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Forum Review

Oxidative Stress and Autophagy

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ABSTRACT

Organisms respond to oxidative injury by orchestrating a stress response to prevent further damage. An increase in the intracellular levels of antioxidant agents, and at the same time the removal of already damaged components, are both part of the oxidative stress response. Lysosomes have been classically considered one of the main targets of the reactive oxygen species. In fact, the destabilization of the lysosomal membrane during oxidizing conditions promotes the leakage of the enzymes contained in these organelles and contributes to cellular damage. However, recent evidence supports a protective role of the lysosomal system, which can eliminate altered intracellular components through autophagy, at least in the first stages of oxidative injury. Consequently, activation of the main intracellular proteolytic systems, the ubiquitin/proteasome, and also the lysosomal/autophagic system occurs during the oxidative stress response. The opposing roles for the lysosomal system under oxidizing conditions are discussed in this review. *Antioxid. Redox Signal.* 8: 152–162.

AUTOPHAGY: THE BASICS

A FINE-TUNED BALANCE between protein synthesis and protein degradation inside cells is responsible for the continuous renewal of the intracellular pool of proteins (20). Two major proteolytic systems contribute to protein removal: the ubiquitin/proteasome system (extensively reviewed in other sections of this focused issue) and the lysosomal system.

The lysosomal system is composed of a series of vesicular compartments with different ultrastructural and biochemical characteristics, which act coordinately to guarantee the degradation of substrate products in the "final" organelle, the lysosome (22). Thus lysosomes receive substrates for degradation, both from outside and from inside the cell, via two processes known as endocytosis and autophagy, respectively (19, 20, 33, 55, 79). This review focuses on the degradation of intracellular oxidized proteins by lysosomes, and consequently we will only discuss the autophagic process.

The term autophagy groups a series of intracellular pathways that lead to the removal of cytosolic components in lysosomes. These pathways, referred to as macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), in the case of mammalian cells, are interconnected and, in some cases, share common components (19, 20, 33, 49, 69, 79). In addition, there is growing evidence for cross-talking between these pathways and also with nonlysosomal mechanisms of degradation. Although many of the molecular players involved in these processes are still unknown, the molecular dissection of macro- and microautophagy, to some extent, has been recently made possible by screening of yeast mutants (33, 39, 79). Added to the newly identified players for this pathway, known generically as autophagy (ATG) genes (39), the introduction of yeast as a study model and, nowadays, many other model systems have contributed to the current expansion of the field. Most of the autophagic components found in yeast have orthologs in other species from amoebae to mammals (53). Genetic studies in worms (*C. elegans*), flies (*D. melanogaster*), and even in some amoebae and plants are also contributing new genes related to autophagy (4, 31, 50, 60). The proteins encoded by these genes are used now as markers for this lysosomal pathway, but in addition, mutations in these genes have revealed a new myriad of cellular functions for autophagy (43, 49, 69). In the case of chaperone-mediated autophagy (CMA) this kind of genetic screening has not been possible,

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because so far, it has only been detected in mammalian cells. A detailed description of the molecular components of the autophagic pathways is beyond the scope of this review. We will only highlight here the main characteristics of each of these pathways. Interested readers are referred to recent comprehensive reviews in this topic (33, 43, 49, 57, 69, 79).

The different autophagic pathways

Macroautophagy, microautophagy, and chaperone-mediated autophagy differ in the way that substrate proteins are delivered to lysosomes (Fig. 1). Macroautophagy is the best characterized form of autophagy. It is activated during starvation to meet the requirement of essential constituents by recycling existing cellular components (33, 53, 79). Cytosolic components and organelles are sequestered through the elongation and sealing of a limiting membrane to form a double membrane vesicle known as an autophagic vacuole or autophagosome (57). This vesicle then fuses with secondary lysosomes, which provide the enzymes required for the degradation of the entrapped cytosolic components. Genetic studies have revealed that the limiting membrane originates *de novo* in yeast but from specific regions of ER in mammals (52, 57). The protein components involved in autophagosome formation (e.g., Atg8, Atg12, Atg5) and elongation (e.g., Atg13, Atg1) initially identified in yeast are all conserved in higher eukaryotes (52, 58). Studies in the amoeba *Dictyostelieum discoideum* and in *Drosophila* have revealed that mutations in macroautophagy genes lead to survival problems during nutrient starvation and to aberrant development (4, 31, 60). Macroautophagy has also been genetically connected to expansion in lifespan in *C. elegans* (50). RNA interference of ATG genes in long-lived mutant worms (daf-2 mutants) shortens their prolonged survival, establishing as well a connection between macroautophagy and the insulin signaling pathway (daf-2 codes for an insulin receptor-like protein). The notch signaling pathway is also related to macroautophagy and it is likely key for the connection of this form of autophagy to cell death, at least during fly metamorphosis (4). Several lines of evidence support, in fact, a close relation between autophagy and cellular death, since blockage of macroautophagy by disruption of Beclin1 (homologue to yeast Atg6 and connected to the anti-apoptotic members of the Bcl-2 family of proteins) leads to massive cell death by apoptosis (68). These findings suggest the autophagic removal of damaged intracellular components as a preventive measure before the final decision of death by apoptosis is made.

As in macroautophagy, substrates (soluble proteins and complete organelles) are delivered to lysosomes for degradation via microautophagy through sequestration of complete regions of the cytosol (20, 33, 79). However, in this case the limiting membrane is the lysosomal membrane, which, thanks to the plastic properties of this vesicular compartment, elongates or invaginates to surround the cytosolic components (Fig. 1). The morphological image of microautophagy is as a

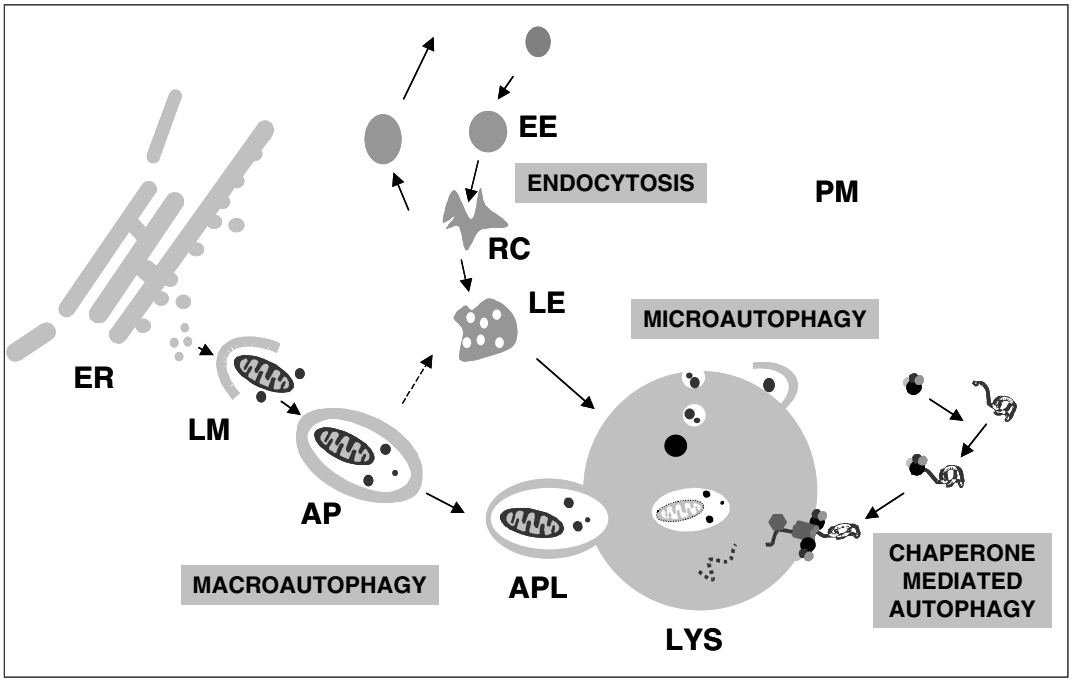

FIG. 1. Schematic model of the main forms of autophagy in mammalian cells. Internalization of complete regions of cytosol first into autophagosomes that fuse then with lysosomes (macroautophagy) or directly by the lysosomal membrane (microautophagy) contrast with the selective uptake in a molecule-by-molecule basis of cytosolic proteins via chaperone-mediated autophagy. This intracellular degradation (autophagy) uses different mechanisms than the degradation of extracellular and plasma membrane components, which it is achieved through endocytosis. Abbreviations: ER, endoplasmic reticulum; LM, limiting membrane; AP, autophagosome; APL, autophagolysosome; LYS, lysosome; EE, early endosomes; RC, recycling compartment; LE, late endosomes; PM, plasma membrane.

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result, the presence of multivesiculated lysosomes or lysosomes containing tubular projections out of their membranes. Although our current understanding of microautophagy is not as good as for macroautophagy, it has significantly advanced through the study of micropexophagy, a specialized form of microautophagy that selectively removes intracellular peroxisomes under specific conditions (5, 38, 76). In a particular yeast strain, *Pichia pastoris,* removal of unnecessary peroxisomes following peroxisomal proliferation, is attained through macroautophagy when the yeast is maintained in the presence of glucose. However, peroxisomes are directly engulfed by the vacuole, equivalent to lysosomes in yeast, if the nutritional support switches toward a nitrogen source (5, 76). Some of the molecular components of micropexophagy are shared with macroautophagy, however, other components are unique for this process (5). In mammals, in contrast to the inducible character of macroautophagy, microautophagy is constitutively activated, being thus responsible for basal lysosomal degradation of cytosolic components (2, 54).

(single letter amino acid annotation for lysine, phenylalanine, glutamic acid, arginine and glutamine)

The third form of autophagy, chaperone-mediated autophagy (CMA), has in common with macroautophagy its inducible-nature (46, 48). Although some basal CMA activity can be detected in most cell types, maximal activation is attained in response to different stresses, as described below. The main difference between this pathway and the two other forms of autophagy is that CMA does not require membrane remodeling, for the formation of vesicles or tubes. Instead, substrate proteins are directly translocated through the lysosomal membrane by a receptor/translocon-like structure (Fig. 1). Also unique is the selectivity of this pathway by which only soluble proteins, containing a particular targeting motif biochemically related to the pentapeptide KFERQ, are degraded (14). This motif is recognized by a cytosolic chaperone and its co-chaperones that target the substrate to lysosomes (15). At the lysosomal membrane the complex chaperones/substrate docks on the cytosolic tail of a type I transmembrane lysosomal protein, and, after unfolding, the substrate crosses the lysosomal membrane assisted by a chaperone resident in the lysosomal lumen (1, 16). The requirement for a targeting motif in the substrates allows selective removal of some proteins by lysosomes without affecting the turnover of neighboring ones. Although CMA has only been described in mammalian cells so far, a somehow related mechanism, the vacuole import and degradation pathway (vid), exists in yeast (9, 32). Vid recapitulates some steps of CMA and of macroautophagy in a single pathway. Similarly to CMA, substrates are translocated in a chaperone-dependent manner inside small vesicles, but gain access to proteases after fusion with the vacuole by a process mechanistically similar to the fusion of autophagosomes with lysosomes (32). Whether *vid* is phylogenetically related to CMA still requires clarification. Dysfunction of CMA has been found in aging and in some familial forms of Parkinson's disease, and it seems to contribute to the accumulation of damaged proteins and the impaired response to stress common to both conditions (48).

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Tracking autophagy

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Along with genetic screenings and, directly as consequence of them, the availability of new methods to monitor autophagy has been a key factor for the current expansion of this field. During the years, electron microscopy (EM) analysis has been the conventional method for tracking autophagy. Double membrane vesicles with identifiable cytosolic components are the morphological manifestation of macroautophagy, while lysosomes with invaginations, tubulations or vesicles inside are a signature of microautophagy. Quantification of autophagic activity by EM is obtained by measuring the volume of vacuoles compared to that of total cytosol (51). The limitation of this method is that it cannot be applied to track chaperone-mediated autophagy, and even for macro- and microautophagy, the characteristic heterogeneity of the lysosomal system, makes it difficult, sometimes, to establish the nature of a particular vesicle. Consequently, there has been a longtime quest for markers for autophagy. Recently, some fluorescent compounds, such as monodansylcadaverin, able to highlight acid compartments or lipid-enriched organelles have been used, but not without raising major concerns about specificity (51). Although available markers are still scarce, the recent advances in our understanding of autophagosome formation in yeast have provided some molecular tools for tracking autophagy. Different *atg* proteins associate transiently to particular stages in the maturation of autophagosomes, thus allowing their identification. These markers are now used fused to fluorescent proteins or detected directly with antibodies conjugated to gold particles in immunoelectron microscopy (53, 71).

A classical, but still widely used method to track the different forms of autophagy is by measuring rates of protein degradation through *pulse and chase* experiments (20, 67). Lysosomal substrates are usually long-lived proteins that can be preferentially labeled by incubation of culture cells with radiolabeled amino acids for a long (> 1 day) pulse period, and then following their degradation for up to 30 hours. Combinations of different autophagic inhibitors and activators allows for the differentiation of the degradation corresponding to each autophagy type. A variation of these assays, often used in yeast, tracks the degradation of particular substrates known to be preferentially delivered to the vacuole (equivalent to lysosomes, in yeast) by a particular form of autophagy (58, 76).

AUTOPHAGY AND OXIDATIVE STRESS

Lysosomes have been considered for years as one of the main intracellular targets for the damaging effect of free radicals. However, recent evidence supports a protective role of the lysosomal compartment, as part of the oxidative stress response. Here, we review this dual lysosomal role and analyze the intracellular consequences of the malfunctioning of this system.

Lipofuscin and the lysosomal function

For the vast majority of substrates transported into the lysosomal lumen, their unalterable fate is rapid breakdown by the battery of proteases, lipases, and endonucleases resident in this compartment (20). However, macromolecules that have been oxidatively modified, often crosslink with other proteins, lipids, carbohydrates, and even metals, becoming resistant to hydrolases and forming a cellularly indestructible byproduct known

as lipofuscin (12, 74). Lipofuscin is an autofluorescent pigment that accumulates during aging in the lysosomal lumen of postmitotic cells and has often been used as a biomarker of aging (34, 73, 74). The dependence on iron as a reducing agent for the formation of lipofuscin supports that lipofuscin forms in secondary lysosomes as a result of iron-catalyzed oxidative reactions involving autophagocytosed materials (81). The biogenesis of lipofuscin in lysosomes is a multi-step process dependent on the presence of hydrogen peroxide and ferrous iron in the lysosomal lumen. These two compounds react to yield highly reactive species that catalyze the crosslinking of various cellular components inside the lysosomal compartment (74). Lipid peroxidation and glucoxidation products have been identified in the proteinaceous components of the lipofucsin accumulation, suggesting that these compounds are also involved in lipofuscinogenesis (66).

In addition to the normal accumulation of lipofuscin seen during senescence, buildup of a related pigment (ceroid) occurs in conditions associated to an increase of reactive oxygen species (ROS), such as in those associated to deficiencies of antioxidant defenses and in certain pathologies grouped under the common term of "ceroids" (34). These also autofluorescent pigments share biochemical properties and generating steps with lipofuscin (10, 34). Increased ceroid/lipofuscin formation in response to oxidative stress has been experimentally demonstrated in human fibroblasts cultured under hyperoxic conditions (74, 75).

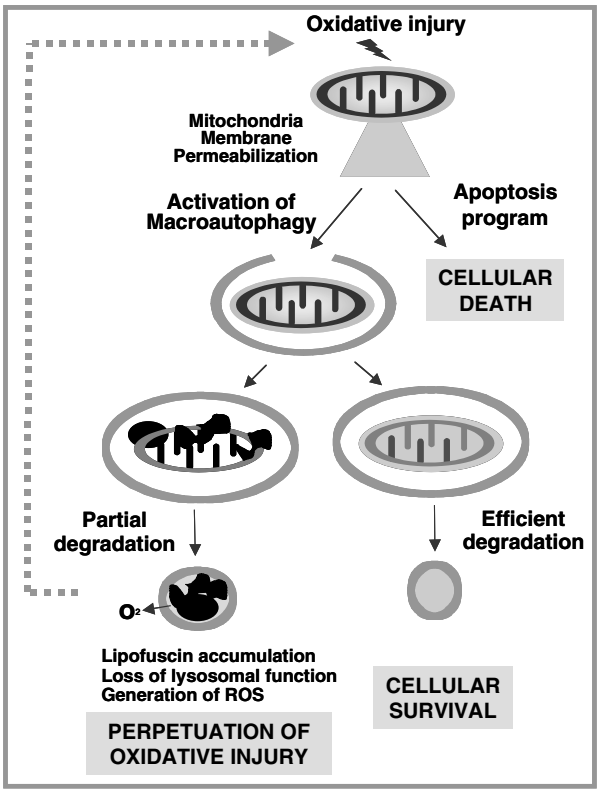

FIG. 2. The dual role of macroautophagy as part of the oxidative stress response. Mild oxidative stress activates macroautophagy to facilitate the removal of damaged organelles (a depolarized mitochondria shown here). Successful autophagy of the damaged components contributes to cellular recovery. However, acute or persistent oxidative stress results in intracellular increase of reactive oxygen species that damage the lysosomal membrane. Alteration of the lysosomal compartment prevents their fusion with autophagic vacuoles containing damaged components, and also results in the release of potent hydrolases, enhancing the degree of cellular damage. If autophagy is not engaged as part of the oxidative stress response, or the oxidative injury overcomes the cellular defenses, cells die after activating the apoptosis program.

Lysosomes as the "bad guys" in the oxidative stress response

Since the early works identifying the accumulation of lipofuscin in lysosomes, numerous studies have analyzed the consequences of this pigment on lysosomal activity and in cellular functioning. Cells experimentally loaded with lipofuscin display an increased susceptibility to oxidative stress and to lysosomal breakage (75). The lysosomal membrane serves a vital function by isolating very potent degradative enzymes from other intracellular components. Direct damage of the lysosomal membrane by ROS during oxidative stress has been extensively reported. Damage of the lysosomal membrane often results in cytosolic leakage of potent hydrolases which could cause intracellular havoc (Fig. 2) (10–12, 73). Besides the lysosomal membrane, a second mechanism that protects cytosolic components from the lysosomal enzymes is the fact that these hydrolases reach maximal activity only at acidic pH, typical of the lysosomal lumen. However, even at the neutral cytosolic pH, most of the lysosomal enzymes still retain significant activity, and consequently, when released cause cytosolic damage. The magnitude of the cellular damage varies with the intensity of the oxidation, which determines the degree of disruption of the lysosomal membrane. Particular conditions seem to facilitate lysosomal breakage during oxidative injury. Thus, nutrient-deprived cells show augmented sensitivity to hydrogen peroxide-induced oxidative stress (59). The enhanced availability of iron in low molecular weight form under these conditions results in an increased potential of intralysosomal Fenton chemistry that causes lysosomal rupture. Augmented autophagic degradation of proteins under these conditions also increases this pool of ferric iron and the sensitivity of cells to oxidative stress (65). Supporting the contribution of lysosomes themselves to the generation of reactive oxygen species is the fact that methylamine or chloroquine (lysosomotropic agents) or 3-methyladenine (an inhibitor of autophagy) prevents ROS formation and generation of lipid peroxidation (62).

Oxidative stress can also influence the permeability of the lysosomal membrane by inducing cross-linking of the lysosomal membrane proteins via disulphide bounds (Fig. 2) (78). This partial aggregation of the lysosomal membrane proteins results in increase of the lysosomal proton permeability, increase in the lumenal pH and membrane potential, and proton leakage. Consequently, lysosomal membrane permeability *in vivo* may depend on the redox states of their membrane thiol groups (78).

Direct damage of the lysosomal membrane by particular oxidized proteins has been reported for proteins such as oxi-

dized ferritin and low-density lipoprotein (LDL) internalized by endocytosis in macrophages. The major cytotoxic components of the oxidized LDL are cholesterol oxidation products that cause lysosomal destabilization, release of cathepsins, apoptosis, and post-apoptotic necrosis (83).

Although not well characterized yet, cells may have a defensive mechanism to cope with moderate lysosome leakage. Thus, Brunk and Dalen (11) showed that cultured fibroblasts exposed to mild oxidative stress remain viable despite leakage of hydrolytic enzymes into the cytosol. However, more intense oxidation further destabilizes the lysosomal membrane and results in apoptosis or cellular necrosis (11). The molecular mechanisms behind the divergent effect of oxidative stress on lysosomal components are unknown. Very little is known about how lysosomes are eliminated when they are no longer functional, but macroautophagic digestion by still functional lysosomes seems a reasonable possibility. We hypothesize that moderate oxidative stress, affecting only a small percentage of lysosomes, may activate macroautophagy to sequester the "leaking" lysosomes out from the cytosol. As described for mitochondria, there could be particular modifications at the lysosomal membrane, such as exposure of membrane protein regions normally in the lysosomal lumen, which allow their selective recognition by the autophagic limiting membrane. Fusion of these autophagic vacuoles with still functional lysosomes would guarantee the elimination of the damaged lysosomal membrane, and allow the reutilization of still functional lysosomal enzymes by the resulting secondary lysosome. In fact, ours and other groups have occasionally reported the presence of "lysosome-like" vesicles, inside autophagic vacuoles (AC Massey and AM Cuervo, unpublished observations). During massive acute oxidative stress, the lack of a significant number of functional lysosomes left after the injury could prevent the removal of the damaged ones. The continuous leakage of lysosomal enzymes, in particular cathepsin D, has been shown to induce apoptosis through caspase-3-like proteases (11).

Lysosomes in removal of damaged cytosolic components

The damage caused by lysosomes during oxidative stress has been the primary focus of interest for decades. Nevertheless, an emerging area of study focuses on the nonharmful participation of the lysosomal system during oxidative stress. Both, macroautophagy (41) and the selective autophagic pathway of CMA (37) perform beneficial functions for the cell under oxidizing conditions.

Though mainly considered as nonselective, under oxidative conditions the macroautophagic pathway has been shown to carry out a discriminatory removal of damaged mitochondria (Fig. 2) (41, 64). Oxidative damage has been shown to increase the permeability of the mitochondrial membrane to various molecules and to result in mitochondrial functional failure. Increased mitochondrial permeability is accompanied by depolarization of the mitochondrial membrane and uncoupling of oxidation and phosphorylation reactions in the mitochondrial lumen. Leakage of intramitochondrial components, such as cytochrome c, constitutes the first step of the activation of different cellular death programs (3). Macroautophagy safeguards the cell against major harm by degrading damaged mitochondria in the very early stages before cytochrome c release occurs (41, 42). In fact, during the first stages of oxidative injury, cells respond to mitochondria and lysosome leakage with a period of recuperative macroautophagy, evinced by an increase in the number of autophagic vacuoles in the affected cells (11). Activation of this protective macroautophagy is tightly related to activation of apoptosis and necrosis, and it is considered to be in the crossroad between cellular death or survival (68). Although the mechanisms coordinating these processes during oxidative stress are still unknown, for other stress conditions such as nutritional stress, activation of macroautophagy prior to the insult has been shown to "prep" the cells making them more resistant to the cellular damage (8). Likewise, blockage of macroautophagy in some types of cancer cells increases their susceptibility to irradiation (61), suggesting that they may use macroautophagy to defend themselves against the irradiating insult. As for other types of injuries, the fate of the cell during oxidative stress is likely determined by the extent of the mitochondrial damage; low levels of mitochondria damage will promote cell repair while higher levels will cause apoptosis, and likely the severity of the lysosomal leakage (41, 64). Two molecules that have been already implicated in this cross-talk between apoptosis and autophagy are the death-associate protein kinase (DAPk) and the tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (61). Both factors are able to activate apoptosis and autophagy in a caspase-dependent or independent manner. Future investigation is required to understand why this double function and the mechanism that determines the switch from their role in one process or the other. One appealing possibility is the regulation through a dose-dependent mechanism. Different stress could result in the transcriptional activation of these factors as part of the cellular response to stress. Low levels of these factors may successfully activate macroautophagy. If autophagy is efficient in protein removal, these factors would be degraded and autophagy would be shut down. If autophagy is activated but it is insufficient for the removal of the cellular damage (massive damage of the lysosomal compartment, or impaired ability for productive autophagy because of the accumulation of damaged products in lysosomes), the intracellular levels of the two death factors (and possibly others) would remain high enough to activate apoptosis and cell death.

Only recently has the contribution of other autophagic pathways in cellular recovery postoxidative insult been explored. Under conditions of mild oxidation, CMA participates in the cellular response to oxidation by facilitating the removal of soluble, oxidatively damaged proteins from the cytosol (37). Oxidization produces a bipartite effect by acting on CMA substrates and on CMA active lysosomes (Fig. 3). Oxidized substrate proteins bind and translocate into lysosomes more efficiently than their unaltered counterparts (37). The partial unfolding associated to oxidative modification of a protein is likely to facilitate its recognition by the CMA-related cytosolic chaperone, by exposing the lysosomal targeting motif, normally buried in the protein core. In addition, partial unfolding should facilitate faster transit through the lysosomal membrane. On the other hand, independent of this effect on the substrates, lysosomes from culture cells and animals subjected to mild oxidative stress show higher ability to bind and internalize substrates via CMA (37). This activation of CMA is at-

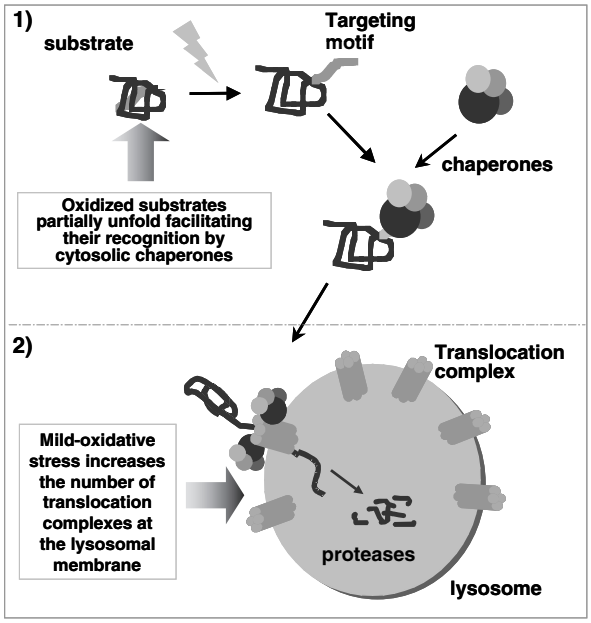

FIG. 3. Chaperone-mediated autophagy participates in the removal of oxidized proteins. (**1**) Soluble cytosolic proteins containing the CMA-targeting motif, when subjected to mild oxidation, are more readily translocated into lysosomes. Uptake may be facilitated by partial unfolding that favors both, their recognition by the cytosolic chaperones and their transport across the lysosomal membrane. (**2**) Lysosomes from cells exposed to mild-oxidative stress are more active for CMA. The enhanced activity correlates with an increase in the number of translocation complexes at the lysosomal membrane.

tained through an increase in the number of translocation units at the lysosomal membrane (Fig. 3). These activated lysosomes display increased levels of the CMA receptor, the chaperone hsc70 and various co-chaperones (37). These changes are similar to the ones detected as a result of starvation, a known activator of CMA (18). In contrast to other stress conditions in which the increase in translocation units results directly from changes in the lysosomal compartment, the activation of CMA during mild-oxidative stress requires *de novo* synthesis of protein, suggesting a transcriptional regulation (37).

The need for this dual activation of two different forms of autophagy during oxidative stress is easy to infer, on the basis of their particular characteristics. Thus, while the removal of damaged organelles as a whole can only occur through macroautophagy, the removal of oxidized soluble proteins in lysosomes without sacrificing still functional ones can only be achieved through a more selective mechanism, such as CMA. More difficult to understand is, perhaps, why cells activate CMA when the activation of the ubiquitin/proteasome system is already a common component of the oxidative stress response. Many studies *in vivo* and *in vitro* have documented the degradation of soluble oxidized proteins by the proteasome (30). In fact, blockage of the proteasome results in accumulation of damaged proteins inside cells, suggesting that it largely contributes to their removal. Therefore, why then a second system for the selective removal of oxidized proteins? It is probably easier to answer this question taking into account the cross-talk among the different pathways. Blockage of a form of autophagy is usually balanced by changes in the others. Thus we have found that selective blockage of CMA results in a compensatory activation of macroautophagy (AC Massey, G Sovak, S Kaushik, R Kiffin, and AM Cuervo, submitted) and likewise, cells lacking a gene required for macroautophagy display higher rates of CMA (S Kaushik, A Massey, N Mizushima, and AM Cuervo, unpublished results). Furthermore, changes in the activity of the ubiquitin/proteasome system have consequences on the lysosomal function and *vice versa*. Chronic blockage of the proteolytic activity of the proteasome deregulates the ability of cells to activate macroautophagy in response to nutritional stress (23). Thus, it is likely that CMA could complement the function of the ubiquitin/proteasome system when necessary, as part of the oxidative stress response, or completely replace this pathway, depending on the cellular conditions and their need for maintaining the proteasome system engaged in other regulatory functions. Ongoing studies aimed at selectively blocking CMA without affecting other proteolytic systems should help to establish the percentage of oxidized proteins removed by one proteolytic system or another, as well as the consequences of the failure of one of these systems in the way that cells respond to an oxidative injury.

Further information on publication? Please add to reference list. Please renumber following references if required.

(still under revision, no changes needed)

Autophagy and oxidative stress in aging and age-related disorders

Rates of intracellular protein degradation decrease with age in almost all organisms and tissues analyzed (29, 80). Numerous studies have reported malfunctioning with age in both the ubiquitin/proteasome system and the lysosomes (17, 19, 25, 35, 36).

Alterations in macroautophagy are evident in the form of an accumulation of autophagic vacuoles as tissues age (72). Macroautophagy malfunctioning results in part from a decrease in the rate of formation of these autophagic vacuoles, but predominantly in the rate at which they are eliminated after fusing with lysosomes (72). Although the main reason for this impaired clearance remains unknown, experimental accumulation of lipofuscin inside secondary lysosomes has been shown to decrease their ability to fuse with autophagic vacuoles (72). In addition, even for autophagic vacuoles fused to lysosomes, the presence in autophagosomes of engulfed materials able to generate reactive oxidative species, such as damaged or malfunctioning mitochondria, could destabilize the lysosomal membrane, alters the lumenal lysosomal pH, and/or directly inactivate lysosomal enzymes (75). The persistence of these undegraded products inside lysosomes leads to the formation of lipofuscin, as described in the previous section, thus perpetuating the inability of this compartment to carry out productive macroautophagy (74). The activation of macroautophagy as part of the early protective response against oxidative stress is thus clearly impaired as organisms age, and this likely contributes to their lower ability to handle cellular damage mediated by oxidation (77).

The activity of the second autophagic pathway shown to be involved in the oxidative stress response, CMA, also declines with age (21). In contrast to macroautophagy, accumulation

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of lipofuscin in lysosomes does not seem to be behind the impairment in CMA. In fact, the lysosomes more active for CMA rarely accumulate lipofuscin in their lumen. The cause of the age-related malfunctioning is a decrease in the levels of the receptor for the CMA substrates at the lysosomal membrane (21). Consequently, the substrates are recognized by the cytosolic chaperone, but the substrate-chaperone complex cannot dock at the lysosomal surface, or enter the lysosomal lumen. Long-term permanence of the substrates in the cytosol is likely to promote their aggregation. This process is accelerated when cells are exposed to oxidative stress. Lysosomes isolated from old animals have lower levels of endogenous oxidized substrates in their lumen, and decreased ability to translocate these proteins when presented to them even *in vitro* (37).

The sequence of events that lead to the failure of the two autophagic pathways with age and how this relates to the accumulation of oxidized proteins, characteristic of old organisms, remains unknown. Based on the previously described cross-talk between these two autophagic pathways, we hypothesize that the primary failure in CMA (because of the reduced levels of the lysosomal receptor with age), results in a progressive increase in the cytosolic levels of oxidized proteins with age. The probability of crosslinking events between oxidized proteins and other soluble cytosolic components increases with the increase in the time that they remain in the cytosol. As any other cytosolic components, these oxidized proteins would be sequestered into autophagic vacuoles each time that macroautophagy is activated (i.e., in between meals in the case of liver) and eventually delivered into lysosomes. However, the increased rates of crosslinking probably decrease the susceptibility of these modified proteins to the attack of the lysosomal enzymes, remaining as undigested lysosomal products (lipofuscin). The presence of lipofuscin in these secondary lysosomes aggravates the situation as it progressively impairs autophagic vacuole clearance (37).

In addition to normal aging, numerous diseases directly relate to oxidative stress, either as part of the evolution of the pathology, or directly, as cause of the pathological condition. Some examples of these oxidation-related pathologies in which the failure of the lysosomal system seems to play an important role, are atherosclerosis, age-related macular degeneration, Alzheimer's disease—a neurodegenerative disorder—and diabetes mellitus. Although in advanced stages all these diseases share a similar phenotype, that is the presence of undigested products in the terminal compartment (the lysosomes), the original oxidized compounds reach the lysosomal compartment following different pathways. Thus, in atherosclerosis and age-related macular degeneration oxidized/crosslinked materials are internalized from the extracellular environment by phagocytosis, whereas in Alzheimer's disease, both endocytosis and macroautophagy, result in the delivery to lysosomes of a protease-resistant product that increases the generation of free radicals. Finally, in diabetes mellitus, reduced CMA activity seems responsible for the decreased turnover of oxidized proteins, leading to their accumulation in the cytosol, and eventual delivery to lysosomes, likely by macroautophagy.

The atherosclerotic plaque, the histological lesion directly responsible for most of the age-related alterations in the vascular system, originates as a result of a problem in the orchestration of the lysosomal response to an original oxidative injury. Oxidation of the low-density lipoproteins that transport cholesterol through the artery walls into the tissues, promotes the retention of such particles in the walls. Macrophages migrate to the affected area to engulf the oxidized proteins and thus promote their degradation in lysosomes (45). Although the percentage of modified cholesterol is relatively low, the most abundant of these modified forms causes lysosomal sequestration of esterified native cholesterol by directly inhibiting the vacuolar ATPase, responsible for maintaining the intralyososmal acidic pH. In addition, the accumulation of large amounts of unmodified cholesterol in the lysosomes is also likely to directly alter the intralysosomal pH (45). The alkalization of the macrophage lysosomes results in the accumulation in their lumen of partially digested materials. With time, the macrophages are filled with loaded lysosomes, becoming "foam cells" with limited phagocytic activity (44). The accumulation of foam cells activates an inflammatory response that induces the arrival of new phagocytic cells. The growth of the lesion results from the continuous arrival of macrophages in the attempt to remove the damaged tissues (44). The failure of macrophage function leads to their death and accumulation in the lesion area (45). The early disruption of lysosomes induced by oxidized cholesterol with resultant autophagocytosis may be a critical event in apoptosis and/or necrosis of macrophages/foam cells during the development of atherosclerotic lesions (83). The amount of ROS generated in the atherosclerotic plaque increases with the accumulation of damaged cells and of extracellular debris, thus perpetuating the lesion (40).

Partial degradation of endocytosed materials in lysosomes also constitutes the basis of the age-related macular degeneration. The retinal pigmented epithelium has a dual role, playing an active part in the visual cycle, and also as support for the photoreceptor cells. The photoreceptor material that has become irreversibly damaged by side-reactions during the normal visual cycle is phagocytosed by the retinal cells and degraded in their lysosomes. Some of the phagocytosed byproducts are resistant to degradation and remain in the lysosomal system of the retinal pigmented epithelium cells, forming accumulations that resemble those of lipofuscin in other cells. Massive accumulation of the pigment results in cellular death and loss of sight in age-related macular degeneration (70). The pigment can itself absorb light, promoting the formation of ROS. As in other oxidizing conditions, these free radicals mediate cellular damage by destabilizing the lysosomal membrane and enzyme leakage (84). In addition, the main component of the pigment binds directly to the vacuolar ATPase perturbing its function (7). The alkalization of the lysosomes impairs even more their ability to handle the engulfed substrates and perpetuates this disease. The decrease in the protective mechanisms against oxidation with age, and the age-related failure of the lysosomal system favors the development of macular degeneration in old organisms (28).

Alzheimer's disease is the most common neurodegenerative disorder preferentially affecting the aged population. The neuronal pathology in Alzheimer's disease is, at least in part, related to the abnormal cleavage of a protein that generates a toxic peptide fragment known as Aβ-42. This peptide is for the most part secreted to the extraneuronal media where it aggregates forming plaques, a histological lesion characteristic

of this neurodegenerative disorder. Recent studies have shown that neurons can internalize soluble Aβ-42 from the extraneuronal medium and accumulate it inside their endosomal/lysosomal system (56). The resistance of soluble Aβ-42 to proteases makes it rapidly reach cytotoxic concentrations, which induce free radical generation within lysosomes and disruption of the lysosomal membrane proton gradient, followed by cell death (24). Changes in the endosome/lysosome system occur very early in the affected neurons of Alzheimer's disease patients (13). In fact, upregulation of macroautophagy is an initial response as a protective mechanism against the intracellular damage (82). Since the half-life of an autophagic vesicle once formed is less than 10 minutes, the observed build up of these vesicles in Alzheimer's patients suggests a possible impairment in the clearance mechanisms. The alterations in vesicular trafficking characteristics of this disorder may prevent the autophagic vacuoles from fusing to mature lysosomes. In addition, direct damage of lysosomes by Aβ-42 or the generated free radicals may block the degradation of the engulfed materials inside the vesicles and lead to their accumulation. Recently, both the enzymes and the substrate protein that generate Aβ-42 have been detected in autophagic vacuoles (82). The extended duration of the autophagic vacuoles in the cytosol of Alzheimer's disease neurons makes them a new source for the generation of intracellular Aβ-42. Although probably this is not the only mechanism responsible for neuronal death in this disease, it could constitute a possible target for future therapeutic interventions, since treatment of neurons in culture with either antioxidants or lysosomotropic amines partially blocks the release of lysosomal contents and cellular death.

Diabetes mellitus, one of the most frequent diseases of the elderly, brings associated different alterations in several organs and systems. Among them, the diabetes-associated protein accumulation in kidney leads to renal hypertrophy and, in the long-term, to renal failure. The large decrease in protein degradation found in diabetic animals compared to wild type could explain, at least in part, the increase in intracellular protein content. Blockage of lysosomal degradation with ammonium chloride levels the degradation rates in both groups, suggesting that the lysosomal system is preferentially affected in diabetic animals. Interestingly, the levels of KFERQ-containing proteins, mainly substrates for CMA, become particularly elevated in cortical cells of diabetic kidneys, pointing toward a primary alteration of CMA in this pathology (26). In fact, follow up of the degradation of Pax2.8, a transcription factor substrate for CMA, has revealed its stabilization in affected cells (27). Stabilization of Pax2.8, and likely of other transcription factors, can lead to excess protein synthesis increasing the mass and size of the diabetic kidney (27). Consequently, CMA malfunctioning contributes indirectly to control kidney development and growth by regulating the degradation of transcription factors. Although the primary reason for the decrease in CMA activity in the diabetic kidney remains elusive, both a decrease in the levels of the lysosomal receptor and chaperones have been described (26). An appealing explanation for the failure of CMA in diabetes affected cells could be that this pathway is exhausted as a consequence of a maintained hyperactivation in response to the oxidizing conditions associated with the disease. Future analysis of CMA activity at different stages of this pathology should shed light in this respect.

CONCLUDING REMARKS AND PENDING QUESTIONS

The resistance to proteolytic cleavage of heavily oxidized intracellular components often results in their intracellular accumulation, either in the cytosol or inside lysosomes when engulfed by phagocytosis or autophagy. The presence of undigested material inside lysosomes alters their normal function and converts them into a new source of free radicals, which destabilize the lysosomal membrane promoting hydrolase leakage and cellular damage. In contrast to this destructive effect of the lysosomal compartment, in the early stages of oxidative damage, or during mild oxidation, lysosomes play a protective role. Activation of macroautophagy and CMA are part of the cellular response to oxidative stress, for removal of the damaged components before further damage/aggregation occurs. The declined autophagic activity with age is probably responsible, at least in part, for the intracellular accumulation of oxidized components and inefficient response to oxidative stress characteristic of aged organisms and of certain age-related pathologies. Based in these recent findings, enhancement of the autophagic response would be desirable as a defensive mechanism against oxidative injuries. Efficient activation of macroautophagy has been described after treatment with the inhibitor of mTOR kinase, rapamycin (63). However, the possible consequences of interfering with the other many intracellular functions mediated by this kinase remain unclear. More physiological approaches may result from the use of caloric restriction, shown to prevent the age-related decrease in macroautophagy in old rodents (6).

A second front of action to promote removal of oxidized components from cells via lysosomes could originate from a better understanding of the origin and nature of lipofuscin. Of special interest is the current utilization of yeast for the study of the effect of lipid oxidation in the activity of the different proteolytic systems, and in particular of autophagy (47). This experimental model combined with the available yeast mutants for autophagy should shed light on the contribution of autophagy malfunctioning to the accumulation of lipid peroxidated products such as lipofuscin.

ABBREVIATIONS

ATG, autophagy genes; CMA, chaperone-mediated autophagy; DAPk, death-associate protein kinase; EM, electron microscopy; LDL, low-density lipoprotein; ROS, reactive oxygen species; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; vid, vacuole import and degradation pathway.

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