several types of autophagy dictate the manner in which macromolecules and organelles arrive at the lysosome (77). Arguably the best-studied type of autophagy is macroautophagy (Fig. 1), which involves the generation of a double-membraned autophagosome that forms nonselectively around bulk cytoplasm, and the shuttling of these contents through a series of vesicular fusion events to the lysosomes for pH-dependent degradation by lysosomal hydrolases (for review, see ref. 131). Autophagosomes may fuse with either late endosomes or lysosomes (131), which both contain lysosomal hydrolases in an acidic environment that facilitates degradation. The fusion of autophagosomes with endosomes forms single-membraned amphisomes (59, 85), which fuse ultimately with lysosomes for terminal degradation. Macroautophagy is induced by intracellular nutrient stress or energy depletion or both and is regulated at multiple levels by upward of 30 known autophagy-related gene (Atg) proteins, including signals that stimulate autophagy induction, the initiation and completion of autophagic vacuole formation, and the recycling of autophagic vacuoles (for review, see ref. 131). Chaperone-mediated autophagy (CMA) is a more-selective form of autophagy in which specific cytosolic proteins with KFERQ sequences are targeted by chaperone proteins such as hsc70 to the lysosome, followed by internalization in lysosomes by the membrane-bound, Lamp2a receptor (45). Microautophagy is a less well-defined type of autophagy in which lysosomes directly ingest cytosolic nutrients by membrane involution (158). Although microautophagy has been identified and studied in simple organisms such as yeast, its occurrence and significance in mammalian cells is unclear. Organelle-specific macroautophagy (*e.g.*, mitophagy, reticulophagy) also has been identified and may selectively target damaged organelles for lysosomal degradation (10, 76, 146). Heterophagy, by definition, is distinct from autophagy because it involves the intracellular degradation of *extracellular* material, which is mediated by endocytosis and the delivery of material to lysosomes from endosomes (120).

Redox-Reactive Iron and Intralysosomal Damage

Lysosomes play a critical role in the breakdown of iron-containing macromolecules on their delivery to lysosomes by autophagy, and as such, the lysosome contains high levels of iron (15, 111, 115, 121). Metalloproteins such as ferritin have been shown to rely on intact lysosome function for their effective degradation and removal of iron, which is thought to provide an important source of free iron for essential intracellular functions (75, 82, 111, 115). Although the compartmentalization of high concentrations of potentially redox-active iron within lysosomes is in theory a protective measure for the rest of the cell, it may also increase the susceptibility for intralysosomal damage and the induction of cell death (167). The brain and neurons, in particular, contain relatively high levels of iron, and iron has been shown to accumulate in neurons with aging (125), which further implicates the potential for iron-mediated damage in age-related neurodegenerative disease. Ferric iron (containing at least one uncoordinated ligand) may react with hydrogen peroxide in forming ferrous iron, along with the deleterious hydroxyl radical, by the Fenton reaction (81). The acidic pH of lysosomes in addition to the presence of reducing equivalents such as cysteine provides a hospitable environment for Fenton chemistry (7), and hydrogen peroxide may readily diffuse into the lysosomal lumen from the cytoplasm, especially under conditions of oxidative stress. In addition, lysosomes do not ordinarily contain reducing enzymes such as catalase or glutathione peroxidase unless they are being degraded by autophagy, which exacerbates the potential for reactive iron-induced damage in lysosomes (81). Hydroxyl radical can oxidize a host of macromolecules, including lipids and proteins, which may not only inhibit their degradation and contribute to the accumulation of intralysosomal lipofuscin as discussed below, but also may inhibit the function of lysosomal hydrolases, further decreasing the degradative capacity of lysosomes (65, 133). In addition, the accumulation of oxidized lipoproteins within lysosomes may negatively affect the integrity of lysosomal membranes and provide a stimulus for the induction of lysosomal membrane permeabilization (LMP), as discussed later.

Conversely, the autophagy of thiol-rich proteins, including metallothioneins, has been proposed to counteract lysosomal damage by binding redox-active iron and other transition metals such as zinc within lysosomes, thus decreasing the probability of Fenton chemistry occurring (7, 32). In addition, under some experimental conditions, the iron chelator desferrioxamine has been shown to attenuate cell damage and cell death through its ability to localize within lysosomes and bind intralysosomal free iron (80, 109, 139, 167).

Lipofuscin and Oxidative Stress

Lipofuscin is an intralysosomal waste material that accumulates in postmitotic cells such as neurons as a function of aging, or in dividing cells whose rate of proliferation has been compromised (reviewed in ref. 17). The makeup of lipofuscin is chemically and morphologically amorphous, consisting of protein and lipid, carbohydrates, transition metals, and autofluorescent pigment (17). The accumulation of lipofuscin in postmitotic cells is closely related to a compromise in its effective degradation, combined with a lack of effective exocytosis (148). Lipofuscin accumulation is associated with age-related neurodegenerative diseases such as Alzheimer (25–27) and in lysosomal storage disorders including NCL/Batten disease (47), which may be related in part to known alterations in the macroautophagy–lysosomal degradation pathway that exist in these diseases. Whereas it is clear that lipofuscin accumulation correlates with lysosome dysfunction, it is not clear the extent to which its accumulation directly contributes to the induction of neuron death, although adverse effects on cell function have been reported (96), with an increased susceptibility of lipofuscin-loaded fibroblasts to apoptosis (147). Regardless, the finding that up to 75% of a neuron's perikarya may contain lipofuscin [reviewed in (149)] suggests that altered lysosome function may exacerbate the sensitivity of neurons to lysosomal death signals.

The inhibition of lipofuscin degradation may result from either the inhibition of lysosomal hydrolases or an increase in oxidative stress or both. Lipofuscin accumulation has been described experimentally by the chemical inhibition of lysosomal hydrolases, either from treatment with protease inhibitors or from the lysosomotropic agent chloroquine (69,

70, 148). Age-related decreases in the activity of lysosomal hydrolases have also been documented, which may contribute to the age-related increase in lipofuscin with normal brain aging (3, 70). Conversely, the overloading of cells with lipofuscin has been shown to cause a decrease in the activity of lysosomal hydrolases (133), suggesting that lipofuscin accumulation *per se* may also initiate a compromise in lysosome function. The ability of oxidative stress to enhance lipofuscinogenesis has been documented in several cell types (137, 151, 159). Lipofuscinogenesis may be caused by proteins that are oxidatively modified outside the lysosome and subsequently delivered to lysosomes for degradation, or may be caused by the intralysosomal formation of reactive oxygen species (ROS), as suggested by the potential for lysosomal lipoproteins to acquire oxidative cross-links (16). The effect of either route would be a net increase in oxidatively modified lipofuscin, with an inherent compromise in its degradative capacity. The importance of oxidative stress in lipofuscin accumulation is further emphasized by its decrease on experimental treatment with antioxidants or the iron chelator desferrioxamine (151). In addition, the inhibition of lysosomal hydrolases may exacerbate the oxidative stress–induced accumulation of lipofuscin, because a compromise in intralysosomal enzymatic protein degradation would provide greater opportunities for such proteins to acquire oxidative modifications that contribute to lipofuscin accumulation. In support of this argument, the accumulation of lipofuscin induced by combined oxidative stress and protease inhibition was shown to be three times greater than that observed by either condition alone (148).

Lipofuscin is formed from a variety of intracellular sources that are delivered to lysosomes by the autophagy degradation pathway (for review, see ref. 131). The induction of macroautophagy may provide a potent stimulus for lipofuscin accumulation (Fig. 2). Nutrient deprivation and resultant oxidative stress are natural stimuli for macroautophagy induction, and as such, may result in the increased delivery of undegradable, oxidatively modified proteins to lysosomes that accumulate as part of lipofuscin. Along these lines, ROS induced by starvation were found recently to regulate macroautophagy induction critically through the cysteine-dependent activity of Atg4, an autophagy-specific protein that regulates autophagosome formation (124). The induction of mitophagy may also increase the lysosomal delivery of oxidatively damaged mitochondrial membranes and proteins, in addition to superoxide anion, which is generated normally in mitochondria by the electron-transport chain (37). In further support of mitophagy contributing to lipofuscin accumulation, subunit c of mitochondrial ATP synthetase has been shown to be a major component of lipofuscin, in particular in aged neurons (47). An increase in intralysosomal redox-active iron also may result from the autophagy-mediated degradation of ferritin (75, 111, 115, 121). Under conditions of oxidative stress, the diffusion of readily available hydrogen peroxide into the lysosomal lumen may drive Fenton chemistry to form the highly reactive hydroxyl radical that would promote oxidative cross-links that enhance lipofuscin accumulation, a hypothesis that has been previously proposed and is further supported by the increase in lipofuscin accumulation on inhibition of lysosomal pro-

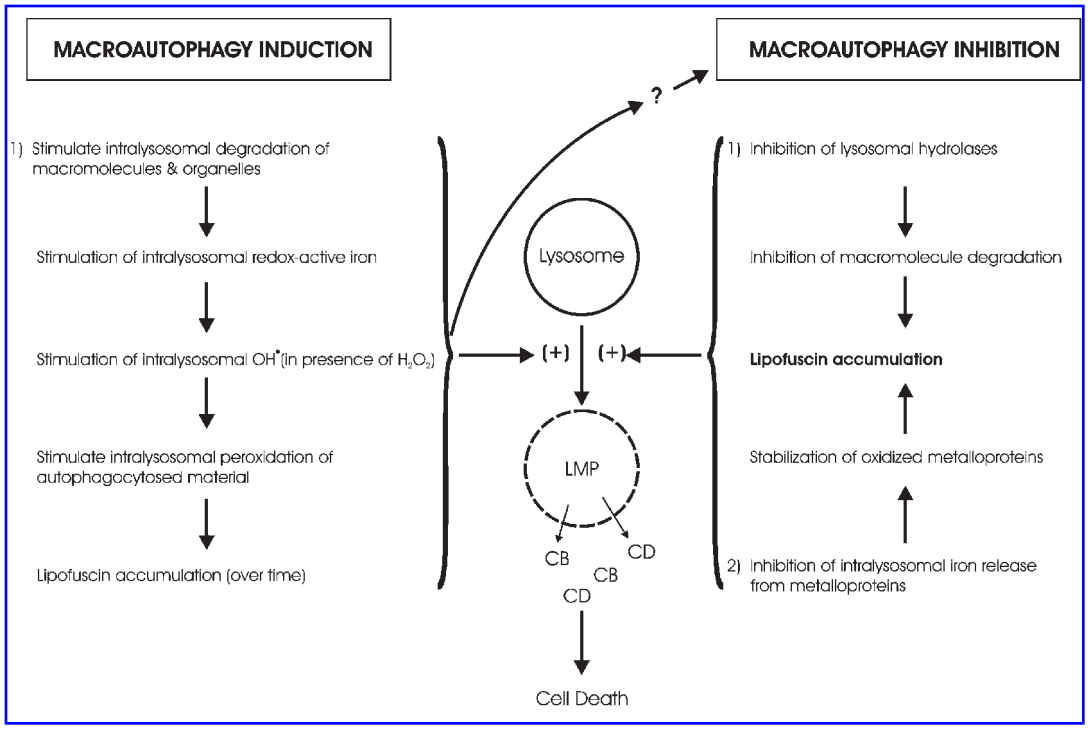

FIG. 2. Macroautophagy induction *versus* inhibition in oxidative stress–induced lysosome damage. The induction of lysosomal membrane damage, LMP, and cell death may be directly influenced by both the aberrant induction and inhibition of macroautophagy, which can lead to the induction of intralysosomal oxidative stress. It has also been proposed that an initial overinduction of macroautophagy induction may lead to an eventual inhibition of macroautophagy, which also may be related in part to the induction of oxidative stress. Please see text for further details.

teases (148). The generation of intralysosomal free radicals may cause peroxidation of membrane polyunsaturated fatty acids to form relatively stable and cytotoxic aldehydes, alkenals, or hydroxyalkenals, including malondialdehyde or 4-hydroxy-nonenal (4-HNE) (50). Treatment of purified protein with 4-HNE, for instance, has been shown not only to form protein cross-links (36, 54, 156) and generate protein-associated fluorescence similar to that found in the autofluorescent lipofuscin (55, 63, 152), but also to cause enzyme inactivation (30, 38, 50, 134, 144, 156) that may further enhance lipofuscin accumulation.

Inhibition of macroautophagy completion may also contribute to the accumulation of lipofuscin (Fig. 2), as was shown previously by treatment with the lysosomotropic agent chloroquine or with protease inhibitors (69, 70, 148). Treatment with lysosomotropic agents and protease inhibitors has been shown to increase intralysosomal ferritin stability and decrease the available pools of redox-active iron (75, 82), which, in contrast to macroautophagy induction, may suggest a limited role for redox-active iron and Fenton chemistry in the intralysosomal production of ROS after macroautophagy inhibition. Rather, the inhibition of lysosomal hydrolases may initially play a more direct role in lipofuscin accumulation after macroautophagy inhibition, because in this setting, it would be logical to predict a more-direct compromise in lysosome function as the initial stimulus for altered macroautophagy. Because oxidized lipoproteins or lipofuscin accumulation has been shown experimentally to decrease the activity of lysosomal hydrolases (65, 133), it is possible that lipofuscin accumulation *per se* may also initiate a compromise in lysosome function that would lead to macroautophagy inhibition, perhaps as a response to initial macroautophagy induction. This explanation is attractive for the etiology of Alzheimer disease neuropathology, as it was hypothesized previously that macroautophagy is induced early in the course of AD onset, which is followed in later stages by macroautophagy inhibition (103, 105).

Lysosomotropic Agents Generate Oxidative Stress

Christian De Duve (42) coined the term "lysosomotropic" in 1974 to delineate a group of uncharged compounds, typically amphiphilic weak bases, that are attracted to acidic compartments within cells, or are, in other words, "acidotropic." Such uncharged molecules diffuse passively through the membranes of acidic organelles, including lysosomes, which have a typical pH range of 4.5 to 5 (106). Once inside lysosomes, these agents become protonated, and their charge effectively precludes their transport across lysosomal membranes, resulting ultimately in an effective increase in intralysosomal pH and the impairment of lysosome-mediated degradation (42, 126). Accumulation of such agents in lysosomes depends initially on the pH gradient between the intra- and extralysosomal compartments and can be prevented by the prior increase in intralysosomal pH.

Chloroquine [7-chloro-4-(4-dimethylamino-1-methylbutylamino)quinoline; see structure, Fig. 3] is a well-known antimalarial agent that has been used for many years to investigate lysosome function. Chloroquine exerts its antimalarial effects by concentrating in the acidic digestive vacuole of *Plasmodium* parasites, where it is hypothesized to complex with ferric heme (ferriprotoporphyrin IX, FPIX) monomer (51), which is produced on parasitic degradation of host hemoglobin. By complexing with FPIX, chloroquine promotes accumulation of the toxic, undimerized form of FPIX, which increases susceptibility to iron-dependent peroxidation of lipid membranes (51), an effect that has been observed with treatment of liposomes with the chloroquine–FPIX complex (143). It is thus reasonable to predict that chloroquine also forms a similar type of lipid peroxidation–generating complex with iron-containing proteins in the lysosomes of mammalian cells. In support of this argument, chloroquine has been shown effectively to inhibit the intralysosomal release of free iron from ferritin, which is known to require intact lysosome function (75, 82). Regardless, chloroquine does induce lipid peroxidation in mammalian cells (11, 68, 112), and future studies are needed to delineate whether this occurrence is specific for lysosomal membranes. Because chloroquine effectively inhibits the intralysosomal release of free iron from ferritin, Fenton chemistry may not play a principal role in the induction of lysosomal damage mediated by chloroquine and subsequent macroautophagy inhibition.

Alternatively, chloroquine-induced oxidative damage to lysosomal membranes and the accumulation of oxidatively modified lipoproteins may result from macroautophagy inhibition combined with its inhibition of lysosomal proteases (4, 43, 53, 62, 163) mechanisms that may be responsible for its induction of lipofuscin, as previously described (70). Chloroquine also was shown recently to reduce intracellular levels of glutathione (110), which could lead to an increased production of cytosolic hydrogen peroxide and concomitant extralysosomal damage of macromolecules and organelle membranes.

The intralysosomal accumulation of chloroquine has been shown to induce profound alterations in lysosome function, including inhibition of both the proteolytic maturation and enzyme activities of CB and CD (4, 43, 53, 62, 101, 163), which may be secondary to chloroquine-induced increase in intralysosomal pH and disruption of pH optima for these enzymes. In our laboratory, we observed similar results in SH-SY5Y cells, such that a death-inducing concentration of chloroquine markedly decreases maturation of CD, as measured by Western blot (Fig. 4). However, recent reports also indicate that chloroquine increased CD levels, as measured by Western blot, but it is unclear from these studies which form of CD (pro *versus* mature, "active" forms) was increased (9, 18). Earlier studies reported an increase in lysosome size or swelling by chloroquine and other lysosomotropic agents

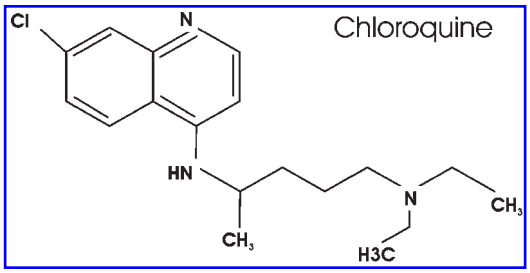

FIG. 3. Chemical structure of chloroquine. Chloroquine [7-chloro-4-(4-dimethylamino-1-methylbutylamino)quinoline] represents the class of fluoroquinolones.

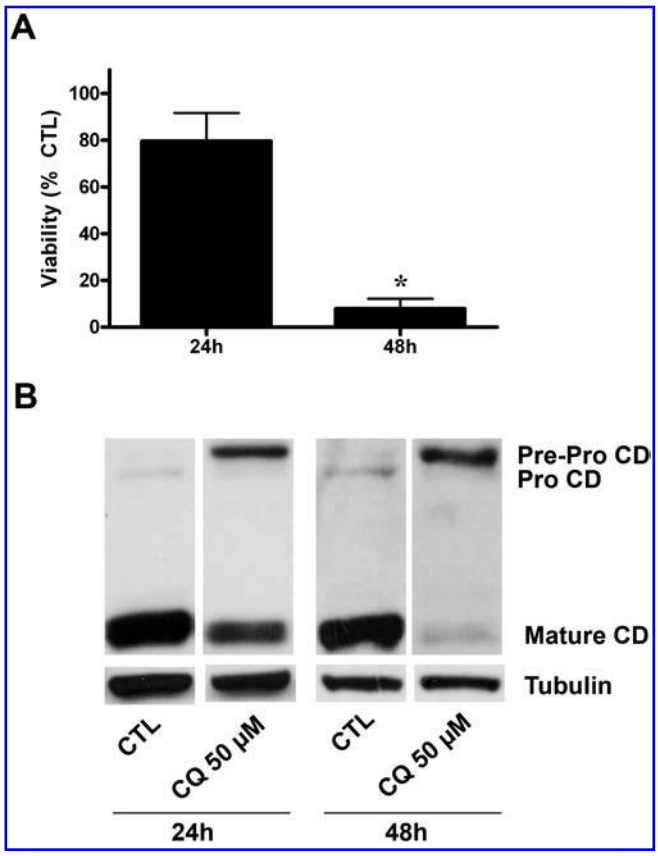

FIG. 4. Chloroquine-induced death of human SH-SY5Y cells follows alterations in the processing of CD. (**A**) Treatment of human SH-SY5Y cells with chloroquine (50 μM) significantly attenuates cell viability at 48 h *vs.* vehicle control but not at 24 h. $^*p < 0.05$ *vs.* vehicle control (Student's unpaired *t* test). (**B**) By 24 h, chloroquine induces a modest decrease in the mature "active" form of CD, migrating at ~30 kDa, along with a marked increase in the inactive, "pre-pro" fragment migrating at ~50 kDa, in comparison to vehicle control. After 48 h of chloroquine treatment, levels of the mature active form of CD appear to be further reduced in comparison to 24 h. Levels of β-tubulin (migrating at ~50 kDa) serve as the loading control.

(97, 107, 138), which results from intralysosomal chloroquine reaching isotonicity with levels in the cytosol and the subsequent increase in water flow into the lysosome. Such "swollen" lysosomes may exhibit increased membrane fragility, as indicated in isolated preparations by their increased latency to release lysosomal enzymes (97, 138) by an increase in lysosomal enzymes in purified cytosolic preparations (89). These findings clearly suggest the induction of LMP and may play a significant role in the induction of cell death after chloroquine treatment, as described later.

ROS, Autophagy, and Lysosomal Membrane Permeabilizations Death Stimuli

The susceptibility of lysosomes to oxidative stress or membrane destabilization or both is thought to play a major role in the induction of LMP, which results in the release of lysosomal enzymes into the cytosol and the potent induction of cell death. Both macroautophagy induction (14, 19, 157) and inhibition (14) have been shown to regulate cell death potently through the induction of LMP, which may involve the generation of reactive oxygen species (Fig. 5). For many years, it was believed that LMP-induced cell death was unregulated and necrotic (40). Today, it is well established that LMP may induce both apoptosis and necrosis, which seems to depend in part on the magnitude of LMP and the amount of proteolytic enzymes released into the cytosol. Many studies have indicated that stimuli that produce LMP tend to induce apoptosis at lower concentrations and necrosis at higher concentrations (84). Because multiple types of cell death can be induced after LMP, it is not surprising that the inhibition of apoptosis after LMP has been shown to shunt the type of death to a more-necrotic nature (57). To this end, we also showed that the inhibition of Bax-dependent neuron death after lysosome dysfunction does not attenuate the degree of neuron loss or neurodegeneration (130).

The cysteine protease CB and the aspartic acid protease CD are two of the most ubiquitous lysosomal enzymes (61), and as such, they have been shown to play a major role in the stimulus-specific induction of cell death after LMP. Because lysosomal hydrolases possess optimal activation at acidic pH, it is fair to question their ability to function once released into the cytosol. However, *in vitro* studies have shown that lysosomal proteases can function for several minutes to more than an hour at neutral pH (154), confirming their potential for activation outside of lysosomes. In addition, recent studies indicated that the cytoplasmic pH is reduced in the course of cell death (98, 99), which increases the potential for lysosomal proteases directly to influence cell death after LMP.

Many studies have used hydrogen peroxide to generate oxidative stress–induced LMP and apoptosis, in both neural (21, 67) and nonneural cell types (5, 33). In addition, studies have indicated the induction of LMP by other stimuli that indirectly induce hydrogen peroxide, including TNF-α (60) and lipopolysaccharide (161). The induction of LMP by hydrogen peroxide is believed to occur through its ability to diffuse freely from the cytosol into iron-rich lysosomes, where it uses Fenton chemistry to induce the production of the highly reactive hydroxyl radical (150). In addition, both hydrogen peroxide and stimuli known to produce hydrogen peroxide indirectly (such as TNF-α) have been shown to induce activation of phospholipase A2 (PLA2), which in theory stimulates the degradation of membrane lipids that could potentially increase lysosome destabilization and LMP (71, 172). LMP-induced apoptosis has also been evidenced after treatment with other oxidative stress–inducing compounds, including naphthazarin (73), which generates ROS through redox cycling, and hypochlorous acid, shown recently to induce lysosome destabilization in cultured cortical neurons (165).

ROS-induced LMP is a potent stimulus that has been shown in many studies to precede the induction of mitochondrial-dependent apoptosis (21), which has also been indicated by treatment with lysosomotropic agents or other agents that mediate indirect production of ROS (14). In addition, several studies have shown that CB (153) and CD (74) mediate mitochondrial apoptosis, findings that strongly implicate LMP in the "lysosomal–mitochondrial axis" theory of cell death, as previously described (150). Further proof of this paradigm came from an elegant study whereby the cy-

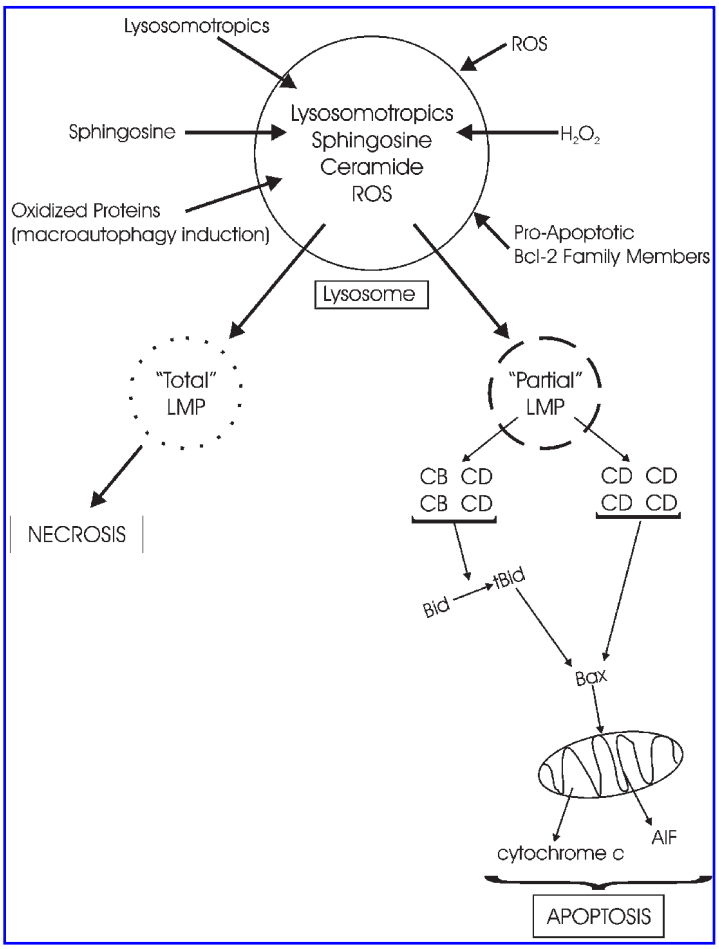

FIG. 5. Oxidative stress, lysosomal membrane permeabilization, and the induction of necrotic *versus* apoptotic death. Agents that promote the direct or indirect production of oxidative stress may lead to lysosome membrane permeabilization (LMP) and cell death. It is thought that the induction of total LMP favors the onset of necrosis, whereas partial LMP favors the onset of apoptosis. LMP is associated with the release of lysosomal cathepsins into the cytosol and the interaction with pro-apoptotic Bcl-2 family members, which leads to the induction of mitochondrial apoptosis. Proapoptotic Bcl-2 family members may also act directly at the lysosomal membrane as a stimulus for LMP. For further details, please see the text.

tosolic microinjection of CD induced caspase-dependent death, an effect that was inhibited by combined microinjection of CD with its inhibitor pepstatin A (114). Conversely, lysosomal enzymes have been shown to increase production of mitochondrial ROS, which may result in further lysosomal destabilization as part of a deleterious feedback loop (171).

Recent studies have shown that one mechanism by which cytosolic cathepsins induce mitochondrial apoptosis is through direct effects on Bcl-2 family members (Fig. 5). This concept was first suggested by Stoka *et al.* (141) in 2001, which reported cleavage of the proapoptotic Bcl-2 family member Bid by lysosomal extracts, and the ability of this cleavage product to induce cytochrome *c* release from mitochondria. Bid cleavage along with induction of mitochondrial apoptosis was first shown to be mediated *via* the cysteine protease caspase-8 (83) . A follow-up study confirmed that CB is directly responsible, at least in part, for Bid cleavage and induction of mitochondrial apoptosis (35). Another study suggested that CD plays a role in apoptosis mediated by Bid cleavage after treatment with ceramide (64). In addition, recent evidence has shown that after the induction of LMP, cytosolic CD interacts directly with proapoptotic Bax in the promotion of mitochondrial apoptosis by a variety of stimuli, including treatment with hydrogen peroxide (21). This CD–Bax–mitochondrial death pathway has also been shown to stimulate downstream mitochondrial release of apoptosis-inducing factor (AIF) (12), a mitochondrial flavoprotein that, on release from mitochondria, is implicated in caspase-independent apoptosis and necrosis (39). Thus, the interaction of cytosolic cathepsins with Bcl-2 family members has the potential to induce multiple types of cell death, and future studies are warranted to determine whether this pathway also plays an important role in the induction of neuron death in acute injury or neurodegenerative disease.

For many years, it was widely believed that the regulation of cell death by Bcl-2 family members was due solely to their manipulation of mitochondrial membrane integrity. However, several intriguing studies over the last few years suggested that other organelles, including the ER and lysosomes, may also be regulated by Bcl-2 family members in the induction of cell death (61). The first reports of Bcl-2 family–mediated regulation of lysosome function were from the laboratory of Ulf Brunk (173), which suggested that lysosome-localized Bcl-2 attenuated hydrogen peroxide–induced apoptosis, at least in part, by promoting lysosome stabilization. Subsequent studies have shown that proapoptotic Bax not only localizes to lysosomal membranes after stressful stimuli but also regulates the induction of LMP (162). The BH3 domain–only molecules Bim and Bad were also shown to localize to lysosomes after a death stimulus and regulate the induction of LMP, although their induction of LMP required the presence of Bax (162). Together these findings support the potential for the additional "upstream" influence of Bcl-2 family members in the regulation of lysosome-

dependent neuron death (Fig. 5), and as a result, the potential for their regulation of multiple types of neuron death.

Chloroquine-induced Neuron Death

One of the most striking observations after treatment of cells or tissues with chloroquine is the massive accumulation of autophagic vacuoles that results from the inhibition in completion of the macroautophagy–lysosomal degradation pathway. We and others have shown that sustained incubation with chloroquine potently induces cell death that is characterized by morphologic and biochemical markers of apoptosis and is preceded by autophagic vacuole accumulation (14, 129, 168). In our laboratory, chloroquine-induced cell death has been evidenced in a variety of cell types, including immature and fully differentiated primary neurons, neural precursor cells, and a variety of neural cell lines (Fig. 4). At present, whether the accumulation of AVs directly mediates chloroquine-induced neuron death has not been thoroughly investigated, although the ability of macroautophagy inhibition to induce cell death was clearly indicated previously in the literature (14, 129). Death induced by macroautophagy inhibition may result from a compromise in homeostatic organelle turnover, thus increasing the accumulation of damaged organelles with compromised function, which could trigger the initiation or completion of death-pathway signaling. Certainly the accumulation of undegradeable oxidized lipoproteins may cause associated damage to lysosomal membranes.

Mitochondrial dysfunction appears to play a major role in chloroquine-induced cell death, as indicated previously by a decrease in mitochondrial membrane potential and an attenuation of cell death by the targeted disruption of proapoptotic *bax* or *bcl-2* overexpression, and the exacerbation of cell death after the targeted disruption of antiapoptotic *bcl-x* (14, 129, 168). Although chloroquine induces robust activation of caspase-3, the targeted genetic disruption of caspase-3 or treatment with general caspase inhibitors does not attenuate chloroquine-induced neuron death (129, 168). Together, these findings suggest either that the commitment point for chloroquine-induced neuron death lies upstream of caspase activation, or indicates that the potential for both caspase-dependent and -independent death pathways triggered by disruption of the macroautophagy–lysosomal degradation pathway. We have also shown that chloroquine-induced death of immature neurons is attenuated by the protooncogene *p53*, an effect that was not observed in cultures of postmitotic neurons (68, 70), which suggests that p53-dependent autophagic cell death may be cell-type or differentiation dependent or both. As such, chloroquine-induced, p53-dependent autophagic death is being actively investigated as a potential therapeutic target in several types of cancers, including glioblastomas (110).

We showed recently that the plecomacrolide antibiotic bafilomycin A1 (BafA1) and other structurally similar compounds significantly attenuate chloroquine-induced neuron death (128, 129), at concentrations ($\leq$1 n*M*) shown previously not to inhibit vacuolar-type ATPase (13). Although a previous study suggested that a high dose of 100 n*M* BafA1 attenuated cell death induced by hydroxychloroquine by attenuating the pH-dependent fusion of chloroquine into the lysosome (14), our results suggest that "neuroprotective" concentrations of BafA1 ($\leq$1 n*M*) do not alter the ability of chloroquine to inhibit macroautophagy, because AVs still accumulate in chloroquine+BafA1–treated cells, concomitant with an absence of apoptotic morphology, and that the chloroquine-induced inhibition of long-lived protein degradation was not affected by 1 n*M* BafA1 (129). Ongoing studies in our laboratory are delineating the potential mechanisms by which plecomacrolide antibiotics attenuate neuron death induced by lysosomotropic agents and whether cell death induced by other disruptions in lysosome function can also be attenuated by plecomacrolides.

Chloroquine-induced cell death was shown previously to involve LMP, as indicated immunocytochemically by the diffuse cytosolic immunoreactivity of the lysosomal protease CB in chloroquine-treated cells (14). LMP was suggested as an upstream mediator of mitochondrial cell death, because selective inhibition of CB significantly attenuated chloroquine-induced mitochondrial dysfunction concomitant with an increase in viability (14). Interestingly, chloroquine also enhances the extracellular secretion of many lysosomal enzymes, including β-hexaminodase, CB, and CD (58, 88, 100, 101, 122), which effectively blocks the delivery of newly synthesized lysosomal hydrolases to lysosomes. It will be important in future studies of chloroquine-induced LMP to confirm results of immunocytochemistry with rigorous biochemical analyses indicating an increased appearance of lysosomal enzymes in purified cytosolic fractions *via* western blot.

Chloroquine has also been shown to inhibit the activities of sphingolipid-metabolizing enzymes, including sphingomyelinase and acid ceramidase (48, 72), which are localized to lysosomes and most likely reflect the deleterious alterations in lysosome function that are induced on chloroquine treatment. Inhibition of these lipid-metabolizing enzymes causes the accumulation of ceramide and sphingosine, two highly reactive lipid mediators that have been shown to mediate oxidative stress–induced apoptosis (123, 170). Sphingosine has been shown to exhibit detergent-like properties toward lysosome membranes, which may contribute to chloroquine-induced LMP and subsequent apoptosis (74). Together, these results suggest that the aberrant production of reactive lipid metabolites not only may mediate cell death induced as a result of lysosome dysfunction mediated during macroautophagy inhibition but also may further exacerbate lysosome dysfunction and stimulate LMP-induced cell death. It should be noted, however, that inhibition of sphingolipid-metabolizing enzymes also increases levels of the antiapoptotic sphingolipid sphingosine-1-phosphate concomitant with proapoptotic sphingolipids (48), which suggests a potential balance of pro- *versus* antiapoptotic lipid mediators that must be addressed appropriately to understand the net contribution of lipid mediators in neuron death regulation.

Oxidative Stress, Autophagy, and Lysosome Dysfunction in CNS Aging and Alzheimer's Disease

Several properties of the aging brain make it uniquely susceptible to age-related oxidative damage. First, neurons are postmitotic; thus, over their life span, age-related macromolecular damage accumulates and compromises their function. This is evidenced by the age-related increase in lipo-

fuscin, which may provide both cause and effect for age-related declines in lysosome function and autophagy signaling (69, 70, 87, 149). Second, neurons have high energy demands compared with other cell types, and they may be more vulnerable to the deleterious effects of mitochondrial dysfunction, combined with the fact that the electron-transport chain of oxidative phosphorylation generates ROS (1). Third, the brain is composed of large amounts of lipids and transition metals including iron (1, 52, 169), which increases the probability of age-related lipid peroxidation. Last, the aging brain contains fewer reducing equivalents that in theory would contribute to an increase in oxidative stress (136). Age-related oxidative stress in the cytoplasm may cause macroautophagy induction, in particular as cytoplasmic macromolecules or organelles become damaged and are delivered to lysosomes for degradation. Conversely, age-related oxidative stress in the lysosome may lead to macroautophagy inhibition if the result of sustained oxidative stress is a net compromise in lysosome function.

Many studies have indicated pronounced alterations in the endosomal–lysosomal pathway in human AD brain, which are some of the earliest reported abnormalities in AD brain neurons and precede the onset of both Aβ-containing plaque and tangle neuropathology (28). Enlarged, Aβ-immunoreactive endosomes have been reported in brains of AD patients (102) before Aβ deposition, which suggests a potential for endosome-mediated Aβ secretion and deposition. Increased levels of CD have also been localized to endosomes of AD patients (23, 24). Endosomal CD may be linked to Aβ formation, in that CD possesses inherent β- and -secretase activity, enzymes that are responsible for the cleavage of amyloid precursor protein into Aβ (31), although APP processing was shown previously to be unaffected by CD deficiency in mice (119). Increased levels and activity of CB and CD in AD brain have been shown to occur concomitant with lysosome proliferation (2, 22, 24, 27, 29) and may reflect a compensatory response to altered macroautophagy, but it is not clear whether such alterations serve a beneficial role to promote protein degradation or death signaling. In addition, both CB and CD have been localized extracellularly to amyloid plaque, which may indicate their potential to regulate plaque deposition (27, 29). In support of this argument, CB has been shown effectively to decrease levels of the more amyloidogenic A$\beta_{1\text{-}42}$, and CB deficiency in mice was shown to cause an increase in extracellular Aβ deposition (94).

Alterations in the autophagy–lysosomal degradation pathway have been indicated in AD by pathologic increases in autophagic vacuoles (AVs) observed in cortical biopsies obtained from AD brain (104, 166). Accumulating AVs in AD brain have been found to localize in large part to dystrophic neurites, which may be related to alterations in intracellular trafficking that either cause AV accumulation or result from AV accumulation. Both immature, double-membraned autophagic vacuoles and mature, single-membraned autophagolysosomes have been shown to accumulate in AD dystrophic neurites, which implicates both macroautophagy induction and inhibition in AD and may reflect both an early and late autophagic response of individual neurons to AD-associated stress. Recent evidence suggests the localization of Aβ in AVs both in human AD brain and in experimental models of AD, and that the processing of APP into Aβ may even occur within AVs (166). The intracellular accumulation of Aβ has also been reported after the chemical inhibition of macroautophagy completion mediated by *in vivo* and *in vitro* treatment with chloroquine (34, 90), providing further evidence that macroautophagy plays a vital role in Aβ processing and degradation.

Taken together, evidence suggests that alterations in both the endosomal–lysosomal and autophagy–lysosomal degradation pathways play an intimate role in the generation of AD neuropathology, and oxidative stress may play a major role in inducing alterations in intracellular recycling pathways. Oxidative stress has been proposed to play a major role in the onset and progression of AD. Many reports of increased oxidative damage have been reported in AD brain (reviewed in refs. 20 and 92), which may have important ramifications in the macroautophagy-lysosome degradation pathway. An increase in mitophagy has been reported in AD brain (93), which is likely a response of autophagy to clear oxidatively damaged mitochondria in postmitotic neurons. Treatment of neuronal cells under conditions of oxidative stress was shown recently to induce macroautophagy of Aβ and promote its localization in lysosomes (174), which may reflect a stimulus early in the progression of AD to clear intracellular levels of Aβ. The effects of oxidative stress on lysosomal function in experimental models of AD have not been directly tested, although as described later, treatment with Aβ produces profound effects on lysosomal function that may be related in part to the generation of oxidative stress.

Aβ-Induced Neuron Death

Although it is obvious that a definite progression of neuron loss occurs in AD, studies of AD brain have in large part shown inconsistent findings regarding a role for apoptosis as an important mechanism of neuron death (reviewed in refs. 108 and 116). This variability of results may be explained by the inherent heterogeneity of the human AD population at the time of tissue biopsy and by differences in the processing of postmortem tissue. In addition, an inherent challenge exists in proving with great confidence the relevance of neuron death mechanisms in age-related neurodegenerative disease, because only a small number of neurons succumb to cell death at any one time. Nevertheless, countless studies focused on delineating the mechanisms of Aβ-mediated neuron death as a contributing factor to neuron loss in human AD brain.

Results of *in vitro* studies indicate a clear link between Aβ, oxidative stress, and cell death (reviewed in ref. 108). Many studies have shown that Aβ-induced cell death and apoptosis is mediated by oxidative stress (145), effects that in many cases were inhibited on treatment with antioxidants (8). Treatment with Aβ has been shown to increase free radical production and markers of oxidative stress (66), and the induction of oxidative stress has been shown to induce the intracellular accumulation of Aβ (173). As discussed earlier, the autophagy–lysosomal degradation pathway is a sensitive target for oxidative stress–induced damage, and treatment with soluble forms of Aβ at death-inducing concentrations has been shown to result in its intralysosomal localization (46) and induction of intralysosomal oxidative stress (46) and the induction of LMP (46). Other alterations in lysosome function, including alterations in levels of CD,

have also been reported after Aβ treatment (18). Although many studies of Aβ-induced cell death suggest a role for apoptosis (44), our laboratory previously showed that Aβ-induced neuron death is Bax dependent but caspase independent (127). Even though we observed activation of caspase 3 after treatment with Aβ, neither inhibition of caspase-3 nor the targeted genetic disruption of caspase-3 attenuated Aβ-induced neuron death (127). Together, these findings suggest that Aβ may play a significant role in oxidative stress–induced neuron death in AD brain, although the role of caspase-dependent apoptosis is still controversial. The likely disruption of the macroautophagy–lysosomal degradation pathway in AD brain, however, suggests the potential contribution of multiple types of neuron death, both caspase dependent and independent, to AD neuropathology.

Regulation of Neuron Death in CD-Deficient Mice as a Model of NCL/Batten Disease

NCL is a heterogeneous group of pediatric lysosomal storage disorders known collectively as Batten disease. Clinical features of NCL/Batten disease include seizures and progressive blindness, with eventual loss of motor control and ultimate death (56). NCLs were classified originally by their age at onset and include congenital (at birth), infantile (INCL, within 1 year of birth); late infantile (LINCL, 2–4 years); juvenile (JNCL, 4–7 years); or the very rare adult form (ANCL). Presently seven gene mutations are known in humans to cause NCL (CLN1, CLN2, CLN3, CLN5, CLN6, CLN8, and CD), which produce distinct biochemical alterations in lysosome function and are also defined by the type of storage protein that accumulates as a result of lysosome dysfunction (91).

A major focus of our laboratory is the study of CD deficiency–induced neuron death as a model of lysosome dysfunction in congenital NCL/Batten disease. It was not until 2006 that human CD mutations were first reported in two separate studies (135, 140). In one study, a complete loss of CD function was reported in four patients with congenital NCL (135), and these patients exhibited perinatal seizures before dying by 2 weeks of age. In the other study, a partial loss of CD enzymatic activity was observed in an adolescent patient diagnosed with NCL-like symptoms at early school age (135). Before the finding of CD mutations in humans, however, sporadic mutations in CD resulting in a characteristic NCL-like phenotype were reported in sheep (155) and more recently in American bulldogs (6). In 1995, the effects of experimental CD deficiency in mice were initially reported in an attempt to characterize further the role of CD in lysosome function (118). CD deficiency was found to inhibit bulk proteolysis, and CD-deficient mice died by postnatal day 26 of a plethora of morbidities including intestinal necrosis, thromboembolia, and seizures (118).

An increase in seizures and blindness in CD-deficient mice led to the subsequent analyses of brain function in these mice, when it was determined that CD deficiency resulted in robust neurodegeneration characterized by the massive accumulation of lipofuscin-laden AVs (78) and significant neuron loss (130). AVs in CD-deficient mice were found to accumulate as early as postnatal day 8 (79), and their accumulation has been shown to precede the induction of apoptosis (78), which occurs as early as postnatal day 16 (78). Although a role for oxidative stress has not been directly verified in the induction of AV accumulation, the accumulation of lipofuscin and likely inhibition of autophagy are strong indicators that the overproduction of oxidative stress plays a prominent role in CD deficiency–induced neuropathology, and further studies are needed to confirm this. Previous studies suggested that the induction of nitrosative stress in CD-deficient brain accelerates CD deficiency–induced neuropathology, arising potentially from an increase in nitric oxide and peroxynitrite from microglial activation (95, 164). Treatment of CD-deficient mice with inhibitors of nitric oxide synthase attenuated the appearance of apoptotic neurons but not neurons exhibiting AV accumulation and lacking apoptotic morphology (95). In particular, such apoptotic neurons were found in many cases to be localized adjacent to neurons undergoing such "autophagic stress," which led to the hypothesis that cells undergoing autophagic neurodegeneration induced microglial activation, which in turn resulted in nitric oxide–dependent apoptotic death of neighboring neurons (95). This hypothesis, if correct, would explain subsequent findings in our laboratory indicating inactivation of prosurvival Akt and activation of proapoptotic GSK-3β in CD-deficient neurons with a time course related to that of apoptosis induction (160), along with findings in our laboratory indicating inactivation of Akt and apoptosis induction in cultured cells on treatment with peroxynitrite (132).

Further to investigate the role of apoptosis in CD deficiency–induced neuron death, we generated mice deficient in both CD and the proapoptotic molecule Bax (130). Whereas Bax deficiency clearly reduced the induction of apoptosis following CD deficiency, no decrease in neuron loss, neurodegeneration, or autofluorescent storage material was found (130). Together these results suggest that although CD deficiency induces apoptosis, the resultant lysosome dysfunction contributes to the induction of multiple types of neuron death and that apoptosis plays a limited role in the neurodegenerative phenotype induced by CD deficiency. Although the relative contribution of apoptosis to neuron death and neurodegeneration is obviously disease specific, it is clear that in the present study of neuron death, a whole host of cell-death mechanisms should be considered, including apoptotic *versus* nonapoptotic, or with different types of apoptosis that are either caspase dependent or independent.

Perspectives

Lysosome dysfunction is quickly emerging as a prominent area of research in which to study potential mechanisms of neuron death. Multiple types of neuron death delineated in the literature appear to involve, at some level, the disruption of lysosome function, and both the induction of oxidative stress and altered autophagy signaling have the capacity to regulate neuron death through the lysosome. Potential therapies such as the phosphodiesterase inhibitor zaprinast, the lysosomal "modulator" Z-Phe-Ala-diazomethylketone (PADK), plecomacrolide antibiotics such as BafA1, calpain inhibitors, cathepsin inhibitors, and metal chelators (9, 18, 33, 67, 128, 129, 165) may act through their direct attenuation of oxidative stress or indirect attenuation of oxidative stress–in-

duced damage. In theory, this would promote the stabilization of lysosome membranes and decrease the onset of LMP in neurons. The use of these agents will undoubtedly receive greater prominence in the near future as the lysosome in turn receives greater attention as a therapeutic target in the onset and progression of neurodegenerative disease.

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Abbreviations

Aβ, beta amyloid; AD, Alzheimer disease; AIF, apoptosis-inducing factor; APP, amyloid precursor protein; Atg, autophagy-related gene; AV, autophagic vacuole; BafA1, bafilomycin A1; CB, cathepsin B; CD, cathepsin D; CMA, chaperone-mediated autophagy; ER, endoplasmic reticulum; FPIX, ferriprotoporphyrin IX; 4-HNE, 4-hydroxy-nonenal; LMP, lysosomal membrane permeabilization; M6P, mannose-6-phosphate; NCLs, neuronal ceroid lipofuscinoses; PLA2, phospholipase A2; ROS, reactive oxygen species; TGN, trans-Golgi network.

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