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Review

Autophagy and Aging

The Importance of Maintaining "Clean" Cells

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ABBREVIATIONS

CMA	chaperone-mediated autophagy
GH	growth hormone
IRS-1	insulin receptor substrate 1
LAMP	lysosome-associated membrane protein
mtDNA	mitochondrial DNA
PI3K	phosphatidylinsositol-3 kinase
PDK1	phosphoinositide-dependent protein kinase-1
TOR	target of rapamycin

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ABSTRACT

A decrease in the turnover of cellular components and the intracellular accumulation of altered macromolecules and organelles are features common to all aged cells. Diminished autophagic activity plays a major role in these age-related manifestations. In this work we review the molecular defects responsible for the malfunctioning of two forms of autophagy, macroautophagy and chaperone-mediated autophagy, in old mammals, and highlight general and cell-type specific consequences of dysfunction of the autophagic system with age. Dietary caloric restriction and antilipolytic agents have been proven to efficiently stimulate autophagy in old rodents. These and other possible experimental restorative efforts are discussed.

INTRODUCTION

Increasing age causes a progressive post-maturational deterioration of tissues and organs, leading to an impairment of cell and tissue functioning, increased vulnerability to challenges and decreased ability of the organism to survive.[1] There is extensive evidence that damage (especially oxidative damage) to proteins, DNA, membrane lipids, and cell organelles plays an important role in aging.[2,3] Accumulation of damaged (e.g., oxidized and/or glycated, i.e., nonenzymatically glycosylated) extracellular, intracellular and membrane proteins may account for the age-associated malfunctioning of many biological processes and has been frequently used as a biomarker of aging.[3] For example, accumulation of damaged 3-hydroxy-3-methylglutaryl coenzyme A reductase in the endoplasmic reticulum may cause hypercholesterolemia[4] and an abnormal increase in the tissue levels of the lipid-soluble anti-oxidant dolichol,[5] which is an excellent biomarker of aging.[6] Similarly, accumulation of oxidized nuclear[7] and mitochondrial DNA[8] (mtDNA) and of damaged organelles (in particular, mitochondria and peroxisomes) may start a vicious circle perpetuating the age-related increase in oxidative stress.[9]

Accumulation of damaged proteins with age was postulated to reflect an unbalance between the rates of protein damage and of protein turnover.[3,10] Reduced protein degradation could thus contribute to the aging process by reducing breakdown of altered proteins and also by prolonging the "dwell time" of proteins in a cell and, consequently, increasing their probability of becoming post-translationally altered.[10] This initial hypothesis has been extensively verified, and studies reporting decreased rates of protein degradation with age in almost all organisms and systems analyzed, are scattered throughout the literature of the last four decades.[11-13]

Two major proteolytic systems contribute to the continuous removal of intracellular components: the ubiquitin/proteasome system and the autophagic/lysosomal system. The ubiquitin-proteasome system is the most important extralysosomal degradative pathway, playing a major role in the maintenance of cellular homeostasis, protein quality control, and in the regulation of essential intracellular processes such as, cell cycle progression, cell division, transcription and signaling (reviewed in refs. 14 and 15). Although many reports have shown differing degrees of decrease in the activity of the ubiquitin/proteasome system with age in several tissues, the decline does not seem to be universal.[16-19] Decreased proteasome activity with age results, in many instances, from the inhibitory effect that oxidized and cross-linked proteins and lipids, abundant in old tissues, exert on this major cytosolic protease.[20] In support of decreased proteasome activity being a consequence, rather than cause, of the accumulation of damaged intracellular products in old cells, changes in proteasome activity do not correlate temporally with the accumulation of protein carbonyl derivatives (irreversible protein modifications generated via a variety of mechanisms including fragmentation and amine oxidation).[21]

Furthermore, the proteasome only has the capacity to degrade proteins; it is unable to degrade damaged organelles. Along these lines, extensive evidence has been produced that the decline in the ability of lysosomes to degrade intracellular components (autophagy) may be the main cause of the reduction of protein degradation during aging (reviewed in refs. 18 and 19). In fact, the temporal pattern of changes in protein carbonyl derivatives correlates with that of the age-related decline in autophagic proteolysis.[21,22] In this review, we recapitulate the main changes that the lysosome/autophagic system undergoes with age, emphasizing the cellular consequences of these functional changes, and highlighting recent advances in experimental restorative approaches.

AUTOPHAGY: THREE DIFFERENT ROUTES FROM THE CYTOSOL TO THE LYSOSOME

Lysosomes, or the vacuole in yeast, are the final compartment where many intracellular constituents are delivered for degradation via a series of pathways, globally referred to as autophagy. The potent battery of hydrolytic enzymes present within the lysosome allows for the degradation of all types of biomolecules (reviewed in refs. 23 and 24). Autophagy is an evolutionarily conserved catabolic mechanism that occurs in all eukaryotic cells and contributes to the turnover of cellular components (long-lived proteins, plasma membrane and organelles),[24-28] playing an important role in the maintenance of cellular integrity in post-mitotic tissues.[26,29,30] Autophagy is also essential for cell survival at times of limited nutrient availability, providing essential elements through the degradation of existing cellular constituents. In mammals, activation of autophagy during fasting is regulated by amino acids and hormones, including glucagon and insulin.[31-33]

The different types of autophagy, namely macroautophagy, microautophagy and chaperone-mediated autophagy, differ in the mechanism by which substrates are delivered to lysosomes, their regulation and their selectivity (Fig. 1). Macroautophagy, frequently referred to simply as autophagy, and microautophagy are conserved from yeast to mammals, while chaperone-mediated autophagy, so far, has been only described in mammals. In this review, we will use the term autophagy to refer to the general process that encompasses all the above mechanisms. Detailed descriptions of the different forms of autophagy can be found in references 24–26, 28 and 34. Macroautophagy is a stress-induced form of autophagy whereby intracellular organelles and cytosol are first sequestered away from the remaining cytoplasm by a double membrane-bound vacuole (autophagosome) (Fig. 1). Acid hydrolases are then introduced by fusion of the initial vacuole with lysosomes to form a single-membrane-bound degradative vacuole (autophagolysosome), which, as degradation of its content progresses, matures to a secondary lysosome or residual body (depending on whether degradation is complete or partial, respectively).[35,36] The different steps in macroautophagy (formation of the limiting membrane, elongation, maturation, lysosomal fusion and degradation), are mediated by a group of more than 14 proteins, first described in yeast, and generically known as Atg proteins.[37] Knock-downs and overexpression of the genes encoding these proteins in different organisms has tremendously advanced our understanding of the role that macroautophagy plays in different physiological and pathological processes (reviewed in refs. 28,29 and 39).

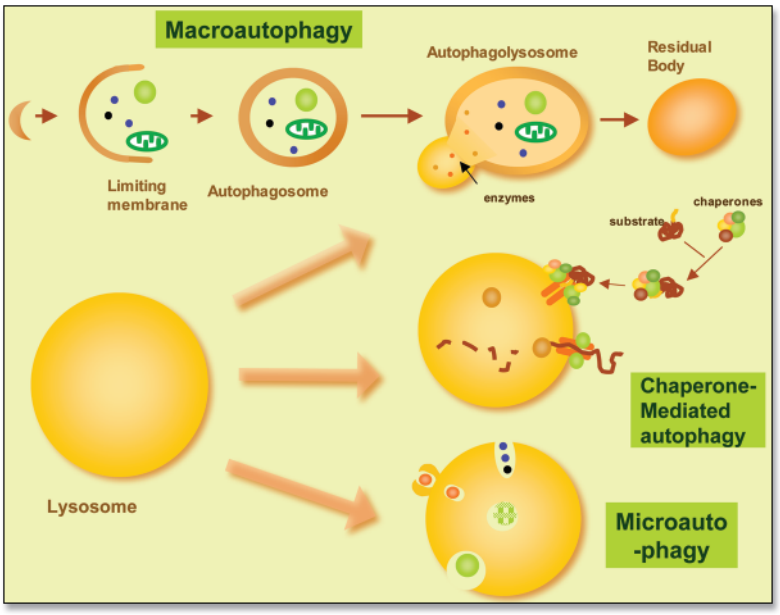

Figure 1. Schematic model of the types of autophagy in mammalian cells. Internalization of complete regions of cytosol first into autophagosomes that then fuse with lysosomes (macroautophagy), or directly by the lysosomal membrane (microautophagy) contrast with the selective uptake on a molecule-by-molecule basis of cytosolic proteins via chaperone-mediated autophagy.

Chaperone-mediated autophagy (CMA) is also an inducible form of autophagy, maximally activated by different stressors such as nutritional stress, exposure to toxic compounds and oxidative stress.[23,24,40,41] Under these conditions, substrate proteins are selectively targeted to lysosomes after interacting with a cytosolic chaperone, hsc70, the constitutive member of the 70kDa family of heat shock proteins (Fig. 1). Interaction of the chaperone with the substrate occurs through a particular amino acid motif (biochemically related with the pentapeptide KFERQ), which is present in all substrates for this pathway.[42] The substrate/chaperone complex docks on a receptor protein at the lysosomal membrane (LAMP-2A or lysosome-membrane associated protein type 2A), and, after unfolding, the substrate crosses the lysosomal membrane assisted by a resident chaperone present in the lysosomal lumen.[43] Although less important quantitatively than macro- and microautophagy, because only soluble cytosolic proteins and not organelles can be degraded by CMA, this is the only autophagic pathway by which particular cytosolic proteins can be selectively degraded by lysosomes.

Less information, particularly in mammals, is currently available about the mechanisms and molecular components that participate in microautophagy.[44,45] In this type of autophagy, cytosolic components are directly sequestered by the lysosomal membrane that deforms to create cytosol-containing invaginations or tubulations, which once pinched-off from the lysosomal membrane are degraded in the lumen (Fig. 1).[44] The lack of methods to directly measure microautophagy, and of specific markers for this process, makes it difficult to evaluate age-related changes in this form of autophagy, and consequently will not be further discussed in this review.

AGE-RELATED CHANGES IN AUTOPHAGY

Decreased macroautophagy proteolysis has been extensively reported (reviewed in refs. 18 and 19). The age-associated changes in liver macroautophagic proteolysis have been extensively studied in

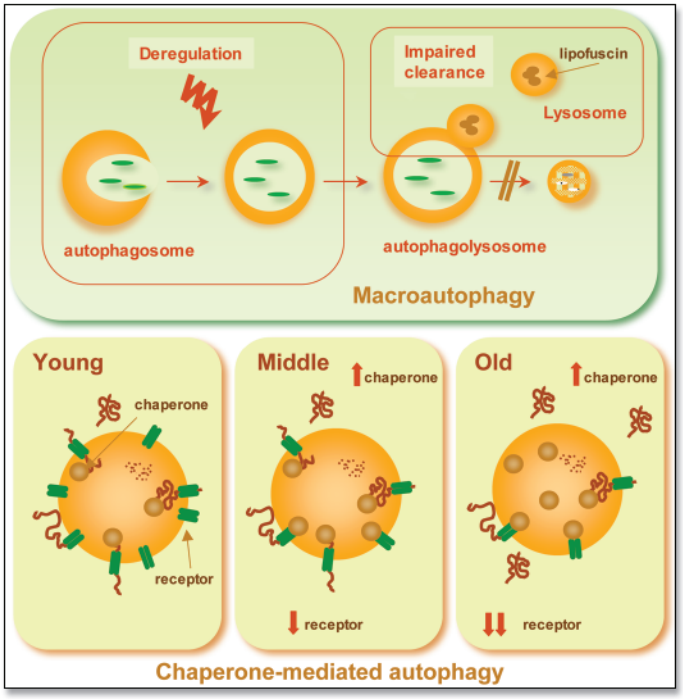

Figure 2. Age-related changes in autophagy. Top: Defective macroautophagy in aged cells results in part from diminished autophagosome formation (deregulation) and from poor clearance of autophagosomes. Presence of undigested materials in lysosomes (lipofuscin) could be responsible for their impaired ability to fuse and/or to degrade the autophagosome contents. Bottom: A decrease in the lysosomal levels of the CMA receptor is the primary defect identified as responsible for the diminished CMA activity during aging. Normal CMA activity is initially maintained (middle age) by increasing the amount of lumenal chaperone. At advanced ages the levels of the receptor are so low that compensation by the chaperone is no longer possible.

vivo in rodents of different ages, and in vitro in isolated hepatocytes from these rodents.[46,47] In this last case, macroautophagy can be modulated by incubation with amino acid mixtures or particular hormones (insulin or glucagon). Electron microscopy studies and metabolic assays (monitoring the release of radiolabeled amino acids from prelabeled resident proteins) revealed that maximum rates of proteolysis after overnight fasting (in vivo) and maximum sensitivity to stimulation by lower levels of amino acids (in vitro) were reached at six months of age and declined remarkably thereafter.[48,49] In contrast, the rate of protein degradation in the presence of high concentrations of amino acids was not affected by aging. The regulation of macroautophagy by hormones was differently affected by age.[46,48,49] Thus, the stimulatory effect of glucagon was blunted, whereas the inhibitory effect of insulin was not altered significantly by age. A similar age-dependent decrease in proteolysis was observed in rats fed ad libitum (i.e., unrestricted diet) when macroautophagy was stimulated by the injection of an anti-lypolitic agent.[47] This drug may affect macroautophagy by increasing the glucagon/insulin ratio and changing levels of amino acids in plasma.[47] Changes in macroautophagy activity, under these conditions, paralleled the age-related changes in the effects of the anti-lipolitic drug on glucose and insulin plasma levels. Thus, early changes in the regulation of macroautophagy could be secondary to age-related alterations of metabolism and/or in the hormonal response to fasting.[46,48,49] This alteration in the regulation of macroautophagic proteolysis, which leads to the accumulation of altered organelles and membranes, may start a vicious pro-aging circle.[50] Recent genetic evidence also supports this critical role of macroautophagy as an anti-aging mechanism. As described below, blockage of macroautophagy genes in long-lived *C. elegans* mutants prevents life-span extension.[51]

CMA activity also decreases with age. In fact, a decrease in the degradation of CMA substrates microinjected in senescent fibroblasts in culture was described before any molecular component was identified for this pathway.[52] The age-related decrease in CMA was later corroborated in old rodents using in vitro transport assays to reproduce translocation of CMA substrates into lysosomes.[53] Lysosomes isolated from different tissues of old rodents had reduced ability for binding and uptake of the cytosolic substrate proteins.[53] Interestingly, the degradation of the substrates once they reached the lysosomal lumen was unperturbed by age, suggesting that the activity of the lysosomal enzymes is preserved until late in life.[53] This finding justifies why current experimental restorative efforts, aimed to prevent/revert the decline in CMA with age, are focused on increasing binding and uptake, since proper degradation is assured if translocation of substrates is attained.

WHO IS TO BLAME FOR THE AUTOPHAGIC DECLINE UPON AGING?

Morphometric studies quantifying the number of autophagosomes formed and eliminated in different tissues of old animals have revealed a decrease with age in both, formation and subsequent elimination of autophagosomes (Fig. 2).[48,54,55] Defective autophagosome clearance may be the consequence of a decrease in the proteolytic activity of lysosomes with age and/or of impaired ability of lysosomes to fuse with autophagosomes. The accumulation of undigested products inside lysosomes (in the form of heavy lipofuscin loading see below) seems to be responsible, at least in part, for reduced autophagosome elimination. Recent studies point toward defective formation of autophagosomes being related to signaling-mediated deregulation of macroautophagy, rather than to a primary defect in any of the molecular components that participate in this process. In particular, the effects of (age-related) oxidative stress on the insulin receptor-signaling pathway seem to play a critical role in decreased macroautophagy in old organisms (Fig. 3).

The life span of the nematode *Caenorhabditis elegans* can be extended by up to 300% by mutations in the insulin receptor homolog Daf-2 or other proteins of the insulin receptor signaling cascade.[56,57] In *Drosophila*, insulin receptor mutations have been shown to cause not only significant life span extension but also an amelioration of the age-related decline in cardiac performance.[58] As mentioned above, RNA interference of macroautophagy genes in the Daf-2 (e1370) mutant of *C. elegans*, have shown that autophagy is essential for life span extension.[51] It should be noted, however, that blocking macroautophagy did not shorten the maximum life-span of wild type nematodes, and that it has not been shown that activation of macroautophagy will extend life span in a wild type nematode.

Classic studies in liver support an inhibitory effect of insulin on macroautophagy, opposite to the stimulatory effect of glucagon.[32] Stimulation of the insulin receptor typically involves autophosphorylation and activation of the insulin receptor kinase (IRK), the subsequent recruitment of insulin receptor substrate 1 (IRS-1) and phosphatidylinsositol-3 kinase (PI3K) which converts phosphatidylinositol (4,5)diphosphate (PI(4,5)P_2) into PI(3,4,5)P_3. This in turn binds and activates the phosphoinositide-dependent protein kinase-1 (PDK1) which phosphorylates the serine/threonine kinase

Akt 1/PKB. Akt 1 is activated by binding to PI (3,4,5)PI_3 at the cell membrane and by phosphorylation. Activated Akt causes activation of the target of rapamycin (TOR, FRAP, or mTOR in mammals), which controls a number of physiological functions including protein synthesis.[59] In addition, Akt 1 is an inhibitor of autophagy, SIRT1, and certain FOXO transcription factors. Insulin receptor signaling activity is downregulated by several phosphatases which dephosphorylate either IRK or PI(3,4,5)P3. Downregulation of the insulin receptor signaling cascade in mammals is typically found in the post-absorptive (fasted) state, implying that substantial induction of macroautophagy is largely restricted to this state (reviewed in ref. 60). As in liver, there is evidence that skeletal muscle tissue of healthy human subjects shows considerable net protein catabolism after overnight fast[61-63] which is essentially the manifestation of macroautophagic activity.[60,64,65]

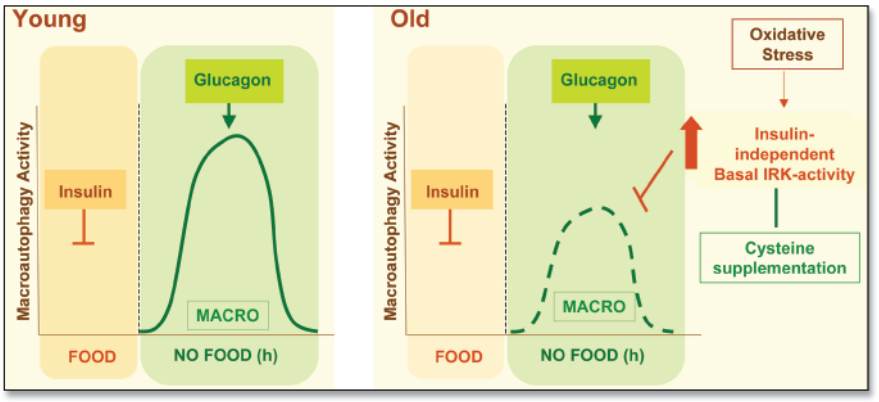

Figure 3. Deregulation of hormonal control of macroautophagy. Insulin suppresses macroautophagy while glucagon activates it in many mammalian cell types. With increasing age, the inhibitory effect of insulin is maintained, but macroautophagy is not properly activated in the presence of physiological levels of glucagon. Activation of the insulin-independent basal activity of the insulin-receptor (probably by oxidation) could be behind the loss of glucagon-mediated activation of macroautophagy with age.

As described in the previous section, the inhibitory effect of insulin on macroautophagy is not significantly altered with age, but the ability of glucagon to upregulate macroautophagy is clearly impaired in old rodents.[47] In view of these facts, it would appear that stimulation of macroautophagy in older animals must focus on downregulation of the basal activity of the insulin receptor signaling during the fasted state. A recent investigation of the human insulin receptor has shown that the insulin-independent basal IRK activity is weak but clearly detectable and strongly increased under oxidative conditions, which could counteract the stimulatory effect of glucacon on macroautophagy (Fig. 3). Specifically, the basal IRK activity is increased by low micromolar concentrations of hydrogen peroxide or by an oxidative shift in the intracellular thiol/disulfide redox status.[66,67] The physiological relevance of this effect has been underscored by the results of a clinical study in non-diabetic obese subjects, indicating that basal insulin receptor signaling can be decreased by supplementation with the cysteine derivative, N-acetylcysteine (a reducing agent) without seriously compromising glucose clearance in the postprandial state.[68]

In addition to the direct effect of hydrogen peroxide on the *basal* IRK activity, hydrogen peroxide further enhances the insulin receptor signaling cascade through inhibition of several phosphatases which normally downregulate its signaling activity by dephosphorylating IRK and PI(3,4,5)P_3.[69,70] In line with these findings, it was shown that the insulin-stimulated generation of hydrogen peroxide plays an integral role in insulin signal transduction.[71] There is a growing body of evidence for an age-related increase in oxidative stress and an oxidative shift in the thiol/disulfide redox status.[3,72,73] For example, serum and tissue concentrations of vitamin E and plasma concentrations of vitamin C were shown to decrease with age,[74-76] and an age-related decrease in the intracellular glutathione content was found in many tissues in rodents.[77,78] In humans, an age-related oxidative shift in the ratio of reduced to oxidized glutathione has been demonstrated in whole blood and peripheral blood mononuclear cells,[79,80] and an age-related oxidative shift in the cysteine/cystine redox status has been shown in the plasma.[81,82] This oxidative shift is accompanied by a decrease in the ratio of reduced vs. oxidized forms of plasma albumin and other thiol/disulfide redox couples.[83,84] Taken together, these findings suggest that any oxidative stress or oxidative shift in the glutathione redox status may be associated with an increase in basal insulin receptor signaling, which thereby compromises the macroautophagic activity in the post-absorptive period (Fig. 3).

In addition to insulin signaling, alterations in the Growth-Hormone-IGF-1 signaling pathway, may lead to extension of life[85] and affect macroautophagy. Although future investigation of this signaling mechanism and its changes with age is required, data have been obtained showing that IGF-1 may inhibit macroautophagic proteolysis (Donati A, Bergamini E, Cavallini G, unpublished) and degradation of mitochondria with deleterious mtDNA mutations.[86]

Finally, as discussed in detail below, independently of all these changes in signaling, heavy lipofuscin loading of lysosomes may also contribute to age related decline of macroautophagy.

In the case of CMA, a decrease with age in the levels of the lysosomal receptor that mediates substrate internalization into lysosomes, LAMP-2A, seems to be responsible for declined CMA activity during aging (Fig. 2).[53] Although reduced levels of receptor are evident at nine months of age in lysosomes from rat liver, CMA activity is initially preserved through an increase in the number of lysosomes involved in this autophagic pathway. Of the different groups of lysosomes present in cells, only those containing the lumenal chaperone, lysosomal hsc70, required for CMA substrate translocation are active for CMA.[41] Under particular circumstances, such as prolonged starvation, the group of lysosomes normally lacking the lumenal chaperone, and consequently inactive for CMA, become competent for substrate uptake/degradation through enrichment of their chaperone content.[40,41] Recruitment of this "reservoir" population of lysosomes for CMA, may be behind the compensatory mechanism that preserves CMA activity when levels of the receptor initially decrease. However, as age progresses, levels of the receptor decrease to such an extent that the CMA defect becomes evident.[53] The reasons why levels of the receptor decrease with age are currently the subject of investigation. Regulation of the lysosomal levels of this receptor is normally attained through changes in its degradation rate in the lysosomal compartment and in its distribution between the lysosomal membrane and lumen.[40,41] Faster degradation, instability of the receptor in lysosomes from old organisms, or impaired ability to retrieve the receptor from the lumen into the lysosomal membrane, could be behind the observed decreased levels of the receptor seen during aging.

GENERAL CONSEQUENCES OF AUTOPHAGIC FAILURE

The most imminent consequence of autophagic insufficiency is probably the age-related 'waste' accumulation within long-lived post-mitotic cells. Age-related cellular alterations reflecting imperfect autophagic turnover of macromolecules and organelles are summarized in Figure 4.

The role of autophagy as a cell repair and turnover mechanism is particularly important for long-lived post-mitotic cells such as neurons, cardiac myocytes and skeletal muscle fibers, which are characterized by a very low (if any) replacement rate. This distinguishes them from short-lived post-mitotic cells, such as peripheral blood and intestinal epithelial cells, which are efficiently renewed due to proliferation and differentiation of stem cells, providing for dilution of oxidatively or otherwise damaged biological structures.[87,88] These damaged structures (so-called biological 'garbage' or 'waste') progressively accumulate within long-lived post-mitotic cells, suggesting that they are not perfectly turned over.[87-89] Intracellular 'waste' material accumulates extra- and intralysosomally, reflecting insufficient autophagic sequestration and degradation, respectively.

Damaged mitochondria and cytosolic protein aggregates: The extralysosomal waste. Senescent mitochondria and indigestible oxidized protein aggregates are well-characterized forms of extralysosomal 'waste'. Many mitochondria in aged post-mitotic cells are enlarged and structurally deteriorated, showing swelling and disintegration of cristae, often resulting in the formation of amorphous material.[90,91] Excessively enlarged mitochondria are usually called 'giant'.[90] Senescent mitochondria are defective in ATP production[92] and are reported to produce increased amounts of reactive oxygen species (ROS),[93] which are harmful for cells and nevertheless cannot be eliminated. The mechanisms underlying age-related mitochondrial changes are still debated. Initial mitochondrial damage can be attributed to ROS injury combined with inadequate functioning of autonomous mitochondrial repair systems including Lon and AAA proteases, as well as (mtDNA) repair.[89] Conceivably, damaged mitochondria should be autophagocytosed and degraded, but their accumulation with age suggests that, they either acquire replicative advantage over normal mitochondria, or somehow escape macroautophagy.

A possible role of enhanced replication (clonal expansion) of defective mitochondria in aging follows from the facts that some senescent cells contain only mitochondria with single-type mtDNA mutations (homoplasmic mutations).[94,95] In support of the clonal expansion hypothesis, the accumulation of homoplasmic mitochondrial mutations has been demonstrated even in malignant cells, which actively proliferate and, thus, dilute damaged structures.[96] These facts, however, do not exclude the possibility of decreased macroautophagy of damaged mitochondria. De Grey postulated that some mutated, poorly respiring mitochondria experience a decreased oxidative damage to their membranes and, therefore, are less targeted for macroautophagy compared to normal mitochondria.[97] Although it remains unproved that mitochondria with oxidatively damaged membranes are selectively autophagocytosed, some data indicate that mitochondrial autophagy can be a nonrandom process. For example, tagged by ubiquitin, sperm mitochondria are selectively autophagocytosed in fertilized oocytes,[98] while yeast mitochondria are recognized for autophagy by the presence of the outer membrane protein Uth1p.[99]

Because mtDNA mutations affect only a portion of senescent cells (for example, one out of seven aged human cardiac myocytes),[95] it is reasonable that age-related mitochondrial changes may also develop independently of mtDNA damage.[100] There is a possibility that senescent mitochondria are poorly autophagocytosed just because of their large size, making their autophagy more energy consuming than that of normal mitochondria. Such a possibility is supported by experimental data on cultured cardiac myocytes, suggesting that small mitochondria are autophagocytosed more efficiently than large ones.[91] Initial mitochondrial enlargement, in turn, may occur because of oxidative damage to proteins responsible for mitochondrial fission or mutations in the corresponding nuclear genes. In agreement with this, enlarged mitochondria show decreased DNA synthesis,[91] which is normally associated with fission. Mitochondrial turnover may also decline in aged cells due to decreased autophagic capacity associated with lipofuscin overload (see below).

Formation of indigestible protein aggregates (aggresomes) occurs secondary to protein damage or mutations that result in protein unfolding and misfolding.[20] Aggresomes most commonly occur within aged post-mitotic cells, differing by composition and morphology in particular cell types such as neurons. For example, Lewy bodies, which are mainly composed of α-synuclein, form within aging dopaminergic neurons of substantia nigra,[101] whereas neurofibrillary tangles and argyrophilic grains (occurring in perikarya and processes of brain neurons, respectively) represent aggregates of the hyperphosphorylated protein tau.[102] While showing moderate increase in normal aging, certain types of aggresomes amass dramatically in specific pathologies, such as α-synuclein aggregates in Parkinson and Lewy body diseases, and neurofibrillary tangles in Alzheimer's disease.[103] A detailed description of the roles that changes in autophagic activity may play in these neurodegenerative disorders can be found in reference 104.

Failure of CMA with age has also been proposed to contribute to the accumulation of damaged proteins characteristic of aged cells. A role for CMA in the removal of damaged proteins has been described after exposure to certain toxin compounds and during mild-oxidative stress.[22,41] Under these conditions both, an increased susceptibility of the modified proteins to be taken up by lysosomes via CMA and a direct upregulation of this autophagic pathway, contributes to the selective removal of the damaged proteins. Decreased ability of lysosomes from old rodents to take up oxidized cytosolic proteins, when compared to younger animals, has recently been reported.[22] Furthermore, impaired degradation of selective cytosolic proteins because of decreased CMA activity with age could contribute to particular aspects of the aging phenotype. For example, specific subunits of the proteasome are normally degraded by CMA;[24] consequently, changes in the turnover of those subunits as CMA activity decreases could explain, at least in part, some of the observed changes in the subunit composition of the proteasome with age.[16]

The concomitant failure of both macroautophagy and CMA probably precipitates the accumulation of damaged cytosolic proteins with age (Fig. 4). Thus, some particular types of aggresomes could form in response to failure in the removal of particular damaged cytosolic proteins by CMA. Aggregation of the modified proteins is believed to be an active process that probably serves two purposes:[105] one, it may prevent the accumulation of oligomeric forms of the modified proteins, proven to be more toxic for the cells than the aggregates; two, it may favor the localization of the damaged proteins in a particular region of the cell, from where the aggresomes could then be trapped in autophagosomes for removal via macroautophagy. The added failure of macroautophagy with age would be responsible for the accumulation of these aggresomes inside cells. In fact, this

seems to be the reason for α-synuclein accumulation in familial forms of Parkinson's disease. Unmodified α-synuclein is, at least in part, degraded by CMA, but mutant forms of this protein are not degraded and instead end up in cytosolic aggregates.[106] Degradation of the mutant aggregate protein is initially possible through macroautophagy, although as the disease progresses the removal of the aggregates also becomes less efficient.[106]

Lipofucsin: The intralysosomal waste. Intralysosomal 'waste' material called lipofuscin, or age pigment, is a brown-yellow, autofluorescent, polymeric substance primarily composed of aldehyde-cross-linked protein and lipid residues.[107] Lipofuscin accumulation within long-lived post-mitotic cells is a recognized hallmark of aging that occurs with a rate inversely related to species longevity.[108] The undegradability of lipofuscin is well demonstrated by the fact that even starving cells, with activated autophagy, cannot get rid of the pigment.[109] This and other observations[110] also argue against any substantial exocytosis of lipofuscin granules.

Mechanisms behind lipofuscinogenesis are explained by results of experimental manipulations influencing the rate of lipofuscin accumulation. In cell culture models, oxidative stress (such as cultivation at 40% ambient oxygen or exposure to low molecular weight iron) enhances lipofuscin accumulation, whereas growth at 8% ambient oxygen or treatment with antioxidants or iron-chelators diminishes it (reviewed in ref. 111). Furthermore, a combination of oxidative stress with lysosomal protease inhibition, allowing prolonged oxidation and consequent cross-linking of autophagocytosed material, dramatically enhances lipofuscin formation.[107] These findings suggest that lipofuscin forms inside lysosomes as a consequence of iron-catalyzed oxidation of autophagocytosed material (Fig. 4). The rate of lipofuscin formation is thus related to the degree of mitochondrial production of hydrogen peroxide and its diffusion into lysosomes, as well as to the amount of intralysosomal redox-active iron. Autophagocytosed mitochondria seem to be a major source for both macromolecular components of lipofuscin and the low mass iron that catalyzes the peroxidative reactions resulting in its formation.[111]

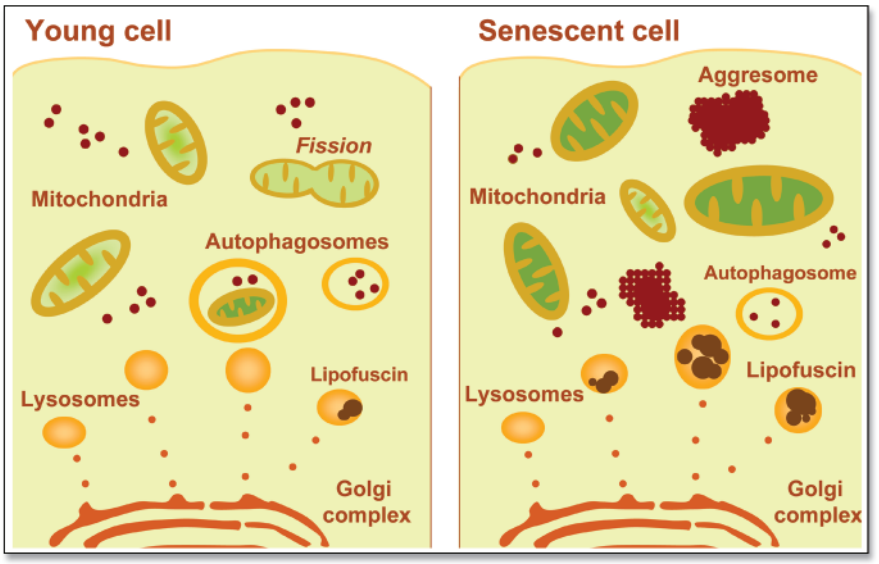

Figure 4. Tentative scheme illustrating age-related accumulation of damaged structures ('waste' material) within long-lived post-mitotic cells as a result of imperfect macroautophagy. Autophagy normally efficiently degrades damaged biological structures, such as mitochondria and cytosolic proteins. Regular mitochondrial fission prevents extreme enlargement of mitochondria, which may otherwise inhibit their uptake by autophagy. Some mitochondria, nevertheless, excessively enlarge apparently as a consequence of oxidative damage to their components involved in fission. Oxidative modification of macromolecules undergoing autophagic degradation results in the formation of an undegradable intralysosomal pigment, lipofuscin. Over time, lipofuscin occupies an increasing portion of the lysosomal compartment, which hampers macroautophagy, in particular due to the fact that large amounts of lysosomal enzymes are transported to abundant dysfunctional lipofuscin-loaded lysosomes rather than active lysosomes. Consequently, the number of excessively enlarged ('giant') mitochondria, usually deficient in ATP production and producing increased amounts of ROS, progressively increases, while oxidatively modified cytosolic proteins form large indigestible aggregates (aggresomes). Dark red dots symbolize cytosolic proteins, while dark green color of mitochondria indicates damage. Detailed explanations and references are given in the text.

Intralysosomal accumulation of undegradable material also occurs independent of age as a manifestation of certain pathological conditions (e.g., lysosomal storage diseases, malnutrition, radiation-induced injury) and it can be induced by many chemical agents including drugs and environmental pollutants. Such pigment, showing morphological and chemical similarity with lipofuscin, is occasionally called 'ceroid', or 'ceroid-type lipofuscin'.[112] This distinction is, however, valid only in terms of etiology but not in terms of properties and basic mechanisms of pigment formation.

Lipofuscin seems to diminish macroautophagic capacity of post-mitotic cells by acting as a sink for newly produced lysosomal enzymes and, therefore, interfering with turnover of cellular components (Fig. 4).[9] With time, lipofuscin forms large intralysomal aggregates that completely fill many lysosomes. Such structures were previously called 'residual bodies' and considered inert structures. It has, however, been shown that such bodies are integrated parts of the cellular vacuolar compartment and constantly receive newly produced lysosomal enzymes by fusing with late endosomes (reviewed in ref. 9). Because lipofuscin is indigestible, the delivery of acid hydrolases to lipofuscin-loaded lysosomes is a waste, which in cells with large quantities of age pigment cannot be compensated for by increased production of lysosomal enzymes. Thus, aging post-mitotic cells finally end up in a situation where most lysosomal enzymes are directed to abundant lipofuscin-loaded lysosomes, leaving little enzymatic activity for useful purposes, such as autophagic turnover of organelles and macromolecules (Fig. 4). The result will be reduced efficiency of the autophagic clearance of damaged structures and progressive functional decay.[9]

Negative effects of lipofuscin accumulation are not limited to the inhibition of macroautophagy in senescent post-mitotic cells. Being rich in heavy metals, particularly in iron, lipofuscin may jeopardize lysosomal stability under severe oxidative stress, causing enhanced lysosomal rupture and consequent apoptosis/necrosis.[109,113] Furthermore, lipofuscin is a fluorochrome and may sensitize lysosomes to visible light, a process potentially important for the pathogenesis of age-related macular degeneration.[114] Lipofuscin, thus, seems to be an important contributor to cellular degeneration, and not only a hallmark of aging as was hitherto believed.

IMMUNOSENESCENCE: A PARTICULAR EXAMPLE OF CONSEQUENCES OF AUTOPHAGIC FAILURE WITH AGE

In addition to the consequences of diminished autophagic activity with age common to all aged cells, described above, failure of this proteolytic system is also responsible for the loss of particular cell functions specific to some cell types. Here we illustrate the contribution of autophagic changes to the decay in function of T-cells with age. Immunosenescence is the generic term for any immunological deficit occurring with aging. For example, aging is associated with a dramatic decline in immune functions involving both B- and T-cells as well as neutrophils[115,116] and with increased morbidity and mortality following infections, higher incidences of cancer and declining antibody responses to specific vaccines.[117] T-cells are the best studied lymphocyte subset in this respect.

Normal somatic cells undergo a finite and predictable number of cell divisions in tissue culture before reaching an irreversible state of growth arrest.[118] Similarly, T lymphocytes possess a limited in vitro lifespan corresponding to replicative senescence.[119] Aging may be considered as the consequence of several cellular metabolic modifications occurring at different levels such as cell cycle and telomeric length regulation, structural organization, cellular interactions, stress response capability, and catabolic functions among which autophagy is grouped.

As in other cell types, an age-related increase in lipofuscin was described in the cytoplasm of lymphocytes in vivo when comparing elderly individuals and centenarians.[120] In long-term culture of lymphocytes, a significant increase with aging in the percentage of cells presenting at least one autophagic inclusion was observed. Furthermore, a progressive increase of lipofuscin specific autofluorescence and more autophagic vacuoles were found as cells acquired replicative senescence characteristics.[121] As mentioned before, under these conditions, lysosomal enzymes of aged post-mitotic cells are mainly associated with undegradable lipofuscin, while enzymes are in short supply in newly formed autophagosomes.[107] The analysis of the expression of *ATG* genes in fibroblast or lymphocyte long-term cultures did not show significant variations with the age of the culture, suggesting that the macroautophagic process does not increase with aging, but that ineffective autophagic vacuoles do accumulate leading to accumulation of lipofuscin.[121,122] This accumulation of undegradable material could be involved in cell death,[113] although the mechanisms leading to the death of senescent cells are not completely understood and experimental results at present still conflict.

An increased apoptosis of T cell subsets has been reported in aging humans.[123,124] This increase was associated with up-regulation of Fas and Fas ligand expression and increased susceptibility to Fas-mediated apoptosis associated with caspase activation.[125] During long-term cultures, repeatedly stimulated $CD8^+$ T-lymphocytes became progressively intolerant to activation. Cell death following stimulations was associated with DNA fragmentation and activation of caspases; two features of apoptotic cell death. The early stages of macroautophagy were shown to be required for apoptotic cellular death induced by TNFα.[126] Furthermore the autophagic process once activated via an apoptotic signal, which causes mitochondrial dysfunction, may also mediate cell death.[127] A detailed review of the relationship between autophagy and apoptosis can be found in reference 128.

Autophagy in in vitro senescent lymphocytes, with accumulation of autolysosomes and lipofuscin, could be associated with cellular fragility, favoring stress-induced cell death by apoptosis or necrosis. A role of autophagy, in vivo, in the decline of immune functions in the elderly, remains to be demonstrated.

RESTORATIVE EFFORTS: A MULTI-FRONT ATTACK?

Anti-aging caloric restrictions prevent the accumulation of altered proteins and the age-related alteration of autophagic proteolysis. Prolonged calorie restriction has been shown to extend both the median and maximal lifespan in a variety of lower species such as yeast, worms, fish, rats, and mice and to improve the health conditions of primates, depending on level and duration.[129] In addition, caloric restriction has also been shown to improve recovery from toxic challenge in lower animals.[130] Restriction of food intake 40% below food consumption of ad libitum fed rats, or every other day feeding, makes animals spend a large part of their time in the state of fasting, with lower glucose and insulin plasma levels and consequently, favoring activation of the two inducible forms of autophagy, macroautophagy and CMA (ref. 131 and unpublished).

Ten years ago it was proposed that autophagy, as a highly regulated cell repair mechanism, could mediate in part the anti-ageing effects of calorie restriction.[132] Extensive evidence may now support this hypothesis, showing that caloric restriction prevents the accumulation of altered proteins in cytosol and membranes,[129] the increase in liver tissue dolichol,[6] and the accumulation of altered mtDNA. Caloric restriction preserves the juvenile function and regulation of macroautophagy.[49,133] Functioning of macroautophagic proteolysis in caloric restricted rats was studied in vivo by the injection of an antilipolytic drug to older rats fasted overnight, which had been on food restriction since early in life. Results showed that caloric restriction prevented the age-related changes in regulatory plasma nutrients and hormones, and in the proteolysis of resident liver proteins.[49] In in vitro incubated hepatocytes from these rats, dietary anti-aging intervention preserved the juvenile amino acid and hormone regulation of autophagy.[133] Further support for the hypothesis came from the observation that the protection of rat liver autophagic proteolysis from the age-related decline covaried with the duration and level of anti-ageing food restriction in agreement with the known effects of the dietary intervention on longevity.[131] Probably, by maintaining plasma insulin at a markedly low level during long periods of fasting throughout life, caloric restriction is in effect increasing autophagic proteolysis. Treatment with insulin was shown to reverse at least some beneficial anti-aging effects of caloric restriction.[134]

The finding that daily stimulation of autophagic proteolysis by fasting may prevent the age-dependent deregulation of the process in life-long caloric restricted rats, invited studies on the effects of chronic pharmacological stimulation of macroautophagy on the age-dependent decline in autophagic proteolysis. Stimulation of macroautophagy was performed by the administration to fasted rats of antilipolytic drugs like 3,5-dimethylpyrazole or *ACIPIMOX*, which is available on the market for human use.[47] Treatment causes a sudden decrease in the availability of lipid fuel and induces a compensatory increase in protein degradation. The chronic weekly administration of the drug, starting from age six months, restored autophagic proteolysis and its hormonal control in 24-month old rats and prevented the accumulation of the biomarker of aging, dolichol in liver tissue.[135]

In principle, it can be predicted that the long-lasting administration of caloric restriction mimetics[136] and stimulators of macroautophagy via mTOR blockage, like rapamycin, should have similar effects (while GH, Insulin, IGF-1 and anabolic steroids should have opposite effects,

and cause aging and the onset of age-associated diseases). Although experimental evidence in mammals is still lacking, studies in *C. elegans* have recently revealed that TOR deficiency doubles its natural lifespan.[137]

In view of the positive role of the insulin receptor signaling cascade in the regulation of protein synthesis and other important metabolic processes, it is reasonable to assume that, neither in *C. elegans* nor in humans, permanent downregulation of this signaling cascade by genetic or other methods is the best strategy to increase life span. In humans, a weak insulin response to food intake, (i.e., in the postprandial state), may even lead to diabetes. It is thus reasonable to assume that any attempt to further downregulate insulin receptor signaling in humans must be restricted to the fasted state and focus on the *basal* activity of the insulin receptor signaling pathway. The fact that oxidative stress may potentiate this basal activity suggests that macroautophagy could be rescued (i.e., relatively enhanced) by cysteine supplementation. This hypothesis remains to be tested. A series of clinical studies on the effects of cysteine supplementation has already shown significant beneficial effects on several parameters that are typically affected by aging, including skeletal muscle functions and body cell mass,[138,139] but autophagic activity has not been analyzed. In human astrocytes and other cell types in culture, supplementation with vitamin C, a well-recognized antioxidant agent, increases the proteolytic efficiency of lysosomes, and consequently protein turnover through any type of autophagy.[140] In part, the vitamin C (ascorbic acid) effect seems to result from the vitamin stabilizing the lysosomal pH at very acid values. However, a second affect on autophagy, through modulating basal insulin signaling, remains an open possibility.

The effects of loss-of-function mutations of insulin and GH-IGF-1 signaling pathway on macroautophagic proteolysis too may deserve to be studied to unravel new ways to restoration. Likewise, the mechanisms that mediate restoration of CMA in caloric restricted animals remain to be elucidated.

CONCLUDING REMARKS AND PENDING QUESTIONS

A decrease in autophagic activity has been convincingly demonstrated in old organisms. Considerable advance has been made too in our understanding of the defective steps that lead to dysfunction of autophagy with age. However, most of the particular molecular components responsible for the loss of autophagic function in aging still remain elusive. Age-related research should very soon benefit from the current advances in the molecular dissection of macroautophagy and CMA and from the availability of novel autophagic markers and better functional tests. Critical at this point is to understand how age-related changes in different hormonal factors and metabolites contribute to the diminished autophagic activity. The growing number of large-scale proteomic- and metabolomic-based studies on aging models should provide valuable clues on this matter. Different transgenic and conditional knock-out mice for several Atg proteins have also been generated recently. Longevity studies on these animal models would help to assess the components of the aging phenotype directly linked to autophagy malfunction.

Although all the attempted restorative efforts on autophagy have been done at the experimental level and there are not direct therapeutic applications yet, particularly sound are the results obtained with caloric restriction, proving not only that restoration of autophagic proteolysis in older animals is feasible, but also that it might help to counteract progression of aging. However, although the ability of caloric restriction to slow down aging has been known for more than 30 years, the mechanisms that mediate this beneficial effect remain unclear. Future studies analyzing the effect of different caloric restriction-mimetics on autophagy should shed light on the mechanistic basis for the preservation of normal autophagic function.

Finally, there is growing evidence supporting a cross-talk among the different forms of autophagy, and even between autophagy and other proteolytic systems. In aging, and different age-related disorders, malfunctioning of several of those systems has been shown to coexist. This makes even more severe the consequences of the failure of a particular autophagic pathway or proteolytic system, since activation of compensatory mechanisms may not be possible. Whether the age-related changes in macroautophagy and CMA occur independently or one is consequence of the failure in the other, and the impact of the declined activity of these two lysosomal pathways on other proteolytic systems will require future investigation.

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